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Response of The American Journal of Clinical Nutrition to the National Institutes of Health Public Access Policy

Charles H Halsted, Editor-in-Chief, and Ronenn Roubenoff, Chair of the Publications Management Committee of the American Society for Clinical Nutrition

INTRODUCTION

On 2 May 2005, the National Institutes of Health (NIH) issued a policy aimed at ensuring rapid public access to the results of NIH-funded research. The new NIH Public Access Policy requests that principal investigators (PIs) of NIH-funded research deposit an electronic form of their completed manuscripts on the NIH National Library of Medicine’s PubMed Central website within 12 mo of journal acceptance (1). However, this new policy may be both redundant and in conflict with the publishing procedures and policies of most independent scientific journals. For example, the electronic version of each American Journal of Clinical Nutrition (AJCN) issue is immediately available at no cost to institutions in developing countries designated as low income by the World Bank, and the public can obtain at no cost all editorials and review articles immediately and all other content 12 mo after publication. At the same time, and to ensure scientific integrity, AJCN policies include ownership of copyright to all accepted material after rigorous copyediting before publication. Our copyright policy provides legal protection to authors, the AJCN, and its sponsor—the American Society for Clinical Nutrition (ASCN)—against commercial advertisers and others who might otherwise profit by distorted use of our published material. Such protection is particularly important in the realm of diet and nutrition, which is the source of a vast commercial enterprise. Although PIs are likely to follow the requests of their primary funding agency, they risk running afoul of our copyright policy and potentially of posting misleading information if manuscripts are submitted to PubMed Central for public access before they have been properly copyedited. The purpose of this editorial is to provide a roadmap to not only help prospective AJCN authors comply with the NIH directive but also to minimize conflict with AJCN copyright and publishing procedures.

THE NIH PUBLIC ACCESS POLICY

According to its Public Access Policy, the NIH now requests that PIs submit, to the National Library of Medicine’s PubMed Central, manuscripts accepted for publication on or after 2 May 2005 that result from currently funded or previously supported NIH research projects. The NIH policy is targeted at recipients of all research grant and career development award mechanisms, cooperative agreements, contracts, institutional and individual National Research Service Awards, and NIH intramural research studies. The NIH policy applies to peer-reviewed original research publications that have been supported directly, in whole or in part, by NIH funds but does not apply to book chapters, editorials, reviews, or conference proceedings (1). NIH-funded PIs are requested to comply with the terms of the NIH manuscript submission system (Internet: http://www.nihms.nih.gov) at the National Library of Medicine’s PubMed Central by submitting an electronic version of the final manuscript at the time of acceptance for publication. The policy states that “the author’s final manuscript is defined as the final version accepted for journal publication, and includes all modifications from the publishing peer review process” (1). When a PI deposits an article in PubMed Central, it will be kept internally at the NIH until a time stipulated by the PI for its release to the public. The NIH encourages PIs to permit public release of accepted manuscripts as soon as possible within the 12-mo time frame after the official date of final publication.

POTENTIAL PITFALLS OF THE NIH POLICY

Because the PI is requested to submit the initially accepted manuscript to PubMed Central, whereas all finalized AJCN manuscripts are published in print or online by HighWire Press, at least 2 different versions of an article will end up on the Internet. The responsibility for meeting the NIH policy falls on the PI, who is requested to submit the accepted version of the manuscript, which has not yet been copyedited, and the NIH does not allow publishers to link the final published version of articles to PubMed Central. Furthermore, the NIH policy contains no safeguard against the possibility that the initially accepted manuscript that is submitted by the PI to PubMed Central contains factual errors that are only caught later and corrected during the Journal’s copyediting process. Our greatest concern is that errors in dosing or in other clinically relevant information in the uncopyedited version may be published via PubMed Central, thereby putting patients at risk and raising liability issues for PIs, the

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2 The full content of this editorial is available on the Internet at http://www.ajcn.org.

3 Reprints not available. Address correspondence to CH Halsted, The American Journal of Clinical Nutrition, 3247 Meyer Hall, University of California, Davis, One Shields Avenue, Davis, CA 95616-8790. E-mail: chhalsted@ucdavis.edu.
THE WASHINGTON DC PRINCIPLES COALITION

Last year, in response to governmental and other efforts to promote immediate public access to scientific material, the AJCN joined a coalition of 104 other not-for-profit publishers and developed the Washington DC Principles for Free Access to Science (DC Principles) (2). First among these principles is the following statement: “As not-for-profit publishers, our mission is to maintain and enhance the independence, rigor, trust, and visibility that have established scholarly journals as reliable filters of information emanating from clinical and laboratory research.” These principles emphasize that we already support 1) the efficient online access to our journal content through HighWire Press, which includes extensive electronic reference linkages to hundreds of other journals; 2) the immediate availability of important articles of interest, such as editorials and review articles, at the time of publication; 3) the immediate availability of all articles to scientists in low-income countries; and 4) the equal opportunity for all scientists worldwide to publish in scientific journals, regardless of economic circumstance. Furthermore, the coalition questions the value of the immediate public distribution of scientific content (3). This stance supports the concepts offered in a previous AJCN editorial, ie, that the public is too often confused by conflicting data and that scientific “facts” are seldom established by a single experiment, require reproducibility, and should only be considered valid after confirmation by independent and unbiased follow-up studies (4).

THE AJCN’S RESPONSE

In meeting the challenges posed by the new NIH Public Access Policy, the AJCN takes the position that the PI is ultimately responsible for any conflicts that may arise from compliance with the NIH policy, ie, from the premature submission of accepted manuscripts. According to the copyright agreement that all authors sign when submitting a manuscript to the Journal, the AJCN owns the copyright to all material destined for publication. To facilitate compliance with the NIH policy, the AJCN will grant the PI permission to deposit an accepted manuscript in PubMed Central with the stipulation that the PI accepts any liability that may arise from the release of the manuscript in its unpublished form. To avoid this potential liability, the AJCN recommends that the PI delay submission of the manuscript to PubMed Central until after the copyediting process is complete. At that point, the PI will be provided with a PDF of the final version of the article that is to be published in the AJCN. Provision of this final version of the article to PubMed Central will avoid any ambiguity that could otherwise result from the existence of one uncopyedited and another copyedited version of the same article.

If the PI decides to submit the uncopyedited version of the manuscript to the NIH, a further stipulation of our permission is that the manuscript must contain the following statement at the top of the title page: “This is an uncopyedited author manuscript that has been accepted for publication in The American Journal of Clinical Nutrition, copyright American Society for Clinical Nutrition, Inc (ASCN). This manuscript may not be duplicated or reproduced, other than for personal use or within the rule of ‘Fair Use of Copyrighted Materials’ (section 107, Title 17, US Code) without permission of the copyright owner, the ASCN. The final copyedited article, which is the version of record, can be found at http://www.ajcn.org. The ASCN disclaims any responsibility or liability for errors or omissions in the current version of the manuscript or in any version derived from it by the National Institutes of Health or other parties.” Regardless of whether the uncopyedited version or the copyedited final version of the manuscript is submitted to the NIH, the PI must abide by the AJCN policy of making articles free online on our website as well as in the NIH repository 12 mo after publication.

Neither author had any financial or other personal conflict with the statements expressed in this editorial.

REFERENCES

The insulin-like growth factor axis: a potential link between glycemic index and cancer\textsuperscript{1,2}

Sudha B Biddinger and David S Ludwig

The question of how diet affects cancer risk has been debated for many years. Dietary fiber is thought to decrease the transit time of food in the colon and to dilute carcinogens, thus minimizing the body’s exposure to toxins. The presence of certain compounds that induce the production of beneficial xenobiotic metabolizing enzymes may be important. In addition, high concentrations of antioxidants, which decrease free radical–induced damage, may play a role. At this time, however, the mechanism by which some foods promote and others protect against cancer remains largely unknown.

The novel dietary factor glycemic index (GI) has been linked to the risk of several types of cancer, including breast, ovarian, endometrial, pancreatic, and colorectal (1), although the mechanisms involved were not previously examined. The GI reflects the way in which a food (meal or diet) affects postprandial blood glucose and, consequently, insulin concentrations. Foods that are rapidly digested in the gut, including most refined-grain products and potato, have a high GI, whereas nonstarchy vegetables, fruit, and legumes tend to have a low GI (2).

In this issue of the Journal, Brand-Miller et al (3) suggest that a high-GI diet may increase cancer risk by modulating the insulin-like growth factor (IGF) axis. Using 10 healthy subjects, the authors compared the effects of a high-GI meal and a low-GI meal on glucose, insulin, and various components of the IGF axis in the serum over 4 h. The meals were well matched for macronutrient composition. As intended, the areas under the curve for glucose and insulin were 40% and 70% lower, respectively, after the low-GI meal than after the high-GI meal. There was no change in either free or total IGF-I. At 4 h, the high-GI meal reduced IGF-binding protein (IGFBP)-1 by 13 ng/mL and reduced IGFBP-3 by 110 ng/mL. The low-GI meal, on the other hand, reduced IGFBP-1 by 55 ng/mL and increased IGFBP-3 by 251 ng/mL. Thus, the low-GI meal produced a greater decrease in IGFBP-1 than did the high-GI meal and produced an increase, rather than a decrease, in IGFBP-3.

To understand the significance of these changes, it is important to consider the complexity of IGF-I signaling. IGF-I has powerful effects; it stimulates anabolic metabolism, cell proliferation, and cell differentiation and can also inhibit apoptosis. IGF-I in the serum is thought to be derived primarily from the liver and complexed to IGFBPs (4). IGFBP-3 is the major IGFBP in the serum, and most circulating IGF-I is present in ternary complexes with the acid-labile subunit and with either IGFBP-3 or -5. IGF-I in the ternary complex is sequestered in the plasma and hence has a prolonged half-life.

Circulating IGF-I is also found in binary complexes of IGF-I and IGFBP-1, -2, -4, or -6, all of which may exit the vasculature more freely. Only a small amount of IGF-I exists freely in the circulation.

At the cellular level, IGF-I signals primarily through the IGF-I receptor, a tyrosine kinase, but it can also interact with the insulin receptor and the IGF-II receptor. The interaction of IGF-I with these receptors is also modulated by IGFBPs, which can either promote or attenuate IGF-I activity. Some IGFBPs are known to have effects on cell growth and proliferation that are independent of either IGF-I or its receptor. Furthermore, IGFBP activity is itself regulated by posttranslational modifications such as phosphorylation and by proteolysis.

Given the redundancy of the IGF system and its many layers of regulation, it is not surprising that IGFBPs are found to have varied biological effects. For example, IGFBP-1 promotes apoptosis of breast cancer cells under some conditions but not under others (5, 6). IGFBP-3 has been shown to promote apoptosis in most in vitro studies. Nonetheless the fact that transgenic mice overexpressing IGFBP-3 in the involuting mammary gland displayed decreased apoptosis suggests that factors other than the amount of IGFBP-3 determine its actions (7).

One finds similar inconsistencies in epidemiologic studies of the role of the IGFBPs in cancer. On the basis of in vitro data, it might be expected that high concentrations of IGFBP-3 would reduce the risk of cancer. Whereas some studies found such an association, a large meta-analysis failed to show any protective effect of serum IGFBP-3 concentrations on the risk of prostate, colorectal, or breast cancer (8). Similarly, IGFBP-1 concentrations have been shown to be both positively and negatively associated with cancer risk (5, 9).

Brand-Miller et al were the first, in the study reported in this issue of the Journal, to document the effects of GI on the IGF axis. Clearly, the magnitude of the changes they found was small: \( \approx 6\% \) for IGFBP-3. Moreover, there was no corresponding change in either total or free IGF-I. It is also surprising that IGFBP-1, which is known to be suppressed by insulin, was reduced to a greater extent after the low-GI challenge. This suggests that the GI may have effects on the IGF system that are independent of insulin.

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Despite these caveats, the fact that any change at all was noted is interesting, given the short duration of the study (4 h). Changes of this magnitude could conceivably affect IGF-I signaling significantly over time, through not only endocrine but also autocrine and paracrine pathways.

In summary, the powerful effects of IGF-I on cell growth and proliferation strongly argue for a role of the IGF system in cancer development. However, because of the complexity of the IGF axis, the exact roles of the different IGFBPs in modulating IGF-I function remain unclear. The study by Brand-Miller et al suggests that the GI can modulate IGF-I signaling within hours. These findings raise the possibility that, by lowering the dietary GI, we may be able to manipulate the IGF axis to prevent cancer.

Neither author had any personal or financial conflicts of interest with the study by Brand-Miller et al or the article concerning it.

REFERENCES
Science-based micronutrient fortification: which nutrients, how much, and how to know?1,2

Irwin H Rosenberg

This issue of the Journal includes a comprehensive biochemical assessment of folate status in a representative sample of the American population in the National Health and Nutrition Examination Survey (NHANES) 1999–2000 (1). This survey represents the largest, but not the first, documentation of folate status since the mandatory folic acid fortification of flour and grain products was initiated in the United States in 1998. As was shown in other populations (2–5), folate status in the NHANES 1999–2000 has improved remarkably since the initiation of folic acid fortification. Mandatory folic acid fortification may be the most important science-driven intervention in nutrition and public health in decades. The study by Pfeiffer et al (1) reminds us that there is still work to be done and a need for protocols that assign responsibility and resources for the evaluation of public health interventions that affect large sections of the total population of the United States and other countries.

A sign in a health food store caught my eye in the 1980s, shortly after I’d agreed to serve on a Federal Drug Administration (FDA) panel to assess communication concerning the over-the-counter marketing of vitamins in the United States. The sign read, “God gave us vitamins and the FDA is taking them away.” Then, as now, we were faced with a passionate certainty about the value of particular interventions and remedies or about the harm of others and with the substitution of transcendent beliefs for scientific analysis. In sharp contrast, the case study of folic acid fortification as an approach to prevent neural tube defects is a latter-day example of the application of meticulously controlled scientific trials to insightful previous hypotheses and observational studies. These controlled trials led the FDA to mandate folic acid fortification of the diet. Although folic acid is not the natural form of the vitamin as it exists in food, and evidence suggests that concentrations of unmetabolized folic acid in the blood after the ingestion of supplements or fortified foods may have different effects on folate metabolism, folic acid has served wonderfully in preventing and treating folate deficiency and for much of the study of folate biology. However, folic acid is not the natural form of the vitamin as it exists in food, and evidence suggests that concentrations of unmetabolized folic acid in the blood after the ingestion of supplements or fortified foods may have different effects on folate binding proteins and transporters (9). The tolerable upper level of folic acid intake, set forth recently by the Institute of Medicine in the Dietary Reference Intakes (10), warrants continuing consideration, especially the publication’s emphasis on the possible before conception, I will not now criticize a process that was imperfect in the sense that it lacked an empirical and science-based plan for monitoring and evaluating the effectiveness and safety of this large-scale intervention. Despite a great deal of effective, although at times discordant, interaction between the 3 sections of the Department of Health and Human Services (FDA, Centers for Disease Control and Prevention, and National Institutes of Health), there was, at the time the decision was made to mandate the addition of folic acid to enriched flour, no coherent plan to monitor the national and regional effect on neural tube defect births, to document actual changes in folate status, or to assess the possible occurrence of untoward effects on the population. Although evaluations of the effect of folic acid fortification on folate status, homocysteine concentrations, and neural tube births were conducted, the relative responsibilities of the 3 federal agencies for the evaluation of this major public health intervention have not been clear. In particular, none of these agencies was assigned the responsibility of monitoring the safety of this intervention. Yet, despite initial positive effects of the folic acid fortification of flour and grain on folate status and on the prevention of neural tube defects, the possibility remains that certain segments of the exposed population may benefit less and may even experience some adverse effects from an increase in folic acid intakes, which have turned out to be even greater than originally modeled in premendatory predictions.

The synthesis of folic acid by Lederle Labs in 1947 was one of the milestones achieved during the era of discovery of vitamins in the first half of the 20th century. This stable and unreduced form of folate has served wonderfully in preventing and treating folate deficiency and for much of the study of folate biology. However, folic acid is not the natural form of the vitamin as it exists in food, and evidence suggests that concentrations of unmetabolized folic acid in the blood after the ingestion of supplements or fortified foods may have different effects on folate binding proteins and transporters (9). The tolerable upper level of folic acid intake, set forth recently by the Institute of Medicine in the Dietary Reference Intakes (10), warrants continuing consideration, especially the publication’s emphasis on the possible
The adverse effects of high folic acid intakes in persons with a poor vitamin B-12 status. Questions raised by these tolerable upper levels emphasize the importance of establishing protocols for evaluating the safety and effectiveness of folic acid fortification interventions with the use of the best scientific information available.

The World Health Organization recently held a consultation in an effort to lay out the kind of decision-making process that many countries will undergo as they consider the fortification of food products with micronutrients. Policy decision making in any country considering fortification with one or several micronutrients should not be limited to the documentation of the indications for fortification, the food or foods used to deliver the nutrients, the expected bioavailability of the nutrient, or even the dose of the fortificant. A plan for evaluating the effectiveness and safety of the intervention is also needed. Although such an evaluation may be costly, the target population of a nationwide or global intervention deserves to be informed about its effectiveness with regard to health promotion, disease prevention, and safety. Nutrition scientists have a major role to play in designing these evaluative protocols. The agencies responsible for national or international implementation of food fortification programs must be ready to accept the responsibility and to provide the funds for these evaluations.

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Is docosahexaenoic acid, an n−3 long-chain polyunsaturated fatty acid, required for development of normal brain function?
An overview of evidence from cognitive and behavioral tests in humans and animals

Joyce C McCann and Bruce N Ames

ABSTRACT
This review is part of a series intended for nonspecialists that will summarize evidence relevant to the question of whether causal relations exist between micronutrient deficiencies and brain function. Here, we focus on experiments that used cognitive or behavioral tests as outcome measures in experimental designs that were known to or were likely to result in altered brain concentrations of the n−3 fatty acid docosahexaenoic acid (DHA) during the perinatal period of “brain growth spurt.” Experimental designs reviewed include observational breastfeeding studies and randomized controlled trials in humans and studies in rodents and nonhuman primates. This review is based on a large number of expert reviews and commentaries and on some 50 recent studies in humans and animals that have not yet been included in published reviews. Expert opinion regarding the strengths and weaknesses of the major experimental systems and uncertainties associated with interpreting results is summarized. On the basis of our reading of this literature, we conclude that evidence from several types of studies, particularly studies in animals, suggests that, within the context of specific experimental designs, changes in brain concentrations of DHA are positively associated with changes in cognitive or behavioral performance. Additional experimental information required to conclude that a causal association exists is discussed, as are uncertainties associated with applying results from specific experimental designs to the question of whether infant formula should be supplemented with DHA. Am J Clin Nutr 2005;82:281–95.

KEY WORDS Long-chain polyunsaturated fatty acids, LCPUFAs, docosahexaenoic acid, DHA, linolenic acid, cognition, learning, memory, perinatal period, breastfeeding, formula feeding, essential fatty acids, n−3 fatty acids, brain, neurology, infants, childhood, rodent studies, nonhuman primate studies

INTRODUCTION
During the last trimester of fetal life and the first 2 y of childhood, the brain undergoes a period of rapid growth termed the “brain growth spurt” (1). A large and dispersed literature suggests that an inadequate supply of any of a number of essential micronutrients during this period, as well as throughout life, can compromise brain function (2−9). In addition to the enormous literature examining the effects of individual micronutrients, some studies have examined the effects of multivitamin and mineral supplementation on cognitive function (10, 11).

If a causal relation between micronutrient deficiencies and suboptimal brain function exists, it has major public health implications. Large segments of the world population (including the United States), particularly the poor, are known to be undernourished in a number of micronutrients (12−19). A major effort to address micronutrient undernutrition, as an adjunct to the various programs underway to improve dietary habits, particularly among the poor, will be well justified. One of us has discussed such an approach as a relatively inexpensive and efficacious adjunct to current public health programs (20, 21). This review is the first of a series intended to provide the nonspecialist with critical summaries of the available experimental evidence pertinent to a discussion of this important public health question (20, 21).

The goal of this review is to provide an overview of human and animal in vivo experiments that link the availability during development of the long-chain polyunsaturated fatty acids (LCPUFAs), particularly the n−3 LCPUFA docosahexaenoic acid (DHA, 22:6n−3), to performance on cognitive or behavioral tests. Primary resources were recent research reports not yet reviewed, key earlier studies, and a large number of expert reviews and commentaries. We searched the literature by using a combination of techniques including key word and author
formulas that are either unsupplemented or supplemented with controlled trials (RCTs) in humans compare groups fed infant formulas that are either breastfed for different periods of time. Most randomized controlled trials (RCTs) in humans, particularly those in very-low-birth-weight (VLBW) and preterm infants, suggest that greater visual acuity in infancy is associated with increased intake of LCPUFAs (66–68), and these results are supported by several meta-analyses (69–71). The conclusions of systematic reviews differ somewhat, however, as to the overall strength of evidence for a positive effect of LCPUFA-supplemented infant formula on visual acuity (30, 69–74). Numerous publications provide additional review (4, 30, 57, 75–80). Effects of DHA availability during development on visual acuity are not a focus of this review but are discussed as a potential confounder of results in some cognitive and behavioral tests.

DHA is not widely distributed in the diet but is present in some foods, particularly fatty fish (30, 81–84). DHA can also be synthesized in the body from the essential fatty acid (EFA) α-linolenic acid (ALA). Although infants are able to convert ALA to DHA (85–88), DHA is known to be supplied directly to the fetus through the placenta and to the postnatal infant via breast milk (89–93). Uncertainty as to whether endogenous synthesis by the infant is sufficient to supply enough DHA during the postnatal phase of perinatal growth has stimulated a great deal of interest in whether infant formula should be supplemented with LCPUFAs. This general issue has been discussed in many reviews and commentaries (4, 30, 54, 79, 94–100). We do not take up this issue directly in this review, but we do comment on the relevance to this important question of the results from the experimental systems we reviewed.

Basic experimental designs

Three types of protocols were most commonly used in the studies reviewed. Each of these protocols differently manipulates the dietary supply of EFAs or LCPUFAs during development, and all result, or are likely to result, in different brain concentrations of DHA in comparison groups, which makes them relevant to a discussion of whether DHA availability in the brain is linked to cognitive or behavioral function.

Most observational breastfeeding studies in humans compare children who were either breastfed or formula-fed or those who were breastfed for different periods of time. Most randomized controlled trials (RCTs) in humans compare groups fed infant formulas that are either unsupplemented or supplemented with LCPUFAs. Most studies in animals compare offspring whose supply of ALA, the precursor of DHA, has been severely limited for different periods of time.

In most rodent ALA-restriction studies, brain concentrations of DHA are dramatically reduced in test animals, by as much as 85% (101), and decreases of 75% in nonhuman primates have been reported (64). It is important, however, that some studies in rodents also used dietary conditions that resulted in smaller decreases in brain DHA (40, 102–105). Human autopsy studies reported significant differences of ≈11% to 40% in DHA concentrations in brain gray matter between breastfed and unsupplemented formula-fed infants (106–110); two further references provide review (4, 111). Direct autopsy evidence that compares brain DHA concentrations in human infants fed unsupplemented and LCPUFA-supplemented formulas is not available. However, a recent autopsy study in nonhuman primates reported ≈30% lower concentrations of DHA in the visual cortex of preterm infants fed unsupplemented formula than in those fed LCPUFA-supplemented formula (112). In humans, significant differences in plasma concentrations of DHA between unsupplemented and supplemented formula comparison groups are well documented (113, 114).

It is important to note that, in all of these common experimental designs that affect DHA status, DHA is not the only variable. Even in RCTs, formulas typically are supplemented with other LCPUFAs, particularly AA, in addition to DHA. Thus, to different degrees, these designs have an inherent lack of specificity that is important to take into account in evaluating the significance of results relative specifically to DHA.

Performance tests

Many different kinds of tests have been used in human and animal studies. In this review, we focus primarily on tests aimed at assessing cognition or related mental functions, but a few tests assessing other correlates of functional development of the nervous system, such as neuromotor activity (115, 116) and sleep patterns (117), are also included. Several reviews address this important topic (4, 48, 57, 118–127). Tests in human studies include standardized global tests that screen broadly for cognitive-related functions, such as the Bayley Scales of Infant Development (128) and the Kaufman Assessment Battery for Children (129), and tests that target more specific functions, such as the development of language and communication skills [eg, the MacArthur Communicative Development Inventory (130)], visual recognition [eg, the Fagan Test of Infant Intelligence (131)], or problem solving (132, 133). As previously reviewed (57, 75, 118), performance tests in nonhuman primate studies have included assessments of behavior patterns (134, 135) and some tests that have also been used in human studies, most notably “look duration” tests of visual attention (136). Commonly used in rodent studies are tests that measure aspects of spatial learning such as the radial maze (137, 138) and Morris water maze (139–141), the elevated plus maze (a test that measures anxiety; 142, 143), and tests that measure stimulus-response type learning, such as brightness discrimination tests (144, 145). Several reviews provide further discussion of performance tests used in rodent studies (119, 144, 146, 147).

The fact that different tests measure different aspects of cognition or behavior must be taken into account in the interpretation and comparison of results across different tests. For example, in humans, it has been suggested that different results might be
expected in global tests (such as the Bayley Scales of Infant Development) and in tests targeted to more specific neural domains (such as look duration tests), which may not equally measure specific neural functions that may be affected by LCPUFA availability (57, 124). In rodent tests, it has been suggested that a version of the Morris water maze that measures working memory may be more sensitive in detecting performance deficits in n−3-restricted animals than is the more commonly used “place” version of the test (148). Evaluation of results in performance tests must also consider potential confounders. For example, most of the tests listed above rely on vision or motor activity, and thus possible effects of DHA on visual or motor development could theoretically influence the outcome.

HUMAN STUDIES

Breastfeeding studies

Information provided by observational breastfeeding studies, although relevant to the question of causality, is limited. As has frequently been noted (4, 80, 91, 126, 127, 149–153), the decision to breastfeed is associated with a number of factors that potentially confound positive results in these studies, such as socioeconomic status, home environment, and maternal intelligence quotient (IQ). Moreover, experimental designs involving breastfeeding are inherently limited in their ability to identify which among a number of potentially active ingredients in breast milk might be responsible for enhancement effects (4, 91, 127, 154, 155).

Five systematic reviews published since 1999 critically evaluated partially overlapping subsets of breastfeeding studies spanning >20 y (4, 152, 153, 156, 157). Most of the studies included in these reviews compared the performance of children who were breastfed or formula-fed. Before adjustment for covariables, most of these studies reported higher scores on performance tests for children who were breastfed. Reviewers were in agreement that a significant number of potentially confounding variables complicated the analysis and interpretation of these results. However, they agreed less as to whether there was some residual enhancement in test performance after adjustment for covariables. For example, one can compare the conclusions of the studies by Anderson et al (156) and Drane and Logemann (157) and of several critical commentaries (149, 150, 158).

Areas that reviewers suggested for future research included improvement in the overall quality of studies; comprehensive confounder analysis (153, 157); examination of longer durations of breastfeeding, with duration of breastfeeding as a dose-response variable (157); and more studies comparing breast milk–fed and formula-fed premature infants (152).

Breastfeeding studies published in the past 5 y (since 1999), along with formula-feeding studies, are listed in Table 1. The table includes an indication of the type of experimental design, the performance tests used, and conclusions of the investigators. Reviewers that have cited these studies are also indicated in the table. The range of experimental approaches is greater in these newer studies than in those reviewed earlier, which is consistent with suggestions of earlier reviewers. Thus, whereas several of these studies compared breastfed and formula-fed children (160–167), 12 studies compared the performance of children who were breastfed for different periods of time (159, 168–178).

The great majority of these studies reinforce conclusions of earlier work. Most studies that compared breastfed and formula-fed groups and those that examined groups that were breastfed for different periods of time observed a weak association between scores on performance tests and longer periods of breastfeeding, even after adjustment for at least some potential confounders. As also indicated in Table 1, positive results were reported across a wide range of different performance tests.

It is important to note, however, that these studies do not adjust for all possible confounders. As an example, one of the strongest potential confounders logically is—and has been shown experimentally to be—maternal IQ. As discussed previously (150), in the 1999 Anderson et al (156) meta-analysis of earlier studies, only 6 of the 20 breastfeeding studies cited included maternal IQ as a potential confounder. Among the more recent observational breastfeeding studies listed in Table 1, only 8 included maternal IQ as a potential confounder (159, 160, 169, 174, 177–180). Among those 8 studies, only 2 (159, 177) reported that better test performance by breastfed youngsters remained significantly different after adjustment for multiple confounders. However, a commentary on 1 of these 2 positive studies, that of Rao et al (177), suggested that additional confounders still might account for the residual positive results (149). Specifically, the commentators suggested the inclusion of a verbal measure of maternal IQ (ie, the Peabody Picture Vocabulary Test) and of a measure of parenting quality (such as the Home Observation Measurement of Environment). These measures were significant confounders in a study reported by the commentators (160) that lost statistical significance with adjustment (Table 1). It may be of interest that only one other study (178) among those discussed here used a verbal measure of maternal IQ [all other studies used the Raven’s Progressive Matrices (203, 204)]. That study also reported, as discussed above, that adjustment almost completely removed significance (178).

Randomized controlled trials

Studies comparing children fed differently supplemented formulas

Randomized controlled trials (RCTs) offer much greater opportunity than do observational studies for the control of experimental variables, including the quantity and composition of LCPUFA supplements. In addition, this design affords the opportunity to avoid many of the potential confounders that complicate the interpretation of observational breastfeeding studies. RCTs, however, are also subject to confounding and to other sources of uncertainty that can complicate the comparison of results in different trials, such as the use of different formulas or oils or the presence of other sources of variability in the DHA status of infant groups. A general review is available in numerous publications (4, 48, 54, 77, 122, 151, 154, 181, 182).

Results of RCTs have been discussed by many investigators and reviewers. We focused on several systematic reviews and meta-analyses involving term infants (69, 71, 74, 122), preterm infants (70, 73), or both (4, 72, 78–80, 182, 183). Although some of these reviews focused only on visual acuity, the most commonly measured outcome in human RCTs, most reviews also critically examined cognitive or behavioral outcomes (4, 72–74, 78–80, 182, 183). Conclusions varied somewhat among these reviewers as to the strength of the evidence for effects of
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<td>Agostoni et al, 2001 (168)</td>
<td>BF duration, 3–12 mo [44]</td>
<td>A (1 y)</td>
<td>Positive trend, adjusted (PDI, ( P = 0.07 ))</td>
<td>150</td>
</tr>
<tr>
<td>Angelsen et al, 2001 (169)</td>
<td>BF duration, &lt;3 to ( \geq ) 6 mo [345]</td>
<td>A (13 mo); B, C (5 y)</td>
<td>Positive, partial adjusted [A (MDI), B]</td>
<td>127, 150</td>
</tr>
<tr>
<td>Auestad et al, 2001 (162)</td>
<td>RCT [239]; BFR [165]</td>
<td>R (2, 4, 6, 12 mo); O (6, 9 mo); A (6, 12 mo); EE (9, 14 mo)</td>
<td>Negative</td>
<td>52, 69, 80</td>
</tr>
<tr>
<td>Bakker et al, 2003 (179); Ghys et al, 2002 (180)</td>
<td>Blood LCPUFA concentrations (umbilical at birth and at 4 or 7 y)</td>
<td>M (4 y) [128]; L (7 y) [306]</td>
<td>Negative, adjusted</td>
<td></td>
</tr>
<tr>
<td>Birch et al, 2000 (189)</td>
<td>RCT [56]</td>
<td>A (18 mo)</td>
<td>Positive</td>
<td>150</td>
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<tr>
<td>Birch et al, 2002 (190)</td>
<td>RCT [65]</td>
<td>BB (17, 26, 39, 52 wk); VEP (6, 17, 26, 52 wk)</td>
<td>Positive (17 wk) (BB); positive (17, 26, 52 wk) (VEP)</td>
<td>73</td>
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<tr>
<td>Bouwstra et al, 2003 (164)</td>
<td>RCT [250]; BFR [147]</td>
<td>D (3 mo)</td>
<td>Positive, adjusted</td>
<td>69</td>
</tr>
<tr>
<td>Makrides et al, 2000 (195)</td>
<td>BF duration, ( \leq ) 4 mo or &gt;4 mo [249]</td>
<td>A (18 mo)</td>
<td>Positive, adjusted (MDI)</td>
<td>4, 72, 78–80, 119, 124, 181–183</td>
</tr>
<tr>
<td>Hoffman et al, 2003 (193)</td>
<td>RCT [61]</td>
<td>BB (4, 6, 9, 12 mo); VEP (4, 6, 12 mo)</td>
<td>Negative (BB); positive (12 mo) (VEP)</td>
<td>69</td>
</tr>
<tr>
<td>Horwood et al, 2001 (176)</td>
<td>BF duration, 0 to &gt;8 mo [280 VLBW]</td>
<td>H (7–8 y)</td>
<td>Positive, adjusted</td>
<td>150</td>
</tr>
<tr>
<td>Innis et al, 2001 (188)</td>
<td>Blood LCPUFA concentrations [83]</td>
<td>A (6, 12 mo); O (6, 9 mo); P, Q (9 mo); R (2, 4, 6, 12 mo)</td>
<td>Positive, adjusted (P, R)</td>
<td>52</td>
</tr>
<tr>
<td>Makrides et al, 2000 (195)</td>
<td>RCT [58]; BFR [85]</td>
<td>VEP (16, 34 wk)</td>
<td>Negative</td>
<td>4, 78, 181</td>
</tr>
<tr>
<td>Morley et al, 2004 (161)</td>
<td>BF [175] versus FF [147]</td>
<td>A (18 mo); U (9 mo)</td>
<td>Positive, adjusted (A)</td>
<td>72, 73, 80, 182, 194</td>
</tr>
<tr>
<td>O’Connor et al, 2001 (196)</td>
<td>RCT [470 PT]; BFR [43]</td>
<td>O (6, 9 mo); A (12 mo); EE (14 mo); R (2, 4, 6 mo); VEP (6 mo)</td>
<td>Positive [VEP, EE, O (6 mo), A (PDI)]</td>
<td>72, 73, 80, 182, 194</td>
</tr>
<tr>
<td>Oddy et al, 2003 (172)</td>
<td>BF duration, 0 to &gt;6 mo [2393]</td>
<td>G (6 y) [1450]; H (8 y) [1375]</td>
<td>Positive, adjusted (G)</td>
<td></td>
</tr>
<tr>
<td>Quinn et al, 2001 (173)</td>
<td>BF duration, 0 to 6 mo [3880]</td>
<td>G (5 y)</td>
<td>Positive, adjusted</td>
<td></td>
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<tr>
<td>Rao et al, 2002 (177)</td>
<td>BF duration, SGA [220]; AGA [299]</td>
<td>A (13 mo); B (5 y)</td>
<td>Positive, adjusted (B)</td>
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TABLE 1 (continued)

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<tr>
<td>Richards et al, 2002 (175)</td>
<td>BF duration, 0 to &gt;7 mo [1739]&lt;sup&gt;16&lt;/sup&gt;</td>
<td>J (15 y); HH (26 y); K (53 y)</td>
<td>Positive, adjusted (J)&lt;sup&gt;19&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Smith et al, 2003 (178)</td>
<td>BF/HMF duration, &lt;1 wk to &gt;6 mo [439 VLBW]</td>
<td>L, G, V, W, X (6–8 y)</td>
<td>Positive, adjusted (X, L)&lt;sup&gt;20&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Williams et al, 2001 (197)</td>
<td>BF duration, 0 to ≥4 mo; MD [435]</td>
<td>BB (3.5 y)</td>
<td>Positive, adjusted</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> n is given as numbers in brackets. AGA, appropriate for gestational age; ALA, α-linolenic acid; BF, breastfed; BFR, breastfed reference group included in an RCT; FF, formula fed; HMF, human milk fed; IQ, intelligence quotient; MD, maternal diet comparison; MDI, mental development index; MS, maternal LCPUFA supplementation; PDI, psychomotor development index; PT, preterm infants; RCT, randomized controlled trial; SGA, small for gestational age; VEP, visual evoked potential; VLBW, very low birth weight.

<sup>2</sup> A, Bayley Mental and Psychomotor Development Index; B, Wechsler Preschool & Primary Scales of Intelligence (WPPSI-R); C, Peabody Developmental Motor Scales; D, general movements; E, Wechsler Adult Intelligence Scale (WAIS); F, Borge Priens Prøve (BPP); G, Peabody Picture Vocabulary Test (PPVT-R); H, Wechsler Intelligence Test for Children–Revised (WISC-R); I, Griffiths Scales of Infant Development; J, Group Ability Test AH4 (verbal and nonverbal intelligence); Watts-Vernon Reading Test, and mathematics test; K, National Adult Reading Test (NART); L, Kaufman Assessment Battery for Children (K-ABC); M, Groningen Developmental Scale (adaptation of the K-ABC); N, McCarthy Scales of Children’s Mental Abilities; O, Fagan Test of Infant Intelligence (novelty preference); P, conditioned head-turn procedure (speech perception); Q, object search task; R, Teller Acuity Card Procedure (visual acuity); S, Wide Range Achievement Test–Revised (spelling and arithmetic subtests); T, Woodcock Reading Mastery Test-Revised (word and passage comprehension subtests); U, Knobloch, Pasamanick, and Sherrard’s Developmental Screening Inventory; V, Clinical Evaluation of Language Fundamentals–Third Edition; W, California Children’s Verbal Learning Test; X, Wide Range Assessment of Visual Motor Abilities (WRAVMA); Z, Stanford-Binet Intelligence Scale Form L–M; AA, Beery Visual-Motor Index Test; BB, Preferential looking test for stereocoria; CC, Clinical Adaptive Test/Clinical Linguistic & Auditory Milestone Scale (CAT/CLAMS); DD, Gross Motor Scale of the Revised Gesell Developmental Inventory; EE, MacArthur Communicative Development Inventories; FF, look duration tests aimed at measuring the development of attention; GG, sleep patterns; HH, highest education or training level.

<sup>3</sup> Confounders evaluated individually, but not combined in the “adjusted scores.” Total population was of relatively high socioeconomic status.

<sup>4</sup> Follow-up of children participating in a 1997/1998 study (198, 199) in which children fed the docosahexaenoic-acid-only–supplemented formula scored lower than did other groups on some tests.

<sup>5</sup> Follow-up of an earlier study (200).

<sup>6</sup> Same infants as in Bouwstra et al (164, 170).

<sup>7</sup> IQs of parents evaluated for 164 infants.

<sup>8</sup> Mental Processing Composite of the K-ABC.

<sup>9</sup> Significantly higher IQ scores at both ages after adjusting for social class and maternal education, but differences were no longer significant after adjustment for maternal IQ and parenting skills.

<sup>10</sup> A subsequent study (201) combined data from this and another study (166, 195) to examine a number of perinatal and nutritional variables in relation to the VEP results.

<sup>11</sup> Comparison of formulas supplemented with different concentrations of ALA.

<sup>12</sup> The breastfed group was SGA; the formula-fed groups were term infants. The study also included a group fed a protein and vitamin–enriched formula.

<sup>13</sup> Duration of breastfeeding was determined by interview with mothers; 2 independent groups were assessed: 2280 men and 973 men and women.

<sup>14</sup> Results for a human milk–fed group were not included in the statistical analysis.

<sup>15</sup> A subsequent study (202) analyzed these data further. PDI results were for infants in the ≤1250 g birth weight subgroup.

<sup>16</sup> Prospective study.

<sup>17</sup> Maternal IQ was evaluated but not included in confounder analysis. The primary goal of the study was to examine effects of various concentrations of organochlorine compounds in cord serum.

As shown in Table 1, only 3 of the RCTs listed that compared LCPUFA-supplemented and -unsupplemented formula-fed children were not discussed in previous reviews. Auestad et al (163), in a 39-mo follow-up study of term infants, did not observe a significant difference in performance on several mental and motor development tests (or in visual acuity). Bouwstra et al (164), also in a study of term infants, reported positive effects (P < 0.05) of LCPUFA supplementation at 3 mo of age by using a test that assessed the quality of general movements considered to be an indicator of brain function. And Fewtrell et al (184) reported weakly significant (P = 0.04) enhancement effects in 18-mo-old children fed fish oil–supplemented formula as preterm infants in the Mental Development Index component of the Bayley Scales.
Other experimental designs

The study of Colombo et al (185) listed in Table 1 adds to the series of positive results obtained in look duration tests. This study reported a positive correlation between maternal erythrocyte phospholipid DHA at birth and performance in look duration tests over the first 18 mo of life. Other studies listed in Table 1 that were not previously discussed by reviewers include a negative study that examined possible correlations between performance in several mental development tests at 4 and 7 y of age and LCPUFA blood concentrations at birth or at the time of testing (179, 180), a study reporting a positive correlation between maternal phospholipid DHA concentrations and the maturity of sleep patterning in infants (186), and a study, some results of which were significant, that compared performance in several mental and motor development tests of ≈1-y-old term infants fed infant formulas differing in ALA content (187).

STUDIES IN ANIMALS

The great strength of animal studies is that they afford the opportunity for more flexibility in design and in the ability to control experimental variables than can be achieved in human studies. The most commonly used experimental design in animals involves limiting the dietary supply of ALA, the precursor of DHA, which is not feasible in human studies because ALA is an EFA. Previous reviewers of animal studies (49, 57, 119, 146, 147) concluded that reproducible effects of dietary restriction of ALA on look duration in nonhuman primates suggested possible involvement of DHA in cortical pathways associated with visual attention (57) and also concluded that effects on cognition in rodent studies were shown in tests that involved sensory pathways other than the visual (31, 49, 209-211).

Most animal studies have been conducted by using rodents. Although a detailed comprehensive methodologic review in 1992 (146) discussed early rodent studies, and more recent reviews have critically discussed some subsequent work (49, 57, 119, 147), there is now quite a large database of rodent behavioral studies that have not been critically reviewed. More than 30 rodent studies (102–105, 148, 212–238) have been published since the 1992 comprehensive methodologic review of Wainwright (146). More than 20 of these reports were published since 1999 (Table 2). (Investigations that focused solely on visual function are not included here.) The results reported in these studies are briefly summarized below.

First, almost all of the 20 rodent studies listed in Table 2 reported significant deficits in test performance by subjects compared with controls on at least some tasks in at least some n–3–restricted groups. These results support earlier studies, previously reviewed (49, 57, 119, 146, 147), that suggested an association between a diet severely restricted in n–3 FAs during development and poorer performance of offspring in tests designed to measure cognitive or behavioral ability. This important basic conclusion is tempered by the fact that performance differences are typically not large, are observed on some tasks and not others, often are not observed across multiple test sessions, and have not been observed in all reports (further discussed below).

Second, performance deficits were reported in a variety of tests, which suggests that the effect on performance is not test-specific. For example, among studies listed in Table 2, tests for which effects were reported included the Morris water maze (105, 212, 215-217, 219, 220, 232), 8-arm radial maze (229, 230), elevated plus maze (220, 234), active avoidance (227, 235), olfactory-cued (214, 216), and brightness discrimination (224, 227) tests.

Third, 8 of the studies in Table 2 supplemented n–3–restricted animals with DHA, DHA+AA, DHA-rich oils, or DHA and additional n–6 FAs and compared the animals’ performance with that of n–3–restricted controls (40, 212, 217, 224, 229, 230, 234, 235). All of these studies reported that performance was significantly enhanced in the supplemented groups.

Fourth, all but 5 studies (218, 219, 233-235) listed in Table 2 also measured brain concentrations of LCPUFAs. In almost all cases, brain DHA concentrations were significantly lower in n–3–restricted groups than in controls, and several investigations pointed to the importance of AA as well (224, 227, 228). In addition to conducting performance tests and determining brain concentrations of LCPUFAs, several studies recorded changes in biochemical indicators of brain function in n–3–restricted and control groups (226, 229, 235).

Fifth, limited evidence of a dose-response effect is reported in 2 studies (215, 240). We also note several reports in which performance deficits were observed in some groups by using dietary protocols that resulted in significantly less reduction in brain DHA than is seen in most multigeneration ALA-restriction studies (38, 40, 105, 148).

Factors involved in assessing and interpreting studies in rodents

An in-depth methodologic review of all of the studies discussed above is beyond the scope of this review. We note, however, that a thorough critical evaluation of results should take into account a number of methodologic and other issues, many of which have been discussed by previous reviewers (49, 51, 52, 55, 57, 118, 119, 146, 147, 209, 241, 242). Several of these issues are briefly summarized below.

Potential confounders not usually evaluated cannot be excluded as alternative explanations for results in many experiments, as is discussed in several reviews (4, 57, 119, 243, 244). Most tests measure a combination of performance and behavioral characteristics in addition to cognition per se; Wainwright (119) and Wainwright et al (148) provide further discussion of this topic. For example, in addition to learning ability, commonly used water and radial maze tests involve locomotor ability and visual recognition of cues, and brightness discrimination tests require visual ability. As discussed above, chronic dietary restriction of ALA adversely affects the development of visual function, which must be considered to potentially influence the results of these tests. Other potential confounders are long-term severe imbalance in the ratio of n–6 to n–3 FAs or other effects of chronic ALA restriction. In addition to its role as precursor of DHA, ALA is an important source of energy, is a precursor for the synthesis of saturated and monounsaturated FAs (51, 245)—that comprise some 75% (by wt) of the FAs present in brain tissue (4), including myelin (246)—and is believed to be required for the development of an adequate arterial supply (245, 247). In addition, the very high n–6:n–3 used to generate n–3–deficient groups in the rat experiments may result in an unnaturally high level of inflammatory activity (248, 249), which could affect the general health or vigor of the animals and thus influence their ability to perform in cognitive and behavioral tests.
n–3 FATTY ACIDS AND COGNITION

Statistical considerations not taken into account can affect conclusions about the positivity (or negativity) of experiments, as well as the interpretation of positive results. Wainwright (250) has discussed several design and analysis issues with respect to multigenerational studies in multiparious species, and she emphasizes that weak or marginal results must be viewed with caution. With reference to the studies included in this review, we point particularly to 3 important statistical considerations. First, as Wainwright discussed (250), the inflation of sample size by using the number of pups instead of the number of litters as the unit of measure results in an increased potential for false positives unless appropriate cluster design adjustments are made. Second, in correlational analyses, if group means for the measures being correlated are significantly different, a simple correlation across groups does not necessarily indicate a correlation for individual animals. Third, because “learning” implies a change over time, it is inappropriate to conclude that there is a learning difference between compared groups unless the statistical analysis indicates a group × time interaction.

As discussed by many reviewers (4, 119, 147), test results obtained in experiments involving severe depletion of brain DHA have uncertain relevance to effects that might be expected under less severe conditions. In nonhuman primates (64) and piglets (38), DHA concentrations in the brain are dramatically reduced after the maternal dietary supply of ALA is limited during one gestational period. In rodents, a longer period of chronic ALA restriction is required to achieve a comparable reduction because of the more efficient biosynthesis of DHA from ALA in rodents (251). Thus, most rodent studies have been conducted with animals whose mothers or even mothers and grandmothers were raised on diets limited in ALA. In animals whose supply of DHA has been limited in this way, during the perinatal period (when rapid accumulation of

### TABLE 2

Animal studies (1999–2004) that tested effects of diets differing in n–3 fatty acid content during development on behavior

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<td>Carrie et al (217, 219, 220); Frances et al (218)</td>
<td>Swiss OF-1 F1 mice (most studies); 3 basic designs: 1) comparison of n–3–deficient animals (ratio of n–6 to n–3 ≥1200 compared with 6) (218, 220); 2) supplementation protocol (219); and 3) comparison of n–3–deficient animals supplemented with DHA at different ages (217)</td>
<td>Reward test aimed at determining the interest level of animals in responding to a pleasurable stimulus (218); Morris water maze (217, 219, 220); elevated plus maze test (220)</td>
</tr>
<tr>
<td>Gamoh et al (229, 230)</td>
<td>Wistar F2 rats; fish oil–deficient diets and then DHA administered intragastrically 5 wk before testing</td>
<td>8-Arm radial maze test at 10 wk (230) or 100 wk (229) of age</td>
</tr>
<tr>
<td>Ikemoto et al (224)</td>
<td>Donryu F1 rats; semipurified diets supplemented with safflower or perilla oil</td>
<td>Brightness discrimination learning test at 11 wk of age</td>
</tr>
<tr>
<td>Levant et al (40)</td>
<td>Long-Evans F1 rats; n–3–deficient diets from conception to weaning</td>
<td>Tests for haloperidol-induced catalepsy, locomotor activity, and withdrawal latency to a thermal stimulus at ≈2 mo</td>
</tr>
<tr>
<td>Moriguchi et al (212, 215); Greiner et al (214, 216); Catalan et al (213)</td>
<td>Long-Evans F1 or F2 rats; n–6:n–3, ≈350 and 5</td>
<td>Morris water maze or an olfactory-cued discrimination test conducted in animals ranging in age in various studies from 8 to 13 wk</td>
</tr>
<tr>
<td>Takeuchi et al (234, 235)</td>
<td>F1 Wistar rats; diets lacking n–3 FAs or with n–6: n–3 of 0.39; at 3 wk of age, DHA was administered to one group</td>
<td>Active avoidance and 3-panel runway test (235) or plus maze test (234) at 8 wk of age</td>
</tr>
<tr>
<td>Umezawa et al (227)</td>
<td>Senescence-resistant F1 mouse strains; supplementation with safflower or perilla oil</td>
<td>Active avoidance (Sidman) and brightness discrimination learning tests at 15 mo of age</td>
</tr>
<tr>
<td>Wainwright et al (105, 231, 233); Clements et al (232)</td>
<td>Long-Evans rat pups artificially reared by gastrotomy with rat milk substitutes variously supplemented with DHA or AA or both (105); F1 B6D2F1 mice; DHA in the brain manipulated by changing the concentration of y-linolenic acid (an n–6 FA) in the diet (231); adequate diets of two rat strains—spontaneously hypertensive rats and their progenitor strain (Wistar-Kyoto)—were supplemented with AA and DHA beginning at weaning (232); F1 B6D2F1 mice; diet with a very low n–6:n–3 (n–6 was linoleic acid; n–3 was DHA) (233)</td>
<td>Morris water maze (105); behavioral development tested at 12 d of age, and adults tested in elevated plus maze (231); a version of the Morris water maze at 8 wk of age (232); a behavioral test battery at 32 d of age (233)</td>
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<td><strong>Piglets</strong></td>
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<td>Ng and Innis (38)</td>
<td>Piglets fed low-PUFA or high-PUFA diets (+DHA and AA) starting at 1 d of age</td>
<td>Elevated plus maze at 18–22 d of age</td>
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<tr>
<td><strong>Monkeys</strong></td>
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<tr>
<td>Champoux et al (239)</td>
<td>Rhesus macaque infants were fed standard formula or standard formula supplemented with DHA and AA</td>
<td>Neurobehavioral assessments at 7, 14, 21, and 30 d of age</td>
</tr>
</tbody>
</table>

1 F1, first generation offspring of treated F0 females; F2, second-generation offspring; DHA, docosahexaenoic acid; FAs, fatty acids; AA, arachidonic acid; PUFA, polyunsaturated fatty acid.
brain DHA would normally occur), the place of DHA is taken primarily by another FA, docosapentaenoic acid (DPA; 22:5n–6), which differs from DHA in just one double bond—the n−3 double bond (101, 252).

**Performance of n−3–restricted rats and mice in the Morris water maze**

The Morris water maze is the most commonly used learning and memory test in the rodent studies reviewed (102–105, 148, 212, 215–217, 219, 220, 223, 232, 236). Below, we briefly discuss the subset of these studies that included a determination of brain concentrations of DHA and compared an n−3–restricted group to “n−3–adequate” controls. It is noted that the 10 studies selected (103–105, 148, 212, 215–217, 220, 223) included additional outcome measures or other comparisons not discussed in this example. Some of these studies have been reviewed elsewhere (49, 119, 241).

In rats, one laboratory reported reproducibly better performance of controls than of severely n−3–restricted animals in the “place” version of the test (212, 215, 216). Using the same version of the test, a different laboratory reported a nonsignificant trend (P > 0.08) in one study (148) and a negative result for a “saturated fat” group in a larger study (105). The latter 2 studies also used the “delayed-matching-to-place” or “working memory” version of the test, and both reported significant positive results [a diet × trials interaction, P < 0.01 (148) or P < 0.05 in a planned comparison (105)].

In mice, a positive result (P < 0.05) was reported for a saturated fat group, but not for an n−3–restricted group in the same study (103). Positive results were also reported in 2 other studies (217, 223). However, in one of those studies (217), significance relied on a post hoc comparison of results in a single session conducted on a data set after a nonsignificant ANOVA, and, in the other study (223), significance was marginal (P = 0.044) and relied on the use of a one-tailed test. Two other mouse studies reported negative results (104, 220).

Although rats are known to perform better than mice in the water maze (253–255), it may be of interest that, among the studies discussed above, positive results were obtained in both species in the place version of the test when reductions in brain concentrations of DHA in the restricted group exceeded 80% (103, 212, 215, 216). Results were negative or marginal when brain reductions were less, ie, 25% (217), 41% (220), 50% (103), 60% (223), and 53% (104). It may also be of interest that positive results were obtained in rats with less severe depletion of brain DHA, ie, 38% (105) and 51% (148), in the delayed-matching-to-place version of the water maze, which, as discussed previously (148), may be more sensitive for detection of effects of n−3 restriction than is the place version.

**Docosahexaenoic acid supplementation studies**

Only a few studies in animals have examined effects of LCPUFA supplementation of normal diets during development on performance in cognitive or behavioral tests. Among the rodent studies listed in Table 2, only one (105) used a study design analogous to that of formula comparison studies in humans. That study did not find that supplementation with DHA or AA (or both) improved subsequent performance on 2 versions of the Morris water maze, despite the fact that feeding diets with normal concentrations of EFAs supplemented with different amounts of AA and DHA to artificially reared pups was found to result in a range of different concentrations of AA and DHA in forebrain phospholipids (256). One other study listed in Table 2 included dietary groups for which n−6:n−3 was within the range considered roughly normal (ie, ≈5–10) or enriched for n−3 FAs (219).

That study reported some changes in performance on the Morris water maze in animals fed the n−3–enriched diet but also reported adverse effects in older animals fed the n−3–enriched diet for extended periods. A study in rhesus macaque infants (239) reported that those fed LCPUFA-supplemented formula had stronger orienting and motor skills than did those fed standard formula.

**SUMMARY AND CONCLUSIONS**

Each of the 3 major experimental designs reviewed contributes some evidence that is relevant to a discussion of possible causal linkages between altered brain concentrations of DHA during the perinatal period and subsequent cognitive or behavioral performance. (As indicated above, effects of DHA availability during development on visual function are not a subject of this review.) Evidence from chronic dietary restriction rodent studies that is most relevant to the issue of causality shows that the addition of DHA to diets of animals whose brain concentrations of DHA have been severely reduced restored control performance levels (40, 212, 217, 224, 229, 230, 234, 235). As discussed in this review, the relevance of these results to effects that might be expected in humans under less severe conditions is uncertain. Formula comparison and maternal supplementation studies in humans and ALA dietary restriction studies in nonhuman primates both link the availability of n−3 LCPUFAs to the development of visual attention (136, 185, 206–208), although, as discussed, it is difficult to exclude as an alternative explanation for these results the possible confounding due to effects of DHA on visual function. We also point to formula supplementation (164, 239) or maternal plasma correlational (186) studies in humans or monkeys that suggest enhanced neuromotor development in infants with higher DHA status. Positive results in breastfeeding studies are seriously eroded by adjustment for multiple covariates, and residual positive effects cannot be attributed to LCPUFAs because of other potentially active constituents in breast milk. Nevertheless, it is of interest that these studies consistently showed a positive association between breastfeeding and performance across a wide range of different tests, even after adjustment for at least some potential confounders.

Clearly, the experiments most capable of providing definitive evidence that is directly relevant to human exposure conditions are RCTs in humans. As reviewed above, these trials have often not shown an effect of LCPUFA supplementation on cognitive or behavioral performance, and some reviewers have considered that, overall, the evidence was insufficient to conclude that LCPUFA supplementation benefited development (73, 74). Understanding why most RCTs have yielded mixed results, particularly in light of more consistently positive effects observed in rodent tests and human breastfeeding studies, is of obvious importance and has been discussed by a number of reviewers and investigators (4, 49, 77, 79, 122). The suggestion of previous reviewers (57, 124) that differences in the sensitivity of global tests, such as the Bayley Scales, and of tests targeted at more
specific neural domains, such as look duration or problem-solving tests, might account for mixed results in RCTs was discussed earlier in this review. Other possible explanations include inadequate supplementation of DHA in formulas (49, 257), poor study quality (77), the ability of term infants fed unsupplemented formulas to synthesize their own DHA (73), an absence of cognitive deficits when differences in brain concentrations of DHA are small due to brain plasticity (ie, the ability of the brain to adapt), or the inability of performance tests to detect subtle differences in performance that result from relatively small differences in brain concentrations of DHA (105, 210).

The relative merits of hypotheses that invoke brain plasticity or difficult-to-detect weak effects due to small decreases in brain DHA are important to follow up. The former hypothesis suggests that there may be no adverse consequences of relatively small reductions in brain DHA, whereas the latter suggests that relatively small reductions could result in subtle performance deficits that are difficult to detect. If experts can agree on a reliable marker, constructing a dose-response curve that relates the degree of reduction in brain DHA to effects on neural function would be of great value. It appears to us from our reading of the literature that cognitive and behavioral tests may not be sensitive or consistent enough to permit such an analysis, but some biochemical markers, such as dopamine inducibility (36, 38), may be useful.

Most of the explanations discussed above focus on the possibility that negative results in RCTs may be false-negative. Following some discussion of this point in the literature (146), we note that it is equally important to consider the possibility that positive results are false positives. In addition to the need to better identify confounders and methodologic sources of possible bias, as discussed, we note the value of independent replication to confirm positive results.

It is of interest to examine the degree to which evidence from the 3 major experimental systems reviewed satisfies conditions of causality. Causal criteria [slightly adapted from the original formulation (258)] require evidence of 1) a consistent association, 2) a plausible biological rationale, 3) an ability to experimentally manipulate the effect, 4) a dose-response relation, and 5) specificity of cause and effect. Whereas some of these 5 criteria are satisfied by some experimental results reviewed, others are not. First, as detailed above in this review, all 3 major experimental systems reviewed (ie, human breastfeeding studies, human formula comparison RCTs, and animal ALA dietary restriction studies) show consistent associations under certain conditions. Second, mechanistic studies are not discussed in this review, but plausible biological rationales have been suggested, although not proven; recent references and reviews are cited in the Introduction. Third, experiments in rodents that restored performance by supplementing severely ALA-restricted animals with DHA provided some evidence of ability to manipulate the effect. Fourth, as indicated above in this review, limited evidence suggesting a possible dose-response effect was provided in 2 rodent studies (215, 240) and in a study that correlated concentrations of DHA in the blood of breastfed infants with enhanced performance (188). The only studies reviewed that provided consistent evidence of a dose-response effect are breastfeeding studies that correlate duration of breastfeeding with improvements in test performance (see Table 1). Fifth and finally, as discussed above, the interpretation of results in all 3 experimental designs is complicated by uncertainty as to the specificity of cause and effect. With respect to “cause,” DHA is not specifically pointed to in breastfeeding studies because there are numerous factors in breast milk besides DHA that could affect the outcome. DHA is also not specifically implicated in ALA-restriction studies in animals because of FA imbalance or other possible effects of severe and chronic ALA restriction. Human formula–comparison studies are the least uncertain with regard to the specificity criterion. However, most of these studies do not supplement formulas only with DHA. With respect to “effect,” possible effects of DHA on endpoints other than cognition or behavior, such as visual function, could bias the results of numerous tests, as indicated above. Thus, on the basis of these 5 causal criteria, the experimental results reviewed here supply some evidence that is not inconsistent with a causal connection between DHA availability and cognitive function, but they do not show causality.

Finally, we comment briefly on our view of the relevance of results reviewed here to the question of whether infant formula should be supplemented with DHA. The experiments most directly relevant to this question obviously are human RCTs that compare performance in children fed LCPUFA-supplemented or -unsupplemented formulas. As discussed, results from these RCTs are mixed and have not consistently shown a positive effect of supplementation on cognitive or behavioral function other than intriguing results from a relatively small number of studies. Results of human breastfeeding studies, though seriously confounded, are not inconsistent with a need for supplementation, but the studies do not provide direct or clear evidence. We consider that animal studies provide the most convincing and consistent evidence linking a decrease in brain concentrations of DHA to altered performance on cognitive or behavioral tests. However, effects are not large, despite the fact that the studies were conducted under severe dietary conditions, and results are difficult to extrapolate to the human situation. In our view, the main contribution of these animal studies to the discussion of infant formula supplementation is that they suggest the possibility, as discussed by others, eg, Wainwright (119), that small differences in brain concentrations of DHA, such as most likely occur between infants fed supplemented or unsupplemented formulas, may result in subtle effects that currently are difficult to detect but could be significant.

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Eating behavior among women with anorexia nervosa

Robyn Sysko, B Timothy Walsh, Janet Schebendach, and G Terence Wilson

ABSTRACT

Background: Disturbed eating and severe caloric restriction are characteristic features of patients with anorexia nervosa (AN). Despite the importance of eating behavior in the presentation of AN, there have been relatively few objective laboratory studies of eating behavior among persons with eating disorders.

Objective: The purpose of the study was to obtain objective measures of eating behavior among patients with AN before and immediately after weight restoration and to compare those measures with measures among control subjects.

Design: Twelve patients hospitalized for AN and 12 control subjects participated in the study. Eleven of the 12 patients were retested at 90% of ideal body weight.

Results: The average meal consumption was 103.97 ± 102.08 g for patients at low weight and 178.03 ± 202.97 g after weight restoration (NS). Control subjects consumed significantly more than did AN patients at both time points, and the average meal size was 489.58 ± 187.64 g. Patients showed significant decreases in psychological and eating-disordered symptoms after weight restoration.

Conclusion: These data suggest that patients with AN show a persistent disturbance in eating behavior, despite the restoration of body weight and significant improvements in eating-disordered and psychological symptoms.


KEY WORDS Eating behavior, anorexia nervosa, dietary restraint, meal consumption, eating disorders, laboratory test meal

INTRODUCTION

Disturbances in eating behavior are defining features of eating disorders. Patients with anorexia nervosa (AN) severely restrict dietary intake, whereas patients with bulimia nervosa (BN) experience recurrent episodes of binge eating. Although disturbed eating is a hallmark of patients with eating disorders, relatively few objective laboratory studies of eating behavior among persons with eating disorders have been conducted.

Most studies of eating behavior in a laboratory setting have focused on patients with BN, and they documented that, when binge eating, patients consume substantially more food and eat faster than do age-, weight-, and sex-matched control subjects (1–4). Findings from studies also suggested abnormalities in meal-related physiologic functioning in BN, including rate of gastric emptying (5–10), release of the satiety hormone cholecystokinin (6, 11, 12), gastric capacity (7), and gastric relaxation (13). In comparison to BN, relatively little is known about disturbances in eating behavior among persons with AN. In addition, although improvements in psychological symptoms and weight have been documented during inpatient hospitalization for AN (14), it is not clear whether a similar normalization occurs for food intake.

The current study was designed to measure total consumption during a laboratory test meal of patients with AN at low weight and after weight restoration in comparison to control subjects. In addition, the study aimed to examine the relation between eating behavior and self-reported clinical characteristics, such as restraint over eating, and the relation between changes in these measures during treatment. Finally, the study aimed to examine the relation between changes in psychological symptoms, measured by interview and self-report, and changes in test meal intake during the course of inpatient hospitalization for patients with AN.

SUBJECTS AND METHODS

Subjects

Twelve women with AN or an eating disorder not otherwise specified, as defined by the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV), participated in the study. Six participants were diagnosed with AN, restricting subtype (AN-R), and 4 participants were diagnosed with AN, binge-purge subtype (AN-B/P), by the Structured Clinical Interview for DSM-IV (SCID-IV; 15). The DSM-IV requires the presence of amenorrhea over a 3-mo period for the diagnosis of AN, and, in the current study, 2 persons with an eating disorder not otherwise
after weight restoration, which was defined as attaining ≥90% of
ideal body weight (IBW; 19). The second test meal occurred a
mean of 54.18 ± 14.48 d (range: 37.0–84.0 d) after initial testing
and a mean of 14.55 ± 6.52 d (range: 6.0–31.0 d) after patients
reached 90% IBW. All patients but one were free from medica-
tions at the time of testing; that one patient was receiving alen-
dronate for osteoporosis at both test meals but was included in the
study because alendronate is not known to influence eating or
weight. All patients were between the ages of 18 and 45 y and
were receiving treatment on an inpatient unit at the New York
State Psychiatric Institute at Columbia University Medical Cen-
ter (New York City). Patients were required to be free of psy-
chotic illnesses, drug abuse, and significant suicidal ideation.

Twelve women without eating disorder symptoms served as
the control group. Control subjects were required to be between
90% and 120% IBW. All control subjects participated in the
study in exchange for monetary compensation and were without a
current psychiatric diagnosis or significant medical illness.
Control subjects participated in only 1 test meal session.

All participants provided written informed consent. This study
was reviewed and approved by the Institutional Review Board of
the New York State Psychiatric Institute.

Procedures

The procedures were identical for all test sessions. Patients and
control subjects consumed a standardized breakfast on the morn-
ing of the test meal. For patients at low weight, the standardized
breakfast consisted of 4 fluid ounces (118.29 mL) apple juice, 8
fluid ounces (236.59 mL) whole milk, 2 ounces (56.70 g) Special
K cereal (Kellogg’s, Battle Creek, MI), one 1.5-ounce (42.52 g)
slice of whole-wheat bread, and 2 tablespoons (30 mL) grape
juice (≈580 kcal); for patients at 90% IBW and control subjects,
the standardized breakfast was 8 fluid ounces (236.59 mL) apple
juice, 8 fluid ounces (236.59 mL) whole milk, 2 ounces (56.70 g)
Special K cereal (Kellogg’s), two 1.5-ounce (42.52 g) slices of
whole-wheat bread, 1 teaspoon (5 mL) butter, and 1 tablespoon
(15 mL) grape juice (≈855 kcal). Participants did not consume
any additional food or liquid, other than water, before reporting
to the laboratory for the meal session 4 h later.

The test meal was a strawberry yogurt shake; this test meal was
used previously in other eating behavior studies (2). A shake (975
g; 1.04 kcal/g or ≈1014 kcal) was provided to participants in a
covered, opaque, 83-fluid-ounce (2454.60-mL) container with a
straw. Before each test meal, participants received instructions
by tape recorder. The instructions specified that participants
should consume as much of the shake as they would like and that
the meal would serve as their lunch for the day. The instructions
asked participants to avoid touching or manipulating the con-
tainer in any way. The meal was placed on a modified version of
an eating monitor (20), which measured intake (in g) every 5 s.
During the meal, participants were observed through a closed-
circuit video monitor.

As in previous eating behavior studies (1, 21, 22), participants
were asked to make ratings before and after the test meal of
hunger, fullness, sickness, loss of control, urge to eat, preoccu-
pation with thoughts of food, and fear of fatness on a 15-cm visual
analog scales (VAS). Patients with AN were also asked whether
they intended to eat less than, as much as, or more than the
amount they were expected to eat for lunch on the inpatient unit.
The VAS was anchored by the phrases “Not at all” and “Ex-
tremely.” After each 50-g increment during the meal, patients
with AN and control subjects rated hunger, fullness, sickness,
feelings of loss of control, and liking of the shake on the VAS.
Participants were signaled to complete the VAS during the meal
at approximately each 50-g increment or after a 5-min interval if
50 g had not yet been consumed. The participant signaled the end
of the meal to the experimenter by pushing a button (doorbell). At
the conclusion of the meal, patients with AN chose whether they
believed they had eaten less than, as much as, or more than they
typically ate for lunch on the unit, and they used a VAS to rate the
difficulty of stopping eating, hunger, fullness, sickness, loss of
control, urge to eat, preoccupation with thoughts of food, and fear
of fatness. Time needed to complete the VASs was subtracted from
the calculation of the total meal duration.

Measure

Patients (at low weight and 90% IBW) and control subjects
completed the Beck Depression Inventory (23), the Beck Anxi-
ety Inventory (24), the Dietary Intent Scale (25), the Eating
Disorder Examination Questionnaire (EDE-Q; 26), the Eating
Disorder Inventory (27), the Three Factor Eating Questionnaire
(TFEQ; 28), the Mizes Anorectic Cognitions Scale (29), and the
Rosenberg Self-Esteem Scale (30). Patients with AN were also
administered the Eating Disorder Examination (EDE), version
12 (31). For the Beck Depression Inventory, scores were calcu-
lated without the weight loss item because of the difficulty in
differentiating between weight loss as a symptom of depression
and as a symptom of AN.

Statistical analyses

Means and SDs were calculated for the Beck Depression In-
ventory, Beck Anxiety Inventory, EDE, EDE-Q, Eating Disorder
Inventory, Rosenberg Self-Esteem Scale, and Mizes Anorectic
Cognitions Scale for control subjects and patients with AN at low
weight and after weight restoration. One-way analysis of vari-
ance (ANOVA) was used to compare the differences between
patients with AN and control subjects at low weight and 90% IBW,
and paired t tests were used to compare the scores between
low-weight and 90% IBW patients with AN. Because the ANO-
VAs and paired t tests compared data from the control subjects
and the patients with AN, both at low weight and 90% IBW, and
between the patients with AN at low weight and 90% IBW, the
P values from the ANOVAs and the paired t tests for these
comparisons were multiplied by 3 in accord with the Bonferroni
correction for multiple comparisons. Effect sizes (d) were cal-
culated as the mean difference between the 2 groups being com-
pared (eg, patients at low weight and patients at 90% IBW) for a
given variable divided by the mean SD of the 2 patient groups on
that variable.

Pearson’s correlation coefficients were calculated between the
changes in psychological symptoms and the change in intake for
patients with AN between low weight and 90% IBW.
coefficients were also used to analyze the relation between self-reported restraint scores from the Dietary Intent Scale, TFEQ, EDE-Q, and total intake. The TFEQ scoring, as described by Stunkard and Messick (28), was used to derive 3 subscales (restraint, disinhibition, and hunger). In addition, 2 subscales (rigid control and flexible control), derived from a factor analysis of the TFEQ (32), were calculated.

For the test meal sessions, initial VAS ratings and postmeal VAS ratings during the meal were analyzed by using one-way ANOVA to compare AN patients at low weight and 90% IBW with control subjects. Paired t tests were also calculated to compare AN patients at low weight with AN patients at 90% IBW for the premeal or postmeal VAS ratings. Because the average VAS ratings of control subjects were compared with those of patients with AN at both low weight and 90% IBW and the VAS ratings of patients with AN were compared between low weight and 90% IBW testing, the P values from the ANOVAs and paired t tests were multiplied by 3 in accord with the Bonferroni correction. Statistical calculations were performed by using SPSS for WINDOWS software (version 11; SPSS Inc, Chicago, IL). All values are means ± SDs.

RESULTS

The mean age of patients with AN was 21.33 ± 2.93 y (range: 18–28 y), and that for control subjects was 23.33 ± 5.03 y (range: 18–37 y) (NS). All participants were white. The mean body mass index (in kg/m²) of all 3 groups is listed in Table 1. One-way ANOVAs indicated that patients at low weight (P < 0.003) and 90% IBW (P < 0.003) had a significantly lower body mass index than did control subjects.

The data on total intake (in g) and the scores on the psychological measures for the 3 groups are presented in Table 1. None of the correlations between changes in psychological measures and changes in intake was significant.

Correlation coefficients were calculated between total intake during the meal and measures of dietary restraint [DIS, TFEQ Restraint Subscale, TFEQ Disinhibition Subscale, TFEQ Hunger Subscale, TFEQ Flexible Control and Rigid Restraint Subscales (32), EDE-Q Restraint Subscale, and EDE Restraint Subscale] for the 3 groups and for the change in restraint scores and the change in intake between low weight and 90% IBW testing in patients with AN. The correlations for the restraint scores and intake at each meal and for the change in restraint scores and change in intake range from −0.016 to −0.561, but none of the correlations was significant.

Patients at low weight overestimated their meal consumption by an average of 945.03 kcal, and patients at 90% IBW overestimated by an average of 111.66 ± 132.49 kcal. Control subjects underestimated their test meal consumption by an average of 217.48 kcal. The estimates of caloric intake at each meal and for the change in restraint scores and changes in intake was significant.

The correlations for the restraint scores and change in intake between low weight and at 90% IBW were analyzed by using one-way ANOVA between 90% IBW AN patients and control subjects, ANOVA between low-weight AN patients and control subjects, and changes in intake was significant.

Paired ANOVA to compare AN patients at low weight and 90% IBW VAS ratings during the meal were analyzed by using one-way ANOVA to compare AN patients at low weight and 90% IBW VAS ratings during the meal were analyzed by using one-way ANOVA between 90% IBW AN patients and control subjects, ANOVA between low-weight AN patients and control subjects, and changes in intake was significant.

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slower rate of eating [total intake (in g)/total time eating (in s)] than did control subjects (low weight: 0.532 ± 0.658 g/s; 90% IBW: 0.462 ± 0.593 g/s; control: 1.90 ± 0.928 g/s). Patients with AN-R showed a faster rate of eating during the meal at low weight than did patients with AN-B/P (AN-R: 0.786 ± 0.648 g/s; AN-B/P: 0.408 ± 0.749 g/s), but patients with AN-B/P ate more quickly at 90% IBW than did patients with AN-R (AN-R: 0.460 ± 0.522 g/s; AN-B/P: 0.753 ± 0.872 g/s); none of these differences were significant. The means, SDs, and ANOVAs of the premeal and postmeal VAS ratings of patients at low weight, patients at 90% IBW, and control subjects are presented in Table 2.

### DISCUSSION

This study found that patients hospitalized for AN consumed substantially less of a single-item test meal than did control subjects, both before and after weight gain. The considerable changes in weight and in psychological and eating-disordered symptoms that occurred during hospital treatment were not paralleled by changes of similar magnitude in food consumption during the test meal. This finding suggests that, immediately after restoration to a normal weight, many patients with AN exhibit a persistent behavioral eating disturbance that may increase vulnerability to relapse.

The few previous studies that explicitly examined the eating behavior of patients with AN under controlled conditions found inconsistent results. Rolls et al (22) found a trend for AN patients to eat less than did control subjects. Halmi and Sunday (21) reported that the average test meal consumption by hospitalized low-weight patients with AN varied considerably and, surprisingly, could be greater than that by control subjects (33). The eating behavior of patients with AN in the current study was more consistent with the total meal consumption of patients with AN in a study by Gwirtsman et al (34), in which inpatients consumed smaller amounts of food than did control subjects when encouraged to eat in a manner similar to their eating before hospitalization.

Previous research has shown a consistent disturbance in the subjective ratings of hunger and fullness of patients with AN during a test meal. Several studies have found that, before the consumption of a test meal, patients with AN-R reported feeling less hungry and more full than did control subjects (21, 22, 33, 35) and that, after the meal concluded, patients with AN continued to report feeling less hungry and more full than did control subjects (21, 33, 35). Weight restoration did not significantly alter subjective satiety ratings, because patients with AN continued to report feeling less hungry and more full than did control subjects after an inpatient hospitalization, which may indicate that these disturbances in hunger and satiety persist even after weight restoration (35). The current study did not find significant differences between patients with AN and control subjects in VAS ratings of hunger or fullness before or after the test meal. Similar to previous research (21), this study did observe that patients with AN at a low weight reported more subjective preoccupation with food than did control subjects. In addition, Halmi and Sunday (21) and Halmi et al (33) found that patients with AN-R ate significantly more slowly at a low weight than did control subjects; this finding was replicated in the current study for the low-weight AN patient group as a whole but not for the AN-R subtype.

Differences in experimental procedures may account for some of the discrepant findings between the current study and those of Halmi and Sunday (21) and Sunday and Halmi (33). For example, in the current study, the test meal occurred in the afternoon, and patients and control subjects were asked to eat the test meal instead of lunch after a 4-h fast. The Halmi and Sunday (21) and Sunday and Halmi (33) studies took place in the morning, instead of breakfast, after a 10-h or overnight fast. In addition, VAS ratings for the meal studies of Halmi and Sunday (33) and Halmi et al (35)...

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### TABLE 2

Premeal and postmeal visual analog scale (VAS) ratings of patients with anorexia nervosa (AN) at low weight and at 90% ideal body weight (IBW) and control subjects.

<table>
<thead>
<tr>
<th>VAS ratings</th>
<th>Patients with AN</th>
<th>Control subjects (n = 12)</th>
<th>Low weight (n = 12)</th>
<th>90% IBW (n = 11)</th>
<th>Low-weight AN patients vs control subjects</th>
<th>90% IBW AN patients vs control subjects</th>
<th>90% IBW AN patients vs 90% IBW AN patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Premeal</td>
<td>Postmeal</td>
<td>Premeal</td>
<td>Postmeal</td>
<td>Premeal</td>
<td>Premeal</td>
</tr>
<tr>
<td>Fullness</td>
<td></td>
<td>4.86 ± 3.76&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9.23 ± 3.66</td>
<td>7.91 ± 5.28</td>
<td>9.72 ± 5.40</td>
<td>7.12 ± 5.58</td>
<td>9.34 ± 4.90</td>
</tr>
<tr>
<td>Hunger</td>
<td></td>
<td>6.35 ± 2.54</td>
<td>1.84 ± 2.40</td>
<td>6.58 ± 5.17</td>
<td>3.39 ± 4.87</td>
<td>4.74 ± 3.95</td>
<td>2.64 ± 3.24</td>
</tr>
<tr>
<td>Loss of control</td>
<td></td>
<td>0.283 ± 0.674 ± 0.459 ± 0.882</td>
<td>10.23 ± 5.38</td>
<td>11.03 ± 5.34</td>
<td>5.78 ± 5.15</td>
<td>9.18 ± 5.17</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Liking of food</td>
<td></td>
<td>9.19 ± 3.11</td>
<td>8.72 ± 3.63</td>
<td>6.59 ± 3.96</td>
<td>8.18 ± 4.70</td>
<td>6.23 ± 5.78</td>
<td>5.28 ± 6.16</td>
</tr>
<tr>
<td>Preoccupation</td>
<td></td>
<td>3.26 ± 4.49</td>
<td>3.09 ± 3.72</td>
<td>11.98 ± 4.20</td>
<td>13.77 ± 1.62</td>
<td>6.92 ± 4.97</td>
<td>8.97 ± 3.91</td>
</tr>
<tr>
<td>Fear of fat</td>
<td></td>
<td>2.89 ± 3.47</td>
<td>3.22 ± 3.70</td>
<td>14.22 ± 1.13</td>
<td>13.93 ± 1.70</td>
<td>12.35 ± 3.49</td>
<td>12.36 ± 3.53</td>
</tr>
<tr>
<td>Difficulty</td>
<td></td>
<td>1.88 ± 2.52</td>
<td>4.85 ± 4.58</td>
<td>2.47 ± 2.55</td>
<td>2.04 ± 2.75</td>
<td>2.04 ± 2.75</td>
<td>2.04 ± 2.75</td>
</tr>
</tbody>
</table>

<sup>1</sup> Bonferroni corrected; multiple-comparison ANOVAs and paired t tests.
<sup>2</sup> ANOVA between AN patients (low-weight or 90% IBW) and control subjects.
<sup>3</sup> Paired t tests between AN patients at low weight and at 90% IBW.
<sup>4</sup> ± SD (all such values).
were obtained 14 min after the conclusion of the meal, whereas the current study provided the last VAS immediately after the termination of the meal. In the current study, patients had no prior exposure to the novel food (yogurt shake) used in the test meal and were not allowed to see the contents of the container as they ate. In contrast, the studies of Halmi and Sunday (21) and Owen et al (36) found that the ability to see the test meal while eating did not affect the average amount consumed by patients with AN; however, the food used in those test meals was Sustacal (Mead Johnson, Evansville, IN), which constituted the main source of energy provided to the patients with AN during treatment.

In the current study, the test meal was novel for both control subjects and patients with AN, and patients may have experienced significant anxiety about consuming an unknown type and quantity of food. It is possible that the significant restriction in food intake among patients with AN resulted from an interaction between fear of weight gain and anxiety about a new or unknown food in an unfamiliar environment. Although it is not clear to what extent such concerns affected total meal consumption among patients with AN, the results of the current study are consistent with those of a recently described model of fear conditioning in the maintenance of AN (37).

The current study also compared self-reported measures of dietary restraint with an index of objective behavior. Research with eating-disordered patients often assesses the construct of dietary restraint; however, dietary restraint can refer to several different attitudes or behaviors. The term usually indicates a cognitive set linked to attempts to diet and tends to be associated with unsuccessful dieting (38). The comparison of behavioral and self-reported measures of the same construct provides an opportunity to validate self-reported measures of dietary restraint against objective dietary restraint in a meal paradigm. One previous study examined the agreement between eating behavior, as a measure of behavioral restraint, and self-reported measures of restraint (39) and found no relation between the amount of food consumed and the measures of dietary restraint in either control subjects or persons with a diagnosis of an eating disorder. The current study similarly found that there was no relation between self-reported measures of dietary restraint and total meal intake in patients with AN, either at low weight or 90% IBW, or in control subjects.

There are several important limitations in the design of this study. The sample size was small, which may have affected the statistical power to detect differences in VAS ratings of hunger or fullness and the correlations between self-reported and behavioral dietary restraint. Some of the patients with AN did not respond to all of the questionnaires, such as the EDE-Q, in which statistical power to detect differences in VAS ratings of hunger or fullness and the correlations between self-reported and behavioral dietary restraint in either control subjects or patients with AN; however, the food used in those test meals was Sustacal (Mead Johnson, Evansville, IN), which constituted the main source of energy provided to the patients with AN during treatment.

In summary, the current study compared the test meal intake by patients with AN at low weight and after weight restoration with that by control subjects. Contrary to previous research (21, 33), the current study found that inpatients with AN consumed significantly less of a test meal than did control subjects at both time points. The small changes in eating behavior observed during inpatient treatment contrast with the significant changes found in weight and in psychological and eating-disordered symptoms within the same time period. Although most hospitalized patients with AN in our program and other programs respond to treatment (14, 40, 41), the nutritional restoration that occurs on an inpatient unit does not necessarily resolve the core eating difficulties for patients with AN. This continued vulnerability during the period after inpatient hospitalization is exemplified by significant relapse rates among patients with AN between 30% and 70% (42, 43). Further study of eating behavior in AN may be worthwhile to better understand the maintenance and outcome of the treatment of AN.

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Energy intakes of children after preloads: adjustment, not compensation1–3

Joanne E Cecil, Colin NA Palmer, Wendy Wrieden, Inez Murrie, Caroline Bolton-Smith, Pete Watt, Deborah J Wallis, and Marion M Hetherington

ABSTRACT

Background: Young children accurately compensate for energy-dense preloads consumed before test meals. The accuracy of compensation seems to deteriorate as a function of age.

Objective: The hypothesis that accurate energy compensation varies by age, body mass index, and individual characteristics of children and their mothers was tested.

Design: Energy intake (EI) from a test meal was measured in 74 children aged 6–9 y 90 min after the ingestion of no-energy (NE), low-energy (LE), or high-energy (HE) preload snacks. The NE preload consisted of 250 mL water, the LE preload consisted of a 56-g muffin + a 250-mL orange drink (783 kJ), and the HE preload consisted of a 56-g muffin + a 250-mL orange drink (1628 kJ).

Results: A significant dose-related reduction in EI was found after the preloads; younger children adjusted more effectively than did older children, although total EI (including preload energy) indicated that the adjustment was not accurate. The compensation index (COMPX) differed by preload and age group; COMPX scores were higher between the NE and LE preloads (younger children: 44.4 ± 9.3%; older children: 57.0 ± 11.6%) than between the NE and HE preloads (39.6 ± 4.9%; 31.3 ± 6.2%) and the LE and HE preloads (35.2 ± 7.8%; 7.4 ± 9.8%). This finding indicates a more consistent response across preloads and a greater sensitivity to energy load by younger than by older children. High interindividual variation and low intraindividual variation in COMPX was found. The tendency to over- or undereat in response to the preloads (deviation from perfect) correlated directly and positively with maternal concerns about child overweight, not with actual BMI.

Conclusions: The children adjusted their EIs in response to different preloads, and the younger children did so more effectively than did the older children. Poor short-term energy compensation may constitute a behavioral marker for positive energy balance.


KEY WORDS Children, eating behavior, energy compensation, food intake, child overweight

INTRODUCTION

Childhood obesity has doubled over the past 20 y (1, 2), and, although there is a general risk of excess weight gain from an increasingly obesigenic environment (3), it is critical to identify behavioral attributes that contribute to the risk of obesity early in development. Given the association between childhood and adult obesity (4–6), early intervention to prevent excessive weight gain (7) is warranted. Positive energy balance, achieved by consuming more energy than is expended, is key to the development of childhood obesity (8). Understanding controls of energy intake in children within and beyond single meals may provide markers for overconsumption in children.

Preschool children appear to be sensitive to the energy density of snacks given before a meal. In several studies, young children were shown to compensate accurately for the energy content of a snack given before lunch (9, 10). A compensation index (COMPX) was calculated for each child by dividing the difference in energy intake after 2 preloads by the difference in the energy content of the preloads, transformed to a percentage (11). A COMPX score of 100% reflects perfect compensation. Young children are generally good at compensating for high-energy snacks and can achieve COMPX scores between 50% (12) and 80% (10). In contrast with children aged <5 y, older children appear to compensate less effectively for high-energy snacks and have COMPX scores of ≈20% (10), which are more similar to adult scores (13). Indeed, some studies have shown no difference in compensation ability between 4- and 6-y-old children, adults, and older adults (14). Poorer self-regulation is associated both with increased adiposity of the child (11, 15) and with more controlling feeding styles of parents as determined by the Child Feeding Questionnaire (11). Clearly, many biological and behavioral factors influence short-term compensation, and these are worthy of further exploration.

Large interindividual variation in COMPX scores have been observed across different studies (14) and, within families, siblings fail to show similar compensation abilities (16). This suggests that some children are more sensitive to the energy content

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The order of preload administration was partially randomized; time at school followed by the test meal at the typical lunchtime. This time profile replicated the children's usual snack habit. Children were asked to consume either a preload designed to increase compliance with the instruction to maintain their usual breakfast. These measures were noted informally to check compliance. These measures were separated by 1 wk and were conducted on a day when there was no physical education. A check on order effects, a fourth water preload (NE2) was administered to 50% of the children. Data analyses were conducted for 3 preloads, because the test-meal intake during the second control preload did not differ from that during the first control.

The heights and body weights of the children were measured at school on the morning of the first test session. The heights and body weights of the mothers were measured at home or in the laboratory. Standing height without shoes was measured to the nearest 0.1 cm with a stadiometer (SECA, Bolton, United Kingdom). Body weight was measured to the nearest 0.1 kg with a mechanical floor scale (SECA) while the subjects were wearing light clothing. Body mass index (BMI) for each child and parent was calculated as weight (kg)/height² (m²).

Parents also completed a number of questionnaires, including the Child Eating Behavior Questionnaire (CEBQ; 17) and the Child Feeding Questionnaire (CFQ; 18). The 35-item CEBQ measures aspects of children's eating styles according to 8 factors that measure the responsiveness to and enjoyment of food, satiety responsiveness, slowness in eating, fussiness, desire to drink, emotional overeating, and undereating. The internal consistency for these 8 factors is high and ranges from 0.74 for satiety responsiveness to 0.91 for enjoyment of food (17). Similarly, the 31-item CFQ measures parental attitudes toward, and strategies in, child feeding through 7 factors: perceived responsibility for child feeding, perceived parent weight, perceived child weight, concern about child weight, pressure to eat, restriction, and monitoring. Cronbach's α, which is used to assess internal consistency, is also high for these factors and ranges from 0.70 for pressure to eat to 0.92 for monitoring. More detailed psychometric properties of these tools were characterized and published elsewhere (17, 18). Items from the CFQ and CEBQ were correlated with COMPX to examine potential associations between these factors and short-term energy compensation.

### Preloads and test meals
The nutrient contents of the 3 preloads are shown in Table 1. The 3 preloads were developed to differ in energy density. The NE control (0 kJ) consisted of 250 mL water, and the LE and HE preloads consisted of an orange drink and a muffin manipulated to differ only in total energy content. The LE preload (187 kcal, or 782.78 kJ) consisted of 250 mL orange drink (200 mL water + 50 mL low-energy orange diluting drink) and 56 g low-energy-dense muffins, and the HE preload (389 kcal, or 1628.35 kJ) consisted of 250 mL orange drink (200 mL water + 50 mL water + 50 mL maltodextrin (used only in the high-energy preload)).

### Table 1

<table>
<thead>
<tr>
<th>Item</th>
<th>Energy</th>
<th>Protein</th>
<th>Fat</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular-energy minimuffins (high-energy preload)</td>
<td>1780</td>
<td>7.0</td>
<td>21.0</td>
<td>52.0</td>
</tr>
<tr>
<td>Reduced-fat muffins (low-energy preload)</td>
<td>1307</td>
<td>6.6</td>
<td>8.6</td>
<td>52.1</td>
</tr>
<tr>
<td>Low-energy orange drink with no added sugar (undiluted)</td>
<td>78</td>
<td>0.3</td>
<td>0.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Regular-energy orange drink with added sugar (undiluted)</td>
<td>783</td>
<td>0.3</td>
<td>0.1</td>
<td>45.0</td>
</tr>
<tr>
<td>Maltodextrin (used only in the high-energy preload)</td>
<td>1615</td>
<td>0</td>
<td>0</td>
<td>95.0</td>
</tr>
</tbody>
</table>
regular orange diluting drink) with the addition of 15 g maltodextrin (Maxijul; SHS International Ltd, Liverpool, United Kingdom) and 56 g regular-energy-dense muffins.

The NE control (water) was used to provide a baseline intake for comparison. Test meal intake was expected to be greatest after ingestion of the NE control, given that it had no energy density and weighed less than the other preloads. Energy density and the weight and volume of a food load are important factors in determining post-ingestive appetite and food intake (19, 20). These preloads were chosen to be familiar to the children and to incorporate a relatively large difference in energy content (202 kcal, or 846 kJ) with minimal differences in sensory properties. Systematic assessments were made to evaluate the pleasantness and likability of the orange drinks before the study began to ensure acceptance by the children. Each child was required to ingest 100% of the preload on each occasion.

The test meal was a self-selected lunch consisting of a variety of cold food items (Table 2) that were served to each child on individual trays. The lunch provided 7.95 MJ (1900 kcal) energy, which was much more energy than the children normally consumed at lunch, and was devised to offer sufficient quantity and variety to maximize choice. The children were allowed to request additional servings. A maximum of 30 min was allowed for the children to eat their chosen food items, and they were notified of the amount of time remaining 10 and 5 min before the 30-min period was reached. The average group size on each occasion was 4, and the children sat together during the lunch as they normally would at school. Research assistants were present to provide assistance and were instructed to lead any conversation away from the topic of food and eating.

### Statistical Analysis

Intake data were analyzed by using repeated-measures analysis of variance, with age group and sex as between-groups factors. When significant main effects were obtained, post hoc tests with a Bonferroni correction factor for multiple comparisons were applied to determine the source of significant effects. Pearson’s correlation coefficient ($r$) was used to correlate between BMI, energy intake, and COMPX values. Spearman’s correlation coefficients ($r_s$) were applied to correlations between intake and questionnaire scores. Deviation scores were calculated to assess how far children deviated from perfect compensation giving an absolute value. This provided a measure of the extent to which children over- or underconsumed at lunch after the preloads. Thus, the greater the deviation from perfect compensation, the higher the absolute (deviation) value. These deviation scores were correlated with child and maternal characteristics, including BMI and items from the CFQ and CEBQ. Cronbach’s $\alpha$ was calculated for subscales on the CFQ and CEBQ to compare against published norms. Data were analyzed by using SPSS for WINDOWS (version 11.5; SPSS Inc, Chicago, IL), and the results are expressed as means ± SEMs unless otherwise stated. Statistical significance was set at $P < 0.05$.

### RESULTS

A summary of the ages, body weights, heights, and BMIs of the children and their mothers and the number of overweight and obese children is provided in Table 3. Data were collected from 37 girls and 37 boys, of whom 15% were overweight and 8% were obese on the basis of age- and sex-appropriate international standards (21).

### Energy intake

Overall, the children adjusted their intakes at lunch in response to the energy content of the preload ($P < 0.0001$; Figure 1). As expected, the older children generally consumed more than did the younger children (2976 ± 119 kJ compared with 2534 ± 96 kJ; $P < 0.001$). The children’s energy intakes decreased linearly as the preload energy content increased (within-subjects contrast for linearity; $P < 0.001$). Post hoc tests showed that energy intake was significantly different between conditions. Thus, on average, the children ate significantly less after the HE preload than after the LE preload and less after both of these preloads than after the NE (water) preload ($P < 0.001$ in all cases; Table 4). No main effects of sex on energy intake were found when adjusted for body weight, so all analyses considered girls and boys together.

Total energy intake (including energy from the preloads) varied according to condition in a linear way (ANOVA: $P < 0.0001$), which indicated that the adjustment in food intake at lunch after the different preloads failed to accommodate precisely the energy content of the preloads (Figure 1). Thus, the total energy intake (including preload energy) was higher after the HE preload than after the LE and NE preloads.

### Assessment of intake

Energy intake at the test meal was assessed by weighing the food items before and after lunch and then using manufacturer’s information to calculate the total amount of energy consumed. The weights and energy contents of individual food items and drinks were calculated to assess food choice. 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. This value was converted to a percentage: \([\text{change in energy intake at the test meal/change between preload energy content} \times 100] \) (11) A score of 100% indicates precise (calorie for calorie) compensation. Values less than 100% reflect undercompensation, values >100% reflect overcompensation. Total energy intake was calculated by adding energy from the preload to the energy intake at lunch.
Given that differences in energy intake emerged by age group, and that this difference was related to body size, energy intake was transformed into energy consumed as a function of body weight (kJ/kg). An interesting interaction emerged between preload and age group (ANOVA: \( P < 0.02 \)), which suggested that the slope of adjustment between the NE and HE preloads was steeper for the younger than for the older children (Table 4). Thus, after energy intake as a function of body weight was accounted for, the younger children more effectively adjusted their energy intakes after the preloads than did the older children.

To test whether the changes in test-meal intake were specific to the preload energy and not due to increasing familiarity with the procedure or other temporal anomaly, we subjected 42 individuals to a fourth test, repeating the water-alone preload. Importantly, no changes in energy intake were observed in these individuals. Intake did not differ significantly between the 2 sessions (3038 ± 132 and 3054 ± 124 kJ), and energy intake in the 2 sessions was highly correlated (\( r_{(41)} = 0.77, P = 0.001 \)). Thus, the children ate the same amounts at lunch when given the water preload, and this provided an appropriate baseline against which to judge compensation after preloads that differed in energy content.

### Table 3

<table>
<thead>
<tr>
<th>Age, height, weight, and BMI of the study cohort, by age group</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group (n = 74)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mo)</td>
<td>92.1</td>
<td>72</td>
<td>118</td>
<td>11.4</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.28</td>
<td>1.14</td>
<td>1.49</td>
<td>0.07</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>28.3</td>
<td>19.5</td>
<td>57.0</td>
<td>6.1</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>17.2</td>
<td>13.8</td>
<td>25.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Younger children, 6–7.8 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mo)</td>
<td>84.5</td>
<td>72</td>
<td>94</td>
<td>6.6</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.24</td>
<td>1.14</td>
<td>1.37</td>
<td>0.05</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>26.1</td>
<td>19.5</td>
<td>39.0</td>
<td>4.2</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>16.9</td>
<td>13.8</td>
<td>24.8</td>
<td>2.11</td>
</tr>
<tr>
<td>Overweight [n (%)](^a)</td>
<td>8 (17.8)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Obese [n (%)](^b)</td>
<td>3 (6.7)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Older children, 8–9.8 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mo)</td>
<td>103.8</td>
<td>96</td>
<td>118</td>
<td>6.2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.33</td>
<td>1.18</td>
<td>1.49</td>
<td>0.07</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>31.5</td>
<td>22.0</td>
<td>57.0</td>
<td>7.11</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>17.7</td>
<td>14.1</td>
<td>25.8</td>
<td>2.55</td>
</tr>
<tr>
<td>Overweight [n (%)](^a)</td>
<td>3 (10.3)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Obese [n (%)](^b)</td>
<td>3 (10.3)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mothers (n = 70)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.63</td>
<td>1.45</td>
<td>1.81</td>
<td>0.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.6</td>
<td>44.0</td>
<td>108</td>
<td>13.4</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>23.8</td>
<td>16.83</td>
<td>40.65</td>
<td>—</td>
</tr>
<tr>
<td>Overweight [n (%)](^a)</td>
<td>14 (20)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Obese [n (%)](^b)</td>
<td>12 (17.1)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Determined by Cole et al (21) using age-appropriate international cutoffs.

### Table 4

<table>
<thead>
<tr>
<th>Age, height, weight, and BMI of the study cohort, by age group</th>
<th>Younger children (n = 45)</th>
<th>Older children (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake at lunch (kJ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE preload(^a)</td>
<td>2864.5 ± 114</td>
<td>3294.4 ± 142</td>
</tr>
<tr>
<td>LE preload(^b)</td>
<td>2517.6 ± 107</td>
<td>2848.9 ± 133</td>
</tr>
<tr>
<td>HE preload(^c)</td>
<td>2220.5 ± 92.5</td>
<td>2786.0 ± 115</td>
</tr>
<tr>
<td>Energy intake (kJ/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE preload(^a)</td>
<td>111.2 ± 4.2(^d)</td>
<td>106.7 ± 5.2(^e)</td>
</tr>
<tr>
<td>LE preload(^b)</td>
<td>97.5 ± 4.0(^f)</td>
<td>91.8 ± 4.8(^g)</td>
</tr>
<tr>
<td>HE preload(^c)</td>
<td>85.3 ± 3.6(^h)</td>
<td>90.8 ± 4.5(^i)</td>
</tr>
<tr>
<td>Compensation index (%)(^j)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE/LE(^j)</td>
<td>44.4 ± 9.3(^d)</td>
<td>57.0 ± 11.6(^f)</td>
</tr>
<tr>
<td>NE/HE(^j)</td>
<td>39.6 ± 4.9(^d)</td>
<td>31.3 ± 6.2(^e)</td>
</tr>
<tr>
<td>LE/HE(^j)</td>
<td>35.2 ± 7.8(^f)</td>
<td>7.4 ± 9.8(^g)</td>
</tr>
</tbody>
</table>

\(^a\) NE, no-energy preload; LE, low-energy preload; HE, high-energy preload. Variables with different superscript letters (a, b, c) are significantly different, \( P < 0.05 \) (ANOVA and Bonferroni-corrected \( t \) tests for main effects of preload). Means within an age group with different superscript letters are significantly different, \( P < 0.05 \) (ANOVA and Bonferroni-corrected \( t \) tests after significant interaction effects between age and preload). Means in the same row with different superscript letters (x, y) are significantly different, \( P < 0.05 \).

\(^b\) Represents the ability of the child to adjust energy intake at lunch in response to a preload given 90 min earlier; calculated as the difference in energy intake between 2 lunches divided by the difference in energy content of the preload and transformed to a percentage.

To test whether the changes in test-meal intake were specific to the preload energy and not due to increasing familiarity with the procedure or other temporal anomaly, we subjected 42 individuals to a fourth test, repeating the water-alone preload. Importantly, no changes in energy intake were observed in these individuals. Intake did not differ significantly between the 2 sessions (3038 ± 132 and 3054 ± 124 kJ), and energy intake in the 2 sessions was highly correlated (\( r_{(41)} = 0.77, P = 0.001 \)). Thus, the children ate the same amounts at lunch when given the water preload, and this provided an appropriate baseline against which to judge compensation after preloads that differed in energy content.
Compensation ability

The COMPX scores, which reflected the children’s ability to compensate accurately for the energy content of the preloads, confirmed the observation that, although adjustment occurred, few children managed perfect compensation (COMPX = 100%; n = 3). On average, with age groups combined, the best level of compensation (51%) was observed between the NE and LE preloads, and this compared with 35% and 21% for the NE/HE and LE/HE conditions, respectively. Compensation differed according to the type of preload eaten (ANOVA: P = 0.001). Post hoc tests showed that the ability to compensate accurately was higher between the LE and HE preloads and the control (water) than between the 2 energy-dense preloads (Table 4). Furthermore, a significant linear decline in COMPX score was found, which indicated that compensation accuracy followed this order: NE/LE > NE/HE > LE/HE (Table 4).

The interaction between preload and age group was significant (ANOVA: P = 0.04). It appeared that the younger children (40 ± 5%) tended to have higher overall COMPX scores than did the older children (32 ± 6%), but the difference was significant only between the LE and HE preloads (P < 0.05; Table 4). Within-groups analyses indicated that the COMPX score differed significantly by preload only for the older children; thus, COMPX was not significantly different between preloads in the younger children and varied significantly in the older children, ie, younger children were consistent in their response across preloads.

To assess the extent to which there were intraindividual differences in the ability to compensate between preloads, COMPX scores were correlated across preloads independently by age group. Overall, intraindividual variation was low, because correlations between COMPX scores by age group showed significant associations in the younger children [NE/LE:NE/HE (r(44) = 0.54, P < 0.001) and NE/HE:LE/HE (r(44) = 0.49, P < 0.001)] and older children [NE/LE:NE/HE (r(28) = 0.72, P < 0.001) and NE/HE:LE/HE (r(28) = 0.57, P < 0.001)]. COMPX scores were also highly correlated for the sample of 42 children who repeated the control condition. Thus, for COMPX NE/LE [NE1 = 53 ± 10%; NE2 = 55 ± 10%; r(44) = 0.38, P < 0.01] and COMPX NE/HE [NE1 = 36 ± 6%; NE2 = 37 ± 4%; r(44) = 0.44, P < 0.01] correlations were positive and significant. Clearly, COMPX was higher after the LE preload than after the HE preload compared with both control (NE) conditions (ANOVA: P < 0.05).

High positive correlations between COMPX scores after the NE preload suggests that compensation for an energy-dense preload occurs regardless of whether the preload is high or low in energy. However, the low COMPX score indicates that, generally, the adjustment was not accurate.

Food choice

To test whether intake of the preloads had a specific or general effect on food choice at lunch, macronutrient intakes and choice of individual food items were analyzed after each of the 3 preloads. Intakes in weight (g) of protein, fat, and carbohydrate decreased significantly as preload energy intakes increased. Post hoc comparisons confirmed that intake decreased in a linear manner with preload content. Children consumed the same percentage of energy as protein across preloads (13 ± 0.5%), but consumed significantly less energy as fat at lunch after the HE preload (37 ± 1%) than after the NE preload (40 ± 1%; P < 0.01) and a greater percentage of energy as carbohydrate after the HE preload (47 ± 1.0%) than after the NE preload (45 ± 1%; P = 0.053). Thus, the children’s energy intakes tended to be lower after the HE preload than after the NE preload, achieved by switching from sources of fat to sources of carbohydrate.

An evaluation of intakes of individual food items indicated that children tended to eat less of both types of cheese, bread, crackers, and raisins but ate the same amount of other foods in all preloads (ham, potato chips, chocolate, grapes, and orange juice). This pattern of intake might reflect preferences for these latter foods, which are not displaced by consuming the preloads.

Correlations

To identify characteristics of either the children or their mothers, which might predict the ability to compensate, a deviation score was calculated to assess the extent to which children deviated from 100% (perfect) compensation. This was then correlated with a range of variables, including the children’s BMI, the mother’s BMI, and scores on the CFQ and the CEBQ.

The BMI of the children was significantly correlated with maternal BMI (r(73) = 0.34, P < 0.01) and with total energy intake after all preloads (P < 0.01 in each case). The deviation score for NE/LE (x ± SE: 65.4 ± 6.9 for the younger children and 66.1 ± 8.8 for the older children) was significantly and positively correlated with only one item of the CFQ, namely concerns for child overweight (r(73) = 0.3, P < 0.01). Concerns for child overweight correlated with child BMI (r(73) = 0.36, P < 0.01). Internal consistency for this subscale of the CFQ was 0.86, which compared favorably with that published by the authors of the CFQ (r = 0.75) (18). Thus, in the present study, higher BMI in children was associated with a greater level of concern by parents about child overweight, and these concerns corresponded to a greater deviation score for NE/LE. However, there was no significant direct relation between child BMI and this particular deviation score. Concern for child overweight correlated positively with items from the CEBQ [food responsiveness (r(72) = 0.29, P < 0.05) and emotional overeating (r(72) = 0.23, P = 0.052)] and negatively with satiety responsiveness (r(72) = −0.36, P < 0.01) and slowness in eating (r(72) = −0.3, P < 0.05). Again, Cronbach’s α for these subscales (0.86, 0.83, 0.83, and 0.85, respectively) compared favorably with those published by the authors of the CEBQ (17). Taken together, these data suggest that concerns about child body weight status are associated with deviation from perfect energy compensation, and features of eating behavior are related to overconsumption of the test meal. There was no significant correlation between other deviation scores (NE/HE, LE/HE) and the CFQ and CEBQ data.

DISCUSSION

Overall, children in the present study adjusted their food intakes at lunch in relation to the energy content of the preloads administered. Thus, after a midmorning snack consisting of 783 or 1628 kJ, the children’s intake at lunch decreased 13% and 18%, respectively, compared with intake after water. Younger children adjusted their intakes in direct response to the energy content of the preload. However, an age difference in intakes indicated that older children generally consumed more than did the younger children, which would be expected. Thus, after body weight was accounted for, it was shown that the younger children...
adjusted their intakes directly in line with the energy content of the preload; they consumed 12% less after the LE preload and 22% less after the HE preload than did the older children, who reduced their intakes by 13% after the LE preload and by 15% after the HE preload. Clearly, younger children were more able to discriminate between the LE and HE preloads, which suggests that they were more sensitive to the energy content of the preloads than were the older children. Therefore, the younger children did not simply eat less after a snack, as expected, but were able to modify their intakes according to the energy content of the snack.

Despite these adjustments in intake at lunch after the midmorning snacks, intakes fell short of accurate compensation. Therefore, on days when the HE preload was consumed, total energy intake was significantly greater (by 34%). Thus, the consumption of a midmorning snack at school promoted energy intake and may have contributed to a positive energy balance.

The average compensation index of \( \approx 30\% \) indicated that children tended to adjust their intakes but not sufficiently to produce accurate compensation. This finding is in contrast with that of a recent study, which reported average compensation indexes of 103% between low-energy (12.55 kJ) and high-energy (627.6 kJ) preload drinks (14). Nevertheless, in both studies, average compensation masked better accuracy in some of the children than in the others. Indeed, Faith et al (16) showed large individual variation and no familial aggregation in COMPX scores between siblings. In the present study, the younger children tended to compensate for the differences in energy intakes better than did the older children. This finding supports previous research on short-term energy compensation from our laboratory (10).

It was noted that, despite high levels of interindividual variation, the ability to compensate within individuals correlated across preloads. This finding suggests that compensation accuracy is trait-related. This was supported when the control condition was repeated, because compensation scores were strongly correlated. Thus, the individual ability to compensate holds for different preloads. The poorest COMPX occurred between the LE and HE preloads compared with after either of these preloads and water. This would be expected because the task of differentiating between a water and an energy-dense preload is easier than discriminating between 2 preloads that share sensory and other properties. Clearly, children responded to the preloads by reducing their food intake at lunch, but they found it difficult to account for the differences in energy contents of the LE and HE preloads. However, there were large intrasibling variations; some children adjusted their intakes well across all preloads.

When the deviation from perfect compensation was correlated with individual and maternal characteristics, concern about the child’s weight status correlated with deviation scores. Indeed, greater concern about overweight was related to many characteristics of eating style associated with overconsumption, ie, faster eating, emotional overeating, food responsiveness, and lack of satiety. Therefore, although there was no direct correspondence between BMI and COMPX in children, concerns about overweight correlated both with deviation from perfect compensation and with markers of overeating. Taken together, these features could form part of a behavioral phenotype that carries an increased risk of overeating and obesity later in life. It is not clear whether a short-term measure of energy compensation relates to future obesity development, but it is possible that repeated exposure to energy-dense snacks, which are not compensated for, could lead to positive energy balance over time, and may provide a marker of overeating.

Previous research has shown that a child’s body weight status is related to self-regulation, with higher levels of adiposity associated with poorer compensation (11). The present study showed that maternal concern for a child’s weight is associated with self-regulation rather than with current BMI. It is difficult to ascertain whether concern for child weight could be a response to poor regulation in the child or whether concern influences poor regulation. However, it is interesting to note that concern for child weight has been shown to be associated with variance in adiposity and explains \( \approx 15\% \) of the variance in fat mass in children (22). Again, the direction of influence was unclear; nevertheless, concern for child weight is a modifiable behavior that could be tackled in childhood obesity-prevention programs.

Research with young children (ages 3–4 y) has shown that, with some training, the compensation index can be increased from 23% to 65% (23). This study used strategies to increase children’s self-regulation and to recognize internal cues of hunger and satiety. These findings could be extrapolated to interventions with older children, who seem less able to compensate accurately in the short-term for energy-dense snacks.

Food intake was adjusted at lunch after the energy-dense preloads, and this was achieved by reducing the percentage of energy as fat after the high-energy preload compared with the low-energy preload. In particular, children reduced their intakes of cheese, crackers, bread, and raisins, but their intakes of potato chips, chocolate, grapes, and orange juice remained the same after both energy-dense preloads. This pattern of choice is not easily understood in relation to sensory-specific satiety (24), which predicts that fewer foods that share sensory properties with the preload will be eaten, ie, orange juice and other sweet food items. It seems that foods that are highly favored by children are eaten in similar amounts, despite the intake of preloads moderately high in fat and carbohydrate. Thus, sweet snacks consumed midmorning did not displace sweet foods at lunch and were added to daily energy intake with the potential for contributing to overconsumption, if not compensated for later in the day.

Our previous research reported that preschool children adjusted their intakes at lunch after a sucrose preload by consuming fewer carbohydrates (10). The present investigation failed to support this finding and suggests that this precise regulation deteriorates in older children. Nevertheless, a recent study showed that children aged 9–12 y adjusted their food intakes at lunch according to the glycemic index of breakfast (25). Thus, children ate less at lunch after eating a breakfast with a low glycemic index than after a breakfasts with a higher glycemic index. Higher palatability and lower satiety experienced by children after the consumption of foods with a high glycemic index failed to reduce intakes at lunch. These observations may further our understanding of energy compensation in the short-term by highlighting the role of glycemic index, pleasantness, and satiety value on subsequent intake.

Future studies would benefit from ascertaining trait and context-related factors that predict compensation ability. It is clear, that there is large individual variation in compensation ability, and some children demonstrate precise compensation regardless of age. Poorer compensation has been associated with increased child adiposity (11) and greater parental control of child feeding (11), but these factors explain only part of the
variance. It is possible that genetic variation may also account for some of the unexplained individual variation in energy compensation and in eating behavior in general, according to research conducted in animals and humans (26, 27). Further studies could facilitate the identification of children most at risk of positive energy balance, through both the transmission of risk from parents and from poor short-term energy regulation.

We thank the parents and children who participated in this study.

CB-S, MMH, CNAP, WW, and PW wrote the proposal submitted to the Biotechnology and Biological Sciences Research Council. JEC, IM, and DJW collected the data. MMH and JEC analyzed the data and wrote the paper. All authors participated in the design and conduct of the experiment and in the data interpretation. None of the authors had personal or financial conflicts of interest.

REFERENCES

Lack of effect of dietary conjugated linoleic acids naturally incorporated into butter on the lipid profile and body composition of overweight and obese men1–3

Sophie Desroches, P Yvan Chouinard, Isabelle Galibois, Louise Corneau, Jocelyne Delisle, Benoît Lamarche, Patrick Couture, and Nathalie Bergeron

ABSTRACT

Background: Dietary conjugated linoleic acid (CLA) is known to reduce atherosclerosis, plasma total and LDL-cholesterol concentrations, and body fat accumulation in several animal species. Of the few studies that investigated the effects of CLA supplementation in humans, all used commercially formulated oral supplements made from a mixture of CLA isomers.

Objective: We compared the effects on plasma lipoproteins and body composition of the consumption of a modified butter naturally enriched with CLA (CLA-B: 4.22 g CLA/100 g butter fat) by the addition of sunflower oil to the diet of dairy cows with the consumption of a control butter (CON-B) that was low in CLA (0.38 g CLA/100 g butter fat).

Design: In a crossover design study including an 8-wk washout period, 16 men [± SD age: 36.6 ± 12.4 y; body mass index (in kg/m²): 31.2 ± 4.4] were fed each of the 2 experimental isoenergetic diets, providing 15% of energy as protein, 45% as carbohydrates, and 40% as lipids, of which >60% was derived from experimental fats, for 4 wk.

Results: Consumption of the CLA-B diet induced a significantly (P < 0.05) smaller reduction in plasma total cholesterol and in the ratio of total to HDL cholesterol (−0.02 mmol/L and −0.00, respectively) than did consumption of the CON-B diet (−0.26 mmol/L and −0.34, respectively). Abdominal adipose tissue area measured by computed tomography showed no difference in accumulation of either visceral or subcutaneous adipose tissue after the 2 experimental diets.

Conclusion: These results suggest that a 10-fold CLA enrichment of butter fat does not induce beneficial metabolic effects in overweight or obese men.

KEY WORDS Conjugated linoleic acids, lipid profile, plasma lipids, lipoproteins, body composition, obesity, functional foods, C-reactive protein, LDL size

INTRODUCTION

Conjugated linoleic acid (CLA) is a term used to describe positional and geometric derivatives of linoleic acid containing conjugated double bonds. CLA is a group of naturally occurring fatty acids that are mainly present in foods from ruminant sources. In contrast to other fatty acids, which are usually present in gram quantities, CLA are present only in milligram quantities in meats and dairy products (1). Most milk-fat CLA is synthesized endogenously via Δ-9 desaturase from trans-vaccenic acid, an intermediate in the biohydrogenation of linoleic and linolenic acids in the rumen. The remainder of the CLA in milk fat arises directly from CLA absorbed from the digestive tract after being produced in the rumen as an intermediate in linoleic acid biohydrogenation (2). Interest in the potentially therapeutic effects of CLA can be traced back to in vitro investigations by Pariza et al (3) that showed the presence of CLA’s mutagenesis-inhibitory activity in extracts from fried ground beef. Subsequent studies established that the extract exhibited anticarcinogenic activity as well and that CLA was the active component responsible for these effects (4). Those first studies of CLA led to hundreds of investigations and the recognition that CLA has multiple biological effects, such as reductions in atherosclerosis (5–7), plasma lipoproteins and lipids (5–8), and body fat accumulation (9–13). CLA has also been reported to have anticarcinogenic activity (14), antiinflammatory effects (15), and antidiabetic effects (16).

To the best of our knowledge, the few studies that have investigated the effects of CLA in humans were conducted by using commercially formulated oral supplements made from a mixture of synthetic CLA isomers (17–23), which have the advantage of providing high doses of CLA (range: ≈3–7 g/d). In contrast, naturally occurring CLA in common foods are present only in small quantities. The extent to which various amounts of CLA provided in the form of food can affect the health profile of humans is unknown. Therefore, the current study was conducted to test the hypothesis that the incorporation of a modified butter

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2 Supported by the Dairy Farmers of Canada, Natural Sciences and Engineering Research Council of Canada, FCAR Fund, NovoNext Inc, the Quebec Ministry of Agriculture, Fisheries and Food, and the Canada Research Chair in Nutrition, Functional Foods and Cardiovascular Health.

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that is naturally rich in CLA into a well-controlled experimental diet induced beneficial effects on the blood lipid profiles of healthy overweight and obese men. In addition, because consumption of CLA has been found to induce a wide range of beneficial health effects in animals, the effects of the experimental diets on variables related to body composition and to nontraditional cardiovascular disease risk factors such as LDL peak particle diameter and C-reactive protein (CRP) were evaluated as secondary outcome measures. To test our hypothesis that a modified butter naturally rich in CLA benefits the lipid profile, we strictly controlled dietary intake by providing all food to the subjects for the duration of the 4-wk dietary interventions.

SUBJECTS AND METHODS

Subjects

Seventeen overweight and obese (BMI: 26.0–29.9 and >30.0, respectively; 24) men in good health were recruited in the Quebec City area to participate in the nutrition study, and 16 men completed the study. The subject who withdrew completed the first phase of the study but could not come back for the second phase because of relocation for his job. Of the 16 men who completed the study, all were white, and most were Canadian. Subjects were initially screened on the basis of a complete physical examination and medical history. Subjects recruited for the study had to be nonsmokers, to be between 18 and 55 y of age, and to have a BMI >26 and a waist circumference >90 cm. We selected both overweight and obese men as a target population that would benefit from CLA supplementation because of studies showing that 90% of men with a BMI >27 also have adipose tissue areas >100 cm², a threshold value above which moderate alterations in metabolic variables predictive of coronary heart disease are thought to begin occurring (25). None of the participants took medication. Exclusion criteria included the presence of a monogenic dyslipoproteinemia; use of medication known to affect lipid metabolism; chronic, metabolic, or acute disease; and significant weight change in the 6 mo before the experiment. Subjects with regular alcohol intake (>1 drink/d or >7 drinks/wk), unusual dietary habits such as vegetarianism, food allergies, or a dislike for foods included in the experimental diets were also excluded.

The study protocol was fully explained to the participants, who gave their written informed consent. The protocol was approved by the Clinical Research Ethics Committee of Laval University.

Test fats

The 2 experimental fats compared in the present study were a modified butter (CLA-B) that is enriched in CLA and a control butter (CON-B) that is low in CLA. Both butters were manufactured from milk produced by dairy cows from a Laval University dairy herd. To produce the experimental butters, cows were first fed a total mixed diet composed of concentrates plus corn and grass silages as the roughage source. After 3 wk, milk samples were obtained, and the CLA content of milk fat was determined. Cows with the lowest concentrations of CLA were identified, and their milk was collected to make CON-B. Once the production of CON-B was completed, the same cows were fed a similar diet to which 5% sunflower oil was added. Milk samples were taken after 3 wk, and cows with the greatest concentration of CLA continued consuming the diet for the purpose of milk collection and the manufacture of the CLA-B, which was rich in CLA. For both butters, raw milk was separated into cream and skim milk (Westfalia Separator AG, Oelde, Germany). Cream was then immediately pasteurized at 75 °C for 16 s by using a plate heat exchanger (type P20-HB; Alpha-Laval, Lund, Sweden) and stored at 14 °C for 24 h. The control and high-CLA creams were churned at 14 °C until butter granules were formed. The butter milk was then drained off, and the butter was salted (2%), transferred to 0.5-kg plastic containers, and kept at −20 °C until use.

Dietary intervention

Before the beginning of the 2 experimental phases, a registered dietitian instructed the selected participants in how to complete a 3-d (2 weekdays and 1 weekend day) food intake record to estimate their usual energy intakes and thereby set proper energy levels for the experimental period. Subjects began the study at the energy level closest to their usual energy intake. Because fluctuations in body weight can affect lipoprotein metabolism, weight was monitored every weekday before lunch, and the energy level was adjusted if body weight was found to have fluctuated >2.0 kg from baseline. During the daily attendance of the subjects at the metabolic kitchen, staffers were present to monitor consumption of the meals and to strongly encourage continued dietary compliance. Subjects were also provided with a daily reminder sheet on which they were required to check all food items consumed and to report any deviation from the diet, as well as any illness or use of medication. Any use of over-the-counter medications required prior approval by the study physician.

The 2 experimental diets were designed to be identical in food composition, except for the test fat, which was either CLA-B (rich in CLA) or CON-B (low in CLA). Both diets provided 40% of energy as fat, >60% of which was derived from experimental fats (Table 1). The experimental butters were incorporated in a variety of recipes, such as those for muffins, cakes, and sauces, and they were also used as a spread. Protein sources included boneless chicken breast (4 meals/wk), extra-lean pork (5 meals/wk), fish (2 meals/wk), veal (1 meal/wk), eggs (1 meal/wk), lean ham (1 meal/wk), and tofu (1 meal/wk). Of possible milk products, only fat-free milk, non-fat yogurt, and 1% fat cottage cheese were used, so that almost no milk fat sources besides the butter were present in the experimental diets. Seven-day cycle menus were developed for each experimental diet and were designed to supply the daily recommended allowances for essential nutrients of the Institute of Medicine, National Academy of Sciences (26). The nutritional composition of the diets and the dietary intake from the 3-d food intake records were assessed with the use of the Canadian government’s Nutrient File database (Health Canada, Ottawa, Canada, 1997) and the NUTRITION DATA SYSTEM FOR RESEARCH (NDS-R) software (version 4.03_31; Nutrition Coordinating Center, Minneapolis, MN).

Because the diets were administered under conditions in which body weight was to be held constant, subjects were asked to eat all the food and only the food that was provided to them by the metabolic kitchen staff for the duration of the 4-wk experimental periods. Consumption of alcohol 1 wk before and during each experimental period was forbidden. Consumption of caffeinated beverages such as soft drinks, coffee, and tea was limited to a maximum of 2 drinks/d; the portion size for soft drinks was a 16-oz can (≈474 mL), and that for coffee or tea was ≈8 oz (≈237 mL). On weekdays, subjects came to the metabolic kitchen daily to consume their lunch meal, and they were then
given their next dinner and breakfast meals in a package to take home. Weekend meals were distributed to the participants on Fridays.

Experimental design

A crossover design was used to compare the effects of the CLA-B diet that was rich in CLA with those of the CON-B diet that was low in CLA on plasma lipids, lipoproteins, and body composition. Eight subjects were randomly assigned to the CLA-B diet for the first 4-wk period and to the CON-B diet for the second 4-wk period. The other 8 subjects were assigned to the nutritional treatment sequence in reverse. Experimental periods were separated by an 8-wk washout period, during which the subjects resumed their usual diets to remove the residual effects of the preceding experimental diet on the tested variables. Subjects were blinded to dietary assignments and were not informed of their lipid or body-composition responses until the study was completed. Principal investigators and the laboratory technicians were also blinded to dietary assignments. Throughout the study, participants were asked to maintain their usual level of physical activity, which was evaluated by a weekly questionnaire completed by the subjects.

Fatty acid analyses

The fatty acid composition of the experimental fats was analyzed by using a gas chromatograph (HP 5890 chromatograph; Hewlett-Packard Co, Palo Alto, CA) equipped with a 60-m DB-23 capillary column (internal diameter: 0.32 mm; film thickness: 0.25 μm film thickness; J and W Scientific, Folsom, CA) and a flame ionization detector. At the time of the sample injection, the column temperature was 150 °C, and it was then ramped up at 5 °/min to 200 °C. Inlet and detector temperatures were 240 and 250 °C, respectively. The split ratio was 100:1. The flow rate for hydrogen carrier gas (Praxair Inc, Vanier, Canada) was 2.8 mL/min. Peak area was measured using a NELSON ANALYTIC SYSTEM 2600 (version 5; PE Nelson, Cupertino, CA). Each peak was identified with the use of pure methyl ester standards (Alltech, Deerfield, IL) on the basis of their retention times. The area-to-concentration ratio for all identified fatty acids was used to determine their respective concentrations after adjustment for the difference in molecular mass between the fatty acids and their methyl esters (27).

The CLA isomers in milk fat were analyzed with silver ion-HPLC according to published procedures by using 3 ChromShper 5 Lipids columns in series (ChromPack, Bridgewater, NJ) (28). The trans 18:1 isomers were separated with the use of silver ion-TLC and analyzed by using gas chromatography with a 100-m CP Sil 88 capillary column (ChromPack, Middelburg, Netherlands) (29, 30).

Composition of test fats

CLA-B was characterized by having more than 10 times as much CLA (4.22 g CLA/100 g fatty acids) as did CON-B (0.38 g CLA/100 g fatty acids). Cholesterol concentrations in CON-B

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TABLE 1

Nutritional composition of habitual and experimental diets

<table>
<thead>
<tr>
<th>Diets</th>
<th>Habitual</th>
<th>CON-B</th>
<th>CLA-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2695.5 ± 536.7</td>
<td>2950.0 ± 388.2</td>
<td>2937.5 ± 382.8</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>101.14 ± 18.84</td>
<td>112.8 ± 14.8</td>
<td>112.3 ± 14.6</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>15.3</td>
<td>15.3</td>
<td>15.3</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>342.69 ± 96.01</td>
<td>342.3 ± 45.0</td>
<td>340.8 ± 44.4</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>50.3</td>
<td>46.4</td>
<td>46.4</td>
</tr>
<tr>
<td>Lipids (g)</td>
<td>103.59 ± 22.70</td>
<td>134.7 ± 17.6</td>
<td>133.4 ± 17.4</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>34.8</td>
<td>40.9</td>
<td>40.9</td>
</tr>
<tr>
<td>SFA (g)</td>
<td>34.74 ± 8.11</td>
<td>63.2 ± 8.3</td>
<td>62.9 ± 8.2</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>11.6</td>
<td>19.3</td>
<td>19.3</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>21.77 ± 9.62</td>
<td>26.2 ± 3.5</td>
<td>26.1 ± 3.4</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>7.3</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>39.05 ± 9.98</td>
<td>35.0 ± 4.6</td>
<td>34.9 ± 4.5</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>13.1</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>303.4 ± 116.7</td>
<td>496.6 ± 65.3</td>
<td>494.5 ± 64.4</td>
</tr>
<tr>
<td>CLA (g)</td>
<td>NA</td>
<td>0.24 ± 0.03</td>
<td>2.59 ± 0.34</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>0.07</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

All values are x ± SD; n = 16. CON-B, control butter low in CLA; CLA-B, modified butter enriched in conjugated linoleic acid; SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; NA, not available.

1 Significantly different from habitual diet, P < 0.05 (paired t test).

2 Significantly different from the CON-B diet, P < 0.0001 (Student’s t test).
and CLA-B were 1.9 and 2.5 mg/g, respectively. A similarly greater cholesterol content was previously observed in butter made from the milk of cows fed sunflower seeds than in butter made from the milk of cows fed a low-fat control diet (31). This effect has been attributed to a greater amount of small fat globules due to the depression of fat in the milk from cows receiving sunflower seeds. A large proportion of cholesterol in milk fat is found at the level of the fat globule membrane. Reducing the size of the fat globule may have increased the membrane surface area and, consequently, the concentration of cholesterol in butter. The size of the fat globules was not ascertained in the current experiment, but the milk-fat content was actually 17% less in cows fed sunflower seeds. A large proportion of cholesterol in milk fat is obtained after precipitation of LDL in the infranatant fluid (density 1.006 g/mL) was isolated by ultracentrifugation. HDL particles were stored at 4 °C until processed.

The relative isomorphic distribution of CLA isomers in both experimental butters is shown in Figure 1. As could be expected from available data in the literature, the most abundant CLA isomer present in CON-B and CLA-B was cis-9, trans-11. Increasing the CLA content of the butter resulted in a smaller proportion of the trans-10, cis-12 isomer. The relative distribution of the trans octadecenoic acid (trans 18:1) isomers shown in Figure 2 indicates that the 18:1 trans-11 isomer (trans-vaccenic acid), a suspected precursor of cis-9, trans-11 CLA isomer, accounted for >50% of the 18:1 trans fatty acids in CLA-B.

**Anthropometric measurements and computed tomography**

At the beginning (day 1) and at the end (day 28) of each experimental period, waist circumference was measured midway between the lowest rib and the iliac crest by using a standard tape measure (32). Height and body weight were measured according to the procedures recommended at the Airlie Conference (33). Measures of variability for waist circumference, height, and body weight were ± 0.1 cm, ± 0.1 cm, and ± 0.1 kg, respectively. In the week after each experimental period (day 30 or 31), visceral adipose tissue accumulation (measure of variability: ± 0.1 mm) was assessed by computed tomography, which was performed on a Siemens Somatom DRH scanner (Erlangen, Germany) and analyzed as described previously by Després and al (34).

**Separation and analysis of plasma lipoproteins**

Fasting blood samples (12-h fast) were collected from an antecubital vein into evacuated tubes containing disodium EDTA at the beginning (day 1) and at the end (day 28) of each experimental period. Samples were then immediately centrifuged at 4 °C for 10 min at 1500 × g to obtain plasma samples, which were then stored at 4 °C until processed.

Triacylglycerol-rich lipoproteins (VLDL; density <1.006 g/mL) were isolated by ultracentrifugation. HDL particles were obtained after precipitation of LDL in the infranatant fluid (density >1.006 g/mL) with heparin and manganese chloride (35). HDL₂ and HDL₃ subfractions were separated by dextran-sulfate precipitation (36). Fasting concentrations of plasma and lipoprotein cholesterol and triacylglycerols were measured enzymatically by using an RA-500 analyzer, as previously described (37). LDL apolipoprotein (apo) B and HDL apo A-I were measured by

---

**TABLE 2**

Fatty acid composition of the 2 experimental butters, the added oils, and the resulting experimental fats

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control butter</th>
<th>Olive oil</th>
<th>Control fat</th>
<th>CLA butter</th>
<th>Coconut oil</th>
<th>Palm stearin</th>
<th>CLA fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:0</td>
<td>1.5</td>
<td>0.0</td>
<td>1.5</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>6:0</td>
<td>1.4</td>
<td>0.0</td>
<td>1.4</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>8:0</td>
<td>0.8</td>
<td>0.0</td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>10:0</td>
<td>1.7</td>
<td>0.0</td>
<td>1.7</td>
<td>0.8</td>
<td>0.1</td>
<td>0.0</td>
<td>0.9</td>
</tr>
<tr>
<td>12:0</td>
<td>2.3</td>
<td>0.0</td>
<td>2.3</td>
<td>1.2</td>
<td>1.1</td>
<td>0.0</td>
<td>2.3</td>
</tr>
<tr>
<td>14:0</td>
<td>6.3</td>
<td>0.0</td>
<td>6.3</td>
<td>4.5</td>
<td>0.4</td>
<td>0.1</td>
<td>5.0</td>
</tr>
<tr>
<td>14:1</td>
<td>0.6</td>
<td>0.0</td>
<td>0.6</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>15:0</td>
<td>0.7</td>
<td>0.0</td>
<td>0.7</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>16:0</td>
<td>17.9</td>
<td>1.3</td>
<td>19.3</td>
<td>11.8</td>
<td>0.2</td>
<td>6.4</td>
<td>18.5</td>
</tr>
<tr>
<td>16:1</td>
<td>0.8</td>
<td>0.1</td>
<td>0.9</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>17:0</td>
<td>0.4</td>
<td>0.0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>6.3</td>
<td>0.3</td>
<td>6.6</td>
<td>6.6</td>
<td>0.1</td>
<td>0.6</td>
<td>7.2</td>
</tr>
<tr>
<td>trans 18:1</td>
<td>0.7</td>
<td>0.0</td>
<td>0.7</td>
<td>4.7</td>
<td>0.0</td>
<td>0.0</td>
<td>4.7</td>
</tr>
<tr>
<td>cis 18:1</td>
<td>11.1</td>
<td>7.0</td>
<td>18.1</td>
<td>14.8</td>
<td>0.2</td>
<td>2.4</td>
<td>17.4</td>
</tr>
<tr>
<td>18:2</td>
<td>1.2</td>
<td>1.8</td>
<td>3.0</td>
<td>1.5</td>
<td>0.0</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td>18:3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>CLA</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>2.2</td>
<td>0.0</td>
<td>0.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Total (g)</td>
<td>54.4</td>
<td>10.6</td>
<td>65.0</td>
<td>52.3</td>
<td>2.3</td>
<td>10.4</td>
<td>65.0</td>
</tr>
</tbody>
</table>

Composition values are based on a 2500-kcal diet. CLA, conjugated linoleic acid.
rocket immunoelectrophoresis, as previously described by Laurell et al (35). The CV for cholesterol, triacylglycerols, and apo-lipoprotein measurements was <5% (38). Distinct subpopulations of LDL particles in whole plasma were separated by size with the use of nondenaturing 2–16% gradient gel electrophoresis (39). Particle size was quantified by densitometric scanning of Sudan Black–stained gels, using IMAGE MASTER 1 D Prime software (version 3.01; Amersham Pharmacia Biotech, Baie d’Urfé, Canada). LDL peak particle diameter was identified as the predominant subclass of LDL in each subject and was calculated from calibration curves by using plasma standards of known diameter. The CV of the calculated particle diameters was estimated as <0.6%. Leptin and C-reactive protein concentrations Plasma leptin concentrations were determined with the use of a highly sensitive, commercial, double-antibody radioimmunoassay (Human Leptin Specific RIA Kit; Linco, St Louis, MO) that detects leptin concentrations ≥0.5 ng/mL. The CV for the repeated assays ranged from 4.0% to 5.5% and from 6.5% to 8.5% for lower and higher plasma leptin concentrations, respectively (40). Plasma CRP concentrations were measured by using a commercially available, highly sensitive immunoassay with a monoclonal antibody coated with polystyrene particles (Behring Latex Enhanced on the Behring Nephelometer BN-100; Behring Diagnostic, Westwood, MA) as described previously (41). The run-to-run CV at CRP concentrations ranging from 1.0 to 10 µg/mL was <5%. Statistical analysis Data were analyzed by using SAS software (version 8.2; SAS Institute Inc, Cary, NC). Repeated-measures analysis of variance, adjusted for crossover designs, using the general linear model was performed to identify differences between experimental treatments (42). In the analysis of variance model, the carryover effects for each of the variables studied were tested by introducing a term referring to the sequence in which the dietary treatments were given. Although no significant sequence effect was found, a significant interaction was observed between treatment and sequence for VLDL-triacylglycerol and LDL apo B, which suggested a different response to the 2 diets across time. Therefore, only the results of the first period were analyzed for those 2 variables. Paired t tests were used to identify differences within the experimental diets only when significant differences were found between dietary treatments. Group averages are reported as means ± SDs. CRP values were logarithmically transformed before statistical analysis to achieve normal distribution. Two subjects who were found, during the course of the study, to have CRP concentrations >10 mg/L, which suggested the presence of bacterial infection or inflammation, were excluded from statistical analysis for that variable (43). Differences were considered significant at P < 0.05. RESULTS Characteristics of subjects at baseline The 16 participants were overweight or obese, but their mean blood lipid profiles were within the normal range (44); that is, the HDL-cholesterol concentrations were slightly low and triacylglycerol concentrations were slightly high (Table 3). Data from the 3-d food record completed by the subjects before study onset were compiled and compared with the nutritional composition of the experimental diets (Table 1). That some subjects lost weight despite the fact that their experimental energy intake was higher than their reported habitual energy intake (Table 1) suggested that, on average, subjects underestimated their energy intake, a behavior that has been reported in 50% of obese persons (45). Anthropometric measures and body composition The diet-induced variations in body weight, waist circumference, and plasma leptin concentrations did not differ significantly between the 2 groups (Table 4). These findings are consistent with those for body fat distribution, which, although taken only at the end of each experimental diet to minimize the subjects’ exposure to radiation, showed that there was no significant difference in the accumulation of visceral and subcutaneous adipose tissue between the diet groups.
TABLE 3
Physical characteristics and plasma lipid profile of the subjects at baseline

<table>
<thead>
<tr>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
</tr>
</tbody>
</table>

All values are \( \bar{x} \pm SD \); range in parentheses. Measurements were obtained on day 1 of the experimental period.

Plasma and lipoprotein cholesterol

When compared with the CON-B diet, the CLA-B diet resulted in a significantly smaller reduction in plasma total cholesterol, but the changes in VLDL, LDL, and HDL cholesterol and HDL subfractions did not differ significantly between the 2 diets (Table 5). The greater reduction in total cholesterol after the CON-B diet was accompanied by a significantly greater decrease in total: HDL and LDL:HDL cholesterol after the CON-B diet than after the CLA-B diet.

Plasma and lipoprotein lipids and apolipoproteins

The magnitude of the changes in plasma total cholesterol and VLDL, LDL, and HDL triacylglycerols did not differ significantly between the 2 diets (Table 6). The CON-B diet resulted in a significantly greater reduction in plasma apo B concentrations than did the CLA-B diet. The magnitude of the variation in VLDL apo B, LDL apo B, and HDL apo A-I concentrations did not differ between the 2 groups (Table 7).

TABLE 4
Body-composition variables and plasma leptin concentrations at the beginning (day 1) and at the end (day 28) of each diet intervention

<table>
<thead>
<tr>
<th></th>
<th>CON-B</th>
<th>CLA-B</th>
<th>( P ) (between diets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>94.4 ± 13.4</td>
<td>94.3 ± 13.3</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>93.1 ± 13.0</td>
<td>92.5 ± 12.9</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–1.31 ± 1.23</td>
<td>–1.77 ± 1.46</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>105.6 ± 9.8</td>
<td>105.5 ± 10.1</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>105.0 ± 10.2</td>
<td>104.3 ± 10.1</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–0.97 ± 1.46</td>
<td>–1.25 ± 2.14</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Visceral adipose tissue area (cm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 30 or 31</td>
<td>163.7 ± 67.6</td>
<td>156.8 ± 70.9</td>
<td>0.34</td>
</tr>
<tr>
<td>Subcutaneous adipose tissue area (cm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 30 or 31</td>
<td>324.46 ± 117.1</td>
<td>320.88 ± 116.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Plasma leptin (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>10.76 ± 5.05</td>
<td>10.77 ± 5.76</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>8.15 ± 4.44</td>
<td>7.72 ± 4.69</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–2.61 ± 3.06</td>
<td>–3.05 ± 3.02</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

\( 1 \) \( n = 16 \), CON-B, control butter low in CLA; CLA-B, modified butter, enriched in conjugated linoleic acid.

\( 2 \) Comparisons between absolute changes induced by the 2 experimental diets (repeated-measures ANOVA).

\( 3 \) \( \bar{x} \pm SD \) (all such values).

\( 4 \) Measured in the week after each experimental period.

LDL peak particle diameter and plasma CRP concentrations

Neither diet affected the LDL peak particle diameter or the concentration of plasma CRP, a marker of low-to-moderate systemic inflammation (Table 8).

DISCUSSION

Improvements in the lipid profile after CLA supplementation were reported in several animal species (7, 8, 46), but little is known about the effect of CLA, particularly when CLA is provided in the form of naturally occurring foods, on the lipid profile of humans. The purpose of our study thus was to compare the effects of a CLA-B, naturally enriched in CLA by the addition of sunflower oil to the diet of dairy cows, and a CON-B, low in CLA, on body composition, the lipid profile, and other cardiovascular disease risk factors in overweight and obese men.

We found that, in overweight and obese men, in contrast with observations in animals, the ingestion of the CLA-B diet for 4 wk did not improve the lipid profile more than did the ingestion of the CON-B diet. If anything, the CLA-B diet resulted in a significantly smaller reduction in plasma cholesterol and in total:HDL cholesterol, presumably in part because of the tendency of the CLA-B diet to induce a smaller reduction in VLDL cholesterol than did the CON-B diet. It is noteworthy that a diet that contained 40% of energy as fat and that was relatively high in saturated fatty acids did not induce the negative effects so often associated with this type of dietary habit. It is interesting that obese men have been found not only to underreport their daily energy intakes but also to selectively underreport their fat intakes (47). This suggests that the habitual dietary intakes of our study participants, calculated to be 34.9%, may have been underreported, which could partially explain why the consumption of an experimental diet providing 40% of energy as fat did not lead to a worsening of their lipoprotein or lipid profile. Although studies...
by others of the effects of CLA in humans (20, 48) remain scarce, their findings are globally consistent with those in our current study.

The lack of amelioration in the health profile in the current study could be related in part to the CLA isomers used. We used a modified butter naturally rich in CLA, in which nearly 80% of total CLA was in the form of cis-9, trans-11 CLA (Figure 1). In more recent animal studies, the desirable effects of CLA on blood lipids and body composition were associated with the trans-10, cis-12 isomer rather than with the cis-9, trans-11 isomer (8, 12, 49–51). Data on the potential benefit of the trans-10, cis-12 CLA isomer for the lipid profile in humans remain scarce, but human studies reported no improvement in blood lipids after supplementation with either trans-10, cis-12 or cis-9, trans-11 CLA isomers (52, 53).

The lack of improvement in the lipid profile after the CLA-B diet could also be partly explained by the stereospecific distribution of fatty acids on the triacylglycerol, which has been suggested to affect the processing of dietary fats. Indeed, the hydrolysis of triacylglycerols by lipoprotein lipase and the uptake of remnant particles by the liver were reported to be slower when the fatty acid in the sn-2 position is saturated than when it is unsaturated (54). Furthermore, fatty acids seem to be absorbed better when they are in the sn-2 position than when they are in the sn-1 or sn-3 position (55, 56). In the current study, palm stearin was added to the CLA-B diet to increase its palmitic acid content to make it more comparable to that of the CON-B diet. However, up to 59% of palmitic acid in palm stearin is in the sn-2 position (57, 58), whereas only 42% of the palmitic acid in milk fat takes up that position (59). Incorporating palm stearin, which contains more cholesterol-raising palmitic acid (60) in the sn-2 position, to the CLA-B may thus have rendered it less likely to improve plasma and LDL-cholesterol concentrations. One could argue that the absence of a decrease in plasma and LDL cholesterol after the CLA-B diet could in part be due to that diet’s greater content of 18:1 trans-11 (trans-vaccenic acid; Figure 2) compared with the content in the CON-B diet. It has indeed been suggested that the consumption of trans fatty acids increases LDL-cholesterol and lowers HDL-cholesterol concentrations (61). In that regard, Willett et al (62) found a positive significant association between the intake of trans fatty acids formed by the partial hydrogenation of vegetable oils and the incidence of

<table>
<thead>
<tr>
<th>TABLE 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma and lipoprotein cholesterol concentrations at the beginning (day 1) and at the end (day 28) of each diet intervention.</td>
</tr>
<tr>
<td>CON-B</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>HDL₂ cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>HDL₃ cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>LDL: HDL cholesterol</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
<tr>
<td>Total:HDL cholesterol</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 28</td>
</tr>
<tr>
<td>Change (day 28 – day 1)</td>
</tr>
</tbody>
</table>

1 n = 16. CON-B, control butter low in CLA; CLA-B, modified butter enriched in conjugated linoleic acid.
2 Comparisons between absolute changes induced by the 2 experimental diets.
3 ± SD (all such values).
4 Significantly different from prediet values, P < 0.01 (repeated-measures ANOVA).
coronary heart disease. However, there was no significant association between the intake of trans isomers from animal sources and the incidence of coronary heart disease. This lack of association was thought to be due to the distinct structure of the predominant trans isomer in ruminant fat, trans-vaccenic acid (62). In addition, 18:1 trans-11 appears to be partly converted to cis-9, trans-11 18:2 (CLA) via the action of the 8-9 desaturase present in the intestinal epithelium, liver, and adipose tissue of humans (63, 64). Hence, in the current study, it is unlikely that the greater amount of trans fatty acids in the CLA-B was responsible for the lack of improvement in the lipid profile, because these trans fats more likely provided an additional source of CLA when subjects consumed the CLA-B diet.

The CLA-induced reduction in adiposity and weight gain observed in animals (9–13) could also be a mechanism for mediating the improvements in the lipid profile often reported with CLA supplementation (5, 7). However, consistent with the lack of an improvement in the lipid profile with the CLA-B diet in the current study, the reductions in body weight and waist circumference, as well as in the visceral and subcutaneous adipose tissue areas after the diet intervention, were comparable with the CLA-B and CON-B diets. Another study, conducted in healthy

### TABLE 6

<table>
<thead>
<tr>
<th></th>
<th>CON-B</th>
<th>CLA-B</th>
<th>P (between diets)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total triacylglycerol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.75 ± 0.82</td>
<td>1.56 ± 0.88</td>
<td></td>
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<tr>
<td>Day 28</td>
<td>1.33 ± 0.73</td>
<td>1.31 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.42 ± 0.39</td>
<td>−0.24 ± 0.54</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>VLDL triacylglycerol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.17 ± 0.69</td>
<td>1.38 ± 0.95</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.72 ± 0.35</td>
<td>0.94 ± 0.82</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.45 ± 0.42</td>
<td>−0.44 ± 0.34</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>LDL triacylglycerol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.24 ± 0.11</td>
<td>0.24 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.21 ± 0.09</td>
<td>0.22 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.02 ± 0.05</td>
<td>−0.02 ± 0.06</td>
<td>0.61</td>
</tr>
<tr>
<td><strong>HDL triacylglycerol (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.20 ± 0.05</td>
<td>0.20 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.20 ± 0.04</td>
<td>0.19 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.01 ± 0.03</td>
<td>−0.01 ± 0.04</td>
<td>0.90</td>
</tr>
</tbody>
</table>

1 n = 16. CON-B, control butter low in CLA; CLA-B, modified butter enriched in conjugated linoleic acid.
2 Comparisons between absolute changes induced by the 2 experimental diets (repeated-measures ANOVA).
3 ± SD (all such values).
4 Only the first period of the study was analyzed for VLDL triacylglycerol (n = 8) because of a significant interaction between the treatment and the sequence in which dietary treatments were given.

### TABLE 7

<table>
<thead>
<tr>
<th></th>
<th>CON-B</th>
<th>CLA-B</th>
<th>P (between diets)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma apolipoprotein B (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.04 ± 0.28</td>
<td>0.98 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.96 ± 0.26</td>
<td>0.98 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.09 ± 0.18</td>
<td>−0.00 ± 0.17</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>VLDL apolipoprotein B (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.16 ± 0.06</td>
<td>0.13 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.11 ± 0.04</td>
<td>0.11 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.04 ± 0.04</td>
<td>−0.03 ± 0.04</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>LDL apolipoprotein B (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.84 ± 0.16</td>
<td>1.04 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.74 ± 0.18</td>
<td>0.87 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.09 ± 0.10</td>
<td>−0.17 ± 0.14</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>HDL apolipoprotein A-I (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.21 ± 0.17</td>
<td>1.22 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>1.08 ± 0.19</td>
<td>1.11 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Change (day 28 − day 1)</td>
<td>−0.13 ± 0.10</td>
<td>−0.11 ± 0.07</td>
<td>0.43</td>
</tr>
</tbody>
</table>

1 n = 16. CON-B, control butter low in CLA; CLA-B, modified butter enriched in conjugated linoleic acid.
2 Comparisons between changes induced by the 2 experimental diets (repeated-measures ANOVA).
3 ± SD (all such values).
4 Only the first period of the study was analyzed for LDL apolipoprotein B (n = 8) because of a significant interaction between the treatment and the sequence in which dietary treatments were given.
women ingesting 3.9 g CLA/d for 63 d and in which only 23% of the CLA was in the form of the trans-10, cis-12 isomer, also showed that CLA had no significant effect on body composition. This lack of effect of CLA was interpreted to be due to the type of CLA isomer used; the most effective in reducing body fat in preadipocytes and animal models reportedly is the trans-10, cis-12 isomer (8, 12, 49–51). In support of this hypothesis, supplementation of 3 g of an isomeric blend containing primarily the cis-9, trans-11 CLA isomer had no effect on the body weight or BMI of nonobese (BMI <25; 23) or overweight and obese subjects (BMI 27–35; 53).

We also failed to observe significant changes in the concentration of CRP, a systemic marker for inflammation and, thereby, for processes leading to atherosclerosis, after either the CON-B or CLA-B diet. In contrast, a study reported that, compared with ingestion of a placebo containing olive oil, trans-10, cis-12 CLA supplementation led to significantly greater plasma CRP concentrations in overweight men (65). This difference from the current study may be related in part to the difference in CLA isomer used, because our butter fat was mostly rich in cis-9, trans-11 CLA.

Whereas our results seem to be in agreement with most of the studies aimed at unraveling the effect of CLA in humans, it must be stressed that some aspects of our study design—such as the number of subjects used, their health profile, and the length of the experimental periods—may not have been optimal for observing the number of subjects used, their health profile, and the length of the experimental periods, it has been shown that blood lipid variables normally stabilize within 4 wk of being subjected to strictly controlled dietary intervention conditions (67). However, the beneficial effects of CLA on blood lipids in animal studies were usually observed after nearly 2 mo, and thus it is possible that a longer experimental period may have been necessary to replicate comparable results in humans.

In conclusion, our results indicate that, in overweight or obese men with only a slight deterioration of their lipid profile, a 10-fold CLA enrichment of butter via the addition of sunflower oil to the feed of dairy cows does not induce significantly greater metabolic effects than are observed with the consumption of a control butter diet low in CLA. It remains unclear whether this lack of improvement in the lipid profile and body composition can be attributed to the nature of the CLA isomer found in butter, to the amount of CLA ingested, or to the length of administration of the experimental diets. Further human studies are needed to evaluate the individual effects of different CLA isomers on lipoprotein metabolism and in persons with a more detrimental lipid profile at study onset.

We are indebted to John KG Kramer for his help in the determination of the conjugated linoleic acid isomers and of the trans octadecenoic acid isomers within the experimental butters. We express our gratitude to the metabolic kitchen staff and participants for their dedication and cooperation. NB and PYC were the principal investigators for the study; IG was a co-investigator; PC was responsible for the screening and medical supervision of the study participants; LC is a research assistant to NB; JD is a research assistant to PYC; BL was responsible for the measurement of the nontraditional risk factors; and SD coordinated the study, performed statistical analyses, analyzed the data, and wrote the manuscript. None of the authors had a personal or financial conflict of interest.

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57. Hunter JE. Studies on effects of dietary fatty acids as related to their position on triglycerides. Lipids 2001;36:655–68.
Increasing dietary palmitic acid decreases fat oxidation and daily energy expenditure

C Lawrence Kien, Janice Y Bunn, and Figen Ugrasbul

ABSTRACT

Background: Oleic acid (OA) is oxidized more rapidly than is palmitic acid (PA).

Objective: We hypothesized that changing the dietary intakes of PA and OA would affect fatty acid oxidation and energy expenditure.

Design: A double-masked trial was conducted in 43 healthy young adults, who, after a 28-d, baseline, solid-food diet (41% of energy as fat, 8.4% as PA, and 13.1% as OA), were randomly assigned to one of two 28-d formula diets: high PA (40% of energy as fat, 16.8% as PA, and 16.4% as OA; n = 21) or high OA (40% of energy as fat, 1.7% as PA, and 31.4% as OA; n = 22). Differences in the change from baseline were evaluated by analysis of covariance.

Results: In the fed state, the respiratory quotient was lower (P = 0.01) with the high OA (0.86 ± 0.01) than with the high-PA (0.89 ± 0.01) diet, and the rate of fat oxidation was higher (P = 0.03) with the high-OA (0.0008 ± 0.0001) than with the high-PA (0.0005 ± 0.0001 mg · kg fat-free mass−1 · min−1) diet. Resting energy expenditure in the fed and fasting states was not significantly different between groups. Change in daily energy expenditure in the high-OA group (9 ± 60 kcal/d) was significantly different from that in the high-PA group (−214 ± 69 kcal/d; P = 0.02 or 0.04 when expressed per fat-free mass).

Conclusions: Increases in dietary PA decrease fat oxidation and daily energy expenditure, whereas decreases in PA and increases in OA had the opposite effect. Increases in dietary PA may increase the risk of obesity and insulin resistance.

SUBJECTS AND METHODS

Study design

Forty-three healthy, nonobese, young adults aged 21–34 y participated in a randomized, double-masked, controlled trial. The study was conducted at the General Clinical Research Center (GCRC) of The Ohio State University Medical Center, Columbus, OH (n = 3), and at the GCRC of the University of Texas Medical Branch (UTMB), Galveston, TX (n = 40). The protocol was approved by each institution’s review committee for human subjects and the General Advisory Committee of the GCRC at the respective institutions. The same solid-food diet and formulas were used at both sites, and procedures for measuring energy expenditure, energy expenditure, and fatty acids.

INTRODUCTION

The term “Mediterranean diet” typically refers to the dietary pattern of people living in Greece and southern Italy in the early 1960s; such persons had a long life expectancy and a low risk of coronary heart disease (1). The Mediterranean diet was low in saturated fatty acids (SFAs; 7–8% of energy) and moderate in fat (25–35% of energy), and the fat source consisted primarily of olive oil. Oleic acid (18:1; OA) constitutes ≈72% of the SFAs in olive oil (1, 2).

As opposed to oxidation, storage of FA in tissues other than adipose tissue may interfere with normal cellular function and lead to an increased risk of the metabolic syndrome (3–6). Studies using labeled FAs have shown that OA and other unsaturated FAs are more readily oxidized than are saturated FAs (SFAs) (7–11). These tracer data, per se, do not necessarily imply that changing the pattern of dietary FAs would affect total FA oxidation, but some studies have shown that humans or animals ingesting diets enriched with polyunsaturated FAs (PUFAs) and monounsaturated FAs (MUFAs) exhibit higher total FA oxidation, energy expenditure, or both than when ingesting diets containing more SFAs and less PUFAs or MUFAs (12–16).

However, the effects of dietary FA composition on energy expenditure in rodents may be mediated by changes in brown adipose tissue metabolism (14, 16) and, thus, are not relevant to humans. Thus, we conducted a dietary trial in humans to determine the effects on FA oxidation and energy expenditure of 2 different dietary FA patterns: one typical of the North American diet (equal contributions of OA and PA to the total dietary FAs) and one more typical of the Mediterranean diet (much higher OA and much lower PA).

Key Words: Palmitic acid, oleic acid, fat oxidation, energy expenditure, fatty acids

1 From the Department of Pediatrics, University of Texas Medical Branch (CLK and FU) and Shriners Hospital for Children (CLK), Galveston, TX, and the Department of Medical Biostatistics, University of Vermont, Burlington, VT (JB).

2 Supported by NIH grant R01 DK55384. The studies were conducted at the GCRC of the University of Texas Medical Branch at Galveston and The Ohio State University, funded by grants M01 RR 00073 and M01 RR 00034 from the National Center for Research Resources, NIH, USPHS. In addition, the University of Vermont General Clinical Research Center (funded by grant RR010109 from the National Center for Research Resources, NIH, USPHS) provided data analysis support. Ross Products Division of Abbott Laboratories, Inc. provided the experimental formula.

3 Reprints not available. Address correspondence to CL Kien, E203 Given Building, 89 Beaumont Avenue, University of Vermont College of Medicine, Burlington, VT 05405-0068. E-mail: cl.kien@uvm.edu.

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expenditure and body composition were also similar. The inclusion criteria were as follows: age 18-35 y; healthy and no need for medication; body mass index (BMI; in kg/m²) >18, <30, and below the 95th percentile (17); and no current use of drugs that could affect lipid metabolism, including nicotine. Subjects with a BMI > 25 were included to extend the clinical relevance of the study to subjects at risk for the metabolic syndrome. Exclusion criteria were as follows: vegetarian diet, habitual fat intake < 30% of energy, pregnancy, fasting glucose, HDL-cholesterol concentration below the 5th percentile for age and sex, and total cholesterol, LDL-cholesterol, and triacylglycerol concentrations above the 95th percentile (18). As part of the screening history and physical examination, the subjects were asked whether any of their family members manifested or died from obesity, type 2 diabetes, or noncongenital heart disease.

Female subjects were initiated into the study regardless of their menstrual cycle, but each was studied at the same phase of the cycle for both the run-in and baseline diets and the experimental diet. There appeared to be only minor effects of the menstrual cycle on FA turnover (19) and thermic effect of feeding but none on resting energy expenditure (REE), FA oxidation, or the average REE after the meal (20). Moreover, one-half of our 22 female subjects were taking oral contraceptives and not ovulating.

Each subject was studied twice for 28 d, first during the consumption of a solid-food, run-in diet (baseline preformula value) and then during consumption of 1 of the 2 experimental liquid-formula diets (postformula value). Randomization to 1 of the 2 formula treatments was stratified for sex. All food and drinks, except for water, during both phases of the study were provided by the GCRC. Energy intake was adjusted during both diet phases to maintain body weight. All subjects were required to eat breakfast at the GCRC every day of the 8-wk study, but many of the subjects chose to eat one or more additional meals in our dining room each day, especially during the week. Subjects who did not keep appointments or who had unusual changes in body weight in relation to energy intake, especially during the formula diet phase of the study (because the run-in diet provided a good approximation of energy needs), were dropped from the study (21). On this basis, one female subject in the high-PA group was excluded from the original data set; thus, although there were originally 44 subjects in the study (n = 22 in each formula group; 11 women and 11 men in each group), the results are reported for 43 subjects (n = 21 in the high-PA group; n = 22 in the high-OA group).

The main purpose of the baseline, solid-food diet was to establish which subjects would comply with the protocol and to establish baseline data on all subjects on the same diet. We analyzed the content of the 9 separate meals composing the run-in diet (Covance Laboratories, Madison, WI). On the basis of the average of these 9 analyses, the composition of this solid-food diet was as follows: 14.6% of energy as protein, 45.1% of energy as carbohydrate, and 40.8% of energy as fat. The FA composition consisted of 13.1% of energy as OA (32.4 g/100 g total fat) and 8.4% of energy as PA (20.8 g/100 g fat). For comparison, a post hoc analysis of the dietary histories obtained from most of the participants for the period preceding the run-in diet indicated the following composition of the habitual diet: 18.0% of energy as protein, 49.6% of energy as carbohydrate, and 33.0% of energy as fat (11.0% of energy as SFA and 11.0% of energy as MUFA).

The macronutrient and FA compositions of the 2 experimental formula diets (Ross Products Division Inc, Abbott Laboratories Inc, Columbus, OH) are shown in Table 1. Compared with the baseline diet, the high-PA diet contained 100% more PA and 25% more OA as percentages of energy (n = 21); the high-OA diet was identical to the high-PA diet, except that, compared with the baseline diet, it contained 80% less PA and 140% more OA as percentages of energy (n = 22). The formulas were patterned after formulas used for nutritional support and contained adequate vitamins and minerals on a per kilocalorie basis. The fat blend in the high-PA formula was 91% palm oil, 6% high-OA sunflower oil, and 3% soy lecithin. The fat blend of the high-OA formula was 90% palm oil, 97% high-OA sunflower oil, and 3% soy lecithin. The 2 oils used in these formulas are naturally occurring and are not intersterified. Although fat absorption was not measured in this study, and fat intake did not prove to be a significant covariate in our statistical analysis, fat absorption is typically >90% for these oils. The positional distribution of FAs on the triacylglycerol molecule was typical for these oils. These oils contain naturally occurring phytochemicals, especially palm oil, but we have no basis for thinking that any of these compounds could affect the processes under study. These oils typically contain >95% triacylglycerol. Safflower oil contains ≈3% 1,3-diacylglycerol, but diacylglycerol is not detectable in palm oil.

On the 28th day of each diet period, after an evening meal at 1800 (≈33% of the daily energy intake), indirect calorimetry was performed overnight in both the fed and fasting states (22). Except for bedside bathroom privileges, the subjects remained in bed from 1700 to 0720. Oxygen consumption and carbon dioxide

### Table 1

<table>
<thead>
<tr>
<th>Composition of the experimental diets¹</th>
<th>High-PA diet</th>
<th>High-OA diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloric distribution (% of energy)</td>
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<tr>
<td>Protein</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Fat</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Fatty acid profile (g/100 g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic</td>
<td>42.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Oleic</td>
<td>41.0</td>
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</tr>
<tr>
<td>Linoleic</td>
<td>11.4</td>
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<td>Stearic</td>
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<td>4.1</td>
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<td>α-Linolenic</td>
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<td>Myristic</td>
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<td>Palmitoleic</td>
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<td>Eicosapentaenoic</td>
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<td>Docosahexaenoic</td>
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<tr>
<td>Arachidonic</td>
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<td>Fatty acid class (%)</td>
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<tr>
<td>Saturated</td>
<td>47.1</td>
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<tr>
<td>Monounsaturated</td>
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<td>78.4</td>
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<tr>
<td>Polysaturated</td>
<td>11.7</td>
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<td>Fractional energy (%)</td>
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<tr>
<td>Total saturated</td>
<td>18.8</td>
<td>3.3</td>
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<tr>
<td>12:0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>16:0</td>
<td>16.8</td>
<td>1.7</td>
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<td>18:0</td>
<td>1.6</td>
<td>1.6</td>
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<tr>
<td>Total monounsaturated</td>
<td>16.5</td>
<td>31.4</td>
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<tr>
<td>18:1n−9</td>
<td>16.4</td>
<td>31.4</td>
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<tr>
<td>Total polysaturated</td>
<td>4.8</td>
<td>5.3</td>
</tr>
<tr>
<td>18:3n−3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>18:2n−6</td>
<td>4.6</td>
<td>5.2</td>
</tr>
</tbody>
</table>

¹ PA, palmitic acid; OA, oleic acid.


production (Vmax SPECTRA 29; Sensor Medics Corp, Yorba Linda, CA) were measured for 20 min each time at 60-min intervals after the meal for 7 h (fed state) and then at 120-min intervals until 13 h after the meal (fasting state) (22). Urinary urea nitrogen was measured during the 14-h interval, and protein oxidation was estimated for both the fed (1720–0120) and the fasting (0120–0720) periods (22). REE and substrate utilization were calculated according to standard procedures by using urinary urea nitrogen measurements as estimates of protein oxidation (22–24) and the Weir equation (25). Urinary urea nitrogen was measured at the UTMB clinical laboratory according to the urease quinolinium dye method (Vitros 250 Chemistry System; Ortho-Clinical Diagnostics, Rochester, NY). Respiratory quotient (RQ), rates of fat and carbohydrate oxidation, and REE were estimated for both the fed and fasting states from the average value for each period (22). The flow sensor was manually calibrated with a syringe before each daily measurement. This procedure was repeated if there seemed to be instability in the measurements during the overnight studies. The gas sensors were calibrated before each 20-min measurement.

Body composition was measured just before the solid-food diet began and at the end of each 28-d diet phase by dual-energy X-ray absorptiometry (Delphi QDR 4500A Bone Densitometer; Hologic, Bedford, MA), and body energy (BE) was estimated by using Atwater conversion values for fat mass (FM; 9.3 kcal/g) and body protein (4.1 kcal/g protein), assuming 0.2 g protein/g fat-free mass (FFM).

\[
BE = (9.3 \text{ kcal/g FM}) \times (1 \text{ g protein/5 g FFM}) + [4.1 \text{ kcal/g protein}] 
\]

BE (kcal) = (9.3 kcal/g × FM; in g) + [4.1 kcal/g protein] × (1 g protein/5 g FFM) × FFM; in g] (I)

In addition, daily energy expenditure (DEE) was determined from the average energy intake (EI) and the change in BE (ΔBE) estimated from the dual-energy X-ray absorptiometry measurements:

\[
\text{DEE} = \text{EI} - \Delta \text{BE}
\]

where ΔBE is the BE at the end of the formula diet period (postformula value) minus the BE at the end of the run-in diet period (preformula value).

In 39 subjects (n = 21 in the high-OA group and n = 18 in the high-PA group), physical activity was estimated for 7d during the third week of each diet phase by using a uniaxial accelerometer worn on the wrist (model 71164; Computer Science and Applications, Manufacturing Technology, Fort Walton Beach, FL). Melanson and Freedson (26) previously showed that this position for the accelerator provided the best index of energy expenditure in the field setting.

### Statistical analysis

Results are expressed as means ± SEMs. The term statistical significance was applied to differences with a 2-tailed P value ≤0.05. The main approach to the statistical analysis was an analysis of covariance; this consisted of examining the change in the respective outcome variable during the experimental diet with the baseline value included as the covariate. All analyses were carried out by using either SAS (SAS System for WINDOWS, version 8.1; SAS Institute Inc, Cary, NC) (27) or SPSS (SPSS Base 10.0; SPSS Inc, Chicago, IL). Our central hypothesis was that the high-OA diet would be associated with a greater rate of fat oxidation and energy expenditure than would the high-PA diet, when corrected for the preformula value obtained from subjects consuming the same, run-in, solid-food diet.

### RESULTS

#### Body composition

BMI was not significantly different between the high-PA and high-OA groups, either at the end of the run-in diet (23.7 ± 0.7 and 24.1 ± 0.6, respectively) or after the formula diet (23.7 ± 0.7 and 23.9 ± 0.6, respectively). At the end of the formula diet, BMI was >25.0 in 8 subjects in the high-PA group and in 6 subjects in the high-OA group (or >25.2 in 6 and 5, respectively). The groups did not manifest statistically significant differences in body composition before the formula diets (Table 2). However, there were group differences in body-composition changes during the experimental formula diets. There was a trend for a larger increase in fat mass in the high-PA group (0.52 ± 0.13 kg) than in the high-OA group (0.14 ± 0.14 kg) (P = 0.06). There was no

### Table 2

Body composition of the high–oleic acid (OA) and the high–palmitic acid (PA) diet groups.

<table>
<thead>
<tr>
<th></th>
<th>Preformula phase</th>
<th>Postformula phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 22)</td>
<td>(n = 21)</td>
</tr>
<tr>
<td>BMI</td>
<td>24.1 ± 0.6</td>
<td>23.7 ± 0.7</td>
</tr>
<tr>
<td>DXA body mass</td>
<td>71.1 ± 2.5</td>
<td>70.0 ± 2.7</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>25.1 ± 1.7</td>
<td>25.1 ± 1.6</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>17.7 ± 1.3</td>
<td>17.5 ± 1.3</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>53.4 ± 2.4</td>
<td>52.4 ± 2.3</td>
</tr>
<tr>
<td>BE (kcal)</td>
<td>209 000 ± 12 000</td>
<td>206 000 ± 13 000</td>
</tr>
</tbody>
</table>

Change from baseline

<table>
<thead>
<tr>
<th></th>
<th>Preformula phase</th>
<th>Postformula phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 22)</td>
<td>(n = 21)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>—</td>
<td>0.41 ± 0.22</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>—</td>
<td>0.14 ± 0.14</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>—</td>
<td>-0.60 ± 0.24</td>
</tr>
<tr>
<td>BE (kcal)</td>
<td>—</td>
<td>835 ± 1207</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Preformula phase</th>
<th>Postformula phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 22)</td>
<td>(n = 21)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>25.1 ± 1.7</td>
<td>0.66 ± 0.18</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>17.7 ± 1.3</td>
<td>0.52 ± 0.13</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>53.4 ± 2.4</td>
<td>-0.39 ± 0.18</td>
</tr>
<tr>
<td>BE (kcal)</td>
<td>209 000 ± 12 000</td>
<td>4542 ± 1182</td>
</tr>
</tbody>
</table>

^1 All values are x ± SEM; BE, total body energy estimated from fat mass (FM) and fat-free mass (FFM) (see text for equation); DXA, dual-energy X-ray absorptiometry.

^2 Significantly different from the preformula high-OA diet (analysis of covariance): ^3P = 0.05; ^4P = 0.06; ^5P = 0.04.
significant difference between the high-PA and high-OA groups in the change in FFM (−0.39 ± 0.18 and −0.60 ± 0.24 kg, respectively; \(P = 0.52\)). This combination of body-composition changes resulted in an increase (\(P = 0.05\)) in body mass in the high-PA group (0.1 ± 2.0 kg) and a decrease in body mass in the high-OA group (−0.5 ± 0.2). There was no significant difference between groups in the increase in percentage body fat. There was a substantially larger increase (\(P = 0.04\)) in BE in the high-OA group (4542 ± 1182 kcal) than in the high-OA group (835 ± 1207 kcal).

Substrate oxidation

Mean RQ in the fed state (RQ fed) decreased in the high-OA group (from 0.87 ± 0.01 to 0.86 ± 0.01) and increased in the high-PA group (0.88 ± 0.01 to 0.89 ± 0.01) \((P = 0.01)\) during the formula diet (Table 3). Similarly, we observed a corresponding increase in the rate of fat oxidation in the fed state (g/min) during the formula diet in the high-OA group and a decrease in the high-PA group \((P = 0.04; \text{Table } 3)\). When fat oxidation in the fed state was expressed as \(g \cdot kg^{-1} \cdot min^{-1}\), the value increased during the formula diet in the high-OA group (from 0.0007 ± 0.0001 to 0.0008 ± 0.0001) and decreased in the high-PA group (from 0.0007 ± 0.0001 to 0.0005 ± 0.0001) \((P = 0.03)\). Conversely, the rate of carbohydrate oxidation in the fed state (g/min) increased less during the formula diet in the high-OA group (from 0.1311 ± 0.0096 to 0.1430 ± 0.0071) than in the high-PA group (from 0.1508 ± 0.0096 to 0.1743 ± 0.0092) \((P = 0.02)\). There was no significant difference between groups in the change in fasting RQ or the fasting rate of fat oxidation (g/min) (Table 3). We also estimated the weighted average daily RQ, assuming that 17 h of the day represented the fed condition; the change in average daily RQ in the high-OA group \((−0.0083 ± 0.0107)\) was significantly different from that in the high-PA group \((+ 0.0085 ± 0.0080)\) \((P = 0.04)\). The change in the average daily rate of fat oxidation (g/min) was not statistically significant \((P = 0.14; \text{Table } 3)\). However, the average daily rate of carbohydrate oxidation increased less in the high-OA group \((0.0107 ± 0.0113)\) than in the high-PA group \((0.0191 ± 0.0095)\) \((P < 0.05)\). There were no significant differences between experimental groups in the fasting rate of carbohydrate oxidation or in the fed or fasting rates of protein oxidation. The addition of a family history of obesity, diabetes, or heart disease as additional covariates did not appreciably affect the \(P\) value for the change in RQ or rate of fat oxidation.

Physical activity, energy intake, and energy expenditure

Physical activity (accelerations/min) was not significantly different between the high-OA and high-PA groups, either during the run-in diet (989 ± 76 and 917 ± 58, respectively) or during the formula period (927 ± 89 and 890 ± 59, respectively). Pre- and postformula energy intakes were not significantly different between the 2 groups, and pre- and postformula fasting and fed REE values and weighted daily averages were not significantly different between the 2 groups (Table 4). However, DEE remained essentially unchanged in the high-OA group (9 ± 60 kcal/d) but decreased in the high-PA group \((−214 ± 69 \text{ kcal/d}; \ P = 0.02; \text{Table } 4)\). Similarly, DEE/FFM (kcal · kg\(^{-1} \cdot \text{d}^{-1}\)) decreased in the high-PA group \((−3.4 ± 1.4)\) but remained essentially the same in the high-OA group \((0.5 ± 1.3; \ P = 0.04; \text{Table } 4)\). The addition of a family history of obesity, diabetes, or heart disease as additional covariates did not appreciably affect the \(P\) value for the change in either DEE or DEE/FFM.

**DISCUSSION**

Increasing the PA intake lowered fat oxidation in the fed state, whereas lowering the PA intake and markedly increasing the OA intake had the opposite effect. Although our subjects who consumed the high-PA diet underwent a large increase in PA intake, the SFA intake of the high-PA group was still considered fairly typical for many residents of North America (28). At least one previous study in humans (12) has suggested that PUFAs, compared with SFAs, tend to increase total FA oxidation. Our study, which involved a long interval of feeding, showed that increasing the ratio of MUFAs to SFAs in the diet also increased fat oxidation without an apparent, partial, compensatory reduction in fasting FA oxidation (12). We should emphasize that our measurements of fat oxidation and REE were carried out in the resting state, overnight, at the end of each study period. Perhaps the results would have been different had these measurements been made while the subjects were carrying out the usual tasks of daily

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**TABLE 3**

Respiratory quotient (RQ) and fatty acid oxidation in the high–palmitic acid (PA) and high–oleic acid (OA) diet groups

<table>
<thead>
<tr>
<th></th>
<th>Preformula phase</th>
<th>Postformula phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-OA diet</td>
<td>High-PA diet</td>
</tr>
<tr>
<td></td>
<td>((n = 22))</td>
<td>((n = 21))</td>
</tr>
<tr>
<td>Average RQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed state(^2)</td>
<td>0.87 ± 0.01</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>Fasting state(^4)</td>
<td>0.87 ± 0.01</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>Fatty acid oxidation (g/min)(^5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed state</td>
<td>0.040 ± 0.006</td>
<td>0.038 ± 0.006</td>
</tr>
<tr>
<td>Fasting</td>
<td>0.034 ± 0.005</td>
<td>0.034 ± 0.004</td>
</tr>
<tr>
<td>Average daily fatty acid oxidation (g/min)</td>
<td>0.038 ± 0.006</td>
<td>0.033 ± 0.004</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm \text{SEM} \).
\(^2\) Estimated from individual measurements obtained 1, 2, 3, 4, 5, 6, and 7 h after the evening meal.
\(^3\) Significantly different from the preformula high-OA diet (analysis of covariance); \(^4\) \(P = 0.01\), \(^6\) \(P = 0.04\).
\(^4\) Estimated from observations made 9, 11, and 13 h after the evening meal.
\(^5\) Estimated from the weighted average of the fat oxidation rate during the fed (17 h) and fasting (7 h) periods of the day.
living in a room calorimeter. These differential effects of dietary FA composition on total FA oxidation may be important because fat oxidation during feeding could affect the accumulation of lipid intermediates in skeletal muscle that, in turn, affect insulin sensitivity (3, 29–32). This study did not examine the mechanisms for the results we observed, but our observations are consistent with the concept that dietary FAs may affect the fundamental mechanisms for mitochondrial fatty acid oxidation (33–37).

Our study also suggests that an increase in the ratio of SFAs to MUFA results in a change in body composition. After being corrected for energy intake, the calculated total DEE decreased in the high-PA group but increased modestly in the high-OA group. These results are partially supported by previous studies in rats, which showed that FAs have differential effects on the thermic effect of food (16). The high-OA group showed little change in which showed that FAs have differential effects on the thermic effect of food (16). The high-OA group showed little change in the high-PA group but increased modestly in the high-OA group. Although there is no apparent reason why the diets would affect physical activity, physical activity was also not different between groups. We do acknowledge that REE was estimated during the fed (17 h) and fasting (7 h) periods of the day.

Finally, the energetic efficiency of muscle contraction can be affected by changes in muscle fiber type or in the expression of specific muscle proteins (45).

In our study, for practical reasons relating to our subjects’ need to work and go to school, REE was measured in the early evening were the prescribed values, and their validity is obviously dependent on subject compliance with the protocol. We can propose no plausible reason for why only one dietary group (high PA) systematically would choose to eat additional food during the formula diet, which was prohibited by the protocol. However, ingestion of unmeasured, additional food energy would lead to a relative gain in BE for the recorded energy intake; thus, calculated DEE would be artifactually low. Thus, it seems unlikely that the difference in DEE between groups would be affected by errors in estimation of food intake, but there still may be inherent uncertainty in the magnitude of the difference between groups.

The experimental diets did not differentially affect REE in either the fed or fasting states, and yet, DEE decreased in the high-PA group. Although there is no apparent reason why the diets would affect physical activity, physical activity was also not different between groups. We do acknowledge that REE was assessed only on the last day of each diet period, whereas the estimation of DEE was based on the entire 28 d of observation in both diet groups. Therefore, it is possible that the REE value did not totally reflect REE during the entire study period. Nevertheless, because DEE, but not REE and physical activity, was apparently affected differentially by the diets, one could surmise that dietary PA and OA may have had differential effects on the energy cost of physical activity. 

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Energy intake (EI) and balance and resting (REE) and daily (DEE) energy expenditure in the high–palmitic acid (PA) and high–oleic acid (OA) diet groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preformula phase</td>
</tr>
<tr>
<td></td>
<td>High-OA diet (n = 22)</td>
</tr>
<tr>
<td></td>
<td>High-OA diet (n = 22)</td>
</tr>
<tr>
<td>Average energy intake (kcal/d)</td>
<td>2704 ± 90</td>
</tr>
<tr>
<td>REE (kcal/min)</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Fasting state</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Average REE (kcal/min)</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td>DEE (kcal/d)</td>
<td>2682 ± 96</td>
</tr>
<tr>
<td>(kcal · kg FFM⁻¹ · d⁻¹)</td>
<td>51.3 ± 1.9</td>
</tr>
<tr>
<td>Change in DEE from baseline</td>
<td></td>
</tr>
<tr>
<td>(kcal/d)</td>
<td>—</td>
</tr>
<tr>
<td>(kcal · kg FFM⁻¹ · d⁻¹)</td>
<td>—</td>
</tr>
</tbody>
</table>

1 All values are x ± SEM.
2 Weighted average of the values during the fed (17 h) and fasting (7 h) periods of the day.
3 Estimated from the equation DEE = EI – ΔBE, where ΔBE is the change in total body energy.
4 Significantly different from the preformula high-OA diet (analysis of covariance): *P = 0.02, **P = 0.04.
and overnight. Although not reported here, REE measured just before the evening meal was sometimes higher than the values measured just after the meal, which suggests that physical activity increased energy expenditure but that this effect was dissipated while the subjects rested in bed during indirect calorimetry. DEE was expressed on a daily basis but was calculated from the average daily energy intake and the changes in body composition over the 28-d study period. So, the marked difference in the results derived from the REE measurement and the DEE estimate suggests that the diets could have differentially affected energy expenditure during the daytime, when the subjects were also physically active to varying degrees. The oxidation of OA, but not of PA, seems to be relatively increased by prior exercise, which in our free-living subjects was apt to occur during the daytime hours (46). Recently, Stavinoha et al (47) provided evidence that the responsiveness of genes targeted by peroxisome-proliferator activated receptor α is enhanced during the dark cycle of rats, when the animals presumably feed or at least search for food in the wild. If these data can be extrapolated to humans, who tend to eat during the day, this mechanism may be relevant to our results, i.e., there could be an interaction between the thermic effect of food and exercise that could not be detected with the design of this study.

In conclusion, increases in dietary PA lowered FA oxidation and increased BE in healthy, nonobese, young adults. Thus, calculated DEE decreased in the high-PA group. In contrast, the change to a Mediterranean-type fat pattern resulted in increased fat oxidation in the fed state and prevented the decrease in DEE that was observed with the high-PA diet. These findings may have relevance to the prevention of obesity, the metabolic syndrome, or type 2 diabetes. The results may have been the consequence of fundamental alterations in gene or protein expression.

We thank our subjects for their thoughtful participation in the study, the nursing and dietary staffs of both The Ohio State University GCRC and the UTMB GCRC, and Travis Solley and Mary Schmitz-Brown for technical assistance. We are grateful to Vikkie Mustad (Ross Products Division, Abbott Laboratories) for her helpful comments regarding the planning of the study, Judah Rosenblatt and Steve Owen (UTMB) for statistical consultation, Steven Heymsfield (consultant on grant) for his overall guidance and advice, bottles Laboratories) for her helpful comments regarding the planning of the study, and assisted in the preparation of the manuscript. None of the authors had any financial conflict of interest to declare.

REFERENCES

Long-chain n–3 polyunsaturated fatty acids in plasma in British meat-eating, vegetarian, and vegan men

Magdalena S Rosell, Zouë Lloyd-Wright, Paul N Appleby, Thomas AB Sanders, Naomi E Allen, and Timothy J Key

ABSTRACT
Background: Plasma concentrations of long-chain n–3 polyunsaturated fatty acids are lower in vegetarians and in vegans than in omnivores. No data are available on whether these concentrations differ between long- and short-term vegetarians and vegans.

Objectives: We compared plasma fatty acid composition in meat-eaters, vegetarians, and vegans and examined whether the proportions of eicosapentaenoic acid (20:5n–3; EPA), docosapentaenoic acid (22:5n–3; DPA), and docosahexaenoic acid (22:6n–3; DHA) were related to the subjects’ duration of adherence to their diets or to the proportions of plasma linoleic acid (18:2n–6; LA) and α-linolenic acid (18:3n–3; ALA).

Design: The present cross-sectional study included 196 meat-eating, 231 vegetarian, and 232 vegan men in the United Kingdom. Information on anthropometry, diet, and smoking habits was obtained through a questionnaire. Total fatty acid composition in plasma was measured.

Results: The proportions of plasma EPA and DHA were lower in the vegetarians and in the vegans than in the meat-eaters, whereas only small differences were seen for DPA. Plasma EPA, DPA, and DHA proportions were not significantly associated with the duration of adherence to a vegetarian or vegan diet. This finding suggests that when animal foods are wholly excluded from the diet, the endogenous production of EPA and DHA results in low but stable plasma concentrations of these fatty acids. Am J Clin Nutr 2005;82:327–34.

KEY WORDS n–3 Fatty acids, vegetarians, vegans, cross-sectional study

INTRODUCTION
Apart from being a source of energy, fatty acids have a wide range of physiologic functions. Many fatty acids can be produced endogenously; however, n–3 and n–6 polyunsaturated fatty acids are essential fatty acids that must be provided in the diet. The long-chain metabolites of these essential fatty acids are needed for cellular membrane functions and the production of eicosanoids, which play a role in inflammatory reactions, blood pressure control, and platelet aggregation. These essential fatty acids also influence gene regulation; for example, they act as ligands of peroxisome proliferator-activated receptors that are involved in growth and development. Of particular interest are the long-chain n–3 fatty acids eicosapentaenoic acid (20:5n–3; EPA) and docosahexaenoic acid (22:6n–3; DHA), which are abundant in oily fish and therefore are also referred to as the marine fatty acids. Although their action is not fully understood, these fatty acids probably account for the inverse relation between fish consumption and the risk of developing coronary heart disease and stroke that has been observed in epidemiologic studies (1–4). Apart from its probable cardioprotective effects, DHA is, with arachidonic acid, one of the 2 most prevalent polyunsaturated fatty acids in brain and retinal phospholipids and plays a role in normal neurotransmission and visual function.

The major dietary source of EPA and DHA is seafood, but they can also be found in lean red meat, in organ meats such as liver and brain, and in eggs (5, 6); very small amounts are found in dairy products. Consequently, vegetarians, who do not eat meat or fish, and vegans, who in addition exclude eggs and dairy products from their diets, have very low or negligible intakes of EPA and DHA. However, vegetarians and vegans have a relatively high intake of linoleic acid (18:2n–6; LA) (7, 8), which is largely derived from plant sources. EPA and DHA can also be synthesized in the body from α-linolenic acid (18:3n–3; ALA); however, this conversion is limited in humans (9), and possibly more in men than in women (10, 11). The conversion may be further suppressed by a high intake of LA, because the desaturation of ALA and LA involves the same rate-limiting Δ6 desaturase enzyme (12). Indeed, trials have shown that ALA supplementation increases plasma EPA and DPA but has little effect on DHA (13–15). As a result, vegetarians and vegans, who have a low dietary intake of DHA (and a correspondingly high intake of LA), have lower amounts of EPA and DHA in blood and adipose tissue than do omnivores (16–19).

1 From the Cancer Research UK Epidemiology Unit, University of Oxford, Oxford, United Kingdom (MSR, PNA, NEA, and TJK), and the Nutrition, Food and Health Research Center, King’s College London, London, United Kingdom (ZL-W and TABS).
2 The EPIC-Oxford study is supported by Cancer Research UK, the Medical Research Council, and the European Commission under the Europe Against Cancer Programme. MSR was supported by a grant from the Swedish Council for Working Life and Social Research.
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Accepted for publication April 21, 2005.
Previous studies that compared the plasma fatty acid composition in different dietary groups involved a small number of subjects, and few studies included both vegetarians and vegans. The present study reports the plasma fatty acid composition in British meat-eating, vegetarian, and vegan men and explores the effect of duration of adherence to a vegetarian or vegan diet on plasma fatty acid composition.

SUBJECTS AND METHODS

Subjects

The subjects of the study were men selected from the Oxford arm of the European Prospective Investigation into Cancer and Nutrition (EPIC-Oxford). The EPIC-Oxford cohort consists of 65,500 participants aged ≥20 y who lived in the United Kingdom between 1993 and 1999. The aim of the EPIC-Oxford study was to recruit participants with a wide range of diets by targeting vegetarians, vegans, and the general UK population (20). The study protocol was approved by a multicenter research ethics committee. The participants were recruited through collaborating general practitioners, vegetarian and vegan societies, and vegetarian and health food magazines or were friends or relatives of other participants. The participants completed a questionnaire that included details on age, anthropometry, diet, smoking, and other lifestyle factors. Blood samples were obtained from 19,700 volunteers. The present study is based on a sample of men who were originally selected to investigate the relation between diet and the hormones involved in the etiology of prostate cancer (21). The sample consisted of all the vegan men who were recruited to EPIC-Oxford between 1994 and 1997 and gave a blood sample and approximately equal numbers of meat-eaters and vegetarians who were randomly selected from the meat-eating and vegetarian men who gave blood samples. The men had no self-reported history of cancer and were not taking medication that influenced hormone concentrations. For the current study, we excluded subjects without data on plasma fatty acid composition (n = 12) and subjects reporting that they were taking fish oil supplements (n = 25), which left 659 men for the analyses (196 meat-eaters, 231 vegetarians, and 232 vegans).

Diet groups and duration of diet adherence

The classification of diet group and duration of adherence to a vegetarian or vegan diet was based on the following 4 questions: 1) “Do you eat any meat (including bacon, ham, poultry, game, meat pies, and sausages)? (Yes or No). If no, how old were you when you last ate meat?”; 2) “Do you eat any fish? (Yes or No). If no, how old were you when you last ate fish?”; 3) “Do you eat any dairy products (including milk, cheese, butter, and yogurt)? (Yes or No). If no, how old were you when you last ate dairy products?”; and 4) “Do you eat any eggs (including eggs in cakes and other baked foods)? (Yes or No). If no, how old were you when you last ate eggs?” Men who reported that they ate meat were classified as meat-eaters. Men who reported that they did not eat meat or fish but reported that they ate dairy products or eggs were classified as vegetarians; the duration of adherence to a vegetarian diet was calculated as the age at recruitment minus the age at which the respondent last ate meat or fish. Men who reported that they did not eat any foods of animal origin were classified as vegans; the duration of adherence to a vegan diet was calculated as the age at recruitment minus the age at which the respondent last ate any foods of animal origin.

Plasma fatty acid composition

Blood samples were obtained from the participants on average 5 mo after they had completed the questionnaire. The blood samples were collected into 10-mL Safety-Monovettes (Sarstedt, Nümbrecht, Germany) containing 1 mL of 3.13% trisodium citrate. The blood samples were then sent by mail to the central laboratory, where they were separated into serum, plasma, buffy coat, and red blood cell fractions. The plasma samples were frozen at -70 °C and stored for ∼4 y until analyzed. The concentration of total plasma esterified and nonesterified fatty acids was measured by transmethylation of the total lipids with methanolic HCl in the presence of a pentadecanoic acid internal standard and then analyzing the methyl esters by capillary gas-liquid chromatography, as described previously (22). The proportions of fatty acids in the plasma were calculated as the concentration of the fatty acid (in mg/L) divided by the sum of the concentrations of all the fatty acids analyzed. The stability of EPA, DPA, DHA, LA, and ALA was assessed by examining the associations between the percentages of these fatty acids in the plasma and the duration of storage at -70 °C; no significant associations were found.

Fish and egg intake

Dietary intake during the 12 mo prior to recruitment was assessed with the use of a 130-item food-frequency questionnaire (FFQ) (18). Six questions related to the consumption of fish: 1) fried fish in batter, such as fish and chips; 2) fish fingers and fish cakes; 3) other fresh or frozen white fish (eg, cod, haddock, plaice, sole, and halibut); 4) fresh or canned oily fish (eg, mackerel, kippers, tuna, salmon, sardines, and herring); tuna was included because fresh tuna is classed as an oily fish, although canned tuna is not); 5) shellfish (eg, crab, prawns, and mussels); and 6) fish roe and taramasalata. One question related to the intake of eggs. Each question had 9 possible answers: never or <1/mo, 1–3/mo, 1/wk, 2–4/wk, 5–6/wk, 1/d, 2–3/d, 4–5/d, and ≥6/d. For each fish food question, the subjects were categorized into 3 groups of intake: never or <1/mo, 1–3/mo, and ≥1/wk. The range of intake of eggs was wider than the intake of fish, and analyses were also performed with egg intake as a continuous variable. The frequency of oily fish intake was also calculated from 7-d food records that were completed by 135 of the meat-eating men.

Other variables

Information on age, height, body weight, smoking habits, and intakes of energy, macronutrients, fiber, and alcohol was obtained through the questionnaire. Body mass index (BMI) was calculated as body wt (in kg)/height2 (in m).

Statistical analysis

The differences between the diet groups in age, BMI, and other characteristics were calculated with an analysis of variance or a chi-square test for categorical variables. Arithmetic means or geometric means [for 14:0, 16:1, 18:0, ratio LA:ALA, cis-9,trans-11-conjugated linoleic acid (CLA), 22:1, 20:3, and 20:5, for which the distributions were more normal when log-transformed] and 95% CIs of the proportions of the fatty acids by
n–3 FATTY ACIDS IN VEGETARIANS AND VEGANS 329

RESULTS

The mean age of the participants was 46.8 y (range: 20–78 y). The meat-eaters were on average 9 y older than the vegans (Table 1). Mean BMI and the prevalence of smoking were highest in the meat-eaters and lowest in the vegans. In the vegetarians, the median duration of time since becoming vegetarian was 11 y and ranged from <1 to 76 y.

Table 1

Characteristics of the men by diet group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Meat-eaters (n = 196)</th>
<th>Vegetarians (n = 231)</th>
<th>Vegans (n = 232)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>52.2 ± 10.87</td>
<td>46.2 ± 12.1</td>
<td>42.8 ± 13.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.2 ± 3.7</td>
<td>23.3 ± 2.9</td>
<td>22.7 ± 3.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Smoking [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>94 (48)</td>
<td>121 (52)</td>
<td>139 (60)</td>
<td>—</td>
</tr>
<tr>
<td>Past</td>
<td>69 (35)</td>
<td>90 (39)</td>
<td>70 (30)</td>
<td>—</td>
</tr>
<tr>
<td>Current, &lt;10 cigarettes/d</td>
<td>19 (10)</td>
<td>13 (6)</td>
<td>14 (6)</td>
<td>—</td>
</tr>
<tr>
<td>Current, ≥10 cigarettes/d</td>
<td>14 (7)</td>
<td>7 (3)</td>
<td>9 (4)</td>
<td>—</td>
</tr>
<tr>
<td>Years since becoming a vegetarian or vegan [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–4 y</td>
<td>—</td>
<td>40 (17)</td>
<td>73 (31)</td>
<td>—</td>
</tr>
<tr>
<td>5–8 y</td>
<td>—</td>
<td>57 (25)</td>
<td>52 (22)</td>
<td>—</td>
</tr>
<tr>
<td>9–15 y</td>
<td>—</td>
<td>52 (23)</td>
<td>73 (31)</td>
<td>—</td>
</tr>
<tr>
<td>≥16 y</td>
<td>—</td>
<td>72 (31)</td>
<td>28 (12)</td>
<td>—</td>
</tr>
<tr>
<td>Daily energy and nutrient intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy (MJ)</td>
<td>10.6 ± 2.7</td>
<td>9.2 ± 2.4</td>
<td>8.5 ± 2.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>34.1 ± 5.4</td>
<td>31.1 ± 5.7</td>
<td>29.8 ± 7.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Saturated fatty acids (% of energy)</td>
<td>11.9 ± 3.0</td>
<td>8.7 ± 2.9</td>
<td>4.9 ± 1.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (% of energy)</td>
<td>10.9 ± 2.3</td>
<td>8.6 ± 2.3</td>
<td>8.6 ± 3.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (% of energy)</td>
<td>5.4 ± 2.0</td>
<td>6.1 ± 2.4</td>
<td>8.2 ± 3.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Carbohydrates (% of energy)</td>
<td>43.5 ± 5.9</td>
<td>51.5 ± 6.3</td>
<td>53.5 ± 7.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>16.7 ± 2.8</td>
<td>13.2 ± 2.0</td>
<td>12.7 ± 1.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>18.6 ± 6.6</td>
<td>25.1 ± 8.0</td>
<td>29.1 ± 10.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>12.1 (5.8, 29.8)²</td>
<td>7.2 (1.8, 14.0)</td>
<td>4.5 (0.4, 13.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Oily fish intake [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 serving/mo</td>
<td>49 (25)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2–3 servings/mo</td>
<td>88 (45)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>≥1 serving/wk</td>
<td>59 (30)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fried fish intake [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 serving/mo</td>
<td>71 (36)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2–3 servings/mo</td>
<td>80 (41)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>≥1 serving/wk</td>
<td>45 (23)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other white fish intake [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 serving/mo</td>
<td>40 (20)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2–3 servings/mo</td>
<td>88 (45)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>≥1 serving/wk</td>
<td>68 (35)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Differences in means for the continuous variables were calculated by using ANOVA, and differences in proportions for the categorical variables were calculated by using a chi-square test.

² Median; 25th and 75th percentiles in parentheses (all such values).

The diet group were calculated from fitted values of multiple linear regression models that were adjusted for age (categorized as 10-y age bands), BMI (categorized by quartiles), cigarette smoking (never, former, current <10 cigarettes/d, current ≥10 cigarettes/d), and alcohol consumption (categorized by quartiles). The mean fatty acid proportions in each diet group were calculated by using a partial Pearson’s correlation that was adjusted for age, BMI, smoking, and alcohol intake. Adjusted means of the proportions of the long-chain polyunsaturated fatty acids by categories of fish intake, together with their 95% CIs and P values for linear trend, were calculated with regression models as described above. All analyses were performed by using version 8.1 of the STATA statistical package (Stata Corp, College Station, TX).
The corresponding median duration for the vegans was 7 y and ranged from <1 to 36 y. There were significant differences in the intakes of energy, macronutrients, fiber, and alcohol between the diet groups. According to the FFQ, 30% of the meat-eaters reported eating 1 serving of oily fish/wk. However, of these men only 13 (6%) reported eating ≥1 serving of oily fish/wk (data not shown). The reported intake of fried fish in the FFQ was slightly lower and the reported intake of other white fish was somewhat higher than the intake of oily fish. Of the meat-eating men, only 7%, 4%, and 3% consumed ≥1 serving of fish cake or fish fingers, shellfish, and fish roe, respectively, per week (data not shown).

The mean concentration of total plasma fatty acids, adjusted for age, BMI, smoking, and alcohol intake, was 15% lower in the vegetarians and 25% lower in the vegans than in the meat-eaters (Table 2). To allow for these differences, the means of the separate fatty acids in the diet groups are presented as proportions, which were calculated as the concentration of each fatty acid divided by the concentration of total fatty acids. Overall, the most prevalent fatty acids were palmitic acid, oleic acid, and LA, which together composed, on average, 79% (range: 41–89%) of the assessed fatty acids. Myristic acid, stearic acid, palmitoleic acid, ALA, dihomo-γ-linolenic acid, arachidonic acid, and DHA each composed ≈1–7% of the assessed fatty acids. The proportions of arachidonic acid, cis-9,trans-11-CLA, trans-10,cis-12-CLA, erucic acid, EPA, and DPA were each <1%.

Of the saturated fatty acids, the proportions of myristic and palmitic acid were 40% and 11% lower, respectively, in the vegans than in the meat-eaters; there were no significant differences in the proportions of plasma stearic or arachidic acid between diet groups. The proportion of palmitoleic acid in the vegans was less than one-half that in the meat-eaters, whereas no significant differences were seen for the other monounsaturated fatty acids. Of the polyunsaturated fatty acids, the proportion of LA was 22% higher in the vegans than in the meat-eaters; there were no significant differences between the diet groups.

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### Table 2

Concentrations of total plasma fatty acids and proportions of plasma fatty acids by diet group.

<table>
<thead>
<tr>
<th>Plasma fatty acids</th>
<th>Meateaters (n = 196)</th>
<th>Vegetarians (n = 231)</th>
<th>Vegans (n = 232)</th>
<th>Difference, vegetarians – meat-eaters</th>
<th>Difference, vegans – vegetarians</th>
<th>Difference, vegans – meat-eaters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fatty acids (mg/L)</td>
<td>3125 (2978, 3271)</td>
<td>2670 (2549, 2791)</td>
<td>2346 (2219, 2472)</td>
<td>−15%</td>
<td>−12%</td>
<td>−25%</td>
</tr>
<tr>
<td>Fatty acid proportions (% of total fatty acids)</td>
<td></td>
<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>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>1.28 (1.16, 1.41)</td>
<td>1.06 (0.98, 1.16)</td>
<td>0.77 (0.70, 0.84)</td>
<td>−17%</td>
<td>−27%</td>
<td>−40%</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>22.82 (22.15, 23.49)</td>
<td>22.07 (21.52, 22.63)</td>
<td>20.25 (19.67, 20.83)</td>
<td>−3%</td>
<td>−8%</td>
<td>−11%</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>6.61 (6.30, 6.95)</td>
<td>6.57 (6.31, 6.84)</td>
<td>6.84 (6.56, 7.14)</td>
<td>−1%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Arachidic acid (20:0)</td>
<td>0.24 (0.22, 0.26)</td>
<td>0.24 (0.22, 0.25)</td>
<td>0.23 (0.21, 0.24)</td>
<td>0%</td>
<td>−4%</td>
<td>−4%</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid (16:1−n−7)</td>
<td>2.00 (1.71, 2.34)</td>
<td>1.23 (1.07, 1.40)</td>
<td>0.92 (0.81, 1.06)</td>
<td>−39%</td>
<td>−25%</td>
<td>−54%</td>
</tr>
<tr>
<td>Oleic acid (18:1−n−9)</td>
<td>22.65 (21.91, 23.40)</td>
<td>22.64 (22.02, 23.25)</td>
<td>22.06 (21.41, 22.70)</td>
<td>0%</td>
<td>−3%</td>
<td>−3%</td>
</tr>
<tr>
<td>Erucic acid (22:1)</td>
<td>0.06 (0.05, 0.08)</td>
<td>0.07 (0.06, 0.09)</td>
<td>0.06 (0.05, 0.08)</td>
<td>17%</td>
<td>−14%</td>
<td>0%</td>
</tr>
<tr>
<td>Polynsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA (18:2−n−6)</td>
<td>30.42 (29.17, 31.67)</td>
<td>33.59 (32.55, 34.62)</td>
<td>37.12 (36.04, 38.21)</td>
<td>10%</td>
<td>11%</td>
<td>22%</td>
</tr>
<tr>
<td>ALA (18:3−n−3)</td>
<td>1.30 (1.20, 1.41)</td>
<td>1.39 (1.30, 1.48)</td>
<td>1.41 (1.32, 1.50)</td>
<td>7%</td>
<td>1%</td>
<td>9%</td>
</tr>
<tr>
<td>LA:ALA</td>
<td>21.86 (18.88, 25.31)</td>
<td>24.53 (21.73, 27.69)</td>
<td>27.48 (24.21, 31.21)</td>
<td>12%</td>
<td>12%</td>
<td>26%</td>
</tr>
<tr>
<td>c9,trans-11-CLA</td>
<td>0.21 (0.17, 0.26)</td>
<td>0.14 (0.11, 0.16)</td>
<td>0.08 (0.06, 0.09)</td>
<td>−33%</td>
<td>−42%</td>
<td>−62%</td>
</tr>
<tr>
<td>cis-9,trans-11-CLA</td>
<td>0.16 (0.14, 0.17)</td>
<td>0.15 (0.14, 0.17)</td>
<td>0.13 (0.12, 0.14)</td>
<td>−6%</td>
<td>−13%</td>
<td>−19%</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid (20:3−n−6)</td>
<td>1.24 (1.12, 1.37)</td>
<td>1.04 (0.96, 1.13)</td>
<td>1.05 (0.96, 1.15)</td>
<td>−16%</td>
<td>1%</td>
<td>−15%</td>
</tr>
<tr>
<td>DPA (22:5−n−3)</td>
<td>0.72 (0.65, 0.80)</td>
<td>0.52 (0.48, 0.57)</td>
<td>0.34 (0.31, 0.37)</td>
<td>−28%</td>
<td>−35%</td>
<td>−53%</td>
</tr>
<tr>
<td>EPA (20:5−n−3)</td>
<td>0.81 (0.77, 0.86)</td>
<td>0.76 (0.72, 0.79)</td>
<td>0.72 (0.68, 0.76)</td>
<td>−6%</td>
<td>−5%</td>
<td>−11%</td>
</tr>
<tr>
<td>DHA (22:6−n−3)</td>
<td>1.69 (1.59, 1.79)</td>
<td>1.16 (1.07, 1.24)</td>
<td>0.70 (0.61, 0.79)</td>
<td>−31%</td>
<td>−40%</td>
<td>−59%</td>
</tr>
</tbody>
</table>

1 All values are arithmetic mean (95% CI). For the meat-eaters, the proportions of 1 serving of oily fish/wk were compared with the meat-eaters, the difference was significant for the other mono-unsaturated fatty acids. The proportions of 1 serving of fish cake or fish fingers, shellfish, and fish roe, respectively, per week (data not shown).

2 P < 0.001.

3 P < 0.05.
between those seen in the meat-eaters and those seen in the vegans.

For each of the long-chain \(n-3\) polyunsaturated fatty acids, the mean proportions in plasma were lower in the vegetarians and lower still in the vegans than in the meat-eaters. EPA was 28\% lower in the vegetarians and 53\% lower in the vegans, and DHA was 31\% and 59\% lower in the vegetarians and vegans, respectively, than in the meat-eaters. The differences in DPA were smaller and were significant only between the meat-eaters and the vegans.

The mean proportions of the long-chain polyunsaturated fatty acids in the vegetarians and the vegans, which were divided into groups based on how many years they had followed a vegetarian or vegan diet, are shown in Figure 1. The mean proportions in the meat-eaters are included for comparison. The proportions of plasma EPA, DPA, and DHA were not significantly different in the vegetarians or in the vegans with regard to how long they had followed their diets.

To investigate the hypothesis that dietary intakes of LA and ALA are related to the endogenous production of the long-chain \(n-3\) polyunsaturated fatty acids, we explored the correlations between the plasma compositions of these fatty acids by diet group (Table 3). The proportion of LA was inversely correlated with EPA in all 3 diet groups, inversely correlated with DPA in the vegans, and inversely correlated with DHA in the vegetarians and in the vegans. Although the correlation between LA and DHA was stronger in the vegans than in the meat-eaters, the correlation coefficients were not significantly different between the diet groups. ALA was positively correlated only with DPA in all 3 diet groups. The ratio of LA:ALA was generally inversely correlated with EPA, DPA, and DHA, most noticeably among the vegans.

When the analysis was restricted to the meat-eaters, no associations were found between the proportions of EPA, DPA, and DHA in plasma and the intake of oily fish that was reported in the FFQ (Table 4); nor were there any associations with the intake of other fish (fried fish, white fish, shellfish, or fish roe) or with the intake of eggs (data not shown). When fish intakes were combined as a total fish intake variable, no associations with the proportions of EPA, DPA, or DHA were seen (data not shown). In the vegetarians, no associations were found between the proportions of EPA, DPA, or DHA and the intake of eggs (data not shown). In the 135 meat-eaters who also completed a 7-d food record, the frequency of oily fish intake reported in the FFQs showed statistically significant agreement with the frequency of oily fish intake reported in the food records (weighted \(k = 0.35\)). No associations were found between the frequency of oily fish intake reported in the food records and the proportions of EPA, DPA, and DHA in plasma (data not shown).

**DISCUSSION**

We measured the concentration of fatty acids in plasma in a large sample of British meat-eating, vegetarian, and vegan men. The plasma samples were stored at \(-70^\circ\text{C}\) for a median of 4 y before the assays were conducted; the percentages of the major fatty acids examined in the present study appeared to be reasonably stable during storage because the percentages were not associated with storage time and because the concentrations of the fatty acids in our study were similar to those observed in freshly collected plasma from the same subjects (23). Overall, the vegetarians and the vegans had substantially lower concentrations of total plasma fatty acids than did the meat-eaters. This is consistent with the lower concentration of total blood cholesterol found in vegetarians and in vegans than in meat-eaters, which has been reported previously (21). Because the concentration of total fatty acids influences the concentrations of individual fatty acids, we compared the fatty acids as proportions of the total fatty acids. The proportions of fatty acids in plasma partly reflect the composition of fatty acids in the diet: the fatty acids found in triacylglycerols reflect the dietary intake of the past few hours, the fatty

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**Figure 1.** Mean proportions of eicosapentanoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) in meat-eaters (\(n = 196\)), vegetarians (\(n = 231\)), and vegans (\(n = 232\)) by duration of time since becoming vegetarian or vegan. The figure shows arithmetic means, or the geometric mean (for EPA), and 95\% CIs adjusted for age, BMI, smoking, and alcohol intake by diet group (\(\bigcirc\), meat-eaters; \(\blacksquare\), vegetarians; and \(\bullet\), vegans). \(P\) values for trend in the mean proportions by duration of adherence to a vegetarian or vegan diet within these subsets were obtained by treating duration of time as a continuous variable in the regression analysis.
The significance of the interaction between diet group and the adjusted linear polyunsaturated fatty acids in plasma by diet group.

PLASMA FATTY ACIDS

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>Meat-eaters (n = 196)</th>
<th>Vegetarians (n = 231)</th>
<th>Vegans (n = 232)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA and EPA¹</td>
<td>-0.22&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-0.19&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-0.32&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.67</td>
</tr>
<tr>
<td>DPA</td>
<td>0.06</td>
<td>0.06</td>
<td>-0.20&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.10</td>
</tr>
<tr>
<td>DHA</td>
<td>-0.10</td>
<td>-0.20&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-0.30&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.85</td>
</tr>
</tbody>
</table>

PLASMA FATTY ACIDS (n = 196)

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>Meat-eaters (n = 196)</th>
<th>Vegetarians (n = 231)</th>
<th>Vegans (n = 232)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA and EPA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.14</td>
<td>0.01</td>
<td>0.13</td>
<td>0.30</td>
</tr>
<tr>
<td>DPA</td>
<td>0.33&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>DHA</td>
<td>0.13</td>
<td>-0.08</td>
<td>0.09</td>
<td>0.04</td>
</tr>
</tbody>
</table>

PLASMA FATTY ACIDS (n = 232)

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>Meat-eaters (n = 196)</th>
<th>Vegetarians (n = 231)</th>
<th>Vegans (n = 232)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA/ALA&lt;sup&gt;2&lt;/sup&gt; and EPA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-0.25&lt;sup&gt;5&lt;/sup&gt;</td>
<td>-0.01</td>
<td>-0.28&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td>DPA</td>
<td>0.10</td>
<td>-0.24&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-0.32&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.11</td>
</tr>
<tr>
<td>DHA</td>
<td>-0.18&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-0.06</td>
<td>-0.19&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.15</td>
</tr>
</tbody>
</table>

¹ EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Values are Pearson correlation coefficients adjusted for age, BMI, smoking, and alcohol intake.

² P for heterogeneity between the diet groups; the P values indicate the significance of the interaction between diet group and the adjusted linear relation between the fatty acids.

³ Log-transformed.

⁴ P < 0.01.

⁵ P < 0.001.

⁶ P < 0.05.

The large number of subjects allowed us to compare subgroups of vegetarians and vegans, and a main objective of the present study was to examine whether the proportions of the long-chain fatty acids vary with the duration of adherence to a vegetarian or a vegan diet. To our knowledge, no previous studies have investigated the differences in plasma fatty acids between long- and short-term vegetarians and vegans. We found no effect of the duration of time since the subjects became vegetarian or vegan, which ranged from <1 y to >20 y, on the proportions of plasma fatty acids. The similar proportions of EPA and DHA in long- and short-term vegans suggest that when foods of animal origin are wholly excluded from the diet, endogenous synthesis of EPA and DHA is not significantly reduced.
DHA is sufficient to result in relatively low but stable plasma concentrations of these fatty acids.

Whether the endogenous production of DHA can be modified by altering the intakes of LA or ALA is not clear. Trials have shown that when dietary ALA is increased, plasma EPA and DPA concentrations increase, whereas DHA concentrations either do not change or increase by a small amount (13–15), which indicates that conversion beyond DPA is limited. In the present study, dietary assessments did not allow us to reliably quantify the intake of specific fatty acids. However, the inverse relations between the proportions of LA and DHA in plasma that we observed in the vegetarians and the vegans suggest that the long-term intake of LA may have an influence on the production of DHA. This finding supports the recommendation that vegetarians and vegans should increase their intake of ALA and limit their intake of LA to optimize their n−3 fatty acid status (8). However, our analyses are based on proportions of plasma fatty acids rather than absolute amounts of fatty acids, and the results should therefore be interpreted with caution.

Although clear differences in the proportions of plasma EPA and DHA were seen between the diet groups, which indicates that dietary intake influences plasma concentrations of fatty acids, we did not find any association between these fatty acids and the intake of either oily fish or other fish, which is in contrast with the findings of other studies (31–35). However, fish intake among the meat-eaters in the present study was relatively low, and the number of meat-eaters and the range of fish intakes may not have been sufficient to detect an association.

The importance of the lower plasma concentrations of DHA seen in vegetarians and in vegans than in omnivores is not known, and plasma concentrations do not necessarily reflect tissue concentrations. Low intakes or low plasma concentrations of n−3 fatty acids have been associated with cardiovascular disease, inflammatory diseases, and neurologic disorders such as schizophrenia and Alzheimer disease (9). However, overall mortality in vegetarians is low compared with that in the general population and is similar to that of health-conscious meat-eaters (36). Indeed, mortality from ischemic heart disease has been shown to be lower in vegetarians than in omnivores (37). This may be explained by favorable differences in several risk factors for ischemic heart disease; for example, plasma LDL-cholesterol concentrations were shown to be lower in vegetarians than in omnivores (38, 39). No differences in platelet function have been found between vegans and omnivores (40), but the concentrations of several clotting factors, notably factor VII, have been found to be lower in vegetarians than in omnivores (39). Few data are available on whether inflammatory or neurologic diseases are more common in vegetarians and in vegans. The importance of long-chain n−3 fatty acids in the diet needs further investigation.

In conclusion, in the present study of 659 British men, the proportions of plasma EPA and DHA were markedly lower in vegetarians and particularly in vegans than in meat-eaters. The proportions of these fatty acids were not affected by the duration of time since the subjects became vegetarian or vegan, which suggests that when animal foods are excluded from the diet, the endogenous production of EPA and DHA results in low but stable plasma concentrations of these fatty acids.

We thank all of the participants in this study and the EPIC-Oxford study staff at the Cancer Research UK Epidemiology Unit.

All of the authors were involved in interpreting the data and contributed to the writing of the manuscript. In addition, MSR performed the statistical analyses and wrote the manuscript; ZL-W performed the plasma fatty acid analyses; PNA helped with the statistical analyses and edited the manuscript; TABS advised on the analyses of the plasma fatty acids; NEA selected the study sample from the EPIC-Oxford cohort; and TJK is the principal investigator of the EPIC-Oxford study. None of the authors had any conflicts of interest.

REFERENCES

A proinflammatory state is associated with hyperhomocysteinemia in the elderly\textsuperscript{1–3}

Anna Maria Gori, Anna Maria Corsi, Sandra Fedi, Alessandra Gazzini, Francesco Sofi, Benedetta Bartali, Stefania Bandinelli, Gian Franco Gensini, Rosanna Abbate, and Luigi Ferrucci

ABSTRACT

Background: The mechanism by which high circulating homocysteine concentrations are a risk factor for atherothrombosis is incompletely understood. A proinflammatory state is related to atherosclerosis, and recent studies suggest that acute phase reactants correlate with circulating concentrations of homocysteine.

Objective: We determined whether high concentrations of inflammatory markers are associated with hyperhomocysteinemia independently of dietary vitamin intakes, vitamin concentrations, and cardiovascular disease risk factors in a large, representative sample of the general population.

Design: Five hundred eighty-six men and 734 women were randomly selected from the inhabitants of 2 small towns near Florence, Italy.

Results: After adjustment for multiple potential confounders, interleukin 1 receptor antagonist (IL-1ra) and interleukin 6 (IL-6) concentrations were significantly ($P < 0.001$) associated with plasma homocysteine concentrations in older ($>65$ y) populations. Compared with participants in the lowest IL-6 tertile, those in the highest tertile had a higher risk of having homocysteine concentrations that were high ($>30$ $\mu$mol/L; odds ratio: 2.6; 95\% CI: 1.1, 5.6; $P = 0.024$) or in the intermediate range 15–30 $\mu$mol/L (odds ratio: 1.6; 95\% CI: 1.2, 2.2; $P = 0.0014$). Sedentary state, intakes of vitamin B-6 and folic acid, and serum folate, vitamin B-12, vitamin B-6, and $\alpha$-tocopherol concentrations were significant independent correlates of homocysteine.

Conclusions: High circulating concentrations of IL-1ra and IL-6 are independent correlates of hyperhomocysteinemia and may explain, at least in part, the association between homocysteine and atherosclerosis.


KEY WORDS Homocysteine, inflammation, cytokines, macronutrients, micronutrients, vitamin concentrations, InCHIANTI Study

INTRODUCTION

Observational studies consistently show that hyperhomocysteinemia is frequent in persons with cardiovascular disease and is a strong, independent risk factor for new cardiovascular events (1–3). The molecular mechanism by which elevated plasma concentrations of homocysteine are related to the pathogenesis of atherothrombotic disease remains incompletely understood.

In vitro and in vivo studies suggest that homocysteine may accelerate the atherosclerotic process by promoting lipoprotein oxidation, exerting procoagulant activity, and enhancing collagen synthesis and smooth muscle cell proliferation (4, 5). More recently, researchers have focused on the relation between homocysteine and inflammation. Evidence shows that concentrations of acute phase reactants, such as fibrinogen, C-Reactive Protein (CRP), and $\alpha$-1 chymotrypsin, correlate with circulating concentrations of homocysteine (6–9). Preclinical studies indicate that interleukin 6 (IL-6) may interact with vitamin B-6 metabolism and compromise cystathionine $\beta$-synthase activity, thereby rising plasma homocysteine concentrations (10). Interestingly, high circulating concentrations of proinflammatory cytokines are associated with a high risk of medical conditions that have also been associated with hyperhomocysteinemia, such as acute ischemic stroke, myocardial infarction, and, more recently, osteoporosis (11–15). Thus, it may be hypothesized that hyperhomocysteinemia and cardiovascular disease risk may be both mediated, in whole or in part, by a proinflammatory state.

The present study used data from a large population-based sample to determine whether circulating concentrations of inflammatory markers are associated with hyperhomocysteinemia, independently of dietary vitamin intakes, circulating vitamin concentrations, and cardiovascular disease risk factors.

SUBJECTS AND METHODS

Data are from the InCHIANTI Study, a population-based epidemiologic study conducted in the Chianti geographic area (Tuscany, Italy). The methodologic details of the study were described elsewhere (16).

1 From the Department of Medical and Surgical Critical Area, Center for the Study at Molecular and Clinical Level of Chronic, Degenerative and Neoplastic Diseases to Develop Novel Therapies, University of Florence, Florence, Italy (AMG, SF, AG, FS, GFG, and RA); the Laboratory of Clinical Epidemiology, Geriatric Department, National Institute of Research and Care on Aging, Florence, Italy (AMC and SB); the Division of Nutritional Sciences, Cornell University, Ithaca, NY (BB); and the Longitudinal Studies Section, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD (LF).

2 Supported as a “target project” by the Italian Ministry of Health.

3 Reprints not available. Address correspondence to AM Gori, Department of Medical and Surgical Critical Area, University of Florence, Section of Cardiology, Viale Morgagni, 85, 50134 Florence, Italy. E-mail: am.gori@dac.unifi.it.

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Briefly, in August 1998, 1616 persons aged 20–102 y were selected from the population registry of Greve in Chianti (a rural area; 11 709 inhabitants; 19.3% ≥65 y of age) and Bagno a Ripoli (Antella village, near Florence; 4704 inhabitants; 20.3% ≥65 y of age). The participation rate was 90% (1453 of 1616).

All subjects gave informed consent to participate in the study, which was approved by the Ethical Committee of the Italian National Institute of Research and Care of Aging. Blood samples were collected in the morning after the participants had fasted for ≥8 h and sat for ≥15 min. The analysis reported here is based on 1320 participants with complete data on homocysteine, inflammatory markers, and other important covariates.

Assays

Commercial enzymatic tests (Roche Diagnostics, GmbH, Mannheim, Germany) were used to measure serum total and HDL-cholesterol and triacylglycerol concentrations. Serum LDL-cholesterol concentrations were calculated by using the Friedewald formula. The interassay CV was <3.8% for total cholesterol, <5% for HDL cholesterol, and <2.5% for triacylglycerols. A low serum HDL-cholesterol concentration was defined as a value ≤40 mg/dL, a high serum LDL-cholesterol concentration as a value ≥160 mg/dL, and a high triacylglycerol concentration as a value ≥200 mg/dL.

Serum concentrations of interleukin 1β (IL-1β), IL-6, soluble IL-6 receptor (sIL-6r, 80 kDa), tumor necrosis factor (TNF-α), and interleukin 1 receptor antagonist (IL-1ra) were quantified by high-sensitivity enzyme-linked immunoabsorbent assays with the use of commercial kits (BioSource Cytoscreen, Camarillo, CA). The minimum detectable concentrations were 0.01 pg/mL for IL-1β, 0.10 pg/mL for IL-6, 0.09 pg/mL for TNF-α, and 0.0 pg/mL for IL-1ra. The interassay CVs were 4.5% for IL-1ra and 7.0% for IL-6, sIL-6r, IL-1β, and TNF-α. Serum concentrations of IL-18 were detected in duplicate by using highly sensitive quantitative sandwich assays (Quantikine HS; R&D Systems, Minneapolis, MN). The minimum detectable concentrations were 0.7 pg/mL, and the interassay CV was 7%.

Serum CRP was measured in duplicate by using an enzyme-linked immunoabsorbent assay and a colorimetric competitive immunoassay that uses purified protein and polyclonal anti-CRP antibodies. The minimum detectable threshold was 0.03 mg/L, and the interassay CV was 5%.

For the measurement of homocysteine, whole venous blood was collected in tubes containing EDTA (0.17 mol/L), immediately placed in ice, and centrifuged within 30 min at 4 °C (2000 × g for 15 min). The supernatant fluid was stored in aliquots at −80 °C until assayed. Plasma concentrations of total homocysteine (free and protein bound) were measured with a fluorimetric polarized immunoassay method (IMX; Abbot Laboratories, Oslo, Norway). The sensitivity of the IMX homocysteine assay was 0.5 μmol/L, and the interassay CV was 4.1% = 0.0987.

Sera for measuring folate, vitamin B-6, and B-12 were obtained by centrifuging blood collected in evacuated tubes without anticoagulant at 2000 × g for 10 min and were stored at −20 °C. Vitamin B-6 was measured by HPLC (Immundiagnostik, Bensheim, Germany) and vitamin B-12 and folates by radioimmunoassay (ICN Pharmaceuticals, New York, NY). The minimum detectable concentrations were 0.6 ng/mL for folate, 0.2 ng/mL for vitamin B-6, and 75 pg/mL for vitamin B-12; the intraassay CVs were 4.1% for folate, 2.8% for vitamin B-6, and 11.2% for vitamin B-12; and the interassay CVs were 7.1% for folate, 4.1% for vitamin B-6, and 12.3% for vitamin B-12. Plasma vitamin E (α-tocopherol) concentrations were measured by reversed-phase HPLC as previously described (17) and expressed in mmol/L. Triplicate analysis of the reference samples provided by the American Association for Laboratory Accreditation (Washington, DC) showed an intrabatch CV of 3% and an interbatch CV of 4.2%.

Covariates

Three consecutive measurements of blood pressure were made according to a standardized protocol after the subjects had rested for 10 min, and the average of the last 2 measurements was used to calculate systolic and diastolic blood pressure. Hypertension was defined as a systolic blood pressure >130 mm Hg and a diastolic blood pressure >80 mm Hg or current antihypertensive treatment. Diabetes mellitus was defined according to American Diabetes Association criteria (18).

Body mass index was calculated as weight (kg)/height² (m) and was dichotomized in the analysis as ≥27 compared with <27. Waist circumference was dichotomized as > or <102 cm in men and 88 cm in women. The average daily intakes of alcohol (<30 compared with >30 g/d) and vitamins were estimated by administering the European Prospective Investigation into Cancer and Nutrition (EPIC) food-frequency questionnaire.

The EPIC food-frequency questionnaire provides a detailed assessment of food consumption during the previous year through a large number of structured and pre-coded questions. Originally, the questionnaire was conceived to be self-administered. However, in a pilot study we realized that in older subjects this method of administration provides ambiguous results, mainly because the questions are misunderstood. Thus, in the InCHIANTI Study, the EPIC questionnaire was administered by the interviewers. The information provided by the questionnaire was transformed into an average daily intake of macronutrients and micronutrients, including vitamins, by using custom software that used for reference a food-composition database for epidemiologic study in Italy, which was edited by the European Institute of Oncology in 1998 (19, 20).

On the basis of responses to multiple questions, a sedentary state was defined as being inactive or performing low-intensity physical activity (ie, walking, light household) <1 h/wk. Smoking status was assessed by self-report. Pack-years, a measure of smoking exposure that combines intensity and duration, was calculated as packs smoked per day multiplied by years of smoking.

Statistical analysis

All analyses were performed with the use of the SAS statistical package (version 8.02; SAS Institute Inc, Cary, NC). P values <0.05 were considered statistically significant. Log-transformed values for homocysteine, IL-1β, IL-1ra, IL-6, IL-6r, IL-18, TNF-α, CRP, vitamin B-6, vitamin B-12, and folate were used in the analyses and back transformed for data presentation.

Differences in characteristics between men and women were determined by using a general linear model or a logistic regression analysis adjusted for age. To evaluate the relation between circulating homocysteine concentrations, vitamin intakes, circulating vitamin concentrations, and cardiovascular disease risk
factors, we divided the study population into tertiles of circulating homocysteine (<12.2, 12.2–15.6, and >15.6 μmol/L). Differences between tertiles were evaluated by general linear models adjusted for sex, age, total energy intake, and serum creatinine.

Furthermore, in the final adjusted model of the multivariate linear regression analysis, we included sex × IL-6 and sex × IL-1ra as well as age × IL-6 and age × IL-1ra (age was coded as <65 yr or ≥65 yr) among the independent variables to test the interaction between inflammatory markers and sex and inflammatory markers and age, respectively.

In addition, we used multivariate linear regression analyses to test the independent association of inflammatory markers with plasma homocysteine concentrations, after adjusting for vitamin intakes, circulating vitamin concentrations, and cardiovascular disease risk factors. Variables that were not significantly and independently associated with homocysteine were removed from the final regression model by backward selection.

High concentrations of IL-1ra and IL-6 were defined as the highest tertile of the distribution of these cytokines. The odds ratio (OR) for having high (15–30 or >30 μmol/L) compared with normal (<15 μmol/L) concentrations of homocysteine, according to cytokine tertiles, was estimated by multivariate logistic regression analysis with the use of homocysteine groups as a polychotomous dependent variable and high IL-6 and high IL-1ra as independent variables. Age; sex; serum creatinine; intakes of energy, folate, and vitamin B-6; circulating concentrations of vitamin B-6, vitamin B-12, α-tocopherol, and folate; and sedentary state as covariates.

### RESULTS

The characteristics of the InCHIANTI participants are shown in Table 1. The InChianti study participants consists of 734 females (55.6%) and 586 males (44.4%); significant differences by sex were found.

Mean (and 95% CI) plasma homocysteine concentrations were significantly higher (P < 0.0001) in men (15.3 μmol/L; 95% CI: 14.9, 15.7 μmol/L) than in women (13.5 μmol/L; 95% CI: 13.2, 13.8 μmol/L) after adjustment for age, serum creatinine, and total energy intake.

Adjusting for sex, serum creatinine and total energy intake, circulating concentrations of homocysteine were significantly higher (P < 0.0001) different across age groups (<65 y: mean 11.7 μmol/L, 95% CI 11.3–12.1 μmol/L, 65–74 y: 13.8 μmol/L, 95% CI 13.5–14.1 μmol/L, 75–84 y: 16.2 μmol/L, 95% CI 15.7–16.7 μmol/L; and >85 y: 18.3 μmol/L, 95% CI 17.4–19.3 μmol/L).

After adjustment for age, sex, total energy intake, and serum creatinine, significantly higher concentrations of IL-6, IL-1ra, TNF-α, and IL-18 were found across homocysteine tertiles (Table 2).

After adjustment for age, sex, serum creatinine, and total energy intake, the average daily dietary intake of many vitamins (all B vitamins, β-carotene, folic acid, and vitamins C, A, and E) and serum concentrations of folate, vitamin B-12, vitamin B-6, and α-tocopherol were progressively and significantly lower according to homocysteine tertiles. After adjustment for the same covariates, participants with higher homocysteine concentrations were significantly more likely to be sedentary and to have low

### Table 1

Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Whole group (n = 1320)</th>
<th>Men (n = 586)</th>
<th>Women (n = 734)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65 y [% (n)]</td>
<td>68.9 ± 15.67</td>
<td>67.7 ± 15.5</td>
<td>69.8 ± 15.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>65–74 y [% (n)]</td>
<td>281 (21.3)</td>
<td>133 (22.7)</td>
<td>148 (20.2)</td>
<td>—</td>
</tr>
<tr>
<td>75–84 y [% (n)]</td>
<td>567 (43.0)</td>
<td>266 (45.4)</td>
<td>301 (41.0)</td>
<td>—</td>
</tr>
<tr>
<td>≥85 y [% (n)]</td>
<td>333 (25.2)</td>
<td>139 (23.7)</td>
<td>194 (26.4)</td>
<td>—</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.91 ± 0.19</td>
<td>1.00 ± 0.18</td>
<td>0.84 ± 0.17</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>2004 ± 621</td>
<td>2278 ± 628</td>
<td>1783 ± 521</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hypertension [% (n)]</td>
<td>591 (44.7)</td>
<td>234 (39.9)</td>
<td>357 (48.6)</td>
<td>0.0133</td>
</tr>
<tr>
<td>Diabetes [% (n)]</td>
<td>93 (7.0)</td>
<td>44 (7.5)</td>
<td>48 (6.5)</td>
<td>0.3454</td>
</tr>
<tr>
<td>Sedentary state [% (n)]</td>
<td>242 (18.3)</td>
<td>66 (11.3)</td>
<td>176 (24.0)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Smoking habit [% (n)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>201 (15.2)</td>
<td>119 (20.3)</td>
<td>82 (11.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Former smokers</td>
<td>363 (27.5)</td>
<td>283 (48.3)</td>
<td>80 (10.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Never smokers</td>
<td>756 (57.3)</td>
<td>184 (31.4)</td>
<td>572 (77.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>134 ± 35</td>
<td>130 ± 35</td>
<td>138 ± 35</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>&gt;160 mg/dL [% (n)]</td>
<td>284 (21.5)</td>
<td>105 (17.9)</td>
<td>179 (24.4)</td>
<td>0.0062</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>56 ± 15</td>
<td>51 ± 13</td>
<td>59 ± 15</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>&lt;40 mg/dL [% (n)]</td>
<td>159 (12.1)</td>
<td>107 (18.3)</td>
<td>52 (7.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>126 ± 76</td>
<td>132 ± 89</td>
<td>121 ± 64</td>
<td>0.0062</td>
</tr>
<tr>
<td>&gt;200 mg/dL</td>
<td>142 (10.8)</td>
<td>82 (14.0)</td>
<td>60 (8.2)</td>
<td>0.0005</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 ± 4.5</td>
<td>27.5 ± 3.7</td>
<td>27.8 ± 5.0</td>
<td>0.239</td>
</tr>
<tr>
<td>&gt;27 [n (%)]</td>
<td>370 (28.0)</td>
<td>145 (24.7)</td>
<td>225 (30.7)</td>
<td>0.040</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>91 ± 11</td>
<td>95 ± 9</td>
<td>89 ± 11</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>&gt;88 cm in women or &gt;102 cm in men [% (n)]</td>
<td>533 (40.4)</td>
<td>115 (19.6)</td>
<td>418 (57.0)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

* Differences between men and women were analyzed by using a general linear model or a logistic regression analysis adjusted for age.

† ± SD (all such values).

‡ Defined as a blood pressure >130 (systolic) and >80 (diastolic) mm Hg, current antihypertensive treatment, or both.

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### Table 2

Characteristics of homocysteine tertiles

<table>
<thead>
<tr>
<th>Homocysteine tertiles (μmol/L)</th>
<th>Whole group (n = 1320)</th>
<th>Men (n = 586)</th>
<th>Women (n = 734)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;12.2</td>
<td>68.9 ± 15.67</td>
<td>67.7 ± 15.5</td>
<td>69.8 ± 15.5</td>
</tr>
<tr>
<td>12.2–15.6</td>
<td>281 (21.3)</td>
<td>133 (22.7)</td>
<td>148 (20.2)</td>
</tr>
<tr>
<td>&gt;15.6</td>
<td>567 (43.0)</td>
<td>266 (45.4)</td>
<td>301 (41.0)</td>
</tr>
</tbody>
</table>

---

#### Mean (and 95% CI) plasma homocysteine concentrations

- Men (15.3 μmol/L; 95% CI: 14.9, 15.7 μmol/L)
- Women (13.5 μmol/L; 95% CI: 13.2, 13.8 μmol/L)

#### Differences by sex

- Men: higher homocysteine concentrations compared to women
- Adjusted for age, total energy intake, serum creatinine

---

#### Adjusting for sex, serum creatinine, and total energy intake

- Circulating concentrations of homocysteine were significantly different across age groups

---

#### After adjustment for age, sex, total energy intake, and serum creatinine

- Significantly higher concentrations of IL-6, IL-1ra, TNF-α, and IL-18
- Across homocysteine tertiles

---

#### After adjustment for age, sex, serum creatinine, and total energy intake

- The average daily dietary intake of many vitamins
- Serum concentrations of folate, vitamin B-12, vitamin B-6, and α-tocopherol

---

#### Significant differences

- By sex
- Across age groups
- Between tertiles of homocysteine concentrations

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#### Conclusion

- Homocysteine and inflammatory markers are significantly associated
- Further studies are needed to explore the mechanisms underlying these associations.

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### References

HDL-cholesterol concentrations (≤40 mg/dL) and high LDL-cholesterol concentrations (≥160 mg/dL) (Table 3). No significant interaction of sex and IL-6 or sex and IL-1ra was found. There was a significant interaction of age and IL-6, but not of age and IL-1ra.

In the multivariate linear regression analyses independent of age, sex, serum creatinine, and total energy intake, circulating concentrations of IL-6 and IL-1ra were strongly associated with homocysteine (models 1 and 2; Table 4) in the whole population as well as in older subject (>65 y) subgroups; the significant association was maintained in a model that included IL-6 and IL-1ra and adjusted for all the variables found to be associated with circulating homocysteine concentrations in the preliminary analyses (model 3; Table 4). When all variables not significantly

### TABLE 2
Mean (and 95% CI) serum concentrations of inflammatory markers according to tertiles of circulating homocysteine concentrations

<table>
<thead>
<tr>
<th>Homocysteine (µmol/L)</th>
<th>&lt;12.2 (n = 430)</th>
<th>12.2–15.6 (n = 439)</th>
<th>&gt;15.6 (n = 451)</th>
<th>P for trend&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 6 (pg/mL)</td>
<td>1.18 (1.04, 1.34)</td>
<td>1.23 (1.09, 1.138)</td>
<td>1.48 (1.30, 1.67)</td>
<td>0.0012</td>
</tr>
<tr>
<td>sIL-6r (ng/mL)</td>
<td>91 (83, 98)</td>
<td>87 (81, 94)</td>
<td>92 (84, 100)</td>
<td>0.90</td>
</tr>
<tr>
<td>Interleukin 1β (pg/mL)</td>
<td>0.13 (0.12, 0.15)</td>
<td>0.14 (0.12, 0.15)</td>
<td>0.14 (0.12, 0.16)</td>
<td>0.58</td>
</tr>
<tr>
<td>IL-1ra (pg/mL)</td>
<td>119 (110, 130)</td>
<td>132 (121, 143)</td>
<td>146 (134, 160)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>4.3 (3.8, 4.9)</td>
<td>4.7 (4.1, 5.3)</td>
<td>4.9 (4.3, 5.6)</td>
<td>0.0234</td>
</tr>
<tr>
<td>Interleukin 18 (pg/mL)</td>
<td>357 (338, 376)</td>
<td>370 (352, 389)</td>
<td>388 (368, 410)</td>
<td>0.0014</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.3 (2.0, 2.8)</td>
<td>2.4 (2.1, 2.8)</td>
<td>2.7 (2.3, 3.2)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values were adjusted for age, sex, total energy intake, and serum creatinine. sIL-6r, soluble interleukin 6 receptor; IL-1ra, interleukin 1 receptor antagonist; CRP, C-reactive protein; TNF-α, tumor necrosis factor α.

<sup>2</sup> From general linear models adjusted for age, sex, creatinine, and total energy intake.

### TABLE 3
Mean (and 95% CI) daily intakes and circulating concentrations of vitamins and cardiovascular disease risk factors according to tertiles of circulating homocysteine concentrations

<table>
<thead>
<tr>
<th>Homocysteine (µmol/L)</th>
<th>&lt;12.2 (n = 430)</th>
<th>12.2–15.6 (n = 439)</th>
<th>&gt;15.6 (n = 451)</th>
<th>P&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily intake of vitamins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>1.36 (1.32, 1.41)</td>
<td>1.33 (1.28, 1.37)</td>
<td>1.26 (1.21, 1.31)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>17.5 (17.0, 18.0)</td>
<td>17.1 (16.7, 17.6)</td>
<td>16.6 (16.1, 17.1)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Thiamine (mg)</td>
<td>0.94 (0.92, 0.97)</td>
<td>0.92 (0.90, 0.94)</td>
<td>0.90 (0.88, 0.93)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Vitamin B-6 (mg)</td>
<td>1.76 (1.72, 1.81)</td>
<td>1.73 (1.69, 1.77)</td>
<td>1.67 (1.62, 1.72)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>124 (117, 131)</td>
<td>114 (107, 120)</td>
<td>110 (103, 117)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>β-Carotene (mg)</td>
<td>2.4 (2.2, 2.6)</td>
<td>2.3 (2.2, 2.5)</td>
<td>2.1 (1.9, 2.3)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>278 (269, 287)</td>
<td>266 (257, 274)</td>
<td>255 (247, 264)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>6.6 (6.4, 6.8)</td>
<td>6.5 (6.3, 6.7)</td>
<td>6.3 (6.1, 6.6)</td>
<td>0.0167</td>
</tr>
<tr>
<td>Retinol (µg)</td>
<td>936 (847, 1026)</td>
<td>920 (837, 1003)</td>
<td>827 (737, 917)</td>
<td>0.0151</td>
</tr>
<tr>
<td>Circulating concentrations of vitamins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B-12 (pg/mL)</td>
<td>467 (430, 506)</td>
<td>392 (364, 423)</td>
<td>334 (308, 363)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Folate (ng/mL)</td>
<td>3.3 (3.1, 3.6)</td>
<td>2.9 (2.7, 3.1)</td>
<td>2.4 (2.2, 2.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vitamin B-6 (ng/mL)</td>
<td>9.0 (7.1, 11.4)</td>
<td>8.4 (6.7, 10.4)</td>
<td>6.0 (4.7, 7.6)</td>
<td>0.0014</td>
</tr>
<tr>
<td>α-Tocopherol (µmol/L)</td>
<td>29.3 (28.0, 30.5)</td>
<td>29.2 (28.1, 30.4)</td>
<td>27.9 (26.7, 29.1)</td>
<td>0.0284</td>
</tr>
<tr>
<td>Cardiovascular disease risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>39.3 (32.0, 47.0)</td>
<td>46.9 (39.5, 54.5)</td>
<td>44.2 (36.6, 52.1)</td>
<td>0.28</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>7.4 (4.1, 12.8)</td>
<td>7.3 (4.4, 12.0)</td>
<td>5.0 (2.8, 8.8)</td>
<td>0.17</td>
</tr>
<tr>
<td>Sedentary state (%)</td>
<td>8.6 (5.7, 12.7)</td>
<td>9.1 (6.1, 13.6)</td>
<td>15.1 (10.3, 21.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Current smoking (%)</td>
<td>13.8 (9.5, 19.7)</td>
<td>10.6 (7.3, 15.3)</td>
<td>15.5 (10.7, 21.9)</td>
<td>0.45</td>
</tr>
<tr>
<td>Alcohol intake (g/d)</td>
<td>21 (11, 16)</td>
<td>15 (13, 18)</td>
<td>15 (12, 18)</td>
<td>0.20</td>
</tr>
<tr>
<td>LDL cholesterol &gt; 160 mg/dL (%)</td>
<td>24.7 (18.6, 32.1)</td>
<td>21.1 (16.0, 27.3)</td>
<td>18.0 (13.3)</td>
<td>0.0242</td>
</tr>
<tr>
<td>HDL cholesterol &lt; 40 mg/dL (%)</td>
<td>8.7 (5.6, 13.5)</td>
<td>8.8 (5.5, 13.7)</td>
<td>15.0 (9.8, 22.3)</td>
<td>0.0046</td>
</tr>
<tr>
<td>Triacetylglycerols &gt; 200 mg/dL (%)</td>
<td>11.1 (7.2, 16.8)</td>
<td>8.5 (5.4, 13.1)</td>
<td>11.4 (7.4, 17.2)</td>
<td>0.72</td>
</tr>
<tr>
<td>BMI &gt; 27 kg/m² (%)</td>
<td>26.4 (20.4, 33.4)</td>
<td>24.6 (19.1, 31.2)</td>
<td>31.6 (24.8, 39.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>Large waist circumference (%)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>36.5 (29.1, 44.6)</td>
<td>37.9 (30.7, 45.6)</td>
<td>39.7 (32.0, 47.9)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

<sup>1</sup> From general linear models adjusted for age, sex, creatinine, and total energy intake.

<sup>2</sup> >102 cm in men and >88 cm in women.
and independently associated with circulating homocysteine were removed from this last model, IL-6 and IL-1ra concentrations as well as age, male sex, serum creatinine, total energy intake, sedentary state, vitamin B-6 and folate intakes, and circulating concentrations of vitamin B-12, vitamin B-6, folic acid, and vitamin B-6 intakes; and circulating concentrations of vitamin B-12, homocysteine concentrations, independent of the dietary intake and circulating concentrations of the vitamins involved in the methionine cycle.

Our results show that subjects with IL-6 concentrations in the top tertile of the distribution had an increased risk of having circulating concentrations of homocysteine that, in clinical practice, are considered a marker of high cardiovascular disease risk. Thus, a common inflammatory factor may explain, at least in part, the association between high circulating concentrations of homocysteine and cardiovascular diseases described in many observational studies (1–3). In fact, a large number of experimental and clinical studies have provided convincing evidence of the crucial role of inflammation in the development and progression of atherosclerosis processes (21, 22); in particular, elevated IL-6 concentrations are closely related to an increased risk of.

### DISCUSSION

This study provides original information about the complex relation between homocysteine, biomarkers of inflammation, vitamin dietary intakes, and circulating concentrations in a large population-based study, which includes >1300 Italian subjects dispersed over a wide age range. We found that, in older subjects, IL-6 and IL-1ra, but not other markers of inflammation (CRP, IL-1β, IL-6r, IL-18, and TNF-α), were independent predictors of homocysteine concentrations, independent of the dietary intake and circulating concentrations of the vitamins involved in the methionine cycle.

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### TABLE 4

Multiple linear regression models for markers of inflammation that influence homocysteine concentrations (log)

<table>
<thead>
<tr>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log(IL-6) (pg/mL)</td>
<td>Log(IL-1ra) (ng/mL)</td>
<td>Log(IL-18) (pg/mL)</td>
<td>Log(TNF-α) (pg/mL)</td>
</tr>
<tr>
<td>Whole population (n = 1320)</td>
<td>Whole population (n = 1320)</td>
<td>Whole population (n = 1320)</td>
<td>Whole population (n = 1320)</td>
</tr>
<tr>
<td>β ± SE</td>
<td>β ± SE</td>
<td>β ± SE</td>
<td>β ± SE</td>
</tr>
<tr>
<td>0.036 ± 0.009</td>
<td>0.040 ± 0.016</td>
<td>0.042 ± 0.024</td>
<td>0.011 ± 0.00</td>
</tr>
<tr>
<td>0.0002</td>
<td>0.0121</td>
<td>0.0089</td>
<td>0.210</td>
</tr>
<tr>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>0.035 ± 0.009</td>
<td>0.050 ± 0.015</td>
<td>0.058 ± 0.014</td>
<td>0.063 ± 0.014</td>
</tr>
<tr>
<td>0.0003</td>
<td>0.0011</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>0.0075</td>
<td>0.0002</td>
<td>0.0005</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

1. IL-6, interleukin 6; IL-1ra, interleukin 1 receptor antagonist; IL-18, interleukin 18; TNF-α, tumor necrosis factor α. Multivariate linear regression analysis was used to test the independent association between homocysteine and inflammatory markers. In all 4 models, the dependent variable is log(homocysteine; μmol/L).
2. Adjusted for age, sex, serum creatinine, and total energy intake.
3. Included only variables significantly associated with homocysteine in model 1.
4. Adjusted for age; sex; serum creatinine; total energy intake; sedentary state; folate and vitamin B-6 intakes; circulating concentrations of vitamin B-12, folic acid, vitamin B-6, α-tocopherol, high LDL cholesterol, and low HDL cholesterol; and smoking habit.
5. All variables not significantly and independently associated with circulating homocysteine in model 3 were removed.

### FIGURE 1

Odds ratios (and 95% CIs) for high (>30 μmol/L and intermediate (15–30 μmol/L) compared with normal (<15 μmol/L) homocysteine concentrations associated with circulating concentrations of interleukin 6 (IL-6) in the highest (3rd) compared with the 2 lowest tertiles (1st and 2nd). Values were estimated in a single polychotomous logistic regression model adjusted for age; sex; serum creatinine; sedentary state; energy, folic acid, and vitamin B-6 intakes; and circulating concentrations of vitamin B-12, vitamin B-6, folate, and α-tocopherol. ○, Homocysteine concentrations >30 μmol/L (n = 45) versus <15 μmol/L (n = 799); P = 0.0024. ●, Homocysteine concentrations 15–30 μmol/L (n = 467) versus <15 μmol/L (n = 799); P = 0.0014.
future myocardial infarction (23) as well as clinical outcome in unstable angina (24, 25).

Interestingly, we found that IL-1ra, a naturally occurring antagonist of the pro-inflammatory cytokine IL-1β, was also an independent correlate of homocysteinemia. Although, biologically, IL-1ra has anti-inflammatory properties, because it competitively binds to IL-1β membrane receptors, it is also an acute phase reactant produced by the liver in large quantities during an inflammatory state (26). Thus, high circulating concentrations of IL-1ra are generally considered to indicate a proinflammatory state. Interestingly, Biasucci et al (24) found that increasing concentrations of IL-1ra and IL-6 predict the risk of in-hospital coronary events in patients with unstable angina.

The tight relation between inflammation and homocysteinemia observed in our study suggest that homocysteine concentrations may be influenced by active inflammatory processes, as suggested by the higher concentrations of plasma homocysteine that are often observed in the days after an acute myocardial infarction (27) or stroke (28). Tissue damage accelerates specific remethylation reactions of DNA, RNA, and various proteins during tissue repair, with consequent generation of S-adenosylhomocysteine and release of homocysteine (29). However, given the cross-sectional nature of our study, a reverse causality, namely homocysteine directly stimulating proinflammatory signaling molecules, cannot be excluded. Studies have shown that homocysteine determines acute and chronic endothelial dysfunction by promoting the production of hydrogen peroxide and other highly reactive oxygen compounds, upregulating cell adhesion molecules and inhibiting the release of nitric oxide (5, 30). Interestingly, homocysteine concentrations ranging from 10 to 100 μmol/L induce the monocyte expression and production of IL-8 and monocyte chemoattractant protein 1, chemokines that are essential in modulating the role of leukocytes in the inflammatory response to different types of vascular injuries. Additionally, folic acid supplementation of hyperhomocysteinemic patients reduces IL-8 release from peripheral blood mononuclear cells (31).

In our study, the association between inflammatory state and homocysteinemia was found in subjects aged ≥65 y but not in subjects aged <65 y. This finding is consistent with several lines of research, which have shown that a large percentage of old and very old persons are affected by a mild proinflammatory state (32, 33). The continuous inflammatory stimulus might determine an increased demand for folate that, if not compensated for by increased intakes, may cause hyperhomocysteinemia. The inflammatory process creates an oxidative stress condition. Interestingly, in our study, low concentrations of α-tocopherol (circulating vitamin E) were significantly associated with higher homocysteinemia, which suggests that noncompensated oxidative stress may contribute to the increase in plasma homocysteine concentrations. The oxidative stress determines nucleic acid damages, including base modifications, double-base lesions, and strand breaks (34). Folate is required for DNA synthesis and repair (35). Interestingly, in animal models, activated macrophages overexpress high-affinity folate receptor (36). Thus, it is likely that in an inflammatory state vitamins involved in the methionine cycle may be mobilized from the liver and peripheral tissues to sites of inflammation (37).

Previous studies that addressed the relation between inflammation and homocysteine reported conflicting findings. A weak association between CRP and homocysteine concentrations was observed in the participants of the Framingham Heart Study (38) and in the apparently healthy middle-aged men enrolled in the Physicians’ Health Study (9). Conversely, inflammatory markers were not statistically associated with plasma homocysteine concentrations, either in 519 healthy middle-aged adults of the Atherosclerosis Risk in Communities Study (39) or in 373 healthy persons aged >65 y examined by Ravaglia et al (40).

An unexpected finding of our study was the significantly low prevalence of participants with LDL concentrations >160 mg/dL in the highest tertile of homocysteine. The reason for this finding remains unknown, but it is noteworthy that after adjustment for multiple potential confounders, LDL-cholesterol was no longer independently associated with homocysteine concentrations.

Our results provide solid evidence of a relation between inflammation and homocysteine, which has been suggested by previous investigations (6–9, 38). The fact that we found a strong independent association of IL-6 and IL-1ra but not of CRP with homocysteine is puzzling because IL-6 increases early in inflammation (41) and is the principal inductor of hepatocyte messenger RNA CRP expression (42, 43). Proinflammatory cytokines are involved in the transcriptional control of CRP production (44) and, additionally, the synthesis of CRP is strongly influenced by mechanisms of posttranscriptional regulation that are independent of IL-6 (45).

In conclusion, the results of our large population-based study showed a strong, independent association between inflammatory markers and homocysteine concentrations and identified the cytokines IL-6 and IL-1ra as major determinants involved in this association. This association, independent of the leading factor, may explain, at least in part, why subjects with high concentrations of homocysteine have a high risk of developing cardiovascular diseases. Future studies of homocysteine as a modifiable risk factor for health outcomes need to consider inflammation as a potential confounder in the analysis. The evaluation of homocysteine concentrations in addition to that of vitamin B-6, vitamin B-12, folate, and interleukins after 6 y of follow-up will allow us to further elucidate the relation between inflammation and homocysteine.

LF, AMG, RA, and GFG were responsible for the study design. AMC, BB, SB, and FS were responsible for the collection of data. LF and SB were responsible for the statistical analysis. LF, SB, and FS were responsible for the clinical evaluation of the subjects. BB was responsible for the administration of the food-frequency questionnaire. AMG, SF, AMC, and AG were responsible for the laboratory investigations. AMG, LF, RA, and GFG were responsible for writing the manuscript. All authors disclose any affiliation with any organization with a financial interest, direct or indirect, in the subject matter or materials discussed in the manuscript that may affect the conduct or reporting of the work submitted.

REFERENCES

Inflammation contributes to low plasma amino acid concentrations in patients with chronic kidney disease

Mohammed E Suliman, A Rashid Qureshi, Peter Stenvinkel, Roberto Pecoits-Filho, Peter Bárány, Olof Heimbürg, Björn Anderstam, Ernesto Rodríguez Ayala, José C Divino Filho, Anders Alvestrand, and Bengt Lindholm

ABSTRACT

Background: Inflammation and malnutrition are common in chronic kidney disease (CKD) patients, and plasma concentrations of free amino acids (AAs) in these patients are often abnormal. Malnutrition contributes to alterations in AA concentrations.

Objective: The objective was to study the effects of inflammation on plasma AA concentrations.

Design: Concentrations of plasma AAs, serum albumin, and several inflammatory markers were analyzed in 200 fasting, nondiabetic CKD patients who were close to the start of renal replacement therapy. The nutritional status of these patients was assessed by a subjective global assessment.

Results: The patients with inflammation [C-reactive protein (CRP) concentrations >10 mg/L] or malnutrition had lower AA concentrations than did the patients with no inflammation or malnutrition. The presence of both inflammation and malnutrition was associated with more marked reductions in AA concentrations than was malnutrition alone. Significant inverse correlations were observed between the plasma concentrations of most of the essential and nonessential AAs and inflammatory markers, whereas serum albumin concentrations were positively correlated with several AA concentrations. A stepwise multivariate regression analysis showed that serum CRP concentrations were independently associated with low concentrations of the sums of both nonessential AAs and all AAs. An analysis of all-cause mortality with a Kaplan-Meier test showed that the patients with higher AA concentrations had significantly better survival than did the patients with lower AA concentrations.

Conclusions: Plasma AA concentrations are low in CKD patients with inflammation and are inversely correlated with concentrations of inflammatory markers. Although inflammation and malnutrition are closely related, CRP concentrations were independently associated with low concentrations of the sums of both nonessential AAs and all AAs, which suggests an independent role of inflammation as a cause of low plasma AA concentrations in CKD patients. Am J Clin Nutr 2005;82:342–9.

KEY WORDS Amino acids, cardiovascular disease, chronic kidney disease, C-reactive protein, inflammation, malnutrition, mortality

INTRODUCTION

Chronic kidney disease (CKD) is characterized by an exceptionally high mortality rate, primarily from cardiovascular disease (CVD). Chronic inflammation is a common feature in CKD patients and is associated with atherosclerotic CVD through various pathogenetic mechanisms (1). Moreover, the prevalence of protein-energy malnutrition in CKD patients is high, and inflammation is more prevalent in malnourished patients than in those with normal nutritional status (2, 3). A syndrome consisting of malnutrition, inflammation, and atherosclerosis is present in a large proportion of CKD patients and is associated with increased mortality (4).

Patients with CKD generally have an abnormal plasma amino acid (AA) pattern, ie, high plasma concentrations of several nonessential AAs (NEAAs) and low concentrations of most essential AAs (EAAs) (5–11). However, the mechanisms behind these abnormalities are not fully understood. Some of the changes are ascribed to derangements in AA metabolism, either because of deficient excretory and metabolic functions of the diseased kidneys or because of uremia per se. Inadequate nutritional intake and malnutrition may also contribute to plasma AA abnormalities. To some extent, the abnormal pattern of AAs seen in CKD patients resembles that seen in protein malnutrition, but the abnormal AA patterns are also observed in CKD patients with normal nutritional status (11). Because the biochemical changes that occur during inflammation exert a demand on AA metabolism (12), we hypothesized that the systemic inflammatory response seen in a large proportion of CKD patients may contribute to AA pattern disturbances.

The systemic inflammatory response stimulates protein catabolism (13, 14), and the release of AAs from muscle protein provides a substrate for the synthesis of acute phase proteins and proteins of the immune system (15), which could result in a general reduction in plasma AA concentrations. Because proinflammatory cytokines can cause anorexia and increased protein catabolism, they represent an important cause of protein wasting in CKD patients (16). Furthermore, proinflammatory cytokines

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activate inflammatory cells to produce reactive oxygen species, which may enhance AA oxidation. CKD patients often have signs of increased oxidative stress, which can also alter AA and protein concentrations in these patients.

In the present study, we hypothesized that inflammation, as evidenced by increased concentrations of C-reactive protein (CRP) and proinflammatory cytokines such as interleukin 6, is an important cause of plasma AA changes in CKD patients. For this purpose, concentrations of AAs and inflammatory markers from the plasma of fasting subjects were measured in a post hoc analysis of the baseline data of an ongoing prospective study in CKD patients who were starting dialysis treatment at baseline (2). To our knowledge, this was the first study to investigate a possible relation between plasma AA concentrations and inflammatory markers in CKD patients. Moreover, we studied the effects of both nutritional status and CVD on plasma AA concentrations. Furthermore, because the relation between AA concentrations and mortality has not been studied in CKD patients, the patients were followed from the start of dialysis therapy over a 5-y period to assess the effects of basal AA concentrations on all-cause mortality.

SUBJECTS AND METHODS

Subjects

The patients in the present study were included in a prospective cohort study of atherosclerosis and lipid metabolism in patients who were beginning dialysis replacement therapy at the Renal Clinic of the Karolinska University Hospital Huddinge, Stockholm, Sweden (2). In the present study, post hoc analyses of 200 CKD patients (120 men) with a median age of 53 y (range: 22–70 y) and a median glomerular filtration rate (GFR) of 7 mL/min (range: 1–16 mL/min) were conducted. The mean (±SD) body mass index (in kg/m²) was 25 ± 4. Exclusion criteria were age >70 y, liver dysfunction, diabetes mellitus, clinical signs of intercurrent infection, and unwillingness to participate in the study. Fifty-four (27%) patients had a clinical history or signs of cerebrovascular, cardiovascular, or peripheral vascular disease at the start of the study and were grouped as having clinical CVD (CVDclin). Of the 54 patients, 15 had a history of cerebrovascular disease (stroke), 31 had a history of CVD (acute myocardial infarction, angina pectoris, or coronary artery bypass surgery), 16 had a history of peripheral ischemic vascular disease, and 2 had a history of an aortic aneurysm. One hundred forty-three patients were studied just after starting dialysis treatment (median time to start: 20 d), and 57 patients were studied just after starting dialysis treatment (median time from start: 8 d; see Results). Most patients were taking antihypertensive medications as well as other drugs that are commonly used by patients with CKD, such as phosphate and potassium binders, diuretics, and vitamin B, C, and D supplements. The protocol was approved by the Ethics Committee of Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden, and informed consent was obtained from each patient.

A population-based, randomly selected group of 39 control subjects (28 men) with a median age of 68 y (range: 38–80 y) was used for comparative analyses of AA concentrations. The control subjects were investigated with a protocol similar to that used for the patient group. The random selection of subjects in the Stockholm region was performed by Statistics Sweden (SCB). No other exclusion criteria, other than an unwillingness to participate in the study, was applied in the selection of the control group.

Blood sampling and laboratory analyses

After the patients fasted overnight, venous blood samples were taken and placed in appropriate tubes for the separation of plasma and serum, which were then stored at −70 °C until analyzed. Plasma AA concentrations were measured with the use of reversed-phase HPLC and fluorometric detection, as described elsewhere (17). The routine procedures used in the Clinical Chemistry Laboratory at Huddinge University Hospital were used to measure serum concentrations of albumin (brom cresol purple), CRP (turbidimetry), fibrinogen, and creatinine and the urinary excretion of creatinine and urea. The detection limit of CRP was 10 mg/L, and all values <10 mg/L were treated as 9 mg/L in the statistical evaluation. High-sensitivity CRP was measured in 39 control subjects by nephelometry. The serum concentrations of tumor necrosis factor α and interleukin 6 were measured with a photometric enzyme-linked immunosorbent assay obtained from Boehringer Mannheim (Mannheim, Germany). Plasma neopterin concentrations were measured with a radioimmunoassay kit (Behring Diagnostic, Rueil-Malmaison, France). The concentrations of both the soluble intracellular adhesion molecule 1 and the soluble vascular cell adhesion molecule 1 were measured (n = 63 patients) with a commercially available enzyme-linked immunosorbent assay kit (R&D Systems Europe Ltd, Abingdon, United Kingdom). A specific radioimmunoassay kit was used to analyze plasma insulin concentrations (Pharmacia, Uppsala, Sweden).

Assessment of nutritional status and protein intake in the patients

A subjective global assessment (SGA) was used to evaluate the overall protein-energy nutritional status of the patients. The SGA included 6 subjective assessments: 3 were based on the patient’s history of weight loss, incidence of anorexia, and incidence of vomiting, and 3 were based on the physician’s grading of muscle wasting, presence of edema, and loss of subcutaneous fat. Each patient was given a score based on those assessments that reflected their nutritional status as follows: 1 = normal nutritional status, 2 = mild malnutrition, 3 = moderate malnutrition, and 4 = severe malnutrition. The patients with an ordinal SGA score of 2, 3, or 4 were grouped together as malnourished. Protein intake was estimated from the protein equivalent of nitrogen appearance (PNA), which was calculated from urea kinetic modeling by using the rate of urea excretion in a 24-h urine collection. Protein intake was estimated from the protein equivalent of nitrogen appearance (PNA), which was calculated from urea kinetic modeling by using the rate of urea excretion in a 24-h urine collection. Ureine was collected from all of the patients before the start of dialysis therapy. PNA was normalized (nPNA) to actual protein intake estimated from nitrogen intake.

Outcome ascertainment

Survival was assessed from the day of examination, with a mean follow-up period of 16.7 mo (range: 0.5–60 mo). The patients were censored at death, when they received a kidney transplant, or when they completed the 5-y follow-up period; all patients participating in the present study were followed up.
Within the follow-up period, 40 (20%) patients died and 80 (40%) patients received kidney transplants.

**Statistical analyses**

Values were expressed as medians (ranges) or means (±SDs), as appropriate. A P value < 0.05 was considered statistically significant. Comparisons between 2 groups were assessed for continuous variables with a Student’s unpaired t test; a Mann-Whitney U test was used when the distribution was skewed. Between-group comparisons were assessed for nominal variables with a chi-square test. Spearman’s rank correlation (ρ) was used to assess the correlations between 2 variables. The difference between 4 groups was analyzed with the Kruskal-Wallis analysis of variance (ANOVA). To measure the degree of association between variables, a Wilks lambda 2-factor ANOVA was used. The model included a test for the effect of order. A generalized linear model was used to identify possible interactions between factors, and a post hoc test was used if there was a significant interaction. A stepwise multivariate regression analysis was used to assess the predictors for the sum of EAA concentrations, the sum of NEAA concentrations, and the sum of all AA concentrations. A survival analysis was made with the Kaplan-Meier test. All analyses were performed with the use of statistical software SAS version 9.1 (SAS Inc, Cary, NC).

**RESULTS**

The clinical and biochemical characteristics of the control subjects and the patients studied are shown in **Table 1**. Fifty-seven patients in the present study had received dialysis therapy for a median of 8 d before the start of the study. However, CRP, AA, serum albumin, and serum creatinine concentrations and the prevalence of inflammation, malnutrition, and diabetes mellitus did not differ significantly between the 57 patients who had already started dialysis and the rest of the patients in the study (data not shown); therefore, all patients were analyzed as one group.

The patients were divided into 2 groups on the basis of inflammatory status (2): patients with CRP concentrations <10 mg/L were classified as having no inflammation (Student’s unpaired t test, Mann-Whitney U test, or chi-square test, as appropriate):
TABLE 2
Comparison between plasma amino acid (AA) concentrations in healthy control subjects and chronic kidney disease (CKD) patients

<table>
<thead>
<tr>
<th>AA</th>
<th>Control subjects (n = 39)</th>
<th>CKD patients (n = 200)</th>
<th>µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>33 (9–89)</td>
<td>43 (9–342)²</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>43 (22–61)</td>
<td>49 (15–97)²</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>88 (60–149)</td>
<td>93 (40–214)</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>633 (364–1013)</td>
<td>567 (299–1010)⁴</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>215 (125–392)</td>
<td>261 (103–742)²</td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>40 (25–76)</td>
<td>84 (23–209)⁴</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>80 (39–130)</td>
<td>89 (10–209)⁴</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>313 (126–610)</td>
<td>317 (84–736)</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>40 (22–62)</td>
<td>57 (13–188)³</td>
<td></td>
</tr>
<tr>
<td>Ornithine</td>
<td>38 (4–93)</td>
<td>80 (6–226)³</td>
<td></td>
</tr>
<tr>
<td>Histidine³</td>
<td>79 (49–120)</td>
<td>74 (12–151)</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>130 (50–225)</td>
<td>103 (50–246)⁶</td>
<td></td>
</tr>
<tr>
<td>Tyrosine³</td>
<td>61 (37–108)</td>
<td>39 (18–91)⁶</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>148 (90–220)</td>
<td>152 (61–286)</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>22 (12–44)</td>
<td>23 (11–83)</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>44 (29–67)</td>
<td>21 (11–101)⁴</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>52 (34–68)</td>
<td>53 (28–168)</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>217 (151–307)</td>
<td>156 (64–398)³</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>53 (26–76)</td>
<td>57 (23–149)</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>109 (64–156)</td>
<td>64 (22–178)⁴</td>
<td></td>
</tr>
<tr>
<td>Sum of NEAAs</td>
<td>1559 (842–2384)</td>
<td>1665 (950–2802)⁶</td>
<td></td>
</tr>
<tr>
<td>Sum of BCAAs</td>
<td>379 (246–532)</td>
<td>284 (133–550)⁵</td>
<td></td>
</tr>
<tr>
<td>Sum of EAAs</td>
<td>901 (588–1242)</td>
<td>748 (400–1412)³</td>
<td></td>
</tr>
<tr>
<td>Sum of AAs</td>
<td>2462 (1430–3625)</td>
<td>2440 (1350–4214)</td>
<td></td>
</tr>
<tr>
<td>NEAAs/EAs</td>
<td>1.7 (1.3–2.3)</td>
<td>2.2 (1.3–4.2)³</td>
<td></td>
</tr>
</tbody>
</table>

¹ All values are medians; range in parentheses. NEAA, nonessential AA; BCAA, branched-chain AA; EAA, essential AA.
² Significantly different from control subjects (Mann-Whitney U test): ² P < 0.01, ³ P < 0.001, ⁴ P < 0.05.
³ Thought to be an EAA in uremia.

0.001) and CVD_clin (38% compared with 21%; P < 0.05) than did the patients with a CRP concentration <10 mg/L; however, sex, body mass index, and GFR were not significantly different between the 2 patient groups. As expected, the serum concentrations of albumin and inflammatory markers differed between the 2 groups. The nPNA was significantly lower in patients with inflammation than in patients with no inflammation when nPNA was normalized to SBW; however, no significant differences were seen between the 2 patient groups when nPNA was normalized to ABW (Table 1). Serum insulin concentrations were significantly higher in the patients with inflammation than in the patients with no inflammation (Table 1) and were lower in malnourished than in nourished patients [11.5 (3.7–33.4) and 15.3 (3.6–50.3) µIU, respectively; P < 0.01].

The plasma concentrations of 7 NEAAs from fasting subjects were significantly higher in the CKD patients than in the control subjects, whereas the plasma concentrations of 5 EAAs and glutamine were significantly lower (Table 2). The other AA concentrations were not significantly different between the patients and the control subjects.

Compared with the 128 patients without inflammation, the 72 patients with inflammation had significantly lower concentrations of 7 NEAAs but not of glutamic acid, taurine, or ornithine. Of the EAAs, the plasma concentrations of histidine, threonine, lysine, methionine, and tryptophan were also significantly lower in the patients with inflammation than in the patients without inflammation.

Moreover, serum CRP concentrations were negatively correlated with plasma concentrations of asparagine, serine, glycine, citrulline, arginine, alanine, histidine, threonine, lysine, methionine, and tryptophan and were also negatively correlated with the sum of NEAA concentrations, the sum of EAA concentrations, and the sum of all AA concentrations [r range: −0.16 (P < 0.05) to −0.40 (P < 0.0001)].

Interleukin 6 concentrations (n = 169) were negatively correlated with plasma concentrations of asparagine, serine, glycine, citrulline, arginine, alanine, histidine, tyrosine, and tryptophan and with the sum of NEAA concentrations and the sum of all AA concentrations [r range: −0.15 (P < 0.05) to −0.28 (P < 0.01)]. Serum concentrations of tumor necrosis factor α (n = 151) showed negative correlations with citrulline, arginine, alanine, histidine, threonine, tryptophan, valine, ornithine, and leucine concentrations and with the sum of branched-chain AA (BCAA) concentrations, the sum of EAA concentrations, and the sum of all AA concentrations [r range: −0.16 (P < 0.05) to −0.25 (P < 0.01)].

Serum fibrinogen concentrations (n = 150) were also negatively correlated with the concentrations of asparagine, serine, glutamine, glycine, citrulline, arginine, alanine, histidine, threonine, methionine and with the sum of NEAA concentrations, the sum of all AA concentrations, and the sum of branched-chain AA (BCAA) concentrations, the sum of EAA concentrations, and the sum of all AA concentrations [r range: −0.16 (P < 0.05) to −0.38 (P < 0.0001)].

Furthermore, serum neopterin concentrations (n = 115) were negatively correlated with concentrations of asparagine, citrulline, arginine, threonine, lysine, tryptophan, and valine and with the sum of NEAA concentrations and the sum of all AA concentrations [r range: −0.19 (P < 0.05) to −0.27 (P < 0.01)]. Serum soluble intracellular adhesion molecule 1 and soluble vascular cell adhesion molecule 1 concentrations were negatively correlated with concentrations of asparagine, citrulline, arginine, ornithine, threonine, lysine, methionine, tryptophan, phenylalanine, and valine and with the sum of NEAA concentrations, the sum of EAA concentrations, and the sum of all AA concentrations [r range: −0.27 (P < 0.05) to −0.35 (P < 0.01)]; whereas the inverse correlations with threonine (ρ = −0.25, P = 0.06), histidine (ρ = −0.24, P = 0.06), and phenylalanine (ρ = −0.24, P = 0.06) were not statistically significant.

Serum albumin concentrations were positively correlated with the concentration of most AAs, including asparagine, glycine, citrulline, arginine, alanine, histidine, threonine, and tryptophan and with the sum of branched-chain AA (BCAA) concentrations, the sum of EAA concentrations, and the sum of all AA concentrations [r range: 0.19 (P < 0.05) to 0.35 (P < 0.0001)]. However, we unexpectedly found that nPNA was not correlated with AA concentrations.

Twenty-nine percent of the patients were found to be malnourished when nutritional status was assessed by SGA (n = 196; data were not available for 4 patients; Table 1). Thirty-two (56%) of the malnourished patients (56%) had inflammation, whereas 38 (27%) of the patients with normal nutritional status had inflammation. The plasma concentrations of all EAAs (except tyrosine), a few NEAAs, and NEAA/EAA were significantly lower in the malnourished patients than in the patients with normal nutritional status (data not shown). In addition, nPNA was significantly lower in the malnourished patients than in the patients with normal nutritional status when nPNA was normalized to SBW (0.63 ± 16 compared with 0.71 ± 16 g·kg SBW⁻¹·d⁻¹, P < 0.01), whereas nPNA was similar between the malnourished
The patients who underwent SGA (Table 3; n = 196) were divided into 4 groups on the basis of the presence of inflammation or malnutrition: group 1 (n = 101) included patients who had neither inflammation nor malnutrition, group 2 (n = 25) included patients who had only malnutrition, group 3 (n = 38) included patients who had only inflammation, and group 4 (n = 32) included patients who had both inflammation and malnutrition. Using a one-factor ANOVA, we found that the plasma concentrations of glutamic acid, aspartic acid, alanine, histidine, threonine, methionine, and tryptophan and the sum of NEAA concentrations, the sum of EAA concentrations, and the sum of all AA concentrations were significantly lower in the patients with CVDclin than in the patients with no CVDclin.

A stepwise multivariate regression analysis was used to determine the factors that were associated with plasma AA concentrations (Table 5) with the use of a model that included age, sex, GFR, SGA, and plasma insulin, serum albumin, and CRP concentrations. In 3 models that included the same variables, the sum of NEAA concentrations was independently associated with both sex and the concentrations of serum albumin and CRP; the sum of EAA concentrations was independently associated with sex, concentrations of serum albumin, and SGA; and the sum of all AA concentrations was independently associated with sex, GFR, and concentrations of serum albumin and CRP.

We assessed the association between all-cause mortality and total AA concentrations using Kaplan-Meier survival curves based on the median concentration of AAs (2440 μmol/L). We noted a significant increase in all-cause mortality in patients with low concentrations of AAs (log-rank: 4.2; P = 0.04; Figure 1).

![Figure 1](image-url)
with kwashiorkor, because the abnormal pattern of AAs in uremic patients resembles that seen in children with protein-energy malnutrition (19).

The present data clearly show that concentrations of most NEAAs and several EAAs are lower in CKD patients with inflammation than in CKD patients with no inflammation and that serum CRP concentrations are associated independently with low concentrations of AAs (Table 5). These findings suggest a
significant effect of inflammation on AA concentrations. This hypothesis is additionally supported by the inverse relation between the concentrations of several AAs and some inflammatory markers. Moreover, a more marked reduction in AA concentrations was observed in CKD patients with both inflammation and malnutrition than in malnourished patients with no inflammation, and inflammation was independently associated with the observed alteration in the concentrations of several AAs (Table 3).

The findings in the present study suggest that inflammation could contribute to malnutrition in CKD patients by reducing the circulating pool of free AAs in patients with inflammation. However, the mechanisms by which inflammation lowers plasma AA concentrations in CKD patients are not clear. Evidence suggests that inflammation leads to increased losses of nitrogen in the urine, increased AA oxidation, and increased metabolic demands of AAs (12, 20–22).

Also, the systemic inflammatory response and accumulation of proinflammatory cytokines may contribute to lower AA concentrations in CKD patients through a variety of other mechanisms, such as inhibition of appetite, changes in gastrointestinal functions and carbohydrate metabolism, increased rate of muscle and protein breakdown, and insulin resistance (23–25). Recently, it was reported that the impairment of protein assimilation in uremic patients (26, 27) is associated with the malnutrition-inflammation-atherosclerosis syndrome (27). However, hyperinsulinemia, which is common in CKD patients, likely causes a shift of AAs from an extracellular to an intracellular compartment, which results in low plasma AA concentrations. In the present study, patients with inflammation had higher insulin concentrations than did patients with no inflammation, which may have contributed to the low plasma AA concentrations that were observed in that group of patients. Although insulin may increase extracellular AA transport into tissues, we have not identified any clinical study that confirms such an association. However, in the present study, we found that malnutrition, which was more prevalent in patients with inflammation, was associated with low concentrations of both insulin and AAs. This suggests that the low plasma AA concentrations in the patients with inflammation were not due solely to a difference in insulin concentrations.

Uremic patients are often anorexic, which leads to a reduced intake of protein. In addition, many patients in the present study were prescribed a protein-restricted diet that further contributed to the observed low intake of dietary protein, which was estimated from npNA (Table 1). Therefore, a low protein intake, which was perhaps the consequence of inflammation, may have contributed to the abnormalities in AA concentrations observed in the present study. However, in the present study, no association was found between npNA and AA concentrations.

After protein intake was normalized to SBW, lower protein intakes were observed in patients with inflammation and malnourished patients than in patients with no inflammation and nourished patients. This finding is similar to the findings in patients undergoing hemodialysis (28) and supports the concept that the use of actual body weight for the normalization of protein intake may be flawed and misleading (29, 30) because it yields inflated npNA measurements in underweight and malnourished patients.

In the present study, fasting CKD patients with malnutrition had lower plasma concentrations of most AAs than did nourished patients, and the changes in BCAA concentrations as well as in the concentrations of several EAAs were more associated with malnutrition than with inflammation (Table 3). Not surprisingly, BCAA concentrations were associated with nutritional status. Dietary protein intake, insulin concentrations, and acid-base balance are important factors in BCAA metabolism. However, in the present study, no significant differences in BCAA concentrations were observed between patients with inflammation and patients with no inflammation, although patients with inflammation had lower protein intakes and higher insulin concentrations than did patients with no inflammation. Malnutrition has an influence on plasma AA concentrations in CKD patients (31), but the present study examined the extent to which this relation was independent of the effect of inflammation. As shown in Table 3, the presence of both inflammation and malnutrition in CKD patients was associated with a more marked reduction in AA concentrations than in the patients with only one of these conditions. However, the limited number in patients in the present study did not allow us to detect possible interactions between AA concentrations, nutritional status, and inflammation (Table 3).

CKD patients may experience ≥2 types of malnutrition (4): type 1 malnutrition is associated with anorexia because of the uremic syndrome per se, whereas type 2 malnutrition is mainly cytokine-driven and characterized by inflammation and protein catabolism. The association between malnutrition, inflammation, and atherosclerosis (4) suggests that patients with type 2 malnutrition also could have a higher prevalence of CVD than patients with type 1 malnutrition. In the present study, patients with both malnutrition and inflammation had a higher prevalence of CVD than patients with only malnutrition or inflammation. Moreover, the plasma concentrations of EAAs and NEAAs were significantly lower in patients with CVD than in patients without CVD, which was probably due to the high prevalence of inflammation and malnutrition in the patients with CVD.

The inverse relation between plasma AA concentrations and the concentrations of inflammatory markers in CKD patients agrees with the findings in nonuremic subjects, which showed an immediate or long-standing reducing effect of AA supplementation on proinflammatory cytokine concentrations and on the systemic inflammatory response (15, 32–35). In CKD patients, oral AA supplements (8, 36–38) and AA-based peritoneal dialysis fluid (39) were used to provide additional AAs to improve protein and energy homeostasis. However, the effect of such AA supplementation on the acute or chronic systemic inflammatory response has not been systematically studied in CKD patients. Nonetheless, it was recently reported that oral EAA supplementation reduced CRP concentrations in patients undergoing hemodialysis (40). Such an effect, if confirmed, may add a new advantage for the use of AA supplements in CKD patients.

The present study showed that patients with higher AA concentrations have a better survival rate than do patients with lower AA concentrations (Figure 1). The higher prevalence of inflammation and malnutrition in the patients with lower total AA concentrations may partly explain the higher mortality rate in these patients than in the patients with higher total AA concentrations.

Some limitations of the present study should be considered. First, the findings were limited by the number of patients. Second, measurements in a single sample at a certain time may not reflect the natural course of the disease. Third, this was a post hoc
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analysis, which may limit the value of the study. Finally, the present study does not provide a mechanistic explanation of whether an inflammatory state causes low AA concentrations. Therefore, additional studies are needed to better understand the mechanisms by which a systemic inflammatory response in uremic patients may result in low concentrations of plasma AAs.

In conclusion, the present findings show, for the first time, an independent association between plasma AA concentrations and the concentrations of circulating inflammatory markers in fasting CKD patients. This suggests a possible role of inflammation as a cause of low plasma AA concentrations in uremic patients. Additional studies are needed to confirm these findings and to assess whether AA supplementation has a beneficial effect on inflammation, malnutrition, and outcome in uremic patients.

We acknowledge the skilled technical assistance of Monica Eriksson and Ann-Christin Bragfors-Helin.

MES, PS, PB, OH, AA, and BL were responsible for the study design. PS, PB, and OH were involved in the patient recruitment and the data collection. RP-F, ERA, and BA provided significant advice on the laboratory procedures. MES and ARQ were responsible for the statistical analyses, which were reviewed by JCDF, AA, and BL. MES wrote the manuscript, which was reviewed by all coauthors. JCDF and BL are affiliated with Baxter Healthcare Inc. None of the other authors had any conflicts of interest.

REFERENCES


The glycemic index of foods influences postprandial insulin-like growth factor–binding protein responses in lean young subjects

Jennie C Brand-Miller, Vicki Liu, Peter Petocz, and Robert C Baxter

ABSTRACT

Background: Growth in normal and malignant tissues has been linked to hyperinsulinemia and insulin-like growth factors (IGFs). We hypothesized that IGF and IGF-binding protein (IGFBP) responses may be acutely affected by differences in the glycemic index (GI) of foods.

Objective: We compared the postprandial responses of IGFs and IGFBP to 2 foods of similar macronutrient composition but with greatly different GIs—pearled barley (GI: 25) and instant mashed potato (GI: 85).

Design: Ten young lean subjects consumed 50-g carbohydrate portions of the 2 foods or water (extended fast) in random order after an overnight fast. Capillary blood was collected at regular intervals over 4 h for measurement of blood glucose, insulin, and components of the IGF system.

Results: Serum IGFBP-1 declined markedly after both meals, but the mean (±SEM) change at 4 h was significantly (P < 0.01) more prolonged after the low-GI meal (−55 ± 20 ng/mL) than after the high-GI meal (−13 ± 15 ng/mL). Conversely, the change in serum IGFBP-3 concentration at 4 h was significantly (P < 0.05) higher after the low-GI meal (251 ± 102 ng/mL) than after the high-GI meal (−110 ± 96 ng/mL); the same pattern was observed at 2 h. Changes in IGFBP-2, free IGF-1, and total IGF-1 responses were minimal and did not differ significantly from those during the 4-h fast.

Conclusion: Acute changes in IGFBP-3 after low-GI and high-GI foods may provide a biologic mechanism linking cell multiplication with greater consumption of high-GI carbohydrates. Am J Clin Nutr 2005;82:350–4.

KEY WORDS Glycemic index, insulin, insulin-like growth factors, cancer

INTRODUCTION

There is increasing recognition that cancer growth may be promoted by hyperinsulinemia (1). Known risk factors for colorectal cancer, eg, physical inactivity, obesity, diabetes, and low-fiber diets, can be linked through a common association with high blood concentrations of insulin. Hyperinsulinemia could promote tumor development by increasing the activity of insulin-like growth factors (IGFs), which in turn stimulate cell proliferation and inhibit apoptosis (programmed cell death) (2). Postprandial hyperglycemia per se, as assessed by high 2-h postchallenge blood glucose concentration, has also been linked to a greater risk of colon cancer (3). Refined sugar and starchy foods that increase postprandial glycemia have been positively associated with colorectal and breast cancer (4, 5). Conversely, fiber, which has variable effects on the rate of carbohydrate absorption, has been associated with a lower cancer risk in some studies (6) but not in others (7). Because total carbohydrate intake per se shows no relation to cancer risk, the glycemic nature of the carbohydrate may be more important than its absolute amount.

The degree of postprandial glycemia is influenced by both the quality and quantity of carbohydrate in the meal (8). The glycemic index (GI) of a food is a summative measure of the carbohydrate quality or “glycemic potential” on a scale on which the reference food (glucose or white bread) by definition has a GI of 100. The GI permits comparisons of carbohydrates in different foods on a gram-for-gram (or weight-for-weight) basis. The average GI of whole diets has been linked to increased cancer risk in several case-control and observational studies (9). The concept of glycemic load (GL), defined as the product of the GI and the carbohydrate content, was introduced to derive a global estimate of postprandial glycemia and insulin demand. In the Women’s Health Study, GL was independently related to colorectal cancer with a relative risk of 2.85 between the highest and lowest quintiles after adjustment for known confounders (10). Similarly, in an Italian case-control study, persons in the highest quintile of GL had a relative risk of 1.8 after adjustment for age, body mass index, physical activity, fiber, and other risk factors (11). Breast cancer development may also be sensitive to hyperglycemia and insulinemia. In the same Italian study (12), the highest quintile of GI and GL had an adjusted relative risk of 1.4 and 1.3, respectively.

The association between dietary GI or GL and cancer risk may be mediated by the IGF system (9). IGF-I circulates while bound to 1 of 6 IGF-binding proteins (IGFBP-1 to -6) that together coordinate cell growth (13). Food intake has direct effects on all aspects of the IGF system. Prolonged fasting and protein-restricted diets are associated with reductions in plasma IGF-I, whereas meal feeding is associated with an acute decline in circulating IGFBP-1 (14). We hypothesized that the IGF axis may be responsive to differences in the GI of foods and thus provides a mechanism by which the growth of both normal and...
aberrant cells may be promoted. The aim here was to measure changes in blood glucose, insulin, IGF-I, and IGFBP-1, -2, and -3 after consumption of 2 foods with different GIs.

SUBJECTS AND METHODS

Study population

Ten young lean subjects were recruited from the University of Sydney student population (Table 1). Inclusion criteria were that subjects have a BMI (in kg/m²) of 19–25 and that they be 18–30 y old, of European white origin, nonsmoking, engaged in moderate physical activity, and not taking medication known to alter glucose tolerance.

All subjects gave written informed consent. The protocol was approved by the University of Sydney human ethics committee.

Research design

Volunteers attended the Human Nutrition Unit on 6 separate occasions after a 10-h overnight fast. In randomized order, 2 visits involved the administration of a 50-g carbohydrate portion of the high-GI food, 2 visits involved the administration of a 50-g carbohydrate portion of the low-GI food, and 2 visits involved an extended period of fasting (water only). The low-GI challenge was an 82-g serving of instant mashed potato reconstituted with 285 mL boiling water (published GI: 83 ± 1; 15). The low-GI challenge was a 160-g serving of cooked barley (published GI: 25 ± 2; 15). Whereas the GI of instant mashed potato was more than 3 times that of pearled barley, the meals were comparable in energy and macronutrients (Table 2) and were consumed within 13 min along with 250 mL water.

During each visit, a total of 11 fingerprick capillary blood samples (≈1 mL each) were collected at 0 time (just before the start of eating) and at 15–30-min intervals over 4 h. On 3 visits (one for each treatment), the samples were collected into Eppendorf tubes containing heparin for analysis of glucose and insulin. In the other 3 sessions, blood samples were collected into tubes not containing heparin for the assay of IGFBP-1 and IGFBP-2 at all 11 time points and the assay of IGFBP-3 and free and total IGF-1 at 120 and 240 min. Plasma and serum were immediately separated by centrifugation, and the samples were stored at –20°C until analysis.

Plasma and serum metabolite and hormone measurements

Plasma glucose concentrations were assayed by using the hexokinase glucose-6-phosphate dehydrogenase enzymatic ultraviolet method (Roche Diagnostics, Basel, Switzerland). Precimat glucose standards (Boehringer Mannheim, Mannheim, Germany) were used to construct the standard curve. The intraassay and interassay CV was 1.0% and 0.5%, respectively. The Coat-A-Count Insulin kit (Diagnostic Products Corporation, Los Angeles, CA) was used to measure plasma insulin concentrations. Total IGF-I was measured by using a radioimmunoassay (RIA) with antiserum Tr10 as previously described (16), except that [125I]des-(1–3)IGF-I was used as the radioligand. Free IGF-I was measured by using the immunoradiometric assay kit from Diagnostic Systems Laboratories (Webster, TX). IGFBP-1 and -2 were measured by using specific RIAs as described previously (17–19), although in this instance IGFBP-1 was assayed by using 50 μL of sample or standard. Serum IGFBP-3 was measured by using an enzyme-linked immunoassay and reagents supplied by the Kolling Institute of Medical Research (St Leonards, Australia). Briefly, goat anti-rabbit polyclonal antibody (at a concentration of 1:50) was used to capture IGFBP-3 antiserum R-100 at a final concentration of 1:20,000, which in turn captured biotinylated IGFBP-3. Antibody-bound biotinylated IGFBP-3 was detected by a streptavidin-horseradish peroxidase conjugate that underwent a color change when tetramethyl benzidine was added. The intraassay and interassay CV was 3.6% and 1.0%, respectively.

Statistical analysis

Changes from baseline in plasma glucose and insulin concentrations and in serum IGF-I and IGFBP concentrations were calculated. Cumulative changes in plasma glucose and insulin responses were quantified as the incremental area under the curve (AUC) that was truncated at the fasting value. Significant differences between the 2 meals were determined by using 2-factor analysis of variance for peak changes and AUC values, with foods as a fixed factor and subjects as a random factor. Significance was attained when F was < 0.05. Data were analyzed by using MINITAB statistical software (version 13; Minitab Inc, State College, PA). All data are presented as means ± SEs unless otherwise indicated.

RESULTS

Fasting concentrations of glucose, insulin, and components of the IGF axis did not differ significantly between groups at the start of each study or during the extended 4-h fast (Table 3). After the ingestion of the high-GI food, plasma glucose concentrations increased from baseline, peaked at 30 min (a change of 3.6 ± 0.3 mmol/L), and returned to baseline within 2 h (Figure 1A). In contrast, the glucose response to the low-GI food was smaller but more sustained; it peaked at 45 min (a change of 1.6 ± 0.3

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.8 ± 10.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.5 ± 1.8</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.73 ± 0.01</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. HOMA-IR, homeostasis modeling assessment of insulin resistance; (fasting glucose × fasting insulin)/22.5.
mmol/L) and remained high at 4 h. The incremental AUC after the low-GI meal (122 ± 16 mmol · L⁻¹ · 120 min⁻¹) was 40% lower than that after the high-GI meal (203 ± 26 mmol · L⁻¹ · 120 min⁻¹; \(P < 0.001\)).

Postprandial insulin concentrations peaked 45 and 30 min after the high- and low-GI meal, respectively (Figure 1B). The peak plasma insulin concentration after the high-GI meal (45.5 ± 7.3 IU/mL) was 4 times that after the low-GI meal (10.2 ± 1.2 IU/mL; \(P < 0.005\)). Plasma insulin concentrations returned to baseline within 2.5 h after the high-GI meal but remained slightly high during the period after the low-GI meal. The AUC after the low-GI meal (918 ± 97 pmol · L⁻¹ · 120 min⁻¹) was 70% lower than that after the high-GI meal (3080 ± 458 pmol · L⁻¹ · 120 min⁻¹; \(P < 0.001\)). During the 4-h extended fast, insulin concentrations gradually decreased, but the difference between those at baseline and at 4 h was not significant (Table 3).

Serum IGFBP-1 concentrations decreased markedly (≈50%) after both carbohydrate meals (Figure 2) but remained depressed for longer in the low-GI trial. Thus, at the 4-h timepoint, IGFBP-1 concentrations were significantly lower with the low-GI meal than with the high-GI meal (−55 ± 20 and −13 ± 15 ng/mL, respectively; \(P < 0.01\)). Changes in serum IGFBP-3 concentrations were also dependent on the carbohydrate source, but, unlike IGFBP-1, IGFBP-3 rose after the low-GI meal (251 ± 102 ng/mL; a 6% change at 4 h) and fell by a similar amount after the high-GI meal (−252 ± 110 ng/mL at 2 h and −110 ± 96 ng/mL at 4 h). At both the 2- and 4-h timepoints, the difference between foods was significant (\(P < 0.05\); Figure 2). Changes in IGFBP-2 and in free and total IGF-1 were minimal (data not shown) and were not significantly different from those shown during the extended fast (Table 3).

**DISCUSSION**

To our knowledge, this is the first study to investigate the acute effect of various carbohydrate sources on the IGF axis. As postulated, the greater glycemic and insulin responses to the high-GI meal elicited changes in the IGF system that may be clinically

---

### TABLE 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>7.5 ± 0.9</td>
<td>6.5 ± 0.7</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Free IGF-1 (ng/mL)</td>
<td>0.72 ± 0.14</td>
<td>0.62 ± 0.09</td>
<td>0.68 ± 0.14</td>
</tr>
<tr>
<td>Total IGF-1 (mmol/L)</td>
<td>65.2 ± 15.1</td>
<td>53.9 ± 6.1</td>
<td>51.9 ± 8.6</td>
</tr>
<tr>
<td>IGFBP-1 (ng/mL)</td>
<td>80 ± 24</td>
<td>103 ± 29</td>
<td>108 ± 34</td>
</tr>
<tr>
<td>IGFBP-2 (ng/mL)</td>
<td>137 ± 19.5</td>
<td>124 ± 15</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>IGFBP-3 (ng/mL)</td>
<td>4360 ± 230</td>
<td>4320 ± 250</td>
<td>4190 ± 240</td>
</tr>
</tbody>
</table>

All values are \(\bar{x} \pm SEM; n = 10\). IGFBP, IGF-binding protein. None of the changes over time were significant (2-factor ANOVA with food as a fixed factor and subject as a random factor).
important. Whereas both meals acutely reduced IGFBP-1 concentrations, the reduction was sustained for significantly longer after the low-GI meal (ie, barley). In contrast, IGFBP-3 concentrations rose after the barley meal, fell after the high-GI potato meal, and remained different at 4 h. Because both meals were closely matched for total energy, carbohydrate, fat, protein, and fiber, differences in macronutrient composition are unlikely to confound the interpretation of the results.

The changes in IGFBP-3, although quantitatively small (rising or falling by \(\approx 6\%\)), were 5- to 10-fold those in IGFBP-1, which suggested that high-GI carbohydrates may contribute to a metabolic environment that is conducive to tumor growth. IGFBP-3 is the major IGF-binding protein in serum and is expressed in many tissues, including normal and malignant breast epithelium. There is increasing evidence that IGFBP-3 has intrinsic antiproliferative and proapoptotic effects on human cancer cells. In case-control studies, lower serum IGFBP-3 concentrations are associated with greater risks of lung, bladder, and pancreatic cancer (20–22). However, the subject is controversial, because some studies show that IGFBP-3 can both augment and inhibit the mitogenic actions of IGF-1 and IGF-2 in breast cancer cells (23).

Acute changes in serum IGFBP-3 probably reflect stability or clearance rather than production (19, 24). Most circulating IGF-1 forms a complex comprising IGF-1, IGFBP-3, and an acid-labile subunit. In this form, the half-life of circulating IGF-1 is increased from a few minutes up to several hours because it traverses the capillary endothelium poorly (25). For this reason, a decrease in IGFBP-3, as occurred after the consumption of the high-GI food, suggests that a greater amount of free, biologically active IGF-1 may be available to the tissues (26). Conversely, the rise in IGFBP-3 concentrations after the low-GI meal implies less availability of free IGF-1. Because IGF-1 is a potent glucose-lowering peptide, circulating IGFBP-3 concentrations may fall in order to restore glucose homeostasis. Indeed, hypoglycemia induced by IGF-1 administration can be blocked by the coadministration of IGFBP-3 (19). Whereas we did not detect changes in free IGF-1 per se in the current study, its measurement, based on changes in total IGF and IGFBP-3, is at best an approximation (24). Free IGF-1 concentrations will depend on changes in all 6 IGFBPs.

In previous studies, carbohydrate consumption was shown to induce an acute decrease in serum concentrations of IGFBP-1 (27). This was also the case in the current study: by 2 h, both meals produced a decrease of \(\approx 50\, \text{ng/mL}\) in IGFBP-1. However, the effect was short-lived after the high-GI meal: by 4 h, the IGFBP-1 concentration had returned to the fasting concentration. In contrast, after the low-GI meal, the decrease was sustained for the remainder of the 4-h period of study. This result was somewhat surprising, given the smaller insulin response to the barley (28). However, our findings might be explained by the dynamics of the rise and fall in insulin. Because the high-GI meal elicits both a rapid rise and a rapid fall in insulin concentrations, the IGFBP-1 concentration may mirror this activity, falling and rising in quick succession. In contrast, the small but sustained rise in glycemia and insulinemia after consumption of the slowly digested food may depress IGFBP-1 concentration longer. Whether the magnitude of these changes is important in a clinical sense remains to be seen. IGFBP-1 has been reported to both inhibit and potentiate IGF-1 action, depending on cellular target and posttranslational modifications (26).

The strengths and weaknesses of the study should be considered. Only 2 foods were compared as single meals rather than as part of mixed meals on a chronic basis. Although the meals were matched in nutrient composition as closely as possible, they were not identical: the low-GI meal contained \(\approx 4\, \text{g}\) more protein and \(3\, \text{g}\) less fat than did the high-GI meal. Such small differences, however, are unlikely to elicit the differences in hormonal responses that we observed. In practice, most meals contain much greater amounts of fat and protein than were fed here. Nonetheless, differences in GI have been found to predict the glycemic response to realistic mixed meals and daylong glycemia (29, 30). In persons with diabetes, low-GI diets have been found to lower glycated hemoglobin, a measure of the average blood glucose concentration over the previous 2–3 mo (31). Our subjects were young and lean and mainly female, and the findings may not apply to persons who are overweight or more insulin resistant. However, despite the small number of subjects, the study had sufficient power to show statistically and perhaps biologically important differences in glycemia, insulinemia, and IGF system responses.

Increased consumption of high-GI carbohydrates could be considered one of the more subtle changes in the food supply over the past 50 y. As nations westernize and industrialize, traditional foods such as minimally processed whole grains and legumes are replaced by more highly processed and digestible foods. In general, traditional foods contain carbohydrates that are slowly digested because the starches and sugars remain closely embedded in the plant’s original botanical structure, where they are surrounded by bran and other barriers that inhibit starch gelatinization (32, 33). In contrast, modern methods of processing that involve the production of fine flours and the use of high temperatures and pressures increase starch gelatinization and hence the rate of digestion in vivo. Together with refined sugars, high-GI starches will increase the glycemic and insulinotropic potency of the diet.

We speculate that the findings of the current study may therefore be relevant to normal growth (34). Small increases in height, weight, and rate of maturation continue to occur over time, even in highly developed nations where nutrition standards have been high for decades (35). The differential effects of carbohydrate foods on the IGF axis might also influence the number of adipocytes and thus overweight and obesity. Pawlak et al (36) showed that rats fed a high-GI diet over an 18-week period gained 70% more body fat than did rats of equal body weight fed a low-GI diet. Increased consumption of milk (a low-GI food) has been associated with lower risk of weight gain over time (37) and, in boys, with higher IGFBP-3 concentrations (38). Milk was also the most consistent dietary correlate with IGFBP-3 in a study of \(>1000\) healthy women (39).

The novel findings of the current study require confirmation in daylong and long-term studies using mixed diets with high- or low-GI carbohydrate sources. Further studies will also be necessary to ascertain whether changes in the IGF system are sensitive to postprandial glycemia and insulinemia in other population groups, particularly those with greater insulin resistance.
University of Sydney (www.glycemicindex.com), and is a coauthor of a series of books under the rubric The New Glucose Revolution (New York: Marlowe and Co).

REFERENCES


Whole-body protein anabolic response is resistant to the action of insulin in obese women\textsuperscript{1–3}

Stéphanie Chevalier, Errol B Marliss, José A Morais, Marie Lamarche, and Réjeanne Gougeon

ABSTRACT
Background: Obesity is associated with insulin resistance of glucose and lipid metabolism.
Objective: We sought to determine the effects of obesity on the insulin sensitivity of protein metabolism.
Design: Whole-body \textsuperscript{13}C]leucine and \textsuperscript{3}H]glucose kinetics were measured in 9 lean and 10 obese women in the postabsorptive state and during a hyperinsulinemic, euglycemic, isoaminoacidemic clamp.
Results: In the postabsorptive state, the leucine endogenous rate of appearance (catabolism), normalized for fat-free mass, was 11\% greater and the nonoxidative rate of disappearance (synthesis) was 8\% greater in the obese than in the lean women, but net balance was 29\% more negative (P < 0.05). Clamp amino acid and glucose infusion rates were significantly lower in the obese women than in the lean women (0.65 ± 0.02 compared with 0.85 ± 0.04 and 5.7 ± 0.3 compared with 9.1 ± 0.5 mg · kg fat-free mass\textsuperscript{−1} · min\textsuperscript{−1}, respectively; P < 0.0001 for both), and their rates correlated positively (r = 0.635, P = 0.005). During hyperinsulinemia, synthesis was stimulated less and net leucine balance was much lower in the obese women than in the lean women (−0.08 ± 0.06 and 0.30 ± 0.03 \textmu mol · kg fat-free mass\textsuperscript{−1} · min\textsuperscript{−1}, respectively; P < 0.0001). The percentage change in net leucine balance correlated negatively with all adiposity indexes. Plasma free fatty acids were less suppressed and the respiratory quotient was lower in the obese women than in the lean women.
Conclusion: Obese women show a blunted protein anabolic response to hyperinsulinemia that is consistent with resistance to the action of insulin on protein concurrent with that on glucose and lipid metabolism.

KEY WORDS Hyperinsulinemic clamp, leucine kinetics, resting energy expenditure, glucose kinetics, amino acids, women

INTRODUCTION
Obesity is associated with insulin resistance of glucose and lipid metabolism (1), but its influence on protein metabolism remains a subject of controversy. Postabsorptive protein kinetics have been reported to be significantly higher in obese than in nonobese subjects (2–4) or similar in the 2 groups (5–9). Because insulin is an anabolic hormone, its action in suppressing protein breakdown and stimulating synthesis would be predicted to be impaired in obesity. Whereas some studies in obese persons showed that protein metabolism during a hyperinsulinemic, euglycemic clamp was normal (6, 9), other studies showed a blunted antiproteolytic effect (2, 8). One limitation in interpreting such kinetic results in both lean and obese persons is that the elevated plasma insulin concentrations were accompanied by reduced plasma amino acid (AA) concentrations (2, 6, 9), which were due to the suppression of proteolysis. Consequently, insulin stimulation of protein synthesis cannot be assessed in the face of decreased substrate availability (10). Luzi et al (8) found normal stimulation of protein synthesis in obese persons during the infusion of AAs but found it to be associated with hyperaminoacidaemia. Because AAs themselves stimulate protein synthesis (8, 11–13), quantifying the relative roles of insulin and of the increases in AA is precluded. One way of dissecting out the role of insulin, and thus of showing insulin resistance, is to “clamp” plasma AAs at their postabsorptive concentrations during a hyperinsulinemic clamp. We recently showed both inhibition of protein catabolism and stimulation of synthesis in healthy male subjects by using this technique (14). Thus, we tested 1) whether increased adiposity interferes with the protein anabolic responses to insulin of suppressing protein breakdown, stimulating protein synthesis, or both, and 2) whether the AA infusion rates during a clamp could serve as an index of insulin resistance of protein metabolism, much as glucose infusion rates define insulin resistance of glucose metabolism. Preliminary results were presented in abstract form (15).

SUBJECTS AND METHODS
Subjects and diet
Nine lean and 10 nondiabetic, normotensive obese women were screened with the use of medical history, physical examination, and laboratory investigation as previously detailed (16). No women were taking medications. They were admitted to the


\textsuperscript{1} From the McGill Nutrition and Food Science Centre, McGill University Health Centre–Royal Victoria Hospital, Montreal, Canada.
\textsuperscript{2} Supported by research grants no. MOP-15487 (to RG) and MOP-42500 (to EB) from the Canadian Institutes of Health Research, a research grant from the Canadian Diabetes Association (to JAM), salary awards from the McGill University Health Centre Research Institute (to RG) and from the Fonds de la recherche en santé du Québec (to JAM), and a fellowship from the Canadian Diabetes Association (to SC).
\textsuperscript{3} Reprints not available. Address correspondence to R Gougeon, McGill Nutrition and Food Science Centre, MUHC–Royal Victoria Hospital, 687 Pine Avenue West, Montreal, PQ H3A 1A1, Canada. E-mail: rejeanne.gougeon@muhc.mcgill.ca.
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McGill University Health Centre–Royal Victoria Hospital Clinical Investigation Unit.

All subjects provided written informed consent. The study protocol was approved by the Human Ethics Review Committee of McGill University Health Centre.

The subjects were classified according to their body mass index (BMI: in kg/m²) as lean (BMI: <25) and obese (BMI: >30). Subjects received for 7 d an individualized, formula-based isoenergetic diet that was based on resting metabolic rate obtained by indirect calorimetry (Deltatrac; Sensor Medics, Yorba Linda, CA) and multiplied by a physical activity factor of 1.7 for the lean and 1.6 for the obese subjects. The diet was recently described fully (14). A 24-h food recall was performed by a registered dietitian to estimate usual protein intake for exclusion of subjects whose intakes would differ quantitatively and qualitatively from our diet protocol of 1.8 g · kg⁻¹ · d⁻¹. Nitrogen balance was measured during the last 3 d of the diet (17). Body composition was assessed by bioelectrical impedance analysis (RJL-101A; RJL Systems, Detroit, MI) by using equations validated for lean (18) and obese (19) subjects. With the use of these equations, the FFM values in each of our groups were close to those of published studies of protein kinetics in subjects who resembled ours with respect to BMI, body weight, or both (6, 7, 18).

### Hyperinsulinemic, euglycemic, isoaminoacidemic clamp protocol

The hyperinsulinemic clamp was performed in the postabsorptive state as recently detailed (14), with plasma glucose at 5.5 mmol/L and at each subject’s own plasma branched-chain AA (BCAA) concentrations. Glucose turnover was studied by using a primed [22 μCi (814 kBq)] continuous infusion [0.22 μCi/min (8.14 kBq/min)] of [3-3H]glucose that was begun 180 min before insulin. Concurrently with the tritiated glucose infusion and after an oral bolus of 0.1 mg/kg NaH¹³CO₃, leucine kinetics were studied by using a primed (0.5 mg/kg), constant (0.008 mg · kg⁻¹ · min⁻¹) infusion of [¹³C]leucine (20). A primed infusion of biosynthetic regular human insulin (Humulin R; Eli Lilly Canada Inc, Toronto, Canada) was started at 0 min and maintained at a rate of 40 mU · m⁻² · min⁻¹ for ≥210 min. At 4 min, sterile 20% (wt:vol) potato starch–derived glucose (AVEBE BA, Foxhol, Netherlands) in water with added [3-3H]glucose was infused [ie, the “hot GINF” method (21)] at variable rates to achieve euglycemia based on measurements every 5 min. We have verified by isotope ratio–mass spectrometry that this glucose has negligible ¹³C content, whereas commercial dextrose solutions have corn-derived glucose with ¹³C enrichment sufficiently high that it would have to be quantified and adjusted for in calculations of leucine kinetics. Plasma BCAAs were kept constant with a variable infusion of a 10% AA mixture (10% TrophAmine without electrolytes; B Braun Medical Inc, Irvine, CA) based on measurements of BCAAs every 5 min. This approach maintains most individual AAs within the normal postabsorptive range in healthy subjects (14).

Blood samples were collected for analyses at baseline and every 10 min for 40 min before the insulin infusion and then every 30 min until the last 40 min, at which time they were again drawn at 10-min intervals. Indirect calorimetry was performed for 20 min before the insulin infusion and during the last 30 min of the infusion (22). Glucose turnover was calculated as specified by Saad et al (23), and substrate oxidation was calculated as specified by Bogardus et al (22). Expired air samples were collected and then transferred to evacuated tubes (Vacutainer; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Leucine kinetics were calculated according to Matthews et al (20) by using plasma α-keto isocaprylic acid (α-KIC) as an index of the precursor pool enrichment (reciprocal model). Protein kinetic data are presented as units per minute, units per kg body weight, and units per kg FFM. The latter measurement represents the denominator most closely related to the tissues responsible for most of the protein turnover. Data are presented as mean values for the baseline period (at 2 points) before insulin and for the steady state of the hyperinsulinemic period (at 4 points) for all variables except free fatty acids (FFAs).

### Assays

Plasma glucose was measured by the glucose oxidase method (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA). Assays have been detailed previously for immunoreactive insulin and glucagon (24) and glucose specific activity (21, 24). Plasma total BCAA concentrations were measured by using an enzymatic fluorometric assay (14). Individual plasma AA concentrations were measured by using ion-exchange HPLC with postcolumn ninhydrin detection (25). FFAs were measured by using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals USA Inc, Richmond, VA). The ¹³C enrichment of plasma α-KIC, reduced to hydroxyisocaproate by NaBH₄, was analyzed by gas chromatography–mass spectrometry (GCMS 5988A; Hewlett-Packard, Palo Alto, CA) after derivatization with N-methyl-(n-propyldimethylsilyl)trifluoroacetamide (tBDMS; Regis Technologies Inc, Morton Grove, IL) to yield a tBDMS derivative of hydroxyisocaproate. Expired air was analyzed for ¹³CO₂ enrichment by isotope ratio–mass spectrometry on a Micromass 903D (Vacuum Generators, Winsfor, United Kingdom).

### Validation studies of background enrichment of expired ¹³CO₂ and plasma [¹³C]KIC, and of recovery of ¹³C bicarbonate

We reported a 10.1 ± 1.6% dilution in the background enrichment of expired ¹³CO₂ in lean young men, mainly due to the infusion of the potato starch–derived, low-¹³C glucose (14). Therefore, this effect was sought in representative lean and obese women, to test for sex or obesity differences, which required the performance of identical clamp studies without tracer infusions. The mean age, BMI, percentage body fat (%BF), and glucose variables except free fatty acids.

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We reported a 10.1 ± 1.6% dilution in the background enrichment of expired ¹³CO₂ in lean young men, mainly due to the infusion of the potato starch–derived, low-¹³C glucose (14). Therefore, this effect was sought in representative lean and obese women, to test for sex or obesity differences, which required the performance of identical clamp studies without tracer infusions. The mean age, BMI, percentage body fat (%BF), and glucose variables except free fatty acids.

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Table 1

Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Lean women (n = 9)</th>
<th>Obese women (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>23.9 ± 1.3</td>
<td>41.7 ± 3.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.9 ± 2.0</td>
<td>162.0 ± 2.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>54.1 ± 1.6</td>
<td>96.2 ± 5.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.6 ± 0.4</td>
<td>36.5 ± 1.6</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>41.2 ± 0.6</td>
<td>50.6 ± 1.7</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>23.5 ± 1.8</td>
<td>46.8 ± 1.4</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>67.2 ± 1.0</td>
<td>107.8 ± 3.8</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>92.2 ± 1.1</td>
<td>122.3 ± 3.6</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.73 ± 0.01</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td>Energy intake (kcal/d)</td>
<td>2122 ± 50</td>
<td>2504 ± 96</td>
</tr>
<tr>
<td>(kcal · kg FFM⁻¹ · d⁻¹)</td>
<td>51.6 ± 1.1</td>
<td>49.5 ± 1.0</td>
</tr>
<tr>
<td>Protein intake (g/d)</td>
<td>73.2 ± 2.0</td>
<td>94.4 ± 3.1</td>
</tr>
<tr>
<td>(g · kg FFM⁻¹ · d⁻¹)</td>
<td>1.78 ± 0.04</td>
<td>1.87 ± 0.03</td>
</tr>
<tr>
<td>Nitrogen balance (g/d)</td>
<td>1.02 ± 0.28</td>
<td>0.53 ± 0.51</td>
</tr>
<tr>
<td>2-h OGTT (mmol/L)</td>
<td>5.4 ± 0.5</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)</td>
<td>3.68 ± 0.32</td>
<td>4.73 ± 0.28</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2.14 ± 0.33</td>
<td>2.63 ± 0.27</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.41 ± 0.06</td>
<td>1.17 ± 0.07</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>0.56 ± 0.02</td>
<td>2.04 ± 0.41</td>
</tr>
</tbody>
</table>

All values are ± SEM. FFM, fat-free mass; OGTT, oral-glucose-tolerance test.

Statistical analysis

Results are presented as means ± SEMs. Different variables, including leucine kinetics, were analyzed after normalization per kg FFM or by repeated-measures analysis of variance, to identify time (baseline or clamp) and group (lean or obese) effects, as well as possible interactions indicating a different response to the hyperinsulinemic clamp between lean and obese women. Leucine kinetics were also analyzed with age as a covariate. For certain variables, differences between groups at a given time were assessed by using an unpaired t test. Pearson’s coefficient was used for all correlations. Stepwise linear regression analysis was used to test the possible variables predicting the anabolic response to hyperinsulinemia. The analyses were performed with SPSS for WINDOWS software (version 10.0; SPSS Inc, Chicago, IL). Significance was defined as P < 0.05.

Results

The obese women were significantly older and had significantly higher FFM than did the lean women (Table 1). They also differed significantly in indexes of adiposity: greater body weight, BMI (by design), %BF, waist and hip circumferences, and waist-hip ratio. They had significantly higher total energy intake but not significantly higher intakes per kg FFM. Protein intake was controlled per kg FFM and thus did not differ significantly between groups. Nitrogen balance was slightly positive only in lean women but did not differ between groups. Fasting plasma glucose was normal in both groups (Table 2) but was higher 2 h after an oral-glucose-tolerance test (OGTT) in the obese women. Four obese women had impaired glucose tolerance (IGT) (ie, 2 h value ≥ 7.8 mmol/L). Serum total cholesterol and triacylglycerol were significantly higher and HDL cholesterol was significantly lower in the obese women than in the lean women, but none of the values was outside the reference range.

Hormonal and metabolic data are reported in Table 2 and Figure 1. Plasma total BCAA determined by HPLC were significantly higher in the obese women at baseline and during the clamp (Table 2). There was no change in BCAAs between basal
and clamp periods. Obese women required significantly less exogenous AA per kg FFM (Figure 1A, Table 2) to maintain BCAA concentrations than did the lean women. Glucose infusion rates to maintain euglycemia per kg FFM (Figure 1B, Table 2) were significantly lower in the obese women than in the lean women. When the data for all of the subjects were combined, a significant positive correlation was found between rates of glucose and AA infusion per FFM (Figure 2). In the obese women, the rate of appearance (Ra) of glucose per kg FFM was not completely suppressed during hyperinsulinemia, as it was in the lean women. The rate of disappearance (ie, utilization) (Rd) of glucose per kg FFM was also significantly lower in the obese women than in the lean women and was increased in both groups during the clamp, but less so in the obese women, a finding that was concurrent with the lower glucose infusion rates. Among the obese women, glucose Rd was significantly less in the women with IGT than in the women with normal glucose tolerance (5.5 ± 0.2 and 6.3 ± 0.2 mg · kg FFM⁻¹ · min⁻¹, respectively; P < 0.001).
Both postabsorptive and clamp plasma insulin concentrations were significantly higher in the obese women than in the lean women, and there was a greater increment in the obese than in the lean (627 ± 48 and 504 ± 25 pmol/L, respectively). Therefore, the obese subjects were exposed to endogenous fasting insulin that was twice that in the lean women and to a 33% higher steady state insulin concentration than were the lean women; these differences represented a 24% greater increment in data reported during the clamp in the obese women than in the lean women. Plasma glucagon did not differ significantly between the 2 groups and decreased to the same extent during the clamp. Therefore, the ratio of glucagon to insulin was significantly lower in the obese than in the lean women and decreased in the obese women to a level similar to that in the lean women during the clamp. Postabsorptive FFA concentrations did not differ significantly (Figure 3) between the groups, but maximal suppression required 30 min in the lean women compared with 60 min in the obese women. From 30 to 180 min after the start of insulin, FFA remained higher in the obese women.

Whereas oxygen consumption (\(V_O_2\)) did not change during the clamp, carbon dioxide production increased significantly in both groups (Table 3). These 2 variables were higher in obese than in lean women except when adjusted for differences in FFM (not shown). Nonprotein respiratory quotient increased significantly in both groups during hyperinsulinemia and was significantly lower in the obese than in the lean women. Resting energy expenditure was significantly higher in the obese women than in the lean women except when adjusted for FFM, and it increased significantly in all of the women during the clamp. The obese women oxidized significantly more lipids than did the lean women. During the clamp, lipid oxidation represented 21.6 ± 4.7% of the total nonprotein oxidation in the obese women and 7.5 ± 2.1% in the lean women (\(P = 0.018\)); as a percentage of energy expenditure, it was 27.0 ± 4.5% compared with 11.4 ± 3.0%, respectively (\(P = 0.012\)). Clamp rates of lipid oxidation correlated with serum FFA concentrations (\(r = 0.609, P = 0.006\)). In both postabsorptive and hyperinsulinemic states, rates of lipid oxidation correlated positively with leucine oxidation (\(r = 0.520, P = 0.022\)) and negatively with glucose oxidation (\(r = -0.689, P = 0.001\)).

Four individual plasma AA values (ie, isoleucine, leucine, ornithine, and glutamate) were significantly higher in obese than in lean women, whereas serine was lower at baseline (Table 4). In both lean and obese subjects, not only were total BCAAs (Figure 1A, Table 2) clamped at baseline concentrations, but so were total AAs. These findings were the result of the small, oppositely directed changes listed being equivalent when totaled. Although the significant percentage changes varied from \(\approx 5\%\) to \(\approx 31\%\) (highest in those with low concentrations), it is noteworthy that none of the clamped values was outside the range of reported

\[\text{TABLE 3}\]

| Nonprotein substrate utilization in lean and obese women at baseline and during the hyperinsulinemic clamp \(P\) for time × group interaction$^2$ |
|-----------------|-----------------|-----------------|
| Lean women \((n = 9)\) | Obese women \((n = 10)\) |
| \(\text{VO}_2 \text{(mL/min)}^4\) | \(193.1 ± 2.7\) | \(242.1 ± 9.7\) |
| Clamp | \(194.2 ± 4.3\) | \(241.3 ± 10.9\) |
| \(\text{VCO}_2 \text{(mL/min)}^4\) | \(154.8 ± 3.2\) | \(189.1 ± 6.7\) |
| Clamp | \(179.3 ± 4.0\) | \(209.5 ± 7.7\) |
| \(\text{npRQ}^2\) | \(0.80 ± 0.01\) | \(0.78 ± 0.01\) |
| Clamp | \(0.96 ± 0.01\) | \(0.89 ± 0.02\) |
| \(\text{REE} \text{(kcal} · \text{kg FFM}^{-1} · \text{d}^{-1})^3\) | \(32.1 ± 0.4\) | \(32.6 ± 0.8\) |
| Clamp | \(33.2 ± 0.5\) | \(33.1 ± 0.7\) |
| \(\text{Glucose oxidative Rd} \text{(mg} · \text{kg FFM}^{-1} · \text{min}^{-1})\) | \(1.40 ± 0.22\) | \(1.04 ± 0.13\) |
| Clamp | \(3.79 ± 0.20^6\) | \(2.76 ± 0.24^6\) |
| \(\text{Glucose oxidative Rd (as % of total Rd)}\) | \(52.2 ± 8.6\) | \(40.4 ± 5.1\) |
| Clamp | \(42.3 ± 3.5\) | \(45.9 ± 3.5\) |
| \(\text{Glucose nonoxidative Rd} \text{(mg} · \text{kg FFM}^{-1} · \text{min}^{-1})\) | \(1.38 ± 0.28\) | \(1.52 ± 0.12\) |
| Clamp | \(5.52 ± 0.57^5\) | \(3.20 ± 0.17^5\) |
| \(\text{Glucose nonoxidative Rd (as % of total Rd)}\) | \(47.9 ± 8.6\) | \(59.6 ± 5.1\) |
| Clamp | \(57.7 ± 3.5\) | \(54.1 ± 3.5\) |
| \(\text{Lipid oxidation (mg} · \text{kg FFM}^{-1} · \text{min}^{-1})^2\) | \(1.26 ± 0.10\) | \(1.42 ± 0.08\) |
| Clamp | \(0.29 ± 0.08\) | \(0.70 ± 0.13\) |

$^1$ All values are \(\bar{x} ± \text{SEM}\). \(\text{VO}_2\), oxygen consumption; \(\text{VCO}_2\), carbon dioxide production; FFM, fat-free mass; REE, resting energy expenditure; npRQ, nonprotein respiratory quotient; Rd, rate of glucose disappearance.

$^2$ Time and group effects and time × group interactions were assessed by repeated-measures ANOVA. A significant interaction term is interpreted as a different response to the hyperinsulinemic clamp between the 2 groups. $^3 P < 0.05$, group effect (ANOVA).

$^4 P < 0.05$, group effect (ANOVA).

$^5 P < 0.05$, time effect (ANOVA).

$^6 P < 0.05$, time effect in a given group (paired t test).

$^7 P < 0.05$, group effect in a given time period (unpaired t test).
reference postabsorptive concentrations. There were significant time × group interactions for 4 AAs: serine decreased in the lean women only, alanine increased in the lean women and decreased in the obese women, and threonine and asparagine decreased in the obese women, and threonine and asparagine decreased in the obese women, but there was no difference between the groups in measurements per kg body weight and body weight. During the hyperinsulinemic clamp, rates of total Ra and nonoxidative Rd increased from baseline and were significantly greater per min but less per body weight in the obese women than in the lean women; per kg FFM, they did not differ between groups. Oxidation also increased and was significantly greater per min and per kg FFM in the obese women than in the lean women. Because age correlated with all postabsorptive and clamp leucine kinetics it was introduced as a covariate in the analysis of variance, which resulted in the disappearance of the difference in postabsorptive oxidation and net balance between the groups. However, when we tested these correlations by control for indexes of adiposity (ie, BMI and waist and hip circumferences), they were no longer significant, and age did not correlate with responses in leucine kinetics, which indicates that, in our cohort, the effects of age, when seen, were due to higher adiposity. Thus age was not introduced as a covariate.

Although lean tissues are the site of most protein turnover, because our estimation of FFM includes many individual tissues with different rates and depends on regression equations that were developed by using different reference populations and methods, we present data as rates per unit of time, per kg body weight/min, and per kg FFM/min (Table 5). In the baseline postabsorptive state, total Ra (total flux and endogenous breakdown) and nonoxidative Rd (synthesis) were significantly greater per min and per kg FFM but significantly less per kg body weight in the obese women (Table 1). Oxidation was significantly greater but net balance was more negative per min and per kg FFM in the obese women than in the lean women. Because age correlated with all postabsorptive and clamp leucine kinetics it was introduced as a covariate in the analysis of variance, which resulted in the disappearance of the difference in postabsorptive oxidation and net balance between the groups. However, when we tested these correlations by control for indexes of adiposity (ie, BMI and waist and hip circumferences), they were no longer significant, and age did not correlate with responses in leucine kinetics, which indicates that, in our cohort, the effects of age, when seen, were due to higher adiposity. Thus age was not introduced as a covariate.

During the hyperinsulinemic clamp, rates of total Ra and nonoxidative Rd increased from baseline and were significantly greater per min but less per body weight in the obese women than in the lean women; per kg FFM, they did not differ between groups. Oxidation also increased and was significantly greater per min and per kg FFM in the obese women than in the lean women, but there was no difference between the groups in measurements per kg body weight. Endogenous Ra decreased in both groups; however, the rates remained significantly higher per min and per kg FFM but significantly lower per kg body weight in the

| TABLE 4 | Individual plasma amino acid (AA) concentrations at baseline and during the hyperinsulinemic clamp† |
|----------|--------------------------------------------------|--------------------------------------------------|
|          | Lean women (n = 9)                                | Obese women (n = 10)                             |
|          | Baseline (μmol/L)                                 | Clamp (μmol/L)                                   |
|          |                                                   |                                                  |
| Essential AA |                                                |                                                  |
| Val.sup   | 190 ± 7                                          | 180 ± 7                                          |
| Ile.sup   | 51 ± 2                                           | 54 ± 2                                           |
| Leu.sup   | 110 ± 4                                          | 121 ± 6                                          |
| Trp       | 42 ± 2                                           | 47 ± 5                                           |
| Thr       | 133 ± 20                                         | 115 ± 17†                                        |
| Phe       | 48 ± 2                                           | 57 ± 2                                           |
| Met       | 21 ± 1                                           | 25 ± 2                                           |
| Lys       | 152 ± 10                                         | 182 ± 14                                         |
| His       | 76 ± 5                                           | 98 ± 7                                           |
| Total of AA | 812 ± 35                                       | 873 ± 41                                         |
| Essential AA |                                                |                                                  |
| Tau       | 38 ± 2                                           | 36 ± 2                                           |
| Orn       | 43 ± 3                                           | 41 ± 3                                           |
| Glu       | 54 ± 6                                           | 49 ± 5                                           |
| Ser       | 101 ± 7                                          | 91 ± 7†                                          |
| Ala       | 252 ± 14                                         | 279 ± 21†                                        |
| Gin       | 502 ± 27                                         | 443 ± 31                                         |
| Gly       | 226 ± 14                                         | 259 ± 18                                         |
| Tyr       | 57 ± 5                                           | 42 ± 3                                           |
| Cit       | 35 ± 2                                           | 25 ± 2                                           |
| Asn       | 42 ± 5                                           | 29 ± 2†                                          |
| Arg       | 68 ± 5                                           | 89 ± 7†                                          |
| Total nonessential AA | 1346 ± 65                              | 1325 ± 73                                       |
| Total of AA | 2158 ± 78                                     | 2197 ± 90                                       |

† All values are ± SEM of 2 values for the baseline period and 4 values for the clamp period.

1 Time and group effects and time × group interactions were assessed by repeated-measures ANOVA. A significant interaction term is interpreted as a different response to the hyperinsulinemic clamp between the 2 groups.

2 P < 0.01, time effect (ANOVA).

3 P < 0.05, group effect (ANOVA).

4 P < 0.05, time effect in a given group (paired t test).

5 P < 0.05, time effect in a given group (unpaired t test).
## Table 5
Leucine kinetics in lean and obese women at baseline and during the hyperinsulinemic clamp

<table>
<thead>
<tr>
<th></th>
<th>Lean women</th>
<th>Obese women</th>
<th>P for time ( \times ) group interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(( n = 9 ))</td>
<td>(( n = 10 ))</td>
<td></td>
</tr>
<tr>
<td>(( \mu \text{mol/min} ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Ra(^{1,4})</td>
<td></td>
<td></td>
<td>0.259</td>
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<tr>
<td>Baseline</td>
<td>101.4 ± 2.9</td>
<td>138.7 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>122.9 ± 4.5</td>
<td>155.7 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>Oxidation(^{3,4})</td>
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<td>0.182</td>
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<tr>
<td>Baseline</td>
<td>17.3 ± 1.2</td>
<td>27.5 ± 1.6</td>
<td></td>
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<tr>
<td>Clamp</td>
<td>24.8 ± 2.6</td>
<td>39.1 ± 2.8</td>
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<tr>
<td>Endogenous Ra(^{3,4})</td>
<td></td>
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<td>0.610</td>
</tr>
<tr>
<td>Baseline</td>
<td>101.4 ± 2.9</td>
<td>138.7 ± 6.1</td>
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<tr>
<td>Clamp</td>
<td>85.5 ± 3.8</td>
<td>120.7 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>Infusion rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>37.2 ± 2.1</td>
<td>35.0 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>35.0 ± 1.7</td>
<td>35.0 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Nonoxidative Rd</td>
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</tr>
<tr>
<td>Baseline</td>
<td>84.1 ± 2.3</td>
<td>111.3 ± 4.8(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>98.1 ± 3.9(^6)</td>
<td>116.6 ± 4.2(^5,6)</td>
<td></td>
</tr>
<tr>
<td>Net balance</td>
<td>-17.3 ± 1.2</td>
<td>-27.5 ± 1.6(^6)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>12.6 ± 1.2(^6)</td>
<td>-4.2 ± 2.7(^5,6)</td>
<td></td>
</tr>
<tr>
<td>(( \mu \text{mol} \cdot \text{kg} \text{BW}^{-1} \cdot \text{min}^{-1} ))</td>
<td></td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>Total Ra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.88 ± 0.03</td>
<td>1.45 ± 0.03(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>2.28 ± 0.08(^6)</td>
<td>1.63 ± 0.03(^5,6)</td>
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<tr>
<td>Oxidation(^3)</td>
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<td></td>
<td>0.568</td>
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<td>Baseline</td>
<td>0.32 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>0.47 ± 0.06</td>
<td>0.41 ± 0.02</td>
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<tr>
<td>Endogenous Ra(^{3,4})</td>
<td></td>
<td></td>
<td>0.094</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.88 ± 0.03</td>
<td>1.45 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>1.58 ± 0.06</td>
<td>1.26 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Infusion rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.69 ± 0.05</td>
<td>0.37 ± 0.02(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>0.69 ± 0.05</td>
<td>0.37 ± 0.02(^5)</td>
<td></td>
</tr>
<tr>
<td>Nonoxidative Rd</td>
<td></td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.56 ± 0.03</td>
<td>1.17 ± 0.03(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>1.81 ± 0.04(^6)</td>
<td>1.22 ± 0.03(^5,6)</td>
<td></td>
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<tr>
<td>Net balance</td>
<td>-0.32 ± 0.02</td>
<td>-0.29 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>-0.23 ± 0.02(^6)</td>
<td>-0.04 ± 0.03(^5,6)</td>
<td></td>
</tr>
<tr>
<td>(( \mu \text{mol} \cdot \text{kg} \text{FFM}^{-1} \cdot \text{min}^{-1} ))</td>
<td></td>
<td></td>
<td>0.049</td>
</tr>
<tr>
<td>Total Ra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.46 ± 0.06</td>
<td>2.74 ± 0.05(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>2.98 ± 0.09(^6)</td>
<td>3.07 ± 0.04(^6)</td>
<td></td>
</tr>
<tr>
<td>Oxidation(^3,4)</td>
<td></td>
<td></td>
<td>0.528</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.42 ± 0.03</td>
<td>0.54 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>0.60 ± 0.06</td>
<td>0.77 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Endogenous Ra(^{3,4})</td>
<td></td>
<td></td>
<td>0.723</td>
</tr>
<tr>
<td>Baseline</td>
<td>2.46 ± 0.06</td>
<td>2.74 ± 0.05</td>
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</tr>
<tr>
<td>Clamp</td>
<td>2.07 ± 0.08</td>
<td>2.38 ± 0.06</td>
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<tr>
<td>Infusion rate</td>
<td></td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>0.90 ± 0.05</td>
<td>0.69 ± 0.02(^5)</td>
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<tr>
<td>Clamp</td>
<td>0.90 ± 0.05</td>
<td>0.69 ± 0.02(^5)</td>
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<td>Nonoxidative Rd</td>
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<tr>
<td>Baseline</td>
<td>2.04 ± 0.05</td>
<td>2.20 ± 0.04(^5)</td>
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<tr>
<td>Clamp</td>
<td>2.38 ± 0.08(^6)</td>
<td>2.31 ± 0.04(^6)</td>
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</tr>
<tr>
<td>Net balance</td>
<td>-0.42 ± 0.03</td>
<td>-0.54 ± 0.02(^5)</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>-0.30 ± 0.03(^6)</td>
<td>-0.08 ± 0.06(^5,6)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) All values are \( \bar{x} \pm \text{SEM} \). Ra, rate of appearance; Rd, rate of disappearance; total Ra, total leucine flux, including exogenous amino acid infusion during the clamp period; endogenous Ra, index of protein breakdown; infusion rate, leucine from the amino acid solution infused; nonoxidative Rd, index of protein synthesis; net balance, synthesis – breakdown; BW, body weight; FFM, fat-free mass.

\(^2\) Time and group effects and time \( \times \) group interactions were assessed by repeated-measures ANOVA. A significant interaction term is interpreted as a different response to the hyperinsulinemic clamp between the 2 groups.

\(^3\) \( P < 0.001 \), time effect (ANOVA).

\(^4\) \( P < 0.01 \), group effect (ANOVA).

\(^5\) \( P < 0.05 \), group effect in a given time period (unpaired \( t \) test).

\(^6\) \( P < 0.05 \), time effect in a given group (paired \( t \) test).
With all subjects combined, the percentage change in net leucine balance correlated negatively with all indexes reflecting adiposity: weight, BMI, fat mass, %BF, waist and hip circumferences, and waist-hip ratio (r from \(-0.582\) to \(-0.849\), \(P < 0.01\)). In a stepwise linear regression model, BMI was the single independent variable predicting the percentage change in anabolic response to hyperinsulinemia; the effect was a lesser response with higher BMI (\(R^2 = 0.704\), \(P\) value = 40.0, \(P < 0.001\)) (Figure 4B). Only BMI correlated significantly with the absolute change in net leucine balance when controlled for FFM (\(r = -0.535\), \(P = 0.022\)). Glucose disposal rates correlated with the percentage changes in nonoxidative leucine Rd (\(r = 0.655\), \(P = 0.002\)) and net leucine balance (\(r = 0.687\), \(P = 0.001\)). Results of OGTT (2 h glycemia) correlated positively with baseline flux and oxidation and negatively with net balance in absolute rates and per kg FFM (\(r\) from 0.485 to 0.533, \(P < 0.05\)) and positively with clamp flux and endogenous Ra in absolute rates and per kg FFM (\(r\) from 0.477 to 0.533, \(P < 0.05\)). Similarly, OGTT (area under the curve) correlated positively with baseline flux and oxidation and negatively with net balance per kg FFM and with clamp endogenous Ra per kg FFM.

**DISCUSSION**

Our clamp protocol showed that, in obese women, the whole-body protein anabolic response is resistant to the action of insulin, that this resistance occurred along with that of glucose and FFA, and that it is associated with adiposity. This blunted anabolic response results mainly from impaired stimulation of protein synthesis, whereas suppression of protein breakdown in obese women is not significantly different from that in lean women. These interpretations are based principally on the data per kg FFM, the compartment where most protein turnover occurs and that includes the tissues that are most responsive to insulin. Furthermore, postabsorptive turnover was highly correlated with FFM with an intercept that was not different from zero (27). Other studies of protein turnover in obesity during hyperinsulinemia have not compensated for the hypoaminoacidemia due to the suppression of breakdown (4, 6, 8, 9). The fact that we obtained comparable inhibition of breakdown, as in these studies, further supports the hypothesis that, without exogenous AAs, insulin could not stimulate protein synthesis because of limited substrate availability.

Hypoaminoacidemia decreases the initiation of protein synthesis via decreased activity of eukaryotic initiation factor 2B (28). The stimulation of protein synthesis by AAs in vivo requires substantial increases in plasma AA (leucine: \(>200\%\)) by infusion (8, 11, 29) or by oral ingestion equivalent to a meal (13, 30, 31), ie, a “fed state.” Although we infused a total of 6 g protein in both groups, with 70 g glucose in the lean and 60 g glucose in the obese, plasma AA changes were trivial (leucine \(\leq 10\%\)), and VO\(_2\) was constant, which only partially mimicked the fed state. Whereas much is known about insulin stimulation of protein synthesis, studies that isolate its effects from those of AAs are difficult to design (13, 32, 33). We produced a state in which, with minimal changes in AA concentrations, an effect on synthesis that was almost exclusively attributable to insulin could be unmasked.

Obesity is associated with higher postabsorptive plasma AAs, especially BCAAs, phenylalanine, and tyrosine, in some (6, 34) but not all (8, 35) studies. Decreases in these AAs (36, 37), which
are thought to reflect protein breakdown suppression, have been used as an index of the resistance of whole-body protein response to the action of insulin in obese persons (38, 39) and in patients with type 2 diabetes (40). However, AA concentrations reflect not only protein breakdown but also the balance among protein breakdown, synthesis, and AA oxidation. Moreover, BCAAs are closely related to FFM (r = 0.630 and P = 0.004 in the present study), which is greater in obesity.

Isotopic studies to define insulin resistance of protein by its failure to suppress protein breakdown (Ra) have reached different conclusions. Significantly less Ra suppression was reported in upper- than in lower-body obesity (2) and at low (8) but not high (6, 8, 9, 40; also in the current study) insulin infusion rates. This suggests that the insulin dose-response of Ra suppression is shifted to the right but that the maximum effect is not altered. Alternatively, by the infusion of sufficient AAs to maintain plasma concentrations without inhibiting insulin action on glucose metabolism (41–44), enough substrate may have been available to contribute to catabolism suppression, which prevented the unmasking of an effect of obesity on catabolism (45).

Our finding of significantly higher insulin concentrations in obese than in lean subjects was also found in numerous hyperinsulinemic clamp studies (2, 4, 6, 9). That we nevertheless found insulin resistance of protein synthesis and net leucine balance made this an even more robust observation.

We propose that clamp AA infusion rates may be an index of the sensitivity of whole-body protein anabolism to the action of insulin. As in the euglycemic clamp, rates of substrate infusion are determined by increased uptake, increased oxidation, decreased endogenous release, or all 3. Whereas glucose production was fully suppressed in the lean women and submaximally suppressed in the obese women, endogenous protein breakdown was not completely blocked. Therefore, the rate of AA infusion required resulted from partial endogenous Ra suppression plus stimulation of (oxidative plus nonoxidative) disposal. Because the 2 groups had similar breakdown suppression and increase in leucine oxidation, the lower AA infusion rates must be attributable to the lesser stimulation of synthesis in the obese. The time required to reach maximum AA infusion rates was significantly longer in the obese women than in the lean women (130 and 90 min, respectively; Figure 1A). This difference probably also reflects a resistance to activation of the pathways involved. A longer time was also required for suppressing FFAs in the obese women than in the lean women (Figure 3). FFA availability is reported to influence whole-body protein metabolism (46). We observed less suppression of lipid oxidation in the obese than in the lean subjects (27% and 11% of energy production, respectively). This greater availability of lipid had no effect on leucine oxidation in the obese (9). Their higher lipid oxidation was “glucose sparing”—48% of energy production compared with 66% in the lean women—but is probably due to muscle insulin resistance of glucose metabolism (47). It is of interest that resting energy expenditure increased only in the lean in whom the increase in synthesis was greater. This could be due to the difference in energy cost of protein synthesis (48).

The obese women in the current study showed typical anthropometric (Table 1) and metabolic (Tables 1–4) features of obesity. We clamped BCAAs at each person’s postabsorptive values because of expected higher concentrations in the obese women than in the lean women (6) and also to avoid a bias toward low AA infusion rates by clamping the BCAAs at lower concentrations. That the obese subjects still required less AA to maintain higher plasma BCAAs than did the lean subjects supports even more convincingly the fact that the whole-body protein anabolic response is resistant to the action of insulin. That the method used is robust is supported by reproducibility in the validation studies. Intraindividual variation (n = 5) did not differ significantly from 0: P = 0.419 and 0.711 for AA and glucose infusion, respectively. Furthermore, we tested for an effect of the absence of $^{13}$C enrichment in our glucose infusate on leucine oxidation, and we verified appropriate bicarbonate recovery factors. The calculations based on these control experiments thus minimize any possible bias from these variables.

All women were selected on the basis of comparable usual protein intakes before admission, and diet was controlled to ensure nitrogen (N) equilibrium. N balance did not differ significantly between the lean and the obese subjects, although it tended to be more negative in the subgroup of obese women with IGT. It correlated with postabsorptive net leucine balance in the whole group, but, when the 4 women in the subgroup were removed from the calculations, N balance no longer correlated with net leucine balance. There were no significant correlations between N balance during the week before the study and leucine kinetic responses to the clamp, which strongly suggests that the different responses between groups were due to differences in body composition and could not be influenced by the slight positive N balance in some.

The reduction by half in the anabolic response to the even greater degree of hyperinsulinemia reinforces the possibility that postprandial insulin secretion is elevated in obesity, not only to maintain glucose homeostasis but also to compensate for the resistance of protein (and lipid) metabolism. This blunted anabolic response appears to be aggravated by IGT. Indeed, we found a positive correlation between OGTT 2-h glucose or area under the curve and postabsorptive leucine flux and oxidation and a negative correlation with net leucine balance. The parallel between the resistance of glucose and that of protein was further supported by a correlation between clamp glucose Rd and the percentage change in net leucine balance and in synthesis. These findings suggest that the continuum of insulin insensitivity of glucose in progressing from glucose tolerance to intolerance and to diabetes also applies to protein.

We (49) and others (50) showed that a greater absolute and percentage of endogenous glucose production are due to gluconeogenesis in obesity. Our work showed a correlation between indexes of increased protein turnover and gluconeogenesis (49), which implicates the liver as well as muscle in protein insensitivity to insulin. The obligate postabsorptive hypersecretion of insulin is accompanied by augmented postprandial responses, not only for glucose and lipid homeostasis but also to promote anabolism from dietary amino acids. Because this hypersecretion of insulin can be maintained only until the beta cell is no longer able to compensate, when diabetes occurs, in both fasting and postprandial states, protein turnover could become sufficiently impaired to lead to a loss of FFM (51). Further studies are needed in subjects with IGT and with type 2 diabetes to quantify these effects.

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All authors contributed to the study design, data collection, analyses and interpretation. RG and SC wrote the manuscript, and all authors read and approved it. None of the authors had financial or personal conflicts of interest.

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Altered interorgan response to feeding in patients with chronic obstructive pulmonary disease1–3

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ABSTRACT

Background: Previously, we reported increased values for whole-body protein turnover in patients with chronic obstructive pulmonary disease (COPD) in the postabsorptive state.

Objective: The objective was to investigate whether intake of a carbohydrate-protein meal influences whole-body protein turnover differently in COPD patients and control subjects.

Design: Eight normal-weight patients with moderate COPD and 8 healthy control subjects were examined in the postabsorptive state and after 2 h of repeatedly ingesting a maltodextrin casein-based protein meal (0.02 g · kg body wt−1 · 20 min−1). Combined simultaneous, continuous, intravenous infusion of t-[ring-2H5]-phenylalanine and t-[ring-2H2]-tyrosine tracer and oral repeated ingestion of 1-13C-phenylalanine were performed to measure whole-body protein synthesis (WbPS) and first-pass splanchnic extraction of phenylalanine. Endogenous rate of appearance of phenylalanine as the measure of whole-body protein breakdown (WbPB) and net-WbPS was calculated as WbPS — WbPB. Arterialized venous blood was sampled for amino acid enrichment and concentration analyses.

Results: Feeding induced an increase in WbPS and a reduction in WbPB. The reduction in WbPB was larger in the COPD group than in the control group (P < 0.05) and was related to the lower splanchnic extraction of phenylalanine in the patients. Consequently, net-WbPS increased more after feeding in the COPD group than in the control group (P < 0.05).

Conclusion: Feeding induces more protein anabolism in normal-weight patients with moderate COPD than in healthy control subjects. This is probably because these COPD patients are characterized by an adaptive interorgan response to feeding to prevent or delay weight loss at this disease stage. Am J Clin Nutr 2005;82:366–72.

KEY WORDS Chronic obstructive pulmonary disease, protein feeding, first-pass splanchnic extraction, whole-body protein turnover, endogenous protein metabolism

INTRODUCTION

Muscle wasting commonly occurs in patients with chronic obstructive pulmonary disease (COPD), but different patterns of tissue depletion are observed. A substantial part of the COPD population is characterized by a normal weight with a shift in body composition toward reduced fat-free mass (FFM) despite a relative or absolute increase of fat mass (1, 2). In this group, functional capacity (ie, exercise capacity, muscle strength) and health status (3) are even more impaired than in the underweight patients with COPD with a relative preservation of FFM. This body-composition pattern is also seen with aging and could therefore be described as (accelerated) sarcopenia that could be reflected in altered whole-body substrate metabolism. Indeed, we showed a reduced β-adrenoceptor-mediated lipolysis rate (4) and significantly higher amounts of whole-body protein turnover [protein synthesis (WbPS) and protein breakdown (WbPB) rates] in patients with COPD than in healthy, age-matched control subjects after overnight fasting (5). These data indicate that changes in intermediary metabolism are present in normal-weight patients with COPD that may trigger or reflect sarcopenia.

Although altered whole-body substrate turnover was observed in the postabsorptive state, no studies have yet examined the acute effect of feeding on substrate metabolism in COPD. Feeding is important because the fed state represents >50% of the 24-h metabolic activity and corresponds to the reconstitution of the protein lost during fasting. In COPD, the efficiency of maintaining body proteins may be declined as a result of a selective loss in the ability of skeletal muscle to efficiently use exogenous amino acids for protein anabolism. However, it is also possible that the splanchnic area is the compartment that is mainly contributing to the previously observed increased whole-body protein turnover in COPD (5, 6). The splanchnic tissues could limit the flow and the availability of alimentary amino acids to the peripheral tissues by influencing the absorption of the alimentary amino acids. In previous studies it has been shown that the first-pass splanchnic uptake of the amino acids leucine (7) and phenylalanine (8) increases with age. This means that if the splanchnic tissues use more amino acids, fewer amino acids will be available for the other (peripheral) tissues. Until now it was unknown whether chronic disease such as COPD further aggravated the age-related disturbances found in splanchnic extraction...
of amino acids, thereby negatively influencing the metabolic response to feeding in these patients.

Therefore, the purpose of the present study was to examine the response of whole-body protein turnover and splanchnic amino acid extraction to a given dose of a maltodextrin protein meal in patients with COPD. Milk-based protein (casein) was used because of its high nutritional value (protein quality) and because casein is the protein mostly used (and to the highest degree) in nutritional supplements.

SUBJECTS AND METHODS

Subjects

A group of 8 male patients with moderate airflow obstruction and 8 healthy male volunteers were studied. The patients had COPD according to American Thoracic Society guidelines (9) and chronic airflow limitation, defined as measured forced expiratory volume in 1 s (FEV₁) < 70% of reference FEV₁. Furthermore, the patients had irreversible obstructive airway disease (<10% improvement of FEV₁ predicted baseline after inhalation of β₂-agonist) and were in clinically stable condition and had not experienced respiratory tract infection or exacerbation of their disease at least 4 wk before the study. The patients with COPD were outpatients, attending the hospital for routine pulmonary control every 6 or 12 mo. Exclusion criteria were malignancy, cardiac failure, recent surgery, and severe endocrine, hepatic, or renal disorder. Also, subjects who were using systemic corticosteroids within 3 mo before the beginning of the study were excluded. The number of former smokers in the COPD and control groups was 5 (average number of years stopped was 10.2) and 2 (average number of years stopped was 2.0) years. Body mass index (BMI; in kg/m²) was not significantly different between the groups (control group: 25.4 ± 0.9; COPD group: 27.2 ± 0.8). The maintenance treatment of the studied patients consisted of inhaled β₂ agonists, inhaled anti-cholinergics, inhaled corticosteroids, oral theophylline, or a combination. Written informed consent was obtained from all subjects, and the study was approved by the medical ethics committee of the University Hospital Maastricht.

Pulmonary function tests

All patients and healthy volunteers underwent spirometry to determine FEV₁, and the highest value from at least 3 technically acceptable assessments was used. Diffusing capacity of the lung for carbon monoxide was measured by using the single-breath method (Masterlab; Jaeger, Wurzburg, Germany). All values obtained were related to a reference value and expressed as percentages of the predicted value (10).

Study protocol

The protocol started at 0715 after an overnight fast from at least 0000. All subjects were in the supine position for 3 h. After insertion of a catheter into the right antecubital vein, the first blood sample was taken for baseline measurements. Immediately thereafter, a primed-constant intravenous infusion of stable isotopes (80 mL/h) was started with the use of a calibrated pump (IVAC Corporation, San Diego, CA). Primed and constant infusion of the stable isotopes [ring-2H₅]phenylalanine ([H₅-Phe; prime: 2.19 μmol/kg body wt; infusion: 0.053 μmol · kg FFM⁻¹ · h⁻¹] and [ring-2H₆]-tyrosine ([H₆-Tyr; prime: 0.95 μmol/kg body wt; infusion: 0.018 μmol · kg FFM⁻¹ · h⁻¹] were given through the catheter in the antecubital vein. Primed infusion of [ring-2H₅]-Tyr ([H₅-Tyr; 0.31 μmol/kg body wt) was given in addition through the same catheter. 1-¹³C-Phe was given orally in the postabsorptive state and together with the liquid meal every 20 min (prime: 0.88 μmol/kg body wt; infusion: 0.055 μmol · kg FFM⁻¹ · min⁻¹). Stable isotopes were purchased from Cambridge Isotopic Laboratories (Woburn, MA).

For sampling arterialized venous blood, a venous catheter was placed in a dorsal vein of the left hand, using the heated box technique (11), a technique to mimic direct arterial sampling. After 1.5 h of stable isotope infusion to reach steady state enrichments, enteral nutrition was started by sip-feeding every 20 min, for a total duration of 2 h. The test meal involved a liquid casein-based protein meal and was given in an amount of 0.018 g · kg body wt⁻¹ · 20 min⁻¹. Total fluid intake was 0.67 mL · kg body wt⁻¹ · 20 min⁻¹ by enteral nutrition. Arterialized venous blood samples were taken at 80, 85, 90, 95, 205, and 210 min into infusion. Body composition was measured with the use of Bioelectrical Impedance Spectroscopy (BIS Xiton 4000B; Xiton Technologies, San Diego, CA) to express protein metabolism data per kilogram of FFM. FFM of the patients with COPD was calculated by using a patient’s specific regression equation as described by Steiner et al (12), whereas FFM of the healthy control subjects was calculated by using a specific equation for elderly men as described by Lukaski et al (13).

Enteral protein meals

To avoid metabolic changes as a result of recent modifications of the diet, the subjects were instructed to eat their usual diet at least 3 d before the study. The dietary protein intake of the study subjects was ascertained retrospectively during 5 d by using the dietary history method (COPD group: 0.95 ± 0.10 g protein · kg body wt⁻¹ · d⁻¹; control group: 0.96 ± 0.07 g protein · kg body wt⁻¹ · d⁻¹).

The test meal on the experimental day contained 29.5 g sodium caseinate (casein protein meal: 4.0 g N) and 68.5 g maltodextrin dissolved in ultrapure water to 1000 mL fluid at 60 °C. In total, 800 mL enteral nutrition and 8.1 g protein (based on a 75-kg subject) was supplied during the study. The protein composition of the casein protein meal was a 1:1:1 mixture of commercially available French, Dutch, and Danish sodium caseinates. All meals were prepared at least 1 h before the start of the experiment. To ensure a complete dissolution of the proteins and to prevent bacterial growth, the meals were kept at 4 °C until use.

Sample processing

Analysis of arterialized venous blood

Promptly after sampling, blood was distributed in prechilled, heparinized tubes (Becton Dickinson Vacutainer System, Franklin Lakes, NJ) and kept on ice to minimize enzymatic reactions. All analyses were performed in plasma, obtained by centrifugation of whole blood at 4 °C for 10 min at 3120 × g. For amino acid analysis, 250 μL plasma was deproteinized by mixing it with 20 mg dry sulfosalicylic acid. For analysis of urea, glucose, lactate, and ammonia, 900 μL plasma was deproteinized by mixing with 90 μL of a 500 g/L trichloroacetic acid solution. All samples were stored at −80 °C until further analysis.
Biochemical analysis

The enrichments (tracer-to-tracee ratios) of the amino acids phenylalanine and tyrosine in arterialized venous plasma were analyzed by a liquid chromatography–mass spectrometry system (Thermoquest LCQ, Veenendaal, The Netherlands) (14). Plasma concentrations of amino acids were determined with the use of a fully automated HPLC (Pharmacia, Woerden, The Netherlands), after precolumn derivatization with ω-phthalaldehyde (15).

Plasma glucose, lactate, urea, and ammonia were analyzed spectrophotometrically on a COBAS Mira S (Roche Diagnostics, Hoffmann-La Roche, Basel, Switzerland) by standard enzymatic methods (16). Plasma insulin was analyzed with a commercially available electrochemiluminescence immunoassay (Hitachi Modular Analyzer; Roche, Mannheim, Germany).

Calculations

The sum of amino acids (SUM AA) represents the sum of measurable α-amino acids (glutamine, glycine, threonine, histidine, citrulline, alanine, taurine, arginine, α-amino butyric acid, tyrosine, valine, methionine, isoleucine, phenylalanine, tryptophan, leucine, ornithine, and lysine). All the metabolic data were determined under steady state conditions. Tracer:tracee of phenylalanine reached an isotopic steady state within 1.5 h of infusion and within 2 h of feeding (data not shown) in both groups.

In the postabsorptive and prandial state, WbPS is calculated as follows (5):

\[
WbPS = \text{whole-body Rd of Phe} - \text{hydroxylation of Phe to Tyr} \quad (1)
\]

Whole-body rate of disappearance (Rd) of phenylalanine is equal to whole body rate of appearance (Ra) of phenylalanine under steady state. Whole-body Ra of phenylalanine (Ra_{2H5-Phe}) is the infusion rate/tracer:tracee of phenylalanine in plasma.

Splanchnic extraction (SPE_{Phe}) represents the fraction (in %) of ingested phenylalanine, taken up by the gut and liver during its first pass, and is calculated as follows (8, 17):

\[
SPE_{Phe} = \left[1 - \frac{(Ra_{2H5-Phe}/Ra_{13C-Phe})}{100}\right] \quad (2)
\]

Ra_{2H5-Phe} and Ra_{13C-Phe} represent whole-body Ra of phenylalanine calculated from intravenous {\textsuperscript{2}H}\textsubscript{5}-Phe and intragastric {\textsuperscript{13}C}\textsubscript{Phe} isotopes, respectively.

Whole-body Ra of phenylalanine, not coming from phenylalanine in protein given by the diet [endogenous phenylalanine (Ra_{end-Phe})], is calculated as in equations (3) and (4).

\[
\text{Corrected Phe intake} = \text{dietary Phe intake} \times [1 - (SPE_{Phe} \times 0.01)] \quad (3)
\]

\[
Ra_{end-Phe} = Ra_{2H5-Phe} - \text{corrected dietary Phe intake} \quad (4)
\]

\[
WbPB = Ra_{end-Phe} \quad (5)
\]

\[
\text{netWbPS} = WbPS - WbPB \quad (6)
\]

Summary model used for the calculation of SPE of phenylalanine and protein kinetics is presented in Figure 1. Phenylalanine clearance is the amount of plasma that is completely cleared from tracee in 1 min and is calculated as follows (18):

\[
\text{Rd (Ra in steady state)/plasma concentration of the tracee} \quad (7)
\]

Statistical analysis

Results are expressed as means ± SEs. The mean value of the measures of protein kinetics and the concentrations of amino acids at the time points 80, 85, and 90 min was used as the postabsorptive state and at 200, 205, and 210 min as the fed state. The unpaired Student’s t test was used to determine differences in general characteristics between the control and COPD groups and to test whether the changes in status (postabsorptive and prandial) in protein kinetics and amino acid concentrations were significantly different from zero. If the normality or equal variance test failed, data were log-transformed where appropriate. Furthermore, the two-factor analysis of variance (ANOVA; general linear model, SPSS version 12; SPSS Inc, Chicago, IL) was performed with a group (control and COPD) and status (postabsorptive and prandial) effect. The level of significance was set at \( P < 0.05 \), and \( P \) values are given for the group effect, status effect, and the group-by-status interaction. When an overall significance for group-by-status interaction was observed, unpaired Student’s t test was performed.

RESULTS

Eight male patients with COPD and 8 male healthy volunteers participated in the study (Table 1). Age, height, body weight, and BMI did not differ significantly between the groups, but a tendency toward a lower FFM index (NS) and higher fat mass index (NS) was found in the COPD group. In the control group, all lung function values were within the normal range. The patients with
Increased glucose and insulin concentrations. The concentration was observed for these variables. A status effect was observed for the patients with COPD and the healthy control subjects. No significant group-by-status interaction were observed in the COPD group. Large range of C-reactive protein values (0.5–32 mg/L) in the COPD group than in the control group, probably because of the mild reduction of diffusing capacity for carbon dioxide. C-reactive protein concentration after feeding (ΔPhe conc) was higher in the COPD group than in the control group (P < 0.05). These findings were also present for SUM AA (data not shown). There was a status effect (P < 0.001), and, in addition, there was a tendency toward a difference in ΔSUM AA between the COPD and control groups (P = 0.085). A group effect was observed for phenylalanine clearance (P < 0.01), indicating that phenylalanine clearance was lower in the COPD group than in the control group.

**DISCUSSION**

The ability to obtain homeostatic regulation of protein metabolic processes during the day is important to preserve muscle mass and to function long term. Insight into the protein metabolic response to feeding is of importance in COPD because low-intensity exercise has been shown to induce an increased amino acid release from muscle (19). This finding suggests that physical activity in daily life may induce protein catabolism in COPD. To maintain protein balance on a daily basis and to prevent muscle wasting in COPD for the longer term, a positive protein metabolic response to feeding is therefore of crucial importance. In the present study, feeding increased net WbPS to a higher extent in normal-weight patients with moderate COPD than in healthy control subjects, indicating an enhanced anabolic response to feeding in this patient group.

**TABLE 2**

Plasma concentrations in arterialized blood in the postabsorptive state and during feeding.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 8)</th>
<th>COPD group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Postabsorptive</td>
<td>Prandial</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.4 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.2 ± 0.4</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Ammonia (µmol/L)</td>
<td>88 ± 3</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>8.4 ± 1.2</td>
<td>21.5 ± 3.4</td>
</tr>
</tbody>
</table>

All values are x ± SEM. COPD, chronic obstructive pulmonary disease. Data show postabsorptive values and values 2 h after the start of feeding. Two-factor ANOVA showed a significant status effect for glucose and insulin (P < 0.001). There was no significant group effect and no significant group-by-status interaction.
Effect of feeding on WbPS

Feeding induced an increase in WbPS, which is in line with data obtained in previous studies that showed a positive effect of mixed feeding on protein synthesis (20, 21). In the present study, 0.11 g protein/kg body wt was ingested in 2 h. On the basis of the fed state of 16 h/d, 0.87 g protein/kg body wt will be ingested, which is in line with the current recommended dietary allowances in the elderly (0.8 g protein · kg body wt⁻¹ · d⁻¹) (22) and slightly lower than the recorded daily dietary protein intake of the study groups. Earlier, it has been shown that to increase peripheral protein synthesis, high amino acid availability is important (23, 24). We observed a status effect for protein synthesis and the concentration of phenylalanine and SUM AA, indicating that feeding increased systemic amino acid availability and protein synthesis. However, despite the lower feeding-induced increase in the phenylalanine concentration in COPD, the increase in protein synthesis was not different between the groups.

First-pass splanchnic extraction of phenylalanine

The splanchnic tissues play an important role in the regulation of protein turnover because these tissues are responsible for absorption of the alimentary amino acids and their release to the peripheral tissues. In a study that compared elderly subjects with young healthy subjects, first-pass SPE of dietary leucine was twice as high in the elderly as in the young men (7). In line, a study by Volpi et al (8) showed that the SPE of oral phenylalanine was higher in the elderly than in the young. The exact reason for the elevated SPE of amino acids in the elderly is still unknown. However, it is believed that it may contribute to the development of sarcopenia because it reduces amino acid availability to the periphery.

We also measured SPE of phenylalanine after 2 h of feeding in the patients with COPD and the healthy control subjects using free L-¹³C-Phe given orally and together with the liquid meal. Because the meal as well as the oral tracers was administered in the same continuous feeding protocol, no differences in absorption kinetics between phenylalanine in the meal and the oral L-¹³C-Phe are expected. The data of Volpi et al (8) on SPE of phenylalanine in the healthy elderly are a bit higher than ours (47 ± 3% compared with 35 ± 7%). However, the meal composition used in the 2 studies was different (oral amino acid mixture compared with maltodextrin protein meal). Interestingly, there was a group effect for SPE of phenylalanine. SPE was lower in the patients with COPD than in the control group, indicating that there is lower phenylalanine extraction by the gut, liver, or both during feeding in the patients, which could lead to a higher peripheral availability of dietary phenylalanine. Therefore, it was expected that the lower SPE in COPD would induce a higher prandial phenylalanine concentration in these patients.

Possible factors inducing a lower first-pass splanchnic extraction in COPD

At present, we can only speculate about possible mechanisms of the reduced SPE in COPD. Besides an adaptation to increased needs in the body elsewhere as mentioned previously, it is also possible that the reduced SPE in COPD is reflecting a reduced
splanchnic protein turnover rate rather than a reduced splanchnic amino acid net utilization. However, the possibility that the splanchnic (liver + gut) protein turnover is reduced in COPD is remarkable when considering that this patient group is generally characterized by a low-grade systemic inflammatory state (25). In line, C-reactive protein concentrations tended to be higher in the studied patients with COPD than in the control subjects. However, because inflammation is associated with an increased hepatic protein synthesis (26), one should expect an elevated (but not reduced) protein synthesis in the splanchnic liver compartment in COPD. Other factors known to influence splanchnic protein turnover are nicotine use and intake of certain drugs. Nicotine can act as a splanchnic circulation constrictor because it has been shown that smoking aggravates liver injury and that intraportal nicotine infusion in rats decreases hepatic blood flow (27). However, smoking status and history were not different between the COPD and control groups. The studied patients were clinically stable for at least 3 mo before the study, exhibiting normal blood gases and only using inhalation medication. Still, it is important to highlight that this patient group is regularly experiencing an acute exacerbation of the disease, which is characterized by an increased inflammatory state, changes in blood gases, and use of systemic medication (ie, oral corticosteroids and antibiotics). Nonsteroidal anti-inflammatory drugs are known to reduce blood flow in the splanchnic region (28). Acute changes in the arterial partial pressures of oxygen and carbon dioxide do not reduce splanchnic blood flow (29, 30) but together with an increased inflammatory state may induce changes in insulin sensitivity and thus influence protein metabolism. A positive association has been found between SPE of dietary leucine and BMI (7). Currently, no relation was found between SPE of dietary phenylalanine and body weight or composition. However, it is important to notice that only normal-weight patients with COPD were studied without evidence of muscle wasting.

More research is warranted to get insight into the underlying factors responsible for the lower SPE of amino acids in COPD. The gut plays an important role as buffer of amino acids during fasting (31). The elevated initial release of amino acids into the circulation in COPD may lead to a reduced buffer of amino acids in a later (fasting) phase. Measurement of protein kinetics after 2 h of feeding is therefore necessary in COPD to examine whether protein balance after this anabolic phase is still positive.

In conclusion, the anabolic response to feeding is higher in weight-stable moderate patients with COPD than in healthy control subjects. This is related to lower first-pass SPE in COPD, resulting in a larger reduction of WbPB after feeding. This study shows that normal-weight patients with COPD are characterized by a pronounced adaptive interorgan response to feeding, apparently sufficient to prevent or delay weight loss in this stage of their disease. More studies are needed to investigate whether this adaptive response to feeding is inadequate or absent in weight-losing patients with COPD.

REFERENCES


The daily valine requirement of healthy adult Indians determined by the 24-h indicator amino acid balance approach¹–⁴

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ABSTRACT
Background: The 1985 FAO/WHO/UNU requirement for valine was set at 10 mg · kg⁻¹ · d⁻¹ on the basis of nitrogen balance studies carried out in Western subjects. It is likely that the requirement is higher, however, because the requirement of another branched-chain amino acid, leucine, was found to be about 3 times as high (40 mg · kg⁻¹ · d⁻¹) as the 1985 FAO/WHO/UNU value (14 mg · kg⁻¹ · d⁻¹).

Objective: We assessed the valine requirement in healthy, well-nourished Indians by using 7 test valine intakes (5, 10, 15, 20, 25, 30, and 35 mg · kg⁻¹ · d⁻¹) and the 24-h indicator amino acid oxidation (24-h IAAO) and balance (24-h IAAB) method, with phenylalanine as the indicator amino acid, while maintaining leucine intake at 40 mg · kg⁻¹ · d⁻¹.

Design: Eighteen healthy, well-nourished Indian men were studied during each of 3 randomly assigned 7-d diet periods supplying valine intakes that were equally placed on either side of a putative mean valine requirement of 20 mg · kg⁻¹ · d⁻¹. Twenty-four–hour IAAO and 24-h IAAB were measured on day 7 by use of a 24-h [¹³C]phenylalanine tracer infusion. The breakpoint in the relation between these values and the valine intake was determined.

Results: Two-phase linear regression of daily phenylalanine oxidation or balance against valine intake estimated a breakpoint in the response curve at a valine intake of 17 mg · kg⁻¹ · d⁻¹.

Conclusion: From the 24-h IAAO/IAAB approach, a mean valine requirement of 17 mg · kg⁻¹ · d⁻¹ is proposed for healthy, well-nourished Indian adults.

KEY WORDS Well-nourished Indian adults, valine requirement, branched-chain amino acids, BCAA requirement, 24-h indicator amino acid oxidation, 24-h indicator amino acid balance

INTRODUCTION

The 1985 FAO/WHO/UNU (1) daily requirement for valine was set at 10 mg · kg⁻¹ · d⁻¹ on the basis of nitrogen balance studies carried out in healthy adults (2, 3). The nitrogen balance technique has several disadvantages (4), however; reanalyses of the nitrogen balance data by Hegsted (5) and Millward (6) gave mean valine requirement estimates of 17 and 14 mg · kg⁻¹ · d⁻¹, respectively. On the basis of an obligatory amino acid loss model, Young et al (4) suggested a value of 24 mg · kg⁻¹ · d⁻¹ for the daily valine requirement. Because the branched-chain amino acids (BCAAs) share common enzymes in their oxidative pathways, the valine requirement can also be theoretically estimated by an assumed proportionality with the leucine requirement on basis of the amino acid composition of body protein (7). Such a procedure yields a value of 26 mg · kg⁻¹ · d⁻¹ when a value of 40 mg · kg⁻¹ · d⁻¹ is used for the leucine requirement (8, 9). The response of plasma valine concentrations to different valine intakes has suggested a requirement value of 20 mg · kg⁻¹ · d⁻¹ (10).

Tracer studies with [¹³C]-labeled valine to determine the valine oxidation rate (also called the direct amino acid oxidation, or DAAO, method) over the short term at different valine intakes in an egg-protein-based amino acid mix have suggested a valine requirement of 16 mg · kg⁻¹ · d⁻¹ or greater (10). However, potential problems with the short-term DAAO approach exist, because the kinetics of the test amino acid are measured only over a few hours with extrapolations made for 24 h, and the test amino acid intake supplied by the intravenously administered tracer is not massless. The other type of tracer study, called the indicator amino acid oxidation (IAAO) method, offers significant advantages over the DAAO method in that the oxidation and balance of an independent amino acid, for which the kinetics are well characterized, are used to plot a response curve to graded intakes of the test (in this case, valine) amino acid. The IAAO method has been used in short-term measurements to measure the total BCAA requirement (11), and the total requirement of these amino acids was found to range from 122 to 144 mg · kg⁻¹ · d⁻¹ depending on the outcome (phenylalanine oxidation or a surrogate based on the fraction of tracer oxidized) used. The daily valine requirement can then be estimated to be 28 to 32 mg · kg⁻¹ · d⁻¹ on the basis of the proportion of valine (22.5%) in the egg protein amino acid mix used in this study (11). A later paper from the same group (12) suggested that the requirements for the BCAs may have been overestimated by 10%; if so, the tentative valine requirement derived from these studies would be in the range of 25 to 29 mg · kg⁻¹ · d⁻¹.

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² VR Young is deceased.
³ Supported by NIH grant DK42101.
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However, these IAAO studies were conducted in adults who were first adapted for 2 days to the experimental diet before the tracer study, which comprised measurements for a few hours in the fed state. We have refined the IAAO technique to include a 7-d adaptation period to the experimental diet as well as a 24-h measurement of IAAO and indicator amino acid balance (IAAB) to assess the requirements of lysine, methionine, and threonine in adult Indians (13–17). This approach might reasonably be considered to be the best method currently available to measure amino acid requirements in adults. Therefore, the present study was designed to assess the valine requirement in healthy, young adult Indian males by using the 7-d dietary adaptation period and the 24-h IAAO and IAAB approach, with [13C]phenylalanine as the indicator amino acid. Furthermore, because it is possible that dietary leucine intake may affect valine kinetics and hence the valine requirement, the dietary leucine intake was kept at its requirement level of 40 mg · kg⁻¹ · d⁻¹ (8, 9).

### SUBJECTS AND METHODS

#### Subjects and anthropometry

Eighteen healthy men participated in this experiment. The subjects were weighed to the nearest 0.1 kg, and their height was measured to the nearest 0.1 cm. The logarithm of the sum of 4 (biceps, triceps, subscapular, and suprailiac) skinfold thicknesses was used in age- and sex-specific equations (18) to obtain an estimate of body density, from which percentage body fat and fat-free mass (FFM) were determined (19; Table 1). The purpose of the study and the potential risks involved were explained to each subject, and written informed consent was obtained from each subject. The Human Ethical Review Board of St John’s Medical College approved the research protocol.

#### Diet and experimental design

Each subject was randomly assigned to 3 separate 6-d experimental diet periods during which he received a weight-maintaining diet based on an L-amino acid mixture as previously described (13–17), such that between 7 and 9 observations were made at each valine intake. The test valine intakes during the respective diet periods were chosen from 7 designated valine intakes of 5, 10, 15, 20, 25, 30, and 35 mg · kg⁻¹ · d⁻¹ (Table 2). The 3 valine intakes assigned to each subject were distributed around a putative intake requirement of 20 mg · kg⁻¹ · d⁻¹. The subjects received their daily intake as 3 isoenergetic, isonitrogenous meals (at 0800, 1300, and 2000), except on days 6 and 7 (see below).

#### Twenty-four–hour tracer-infusion protocol and sample collection

The indicator amino acid used was phenylalanine. A primed, 24-h, intravenous [13C]phenylalanine approach was used.

### TABLE 1

Characteristics of well-nourished Indian men studied for their valine requirements

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>21.5 ± 2.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.3 ± 5.4</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.4 ± 1.6</td>
</tr>
<tr>
<td>MUAC (cm)</td>
<td>27.8 ± 1.5</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>14.3 ± 4.0</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>53.1 ± 4.3</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. n = 18. MUAC, midupper arm circumference; FFM, fat-free mass.

### TABLE 2

Composition of the amino acid mixtures used to supply 7 different valine intakes per day

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/g mixture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>17.47</td>
<td>17.38</td>
<td>17.33</td>
<td>17.27</td>
<td>17.30</td>
<td>17.30</td>
<td>17.23</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>52.73</td>
<td>52.46</td>
<td>52.30</td>
<td>52.14</td>
<td>52.23</td>
<td>52.23</td>
<td>52.01</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>60.85</td>
<td>60.77</td>
<td>60.68</td>
<td>60.59</td>
<td>60.45</td>
<td>60.33</td>
<td>60.25</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>38.64</td>
<td>38.59</td>
<td>38.53</td>
<td>38.46</td>
<td>38.39</td>
<td>38.31</td>
<td>38.26</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>93.66</td>
<td>93.19</td>
<td>92.90</td>
<td>92.61</td>
<td>92.76</td>
<td>92.76</td>
<td>92.38</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>33.23</td>
<td>33.06</td>
<td>32.96</td>
<td>32.86</td>
<td>32.91</td>
<td>32.91</td>
<td>32.78</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>24.64</td>
<td>24.52</td>
<td>24.45</td>
<td>24.37</td>
<td>24.41</td>
<td>24.41</td>
<td>24.31</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>18.88</td>
<td>18.86</td>
<td>18.83</td>
<td>18.80</td>
<td>18.76</td>
<td>18.72</td>
<td>18.69</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>L-Valine</td>
<td>4.83</td>
<td>9.64</td>
<td>14.45</td>
<td>19.24</td>
<td>23.99</td>
<td>28.73</td>
<td>33.47</td>
</tr>
<tr>
<td>L-Histidine HCl</td>
<td>34.34</td>
<td>34.16</td>
<td>34.06</td>
<td>33.95</td>
<td>34.01</td>
<td>34.01</td>
<td>33.87</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>84.63</td>
<td>84.20</td>
<td>83.95</td>
<td>83.69</td>
<td>83.82</td>
<td>83.82</td>
<td>83.48</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>214.39</td>
<td>213.32</td>
<td>212.67</td>
<td>212.01</td>
<td>212.35</td>
<td>212.34</td>
<td>211.48</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>33.04</td>
<td>32.87</td>
<td>32.77</td>
<td>32.67</td>
<td>32.72</td>
<td>32.72</td>
<td>32.59</td>
</tr>
<tr>
<td>Glycine</td>
<td>139.92</td>
<td>138.94</td>
<td>136.55</td>
<td>134.21</td>
<td>128.53</td>
<td>124.05</td>
<td>122.44</td>
</tr>
<tr>
<td>L-Proline</td>
<td>45.14</td>
<td>44.91</td>
<td>44.77</td>
<td>44.63</td>
<td>44.71</td>
<td>44.70</td>
<td>44.52</td>
</tr>
<tr>
<td>L-Serine</td>
<td>90.27</td>
<td>89.82</td>
<td>89.54</td>
<td>89.27</td>
<td>89.41</td>
<td>89.41</td>
<td>89.05</td>
</tr>
<tr>
<td>Total¹</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

1 19.45 mg phenylalanine · kg⁻¹ · d⁻¹ was added to each mix every day, except on the infusion day, when this amount of phenylalanine was infused as the tracer.

2 1.046 g · kg⁻¹ · d⁻¹ of the mixture was given to the subjects; the mixture provided 160 mg N · kg⁻¹ · d⁻¹.
used, with the protocol of indirect calorimetry and blood and breath sampling as previously described (13–17). Briefly, [1-13C]phenylalanine (99 atom%; Cambridge Isotope Laboratories, Inc, Andover, MA) was given as a primed, constant intravenous infusion at a known rate of \( \approx 4.5 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) (the priming dose was \( \approx 4.5 \, \mu\text{mol/kg} \)) into an antecubital vein. The bicarbonate pool was primed with 0.8 \( \mu\text{mol/kg} \) of [13C]sodium bicarbonate (99.9 atom%; MassTrace, Woburn, MA). The tracer administration began at 1700 on day 6, with the subjects having consumed their last meal of that day at 1500, and lasted until 1800 on day 7. Therefore, the tracer infusion was given for 25 h, although only the data from the last 24 h were used in calculating daily phenylalanine oxidation and balance. On the day of the infusion study, the subjects received, at hourly intervals, 10 isoenergetic, isonitrogenous small meals beginning at 0600 on day 7 and lasting until and including 1500 (which together were equivalent to the 24-h dietary intake for that day). A similar feeding pattern was imposed on the subjects on day 6, so that the infusion day feeding pattern was not suddenly different from the pattern on the previous day.

The analyses of breath for 13CO2 enrichment by isotope ratio mass spectrometry (EUROPA Scientific Ltd, Crewe, United Kingdom) were as previously described (13), and blood samples for the 13C enrichment of plasma phenylalanine were analyzed as described by Sanchez et al (20) with minor modifications by using a Varian 2000 ITD Mass Spectrometer coupled to a Varian 3800 Gas Chromatograph (Varian Inc, Palo Alto, CA).

**Calculation of phenylalanine oxidation and balance**

Phenylalanine oxidation (\( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{30 min}^{-1} \)) was computed for consecutive half-hourly intervals as the ratio of the 13CO2 production rate (\( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{30 min}^{-1} \)), corrected for recovery, to the isotopic abundance of the intracellular phenylalanine pool that was undergoing oxidation at that time, which was taken to be represented by plasma [13C]phenylalanine enrichment (mole percent excess). A surrogate measure of the rate of phenylalanine oxidation in the fed state was also computed as the proportion of administered tracer oxidized (\( F^{13}\text{CO}_2 \)), and was calculated as the ratio of the 13CO2 production rate (\( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{30 min}^{-1} \)) corrected for recovery to the [13C]phenylalanine infusion rate (\( \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{30 min}^{-1} \)). The \( F^{13}\text{CO}_2 \) was calculated for the first 6 h after feeding commenced. Phenylalanine balance (mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\)) was computed as the difference between phenylalanine input (dietary phenylalanine + intravenous tracer) and phenylalanine output (sum of phenylalanine oxidation over 24 h).

**Statistical methods and data evaluation**

Data are presented as means ± SDs. Weight change and phenylalanine flux were analyzed by using mixed-models analysis of variance. The model for weight change over the 6-d experimental diet periods included a factor for diet period. The model for 12-h phenylalanine flux included diet period, metabolic phase (fasted compared with fed), valine intake, and the intake-by–metabolic phase interaction. For the relations between valine intake and 24-h IAAO (phenylalanine), 12-h fed IAAO (phenylalanine), 24-h IAAB (phenylalanine), and \( F^{13}\text{CO}_2 \), two-phase linear random effects regression models were fit. The intercept and slope of the first line segment and the intercept of the second line segment were estimated; although biologically the slope of the second line segment should be restricted to be zero, it was tested versus zero slope before making this restriction. The models were constrained such that the 2 line segments intersect at the unknown breakpoint. The breakpoint parameter was estimated as \(-1 \) times the ratio of the difference between intercepts divided by the difference between slopes (21). The 95% CI for the breakpoint was calculated by using Fieller’s theorem. Model estimates and SEs are presented.

Model contrasts were used to make pairwise comparisons of interest as appropriate on the basis of the statistical significance of the model parameters and to make comparisons versus zero balance. A two-sided \( P \) value of 0.05 indicated significance for all tests of interaction and main effects; \( P \) values of pairwise comparisons were adjusted by using Holm’s method (22). The data were analyzed by using SAS version 9.1 (SAS Institute Inc, Cary, NC).

**RESULTS**

**Anthropometry**

The subjects’ anthropometric measurements (Table 1) were comparable with those of subjects in our previous series of studies (13–17). During the 6-d experimental diet periods, the subjects experienced a small but significant (\( P < 0.01 \)) weight loss of 0.42 ± 0.34 kg on average across diet periods. There was no significant difference in weight loss between diet periods.

**Phenylalanine oxidation and breakpoint analysis**

Phenylalanine oxidation and balance at the 7 valine intakes are shown in Table 3. Two-phase linear regression models were fit to the valine intake–24-h IAAO (phenylalanine) and valine intake–12-h fed phenylalanine oxidation relations; these results are summarized in Table 4. For 24-h IAAO there was no evidence of a nonzero slope in the second line segment (\( \beta_2 = -0.07 \pm 0.16; P = 0.69 \)) and thus this was restricted to be zero. The breakpoint estimated from this model was 17 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) (95% CI: 11, >35 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\)), which indicates that 24-h IAAO (phenylalanine) decreased linearly until a valine intake of 17 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) above which 24-h phenylalanine oxidation was estimated as 29 ± 1.2 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) at all higher valine intakes. Within this model, 24-h phenylalanine oxidation at the 5- and 10-mg valine intakes was also significantly higher than oxidation above the breakpoint (each \( P < 0.05 \)) but at the 15-mg valine intake was not significantly different from oxidation above the breakpoint.

Results for 12-h fed phenylalanine oxidation were similar to 24-h oxidation. There was no evidence of a nonzero slope in the second line segment (\( \beta_2 = -0.08 \pm 0.09; P = 0.39 \)) and thus this was restricted to be zero. The breakpoint was estimated as 18 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) (95% CI: 13, >35 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\)), which indicates that 12-h fed phenylalanine oxidation decreased linearly until a valine intake of 18 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) above which it was estimated as 14 ± 0.6 mg \cdot kg\(^{-1}\) \cdot d\(^{-1}\) at all higher valine intakes. Within this model, 12-h fed phenylalanine oxidation at the 5- and 10-mg valine intakes was also significantly higher than oxidation above the breakpoint (each \( P < 0.01 \)) but at the 15-mg valine intake was not significantly different from oxidation above the breakpoint.

**Phenylalanine balance and breakpoint analysis**

The results of fitting a two-phase linear regression model to the valine intake–phenylalanine balance relation are summarized in
The model with a fixed slope of zero in the second line segment was restricted to be zero. The breakpoint estimated from this model was 17 mg·kg⁻¹·d⁻¹ (95% CI: 11, 28 mg·kg⁻¹·d⁻¹), which indicates that daily IAAB (phenylalanine) increased linearly until a valine intake of 17 mg·kg⁻¹·d⁻¹, above which phenylalanine balance was estimated as 9.3 ± 1.2 mg·kg⁻¹·d⁻¹ at all higher valine intakes. Within this model, phenylalanine balance was not significantly different from zero balance at an intake of 5 mg valine kg⁻¹·d⁻¹ (P = 0.06) but was significantly different from zero balance at the 10- and 15-mg valine intakes and intakes above the breakpoint (each P < 0.01). Phenylalanine balance at the 5- and 10-mg valine intakes was also significantly lower than balance above the breakpoint (each P < 0.05) but at the 15-mg valine intake was not significantly different from balance above the breakpoint.

Indicates that the daily F¹³CO₂ ratio decreased linearly until a valine intake of 20 mg·kg⁻¹·d⁻¹, above which the F¹³CO₂ ratio was estimated as 0.11 ± 0.007 at all higher valine intakes. Within this model, the F¹³CO₂ ratio at the 5- and 10-mg valine intakes was also significantly lower than the F¹³CO₂ ratio above the breakpoint (each P < 0.05) but at the 15-mg valine intake was not significantly different from the F¹³CO₂ ratio above the breakpoint.

**Phenylalanine flux**

There was no significant effect of valine intake on phenylalanine flux, but flux was significantly higher in the fasted than in the fed phase (Table 3; $P = 0.02$).

**DISCUSSION**

The findings of the present study add to the earlier tracer-derived balance data that we generated by using the diet-adapted, 24-h IAAO and IAAB paradigm to quantify adult amino acid requirements (8, 9, 13–17, 23) in South Asian (Indian) and American subjects. This pattern of amino acid requirements (for leucine, lysine, methionine, and threonine) is similar to the amino acid pattern recommended for adults by a recently convened WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements (24).

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**Table 4**

Two-phase regression analysis of the relation between valine intake and phenylalanine oxidation, phenylalanine balance, and the rate of phenylalanine oxidation in the fed state (F¹³CO₂).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Breakpoint estimate</th>
<th>Equation for variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h Phenylalanine oxidation (mg·kg⁻¹·d⁻¹)</td>
<td>17 (11, ≥35)</td>
<td>Below breakpoint: Oxidation = 36 - 0.45 × intake Above breakpoint: Oxidation = 29 ± 1.2</td>
</tr>
<tr>
<td>12-h Fed phenylalanine oxidation (mg·kg⁻¹·d⁻¹)</td>
<td>18 (13, ≥35)</td>
<td>Oxidation = 21 - 0.37 × intake Oxidation = 14 ± 0.6</td>
</tr>
<tr>
<td>24-h Phenylalanine balance (mg·kg⁻¹·d⁻¹)</td>
<td>17 (11, 28)</td>
<td>Balance = 1.6 ± 0.45 × intake Balance = 9.3 ± 1.2</td>
</tr>
<tr>
<td>F¹³CO₂</td>
<td>20 (12, ≥35)</td>
<td>F¹³CO₂ = 0.14 - 0.0016 × intake F¹³CO₂ = 0.11 ± 0.007</td>
</tr>
</tbody>
</table>

¹ Estimate (95% Fieller’s CI); an upper limit of ≥35 denotes an upper CI limit that was beyond the highest intake of 35 mg Val·kg⁻¹·d⁻¹ and that was not reliably estimated.

² Estimate ± SE. The two line segments intersect at the breakpoint.
A different indicator amino acid (phenylalanine) was used in the present study at an intake of \( \approx 38 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \), which was based on direct amino acid oxidation and balance (DAAO and DAAB) studies of the phenylalanine requirement with a tyrosine-free diet (20, 25). The phenylalanine balances in the present study were largely positive, which could be because balance was measured as the difference between phenylalanine intake and oxidation rather than the hydroxylation rate. At a phenylalanine intake that was similar to that in the present study (with zero tyrosine except for that administered as a tracer), the ratio between phenylalanine hydroxylation and oxidation rates was 0.9 in the 12-h fasted state and close to 1.0 in the 12-h fed state (25). Nevertheless, in that study, the difference in daily phenylalanine balance calculated from the phenylalanine oxidation rate was higher by \( \approx 10\% \) of the phenylalanine intake (\( \approx 4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \)) than when the phenylalanine hydroxylation rate was used as the calculation parameter. The positive phenylalanine balances at valine intakes that were above the requirement could have also been due to the underestimation of phenylalanine oxidation with intravenous administration of the tracer (20). Unlike with leucine, for which there are no major differences in whole-body leucine oxidation when the tracer is given orally or intravenously (26), phenylalanine oxidation occurs predominantly in the liver (27, 28), and the first-pass uptake of this amino acid is greater than it is for leucine (20). However, in this initial investigation into the valine requirement, we preferred to use the intravenous route because it involved a relatively undisturbed period of sleep for the subjects, in contrast with an intermittent oral dosing regimen. We also used the enrichment of plasma phenylalanine to represent the precursor pool enrichment, because in long-term intravenous infusions of \([\text{ring}^{13}\text{C}]\)phenylalanine, it has been found that the ratio of enrichment of phenylalanine in a hepatic secretory protein (apolipoprotein B-100) to the enrichment in plasma phenylalanine approached unity in the fasted state (29).

A tyrosine-free diet was used in the present study, in contrast with earlier IAAO studies of the BCAA requirement, in which a generous intake of tyrosine was provided in the diet to reduce the conversion of phenylalanine to tyrosine (11). The labeled tyrosine formed from the hydroxylation of labeled phenylalanine tracer was partitioned into oxidation and protein synthesis and this partitioning could be variable depending on the needs of protein synthesis. In this respect, the provision of a tyrosine-free diet does not reduce the conversion of phenylalanine into tyrosine, and adds an additional variability to determination of the breakpoint estimate from estimates of phenylalanine oxidation. This might be truer particularly in respect of short-term fed state measurements or the use of surrogate indicators of oxidation such as the \( F^{13}\text{CO}_2 \). However, the desired primary aim of the present study was to assess 24-h phenylalanine oxidation and balance as an outcome of varying dietary valine intakes, and because the provision of \( \approx 38 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \) with a zero tyrosine intake has been shown to result in fairly neutral balances (25), it was our hypothesis that the net phenylalanine oxidation and balance under a defined and constant set of conditions would serve effectively as an indicator of valine equilibrium.

The valine requirement in the present study was similar to that suggested from earlier DAAO studies at different valine intakes (10). We chose to measure the valine requirement when a maintenance intake of leucine (8, 9) was supplied in the diet, because it is possible that the dietary leucine intake could influence the requirement for valine. This maintenance requirement of leucine is not a safe requirement that would meet the needs of 97.5% of the population. Because the BCAAs share common membrane transport systems (30, 31) and metabolic enzymes (32, 33), it is possible that the valine requirement was underestimated. In short-term animal experiments, a high leucine intake has been shown to result in increased valine oxidation (34), while also depressing free valine concentrations in plasma and tissue amino acid pools. Similar findings have been observed in humans, with leucine intake having an effect on plasma concentrations of valine and isoleucine, but with valine and isoleucine intake having little effect on the plasma leucine concentration (35, 36) or oxidation (37).

In contrast, however, it has been shown that varying leucine intake in the range supplied by normal diets (40 to 80 mg · kg\(^{-1} \) · d\(^{-1} \)) has no effect on valine oxidation (38). This is corroborated by comparing the findings of the present study with those of an earlier study (10) in which the valine requirement was estimated by direct measurements of valine oxidation in the presence of generous amounts (egg-protein-based amino acid mixture) of leucine. In these 2 experiments that were based on different principles (24-h IAAO/IAAB and short-term DAAO), the valine requirement estimate was similar, even though the amount of leucine supplied in the diet varied twofold. Therefore, it appears that there may be no regulatory effect of dietary leucine on valine catabolism within the physiologic range of leucine intakes and that the estimated value of the valine requirement is likely to be the same with a range of normal diets.

An important feature of the present study is that we were able to compare short-term fed state measurements of the proportion of tracer phenylalanine oxidized (\( F^{13}\text{CO}_2 \) ratio, short-term IAAO) with conventional estimates of phenylalanine oxidation and balance measured over a day (24-h IAAO/IAAB). We preferred to use the \( F^{13}\text{CO}_2 \) ratio rather than the \( F^{13}\text{CO}_2 \) value (\( \mu\text{mol} \cdot \text{kg}^{-1} \cdot 30 \text{ min}^{-1} \)) alone, because the ratio normalized the data for small differences in tracer infusion rates. The short-term IAAO technique, which was developed initially to study amino acid requirements during growth (reviewed in 39), has been extensively used to measure the amino acid requirements of adults (11, 40–42). Its advantages lie in the short nature of the protocol and its relative noninvasiveness. However, there are critical differences between the short-term IAAO method and the
longer-term 24-h IAAO and IAAB method. One of these is the length of the dietary adaptation period before the tracer study; it is 2 d in the short-term IAAO method but 7 d in the 24-h IAAO/IAAB method. A second important difference is that only fed state measurements are made in the short-term IAAO method, whereas the 24-h IAAO/IAAB method uses oxidation measurements over the whole day divided into 12-h fasted and 12-h fed states, such that daily amino acid balance can be accurately calculated.

The results of the present study show that the breakpoint estimate of the valine requirement, based on the $F^{13} CO_2$ data, was numerically $\approx 20\%$ higher than the estimates obtained from 24-h IAAO/IAAB within the same experiment (20 compared with 17 mg·kg$^{-1}$·d$^{-1}$, respectively); however, this difference was not significant because the 95% CIs of the 2 estimates completely overlapped. Given such large CIs, it is clear that currently designed tracer investigations cannot be powered sufficiently to detect even 20% differences because of the costs involved. There are several theoretical reasons as to why a 24-h tracer protocol may be preferable to a short-term tracer protocol. For instance, earlier 24-h studies of leucine (13) and phenylalanine oxidation (20) showed that the oxidation rate changes in a complex manner throughout the fed period; thus, it is possible that the “window” of the fed period measurement in the short-term IAAO method can confer some variability in the breakpoint estimate. Furthermore, because adaptive changes in amino acid oxidation also occur in the postabsorptive state in response to changes in test amino acid intake, this can modulate the 24-h response pattern. However, given the variability in the estimates of amino acid kinetics, it would appear that in the present state of the art, the short- and 24-h tracer approach gave similar findings, and that breakpoint estimates from 12-h fed state oxidation rates were similar to those obtained from the 24-h estimates.

The estimate of the valine requirement from either the $F^{13} CO_2$ ratio or the 24-h IAAO/IAAB method in the present study was lower than that predicted from the total BCAA requirement by the short-term relatively unadapted IAAO technique (11) or by the obligatory amino acid loss (OAAL) method (4). It is not possible to state with any certainty the reason for this discrepancy. One possibility is that the short-term IAAO study (11) determined the total BCAA requirement and from that predicted the valine requirement based on a similar proportionality in the amino acid composition of body protein. A similar higher valine requirement value is predicted from the OAAL method based on the same principle (4), and it may be that the prediction of the valine requirement based on an assumed proportionality of the BCAAs in body protein is unwarranted. Another possibility is the length of dietary adaptation before the tracer study, because it is possible that the effect of a lack of adequate dietary adaptation might give a higher value for the requirement of the test amino acid, particularly if habitual intake were high.

In summary, the present investigation of 24-h $[13C]phenylalanine$ tracer kinetics in healthy Indian subjects studied with 7 test valine intakes and at a maintenance intake of leucine indicates that the 1985 WHO/FAO/UNU requirement value of 10 mg·kg$^{-1}$·d$^{-1}$ is not adequate for the healthy Indian population.

This manuscript is dedicated to the memory of VR Young.

AVK was involved in study design, data collection, sample and data analysis, and writing of the manuscript. VNR and TDSR were involved in looking after the subjects, data collection, and analysis. JVG was involved in data collection and sample analyses. MMR was involved in study design, data analysis, and writing of the manuscript. The authors had no conflicts of interest.

REFERENCES


Only a small proportion of anemia in northeast Thai schoolchildren is associated with iron deficiency\textsuperscript{1–3}

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ABSTRACT

Background: Iron deficiency is assumed to be the major cause of anemia in northeast Thailand, but other factors may be involved.

Objective: We determined the prevalence of anemia among schoolchildren in northeast Thailand and the role of hemoglobinopathies, selected micronutrient deficiencies, and other factors in hemoglobin status.

Design: Blood samples were collected from 567 children aged 6–12.9 y attending 10 primary schools for the determination of a complete blood count and hemoglobin type [Hb AA (normal hemoglobin), Hb AE (heterozygous for Hb type E), and Hb EE (homozygous for Hb type E)] and the measurement of serum ferritin, transferrin receptor, retinol, vitamin B-12, and plasma and erythrocyte folate concentrations. Children with a C-reactive protein concentration ≥10 mg/L (n = 12), which indicated infection, were excluded.

Results: The prevalence of anemia was 31%. Age, hemoglobin type, and serum retinol were the major predictors of hemoglobin concentration. Hb AA and Hb AE children with anemia had lower (P < 0.01) hematocrit, mean cell volume, and serum retinol values than did their nonanemic counterparts; no significant differences in serum ferritin were found by hemoglobin type. Only 16% (n = 22) of the anemic Hb AA and Hb AE children were iron deficient. Hb AA and Hb AE children with a serum retinol concentration <0.70 μmol/L (n = 14) had a significantly higher geometric mean serum ferritin concentration than did those with a retinol concentration ≥0.70 μmol/L (P = 0.009); no significant difference in transferrin receptor concentrations was found between these 2 groups.

Conclusions: Hemoglobinopathies, suboptimal vitamin A status, and age were the major predictors of hemoglobin concentration. The contribution of iron deficiency to anemia was low, and its detection was complicated by coexisting suboptimal vitamin A status. Am J Clin Nutr 2005;82:380–7.

KEY WORDS Anemia, northeast Thailand, school-aged children, serum retinol, hemoglobin type E, iron

INTRODUCTION

Based on the most recent 1995 Thai National Nutrition Survey, the prevalence of anemia among school-aged children in northeast Thailand is 24% (1). Nutritional iron deficiency is assumed to be a major etiologic factor for anemia (2, 3), which is induced by rice-based diets and low intakes of readily available heme iron from cellular animal protein. The etiology of anemia, however, is multifactorial; genetic hemoglobinopathies, other micronutrient deficiencies, chronic inflammatory disorders, and parasitic infections may also play a role (4), although their relative importance in northeast Thailand is uncertain.

In many countries in Southeast Asia, including Thailand, hereditary disorders affecting the production of hemoglobin are widespread (5–8). There are 2 genetically distinct groups: hemoglobinopathies and thalassemias, each of which results in a slower rate of hemoglobin synthesis and hypochromic, microcytic anemia of varying severity and pathophysiology (6). Among populations in northeast Thailand, the most common hemoglobinopathy is hemoglobin type E (7, 8)—a genetically caused amino acid substitution in the β chain of hemoglobin A that changes the structure of the hemoglobin tetramer. Hemoglobin type E is relatively benign and has few clinical symptoms; therefore, those individuals who are homozygous for hemoglobin type E (Hb EE) or heterozygous for hemoglobin type E (Hb AE) are often not diagnosed (6). Unfortunately, it is difficult to discriminate between the hypochromic, microcytic anemia that is due to these hereditary disorders and iron deficiency anemia (IDA). In both cases, hemoglobin and mean cell volume (MCV) are reduced.

Several other micronutrients besides iron, notably vitamin A, riboflavin, folic acid, and vitamin B-12, are also required for normal hematopoiesis (9). Vitamin A deficiency is well documented among children in northeast Thailand (10–12), but data on deficiencies of the other vitamins are more limited.

Clearly, to design effective intervention strategies, the major predictors of anemia among schoolchildren in northeast Thailand must be identified. Therefore, in this cross-sectional study, we determined the prevalence of anemia among a selected sample of rural primary school children in northeast Thailand and investigated the role of hemoglobinopathies, selected micronutrient deficiencies (iron, vitamin A, folic acid, and vitamin B-12), and sociodemographic factors on hemoglobin concentrations.

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\textsuperscript{2} Supported by the Micronutrient Initiative Fund and the University of Otago Research Fund.

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Anemia was assessed by hemoglobin and MCV and iron deficiency by using various biochemical iron indexes. Vitamin A status was based on serum retinol, folate status on plasma and red blood cell folate, and vitamin B-12 status on serum vitamin B-12 concentrations.

**SUBJECTS AND METHODS**

**Subjects**

This cross-sectional study was conducted in June and July 2002 in Ubon Ratchathani province, northeast Thailand. The participating children (281 boys and 286 girls) were aged 6.0–12.9 y and resided in 10 poor rural subdistricts that served a large rice growing area surrounding the town of Trakarn Phutphon. Ten primary schools with the largest student enrollment in each subdistrict were selected to participate in this study. Eligible children from each school were stratified by age and sex, and equal numbers of children were randomly selected from each strata.

Ethical approval of the study protocol was obtained from Human Ethics Committees of Mahidol University (Nakhonpathom, Thailand) and the University of Otago (New Zealand). Written informed consent was obtained from the parents or guardians of each child in the survey. In addition, permission was sought from local school boards and Thai health workers after meetings in which the purpose and methods of the study were clearly explained by one of the principal investigators (PW).

**Sociodemographic and health-status assessment**

Trained Thai research assistants administered a pretested sociodemographic and health-status questionnaire to an adult member of each participating household. Sociodemographic variables assessed included ethnicity, household size, education, income, employment of the head of the household, and level of education for the mother or caregiver. Health-status indicators included self-reported infection status (diarrhea, respiratory, and parasite), and the use of vitamin or mineral supplements or both.

**Biochemical assessment**

Morning, nonfasting peripheral venipuncture blood samples were drawn by trained nurses from children in the recumbent position into either a trace element–free evacuated tube protected from exposure to light or an evacuated tube containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ). All blood samples were refrigerated immediately after collection. Aliquots of serum for ferritin, transferrin receptor, retinol, vitamin B-12, and C-reactive protein (CRP) measurement were frozen for a complete blood count and hemoglobinopathies. The remainder of the whole blood was then centrifuged (2500 \( \times \) g, 10 min, room temperature), and the plasma was separated as quickly as possible. For the folate analysis, an aliquot of plasma was diluted 1 in 20 with 0.5% sodium ascorbate solution before being frozen. A complete blood count was determined by using an electronic Coulter Counter (Beckman, Fullerton, CA) in Trakarn Phutphon District Hospital. Hemoglobinopathy analysis was performed by using the Variant \( \beta \)-Thalassemia Short Program (Bio-Rad Laboratories Inc, Hercules, CA) in the Thalassemia Research Centre (Institute of Science and Technology for Research and Development, Mahidol University). Hemoglobin type was determined in each subject on the basis of hematologic indexes: Hb AA (normal hemoglobin type), Hb AE (trait for hemoglobin E disease), or Hb EE (hemoglobin E disease).

Serum retinol was analyzed by using HPLC according to the method of the International Vitamin A Consultative Group (13). Serum ferritin and vitamin B-12 concentrations were determined by using an IMx analyzer that uses Microparticle Enzyme Immunoassays technology sera (Abbott Laboratories, Abbott Park, IL). Serum transferrin receptor was analyzed with an enzyme immunoassay by using commercial kits (Ramco Laboratories Inc, Houston, TX). Serum folate and whole-blood folate were measured by using the microtiter technique described by O’Broin and Kelleher (14) with chloramphenicol-resistant *Lactobacillus casei* as the test microorganism. Erythrocyte folate was calculated from whole-blood folate by subtracting serum folate and correcting for hematocrit. An external whole-blood standard (National Institute for Biological Standards and Control, South Mumps, United Kingdom) with a certified folate concentration of 29.4 nmol/L was used to generate the standard curve. Infection was determined on the basis of the CRP concentration by using the Behring Turbitimer System (Behringwerke AG Diagnostica, Germany); a cutoff of \( \geq 10 \) mg/L was used to indicate the presence of inflammation or infection as recommended by the manufacturer.

A pooled serum sample and manufacturer’s controls were used to check the precision and accuracy of all the analytic methods. The certified values for the manufacturer’s controls for serum ferritin supplied for the IMx analyzer system had been calibrated by the manufacturer with the World Health Organization International Reference Material. The between-assay CVs for serum ferritin, transferrin receptor, serum retinol, vitamin B-12, and serum and red blood cell folate were 5.1%, 3.3%, 5.4%, 7.2%, and 11.8%, respectively. Values for the controls fell within the certified ranges for serum ferritin, transferrin receptor, vitamin B-12, and CRP.

Anemia was defined as a hemoglobin concentration of <115 g/L for children aged 6–11 y and <120 g/L for those aged \( \geq 12 \) y (15). Iron deficiency was defined on the basis of an elevated serum transferrin receptor concentration (>8.5 mg/L) (16) or an elevated ratio of transferrin receptor to ferritin (500 \( \mu \)g/L) (17). For storage iron depletion, the commonly used cutoff for serum ferritin of <12 \( \mu \)g/L was used. IDA was defined as iron deficiency concurrent with anemia.

**Statistical analyses**

All data were analyzed by using the STATISTICAL PACKAGE FOR SOCIAL SCIENCES (SPSS for WINDOWS, version 10.0; SPSS, Chicago, IL). Data were checked for normality by using the Kolomogrov-Smirnov test. One-way analysis of variance with post hoc analysis (Tukey’s honestly significant difference) was used to determine the source of differences in hematologic and biochemical indicators between the 3 hemoglobin types. ANOVA was also used to show the effect of anemia and vitamin A deficiency on selected hematologic and iron-status indexes and to examine the predictors of hemoglobin concentration. Statistically significant differences are indicated by \( P < 0.05 \).
Sociodemographic characteristics of the participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>286 (50.4)</td>
</tr>
<tr>
<td>Male</td>
<td>281 (49.6)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>6.00–7.99 y</td>
<td>175 (30.9)</td>
</tr>
<tr>
<td>8.00–9.99 y</td>
<td>180 (31.7)</td>
</tr>
<tr>
<td>10.00–12.99 y</td>
<td>212 (37.4)</td>
</tr>
<tr>
<td>Male head of household</td>
<td></td>
</tr>
<tr>
<td></td>
<td>491 (86.6)</td>
</tr>
<tr>
<td>Estimated annual yearly income ≤ 30000 baht</td>
<td>299 (52.7)</td>
</tr>
<tr>
<td>Occupation of head of household</td>
<td></td>
</tr>
<tr>
<td>Farming</td>
<td>503 (88.7)</td>
</tr>
<tr>
<td>Education level of head of household</td>
<td></td>
</tr>
<tr>
<td>Primary school</td>
<td>473 (83.4)</td>
</tr>
<tr>
<td>High school or beyond</td>
<td>87 (15.2)</td>
</tr>
<tr>
<td>Education level of caregiver or mother</td>
<td></td>
</tr>
<tr>
<td>Primary school</td>
<td>501 (88.4)</td>
</tr>
<tr>
<td>High school or beyond</td>
<td>15 (10.2)</td>
</tr>
</tbody>
</table>

1 There was no relation between the 3 hemoglobin types (Hb AA, Hb AE, and Hb EE) and the sex or age of the children or with the estimated annual yearly income of the head of household.

2 US$1.0 was equivalent to ≈40 Thai baht in 2002.

RESULTS

Sociodemographic and health status

Selected sociodemographic results are presented in Table 1. A total of 567 children (286 girls and 281 boys), all of Thai ethnicity, participated in the survey. The participants were predominantly of low socioeconomic status. Farming, primarily rice growing, was the occupation of 89% of the households, and the median estimated annual household income was 28,400 baht. More than 80% of those identified as either head of household or the primary caregiver had received only primary school education; <3% of the children (n = 12) had an elevated serum CRP concentration (ie, CRP ≥ 10 mg/L), which indicated inflammation or infection; >90% (n = 531) of the sample had never used a nutritional supplement; and <2% (n = 5) of the girls reported that menstruation had commenced.

Of the adults responding to the health-status questionnaire, 35% (n = 201) reported that their child currently had a parasitic infection, and 60% (n = 340) reported that their child had received the appropriate treatment for helminth infections.

Assessment of hemoglobinopathies and hematologic indexes

Of the 548 children examined for hemoglobin type, 57% (n = 321) had Hb AA, 33% (n = 186) had Hb AE, and 5% (n = 28) had Hb EE. One child had evidence of Hb EE and beta thalassemia disease, and the remaining 12 children had either an ambiguous result or a rare hemoglobin variant. These 13 children were excluded from any further statistical analyses. There was no relation between the hemoglobin type and the age or sex of the children or the socioeconomic status of the family as measured by the estimated annual yearly income of the head of household.

Overall, 31% (n = 175) of the children were anemic. At the 95% confidence level, the sample size for this cross-sectional study (n = 567) was sufficient to detect a 30% prevalence of anemia with an absolute precision of =0.04% of the proportion. The prevalence of anemia decreased with increasing age (P < 0.001) but was independent of sex. The medians (1st and 3rd quartiles) for selected hematologic indexes of the children by hemoglobin type are shown in Table 2. Marked differences in all these indexes occurred by hemoglobin type. Hb AA children had significantly higher median values for hemoglobin, hematocrit, and MCV than did Hb EE children. Likewise, Hb AE children had significantly higher median hemoglobin, hematocrit, and MCV values than did the Hb EE children (Table 2). Consequently, the prevalence of anemia, based on low hemoglobin, was lowest in the Hb AA group (21%; n = 68) and second lowest in the Hb AE group (37%; n = 68). Eighty-six percent (n = 24) of the Hb EE group had hemoglobin concentrations indicative of anemia.

The anemia observed in these children was predominantly microcytic hypochromic, as indicated by an MCV < 80 fL in the 6–11 y age group or <82 fL in the >12 y age group (18). The prevalence of a low MCV was dependent on hemoglobin type as shown in Figure 1 and was negatively associated with age. Specifically, 40% (n = 126), 99% (n = 184), and 100% (n = 28) of the Hb AA, Hb AE, and Hb EE children, respectively, had a low MCV. Within each of the specific hemoglobin types, we observed various degrees of microcytic anemia, as indicated by the following correlations between hemoglobin and MCV within each specific hemoglobin type: Hb AA (n = 310; r = 0.420, P =

| Table 2 |

Selected hematologic and biochemical variables of the children by hemoglobin type (Hb AA, Hb AE, and Hb EE)

<table>
<thead>
<tr>
<th></th>
<th>Hb AA (n = 321)</th>
<th>Hb AE (n = 186)</th>
<th>Hb EE (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)²</td>
<td>121.6 ± 9.8 ²</td>
<td>118.4 ± 9.4 ²</td>
<td>106.6 ± 7.4 ²</td>
</tr>
<tr>
<td>Hematocrit ²</td>
<td>36.6 ± 2.8 ²</td>
<td>35.8 ± 2.7 ²</td>
<td>32.8 ± 2.2 ²</td>
</tr>
<tr>
<td>Mean cell volume (fL) ²</td>
<td>80.1 ± 5.8 ²</td>
<td>72.6 ± 3.4 ²</td>
<td>58.4 ± 2.1 ²</td>
</tr>
<tr>
<td>Serum ferritin (μg/L) ²</td>
<td>37.5 (19.6, 71.8) ²</td>
<td>37.4 (20.3, 68.9) ²</td>
<td>59.3 (35.5, 99.1) ²</td>
</tr>
<tr>
<td>Serum transferrin receptor (mg/L) ²</td>
<td>6.8 ± 2.4 ²</td>
<td>6.5 ± 1.9 ²</td>
<td>8.8 ± 2.1 ²</td>
</tr>
<tr>
<td>Serum retinol (μmol/L) ²</td>
<td>1.31 ± 0.38 [312] ²</td>
<td>1.25 ± 0.30 [178] ²</td>
<td>1.27 ± 0.28 [28] ²</td>
</tr>
</tbody>
</table>

1 Means in a row with different superscript letters are significantly different, P < 0.01 (Tukey’s post hoc test for differences between means).

2 All values are ± SD.

3 All values are geometric ± SD.

4 n in brackets.
0.001), Hb AE (n = 177; r = 0.273, P = 0.001), and Hb EE (n = 27; r = 0.329, P = 0.094).

Assessment of iron deficiency and iron deficiency anemia

Children with an elevated serum CRP indicative of inflammation or infection (≥10 mg/L; n = 12) had a significantly higher (P < 0.001) geometric mean (–SD, +SD) serum ferritin concentration (109.4; 20.3, 72.6 μg/L) than did those with a serum CRP concentration <10 mg/L (38.4; 62.5, 191.3 μg/L) and were thus excluded from any further statistical analyses. There was no significant difference between the mean (±SD) serum transferrin receptor concentrations of those children with a serum CRP concentration ≥10 mg/L and those with a concentration <10 mg/L (6.9 ± 2.6 compared with 6.8 ± 1.8 mg/L, respectively).

In children with Hb AA and Hb AE, there was an increase in serum ferritin (P = 0.001) and a decrease in serum transferrin receptor (P < 0.001) with increasing age. After age was controlled for in the Hb AA and Hb AE groups, there were no significant differences in serum ferritin or transferrin receptor concentrations between boys and girls.

Geometric mean serum ferritin and mean transferrin receptor concentrations by hemoglobin type are shown in Table 2. There were no significant differences in these indexes between the Hb AA and Hb AE groups (Table 2). However, Hb EE children had significantly higher (P < 0.001) serum ferritin and transferrin receptor concentrations than did the Hb AA and Hb AE children.

Very little of the anemia in the northeast Thai schoolchildren with Hb AA and Hb AE was associated with iron deficiency. Indeed, the prevalence of IDA on the basis of a low hemoglobin concentration and an elevated transferrin receptor was only 4.4% (n = 14) in the AA group and 4.3% (n = 8) in the AE group. The prevalence of iron deficiency (without anemia) was also low in both groups: 12.1% (n = 39) in the Hb AA group and 5.4% (n = 10) in the Hb AE group, and these differences were significant (P = 0.008; Fisher’s exact test). Even fewer children with Hb AA and Hb AE had evidence of depleted iron stores, on the basis of low serum ferritin concentrations (<12 μg/L); the corresponding prevalences were 0.6% (n = 2) and 2.2% (n = 4) in the Hb AA and Hb AE groups, respectively, and these differences were not significant. When IDA was assessed in these 2 groups on the basis of an elevated ratio of transferrin receptor to serum ferritin (500 μg/L) and coexisting anemia, the prevalence of IDA was only 1.6% in both the Hb AA (n = 5) and Hb AE (n = 3) groups, respectively; the prevalence of iron deficiency alone was 3.7% (n = 12), and 3.2% (n = 6) in the Hb AA and Hb AE groups, respectively. Note that the prevalence of IDA was slightly higher in both the Hb AA and Hb AE groups when defined as anemia plus an elevated serum transferrin receptor concentration.

It is noteworthy that of the children with Hb EE, 54% (n = 15) had both an elevated serum transferrin receptor and a low hemoglobin concentration, whereas only 3.6% (n = 1) had an elevated serum transferrin receptor concentration but no evidence of anemia. Ineffective erythropoiesis is known to accompany the hemoglobin type E variant and was thus responsible for the elevated transferrin receptor concentrations observed (19, 20). None of the Hb EE children had a serum ferritin concentration <12 μg/L, and only one child in the Hb EE group had a ratio of serum transferrin to serum ferritin >500 μg/L. High serum ferritin concentrations may occur in persons who are homozygous for hemoglobin E because they have erythrocytes with a reduced survival time (19). Hence, the iron released from the erythrocytes may accumulate as storage iron because of the slow rate of synthesis of hemoglobin E.

Assessment of folate and vitamin B-12 deficiency

Unlike the hematologic and biochemical iron variables, there were no significant differences in mean plasma or erythrocyte folate or serum vitamin B-12 concentrations by hemoglobin type. The geometric mean (–SD, +SD) plasma and erythrocyte folate concentrations were 21.4 (13.5, 33.9) and 842 (599, 1182) nmol/L, respectively. Mean serum vitamin B-12 was 514 (355,
744) pmol/L. Plasma folate was positively correlated with erythrocyte folate \((r = 0.259, P < 0.05)\). There are no pediatric interpretive values for plasma and erythrocyte folate and serum vitamin B-12. Accordingly, the interpretive values for “at risk” adults were used for plasma folate and erythrocyte folate and serum vitamin B-12 concentrations, ie, <6.8 and <317 nmol/L (21) for plasma and erythrocyte folate, respectively, and <150 pmol/L for serum vitamin B-12 (22). On the basis of these cut-offs, 4 and 5 of the children, respectively, had low blood folate concentrations, but none of the children had a serum vitamin B-12 concentration indicative of vitamin B12 deficiency (22).

Assessment of vitamin A deficiency

Serum retinol concentrations were independent of hemoglobin type (Table 2) and sex but increased significantly with increasing age \((P < 0.001)\). Very few of the children (3%) had a low vitamin A status (serum retinol: <0.7 μmol/L; 23), but 20% \((n = 98)\) had marginal vitamin A status (serum retinol: 0.7–1.05 μmol/L; 24).

Interrelations among laboratory, sociodemographic, and health-status variables

ANOVA showed that in children with Hb AA and Hb AE, age was the most important predictor of hemoglobin concentration, followed by hemoglobin type and serum retinol; serum ferritin was not a significant predictor (Table 3). Moreover, ANOVA (adjusted \(r^2 = 0.255\)) showed that when serum transferrin receptor was treated as a dependent variable, it was inversely related to log serum ferritin \((P < 0.001)\) after adjustment for age and school (data not shown). ANOVA results also showed that, after adjustment for the significant effects of age, anemic children had significantly lower \((P < 0.01)\) mean hematocrit, MCV, and serum retinol concentrations than did those with serum retinol concentrations ≥0.70 μmol/L, but no significant difference in mean serum transferrin receptor concentrations after adjustment for age (Table 5). Sex, estimated annual income, and self-reported parasitic infection status were not associated with any of the laboratory indexes investigated.

TABLE 3
ANOVA showing predictors of hemoglobin concentration in a sample of school-aged children from northeast Thailand

<table>
<thead>
<tr>
<th>Variable</th>
<th>Anemic children ((n = 122))</th>
<th>Nonanemic children ((n = 340))</th>
<th>(\beta)</th>
<th>95% CI</th>
<th>Partial (\eta^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.096</td>
<td>0.054, 0.137</td>
<td>0.033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin type(^2)</td>
<td>2.940</td>
<td>1.311, 4.569</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum retinol (μmol/L)</td>
<td>4.276</td>
<td>1.952, 6.601</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log serum ferritin (μg/L)</td>
<td>1.024</td>
<td>-0.227, 2.275</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) \(n = 450\). Children with hemoglobin type EE and a C-reactive protein concentration ≥10 mg/L were excluded. The interaction term hemoglobin type × log serum ferritin and hemoglobin type × serum retinol are not statistically significant.

\(^2\) Hb AA or Hb AE.

**Discussion**

Our results showed a high prevalence of anemia and emphasized the importance of hemoglobinopathies in the etiology of anemia in northeast Thai schoolchildren. Indeed, more than one-third of these children carried a genetic hemoglobinopathy, specifically the \(\beta\) chain variant hemoglobin type E. Of the children who were homozygous (Hb EE) for this variant, nearly all (86%) were anemic, compared with more than one-third (35%) who were heterozygous (Hb AE) and 21% who had normal hemoglobin (Hb AA).

TABLE 4

<table>
<thead>
<tr>
<th>Variable and hemoglobin type</th>
<th>Anemic children ((n = 122))</th>
<th>Nonanemic children ((n = 340))</th>
<th>(P^2)</th>
<th>(P) for interaction(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA + AE</td>
<td>33.6 (33.3, 33.9)</td>
<td>37.1 (36.9, 37.3)</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Mean cell volume (fl)</td>
<td></td>
<td></td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>AA</td>
<td>76.2 (75.0, 77.4)</td>
<td>81.0 (80.4, 81.6)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AE</td>
<td>71.6 (70.4, 72.8)</td>
<td>73.3 (72.4, 74.2)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Serum ferritin (μg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA + AE</td>
<td>37.3(^3) (33.2, 41.8)</td>
<td>37.4(^3) (34.8, 40.2)</td>
<td>0.945(^5)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum transferrin receptor (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>7.38 (6.83, 7.92)</td>
<td>6.55 (6.27, 6.82)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AE</td>
<td>6.40 (5.87, 6.94)</td>
<td>6.51 (6.11, 6.91)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Serum retinol (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA + AE</td>
<td>1.22 (1.16, 1.28)</td>
<td>1.31 (1.27, 1.35)</td>
<td>0.017</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) Children with Hb EE and a C-reactive protein concentration ≥10 mg/L were excluded from the analysis; anemia was defined as a hemoglobin concentration <115 g/L for children aged 6–11 y and <120 g/L for children aged ≥12 y. The mean values were adjusted for age by using a general linear model.

\(^2\) Significance of the difference between the anemic and nonanemic children.

\(^3\) Interaction of hemoglobin type and anemia.

\(^4\) Geometric \(\bar{x}\).

\(^5\) Calculated from natural log values.
The anemia reported in the Thai children was predominantly microcytic, hypochromic anemia (Figure 1), as indicated by a low MCV. Anemia of this type is known to be associated with hemoglobinopathies and thalassemias (6, 25) as well as with deficiencies of iron (26) and vitamin A (27, 28).

It is of interest that little of the observed anemia reported here appeared to be associated with storage iron depletion. Indeed, concentrations of serum ferritin in the anemic and nonanemic Hb AA and Hb AE children were not significantly different (Table 4) and were within the range reported by other Thai investigators (29, 30). This finding was unexpected in light of the assumed role of nutritional iron deficiency in the etiology of anemia in Thailand (2, 3) but was nevertheless consistent with earlier findings in school-aged children from northern Thailand (27, 29, 31).

Instead, ANOVA results confirmed that age, hemoglobin type, and serum retinol were the most important factors predicting hemoglobin concentration (Table 3). Furthermore, the increase in hemoglobin concentration with age was independent of hemoglobinopathies (ie, there was no significant interaction).

The positive association between serum retinol and hemoglobin observed was reported by others in studies of children in Southeast Asia and elsewhere (32–36). Specifically, the anemic children with Hb AA and Hb AE in our study had a significantly lower mean serum retinol concentration than did their nonanemic counterparts (Table 4). Nevertheless, the effect of suboptimal vitamin A status on the prevalence of anemia has received little attention by policy makers in Thailand, perhaps because the high prevalence of hemoglobinopathies has confounded the detection of a positive relation between hemoglobin and serum retinol in earlier studies in northeast Thailand (27). In addition, there has been a dramatic decrease in the prevalence of vitamin A deficiency in Thailand in recent years. Indeed, only 3% of the northeast Thai children studied had serum retinol concentrations indicative of low vitamin A status (23). Nevertheless, 20% had concentrations indicative of marginal vitamin A status (0.7–1.05 μmol/L) (24), which have also been shown to be positively associated with low hemoglobin concentrations (35–37).

Several mechanisms whereby vitamin A interacts with iron and thus affects hemoglobin concentrations have been proposed. Whether the reduction in hematopoiesis observed in vitamin A deficiency is due to decreased incorporation of iron into hemoglobin or to impaired mobilization of iron from spleen or liver stores into the circulation is still uncertain (38, 39). In our study, the significantly higher serum ferritin concentrations observed in the children classified with low vitamin A status (Table 5) lends support to the hypothesis that a decrease in the mobilization of iron may be involved. Certainly, in earlier studies of children in Thailand in whom vitamin A deficiency anemia has been reported, the absence of low serum ferritin concentrations complicates the detection of storage iron depletion (27, 29, 31). Indeed, in many cases, the existence of relatively elevated serum ferritin concentrations similar to those reported here has often been assumed to result from inflammation or infection (40). This conclusion is not supported by our data; only 2% of the children studied had elevatd CRP concentrations indicative of inflammation or infection. More studies involving the simultaneous determinations of serum retinol, iron-status indexes, hemoglobin, hemoglobin type, and inflammation and infection status in population groups in Thailand and other regions of Southeast Asia are needed to further elucidate these interrelations.

Our results indicated that some of the children in the current study had a biochemical iron deficiency; however, the prevalence was low. In 2 earlier studies of Thai school-aged children (30, 31), serum ferritin, hemoglobin, or both indicators showed a positive response to iron supplements, although the effect was lower than expected in some cases (31). It appears that the detection of iron deficiency or IDA in northeast Thai schoolchildren is complicated by the coexistence of concomitant hemoglobinopathies and suboptimal vitamin A status.

Additional nonnutritional factors implicated in the etiology of anemia include parasitic infections, such as malaria and hookworm. Malaria no longer exists in northeast Thailand, but hookworm infestation does (30). However, we observed no relation between hemoglobin and self-reported helminth status in these Thai schoolchildren, although no objective measure of hookworm infestation was obtained. Unlike some other studies (41), we could not detect any effect of socioeconomic status on hemoglobin concentrations in this study, after controlling for age and hemoglobin type, probably because all the children were selected from schools in the poorer subdistricts in Ubon Ratchathani province.

Nutritional deficiencies of folate and vitamin B-12 may also cause anemia. However, deficiencies of folate and vitamin B-12 are associated with the development of macrocytic anemia, which is characterized by an elevated MCV (26), rather than the microcytic anemia noted here (Figure 1). Moreover, only ≈1% of the children had biochemical evidence of folate deficiency on the basis of plasma and erythrocyte folate concentrations, and none of the children had vitamin B-12 deficiency. The absence of vitamin B-12 deficiency was attributed to the consumption of fish sauce and possibly fermented tempeh by these Thai schoolchildren, both of which foods are known to provide significant amounts of vitamin B-12 in the diet (42).

### Table 5

<table>
<thead>
<tr>
<th>Variable</th>
<th>Serum retinol &lt;0.7 μmol/L (n = 19)</th>
<th>Serum retinol ≥0.7 μmol/L (n = 462)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ferritin (μg/L)</td>
<td>56.92 (41.4, 78.3)</td>
<td>37.02 (34.9, 39.2)</td>
<td>0.009*</td>
</tr>
<tr>
<td>Serum transferrin receptor (mg/L)</td>
<td>7.00 (6.02, 7.99)</td>
<td>6.61 (6.41, 6.81)</td>
<td>0.441</td>
</tr>
</tbody>
</table>

* Children with hemoglobin type EE and a C-reactive protein concentration ≥10 mg/L were excluded from the analysis. The mean values were adjusted for age by using a general linear model.

1 Geometric x.

2 Calculated from natural log values.
It is possible that riboflavin deficiency contributed to the anemia reported in the present study (9), although, unfortunately, we did not measure any biomarker of riboflavin status. Riboflavin deficiency is known to occur among rice-eating populations (43, 44), and the riboflavin intake of a subsample of these children was low in relation to the Thai recommended dietary allowance, consistent with findings of the 1995 National Nutrition Survey for the northeast region of Thailand (1). Furthermore, in an earlier study in Thailand (45) supplementation with both iron and riboflavin was more efficient at restoring hematologic indexes than was an iron supplement alone.

In conclusion our results indicate that hemoglobinopathies and suboptimal vitamin A status, together with age, were the major predictors of hemoglobin concentration in the population of primary school children from northeast Thailand that we studied and should be taken into account in the planning of anemia control programs. Little of the anemia noted in the present study was associated with iron deficiency, which indicates that hemoglobin alone is not a reliable indicator of the prevalence of IDA in this setting. Some iron deficiency probably occurs but its detection is complicated by the coexistence of suboptimal vitamin A status in this region.

We thank the school teachers and the children and families who participated in this survey, Jane Campbell for her excellent laboratory expertise, and the dedicated research assistants. We also thank Pranee Fucharoen for the analysis and interpretation of the hemoglobinopathy data and Ian L Gibson for his logistical, computing, and statistical support throughout the project. RAT participated in the collection, analyses, and interpretation of the laboratory and statistical data. RAT and RSG were the primary writers of the manuscript but received input from the other authors. PW and RSG were responsible for the study concept and design, for securing the funding for the study, and for the acquisition, statistical analysis, and interpretation of the data. TP was the overall project field coordinator and participated in the data acquisition, analysis, and interpretation. KB conducted most of the biochemical analysis in New Zealand and some of the statistical analysis. EW supervised the analysis and interpretation of the serum retinol values. None of the authors had any conflicts of interest in connection with this study.

REFERENCES


Increasing the iodine concentration in the Swiss iodized salt program markedly improved iodine status in pregnant women and children: a 5-y prospective national study

Michael B Zimmermann, Isabelle Aeberli, Toni Torresani, and Hans Bürgi

ABSTRACT

Background: Many industrialized countries struggle to maintain adequate iodine intake because of changes in dietary habits and the food supply. In Switzerland, because of declining iodine intakes in children and pregnant women, the iodine concentration in table salt was increased from 15 to 20 mg/kg.

Objective: We evaluated Swiss iodine nutrition after the 1999 increase in the salt iodine concentration.

Design: In 1999 and 2004, a 3-stage probability proportionate-to-size cluster sampling was done to obtain a representative national sample of primary schoolchildren and pregnant women. Urine and household salt were collected for iodine measurement. The frequency of elevated thyrotropin concentrations found in the newborn screening program was evaluated before and after the increase.

Results: In 1999, median urinary iodine (UI) concentrations among children (n = 610) and pregnant women (n = 511) were 115 μg/L (range: 5–413 μg/L) and 138 μg/L (range: 5–1881 μg/L), respectively, which indicated marginal iodine status. In 2004, median UI concentrations among children (n = 386) and pregnant women (n = 279) were 141 μg/L (range: 0–516 μg/L) and 249 μg/L (range: 8–995 μg/L), respectively (P < 0.01). Newborn thyrotropin concentrations >5 mU/L decreased from 2.9% in 1992–1998 (n = 259 035) to 1.7% in 1999–2004 (n = 218 665) (P < 0.0001).

Conclusions: A 25% increase in iodine concentration in iodized table salt markedly improved iodine status in Switzerland, which showed the value of monitoring and adjusting iodine concentrations in national salt programs. The frequency of newborn thyrotropin concentrations >5 mU/L appears to be a sensitive indicator of iodine nutrition during pregnancy.


KEY WORDS Salt, iodine, monitoring, Switzerland, children, newborns, thyrotropin, pregnancy

INTRODUCTION

Because dietary iodine supply in many countries depends on several shifting commercial, agricultural, and societal factors, regular monitoring of iodine nutrition is necessary. Industrialized countries with long-standing salt iodization programs, including the United States, Netherlands, New Zealand, France, and Australia, have reported declining or low concentrations of urinary iodine (UI) among their populations (1–5). The World Health Organization (WHO) has emphasized the importance of periodic monitoring and adjustment of salt iodide concentrations, but few developed or developing countries have established regular and systematic programs (6, 7). Although the WHO has suggested that the frequency of moderately elevated thyrotropin concentrations in newborn screening programs can be used to assess the severity of iodine deficiency in a population, the cutoff values for defining severity are uncertain (6, 7).

Since 1952, iodized salt has been available nationwide in Switzerland. The concentration of iodine in table salt was increased from 3.75 mg/kg to 7.5 mg/kg in 1962 and then to 15 mg/kg in 1980 (8, 9). During the 1980s, iodine status in Switzerland was adequate (8, 9). However, in the 1990s, studies began to suggest marginal iodine deficiency among schoolchildren and pregnant women (10–13). In 1994, among pregnant women in Lausanne, the mean UI concentration was 83–100 μg/g creatinine (12), and, in 1997, the median UI concentration in primary schoolchildren from Zürich and the Engadine valley was 96 μg/L (13). In response, the Swiss federal government increased the concentration of iodine in table salt to 20 mg/kg in 1998. Considering retail and household turnover of salt, it is thought that, by the end of 1999, most of the salt being consumed in Swiss households was iodized at the new concentration.

However, it was not certain what effect this increase would have on iodine nutrition in Switzerland. Approximately 95% of household salt and 70% of salt for industrial food production in Switzerland is iodized, although iodized salt use is voluntary, and manufacturers and retailers must offer both iodized and non-iodized salt (8). Most dietary intake of salt in industrialized countries comes from processed foods (14), and a significant proportion of salt consumed in processed foods in Switzerland is...
iodized at low concentrations (5–10 mg/kg) or noniodized (8, 10). Export-oriented Swiss food producers are reluctant to use iodized salt because it may limit their markets, and an increasing number of imported processed foods contain noniodized salt. In addition, current guidelines from the Ministry of Health recommend that Swiss adults reduce their intake of salt. In this report, we compare data from the national study done in 1999, at the time of the increase, with findings 5 y later, in 2004.

SUBJECTS AND METHODS

In 1999 and 2004, we used a 3-stage probability proportionate-to-size cluster sampling to obtain a representative national sample of primary schoolchildren and pregnant women. Proportionate-to-size cluster sampling is the recommended method for monitoring national salt iodization programs (7). Census data were used to provide a systematic sampling of urban and rural communities on the basis of the cumulative population. In stage 1 of the sampling, primary schools and obstetric clinics were recruited with the use of stratified random selection. If a school or clinic declined participation, a replacement was randomly selected from the same stratum. The ratio of pregnant women in Switzerland who receive their prenatal care in hospitals to those who receive their prenatal care in private clinics is ≈ 1:2 (Swiss Society for Obstetrics and Gynecology, written communication, 1999), and this ratio was used to sample prenatal care clinics. In stage 2, classrooms were randomly selected from each school. Finally, all children whose parents provided consent were enrolled from the classroom, and the clinic physician sequentially enrolled the desired number of pregnant women. In 1999, ≈ 30 children and pregnant women were sampled from 20 clusters (15); in 2004, ≈ 20 children and pregnant women were sampled from 20 and 15 clusters, respectively.

At the schools, the age of the children was obtained from the school record, and weight and height were measured (16). For the pregnant women, age, week of gestation, type of salt used in the home, and use of vitamin-mineral supplements were recorded by the clinic staff. Spot urine samples were collected from children and pregnant women. In 1999, 30-g salt samples were collected from households in the Zürich metropolitan area. In 2004, every fourth child from the school lists participating in the study brought a 30-g salt sample from home. In 1999, data were collected from April through December; in 2004, data were collected from April through September. Data on newborn thyrotropin concentrations were obtained from the newborn screening program for eastern Switzerland at the University Children’s Hospital in Zürich. In this program, whole-blood samples obtained on day 3 or 4 (72-96 h) after birth are spotted and dried on filter paper (grade 903; Schleicher & Schuell, Dassel, Germany) and sent to a central laboratory for analysis. Because the iodine supply is uniform across Switzerland and a standardized collection protocol is used for newborn screening throughout the country, these thyrotropin data are likely to be nationally representative.

Written informed consent was obtained from the parents of the children and from the pregnant women. Ethical approval for the study was obtained from the Swiss Federal Institute of Technology in Zürich.

### Table 1

<table>
<thead>
<tr>
<th>Study</th>
<th>1999 (n = 610)</th>
<th>2004 (n = 386)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>9.5 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.9 ± 1.8 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male:female</td>
<td>307:303</td>
<td>184:202</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>139 ± 14</td>
<td>141 ± 12</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>34.8 ± 10.2</td>
<td>35.7 ± 9.6</td>
</tr>
<tr>
<td>Urinary iodine concentration (µg/L)</td>
<td>115 (5–413)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141 (0–516)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prevalence of urinary iodine &lt;100 µg/L (%)</td>
<td>40</td>
<td>24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ± SD (all such values).
<sup>b</sup> Median; range in parentheses (all such values).
<sup>c</sup> Significantly different from 1999, P < 0.01 (t test).
<sup>d</sup> Significantly different from 1999, P < 0.01 (chi-square test).

Data analysis

Salt and urine samples were stored at −20 °C until they were analyzed. UI concentration was measured at the Human Nutrition Laboratory in Zürich by using a modification of the Sandell-Kolthoff reaction (17). By this method, the CV for UI concentration in our laboratory is 10.0% at 47.4 ± 0.6 µg/L and 12.7% at 79.5 ± 0.8 µg/L. Salt iodine concentration was measured by colorimetric titration (7). Dried blood spots on filter paper were analyzed for newborn thyrotropin with the use of an immunoassay (18). Normal reference values are <15 mU/L whole blood.

Statistical analysis

We used SPLUS-2000 (Insightful Corporation, Seattle, WA), PRISM (version 3; GraphPad, San Diego, CA), and EXCEL (XP 2002; Microsoft, Seattle, WA) software for data processing and statistics. Normally distributed data were expressed as means ± SDs; nonnormally distributed data were expressed as medians (ranges). Unpaired t tests and chi-square tests were used for comparisons of normally distributed data. UI and thyrotropin concentrations were not normally distributed and were log transformed for comparisons. P values < 0.05 were considered significant.

RESULTS

Data from the children in 1999 and 2004 are shown in Table 1. The median UI concentration in 2004 was 23% higher than that in 1999 (P < 0.01). As shown in Figure 1, the proportion of children with a UI concentration >100 µg/L [the cutoff for iodine sufficiency (6)] increased from 60% to 86%, whereas the proportion with a UI concentration >300 µg/L, which indicates iodine excess (6), increased from 2% to 4%. Significant sex or age differences in median UI concentration were not observed in either year among children (data not shown). Data from the pregnant women in 1999 and 2004 are shown in Table 2. The median UI concentration in 2004 was 80% higher than that in 1999 (P < 0.001). The UI concentration in pregnancy that corresponds to the current recommendations for iodine intake in pregnancy (200–220 µg/d) is estimated to be ≈140 µg/d (19).
As shown in Figure 2, the proportion of women with a UI concentration >140 μg/L increased from 48% to 77%, whereas the proportion of those with a UI concentration >500 μg/L increased from 6% to 8%. In 1999, 70% of the pregnant women were taking a prenatal multivitamin-mineral supplement, but in 2004, the pattern was reversed (Table 2). In 1999, women taking an iodine-containing supplement and women not taking an iodide-containing supplement had a higher median UI concentration than did women not taking a supplement, but in 2004, the pattern was reversed (Table 2). In 1999 and 2004, 82–86% of pregnant women reported using iodized salt in the home.

As shown in Table 3, a comparison of the period before the increase in salt iodine (1992–1998) with the period afterward (1999–2004) found that the frequency of newborn thyrotropin concentrations >5 mU/L decreased from 2.9% to 1.7% (P < 0.0001). From 1992 to 2004, no significant difference was observed in the mean thyrotropin concentrations when samples obtained on day 3 (1.60 mU/L) were compared with samples obtained on day 4 (1.59 mU/L) (P = 0.27). However, the frequency of thyrotropin concentrations >5 mU/L was slightly but significantly (P < 0.0001) higher on day 3 (2.4%) than on day 4 (2.2%).

In 1999 and 2004, 13% and 15% of household salt samples, respectively, contained <10 mg I/kg. Among the samples of iodized household salt, the mean salt iodine concentration in 1999 (n = 91) and 2004 (n = 72) was 13.7 ± 1.9 and 18.3 ± 3.0 mg/kg, respectively (P < 0.01).

**DISCUSSION**

In this study, schoolchildren, pregnant women, and newborns were used as target groups for iodine monitoring. Schoolchildren are recommended for monitoring iodine nutrition in a population because of their easy availability as subjects and their vulnerability to the adverse effects of iodine deficiency (6, 7). An indicator of optimal iodine nutrition in a population is a median UI concentration of 100–200 μg/L in school-age children (6). During pregnancy, adequate iodine is essential for optimal neurologic development of the fetus (6), and the recommended monitoring indicator is thyrotropin concentrations in newborns (7). Older recommendations from WHO suggest that a <3% frequency of newborn thyrotropin values >5 mU/L indicates iodine deficiency (6, 7).

**TABLE 3**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Newborn whole blood thyrotropin (mU/L)</td>
<td>1.2 (0.8, 1.9)</td>
<td>1.2 (0.8, 1.8)</td>
</tr>
<tr>
<td>Prevalence of newborn thyrotropin &gt;5 mU/L (%)</td>
<td>2.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

1 Median; interquartile range in parentheses (all such values).
2 Significantly different from 1999, P < 0.0001 (chi-square test).
sufficiency (7). Although no criteria are established for median UI concentration during pregnancy, the UI concentration in pregnancy that corresponds to current recommendations for iodine intake in pregnancy has been estimated to be ≈140 μg/d (19). In 1999, on the basis of these 3 indicators, iodine intake in Switzerland was marginal.

The 25% increase in the content of Swiss iodized salt markedly improved iodine intakes in schoolchildren and pregnant women. The median UI concentration has increased 23% in schoolchildren and 80% in pregnant women. The reason for the larger increase in the median UI concentration in pregnancy is unclear. It was not due to an increase in iodine-containing supplements taken during pregnancy (19): the proportion of women taking a supplement containing 150 μg iodine was 13% in 1999 and 9% in 2004. Moreover, in 2004, women receiving an iodine-containing supplement had a significantly lower median UI concentration than did women not supplementing with iodine. However, the number of women taking an iodine-containing supplement was small (n = 25), which made comparisons difficult. The food additive erythrosine is rich in iodine and is commonly used as a coloring agent in pharmaceuticals (20). However, it is not used in the prenatal supplements taken by Swiss women. Because the 2 studies (1999 and 2004) were conducted at the same time of year, seasonal variation is an unlikely confounder. Because urine samples were collected in the summer months, UI was at a low concentration in its seasonal variation; iodine intake tends to be higher during winter months in Switzerland because of higher concentrations of iodine in cow milk from feed additives used during the winter (21).

Thyrotropin screening in newborns has been used to assess the severity of iodine deficiency in populations (22–27). In iodine-sufficient populations in Australia and Canada, the prevalence of elevated thyrotropin concentrations (>5 mU/L, with the use of a sensitive monoclonal antibody assay) in blood filter paper specimens collected ≥3 days after birth was between 3% and 5% (23). However, multiple factors other than maternal iodine status can influence measurements of thyrotropin concentrations in newborns, including timing of specimen collection, maternal or newborn exposure to iodine-containing antiseptics, and the thyrotropin assay and collection paper used (26). Because of these uncertainties, the cutoffs for defining severity of iodine deficiency on the basis of newborn thyrotropin concentrations originally proposed by the WHO (7) were not included in the most recent recommendations (6). Our data, obtained with the use of a sensitive thyrotropin assay on samples collected 3–4 d after birth, generally support the original WHO recommendation that a <3% frequency of thyrotropin values >5 mU/L indicates iodine deficiency in a population (7).

The findings of this study indicate that iodine nutrition in children and pregnant women in Switzerland has improved from marginal to clearly sufficient. This improvement illustrates the value of periodic monitoring and adjustment of iodine concentrations in national iodized salt programs. The 1999 and 2004 national surveys are the first of a series that will monitor iodine status in the Swiss population every 5 y. Swiss legislation is flexible in that it specifies a range of 20–30 mg/kg for salt iodization. On the basis of new scientific evidence, a federal decree can change the concentration without the need for a lengthy parliamentary process (28). This approach could serve as a model for other countries that are struggling to maintain adequate iodine intake in the face of shifting dietary habits and changes in the food supply.

We thank the children and women who participated in the study, the medical staff of the obstetric centers, and the teachers. We thank S. Schach et al. (Pratteln, Switzerland), N. Hurrell, and S. Geisselhardt (Zürich, Switzerland) for help with the data analyses and I. Molinari (Zürich, Switzerland) for help with the statistical analyses.

Each of the authors contributed to the study design. MBZ and IA collected the data; MBZ and TT supervised the laboratory analyses and performed the statistical analysis; and all authors contributed to the writing and editing of the paper. None of the authors had a personal or financial conflict of interest.

REFERENCES


Association of maternal smoking with overweight at age 3 y in American Indian children1–3

Alexandra K Adams, Heather E Harvey, and Ronald J Prince

ABSTRACT

Background: Prevalence rates of overweight are higher among American Indian children than among any other ethnic group, but little research has explored contributing influences.

Objective: The objective was to determine the prevalence and predictors of body mass index (BMI; in kg/m²) ≥ 85th percentile in American Indian children in Wisconsin.

Design: A retrospective analysis was conducted with linked pediatric and pregnancy nutrition surveillance systems and birth records from 1997 through 2001. Participants were American Indian mothers and children (aged 0–3 y) who were participating in the Special Supplemental Nutrition Program for Women, Infants, and Children (WIC) operated by the Food and Nutrition Service Area comprising the states of Minnesota, Wisconsin, and Michigan (5). However, little research has been conducted on the prevalence or predictors of obesity among American Indians in this area. Because of the inherent difficulty of treating overweight and obesity and because of the link of overweight and obesity to adult disease, it is imperative that preventive measures are employed (6, 7).

Previous research on contributors to childhood obesity focused primarily on older children and white children and identified genetic, neonatal, environmental, and lifestyle factors related to overweight. These included sex (8–11), race (11), maternal BMI (8–10, 12), paternal BMI (8, 12), gestational diabetes (13, 14), smoking during pregnancy (12, 15, 16), birth weight (8, 10, 12, 17), breastfeeding (16–19), television watching in h/d (11, 12), sleep in h/d (8, 12), rate of weight gain during the first 6 mo of life (10), and family socioeconomic status (11, 16).

To examine some of these factors in a younger and underrepresented American Indian population, this study used linked data from 5 y of Wisconsin Pediatric Nutrition Surveillance System (PedNSS), Pregnancy Nutrition Surveillance System (PNSS), and birth records to identify predictors of overweight in American Indian children at age 3 y. Maternal and child predictors included were birth weight, breastfeeding, maternal prepregnancy body mass index (BMI; in kg/m²), family income, maternal weight change during pregnancy, smoking, and education. This information will help in the design and evaluation of community-based obesity prevention programs in American Indian tribes in Wisconsin.

SUBJECTS AND METHODS

Subjects

The Special Supplemental Nutrition Program for Women, Infants, and Children (WIC) operated by the Food and Nutrition Service Area comprising the states of Minnesota, Wisconsin, and Michigan (5). However, little research has been conducted on the prevalence or predictors of obesity among American Indians in this area. Because of the inherent difficulty of treating overweight and obesity and because of the link of overweight and obesity to adult disease, it is imperative that preventive measures are employed (6, 7).

Previous research on contributors to childhood obesity focused primarily on older children and white children and identified genetic, neonatal, environmental, and lifestyle factors related to overweight. These included sex (8–11), race (11), maternal BMI (8–10, 12), paternal BMI (8, 12), gestational diabetes (13, 14), smoking during pregnancy (12, 15, 16), birth weight (8, 10, 12, 17), breastfeeding (16–19), television watching in h/d (11, 12), sleep in h/d (8, 12), rate of weight gain during the first 6 mo of life (10), and family socioeconomic status (11, 16).

To examine some of these factors in a younger and underrepresented American Indian population, this study used linked data from 5 y of Wisconsin Pediatric Nutrition Surveillance System (PedNSS), Pregnancy Nutrition Surveillance System (PNSS), and birth records to identify predictors of overweight in American Indian children at age 3 y. Maternal and child predictors included were birth weight, breastfeeding, maternal prepregnancy body mass index (BMI; in kg/m²), family income, maternal weight change during pregnancy, smoking, and education. This information will help in the design and evaluation of community-based obesity prevention programs in American Indian tribes in Wisconsin.
Service of the US Department of Agriculture collects information on maternal prenatal and postnatal characteristics and demographics and also performs child growth and nutrition measurements from birth to age 5 y. These data, along with Head Start and maternal and child health data, are reported to the Centers for Disease Control and Prevention (CDC) by the states and stored as 2 data sets, the PNSS and the PedNSS. In Wisconsin, only WIC data are reported to the CDC. These data sets offer an opportunity to look at familial and environmental determinants of overweight in children from lower socioeconomic environments. WIC serves 48% of American Indian infants and children and 65% of American Indian women (20).

The PedNSS and PNSS data sets were obtained from the CDC for all Wisconsin records for the years 1997 through 2001. In Wisconsin, PedNSS data are collected by local WIC clinics, amalgamated, and submitted monthly to the CDC. Information on child growth, nutrition, and general health is included. The PNSS data set contains information on maternal factors related to gestational and postnatal health. Permission for the use of Wisconsin PedNSS and PNSS data was obtained from the Bureau of Family and Community Health, Division of Public Health, Wisconsin Department of Health and Family Services. Birth records for all American Indian births from 1997 through 2001 were obtained from the Bureau of Health Information, Division of Health Care Financing, Wisconsin Department of Health and Family Services. These records included demographic and birth data for both the mother and the child. For the purposes of this study, mothers were identified as American Indian if they self-selected “American Indian” on any of the PedNSS, PNSS, or birth records. In addition, children were identified as American Indian if one or both parents self-selected the child as “American Indian” on the birth record.

Procedures followed were in accordance with the ethical standards of the institutional committee on human experimentation. Approval was obtained from the state WIC office and the University of Wisconsin Institutional Review Board.

An employee of the Wisconsin Department of Health and Family Services matched mothers’ PNSS and children’s PedNSS records to birth records. We obtained 1649 PNSS maternal records and 21 525 American Indian PedNSS records (representing unique child visits; there were multiple visits per child) for children between the ages of 0 and 60 mo. A total of 6769 records were noted (2797) information was missing were excluded. PedNSS assessments occurring at 36 mo and weight-for-length (WFL) (kg/cm) z scores at birth and 36 mo. Persons at risk of overweight were defined as those with BMI ≥ 95th percentile for age- and sex-specific CDC standards (21). Because CDC BMI values for children begin at age 2 y, WFL z scores were used as an outcome measure to allow for comparisons between size at birth and that at 36 mo and for the measure of the change between these 2 time points. WFL z scores were computed by using variables from the CDC that were based on national standards (22). According to WIC protocols, infants and children were weighed to the nearest half-ounce (14 g) and the nearest quarter-pound (110 g), respectively, while wearing underclothes or light clothing at routine visits. At the same time, height was measured to the nearest 1/8 inch (0.31 cm) while the subject was not wearing shoes (23). Large-for-gestational age (LGA) status was defined as >4000 g and small-for-gestational age status was defined as <2500 g at birth.

Maternal predictor variables analyzed (n = 252, unless otherwise noted) were age (in years), prepregnancy BMI (n = 226), weight change (kg gained or lost) during pregnancy (n = 239), smoking before pregnancy (no. of cigarettes/d), smoking at initial WIC visit (no. of cigarettes/d), smoking at first postpartum visit (no. of cigarettes/d), education (no. of years), and income (n = 242). At the initial WIC visit, maternal height was measured to the nearest 1/8 inch (0.31 cm) while the subject was not wearing shoes, and pregravid weight was recorded to the nearest pound (450 g) via self-report or referral data. Smoking before pregnancy was ascertained retrospectively at the initial WIC visit and was classified as any smoking during the 3 mo before the pregnancy (23). All smoking variables were reduced to a bivariate measure of whether (yes or no) the mother ever smoked at each of the 3 time points (ie, smoking before pregnancy, assessed retrospectively at the first WIC visit; smoking at the initial WIC visit, which was used as “smoking during pregnancy”; and smoking at first postpartum visit). The initial WIC visit was defined as the first visit to WIC for a single pregnancy. Most initial WIC visits occur during the first or second trimester (24).

Child predictor variables (n = 252, unless otherwise noted) were birth weight (g), birth length (cm), sex, clinical gestational age (no. of weeks), and breastfeeding (no. of days/wk) (n = 192).

<table>
<thead>
<tr>
<th>Table</th>
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<tbody>
<tr>
<td><strong>WI Al Birth Records 1997 through 2001 (n=6769)</strong></td>
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<tr>
<td><strong>AI status based on Birth or WIC.</strong></td>
</tr>
<tr>
<td>3439 Al mother-child pairs</td>
</tr>
<tr>
<td>3015 Al pairs with gestation ≥36 wk</td>
</tr>
<tr>
<td>494 Al pairs with 36-mo data</td>
</tr>
<tr>
<td>252 Al pairs with complete data on all measures</td>
</tr>
<tr>
<td><strong>WI Al PNSS Records 1997 through 2001 (n=3011). n=1649 have WIC Child ID.</strong></td>
</tr>
<tr>
<td><strong>WI Al PedNSS Records 1997 through 2001 (n=21525). Multiple visits per child.</strong></td>
</tr>
</tbody>
</table>

**FIGURE 1.** Construction of final sample (n = 252) from Pregnancy Nutrition Surveillance System (PNSS), Pediatric Nutrition Surveillance System (PedNSS), and Wisconsin birth records. WI, Wisconsin; AI, American Indian; ID, identification.
Breastfeeding was reduced to a bivariate measure of ever breastfed (yes or no) if the child was breastfed for $\geq 1$ d. Other variables were the changes in weight and height from birth to 36 mo, expressed as percentages of increase.

### Statistical analysis

The relations among maternal and child predictor variables and risk of overweight and overweight at 36 mo were examined by using zero-order Pearson’s correlations. Predictor variables with significant correlations at $P < 0.05$ were combined in a binary logistic regression model of risk of overweight and overweight at 36 mo. Results are reported as odds ratios and 95% CIs. Change in WFL $z$ scores (birth to 36 mo) comparing children of smokers and nonsmokers was examined by using a two-way repeated-measures analysis of variance (ANOVA) with time (birth or 36 mo) as a within-group factor and smoking or nonsmoking as a between-group factor. Differences in the percentage change in height and weight were tested with univariate ANOVAs. Analyses were performed with SPSS software (version 12; SPSS Inc, Chicago, IL).

### RESULTS

Seventy-three percent of mothers who were matched to children (i.e., enrolled in WIC) were single, and 40.6% smoked during pregnancy. The average length of education was 12.1 y. In a comparison of matched WIC mothers with non-WIC mothers who had birth records, chi-square analysis found that the WIC mothers were more likely than were the non-WIC mothers to be single (73.0% and 41.0%, respectively; $P < 0.001$), to have smoked during their pregnancy (40.6% and 29.9%, respectively; $P < 0.001$), and to have less education (12.1 and 12.6 y, respectively; $P < 0.001$). A comparison of the characteristics of our final sample, i.e., mothers who were not enrolled in WIC, and those of the larger sample of matched WIC American Indian mother-child pairs, is shown in Table 1. There were no significant mean differences in predictor variables between our final sample of 252 mother-child pairs and other American Indian mother-child pairs enrolled in WIC ($n = 3015$).

Of the children from the 252 mother-child pairs analyzed, 22.2% of 3 y olds were overweight, and an additional 18.7% were at risk of overweight. Most children had a normal birth weight, but 18.7% of children were LGA. Of the mothers, 54.0% ever breastfed, and 42.5% smoked during pregnancy. Most (57.9%) mothers were either overweight or obese before pregnancy (Table 1).

Child and mother characteristics that showed significant intercorrelations were entered simultaneously into a binary logistic regression model predicting BMI $\geq 85$th percentile at 36 mo. Only smoking at initial WIC visit (odds ratio (OR): 2.16; 95% CI: 1.05, 4.47) was a significant predictor of children at risk of overweight or overweight at age 3, although birthweight (OR: 1.82; 95% CI: 0.09, 3.71) and ever breastfed (OR: 0.53; 95% CI: 0.26, 1.06) tended toward significance (Table 2).

Children were divided into birth weight sextiles to examine the relative size of effects across birth weights. Children with higher birth weights had higher WFL $z$ scores at 36 mo, and this was seen across all birth weight groups when 2-way repeated measures ANOVA was performed ($P < 0.01$). For all birth weight groups except small-for-gestational-age children, change in WFL $z$ scores at 36 mo was positive. LGA children had WFL $z$ scores $\geq$
Overall, the mean increase in WFL $z$ score increase from birth to 36 mo was significantly ($P = 0.009$) more pronounced in children of mothers who smoked (1.33) than in children of mothers who did not smoke (0.88). These children of smokers were significantly smaller at birth, but, at 36 mo, they were significantly larger than were the children of nonsmoking mothers, independent of birth weight, as indicated by the significant difference in the increase in $z$ score between the 2 groups of children ($P = 0.009$; Figure 2). This relation was also found at multiple time points between birth and 36 mo when a subset of children with $\geq 6$ measurements was analyzed ($n = 183; P = 0.035$).

Changes in WFL can be based on either a relatively greater increase in weight or a relatively slower increase in length. Birth weights were significantly higher in the nonsmoking group (3460 and 3622 g, respectively; $P < 0.05$), but birth lengths did not differ significantly between the 2 groups of children (50.4 and 50.8 cm, respectively), which indicated that the lower WFL $z$ scores at birth in children of smokers were due to lower relative weight and not to greater relative length. When changes in WFL $z$ scores were considered relative to changes in weight and height by using univariate ANOVAs, only the mean percentage change in weight differed significantly between the children of smokers and those of nonsmokers (Figure 3 and Figure 4), which indicated that the larger increase in the WFL $z$ score of children of smoking mothers was due to relatively greater increases in weight and not to slower increases in height. In addition, birth weight was negatively correlated with mean percentage change in both weight and height (Figures 3 and 4).

We also examined the association of postnatal smoking (women who began smoking after delivery) on child weight at age 36 mo. Prenatal and postnatal smoking correlated highly ($r = 0.80$). However, when we compared child growth between children of postnatal smokers and children of nonsmokers, no significant differences were found.

### DISCUSSION

This retrospective analysis of linked PedNSS, PNSS, and birth record data for Wisconsin American Indians documented high rates of overweight risk status and overweight at age 3 y. Maternal smoking was a significant predictor of overweight risk status.
and overweight. Children of mothers who smoked during pregnancy showed significantly greater rates of weight gain than did children of nonsmokers, which resulted in significantly greater increases in WFL z score between birth and age 3 y.

In our study population, 22.2% of children were at risk of overweight and 18.7% were overweight. These rates are higher than those of overweight reported nationally for 3-y-old American Indian WIC participants—14.4% (20). Moreover, 18.7% of the children in the sample in the current study were LGA, whereas national American Indian and Wisconsin all-race proportions are 11.3% and 8.7%, respectively (1). This high rate of LGA is especially troubling, given the correlation between birth weight and later BMI seen in this study and in others. Rates of breastfeeding were comparable to reported all-race national and state rates of 52.5% and 55.0%, respectively, but were slightly below national rates of 59% for American Indians participating in WIC (25).

In our population, children of mothers who smoked at the initial WIC visit were almost twice as likely as children of nonsmokers to have a BMI ≥ 85th percentile at age 36 mo. The increased overweight risk and incidence of overweight among children of smokers seen in our study was similar to, if not slightly higher than, that seen in other populations (15, 26-28). However, because of the nature of our data, we were not able to establish a dose-dependent relation for smoking as seen in studies by Power and Jefferis (26) and von Kries et al (15).

The prevalence of maternal smoking during pregnancy seen in the current study is higher than that reported in other studies (15, 26, 27, 29, 30). However, our data corresponded to those from a recent report indicating that 40% of Wisconsin American Indian mothers smoked during pregnancy (28). Mothers enrolled in WIC were significantly more likely to have smoked during pregnancy than were mothers not enrolled in WIC. This agrees with national trends for WIC or lower-income mothers (24, 25).

In the current study, we used smoking status at initial WIC visit to establish whether the mother smoked during pregnancy. Almost half (48.4%) of the mothers who visit WIC do so within the first trimester of their pregnancy, and 39.8% visit WIC during the second trimester (24). By using smoking at initial WIC visit, we captured both mothers who smoked throughout the pregnancy and mothers who smoked during the first part of their pregnancy and quit thereafter. Toschke et al (31) showed an equal effect of smoking in the first trimester only and of smoking throughout pregnancy on overweight at age 5–6 y.

Children of mothers who smoked during pregnancy were, on average, 160 g smaller at birth than were children of nonsmoking mothers, which is consistent with findings of other studies (26). In our sample, however, the children of mothers who smoked were not shorter at birth than were the children of nonsmokers, and this finding is at odds with the literature. Furthermore, the children of mothers who smoked during pregnancy did not show any significant differences from the children of nonsmoking others in height at 36 mo, whereas other studies found that the children of smokers were shorter than the children of nonsmokers at ages 2 and 7 y (31, 32). Nevertheless, these results agree with other studies that showed no significant difference in height at age 3 y after adjustment for maternal, environmental, and birth characteristics (33–35). The lower birth weights of infants of mothers who smoked is important because studies have shown both a greater risk of morbidity in obese persons who were small at birth than in those with a normal birth weight (36) and a greater number of risk factors for adult disease in children who displayed “catch-up” growth between ages 1 and 2 y (34). Paradoxically, other studies show that lower birth weights are correlated with lower BMIs, which suggests that the decreased birth weight of children of smokers may attenuate the magnitude of their later overweight (37).

Our results remained robust after we considered several additional variables, including size at birth and the mother’s prepregnancy weight. The significance of a relation between maternal smoking and child overweight at age 3 y, independent of these factors, suggests mechanisms separate from growth restriction through which smoking affects early childhood growth. Mechanisms relating to alterations in the fetal environment that affect endocrine balance or metabolic functions or the mechanisms of the direct effect of nicotine on brain development have been put forth by others (15, 31, 38).

The correlation of smoking with weight gain from birth to 36 mo, independent of birth weight, could also be explained by lifestyle factors that correlate with smoking—eg, poor nutritional choices and reduced physical activity—and promote weight gain. However, we did not see an association between maternal postnatal smoking and child overweight at age 3 y. Thus, the association between maternal smoking and later growth may be due to the in utero effect of smoking and not to other variables that may be associated with smoking. Toschke et al (31) postulated that smoking in early pregnancy has a direct metabolic effect on the offspring, whereas later smoking may be a marker for other lifestyle factors.

Similar to studies in other populations (8, 10, 39), the current study did not find a significant effect of breastfeeding on overweight at age 3 y. A reason for this may be that the percentage of mothers who exclusively breastfed was not comparable between our study and other studies that showed an association (17, 18). Alternatively, the effects of breastfeeding may not become apparent until later childhood. For example, Bergmann et al (16) showed a protective effect of breastfeeding on overweight at age 6 y, but not at age 3 y.

Limitations of the current study included the large reduction in number of subjects because of nonmatching and missing data. The greatest loss of mother-child pairs was due to the requirement of WIC visits until age 36 mo. It is possible that mothers who remained in WIC long enough to be included differed in some way (eg, nutritional status) that would affect child growth, but none of the relevant measures we examined reflected such differences. An additional obstacle was the standards for collecting PedNSS and PNSS data, which are geared toward easy reporting by WIC personnel rather than toward scientific analysis. Also lacking were data on paternal smoking, which was correlated to child overweight in another study (40). Finally, key differences were noted between WIC and non-WIC participants. However, when differences were considered in more detail, income, education, maternal weight gain, and age did not correlate with the change in WFL z score. This suggests that the relation between smoking and change in WFL z score is independent of these factors and may hold true in a non-WIC population.

To our knowledge, this is the first study to show a relation between smoking in pregnancy and later overweight in American Indian children. Given the limitations and potential biases inherent in retrospective analysis, prospective cohort studies would be an ideal next step in evaluating the suggested relation between
smoking and overweight. Our results have important implications for health care and point to the need for targeted interventions to reduce smoking in pregnant women and women of childbearing age. A similar message should be communicated at the initial WIC visit and at subsequent WIC visits throughout a pregnancy.

We thank Richard Miller of the Wisconsin Bureau of Health Information for his invaluable support in linking the data. We also thank Connie Welch and Pattie Herrick of the Wisconsin WIC Program for their assistance in obtaining these data. We thank Judith S. Kaur, of the “Spirit of E.A.G.L.E.S.” program at the Mayo Clinic, Rochester, MN, for assistance in obtaining project funding. Finally, we thank David Brown for his comments and suggestions throughout this process. None of the authors had personal or financial conflicts of interest.

AKA obtained study funding, established the study concept and design, acquired data, supervised the execution of the study, reviewed and revised the manuscript, and provided critical intellectual content. HEW contributed to the study concept and design, provided administrative support throughout the study, and wrote the manuscript draft. RJP provided statistical expertise and analyzed and interpreted data, revised the manuscript, and provided critical intellectual content.

REFERENCES


Zinc supplementation and psychosocial stimulation: effects on the development of undernourished Jamaican children\textsuperscript{1–3}


ABSTRACT

Background: Undernourished children have poor levels of development that benefit from stimulation. Zinc deficiency is prevalent in undernourished children and may contribute to their poor development.

Objective: We assessed the effects of zinc supplementation and psychosocial stimulation given together or separately on the psychomotor development of undernourished children.

Design: This was a randomized controlled trial with 4 groups: stimulation alone, zinc supplementation alone, both interventions, and control (routine care only). Subjects were 114 children aged 9–30 mo and below \(-1.5\) $z$ scores of the National Center for Health Statistics weight-for-age references who were recruited from 18 health clinics. Clinics were randomly assigned to receive stimulation or not; individual children were randomly assigned to receive zinc or placebo. The stimulation program comprised weekly home visits during which play was demonstrated and maternal-child interactions were encouraged. The supplementation was 10 mg Zn as sulfate daily or placebo. Development (assessed by use of the Griffiths Mental Development Scales), length, and weight were measured at baseline and 6 mo later. Weekly morbidity histories were taken.

Results: Significant interactions were found between zinc supplementation and stimulation. Zinc benefited the developmental quotient only in children who received stimulation, and benefits from zinc to hand and eye coordination were greater in stimulated children. Zinc supplementation alone improved hand and eye coordination, and stimulation alone benefited the developmental quotient, hearing and speech, and performance. Zinc supplementation also reduced diarrheal morbidity but did not significantly improve growth.

Conclusion: Zinc supplementation benefits development in undernourished children, and the benefits are enhanced if stimulation is also provided. \textit{Am J Clin Nutr} 2005;82:399–405.

KEY WORDS Zinc, deficiency, supplementation, child development, psychosocial stimulation, play programs, infants, young children, undernutrition, malnutrition, Jamaica

INTRODUCTION

Childhood undernutrition affects \(>180\) million children under the age of 5 y (1). Undernourished children are at high risk not only of increased mortality and morbidity (2) but also of poor cognitive development and subsequent school failure (3). The mechanism linking undernutrition to poor development is not well established. One hypothesis (functional isolation) is that undernourished children’s low levels of activity and exploration restrict their ability to acquire skills (4). This altered behavior induces less stimulating behavior from their caretakers toward them, thus further reducing the amount of interaction the child has with his or her environment (4). Zinc deficiency is thought to be highly prevalent in undernourished children and has also been linked to reduced activity and play (5, 6) and may exacerbate undernourished children’s poor development. Zinc deficiency is associated with growth retardation (7) and increased rates of diarrhea and pneumonia in children (8); however, evidence of links with psychomotor development in young children is inconsistent (9).

At least 7 trials (10–16) have examined the effects of zinc supplementation on young children’s psychomotor development. In one (10), zinc and copper were given to very-low-birth-weight children and their motor development benefited. Four of the trials showed no benefit to developmental levels (11–14), but 2 of these found improvement in behavior (11) or motor quality (12), whereas in one (14), children with the lowest birth weights were more irritable when given zinc. One Bangladeshi study found no benefit to mental or motor development from iron or zinc given alone but improved motor development when iron and zinc were combined (15). In contrast, in a further study (16), Bangladeshi infants supplemented with zinc had slightly lower developmental scores, possibly because of micronutrient imbalance. Zinc supplementation in older children has also had inconsistent benefits on cognition (17–20).

It is clear that the role of zinc deficiency in children’s development needs to be clarified. Zinc status is difficult to measure, 

\begin{itemize}
\item \textsuperscript{1} From the Epidemiology Research Unit, Tropical Medicine Research Institute, University of the West Indies, Mona, Kingston, Jamaica (JMMG, CAP, and SPW), and the Centre for International Child Health (HBH and SMG-M) and Paediatric Epidemiology and Biostatistics (TJC), Institute of Child Health, University College London, London, United Kingdom.
\item \textsuperscript{2} The stimulation trial was funded by The Thrasher Research Fund; the zinc supplementation trial was funded by the Nestle Foundation. The Grace, Kennedy Foundation (Jamaica); Dr Jeffrey Meeks; and the Matalon and Melhado families provided further financial assistance. The zinc supplement and the vitamin preparation were donated by Federated Pharmaceuticals Limited.
\item \textsuperscript{3} Address reprint requests to JM Meeks Gardner, Caribbean Child Development Centre, School of Continuing Studies, University of the West Indies, Mona, Kingston 7, Jamaica. E-mail: julie.meeksgardner@uwimona.edu.jm. Received June 18, 2004. Accepted for publication April 11, 2005.
\end{itemize}
and response to supplementation is often used as an indicator of deficiency (9). Possible explanations for the lack of response in development in some studies are that the children were not zinc deficient initially or that additional zinc produced imbalances in other micronutrients, such as iron. A further possibility is that even if children become more exploring and active with zinc supplements, this may not lead to better developmental levels if their environment is unstimulating. Additional stimulation has been shown to improve the development of stunted children who received food supplementation (21). Thus, zinc-deficient children living in unstimulating environments may need improved stimulation as well as zinc to improve in development.

To test the latter hypothesis, we conducted a randomized double-blind trial to determine the effect of zinc supplementation with or without psychosocial stimulation on young children’s psychomotor development. We also examined the effect of zinc on growth and morbidity, because an improvement with supplementation would confirm that the children were initially zinc deficient.

SUBJECTS AND METHODS

Sample

The project was conducted in the parishes of Kingston, St Andrew, and St Catherine, Jamaica. Children attending government health centers who are underweight are referred to special nutrition clinics at the centers, where their growth is monitored and their caregivers are counseled about child feeding. All children aged 9–30 mo who were attending 18 nutrition clinics were identified. Those with current weight-for-age $z$ scores below $-1.5$ SDs of the National Center for Health Statistics references (22) and who had weight-for-age below $-2$ SDs in the previous 3 mo were enrolled into the study. Twins or children with physical or mental impairments that could affect development were excluded.

It was not considered feasible to have both the stimulated and the nonstimulated groups of children at the same clinic; therefore, the 18 clinics were randomly assigned to stimulation or control. Within each clinic, the children were stratified into 2 age groups (9–18 and 19–30 mo) and were then randomly assigned to receive the zinc supplement or the placebo. To detect a difference of 0.5 SD in developmental levels at $P < 0.05$ with 80% power, 64 children were required in each arm of the study (supplemented and placebo). Ninety-nine children were identified and enrolled over a period of 6 mo. For logistic reasons, we could not extend the stimulation program. To achieve sufficient power to detect an effect of zinc, we continued enrolling children for a further 2 mo to the zinc trial only. A further 27 children were enrolled from all the clinics in this time period. As shown in Figure 1, the 4 groups were as follows: stimulation only group ($n = 23$), zinc only group ($n = 35$), combined treatment group ($n = 26$), and a control group ($n = 42$). Therefore, 61 children received zinc and 65 received placebo. Informed consent was obtained from parents or guardians, who were unaware of the children’s assignment to zinc or placebo. Ethical approval was obtained from the Ethics Committee of the University of the West Indies and the Jamaican Ministry of Health.

Interventions

Zinc and placebo supplementation

Zinc supplementation consisted of 10 mg elemental zinc (the recommended dietary allowance for children aged >12 mo) as sulfate in a flavored syrup or a placebo (syrup only) given daily for 6 mo. The supplement or placebo was delivered by a community health worker to the homes weekly in 7 vials, each containing one dose. At the end of each week, the community health worker returned with another set of supplement or placebo vials and collected the vials from the previous week. A record was kept of the number of empty vials. All children received a proprietary brand of micronutrients containing iron and vitamins, and caregivers were instructed to give them 0.5 mL daily (Table 1).

Psychosocial stimulation

The main focus of the psychosocial stimulation program was to improve maternal-child interactions. The children and their mothers were visited weekly for half an hour by a specially trained community health worker who showed the mother ways to play with her child. We used a detailed curriculum based on Piagetian concepts for children aged <24 mo and concepts of shape, size, position, and color for children aged >24 mo. In addition, activities to facilitate language development, fine motor skills, and problem solving were included. The curriculum was culturally appropriate and was used successfully with Jamaican mothers and children in previous studies (23). The community health worker also left simple homemade toys with the

<table>
<thead>
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<tr>
<td>Vitamin A, retinyl palmitate (IU)</td>
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1 Per 0.5 mL.
mother each week, which were exchanged at the next visit for other toys in an attempt to keep the intervention low cost.

**Measurements**

Developmental levels and anthropometric variables were measured at enrollment and after the 6-mo trial period.

**Developmental levels**

These were measured by using 4 subscales of the Griffiths Mental Development Scales (24): locomotor (large muscle activities such as walking or jumping), hand and eye coordination, hearing and speech, and performance (shape recognition, block construction, and block patterns). The Griffiths scales have been used in several studies in Jamaica and have been shown to have good test-retest reliability and to predict long-term development (25). All tests were carried out by a single tester, who was unaware of the children’s group assignment. Reliability between the tester and the trainer was high before the study started (intraclass correlation: \( r = 0.98; n = 10 \) children) and in ongoing quality-control assessments (\( r = 0.99; n = 16 \)).

**Anthropometry**

Weights and lengths or heights were measured by using standard techniques (26) by 2 research assistants. Their interobserver reliabilities with the trainer were 0.98 for all measurements (intraclass correlations; \( n = 10 \)). Many of the children (\( n = 79 \)) had both length and stature measured on one occasion. The linear regression of these lengths on stature was used to convert stature to estimated length wherever stature only had been measured [estimated length (cm) = 6.505 + 0.936 stature]. The length-stature regression indicated a close correspondence between the 2 measurements (\( r = 0.98 \)). All analyses were then carried out with the measured or estimated length.

**Social background**

The children’s caregivers were interviewed at enrollment to assess their home backgrounds. The level of stimulation in the home was assessed by questionnaire and observations by using the Bettye Caldwell HOME inventory (27), which was previously modified for Jamaica. Examples of the modifications made include removing questions that were considered inappropriate or ambiguous for the culture, rescaling questions to obtain variation, and changing the scoring to include the frequency of some of the activities the mother did with her child. The final instrument comprised 46 items. Two interviewers carried out these measurements. Interobserver reliabilities were \( >0.9 \) before the start of the study and continued at this level in 10% of all interviews throughout the study. The caregiver’s height and verbal IQ on the Peabody Picture Vocabulary Test (28) were also measured.

**Morbidity**

Throughout the 6 mo of the zinc supplementation trial, weekly visits were made to the children’s homes by the community health worker to take the children’s morbidity history for the previous 7 d from the parents. Clinic visits for illness (not routine visits) and hospitalizations were also recorded. Any symptoms that occurred during the previous 7 d, and the day they were present, were recorded. Ten symptoms were specifically asked about: apathy, anorexia, fever, coughing, nasal discharge, diarrhea (loose or frequent stools identified by the mother as “running belly”), vomiting, rapid or difficult breathing, pain or discharge from the ear, and skin conditions including any rashes or sores. Other symptoms were recorded as miscellaneous and were described. The mother’s judgement was accepted for all symptoms and no attempt was made to reach a diagnosis. Further details of the instrument are reported elsewhere (29). The community health workers were trained in giving the questionnaire and achieved a minimum of 90% concordance with the trainer in 10 consecutive interviews before beginning the study. In addition, each month the supervisor observed 5 interviews to ensure quality control, and a high concordance was maintained.

**Statistics**

We compared the children’s baseline characteristics by using analysis of variance (ANOVA) or chi-square tests. Correlational analyses were conducted to determine which variables were related to the final developmental scores. The effects of the interventions on developmental levels were examined by using multilevel analyses because of the hierarchical structure of the study. The random variables were clinic and child. Analyses were conducted with SPSS for WINDOWS version 11.5 (SPSS Inc, Chicago, IL) and MLwiN version 1.1 (Institute of Education, London, United Kingdom) for multilevel modeling.

We did not hypothesize that the stimulation intervention would affect growth or morbidity, so these variables were compared by zinc supplement status only. The effect of the zinc supplement on the children’s growth was examined by multiple regression of final anthropology status. The independent variables were status at enrollment, age, sex, and supplementation status (zinc supplemented or placebo).

To describe the children’s morbidity, the number of episodes and the total duration of each symptom was recorded. These were corrected for the actual number of days when morbidity was recorded, because some information was unavailable because of the child or mother being away from home. An episode was defined as ended after 3 d without the symptom. The mean duration of each episode was calculated for each symptom. The data could not be normalized and were analyzed by using non-parametric statistics (Mann-Whitney \( U \) tests).

**RESULTS**

Twelve children (9%) did not complete the study. Reasons for withdrawal given by the parents from the zinc-supplemented group were as follows: children became anorexic (\( n = 2 \)), child would vomit after the supplement (\( n = 1 \)), the fathers refused to allow participation after the mother had given consent (\( n = 2 \)), and family moved away (\( n = 1 \)). From the placebo group, parents reported illness (jaundice and liver problems; \( n = 2 \)), families moved away (\( n = 2 \)), the mother was unhappy with the doctors from the research unit (\( n = 1 \)), or the mother felt that giving the supplement daily was too onerous (\( n = 1 \)). The children who withdrew were not significantly different from those who completed the study in any of the enrollment characteristics shown in Table 2 or in baseline developmental quotients, weights, or lengths.

Compliance with the supplementation was indicated by the number of vials returned at the end of the week still containing supplement or placebo. Of the 180 vials that were delivered to
were included as covariates for their fixed effects in all models and the random parameters were clinic and child. Intervention
groups were entered as follows: for zinc supplementation, sup-
plemented = 1, not supplemented = 0; and for stimulation, stimulated = 1, not stimulated = 0. A third term was included (in
the stimulation trial) to control for any effect of being in the
stimulation trial or not (Figure 1). An interaction term (supple-
mentation status × stimulation status) was also offered. The
following possible confounding variables, ie, those that were
significantly different between the groups or were correlated
with the developmental quotient, were also considered: maternal
height, initial length-for-age \( z \) score, HOME score, possessions,
crowding, birth order, and mother’s score on the Peabody Picture
Vocabulary Test. None of these variables was significantly re-
educed to change in developmental quotient.

### Group characteristics at enrollment

The characteristics of the groups at the time of enrollment are
shown in Table 2. Differences among the groups were examined
by 2-factor ANOVA, except for sex, which was compared by
chi-square analysis. Maternal height was significantly different
among the groups, with the mothers in the control and stimulation
groups being taller than those in the both and the zinc only
groups. The children’s anthropometric variables at the time of
enrollment are shown in Table 3. Children who received placebo
were significantly taller than those who received zinc.

The developmental quotient and subscale scores (Table 4)
were not significantly different across the groups at the time of
enrollment. The initial developmental quotient score was
strongly negatively correlated with age (Pearson’s \( r = -0.75,\)
\( P < 0.001 \)) and was significantly related to the Caldwell HOME
score (\( r = 0.26,\) \( P < 0.01 \)) but was not related to other measures
of social background or other maternal characteristics.

### Treatment effects

#### Development

In all groups, there was an overall decline in developmental
quotient and all subscale scores (Table 4). There were significant
differences among the groups in the overall developmental quo-
tient and in the hearing and speech subscale scores (Table 4). The
intervention effects on development were evaluated by use of a
multilevel modeling procedure. Separate analyses were carried
out with the final developmental quotient or each subscale score
as the dependent variable. Initial score and children’s initial age
were included as covariates for their fixed effects in all models

---

**Table 2**

Characteristics of the children at enrollment by group

<table>
<thead>
<tr>
<th></th>
<th>Stimulation and zinc group</th>
<th>Stimulation only group</th>
<th>Zinc only group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n = 11\ M, 14\ F))</td>
<td>((n = 11\ M, 10\ F))</td>
<td>((n = 13\ M, 17\ F))</td>
<td>((n = 9\ M, 29\ F))</td>
</tr>
<tr>
<td>Age (mo)</td>
<td>19.2 ± 5.0</td>
<td>18.8 ± 5.1</td>
<td>18.7 ± 5.8</td>
<td>18.6 ± 6.6</td>
</tr>
<tr>
<td>Birth order</td>
<td>2.6 ± 1.7</td>
<td>2.1 ± 1.5</td>
<td>2.3 ± 1.7</td>
<td>2.1 ± 1.3</td>
</tr>
<tr>
<td>Crowding(^2)</td>
<td>3.1 ± 1.7</td>
<td>2.4 ± 1.3</td>
<td>3.3 ± 1.8</td>
<td>3.2 ± 2.0</td>
</tr>
<tr>
<td>Sanitation(^3)</td>
<td>7.3 ± 3.2</td>
<td>8.1 ± 3.4</td>
<td>8.6 ± 2.9</td>
<td>7.3 ± 3.2</td>
</tr>
<tr>
<td>Possessions(^4)</td>
<td>4.9 ± 2.4</td>
<td>5.1 ± 2.1</td>
<td>5.2 ± 2.0</td>
<td>5.6 ± 2.1</td>
</tr>
<tr>
<td>Mother’s age (y)</td>
<td>26.4 ± 7.8</td>
<td>26.1 ± 7.4</td>
<td>25.5 ± 8.2</td>
<td>26.0 ± 6.3</td>
</tr>
<tr>
<td>Maternal height (cm)</td>
<td>157.4 ± 6.0(^a)</td>
<td>159.9 ± 5.4(^b)</td>
<td>157.2 ± 4.6(^c)</td>
<td>160.8 ± 4.9(^d)</td>
</tr>
<tr>
<td>Maternal PPVT(^5)</td>
<td>89.4 ± 24.4</td>
<td>98.0 ± 24.1</td>
<td>89.9 ± 23.3</td>
<td>93.9 ± 20.2</td>
</tr>
<tr>
<td>HOME score(^6)</td>
<td>45.0 ± 11.0</td>
<td>46.0 ± 13.1</td>
<td>40.9 ± 10.8</td>
<td>45.8 ± 10.3</td>
</tr>
</tbody>
</table>

\(^1\) All values are \( \bar{x} \pm SD \). There were no significant differences in the numbers of males and females in each group by chi-square test. Means in a row with
different superscript letters are significantly different, \( P < 0.05 \) (2-factor ANOVA and Tukey’s test).

\(^2\) Number of persons per habitable room.

\(^3\) Comprises a rating of water source and toilet facilities (range: 0–12).

\(^4\) Number of household possessions (range: 0–10).

\(^5\) Peabody Picture Vocabulary Test, raw score (28).

\(^6\) Based on the Bettye Caldwell Stimulation in the Home scale (27). Higher scores represent more stimulation.

---

**Table 3**

Anthropometric indexes of the children at enrollment and 6 mo later

<table>
<thead>
<tr>
<th></th>
<th>Zinc-supplemented group ((n = 55))</th>
<th>Placebo group ((n = 59))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>8.65 ± 0.92</td>
<td>8.58 ± 1.15</td>
</tr>
<tr>
<td>Enrollment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>10.03 ± 1.02</td>
<td>10.02 ± 1.19</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>76.9 ± 5.4</td>
<td>77.3 ± 6.3</td>
</tr>
<tr>
<td>Enrollment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>83.6 ± 4.4</td>
<td>84.3 ± 5.4</td>
</tr>
<tr>
<td>Weight-for-age ( z ) score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>-2.17 ± 0.41</td>
<td>-2.15 ± 0.55</td>
</tr>
<tr>
<td>6 mo</td>
<td>-2.04 ± 0.56</td>
<td>-2.03 ± 0.58</td>
</tr>
<tr>
<td>Length-for-age ( z ) score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>-1.60 ± 0.82</td>
<td>-1.25 ± 0.81(^2)</td>
</tr>
<tr>
<td>6 mo</td>
<td>-1.26 ± 0.71</td>
<td>-1.08 ± 0.80</td>
</tr>
<tr>
<td>Weight-for-length ( z ) scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>-1.59 ± 0.65</td>
<td>-1.70 ± 0.68</td>
</tr>
<tr>
<td>6 mo</td>
<td>-1.56 ± 0.51</td>
<td>-1.70 ± 0.53</td>
</tr>
</tbody>
</table>

\(^2\) Significantly different from the zinc-supplemented group, \( P < 0.05 \) (t test).

---

Each child, the median return of full vials was 7.25 (quartiles:
2.0–21.37), or 4.0%, for the children receiving zinc, and 9.25
(quartiles: 3.08–21.37), or 5.1%, for those receiving placebo.
The number of full vials returned was not significantly different
between the groups (Mann-Whitney \( U \) test).

The number of intended home visits for the stimulation inter-
vention was not always attained. Sixty-two percent of the chil-
dren received 3 or 4 visits per month, whereas the others were
visited 1–2 times per month.

---
The estimates and 95% CIs of these models are shown in Table 5. Where the interaction between stimulation and zinc supplementation entered the model, the stimulated coefficient is the difference between the stimulation alone group and the control group means, and the zinc coefficient is the difference between the zinc alone group and the control group means. There was a significant interaction between stimulation and zinc supplementation on developmental quotient, and children who received both treatments had the highest developmental quotients after the intervention. There was no main effect of zinc supplementation alone. There was a significant main effect of stimulation alone on developmental quotient. There was also a significant interaction between zinc and stimulation on the hand and eye subscale, which also indicated that children having both treatments had the highest scores. Zinc alone, however, also benefited the hand and eye subscale. There were significant main effects of stimulation alone on hearing and speech and performance. Neither intervention benefited locomotor development.

**Growth**

The initial and final anthropometric measurements are shown in Table 3. It was not hypothesized that the stimulation intervention would affect growth, so only the supplementation intervention was considered for the growth analyses. In multiple regression analyses, the effect of zinc supplementation on final status was determined, including as independent variables initial measurements, sex, and age. The following covariates were also offered stepwise: maternal height, crowding, sanitation, and possession. Supplementation had no significant effect on any of the anthropometric indexes.

**Morbidity**

The median and range of the cumulative episodes, and the total days ill, are shown in Table 6 for the supplemented and placebo groups. The number of episodes of diarrhea \((P = 0.019)\) and the total days of diarrhea \((P = 0.033)\) were significantly fewer in the zinc-supplemented group. There were no significant differences between the groups in number of episodes or duration of any of the other symptoms.

**DISCUSSION**

Zinc supplementation benefited overall development (developmental quotients) in stimulated children but not in unstimulated children. All children who received zinc benefited in hand and eye coordination, but those who received both stimulation and zinc benefited the most. Zinc supplementation was randomly assigned, testers were unaware of the assignment to interventions, and there was little loss from the sample. The zinc-supplemented children also had reduced diarrheal morbidity.

### TABLE 4
Developmental scores at enrollment and 6 mo later\(^1\)

<table>
<thead>
<tr>
<th>Developmental quotient</th>
<th>Stimulation and zinc group (n = 25)</th>
<th>Stimulation only group (n = 21)</th>
<th>Zinc only group (n = 30)</th>
<th>Control group (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrollment</td>
<td>102.4 ± 9.0</td>
<td>105.5 ± 8.8</td>
<td>100.9 ± 12.0</td>
<td>106.7 ± 13.9</td>
</tr>
<tr>
<td>6 mo</td>
<td>99.2 ± 9.0(^a)</td>
<td>96.2 ± 7.6(^b)</td>
<td>92.1 ± 10.1(^b)</td>
<td>96.5 ± 9.3(^b)</td>
</tr>
<tr>
<td>Locomotor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>106.6 ± 9.6</td>
<td>106.9 ± 11.1</td>
<td>105.1 ± 15.3</td>
<td>112.1 ± 16.6</td>
</tr>
<tr>
<td>6 mo</td>
<td>101.8 ± 10.7</td>
<td>98.6 ± 11.8</td>
<td>97.4 ± 12.7</td>
<td>102.6 ± 9.9</td>
</tr>
<tr>
<td>Hearing and speech</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>105.3 ± 12.2</td>
<td>108.1 ± 9.8</td>
<td>102.1 ± 14.7</td>
<td>107.5 ± 17.0</td>
</tr>
<tr>
<td>6 mo</td>
<td>100.4 ± 11.5(^b)</td>
<td>100.4 ± 16.0(^d)</td>
<td>89.1 ± 11.1(^d)</td>
<td>96.6 ± 13.9(^d)</td>
</tr>
<tr>
<td>Hand and eyes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>103.7 ± 9.2</td>
<td>107.2 ± 8.9</td>
<td>100.9 ± 12.6</td>
<td>108.3 ± 13.3</td>
</tr>
<tr>
<td>6 mo</td>
<td>102.0 ± 9.4</td>
<td>94.6 ± 8.8</td>
<td>95.3 ± 11.2</td>
<td>98.3 ± 11.3</td>
</tr>
<tr>
<td>Performance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>93.8 ± 12.0</td>
<td>99.7 ± 13.0</td>
<td>95.7 ± 13.6</td>
<td>98.6 ± 15.2</td>
</tr>
<tr>
<td>6 mo</td>
<td>92.4 ± 16.7</td>
<td>91.3 ± 11.2</td>
<td>86.5 ± 12.2</td>
<td>88.6 ± 12.2</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SD\). Means in a row with different superscript letters are significantly different, \(P < 0.01\) (ANOVA and Tukey’s test).

### TABLE 5
Multilevel analysis of the developmental quotient (DQ) and subscale scores showing the effects of the interventions\(^1\)

<table>
<thead>
<tr>
<th>Fixed variables</th>
<th>DQ</th>
<th>Locomotor</th>
<th>Hearing and speech</th>
<th>Hand and eye</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial score</td>
<td>0.72(^a)((0.56, 0.88))</td>
<td>0.54(^a)((0.39, 0.69))</td>
<td>0.46(^a)((0.27, 0.66))</td>
<td>0.58(^a)((0.41, 0.76))</td>
<td>0.52(^a)((0.32, 0.72))</td>
</tr>
<tr>
<td>Child’s age</td>
<td>0.43(^b)((0.11, 0.75))</td>
<td>0.58(^b)((0.23, 0.93))</td>
<td>-0.24((-0.72, 0.24))</td>
<td>0.15((-0.19, 0.50))</td>
<td>-0.07((-0.53, 0.39))</td>
</tr>
<tr>
<td>Stimulated</td>
<td>2.52((-0.05, 5.00))</td>
<td>1.94((-3.58, 7.46))</td>
<td>7.27((2.81, 11.73))</td>
<td>1.05((-2.64, 4.74))</td>
<td>6.01((1.58, 10.44))</td>
</tr>
<tr>
<td>Zinc supplemented</td>
<td>1.82((-0.63, 4.26))</td>
<td>1.46((-1.90, 4.81))</td>
<td>-2.44((-6.58, 1.71))</td>
<td>4.43((1.17, 7.69))</td>
<td>1.59((-2.54, 5.72))</td>
</tr>
<tr>
<td>Stimulated × zinc supplemented</td>
<td>5.18((0.34, 10.03))</td>
<td>—</td>
<td>—</td>
<td>10.13((3.71, 16.55))</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^1\) 95% CI in parentheses. \(n = 114\). Developmental scores were analyzed by multilevel analysis with clinic and child as random variables. Initial score, age, supplementation group, stimulation group, and whether the child was enrolled in the stimulation trial were entered and an interaction term (supplementation status × stimulation status) was offered.

\(^2\) \(P < 0.05\).
TABLE 6
Number of episodes and duration of illness in the zinc-supplemented and placebo groups during the 6-mo trial

<table>
<thead>
<tr>
<th>Reported symptoms</th>
<th>Number of episodes</th>
<th>Duration</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zinc-supplemented group</td>
<td>Placebo group</td>
<td>Zinc-supplemented group</td>
<td>Placebo group</td>
</tr>
<tr>
<td></td>
<td>(n = 55)</td>
<td>(n = 59)</td>
<td>(n = 55)</td>
<td>(n = 59)</td>
</tr>
<tr>
<td>Apathy</td>
<td>0.0 (0–2)</td>
<td>0.0 (0–3)</td>
<td>0.0 (0–7)</td>
<td>0.0 (0–8)</td>
</tr>
<tr>
<td>Fever</td>
<td>2.0 (0–6)</td>
<td>1.0 (0–8)</td>
<td>3.8 (0–7)</td>
<td>2.2 (0–6)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>0.8 (0–4)</td>
<td>1.0 (0–6)</td>
<td>1.7 (0–9)</td>
<td>1.2 (0–22)</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>3.0 (0–15)</td>
<td>3.0 (0–9)</td>
<td>15.0 (0–14)</td>
<td>17.0 (0–20)</td>
</tr>
<tr>
<td>Cough</td>
<td>2.0 (0–8)</td>
<td>2.0 (0–9)</td>
<td>8.2 (0–16)</td>
<td>12.2 (0–21)</td>
</tr>
<tr>
<td>Rapid or difficult breathing</td>
<td>0.0 (0–3)</td>
<td>0.0 (0–6)</td>
<td>0.0 (0–1)</td>
<td>0.0 (0–1)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0.0 (0–3)</td>
<td>0.8 (0–9)</td>
<td>0.0 (0–6)</td>
<td>0.2 (0–10)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0.0 (0–5)</td>
<td>0.0 (0–4)</td>
<td>0.0 (0–5)</td>
<td>0.0 (0–7)</td>
</tr>
<tr>
<td>Ear infection</td>
<td>0.2 (0–5)</td>
<td>0.0 (0–2)</td>
<td>0.0 (0–28)</td>
<td>0.0 (0–15)</td>
</tr>
<tr>
<td>Skin problems</td>
<td>0.0 (0–7)</td>
<td>0.0 (0–5)</td>
<td>0.0 (0–31)</td>
<td>0.0 (0–25)</td>
</tr>
</tbody>
</table>

1 All values are median; range in parentheses. Groups were compared by nonparametric statistics.
2 Episodes and duration of diarrhea were significantly less in the zinc-supplemented group, P < 0.05 (Mann-Whitney U test). None of the other symptoms were significantly different between groups.

which suggests that they were initially zinc deficient. Therefore, it is probable that zinc was responsible for the improvements.

Some other studies of zinc supplementation failed to show a benefit to developmental levels (13–15). Possible reasons for finding benefits in the present study may be that the children were zinc deficient or that the children received small doses of iron, which may have reduced the chances of zinc affecting their iron status through competitive interactions (30). Unfortunately, we were unable to take blood from the children, so we have no measure of hemoglobin or iron status. However, the provision of additional stimulation appears to be the most important factor contributing to the positive effects on development. It is possible that encouraging the mothers to be more responsive to their children was particularly effective in those children who received zinc and were thus more active and exploring.

Stimulation benefited developmental levels and language development and also interacted with zinc to produce further benefits to developmental quotient and hand and eye coordination. The preferred design would have been to randomly assign children to stimulation as well as zinc, but this was not feasible. We therefore assigned them to stimulation by clinic, and this was taken into account in the analyses. We have found benefits from stimulation interventions in several previous studies (21, 31), but the benefits were usually larger. The duration of the program was shorter than previous ones, which probably explains the smaller benefits. The global benefit of some 5 developmental quotient points is approximately equivalent to 0.4 of an SD score. Change in maternal behavior and other aspects of stimulation in the children’s home may be one mechanism through which the stimulation intervention may have improved developmental outcomes. Unfortunately, we did not measure the levels of stimulation in the home after the intervention; however, similar interventions have shown improvements in the levels of home stimulation (32). This may be an important measure to include in future studies.

All groups declined in developmental levels over the 6 mo of the study, but the group receiving both interventions declined the least. The children came from poor backgrounds, and declines in scores on developmental tests are common in disadvantaged populations at this age and have been shown before in Jamaica (21) and elsewhere (33). Jamaican children from middle socio-economic backgrounds do not show declines in scores at this age (34). Although the Griffiths test is not standardized in Jamaica, the scores are reasonably stable over time, and the scores are predictive of later IQ and school achievement (25).

The children receiving zinc had fewer episodes of diarrhea and fewer days ill with diarrhea. These findings add further to the consensus (8) that zinc deficiency plays an important role in diarrheal morbidity. There were no significant benefits of zinc supplementation on the children’s growth. It may be that the zinc deficiency was only mild and that morbidity is more sensitive to this level of deficiency than is growth. Alternatively, it may be that zinc was not the growth-limiting nutrient in this population or that the study was too short to produce a growth response.

We are unaware of other studies that have combined zinc supplementation with psychosocial stimulation. A possible mechanism underlying the interaction is that the children would be more alert and active with zinc supplementation and would be more able to benefit from stimulation. Unfortunately, we were not able to observe the children’s behavior. The findings of interactions between zinc and stimulation have important implications for policy and indicate clearly that integrated programs of nutrition and child development activities are needed to support optimal child development in disadvantaged populations with nutrient deficiencies.

We thank the Ministry of Health, Jamaica; Michael Ennis; Carla Redding; Joan Thomas; Pauline Alcott; and the community health workers for assistance.

JMMG, CAP, and SMG-McG were responsible for the conceptualization and design of the study. JMMG was responsible for the implementation of the zinc trial and the writing of the manuscript. CAP had overall responsibility for the stimulation trial, HB-H was responsible for the stimulation intervention, SPW assisted with the data analysis, and TJG provided statistical advice. All authors contributed to the critical review of the manuscript and approved the final draft for publication. None of the authors had any financial or personal relationship with any of the companies sponsoring the research.

REFERENCES


Short-term effects of vitamin A and antimalarial treatment on erythropoiesis in severely anemic Zanzibari preschool children

Sarah E Cusick, James M Tielsch, Mahdi Ramsan, Jape K Jape, Sunil Sazawal, Robert E Black, and Rebecca J Stoltzfus

ABSTRACT

Background: The pathophysiology of anemia in coastal East Africa is complex. Impaired erythropoietin production is one possible mechanism. Plasmodium falciparum malaria has been found to blunt erythropoietin production, whereas vitamin A stimulates erythropoietin production in vitro.

Objective: We investigated the 72-h effects of vitamin A and the antimalarial drug sulfadoxine pyramethamine (SP) on erythropoietin production in severely anemic (hemoglobin ≤ 70 g/L) preschool children in Zanzibar, a region of known vitamin A deficiency. We hypothesized that both treatments would stimulate erythropoietin production directly, within 72 h, before a change in hemoglobin would occur.

Design: One hundred forty-one severely anemic children were identified during the baseline assessment of a morbidity substudy of a community-based micronutrient supplementation trial. All severely anemic children were randomly assigned to receive either vitamin A (100 000 or 200 000 IU depending on age) or SP at baseline; 72 h later they received the opposite treatment plus daily hematinic syrup for 90 d. Erythropoietic and parasitic indicators were assessed at baseline and again after 72 h.

Results: After 72 h, SP reduced the malaria parasite density (by 50.29 parasites/μL; P < 0.001), CRP concentrations (by 10.6 mg/L; P = 0.001), and the proportion of children infected with malaria (by 32.4%; P < 0.001). Vitamin A reduced CRP (by 9.6 mg/L; P = 0.011), serum ferritin (by 18.1 μg/L; P = 0.042), and erythropoietin (by 194.7 mIU/mL; P = 0.011) concentrations and increased the reticulocyte production index (by 0.4; P = 0.041).

Conclusions: Contrary to our hypothesis, vitamin A significantly decreased erythropoietin concentration. The most important effect of both vitamin A and SP was the rapid reduction of inflammation. Vitamin A also mobilized iron from stores and stimulated the production of new erythrocytes. Am J Clin Nutr 2005;82:406–12.

KEY WORDS Anemia, vitamin A, malaria, erythropoiesis, erythropoietin, children, inflammation

INTRODUCTION

Nutritional deficiencies and parasitic infections coexist in coastal East Africa, which results in severe multifactorial anemia that mainly afflicts young children. In one hospital in a malaria hyperendemic region of Tanzania, severe anemia (hemoglobin < 80 g/L) was the stated cause of 20% of infant admissions and 27% of infant deaths (1). Elucidating the primary physiologic mechanism of anemia is difficult in this setting because nutritional deficiencies, including those of iron and vitamin A, and parasitic infections, including malaria and helminth infection, often coexist in the same child and potentially cause anemia via multiple physiologic pathways.

Although vitamin A deficiency and malaria infection can each cause anemia by several mechanisms, recent evidence suggests that both conditions may affect erythropoietin production by the kidney. Erythropoietin is the hormone secreted by the kidney in response to hypoxia that stimulates marrow production of red blood cells (2).

Suppression of erythropoietin production is one mechanism by which Plasmodium falciparum malaria has been proposed to cause anemia, although studies have had conflicting results. Several have found a blunted erythropoietin response in malaria patients (3–5), while others (6–8) have found that erythropoietin production was normal or up-regulated in children with P. falciparum malaria.

Vitamin A stimulates erythropoietin production in vitro. Okano et al (9) found that retinoic acid up-regulated erythropoietin production 3-fold in HepG2 cells and further found elevated serum erythropoietin concentrations in rats intragastrically injected with retinoic acid. Jelkmann et al (10) similarly found that vitamin A, not vitamin C or vitamin E, increased the rate of erythropoietin production in HepG2 and Hep3B cells.

In the present study, we investigated the 72-h effects of vitamin A and the antimalarial sulfadoxine pyramethamine (SP) on erythropoietin production in severely anemic (hemoglobin ≤ 70 g/L) preschool children living on Pemba Island, Zanzibar, where vitamin A deficiency is severe (RJ Stoltzfus, unpublished observations, 1999) and P. falciparum malaria is holoendemic (11). All children received vitamin A, SP, iron, and B vitamins, but for the first 72 h, each child was randomly assigned to receive either vitamin A or SP. We believed that the 72-h interval would provide sufficient time to observe changes in erythropoietic indicators that...
were exacted directly by the intervention and not indirectly by a change in hemoglobin (2, 12). In light of repeated findings of blunted erythropoietin production in patients with *P. falciparum* malaria and because of the specific stimulatory effect that vitamin A has on erythropoietin production in vitro, we hypothesized that both treatments would increase erythropoietin production and stimulate erythropoiesis in these severely anemic children.

**SUBJECTS AND METHODS**

**Setting**

Pemba Island, Zanzibar, United Republic of Tanzania is located in the Indian Ocean, just off the Tanzanian coast. Pemba is almost entirely rural, and the economy is sustained largely by the cultivation and exportation of cloves and seaweed. The staple foods of the Pemban diet are rice and cassava, which are usually eaten with a small amount of legumes or green vegetables. Meat and large fish are expensive and are not regularly consumed. Fruit, including mangoes, pineapples, and oranges, are seasonal.

A recent community-based survey found that 38% of children aged 6–71 mo were stunted, and 31.2% were underweight (13). More than 25% of the children in the same survey had a serum retinol concentration <20 μg/dL (RJ Stoltzfus, unpublished observations), which classifies vitamin A deficiency in Pemba as a public health problem (14).

*P. falciparum* malaria is holoendemic to Pemba and is transmitted throughout the year. A peak in malaria parasite density is observed from June to September, following the long rainy season that occurs from April to June (11). The soil-transmitted helminths *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm are endemic (15).

**Participants**

Children enrolled in the study were those found to have a hemoglobin concentration ≤ 70 g/L by HemoCue (HemoCue AB, Angelhom Sweden) during the baseline assessment of a morbidity substudy of a larger, ongoing, community-based micronutrient supplementation trial known as the Zanzibar Infant Nutrition Campaign (Figure 1). The substudy assessed morbidity, dietary intake, micronutrient status, and growth. Substudy clinics were selected on the basis of geographic location. Of 2994 children assessed at 10 substudy baseline clinics in the Wete and Mkoani districts between March and September 2002, 225 children aged 4–43 mo were found to be severely anemic. These children did not receive a randomized treatment assignment in

**FIGURE 1.** Study flow chart. Hb, hemoglobin; SP, sulfadoxine pyramethamine. HemoCue AB (Angelhom Sweden).
the larger trial but, instead, were enrolled in the severe anemia component (SAC). After an informed consent process conducted in Swahili by a nurse, each mother gave written consent for the child’s participation in the SAC. This study was approved by the institutional review boards of the Johns Hopkins University Bloomberg School of Public Health and the Ministry of Health and Social Welfare in Zanzibar.

**Baseline clinic (day 0)**

For children enrolled in the SAC, a venous blood sample was drawn into 2 tubes—one was coated with EDTA and the other was uncoated. Whole blood from the EDTA-coated tube was used to prepare a malaria blood film. Axillary temperature was recorded assuming 8000 leukocytes/μL blood (20).

**Laboratory assessments**

Blood and stool samples were placed in a cooler and brought back to the Public Health Laboratory within 4 h. Erythrocyte protoporphyrin was measured in whole blood from the EDTA-coated tubes with the use of a fluorometer (Aviv Biomedical, Lakewood, NJ) and for manual counting of reticulocytes according to the method of Sigma Diagnostics (18).

Malaria blood films prepared in the field were fixed with ethanol and stained with Giemsa (19). The number of malaria parasites per 200 leukocytes was counted on thick films. If no parasites were detected, the microscopist continued until 500 leukocytes were counted. Malaria parasite densities were calculated assuming 8000 leukocytes/μL blood (20).

From the tubes of blood not coated with EDTA, serum was collected, divided, and frozen up to 4 mo at −20 °C until analyzed. Serum concentrations of erythropoietin (R&D Systems, Minneapolis, MN), transferrin receptor (Ramco Laboratories Inc, Stafford, TX), serum ferritin (Ramco Laboratories Inc), and C-reactive protein (CRP; Alpha Diagnostic International, San Antonio, TX) were measured by enzyme-linked immunosorbent assay. During the assay, the identity of each child and the day on which the sample was collected (day 0 or day 3) were concealed. The average CV for the erythropoietin assay was 4.2% (range: 0.93–7.7%), for the TIR assay was 4.4% (range: 0.68–17.1%), for the serum ferritin assay was 6.2% (range: 1.3–22.2%), and for the CRP assay was 8.7% (range: 0.1–19.4%). Stool samples were refrigerated overnight and were analyzed for helminth eggs the following morning with the Kato-Katz technique (19).

**Statistical analysis**

To accomplish our primary aim of evaluating the 72-h effects of vitamin A and SP on erythropoietin and other hematologic indicators, we compared day 0 and day 3 indicator values within each treatment group with the use of a paired t test. In this paired analysis, all variables were analyzed in their natural units. A 95% CI was calculated around the mean 72-h change for each indicator. A CI that did not include zero represented a statistically significant within-group change.

In a secondary analysis, we used multivariate regression to compare the mean 72-h difference for each indicator between the 2 treatment groups. For this between-group comparison, the mean 72-h difference for each indicator was modeled as the dependent variable, with the baseline value of the indicator and treatment group (vitamin A or SP) as independent variables. A 95% CI was calculated for each adjusted group difference. A CI that did not include zero indicated that the mean 72-h change for a given indicator was statistically significantly different between the 2 treatment groups.

For all analyses, reticulocyte counts were used to calculate a reticulocyte production index for each child as follows: (number of reticulocyte/erythrocytes) × (hematocrit/normal hematocrit) × 100. This index adjusts the reticulocyte count for the degree of anemia (21). A value of 31.5 was considered to be the normal hematocrit value (10.5 g/dL × 3) and was used to normalize the reticulocyte counts in our sample.

For the anthropometric calculations, we defined stunted as a weight-for-age z score less than −2 and wasted as a weight-for-height z score as less than −2. Height-for-age and weight-for-height z scores were calculated by using ANTHRO version 3.0 (Centers for Disease Control and Prevention, Atlanta, GA). All other analyses were performed with the use of STATA 6 statistical software (Stata Corp, College Station, TX).

**RESULTS**

**Baseline characteristics**

Of the 225 children found to be severely anemic, 141 had sufficient serum on both day 0 and day 3 for a complete paired analysis (Figure 1). None of the characteristics were significantly different between the 2 treatment groups at baseline (Table 1).

Before therapy began, baseline erythropoietin concentrations were well above the normal range of 8–18 mIU/mL (22), and TIR concentrations were above the normal upper-limit cutoff of 11 mg/L (23); these findings indicated that erythropoiesis was activated (Table 1). Erythrocyte protoporphyrin was also highly elevated at baseline, well above 90 μmol/mol heme—the upper limit of the normal range (23). CRP concentrations in both groups
were more than twice the normal upper limit of 10 mg/L (24). Malaria was prevalent and relatively severe; ≈80% of the children had malaria, ≈33% of whom had ≥5000 parasites/μL. Nearly half (47.4%; 95% CI: 38.1, 56.7) of the children were stunted, and 5.3% (95% CI: 1.1, 9.4) of the children were wasted. The prevalence of helminth infection was low: 2.4% for Ascaris, 9.1% for Trichuris, and 4.2% for hookworm for the combined treatment groups (data not shown).

### 72-h changes: SP

As we expected, hemoglobin did not change significantly over 72 h in the SP group (Table 2). However, SP did significantly reduce all indicators of malaria infection. Malaria parasite density decreased by >5000 parasites/μL, the proportion of malaria-positive children decreased from >80% to 50%, and the proportion of children with malaria infection of ≥5000 parasites/μL decreased nearly 30%. SP also significantly reduced CRP concentrations by >10 mg/L. Although erythropoietin concentrations decreased slightly in children who received SP at baseline, the reduction was not statistically significant.

### 72-h Changes: vitamin A

As we predicted, hemoglobin did not change significantly over 72 h in the vitamin A group (Table 2). Although vitamin A did not significantly change any indicator of malaria infection, it did significantly decrease CRP and serum ferritin concentrations. Contrary to our hypothesis, vitamin A significantly decreased erythropoietin concentrations by nearly 200 mIU/mL.
Vitamin A effects

Vitamin A rapidly and significantly reduced inflammation, on the basis of CRP concentrations, in 72 h. Vitamin A deficiency in mice has been shown to be associated with increased production of transcripts for the proinflammatory cytokines IL-12 and γ-interferon and with the down-regulated production of transcripts for anti-inflammatory cytokines IL-4 and IL-10 (33). Recent in vitro evidence further suggests that vitamin A may reduce the inflammation caused by malaria infection. Human monocytes cotreated with *P. falciparum* malaria and 9-cis-retinoic acid secreted significantly less TNF-α than did control monocytes (34). The authors proposed that this in vitro evidence provides a physiologic explanation for the recent finding by Shankar et al (35) that vitamin A significantly reduced the number of *P. falciparum* febrile episodes by 30% in young children in Papua New Guinea.

In addition to reducing CRP, vitamin A also significantly reduced serum ferritin, an iron storage and positive acute phase response protein whose concentration increases during inflammation regardless of true iron status (36). This decrease in serum ferritin may reflect both reduced inflammation and the mobilization of iron from storage to functional compartments. The significant increase in the reticulocyte production index observed in children who received vitamin A provides functional evidence for such a mobilization of iron and subsequent incorporation of iron into new erythrocytes.

Mobilization of stored iron may also provide an explanation for the observed significant decrease in serum erythropoietin concentrations following supplementation with vitamin A. In both in vitro and in vivo studies, Kling et al (37) found that cellular iron deprivation stimulated erythropoietin production. Using Hep3B cells, Kling et al found that chelation with deferoxamine and blockade of transferrin receptor–mediated iron uptake stimulated erythropoietin production, whereas administration of hemin impaired erythropoietin production. Similarly, in healthy volunteers, high-dose treatment with deferoxamine coupled to hydroxethyl starch significantly increased erythropoietin concentrations (37).

Furthermore, in a study in Uganda, Totin et al (38) found that the log erythropoietin/hemoglobin regression curve was significantly

### TABLE 2

72-h Changes in erythropoietic, iron-status, and parasitic indicators in the 2 treatment groups

<table>
<thead>
<tr>
<th>Indicator and group</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Within-group mean change</th>
<th>Adjusted between-group difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP (n = 68)</td>
<td>64.6</td>
<td>65.3</td>
<td>0.72 (−1.3, 2.7)</td>
<td>0.50 (−3.6, 2.6)</td>
</tr>
<tr>
<td>Vitamin A (n = 71)</td>
<td>63.1</td>
<td>63.7</td>
<td>0.59 (−1.8, 2.9)</td>
<td></td>
</tr>
<tr>
<td>Malaria parasite density (parasites/μL)</td>
<td>5828 (3544, 8112)</td>
<td>800 (323, 1276)</td>
<td>−5029 (−7385, −2672)</td>
<td>−2502 (−1142, −3862)</td>
</tr>
<tr>
<td>SP (n = 68)</td>
<td>4737 (3204, 6269)</td>
<td>3238 (1983, 238)</td>
<td>−1498 (−3235, 238)</td>
<td></td>
</tr>
<tr>
<td>Vitamin A (n = 72)</td>
<td>79.2</td>
<td>75.0</td>
<td>−4.2 (−16.9, 8.6)</td>
<td></td>
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<tr>
<td>Malaria positive (%)</td>
<td></td>
<td></td>
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<tr>
<td>SP (n = 68)</td>
<td>82.4</td>
<td>50.0</td>
<td>−32.4 (−46.5, −18.2)</td>
<td>−25.6 (−41.1, −10.0)</td>
</tr>
<tr>
<td>Vitamin A (n = 72)</td>
<td>79.2</td>
<td>75.0</td>
<td>−4.2 (−16.9, 8.6)</td>
<td></td>
</tr>
<tr>
<td>Malaria density ≥5000 parasites/μL (%)</td>
<td>33.8 (22.3, 45.4)</td>
<td>5.9 (0.14, 11.6)</td>
<td>−27.9 (−40.4, −15.5)</td>
<td>−17.8 (−29.2, −6.4)</td>
</tr>
<tr>
<td>SP (n = 68)</td>
<td>33.3 (22.2, 44.5)</td>
<td>23.6 (13.6, 33.7)</td>
<td>−9.7 (−22.3, 2.8)</td>
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<tr>
<td>CRP (mg/L)</td>
<td></td>
<td></td>
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<tr>
<td>SP (n = 57)</td>
<td>21.8</td>
<td>11.2</td>
<td>−10.6 (−16.7, −4.4)</td>
<td>−2.6 (−2.5, 7.5)</td>
</tr>
<tr>
<td>Vitamin A (n = 57)</td>
<td>23.6</td>
<td>13.9</td>
<td>−9.6 (−17.0, −2.3)</td>
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<tr>
<td>Serum ferritin (μg/L)</td>
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<tr>
<td>SP (n = 69)</td>
<td>127.5 (85.0, 170.0)</td>
<td>116.1 (71.7, 160.4)</td>
<td>−11.5 (−48.2, −25.2)</td>
<td>6.8 (−42.2, 28.7)</td>
</tr>
<tr>
<td>Vitamin A (n = 72)</td>
<td>127.1 (82.5, 171.6)</td>
<td>109.0 (74.4, 143.6)</td>
<td>−18.1 (−35.5, −0.67)</td>
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<tr>
<td>Erythropoietin (mIU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP (n = 69)</td>
<td>498.0 (309.0, 687.0)</td>
<td>441.1 (240.6, 641.5)</td>
<td>−56.9 (−215.7, 101.9)</td>
<td>77.9 (−247.6, 91.8)</td>
</tr>
<tr>
<td>Vitamin A (n = 72)</td>
<td>634.3 (391.4, 877.2)</td>
<td>439.6 (299.1, 580.1)</td>
<td>−194.7 (−343.9, −45.5)</td>
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<tr>
<td>Transferrin receptor (mg/L)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>SP (n = 69)</td>
<td>21.1</td>
<td>22.7</td>
<td>1.7 (−0.84, 4.2)</td>
<td>1.6 (−4.4, 1.2)</td>
</tr>
<tr>
<td>Vitamin A (n = 72)</td>
<td>19.3</td>
<td>19.7</td>
<td>0.41 (−1.0, 1.9)</td>
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</tr>
<tr>
<td>Reticulocyte production index</td>
<td></td>
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<tr>
<td>SP (n = 39)</td>
<td>2.1</td>
<td>2.2</td>
<td>0.11 (−0.44, 0.67)</td>
<td>−0.05 (−0.6, 0.5)</td>
</tr>
<tr>
<td>Vitamin A (n = 47)</td>
<td>1.5</td>
<td>1.9</td>
<td>0.40 (0.02, 0.78)</td>
<td></td>
</tr>
<tr>
<td>Erythropoietin protoporphyrin (μmol/mol heme)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SP (n = 61)</td>
<td>285.5 (253.2, 317.8)</td>
<td>263.1 (227.7, 298.6)</td>
<td>−22.4 (−55.5, 10.7)</td>
<td>−16.0 (−16.5, 48.6)</td>
</tr>
<tr>
<td>Vitamin A (n = 66)</td>
<td>293.7 (251.4, 336.1)</td>
<td>286.1 (243.5, 328.7)</td>
<td>−7.6 (−18.7, 3.5)</td>
<td></td>
</tr>
</tbody>
</table>

1 All values are means; 95% CIs in parentheses. The sample size varied because of insufficient blood samples. SP, sulfadoxine pyramethamine.

2 72-h difference (day 3 − day 0). CIs that do not contain 0 indicate a statistically significant within-group change.

3 Adjusted difference in mean 72-h change between treatment groups (mean change in SP group − mean change in vitamin A group). The differences were adjusted for the baseline value of each indicator. CI intervals that do not include 0 indicate a statistically significant between-group change.

4 P < 0.05. CIs do not contain 0.
steep in iron-deficient children than in non-iron-deficient children, which indicated that the erythropoietin response is up-regulated during iron deficiency. These studies perhaps help explain the observed reduction in erythropoietin in our study after treatment with vitamin A. If vitamin A indeed mobilized stored iron, more iron would become available for erythropoiesis, which would lower erythropoietin concentrations. Such inferences with regard to the direct effects of vitamin A and SP on the indicators we measured, however, are somewhat limited by the ethically dictated omission of a placebo or control group. Our inclusion of only children with extremely low hemoglobin concentrations means that some regression to the mean was likely. However, the short time interval between the 2 measurements (72 h) makes substantial regression to the mean less likely. Indeed, the lack of a significant change in many indicators from day 0 to day 3 (eg, hemoglobin, transferrin receptor, and malaria indexes in the vitamin A group) and the finding that different indicators changed within each treatment group increase the plausibility that the significant changes that we observed were in fact due to the treatments themselves.

In conclusion, in a setting where malnutrition is severe and malaria is holoendemic, treatment of severely anemic children with vitamin A was associated with rapid changes indicative of reduced inflammation, iron mobilization, and stimulated erythropoiesis. Antimalarial treatment reduced circulating malaria parasites and inflammation but did not cause significant changes in other measures. Contrary to our hypothesis, neither vitamin A nor SP stimulated erythropoietin production. Rather, vitamin A significantly reduced erythropoietin, perhaps by increasing cellular iron availability after the observed reduction in inflammation. More research on the role of vitamin A in the treatment of severe anemia is needed, including an investigation of whether the beneficial short-term effects that we observed are sustained.

RJS, SEC, JMT, REB, and SS were involved with the study design. SEC, JKJ, and MR were responsible for the data collection. SEC helped conduct the laboratory analysis and prepared the manuscript. SEC, RJS, and JMT performed the data analysis. None of the authors had a known conflict of interest.

REFERENCES

Body-composition alterations consistent with cachexia in children and young adults with Crohn disease1–3

Jon M Burnham, Justine Shults, Edisio Semeao, Bethany J Foster, Babette S Zemel, Virginia A Stallings, and Mary B Leonard

ABSTRACT

Background: Crohn disease (CD) in children is associated with low body mass index (BMI), poor growth, and delayed maturation; alterations in lean and fat mass, however, are poorly characterized.

Objective: The objective was to quantify lean mass and fat mass in children and young adults with CD and in healthy control subjects, relative to height and pubertal maturation.

Design: This cross-sectional study assessed whole-body lean and fat mass by using dual-energy X-ray absorptiometry in 104 subjects with CD and in 233 healthy control subjects aged 4–25 y. Linear regression was used to determine the effect of CD on body composition and to generate sex-specific SD scores (z scores) for lean and fat mass relative to height.

Results: Subjects with CD had lower height-for-age and BMI-for-age z scores (P < 0.001 for both) than did control subjects. CD was associated with significant deficits in lean mass after adjustment for height, age, race, and Tanner stage (P = 0.003); deficits in fat mass were not observed. The mean (±SD) lean mass–for-height and fat mass–for-height z scores in the subjects with CD were −0.61 ± 0.92 and −0.04 ± 0.86, respectively. Within the control group, fat mass–for-height was positively correlated with lean mass–for-height (r = 0.41, P < 0.0001); this association was absent in the subjects with CD.

Conclusions: Children and young adults with CD had significant deficits in lean mass but preserved fat mass, which is consistent with cachexia. Further research is needed to identify physical activity, nutritional, and antiinflammatory interventions to improve body composition in persons with CD. Am J Clin Nutr 2005;82:413–20.

KEY WORDS Crohn disease, body composition, cachexia, lean mass, fat mass

INTRODUCTION

Crohn disease (CD) in children and adolescents is characterized by gastrointestinal tract inflammation with malabsorption, nutritional deficiencies, anemia, pubertal delay, growth failure, and osteopenia. Low body mass index (BMI; in kg/m²) is a well-recognized complication of CD in children (1, 2). However, previous studies evaluating fat mass and lean mass in children and adults with CD were based on small numbers of subjects and yielded conflicting results. Some studies reported that the lower body weight in subjects with CD reflected significant fat mass deficits with sparing of lean mass (3–7), whereas others reported significant deficits in lean mass (2, 8–13). Furthermore, the relations between body composition and disease characteristics, such as CD activity and type of therapy, have not been examined in a large cohort of children and adolescents with CD.

In adults, lean mass deficits are associated with demonstrable morbidity, including loss of muscle strength, altered energy metabolism, and increased susceptibility to infections (14). Inflammatory cachexia, which is defined as loss of lean mass without loss of fat mass, has been described in rheumatoid arthritis (15) and has been attributed to muscle-active cytokines, increased resting energy expenditure (REE), and physical inactivity. Although malnutrition is a well-recognized complication of CD, the prevalence of cachexia has not been determined in children or adults.

In children and adolescents, muscle forces are critical determinants of bone mineral accrual (16, 17). We recently evaluated whole-body bone mineral content in children and young adults with CD and in healthy control subjects (18). In that study, CD was associated with significant deficits in whole-body bone mineral content relative to height and maturation. However, adjustment for lean mass eliminated the bone mineral content deficit in the CD group compared with the control group. Therefore, deficits in lean mass and fat mass may have distinct effects on bone accrual, physical function, susceptibility to infection, and quality of life in children with CD.

Analysis and interpretation of body-composition data in children with a chronic illness requires careful attention to sex-, maturation-, and race-related differences in lean and fat mass relative to body size. Dual-energy X-ray absorptiometry (DXA) provides precise and accurate measures of lean mass and fat mass in children and adults (19, 20). The objectives of the present study were to assess lean mass and fat mass in children and young
adults with CD and in concurrent healthy control subjects and to identify risk factors for alterations in fat mass and lean mass in subjects with CD.

SUBJECTS AND METHODS

Study subjects

Persons aged 4–25 y with CD who were being treated at the Children’s Hospital of Philadelphia or the Hospital of the University of Pennsylvania were eligible for the study. Diagnosis was confirmed by radiographic, histologic, and clinical information. Persons with other medical conditions unrelated to CD that potentially affect growth or body composition, such as underlying renal insufficiency, thyroid disease, or known growth hormone deficiency, were excluded. Lumbar spine and whole-body bone measures, vitamin D concentrations, growth, and maturation have been reported in these subjects (18, 21–24).

Healthy control subjects were recruited from general pediatric clinics in the surrounding community and through newspaper advertisements. Control subjects were excluded for any coexisting conditions known to affect growth, nutritional status, dietary intake, or development. The protocol was approved by the Institutional Review Board at the Children’s Hospital of Philadelphia. Informed consent was obtained from the young adult participants and the parents or guardians of those participants aged <18 y. Assent was obtained from those younger than 18 y.

Crohn disease characteristics

Medical records were reviewed for age at disease onset and diagnosis; disease characteristics; medical, nutritional, and surgical interventions; and hematologic and biochemical values after diagnosis. Site of disease was classified as upper gastrointestinal tract only (ie, proximal to the colon), colon only, or both. History of laboratory abnormalities was defined as one or more values outside the normal age and sex reference range after the diagnosis of CD had been established. CD severity was assessed by using the Pediatric Crohn’s Disease Activity Index (PCDAI), which is based on history (30%), physical examination (30%), laboratory data (20%), and height velocity (20%) (25, 26). PCDAI scores are categorized as follows: no disease activity (0–10), mild disease activity (11–30), and moderate to severe disease activity (>30). PCDAI scores were measured at the study visit. Additionally, a cumulative PCDAI score was assigned by averaging all prior PCDAI scores documented in the medical record.

Medications

Use of the following medications was documented: 6-mercaptopurine, sulfasalazine, mesalamine [as Pentasa (Shire US Inc, Newport, KY) or Asacol (Proctor & Gamble Pharmaceuticals, Cincinnati, OH)], metronidazole, calcium suplementation, and corticosteroid enemas. All doses of enteral and parenteral corticosteroids were noted and were converted to prednisone equivalents. Corticosteroid exposure was summarized as lifetime cumulative prednisone dose (g) and as cumulative mg/kg based on body weight at the time of each dose. Average doses during intervals of corticosteroid therapy were summarized as mg/d and mg · kg⁻¹ · d⁻¹. This study was conducted before the use of tumor necrosis factor α inhibitors in pediatric CD.

Anthropometry and pubertal development

Weight and height were measured with a digital scale to the nearest 0.1 kg (Scaltronix, White Plains, NY) and with a stadiometer to the nearest to 0.1 cm (Holtain Ltd, Crymnych, United Kingdom), respectively. Age- and sex-specific SD scores (z scores) for weight, height, and BMI were calculated by using the National Center for Health Statistics 2000 Centers for Disease Control and Prevention growth data and the LMS method (27). Pubertal stage was assessed according to the method of Tanner by a single investigator at the time of the study visit (28).

Dual-energy X-ray absorptiometry

Whole-body DXA scans were performed by using a Hologic QDR 2000 bone densitometer (Hologic Inc, Bedford, MA) with a fan beam in the array mode. The measurements were performed by using standard supine positioning techniques and were analyzed to generate estimates of lean mass (kg) excluding bone, and fat mass (kg). The skull was excluded from measures of lean and fat mass (postcranial). The instrument was calibrated daily with a hydroxyapatite phantom. The precision of lean and fat mass measures made with the use of DXA is reported as 2–3% and 3–4%, respectively (19).

Laboratory studies

Blood samples were obtained from subjects with CD at the time of the study visit. Serum albumin (g/dL), total protein (g/dL), hemoglobin (g/dL), and erythrocyte sedimentation rate (mm/h) were measured (Clinical Laboratory, Children’s Hospital of Philadelphia, Philadelphia, PA) by using standard techniques.

Statistical analysis

Analyses were conducted by using STATA 8.2 (Stata Corporation, College Station, TX). Two-sided tests of hypotheses were used, and P values < 0.05 were considered significant. Differences in means were assessed by using Student’s t test if the data were normally distributed and Wilcoxon’s rank sum test if the data were not normally distributed. Group differences in categorical variables were assessed by using the chi-square or Fisher’s exact test, where appropriate.

The primary outcomes were postcranial fat mass and lean mass in kg. CD is associated with poor linear growth; therefore, these measures were adjusted for stature. To test for body-composition differences between the CD and control groups, natural log-transformed multivariable linear regression models for fat mass and lean mass relative to natural log–transformed height were adjusted for the following covariates that may confound this comparison: age, race (African American versus all others), and Tanner stage of pubertal maturation (stage 1 as the referent group, with indicator variables for Tanner stages 2 through 5 given a value of zero or one if absent or present, respectively). Given the known sex differences in body composition during growth, all results are presented stratified by sex. In healthy children and adolescents, greater fat mass was associated with greater lean mass for height (29). Therefore, a multiplicative interaction term was used to determine whether the relation between fat mass and lean mass differed in the CD group compared with the control group. The assumptions of the regression models were assessed via graphical checks, the Shapiro-Wilk test of
normality of residuals, the Ramsey omitted variable test, and the Cook-Weisburg test for heteroscedasticity.

Because the outcome variable was log-transformed, the independent effect of CD in each multivariate model was summarized as the adjusted ratio of the outcome measure in the subjects with CD divided by the outcome measure in the control subjects, along with 95% CIs; note that these ratios have no units. The adjusted ratios and 95% CIs were calculated as the exponentiated estimate of the regression parameters.

To assess the effect of CD-specific effects on body composition within the subjects with CD, fat mass and lean mass were converted to sex-specific $z$ scores relative to height. For example, a lean mass–for-height $z$ score of $-1.0$ indicates a whole-body lean mass that is 1 SD below that of control subjects of the same height and sex. Data from the control subjects were used to derive the predicted value of lean or fat mass relative to height by using linear regression. The model for lean mass relative to height did not exhibit heteroscedasticity; therefore, the root mean square error served as the SD. The model for fat mass relative to height did exhibit heteroscedasticity; therefore, height-specific SDs were estimated by regressing absolute residuals on height (30).

Factors associated with deficits in lean mass–for-height and fat mass–for-height within the group of subjects with CD were identified by using simple logistic regression; body-composition $z$ scores were considered low if less than $-1.0$ (equivalent to the 16th percentile). Factors found to be significantly $(P \leq 0.05)$ associated with low body-composition $z$ scores were entered into a multivariable logistic regression model. The correlation between body-composition $z$ scores and continuous variables was assessed by using Pearson product-moment estimates.

RESULTS

Subject characteristics and body composition

A total of 104 subjects with CD and 233 healthy control subjects completed the study. Subject characteristics are summarized in Table 1. Control subjects were significantly younger than the subjects with CD. The subjects with CD were predominantly white, which is consistent with the demographics of the disease, and had delayed maturation: within Tanner stages 2 and 4, subjects with CD were an average of 1.4 and 1.5 y older than the control subjects ($P < 0.05$ and $< 0.01$, respectively), adjusted for sex and race.

The subjects with CD had significantly lower height-for-age, weight-for-age, and BMI-for-age $z$ scores than did the healthy control subjects (all $P < 0.0001$). The BMI $z$ score distribution within the control subjects was consistent with a recent report of the US population (31). There were no sex differences in age, Tanner stage, height-for-age $z$ score, or BMI-for-age $z$ score within the control or the CD group.

Disease characteristics

Disease characteristics, medications, and laboratory values were available in 56–88% of the subjects with CD and are detailed in Table 2. Among the 88% with complete medication data, 90% had a history of treatment with corticosteroids. Treatment and disease characteristics were compared between the male and female subjects with CD. Males were exposed to corticosteroids for a greater duration than were females [median duration: 15 mo (range: 0–128 mo) compared with 8 mo (range: 0–79 mo); $P = 0.01$] and thus received a greater total dose over the course of their disease [median dose: 7.9 g (range: 0–74.0 g) compared with 6.2 g (range: 0–41.7 g); $P = 0.01$]. During the exposure to corticosteroids, the doses for each sex (mg/d and mg · kg$^{-1}$· d$^{-1}$) were similar. Corticosteroid dose (total mg/kg) was inversely correlated with height-for-age $z$ score ($r = -0.36$, $P = 0.0005$). Males were more frequently treated with Pentasa than were females (33% compared with 16%; $P = 0.01$) and were more likely to have a history of hypoalbuminemia (27% compared with 8%; $P = 0.02$).

Lean mass

Lean mass (kg) and unadjusted lean mass–for-age and lean mass–for-height $z$ scores for the subjects with CD and the healthy controls are shown in Table 3. The initial assessment of lean mass was adjusted for age and race. The ratio for lean mass in subjects with CD compared with that in the controls was 0.85 (95% CI: 0.80, 0.90; $P < 0.001$) in males and 0.88 (95% CI: 0.83, 0.94; $P < 0.001$) in females. Subsequently, potential confounders were added to the model; these included height and Tanner stage. The final model is summarized in Table 4, which shows that the ratio of lean mass in subjects with CD compared with that

<table>
<thead>
<tr>
<th>Variable</th>
<th>Crohn disease subjects (n = 104)</th>
<th>Control subjects (n = 233)</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>$15.4 \pm 4.3^2$</td>
<td>$11.9 \pm 5.7$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>63</td>
<td>41</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Race (% black)</td>
<td>3</td>
<td>31</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Tanner distribution (no. at stages 1, 2, 3, 4, and 5)</td>
<td>17, 16, 22, 18, 31</td>
<td>117, 24, 16, 33, 43</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>$154.7 \pm 16.7$</td>
<td>$144.1 \pm 22.0$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Height-for-age $z$ score</td>
<td>$-0.74 \pm 1.2$</td>
<td>$0.28 \pm 1.1$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>$47.7 \pm 14.4$</td>
<td>$43.3 \pm 21.2$</td>
<td>0.003</td>
</tr>
<tr>
<td>Weight-for-age $z$ score</td>
<td>$-0.66 \pm 1.1$</td>
<td>$0.36 \pm 1.1$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>$19.4 \pm 3.2$</td>
<td>$19.5 \pm 4.9$</td>
<td>$&gt;0.2$</td>
</tr>
<tr>
<td>Range</td>
<td>$12.5–31.7$</td>
<td>$13.0–40.9$</td>
<td></td>
</tr>
<tr>
<td>BMI-for-age $z$ score</td>
<td>$-0.35 \pm 1.0$</td>
<td>$0.27 \pm 1.12$</td>
<td>$&lt;0.0001$</td>
</tr>
</tbody>
</table>

1 Statistical analysis was performed by using $t$ tests for normally distributed variables; otherwise, the Wilcoxon rank-sum test was used.

2 $x \pm SD$ (all such values).
### TABLE 2
Disease characteristics, medication exposure, and laboratory assessments in subjects with Crohn disease

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease characteristics</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (y)</td>
<td>12.0 ± 3.8 (0.3–20.1) [92]</td>
</tr>
<tr>
<td>Duration of disease (y)</td>
<td>4.0 ± 3.4 (0.1–16.0) [92]</td>
</tr>
<tr>
<td>Age at symptom onset (y)</td>
<td>10.9 ± 3.7 (0.2–19.9) [92]</td>
</tr>
<tr>
<td>PCDAI at study visit</td>
<td>12.0 ± 11.9 (0–52.5) [60]</td>
</tr>
<tr>
<td>PCDAI average</td>
<td>13.5 ± 9.7 (0–40.0) [64]</td>
</tr>
<tr>
<td>Total hospital admissions (n)</td>
<td>2.4 ± 1.9 (1–5) [58]</td>
</tr>
<tr>
<td>Total hospital days (d)</td>
<td>24.7 ± 37.0 (2–207) [58]</td>
</tr>
<tr>
<td>Corticosteroid use</td>
<td></td>
</tr>
<tr>
<td>Total (g)</td>
<td>10.3 ± 12.1 (0–74) [91]</td>
</tr>
<tr>
<td>(mg/d)</td>
<td>19.0 ± 12.2 (0–60) [91]</td>
</tr>
<tr>
<td>(mg·kg⁻¹·d⁻¹)</td>
<td>0.5 ± 0.3 (0–2) [91]</td>
</tr>
<tr>
<td>Duration (mo)</td>
<td>19.6 ± 24.5 (0–128) [91]</td>
</tr>
<tr>
<td>Laboratory values at the time of the study visit</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.0 ± 0.6 (2.9–4.9) [77]</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>12.9 ± 1.1 (9.9–15.2) [77]</td>
</tr>
<tr>
<td>Sedimentation rate (mm/h)</td>
<td>17.2 ± 17.0 (0–69) [75]</td>
</tr>
<tr>
<td>Site of disease (%)</td>
<td></td>
</tr>
<tr>
<td>Isolated upper tract</td>
<td>8 [92]</td>
</tr>
<tr>
<td>Isolated colonic</td>
<td>32 [92]</td>
</tr>
<tr>
<td>Upper tract and colonic</td>
<td>69 [92]</td>
</tr>
<tr>
<td>Perianal</td>
<td>51 [92]</td>
</tr>
<tr>
<td>History (%)</td>
<td></td>
</tr>
<tr>
<td>Joint symptoms</td>
<td>13 [92]</td>
</tr>
<tr>
<td>Anemia</td>
<td>39 [92]</td>
</tr>
<tr>
<td>Hypoalbuminemia</td>
<td>20 [92]</td>
</tr>
<tr>
<td>Surgical resection</td>
<td>21 [91]</td>
</tr>
<tr>
<td>Therapies (%)</td>
<td></td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>34 [92]</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>57 [92]</td>
</tr>
<tr>
<td>Corticosteroid enemas</td>
<td>11 [92]</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>51 [92]</td>
</tr>
<tr>
<td>Mesalamine (Asacol)</td>
<td>60 [92]</td>
</tr>
<tr>
<td>Mesalamine (Pentasa)</td>
<td>26 [92]</td>
</tr>
<tr>
<td>Parenteral nutrition</td>
<td>47 [92]</td>
</tr>
<tr>
<td>Nasogastric feeding</td>
<td>28 [92]</td>
</tr>
</tbody>
</table>

1 SD: range in parentheses; n in brackets (all such values).
2 PCDAI, Pediatric Crohn’s Disease Activity Index. PCDAI scores are as follows: no disease activity (0–10), mild disease activity (11–30), and moderate to severe disease activity (>30).
3 Asacol (Proctor & Gamble Pharmaceuticals, Cincinnati, OH).
4 Pentasa (Shire US Inc, Newport, KY).

### TABLE 3
Unadjusted body-composition measures in subjects with Crohn disease and in healthy control subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Crohn disease subjects</th>
<th>Control subjects</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean mass (kg)</td>
<td>32.2 (12.1–60.1) ⁴</td>
<td>24.7 (9.9–84.1)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Lean mass–for-age ( z ) score</td>
<td>−1.10 ± 1.11 ⁴</td>
<td>0.00 ± 0.99</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lean mass–for-height ( z ) score</td>
<td>−0.61 ± 0.92</td>
<td>0.00 ± 0.99</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>9.1 (1.3–39.2) ⁴</td>
<td>7.3 (1.2–51.6)</td>
<td>0.13</td>
</tr>
<tr>
<td>Fat mass–for-age ( z ) score</td>
<td>−0.21 ± 0.77</td>
<td>0.00 ± 0.95</td>
<td>0.051</td>
</tr>
<tr>
<td>Fat mass–for-height ( z ) score</td>
<td>−0.04 ± 0.86</td>
<td>0.00 ± 0.96</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

¹ Statistical analysis was performed by using t tests for normally distributed variables; otherwise, the Wilcoxon rank-sum test was used.
² Median; range in parentheses (all such values).
³ SD (all such values).

in controls was 0.94 (95% CI: 0.91, 0.98) for males and females, adjusted for height, age, Tanner stage, and race. Therefore, CD is associated with a 6% reduction in lean mass, independent of stature, age, maturation, and race. In a similar model that excluded black subjects in both the control and CD groups, the same 6% deficit in males and females was identified. Given the predominance of Tanner stage 1 control subjects, we performed a similar analysis in the Tanner stage 2–5 participants, stratified by sex, excluding black subjects. In this model, an 8% lean mass deficit in males \((P = 0.003)\) and females \((P = 0.001)\) was noted. Assessment of lean mass as a sex-specific \( z \) score relative to height also showed significant deficits in the CD group, as shown in Figure 1. The mean (±SD) lean mass–for-height \( z \) scores in...
the male and female control subjects were 0.00 ± 1.00, by definition. The lean mass–for-height z-scores were significantly lower in the CD subjects: −0.58 ± 0.87 (P = 0.0002) in males and −0.65 ± 1.03 (P = 0.0004) in females. When the z scores were assessed relative to age, the deficits were greater: −1.16 ± 1.19 in males and −0.98 ± 0.97 in females (both P < 0.0001); lean mass–for age z-scores were significantly lower than lean mass–for-height z-scores in the subjects with CD (P < 0.0001).

**Fat mass**

Fat mass (kg) and unadjusted fat mass–for-age and fat mass–for-height z scores for the subjects with CD and the healthy controls are shown in Table 3. An initial model evaluated fat mass adjusted for age and race. The ratio for fat mass in subjects with CD compared with controls was 0.82 (95% CI: 0.66, 1.02; P = 0.08) in males and 1.01 (95% CI: 0.81, 1.25; P > 0.2) in females. Subsequently, potential confounders were added to the model, including height and Tanner stage. The final model, stratified by sex, which is summarized in Table 5, failed to detect significant deficits in fat mass in males or females with CD. The ratios of fat mass in subjects with CD compared with the controls, adjusted for height, age, Tanner stage, and race, were 0.93 (95% CI: 0.75, 1.16) in males and 1.12 (95% CI: 0.91, 1.38) in females. Similar results were found in a model that excluded black subjects from both the control and CD groups. No fat mass deficits were noted in a model including only Tanner stage 2–5 subjects and excluding black subjects.

Similarly, expression of fat mass as a sex-specific z score relative to height did not reveal significant deficits in CD. The fat mass–for-height z scores in males and females with CD were −0.10 ± 0.89 in males and 0.06 ± 0.79 in females (both P > 0.2 compared with controls), as shown in Figure 1. Fat mass–for-age z scores were −0.22 ± 0.79 (P = 0.11) in males and −0.18 ± 0.75 (P > 0.2) in females with CD and were significantly lower than the fat mass–for-height z scores (P < 0.0001).

**Lean and fat mass interactions**

To evaluate the relations between lean mass and fat mass in CD and in the controls, the model assessing lean mass (Table 4) was tested for a CD-by–fat mass interaction. The interaction term was significant in males (P = 0.004) and females (P = 0.007). When this relation was explored in separate models for the controls and the CD patients, lean mass was positively associated with fat mass in male and female healthy controls (both P < 0.001). However, males and females with CD did not have greater lean mass with greater fat mass. This relation is detailed in Figure 2 and demonstrates the positive slope of lean mass–for-height z score relative to fat mass–for-height z score in the control subjects only. No significant correlation was observed between lean mass–for-height z score and fat mass–for-height z-score in the CD subjects.

**Table 5**

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>Ratio (e^β)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males (R^2 = 0.96; n = 161)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn disease</td>
<td>−0.07</td>
<td>0.93 (0.75, 1.16)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Log (height)</td>
<td>3.65</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>1.02 (0.97, 1.07)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 2</td>
<td>−0.17</td>
<td>0.84 (0.56, 1.27)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 3</td>
<td>−0.26</td>
<td>0.77 (0.50, 1.18)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 4</td>
<td>−0.31</td>
<td>0.73 (0.45, 1.20)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 5</td>
<td>−0.60</td>
<td>0.55 (0.29, 1.02)</td>
<td>0.06</td>
</tr>
<tr>
<td>Race</td>
<td>−0.16</td>
<td>0.85 (0.67, 1.08)</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Females (R^2 = 0.94; n = 176)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn disease</td>
<td>1.12</td>
<td>1.12 (0.91, 1.38)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Log (height)</td>
<td>3.13</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>0.98 (0.94, 1.02)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 2</td>
<td>0.16</td>
<td>1.17 (0.88, 1.58)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 3</td>
<td>0.10</td>
<td>1.10 (0.76, 1.60)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Tanner stage 4</td>
<td>0.36</td>
<td>1.34 (0.99, 2.06)</td>
<td>0.06</td>
</tr>
<tr>
<td>Tanner stage 5</td>
<td>0.56</td>
<td>1.75 (1.09, 2.81)</td>
<td>0.02</td>
</tr>
<tr>
<td>Race</td>
<td>0.26</td>
<td>1.29 (1.06, 1.57)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

^95% CI in parentheses. The ratio represents the exponentiated regression coefficient (β) of each non-log-transformed covariate. Because height was log transformed, the regression coefficients for height are interpreted as follows: fat mass increases as height to the 3.65 power in males and 3.13 power in females.

```plaintext
FIGURE 1. Lean mass–for-height and fat mass–for-height z-scores in subjects with Crohn disease (CD) and in healthy control subjects. There was a significant deficit in sex-specific lean mass–for-height z-scores in both male and female subjects with CD (z ± SD: −0.61 ± 0.92 in subjects with CD and 0.00 ± 0.99 in healthy control subjects; P < 0.0001) but no significant alteration in fat mass–for-height z-scores (P > 0.2) by t test. The center of each box represents the mean value for the specified stratum. The upper and lower boundary of each box represents the 95% CI.

FIGURE 2. Lean mass relative to fat mass in subjects with Crohn disease (CD) and in healthy control subjects. There was a positive correlation between sex-specific lean mass–for-height z-score and fat mass–for-height z-score in healthy control subjects (r = 0.41, P < 0.0001) by Pearson correlation. This relation was absent in children and young adults with CD (r = 0.05, P > 0.2). With the use of multivariable linear regression to examine lean mass, a CD-by–fat mass interaction term was significant in both males (P = 0.004) and females (P = 0.007).
```
DISCUSSION

These data showed significant lean mass deficits and normal fat stores in a large, clinically heterogeneous sample of children and young adults with CD. Lean mass was inversely correlated with the PCDAI, the most widely used estimate of disease activity in pediatric CD. Furthermore, the expected positive association between fat and lean mass observed in healthy control subjects was absent in this sample of subjects with CD. During normal growth and development, lean mass increases steadily, particularly during the pubertal growth spurt (32). As adiposity and body weight increase, additional muscle is required to retain normal function (33, 34). The independent effects of pubertal maturation on lean mass in males and females are confirmed in Table 4.

This pattern of lean mass deficits without fat deficits is termed cachexia (15). In contrast, wasting represents deficits in fat and lean mass, which are typically due to inadequate dietary intake. Roubenoff et al (35) showed that inflammatory cachexia in rheumatoid arthritis was the result of altered energy and protein metabolism (greater REE and protein catabolism and reduced total energy expenditure), accompanied by inflammatory cytokine production. The muscle-active cytokines in rheumatoid cachexia, including tumor necrosis factor $\alpha$, interleukin 1$\beta$, and interleukin 6 (15), are also significantly elevated in CD (36). These cytokines stimulate protein degradation, inhibit myogenic differentiation (37, 38), and induce myoblast apoptosis (39).

Corticosteroids, which were used in 90% of the CD subjects in our study, may directly affect muscle and fat mass. Adiposity is a well-recognized complication of corticosteroid use (40). Studies in animal models have shown that corticosteroids increased myostatin, which is a negative regulator of skeletal muscle mass (41). However, corticosteroid-induced reductions in muscle-active cytokines and disease activity may counteract direct effects on muscle. We recently reported that high-dose corticosteroids resulted in marked obesity without lean mass deficits in childhood nephrosis (42). Vaisman et al (43) documented similar findings in renal transplantation. In that study, recently transplanted subjects experienced initial increases in fat mass, which were likely secondary to corticosteroid use. Lean mass subsequently increased, which may be attributable to the decreasing corticosteroid dose, improved nutrition, and the normal association between fat and lean mass, as described in Figure 2. Our data in CD suggested a negative correlation between corticosteroid dose and lean mass, but this was not statistically significant. This may have been due to the limitations of retrospective assessment of corticosteroid exposure; the cumulative dose did not capture fully the intra- and intersubject variability in corticosteroid regimens. Also, CD severity confounds the potential association between corticosteroid dose and muscle deficits; PCDAI was associated with lean mass deficits, and corticosteroids were administered to patients with more severe disease.

Pentasa therapy was associated with both a higher cumulative corticosteroid dose and lower lean mass. Pentasa is a formulation of 5-aminosalicylic acid that is released throughout the small intestine and colon. In contrast, Asacol, another 5-aminosalicylic acid compound, is delivered primarily to the colon. Asacol is used to treat colitis and Pentasa is used primarily for small-bowel disease. Both drugs limit mucosal inflammation with minimal systemic absorption (44). Therefore, it is unlikely that Pentasa directly affected body composition. Rather, Pentasa therapy is likely a marker of small-intestine disease. Small-intestine disease is associated with micronutrient deficiencies, such as deficiencies of zinc and selenium, which may compromise growth and muscle mass (45, 46). These micronutrients were not measured in this study.

### TABLE 6
Univariate and multivariate logistic regression analysis of factors associated with low lean mass–for-height $z$ score in subjects with Crohn disease

<table>
<thead>
<tr>
<th>Covariate</th>
<th>OR for $z$ score $&lt;-1.0$ (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>2.8 (1.1, 7.0)</td>
<td>0.03</td>
</tr>
<tr>
<td>Nasogastric feeding</td>
<td>2.7 (1.0, 7.0)</td>
<td>0.04</td>
</tr>
<tr>
<td>Isolated upper tract disease</td>
<td>6.7 (1.2, 37.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Mesalamine (Pentasa) therapy$^2$</td>
<td>7.1 (2.5, 19.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Multivariate model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>1.1 (0.4, 3.6)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Nasogastric feeding</td>
<td>2.7 (0.9, 8.5)</td>
<td>0.09</td>
</tr>
<tr>
<td>Isolated upper tract disease</td>
<td>4.5 (0.7, 27.8)</td>
<td>0.11</td>
</tr>
<tr>
<td>Mesalamine (Pentasa) therapy$^2$</td>
<td>6.1 (2.0, 18.8)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

$^1$ The analysis was performed in 92 subjects with Crohn disease for whom exposure data were complete. OR, odds ratio.  
$^2$ Pentasa (Shire US Inc, Newport, KY).
Whereas inflammation and drug therapy may compromise muscle accrual directly through sarcoactive cytokines, indirect effects on REE, nutrition, and physical activity may contribute to cachexia in CD. Energy balance is largely determined by the difference between dietary intake and total energy expenditure. In children, total energy expenditure comprises mainly physical activity energy expenditure and REE, with a minor contribution of diet-induced thermogenesis and growth. Azcue et al (10) showed that, compared with anorexia nervosa subjects, children with CD fail to down-regulate REE with malnutrition. However, greater REE has not been consistently shown in adults with CD (9, 10, 13, 47–50). Elevated REE has been shown in children with cystic fibrosis, who are susceptible to chronic inflammation, malabsorption, and malnutrition (51, 52).

Lower levels of physical activity may contribute to lean mass deficits in CD. Children with CD may have decreased physical activity as a result of fatigue, muscle weakness, and chronic abdominal or musculoskeletal pain. Unfortunately, physical activity was not assessed in the present study. However, physical activity interventions have been shown to improve muscle mass and function in other chronic inflammatory conditions, such as rheumatoid arthritis and chronic kidney disease (53, 54). Additionally, we did not assess dietary intake in our study population. Total energy intake, along with the carbohydrate, protein, and fat composition of the diet, may affect energy balance and body composition. For example, decreased dietary intake of protein or increased protein catabolism may adversely affect lean mass (55). Elevated diet-induced thermogenesis has been reported in adults with inactive CD involving the small intestine, which may further contribute to a negative energy balance (6).

An accurate determination of body composition in CD is of critical importance given the therapeutic goals: to induce disease remission and support normal growth and nutritional status. The analyses presented here illustrate the importance of a concurrent control group, with consideration of the confounding effects of short stature, delayed maturation, and race. Studies documenting altered body composition in children with inflammatory bowel disease are few. Boot et al (2) studied body composition in 55 children with inflammatory bowel disease (22 with CD, 33 with ulcerative colitis) by using DXA and documented low lean mass–for-age (−1.04 ± 1.41) and fat mass–for-age (−0.64 ± 1.02) z scores. Similar to our population, height-for-age z score deficits were present (−0.54 ± 1.25), and pubertal delay was noted. Azcue et al (10) studied anthropometric indexes, bioelectrical impedance measures, and total body potassium in 24 children with CD and 22 controls. Intracellular and extracellular body water was estimated by use of deuterium dilution and the corrected bromide space. Lean mass deficits were variable and depended on both the measurement method and the expression of lean mass as either an unadjusted variable or as a percentage of body weight. Fat mass deficits were present, expressed as a percentage of body weight. The authors compared 20 CD subjects treated with corticosteroids or enteral nutrition alone over a 3-mo interval. Both groups had significant increases in body weight and lean mass with treatment. The increases in lean mass and height were greater in the enteral nutrition group, and there was a trend toward increased percentage body fat in the corticosteroid group (P = 0.07). In both of these studies, failure to adjust for height-for-age may have resulted in an overestimation of the lean mass and fat mass deficits. Our analyses showed that lean mass deficits relative to age are significantly greater than lean mass deficits relative to height.

In summary, in children and young adults with CD, inflammatory cachexia may be due to muscle-active cytokines; corticosteroids; macro- and micronutrient deficits (56, 57); endocrinologic factors, such as insulin-like growth factor 1 (IGF-1) deficiency (58); increased REE; and decreased physical activity. Little is known about the long-term clinical significance of cachexia during childhood. However, poor prenatal and postnatal nutritional status have been associated with adverse consequences on body composition, muscle strength, and mortality during the adult years (59, 60). One likely consequence of decreased lean mass in children and adolescents with CD is impaired bone accrual (18). We recently reported that this same cohort of children and young adults with CD had significant deficits in whole-body bone mineral content, and bone deficits were strongly associated with lean mass deficits. Osteopenia in CD may result in an increased risk of osteoporosis-related morbidity and disability throughout adulthood (61, 62). Future studies are needed to evaluate the efficacy of nutrition and physical activity interventions to improve lean mass in children and young adults with CD. In addition, studies are required to explore the effects of newer biological therapies on growth and body composition in pediatric CD.

JMB designed and performed the statistical analysis, did the background research, and wrote the manuscript. JS provided statistical expertise and assisted in manuscript preparation. ES participated in study design, patient recruitment, and data collection. BJJ assisted in the statistical analysis and manuscript preparation. BSZ provided critical expertise in the design of the study and analysis of the body-composition data and assisted in manuscript preparation. VAS led in study design and data collection and aided in manuscript preparation. MBL was instrumental in the data analysis and manuscript preparation. The authors had no conflicts of interest to report.

REFERENCES


Effect of sucrose on inflammatory markers in overweight humans

Lone B Sørensen, Anne Raben, Steen Stender, and Arne Astrup

ABSTRACT
Background: Observational studies have found that dietary glycemic load is positively associated with C-reactive protein (CRP) concentrations in healthy humans, which suggests that the type of carbohydrate ingested influences inflammatory activity.

Objective: We investigated the effect of a diet with a high content of sucrose or artificial sweeteners on the inflammatory markers CRP, haptoglobin, and transferrin in overweight subjects.

Design: Overweight men and women consumed daily food and drink supplements containing either sucrose (n = 21; body mass index (BMI, in kg/m²): 28.0) or artificial sweeteners (n = 20; BMI: 27.6), predominantly from soft drinks (70%; average = 1.3 L/d) for 10 wk.

Results: During the intervention, sucrose intake increased by 151% in the sucrose group and decreased by 42% in the sweetener group, resulting in a 1.6-kg weight gain in the sucrose group and a 1.2-kg weight loss in the sweetener group over 10 wk (P < 0.001). Concentrations of haptoglobin, transferrin, and CRP increased by 13%, 5%, and 6%, respectively, in the sucrose group and decreased by 16%, 2%, and 26%, respectively, in the sweetener group (between-group differences: P = 0.006, P = 0.01, and P = 0.1, respectively). Adjustment for changes in body weight and energy intake did not substantially influence this outcome.

Conclusions: The study shows that in the present group of overweight subjects a high consumption of sugar-sweetened foods and drinks increased haptoglobin and transferrin but had, at best, only a limited influence on CRP.

KEY WORDS C-reactive protein, CRP, haptoglobin, inflammatory markers, arteriosclerosis, cardiovascular disease, artificial sweeteners, sugar, sucrose, soft drinks, overweight, diabetes

INTRODUCTION

A biological marker of inflammation, C-reactive protein (CRP), has been shown in several prospective, nested, case-control studies to be associated with an increased risk of myocardial infarction, stroke, sudden death from cardiac causes, and peripheral arterial disease (1–3). Moreover, CRP and LDL-cholesterol concentrations measured at baseline in 27,939 apparently healthy women were found to be independent risk factors for first cardiovascular event at 8-y follow-up (4). The importance of CRP as a predictor of coronary heart disease is not clear, because different studies have provided varying results (4, 5). Elevated plasma concentrations of CRP have also been found to be a risk factor for type 2 diabetes independent of the classic risk factors, ie, obesity, smoking, exercise, and family history of diabetes (6). High concentrations of other inflammation-sensitive plasma proteins, including haptoglobin, have also been associated with type 2 diabetes, myocardial infarction, and stroke (7, 8). Recent evidence also links inflammation-sensitive plasma proteins to weight gain and obesity (9).

Although it is not known whether inflammation is a primary event that causes arteriosclerosis, type 2 diabetes, and perhaps obesity or whether it is a secondary event, it is important to identify environmental and lifestyle factors that may be influential. An observational study reported that a high glycemic load, ie, a high intake of rapidly digested and absorbed carbohydrates, was positively associated with CRP in healthy women (10). This finding was especially true in the overweight women, and the relation was independent of other risk factors (10). These results suggest that a proinflammatory process that may increase the risk of cardiovascular disease and type 2 diabetes may be exacerbated by a high intake of rapidly digested and absorbed carbohydrates. However, because of the observational nature of that study, it cannot be ruled out that the association was due to residual confounding caused by unmeasured and immeasurable factors. In the present randomized, controlled trial we tested the hypothesis that overweight men and women who increase their intake of sugar-sweetened drinks and foods experience an increase in the inflammatory serum markers CRP and haptoglobin and a decrease in transferrin compared with a control group that consumed similar amounts of artificially sweetened beverages and foods.

SUBJECTS AND METHODS

The main part of the study, with emphasis on the effect of energy intake, body weight, and body composition, has been described in detail elsewhere (11).

1 From the Department of Human Nutrition, Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark (LBS, AR, and AA), and the Gentofte Hospital, University of Copenhagen, Hellerup, Denmark (SS).

2 Supported by the Danish Research and Development Program for Food Technology (no. 93s-2464-å92-00152) and Danisco Sugar, Coca Cola (Nordic and Eurasia Divisions) generously provided soft drinks for the study.

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Study design

The study had a parallel design with 2 intervention groups. For 10 wk one group received supplemental drinks and foods containing sucrose, and the other group received similar drinks and foods containing artificial sweeteners.

Subjects

Forty-two healthy, overweight subjects were included, and 41 (6 men and 35 women) completed the study. None of the subjects were dieting, and none of the female subjects were pregnant or lactating. Approval was obtained from the Ethical Committee of Copenhagen and Frederiksberg, and the study was performed in accordance with the Helsinki II Declaration. Each subject signed an informed consent document before the start of the study.

Diets

During the 10-wk intervention the subjects consumed a specific minimum amount of either sucrose-sweetened or artificially sweetened foods and drinks daily. The subjects were assigned to 3 different levels of intake according to their initial body weight: level 1, 2, or 3 corresponding to 60–75 kg, 75–90 kg, and >90 kg, respectively. The minimum intake of the experimental diet was regulated by the sucrose intake and corresponded to a sucrose intake of 125 g/d (level 1), 150 g/d (level 2), and 175 g/d (level 3). This corresponded to a total energy intake from sucrose supplements of 2738 kJ/d, 3285 kJ/d, and 3833 kJ/d, respectively. The sweetener group received an equivalent amount (by weight) of foods containing artificial sweeteners.

In the sucrose group ≈70% of the sucrose came from drinks (average: ≈1.3 L/d), and ≈30% came from solid foods. About 80% by weight of the supplements were beverages, and ≈20% by weight were solid foods. The beverages consisted of several soft drinks and fruit juices, and the solid foods consisted of yogurt, marmalade, ice cream, and stewed fruits. Some of the artificially sweetened products were low-fat, so the subjects in the sweetener group were given additional butter or corn oil to keep the fat intake in the 2 intervention diets as similar as possible.

The subjects were supplied with all the drink and food supplements at the Department of Human Nutrition. In addition to the food and drink supplements, the subjects were free to consume their habitual diet ad libitum.

Measurements

Several measurements were performed before, during, and at the end of the 10-wk intervention period. Body weight, fat mass (FM), and fat-free mass were measured at weeks 0, 2, 4, 6, 8, and 10. Subjects completed 7-d dietary records, 7-d diaries (for monitoring hunger, fullness, palatability of the food, and well being), week diaries (for recording illness, menstruation, altered diet, medication, or altered physical activity pattern), 24-h urine collections (to validate the dietary records), and diurnal appetite scores at weeks 0, 5, and 10. Waist and hip circumferences, sagittal height (height of abdomen when lying in a supine position), and blood pressure were measured, and blood samples were taken at weeks 0 and 10. In addition, subjects completed a 3-factor questionnaire about eating behavior (12) as well as a questionnaire about habitual physical activity before and after the intervention. The recordings of physical activity were used to match the 2 groups before the intervention and to monitor any changes after the intervention. After the intervention, subjects also completed a questionnaire about the experimental diet. Results concerning the 7-d diaries, the questionnaires, and the effects of the diets on fat-free mass and sagittal height have been reported elsewhere (11).

Anthropometry

Height was measured to the nearest 0.5 cm by using a wall-mounted stadiometer at the screening visit. All other measurements were done in the morning after a 12-h fast. After voiding, body weight was measured to the nearest 0.1 kg on a digital scale (Seca model 708; Seca Mess und Wiegetechnik, Vogel & Halke GmbH & Co, Hamburg, Germany). Body composition was estimated by bioelectrical impedance by using an Animeter (HTS-Engineering Inc, Odense, Denmark). FM and fat-free mass were calculated as described previously (13). Waist and hip circumferences were measured with a tape measure. Blood pressure was measured in the supine position, after 10 min of rest, using an automatically inflating cuff (UA-743; A&D Company Ltd, Tokyo, Japan).

Seven-day weighed dietary records

To monitor the subjects’ food consumption, three 7-d weighed dietary records were completed: in week 0 (before the intervention), and in weeks 5 and 10 (during the intervention). A 7-d dietary record was also completed 1–2 mo before the intervention to accustom the subjects to the method. Digital scales were used to weigh the food (Soehnle 8020 and 8009; Soehnle-Waagen GmbH & Co, Murrhardt, Germany), and the computer database of foods from the National Food Agency of Denmark (DANKOST 2.0) was used to calculate energy and nutrient intakes (14).

Urine samples

Subjects collected 24-h urine samples during the sixth day and night of every dietary record period (weeks 0, 5, 10) to validate the dietary records. During these 24-h periods, subjects ingested a paraaminobenzoic acid (PABA) pill with the 3 main meals (a total of 240 mg PABA/d) to serve as an indicator of complete urinary collection (15). Urine samples containing <85% recovered PABA were excluded from further analyses. The volume and density of each 24-h urine collection were determined, and a 2-mL sample was frozen at −20 °C until further analyses. The nitrogen content was determined in 30 μL urine on a nitrogen analyzer (NA 1500 Carlo Erba; Fisons Instruments, Milano, Italy). PABA was determined spectrophotometrically (Bodenseewek Perkin-Elmer & Co GmbH, Überlingen, Germany). The conversion of urinary protein to ingested protein and calculation of dietary protein recovery has been reported elsewhere (11).

Assays

Blood samples for glucose analysis were drawn into a test tube containing EDTA and fluoride, and blood samples for insulin, triacylglycerol, CRP, haptoglobin, and transferrin analyses were drawn into tubes with no additives. The tubes (except for insulin analyses) were kept on ice. Plasma glucose was analyzed by
standard enzymatic methods (Boehringer Mannheim GmbH Diagnostic, Copenhagen, Denmark). Insulin concentrations in serum were measured by using radioimmunoassay (AutoDelfi Automatic Immunoassay System; Wallac Oy, Turku, Finland). Triacylglycerol concentrations in serum were analyzed by using the Test-Combination Triacylglycerols GPO-PAP method (Boehringer Mannheim GmbH Diagnostic). CRP concentration was measured by using the CRP (Latex) ultrasensitive assay (Roche Diagnostic Systems, Basel, Switzerland). Haptoglobin and transferrin were analyzed by using the immunoturbidimetric method (Roche Diagnostic Systems).

To assess insulin resistance the homeostasis model (HOMA IR) was used. The formula for the HOMA IR model is fasting insulin (in μU/mL) × fasting glucose (in mmol/L)/22.5 (16).

Statistical analyses

Unpaired t test was used to test initial group differences. Repeated measures analysis of covariance (ANCOVA) was used to test interaction between diet groups (sucrose and sweetener) and time (5 and 10 wk). The mixed procedure in the STATISTICAL ANALYSIS SYSTEM (SAS) software package, version 8.2 was used (SAS Institute, Cary NC). Baseline values were included as covariate. When interaction of diet group and time was significant, Tukey’s post hoc tests were applied.

The effect of diet on body weight, FM, CRP, haptoglobin, transferrin, triacylglycerol, HOMA IR, and blood pressure was tested by using ANCOVA with the mixed procedure in SAS; with week 10 values as response and week 0 values as covariate and subjects as random factor (Table 3). When adjusting for body weight changes or changes in energy intake, ANCOVA with week 10 values as response and week 0 values and body weight changes or changes in energy intake as covariate was used. Log transformation of data with skewed distributions was done when necessary.

RESULTS

The subjects were randomly assigned to 2 intervention groups, which turned out to be well matched for sex, age, body size and composition, and habitual levels of physical activity (Table 1). Forty-one subjects completed the 10-wk study, but 4 subjects (2 from each group) had concentrations of CRP > 10 mg/L, either at baseline or at the end of the intervention, which could indicate infection. Therefore, statistical analyses were done both with and without these 4 subjects. Only the results for inflammatory markers varied with and without the 4 subjects; therefore, only these results are presented with the outcome from both analyses.

<table>
<thead>
<tr>
<th></th>
<th>Sucrose group (n = 21)</th>
<th>Sweetener group (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>33.4 ± 9.0</td>
<td>37.1 ± 10.0</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>82.5 ± 7.7</td>
<td>79.2 ± 8.9</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.71 ± 0.07</td>
<td>1.69 ± 0.07</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.0 ± 2.3</td>
<td>27.6 ± 2.1</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>29.1 ± 4.5</td>
<td>27.9 ± 4.5</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.79 ± 0.02</td>
<td>0.77 ± 0.01</td>
</tr>
</tbody>
</table>

* All values are $\bar{x} \pm SD$. No significant differences were observed between the groups (unpaired t test).

Food intake

The records of ad libitum food intake (including food and drink supplements) showed that there were no significant differences between week 5 and week 10 values for any of the variables in the same diet group (Table 2). Although a significant interaction was observed between diet and time for several of the variables, the Tukey-Kramer adjusted post hoc tests showed that there were no within-group changes from week 5 to week 10 (Table 2).

The food records showed that there was a significantly higher intake of carbohydrate (both in gram and percent of energy) in the sucrose group than in the sweetener group during the intervention (Table 2). The increase in carbohydrate intake in the sucrose group was mainly due to an increased sucrose intake. Sucrose intake (both in gram and percent of energy) and the percentage of energy from fat and protein were higher in the sucrose group than in the sweetener group during the intervention (Table 2). The average difference in total energy intake between the 2 groups was 2.7 MJ/d during the intervention (Table 2). Changes in energy intake correlated positively with changes in sucrose intake ($r = 0.73, P < 0.0001$). The energy density of the diet was significantly lower in the sweetener group than in the sucrose group during the intervention. No differences were observed between the groups in intake of dietary fiber (g/d); total fat (g/d); saturated, monounsaturated, and polyunsaturated fat (g/d); protein (g/d); alcohol (g/d; percent of energy); and total weight of food (g/d).

Validation of protein intake

Urinary protein excretion was estimated in 91 urine samples after excluding 32 samples (17 from the sucrose group and 15 from the sweetener group) that were incomplete, as indicated by a recovery < 85% of PABA. Data were available from 15 subjects in each group at each time point, with the exception of the sucrose group at week 10 ($n = 16$). The differences between urinary protein and self-reported dietary protein ranged from 1 to 13 g/d with no significant differences between groups and times. Dietary protein recovery ranged from 103% to 119%, also with no significant differences between groups and times. Urinary protein correlated significantly with dietary protein at all 3 time points, with the strongest correlations at weeks 5 and 10 (week 0: $r = 0.39, P < 0.05$; week 5: $r = 0.52, P < 0.01$; week 10: $r = 0.53, P < 0.01$).

Serum concentrations of inflammatory markers

At baseline no differences were observed in clinical and biochemical characteristics between the sucrose group and the sweetener group (Table 3). After 10 wk, intervention mean serum CRP concentrations had decreased by 13% in the sucrose group and by 22% in the sweetener group (between-group difference: $P = 0.32$). After excluding 4 subjects with CRP concentrations > 10 mg/L, an increase in mean CRP concentrations by 6% in the sucrose group and a decrease by 26% in the sweetener group were found (between-group difference, $P = 0.1$) (Table 3). No correlation was observed between dietary changes and changes in CRP.

After 10 wk, serum concentrations of haptoglobin, using data from all subjects, had increased by 6% in the sucrose group and decreased by 15% in the sweetener group (between-group differences: $P = 0.02$). Serum transferrin concentrations increased.
Serum concentrations of triacylglycerol and HOMA IR and measurement of blood pressure

No differences were seen between the sucrose and the sweetener groups in triacylglycerol and HOMA IR concentrations during the intervention, both before and after exclusion of the 4 subjects (Table 3). No association was observed between changes in CRP and changes in triacylglycerol and HOMA IR. After 10 wk of intervention, systolic and diastolic blood pressure had increased in the sucrose group and decreased in the sweetener group, resulting in significant between-group differences (Table 3). No association was observed between changes in blood pressure and changes in CRP, haptoglobin, or transferrin.

Body weight

Body weight and FM increased in the sucrose group (1.6 ± 0.5 and 1.3 ± 0.5 kg, respectively) and decreased in the sweetener group (1.2 ± 0.5 and 0.3 ± 0.4 kg, respectively) during the 10-wk intervention. This finding resulted in significant between-group differences, amounting to 2.8 ± 0.7 kg body weight (P < 0.001) and 1.6 ± 0.7 kg body fat (P < 0.01) after 10 wk.
TABLE 3
Inflammatory markers, triacylglycerol, homeostasis model for insulin resistance (HOMA IR), and blood pressure before (week 0) and after 10 wk of intervention in the sucrose group and the sweetener group after the exclusion of 4 subjects.

<table>
<thead>
<tr>
<th></th>
<th>Week 0 (baseline)</th>
<th>Week 10</th>
<th>Week 10 adjusted for baseline values and body weight changes</th>
<th>Week 10 adjusted for baseline values and changes in energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.8 (0.9–3.0)</td>
<td>2.2 (1.2–3.0)</td>
<td>2.1 (1.5, 2.8)</td>
<td>0.10</td>
</tr>
<tr>
<td>Sweetener</td>
<td>1.8 (0.8–4.5)</td>
<td>1.3 (0.8–2.8)</td>
<td>1.4 (1.0, 1.9)</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>138 ± 13</td>
<td>156 ± 15</td>
<td>149 (130, 171)</td>
<td>0.006</td>
</tr>
<tr>
<td>Sweetener</td>
<td>156 ± 15</td>
<td>132 ± 14</td>
<td>112 (98, 129)</td>
<td></td>
</tr>
<tr>
<td>Transferrin (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>296 (264–344)</td>
<td>312 (288–344)</td>
<td>316 (305, 328)</td>
<td>0.01</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.1 (0.8–1.8)</td>
<td>1.2 (0.9–2.3)</td>
<td>1.2 (1.0, 1.4)</td>
<td>0.28</td>
</tr>
<tr>
<td>Sweetener</td>
<td>1.1 (0.8–1.2)</td>
<td>0.9 (0.8–1.1)</td>
<td>1.1 (0.9, 1.3)</td>
<td></td>
</tr>
<tr>
<td>HOMA IR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.0 (0.8–1.5)</td>
<td>1.3 (0.9–1.8)</td>
<td>1.3 (1.1, 1.6)</td>
<td>0.17</td>
</tr>
<tr>
<td>Sweetener</td>
<td>1.1 (0.7–1.5)</td>
<td>1.2 (0.7–1.9)</td>
<td>1.1 (0.9, 1.3)</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>117.2 ± 1.9</td>
<td>122.3 ± 2.4</td>
<td>121.8 (118.3, 125.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Sweetener</td>
<td>116.1 ± 2.1</td>
<td>113.1 ± 2.2</td>
<td>113.6 (110.0, 117.1)</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>71.2 ± 1.6</td>
<td>75.6 ± 2.0</td>
<td>76.2 (73.3, 79.1)</td>
<td>0.037</td>
</tr>
<tr>
<td>Sweetener</td>
<td>73.3 ± 2.2</td>
<td>72.4 ± 1.5</td>
<td>71.7 (68.8, 74.7)</td>
<td></td>
</tr>
</tbody>
</table>

1 Four subjects were excluded because their C-reactive protein concentrations were >10 mg/L, indicating possible infection. CRP, C-reactive protein; BP, blood pressure.
2 Values are ± SEM or median (interquartile range) for variables with skewed distributions. n = 19 in the sucrose group and n = 18 in the sweetener group.
3 There were no significant differences in baseline values between the 2 groups (unpaired t test).
4 Values are least-squared means of 10-wk values adjusted for baseline values; 95% CI in parentheses.
5 P values were derived by analysis of covariance with week 10 values as the response and week 0 values as the covariate.
6 Values are least-squared means of 10-wk values adjusted for baseline values and body weight changes; 95% CI in parentheses.
7 P values were derived by analysis of covariance with week 10 values as the response and week 0 values and changes in body weight as the covariates.
8 Values are least-squared means of 10-wk values adjusted for baseline values and changes in energy intake; 95% CI in parentheses.
9 P values were derived by analysis of covariance with week 10 values as the response and week 0 values and changes in energy intake as the covariates.
10 HOMA IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.

DISCUSSION

In the present study the sucrose diet produced a nonsignificant 6% increase and the sweetener diet produced a 26% decrease in CRP concentrations (P = 0.1). This trend toward an association between a diet high in rapidly digested and absorbed carbohydrates and CRP supports the findings of an earlier observational study (10). In the observational study the CRP concentrations were measured in 244 apparently healthy women, and the association between the subjects’ dietary glycemic load and CRP concentrations was examined (10). It was found that the median CRP concentration for the lowest quintile of dietary glycemic load was significantly lower than the CRP concentration for the highest quintile. This result remained after adjusting for several confounders such as age, BMI, history of hypertension, high cholesterol or diabetes mellitus, and lifestyle factors.

The lack of statistical significance in the present study may be due to a type II error as a result of the small number of subjects, which was only 19 and 18, respectively, in the 2 groups. However, the changes in CRP were relatively small, and, by taking into consideration that the reliability of CRP is poor (17), the results suggest that sucrose has little effect on CRP.

The sucrose diet increased concentrations of haptoglobin, and changes in haptoglobin concentrations were positively associated with changes in sucrose intake. This finding could indicate increased inflammation in the sucrose group and decreased inflammation in the artificial sweetener group. However, changes in haptoglobin concentrations were positively correlated with changes in energy intake, which suggests that haptoglobin may respond to the body’s energy flux rather than to the sucrose intake. However, it is not possible to reach a firm conclusion on this aspect because of the close covariation between energy and sucrose intakes.

Transferrin is a negative acute-phase protein, and a drop in transferrin concentrations therefore reflects increased inflammation. It was therefore contrary to our expectations that transferrin increased in the sucrose group and decreased in the sweetener group. Changes in transferrin concentrations were also positively associated with changes in energy intake, which could indicate
that transferrin responds to the body’s energy flux. Adjusting for changes in energy intake did not eliminate the difference between the 2 groups, in either haptoglobin or transferrin, although the differences became less significant.

Earlier studies that investigated the effect of calorie-restricted diets (18), partial fasting (19), and total fasting (20), on various blood markers, have shown that concentrations of haptoglobin and transferrin decrease when subjects are in negative energy balance. In the study that investigated total fasting (18), transferrin concentrations rose again when the subjects received an 800-kcal diet (re-alimentation), although they were still in negative energy balance. Furthermore, haptoglobin started to increase already during the last period of fasting and increased further during re-alimentation.

The present study was originally designed to test the effects of sucrose compared with artificial sweeteners on changes in intakes of ad libitum energy and macronutrients, body weight, and body composition (11). The decision to analyze frozen plasma was taken >5 y after the study was completed and after the results of Liu et al (10) had been published, which showed an association between glycemic load and CRP. It should be noted that CRP, transferrin, and haptoglobin are stable over long periods and have no diurnal variation (21). Using the original design to test the effect of a high glycemic load on inflammatory activity introduced a confounding difference in energy intake and change in body weight between the 2 diets. This difference could give rise to problems in interpreting the results, because overweight and obesity have been shown to be associated with elevated concentrations of inflammatory markers (19, 20, 22–24). The sucrose diet in our study caused an increase in total caloric intake and a weight gain of 1.6 kg in contrast to a weight loss of 1.2 kg in the group receiving the artificially sweetened drinks and foods. The minor changes in body weight did not have any significant effect on the differences in inflammatory markers between the 2 groups. In a recent study by Engström et al (9) inflammatory markers were linked to weight gain and obesity, and the researchers suggested that relations between inflammatory markers and weight gain reflect dietary factors that increase both weight and inflammatory markers. This suggestion is supported by our results, showing that changes in CRP and haptoglobin were unaffected when adjusted for body weight changes.

We conducted the present study in overweight subjects, because increased fatness is associated with increased inflammation (19, 20, 22–24). It may be possible that the subjects’ inflammatory response is dampened by previous exposure to sucrose, but the group receiving artificial sweetener acted as a control group. One could expect a decrease in CRP if sucrose intake has an influence on inflammatory activity.

Whereas the strength of the randomized design is a minimization of possible confounders, dietary trials such as the present one cannot be conducted in a double-blind fashion, which gives rise to other limitations. Although we aimed to conduct the trial in a blinded fashion (no information about the type of sweetener on the packaging of the drinks or foods), it was obvious that many participants could identify the artificial sweeteners by taste (11). Exercise is suggested to reduce inflammation (25), and, if the subjects who knew that they were getting the sucrose diet were more likely to exercise to compensate for the higher energy intake, then the markers of inflammation in the sucrose group would be artificially low. It does not seem to be a problem in this study because there was no significant between-group differences in the changes in the amount of physical activity or in the level of physical activity as recorded by the subjects after the 10-wk intervention (11).

In the present study no biological markers were available for objective information about dietary intake of fat and carbohydrate. The 24-h urinary nitrogen excretion was used as a marker to validate the dietary protein records, and the results show that significant agreement existed between the reported dietary protein intake and the urinary protein. A significant increase in body weight in the sucrose group was observed, which corresponds to the increased energy intake recorded during the intervention. In the sweetener group a significant decrease in body weight was observed, even though no significant decrease was observed in energy intake (594 kJ). However, 594 kJ/d over 70 d (41.6 MJ) would give rise to a weight loss of 1.2 kg (assuming 34 MJ/kg weight loss) (26), which corresponds to the observed weight loss of 1.2 kg. Thus, these results suggest that the compliance was good in both intervention groups.

The question is whether the intake of sucrose from soft drinks in the present study is realistic. In the sucrose group ≈70% of the sucrose came from drinks (average: ≈1.3 L/d) and ≈30% came from solid foods. This is actually similar to the mean volume and sugar content of the diet of American boys in 1996, slightly above that of American girls (27), and somewhat below the mean consumption of American adults (28). The results should therefore be applicable to a large proportion of the American population.

In conclusion, this study shows that, apart from causing weight gain and increasing blood pressure, a high consumption of sugar-sweetened drinks and foods may increase inflammatory activity in overweight subjects. This finding was independent of weight changes. The relative changes in inflammatory markers in the present study were small. Even though the between-group differences in haptoglobin and transferrin were statistically significant, it is doubtful that these differences are biologically important.

We thank Tatjana H Vasilaras and Christina Møller for conducting the study and the technical staff and students at the Department of Human Nutrition and the Department of Cardiology for help with data collection and analyses. We gratefully acknowledge the participation of all the persons involved in the study.

AR and AA designed the core study, AR lead the conduct of the study, and both participated in the interpretation of the results. AA and SS generated the idea to analyze inflammatory markers in this study. LBS was responsible for the statistical analysis, and AA and LBS wrote the initial draft of the manuscript. All authors have read and corrected the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES


Sarcopenia, obesity, and inflammation—results from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors study1–3


ABSTRACT

Background: Age-related body-composition changes are associated with health-related outcomes in elders. This relation may be explained by inflammation and hemostatic abnormalities.

Objectives: Our objectives were to evaluate the relation between body-composition measures [body mass index (BMI), total fat mass, and appendicular lean mass (aLM)] and C-reactive protein (CRP), interleukin 6 (IL-6), and plasminogen activator inhibitor 1 (PAI-1) and to explore the effect of obesity and sarcopenia on CRP, IL-6, and PAI-1 concentrations.

Design: The data are from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors (TRAIN) study baseline visit (n = 286; mean age = 66.0 y). Total fat mass and aLM were assessed with a dual-energy X-ray absorptiometry scan. Linear regressions were performed between body-composition measures and CRP, IL-6, or PAI-1 concentrations. The effect of sarcopenia and obesity (defined as the percentage of fat mass) on CRP, IL-6, and PAI-1 concentrations was evaluated with the use of analyses of covariance.

Results: CRP and IL-6 were positively associated with both BMI [β = 0.027 (P = 0.03) and β = 0.048 (P < 0.001), respectively] and total fat mass [β = 0.049 (P < 0.001) and β = 0.055 (P < 0.001), respectively] and were inversely associated with fat-adjusted aLM [β = −0.629 (P = 0.002) and β = −0.467 (P = 0.02), respectively]. PAI-1 was positively associated with both BMI (β = 0.038, P = 0.005) and total fat mass (β = 0.032, P = 0.007). No significant interaction was found between either obesity or sarcopenia and CRP, IL-6, and PAI-1 concentrations. Obesity remained significantly associated with high CRP and IL-6 concentrations after adjustments for sarcopenia.

Conclusions: CRP and IL-6 are positively associated with total fat mass and negatively associated with aLM. Obesity-associated inflammation may play an important role in the age-related process that leads to sarcopenia. The relation of inflammation with sarcopenia was not independent of any of the considered obesity indexes. Am J Clin Nutr 2005;82:428–34.

KEY WORDS Sarcopenia, obesity, inflammation, fibrinolysis, skeletal muscle, aging

INTRODUCTION

Sarcopenia, defined as the involuntary loss of skeletal muscle that occurs with advancing age (1, 2), is an important correlate of impairment and physical disability in older persons (1–5) and is associated with a decrease in muscular strength and endurance and a loss of autonomy in older persons (5–9). The age-related decrease in muscle mass and strength is mainly caused by atrophy of muscle fibers, especially the type IIa fibers (1). This is associated with a decline in protein synthesis, particularly in the synthesis of myosin heavy chains (1). It has been suggested that this loss of muscle mass is not isolated, but is strongly connected with a parallel increase in fat mass (10). This mechanism may lead to the concomitant presence of sarcopenia and obesity (2, 10).

Inflammation is linked not only to physical disability (11–13), but also to obesity (14, 15) and to body composition (16). Cytokines, which are produced by adipocytes, may have a direct effect on physical function by accelerating the changes in body composition that are typical of the aging process, namely fat gain and loss of muscle mass (12). Moreover, the endocrine role played by adipose tissue has been suggested as an explanation for the health-related events that are associated with increased fat mass, such as cardiovascular diseases (14, 17). Furthermore, a strong relation between inflammation and hemostatic abnormalities has been shown in clinical conditions that are characterized by body-composition changes, such as insulin resistance syndrome (18).

Despite the growing interest in age-related body-composition changes that may explain major negative outcomes in older...
persons, limited evidence is available about the relation of sarcopenia and obesity with markers of inflammation and fibrinolysis. Of the few studies available, Visser et al (19) reported an inverse association of muscle mass and strength with concentrations of interleukin 6 (IL-6) and tumor necrosis factor \( \alpha \).

The present study evaluated the cross-sectional relations between 2 measures of sarcopenia that are based on appendicular lean mass (20, 21) and a proinflammatory cytokine (IL-6), an acute phase protein [C-reactive protein (CRP)], and an inhibitor of the fibrinolytic process [plasminogen activator inhibitor 1 (PAI-1)]. We also investigated the relation between markers of inflammation and fibrinolysis and body mass index (BMI) and total fat mass. Moreover, we investigated the effects of sarcopenia and obesity (as dichotomous variables) on CRP, IL-6, and PAI-1 concentrations. To provide a more accurate estimate of fat mass than the one provided by BMI, obesity was defined in this instance according to age-, sex-, and race-specific cutoffs of body fat mass (22).

SUBJECTS AND METHODS

Subjects

The present analyses were performed with the use of data from the baseline evaluation of participants who were enrolled in the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors (TRAIN) study. The TRAIN study is a double-blind, crossover, randomized, placebo-controlled trial aimed at assessing the biological mechanisms by which angiotensin-converting enzyme (ACE) inhibition may improve clinical outcomes in persons aged \( >55 \) y who have a high cardiovascular disease risk profile.

To be included in the TRAIN study, the participants had to meet more than one of the following criteria: 1) coronary heart disease, 2) peripheral vascular disease, 3) history of stroke \( (>6 \) mo), 4) diabetes along with more than one other cardiovascular disease risk factor [hypertension, total cholesterol \( >200 \) mg/dL, HDL cholesterol <40 mg/dL, or men or <50 mg/dL for women, triacylglycerols \( \geq 150 \) mg/dL, current cigarette smoking, BMI (in kg/m\(^2\)) \( \geq 30 \)], known microalbuminuria, or any evidence of previous vascular disease), and 5) evidence of clinical or subclinical cardiovascular disease and another risk factor (same as in point 4). Exclusion criteria for the TRAIN study were the current use of or a known hypersensitivity to ACE inhibitors, a diagnosis of specific cardiovascular conditions (including previous myocardial infarction, ejection fraction <40%, syncopal episodes that were likely because of life-threatening arrhythmias, or planned cardiac surgery or angioplasty within 3 mo), conditions that would affect results of the trial (significant renal disease, life-threatening illness, recent surgical procedure, or simultaneous enrollment in another experimental drug trial), or plans to leave the area in the next 3 mo. All the inclusion and exclusion criteria were based on medical history, reviews of medical records, physical examinations, and the laboratory data of participants.

The participants were recruited from the communities of Winston-Salem and Greensboro, NC, through several recruitment strategies. The participants were first screened by a phone interview for eligibility \( (n = 2347) \); Figure 1. A clinical prescreening visit, which was aimed at reviewing medical history and records of potential participants, was arranged for those participants who successfully completed the phone interview \( (n = 576) \). The subjects who successfully completed the prescreening visit \( (n = 401) \) were entered in a screening and single-blind run-in phase, in which compliance and the tolerability of the ACE inhibitor fosinopril were evaluated. The participants who successfully completed all of the preliminary phase interviews and visits \( (n = 295) \) were then randomly assigned to either the placebo or the intervention branch (treatment with fosinopril) of the study during the baseline clinical visit. After 6 mo and 3 midterm follow-up clinical visits, the crossover occurred. After a further 6 mo and 3 midterm clinical visits, the participants made closeout visits. For the present analysis, we used baseline cross-sectional data from 286 participants, after excluding participants \( (n = 9) \) with missing values for inflammatory markers or body-composition variables. All of the participants signed an informed consent form for the study at the screening visit. The Institutional Review Board of Wake Forest University approved the study protocol.

Markers of inflammation and fibrinolysis

To minimize circadian rhythm and other fluctuations in the markers’ concentrations, all of the participants had their blood drawn by venipuncture after fasting for 6 h in the mornings of the screening and of each clinical visit. After the specimens were processed in the cold within 1 h of collection, they were divided into aliquots and placed into cryovials, which were then frozen and stored at \(-70^\circ C\) until analyzed.

Serum concentrations of IL-6 were assessed with a high-sensitivity immunoassay kit (R&D Systems, Minneapolis, MN), which has synthetic peptides and highly specific monoclonal antibodies. The immunoassay had a sensitivity of <0.10 pg IL-6/mL and an expected detection range of 0.15–10.0 pg IL-6/mL.

FIGURE 1. Crossover design for the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors (TRAIN) study.
Serum concentrations of CRP were measured with an enzyme-linked immunosorbent assay with the use of purified protein and polyclonal anti-CRP antibodies (Calbiochem, San Diego, CA). The CRP assay had a sensitivity of 0.08 μg/μL and a standard reference range of 0.5-2.5 mg/L. In our previous studies, the mean CV for CRP across assay runs was 4.2% (23). Serum concentrations of PAI-1 antigen were measured with a 2-site enzyme-linked immunosorbent assay. The analytic CV for this assay is 3.47%.

**BMI and body-composition measures**

For the present analyses we related CRP, IL-6, and PAI-1 concentrations to BMI and total fat mass. BMI was defined as body weight (in kg)/height² (in m). Weight was measured with the use of a Delphi (Hologic Inc, Bedford, MA) dual-energy X-ray absorptiometry (DXA) scan. Height was self-reported by the participants. Total fat mass was defined as the percentage of fat mass, relative to whole body mass, that was determined by the DXA. These 2 measures of obesity and adipose tissue were considered as predictive variables in our models because the production of inflammatory and fibrinolysis markers from adipocytes was already shown (18, 24).

We also explored the relation between markers of inflammation and fibrinolysis and 2 different measures of sarcopenia based on appendicular lean mass (20, 21). Appendicular lean mass was defined as the sum of the lean mass (in kg) that was measured in each participant’s arms and legs with a DXA scan. The first measure, fat-adjusted appendicular lean mass, was defined with the use of the residuals of a linear regression model that predicted the dependent variable appendicular lean mass (in kg) from height (in m) and total fat mass (in kg) (20). Newman et al (20) suggested that for a better estimate of sarcopenia, especially in women and obese individuals, fat mass should be considered together with muscle mass. Moreover, the measure of muscle mass adjusted for fat mass was shown to be associated with lower extremity functional limitation (20). A positive fat-adjusted appendicular lean mass indicates a relatively muscular individual, whereas negative values indicate relatively sarcopenic individuals. A second measure of sarcopenia was defined as the residuals of a linear regression analysis between the dependent variable appendicular lean mass (in kg) and height (in m). Fat-adjusted and non-fat-adjusted appendicular lean mass were considered as dependent variables in our analyses, which explored their relation with CRP, IL-6, and PAI-1 because of the reported negative effects of inflammation on muscle mass (16, 25, 26).

To provide an accurate definition of obesity, we used the age-, sex-, and race-specific cutoffs for percentage total body fat (assessed with a DXA scan) previously reported by Gallagher et al (22). We defined obesity on the basis of percentage total body fat rather than on the basis of the more common BMI ranges to avoid misclassification as a result of 1) increases in fat that occur with aging even when BMI remains constant, 2) ethnic differences, or 3) differences in health status between the subjects and their counterparts with similar BMIs. On the basis of these cutoffs (22), persons aged <60 y were defined as obese if the percentage of body fat was >29% in white men, >27% in African American men, >29% in Asian men, >41% in white women, >39% in African American women, and >41% in Asian women. Participants from other ethnic groups or aged >80 y (n = 17), for which these estimates were not available, were excluded from these analyses.

**Covariates**

Sociodemographic characteristics (age, sex, race, and smoking status), self-reported clinical conditions (diabetes, angina, myocardial infarction, cancer, and stroke), and serum albumin concentrations were considered potential confounders of the relation between body-composition measures and markers of inflammation and fibrinolysis. Serum albumin concentrations were measured with a Roche-Hitachi Modular System-Modular D (Roche Diagnostics, Tokyo, Japan).

**Statistical methods**

Means (±SDs) and proportions were calculated for all of the variables of interest. Median values with 25th-75th percentile ranges were reported for nonnormally distributed variables. Given the nonnormal distribution of CRP, IL-6, and PAI-1, these marker concentrations were log transformed to make them normally distributed. To permit direct comparisons between CRP, IL-6, and PAI-1 concentrations, all of the results are shown per SD increases in log biomarker. Unadjusted and adjusted linear regression analyses were used to identify regression coefficients (with SE) in BMI and body-composition measures for CRP, IL-6, and PAI-1 concentrations. In the analysis of the relation between fat measures (BMI and percentage of total fat) and markers of inflammation and fibrinolysis, the latter were used as dependent variables. Fat-adjusted and nonfat-adjusted appendicular lean masses were considered dependent variables when analyses were performed between lean measures and markers of inflammation and fibrinolysis. Race and sex interactions were assessed by adding the interaction term for sex (or race) × marker concentrations in the adjusted model. Two-factor analyses of covariance were used to assess the possible interaction of sarcopenia and obesity with CRP, IL-6, or PAI-1 (in log values). If the interaction term was not significant, the models were reperformed after the term exclusion. Obesity was defined as previously described (22). Sarcopenia was defined by the lowest sex-specific tertile of fat-adjusted appendicular lean mass; the cutoffs for residuals of fat-adjusted appendicular lean mass were −0.47135 for the men and −2.79396 for the women. The statistical software SPSS version 10.1.0 (SPSS Inc, Chicago, IL) was used for the statistical analyses.

**RESULTS**

The main sociodemographic characteristics of the sample population (n = 286) are shown in Table 1. The mean (±SD) age of the participants was 66.0 ± 7.4 y. Male sex (57.0%) and white race (75.2%) were the predominant characteristics.

Unadjusted and adjusted linear regression models were fit between body-composition measures and markers of inflammation and fibrinolysis (per SD increase). The association between the inflammatory and the fibrinolysis markers (the dependent variables) and obesity and adipose tissue measures (BMI and percentage of total fat mass) are presented in Table 2. Even after adjustment for potential confounders (age, sex, race, smoking, diabetes, angina, myocardial infarction, cancer, stroke, and serum albumin concentrations), serum concentrations of CRP were
positively and significantly associated with both BMI ($\beta = 0.027, SE = 0.013, P = 0.03$) and total fat mass ($\beta = 0.049, SE = 0.011, P < 0.001$); serum concentrations of IL-6 were also positively and significantly associated with both BMI ($\beta = 0.048, SE = 0.013, P < 0.001$) and total fat mass ($\beta = 0.055, SE = 0.012, P < 0.001$). Significant results were also found from adjusted linear regression models between PAI-1 and both BMI ($\beta = 0.038, SE = 0.013, P = 0.005$) and total fat mass ($\beta = 0.032, SE = 0.012, P = 0.007$).

The results from linear regression models that explored the relation between appendicular lean mass measures (dependent variables) and inflammatory and fibrinolysis markers are reported in Table 3. Negative and statistically significant associations were reported between fat-adjusted appendicular lean mass and both CRP ($\beta = -0.629, SE = 0.199, P = 0.002$) and IL-6 ($\beta = -0.467, SE = 0.193, P = 0.02$) and between appendicular lean mass and CRP ($\beta = -0.428, SE = 0.210, P = 0.04$).

No significant race or sex interactions were found for the relation of CRP, IL-6, and PAI-1 with either BMI or measures of body composition ($P > 0.05$ for all).

Finally, we performed 2-factor analyses of covariance to evaluate the interaction between sarcopenia (defined as the lowest sex-specific tertile of fat-adjusted appendicular lean mass) and obesity [defined as the age-, sex-, and race-specific percentage of total fat mass (22)] and CRP, IL-6, and PAI-1 concentrations, but no significant interactions were found ($P > 0.1$ for all interactions). In models that included only sarcopenia and obesity as factors, obese participants had significantly ($P < 0.01$ for all) higher concentrations of both CRP (log value: 1.349; 95% CI: 1.125, 1.574) and IL-6 (log value: 1.258; 95% CI: 1.156, 1.359) than did nonobese participants [log values (95% CI) for CRP and IL-6: 0.770 (0.602, 0.938) and 1.074 (0.997, 1.151), respectively]. No significant differences for PAI-1 or between sarcopenic and nonsarcopenic participants were found.

**DISCUSSION**

In the present study we explored the relations between inflammatory and fibrinolysis markers (CRP, IL-6, and PAI-1) and BMI, total fat mass, and 2 measures of sarcopenia. Our findings showed that all of the considered markers of inflammation and fibrinolysis were strongly correlated with obesity and total fat mass. Concentrations of CRP and IL-6, but not of PAI-1, were inversely associated with fat-adjusted appendicular lean mass. In our sample population, CRP concentrations showed a slightly stronger association with sarcopenia than did IL-6 concentrations. Our findings also showed that the association of higher concentrations of inflammatory markers with sarcopenia was mostly explained by the concurrent presence of adipose tissue.

### Table 1
Sociodemographic characteristics of the sample population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value (n = 286)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>66.0 ± 7.4$^1$</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>43.0</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>75.2</td>
</tr>
<tr>
<td>African American</td>
<td>22.7</td>
</tr>
<tr>
<td>Asian</td>
<td>0.7</td>
</tr>
<tr>
<td>Other</td>
<td>1.4</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>11.5</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>22.0</td>
</tr>
<tr>
<td>Angina (%)</td>
<td>7.7</td>
</tr>
<tr>
<td>Myocardial infarction (%)</td>
<td>5.2</td>
</tr>
<tr>
<td>Cancer (%)</td>
<td>14.7</td>
</tr>
<tr>
<td>Stroke (%)</td>
<td>8.0</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>2.51 (1.06–5.04)$^2$</td>
</tr>
<tr>
<td>Interleukin 6 (pg/mL)</td>
<td>2.91 (2.24–3.93)</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1 (ng/mL)</td>
<td>31.67 (16.53–53.51)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>28.5 ± 4.8</td>
</tr>
<tr>
<td>Total fat mass (%)</td>
<td>32.1 ± 8.3</td>
</tr>
<tr>
<td>Appendicular lean mass (kg)</td>
<td>24.1 ± 5.9</td>
</tr>
</tbody>
</table>

$^1$ ± SD (all such values).

$^2$ Median; 25th–75th percentile range in parentheses (all such values).

### Table 2
Association between body fat measures and concentrations of C-reactive protein, interleukin 6, and plasminogen activator inhibitor 1 (dependent variables) per SD increase in log value$^3$

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>Adjusted$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ (SE)</td>
<td>$P^4$</td>
</tr>
<tr>
<td>C-reactive protein$^4$</td>
<td>0.036 (0.012)</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>0.043 (0.007) &lt;0.001</td>
<td>0.049 (0.011) &lt;0.001</td>
</tr>
<tr>
<td>Total fat mass (%)</td>
<td>0.039 (0.012) 0.002</td>
<td>0.048 (0.013) &lt;0.001</td>
</tr>
<tr>
<td>Interleukin 6$^5$</td>
<td>0.022 (0.007) 0.003</td>
<td>0.055 (0.012) &lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>0.037 (0.012) 0.003</td>
<td>0.038 (0.013) 0.005</td>
</tr>
<tr>
<td>Total fat mass (%)</td>
<td>−0.001 (0.007) 0.99</td>
<td>0.032 (0.012) 0.007</td>
</tr>
</tbody>
</table>

$^1$ n = 286.

$^2$ Adjusted for age, sex, race, smoking, diabetes, angina, myocardial infarction, cancer, stroke, and serum albumin concentrations.

$^3$ Linear regression models.

$^4$ SD for log = 1.09424.

$^5$ SD for log = 0.48534.

$^6$ SD for log = 0.83576.
Sarcopenia, which is the involuntary loss of skeletal muscle that occurs with advancing age (1), is characterized by a decrease of muscular strength and endurance and is significantly associated with a loss of autonomy in older persons (1, 27). In a review, Roubenoff (10) suggested the presence of a vicious cycle between fat gain and loss of muscle, which act synergistically to lead to physical disability (2). In fact, even if the muscle loss is the result of several different underlying causes, muscle loss still represents the major contributor to fat gain, which in turn reinforces muscle loss. The loss of metabolically active cell mass and the weakness that results from the loss of muscle lead to a reduction in resting metabolic rate and physical activity. With an increased fat mass and a reduced muscle mass, physical activity becomes progressively more difficult, and the habitual level of physical activity decreases further. This mechanism leads to sarcopenic obesity, a major risk factor for the onset of physical disability (2, 7, 28). Our findings from the present study support this hypothesis. In fact, inflammatory markers were strongly and negatively associated with fat-adjusted appendicular lean mass; only CRP showed low significance when we tested a lean mass measure without taking fat mass into account. This finding supports evidence that suggests that fat mass should be considered in estimates of the prevalence of sarcopenia (2, 20).

In the present study, we also explored the possible presence of an interaction of sarcopenia and obesity with concentrations of inflammatory markers. No interactions were found. Our analyses showed that obesity explains most of the association between sarcopenia and inflammation. In fact, when the dichotomous variables of obesity and sarcopenia were simultaneously added to the models, only the former remained significantly associated with inflammatory marker concentrations.

Inflammation has been indicated as a potential explanation to the age-related changes in body composition that lead to sarcopenia (1, 9, 16, 26, 29, 30). Several theories hypothesize that inflammation plays an important role during the aging process (31, 32), and a significant increase in production of IL-6 has been reported with advancing age (33). However, the relation between inflammation, adipose tissue, and muscle mass is very close. Major inflammatory markers, such as cytokines, are secreted by adipocytes, and their concentrations have been shown to be associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40).

Previous studies have reported that weight gain and lean tissue anabolism are associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40). Previous studies have reported that weight gain and lean tissue anabolism are associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40). Previous studies have reported that weight gain and lean tissue anabolism are associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40). Previous studies have reported that weight gain and lean tissue anabolism are associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40). Previous studies have reported that weight gain and lean tissue anabolism are associated with obesity (14, 24, 34, 35). At the same time, evidence from humans (25, 36) as well as animal models (37–39) shows a direct negative influence of cytokines on muscle mass, and increased concentrations of inflammatory markers have been associated with a reduced lean mass (19, 36, 40).

Inflammation may enhance catabolic mechanisms (16), which promotes the onset of insulin resistance (41, 42), reduces the dietary energy intake (16), or lowers concentrations of insulin-like growth factor 1 (9, 26). It has also been suggested that age-related changes in body composition might be related to reduced physical activity, which is inversely related to inflammation (43).

In a well-functioning population aged 70-79 y, Visser et al (19) showed that plasma concentrations of IL-6 and tumor necrosis factor α were inversely associated with muscle mass and strength. Our study extends these findings to CRP, an inflammatory marker that is more widely used, and in a population characterized by a higher cardiovascular disease risk. Our results also confirm evidence from previous studies that showed a relation between BMI and total fat mass and markers of inflammation and fibrinolysis (14, 18, 24, 35). Given the well-established relation between BMI and total fat mass and cardiovascular diseases (17, 44), our results indirectly provide further support for the role played by CRP, IL-6, and PAI-1 as risk factors for cardiovascular disease.
SARCOPENIA, OBESITY, AND INFLAMMATION

Some limitations of the present study should be mentioned. The cross-sectional design of the study did not allow us to investigate the cause-effect relation between markers of inflammation and fibrinolysis and body-composition measures. The TRAIN study population is composed of highly selected subjects with a high cardiovascular disease risk profile. Therefore, our findings might have been driven by the presence of subclinical conditions that predispose the subjects to cardiovascular diseases. Additional studies are needed to confirm our results in the general population. Finally, we used self-reported height to calculate BMI and to derive residuals of appendicular lean mass (both unadjusted and fat-adjusted). Self-reported height may not be as accurate as measured heights, especially in older persons. However, the high correlations reported between self-reported and measured heights may have limited a potential bias in our findings (45, 46).

In conclusion, our study showed that obesity, a major risk factor for health-related outcomes, is strongly and positively associated with CRP, IL-6, and PAI-1 concentrations. Inflammatory markers are also inversely associated with appendicular lean mass independent of fat mass, which is consistent with an effect of inflammation on muscle mass. Our findings suggest a possible role of obesity-associated inflammation in the age-related process that leads to sarcopenia.

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Different modes of weight loss in Alzheimer disease: a prospective study of 395 patients\textsuperscript{1–3}

Olivier Guérin, Sandrine Andrieu, Stéphane M Schneider, Morgan Milano, Rabia Boulahssass, Patrice Brocker, and Bruno Vellas

ABSTRACT

Background: Alzheimer disease is often accompanied and worsened by malnutrition. Patterns of weight loss can differ by the patients concerned and by the outcome and interventions required.

Objective: Our aim was to describe and analyze 2 modes of weight loss (progressive and severe) in the course of Alzheimer disease.

Design: This was a prospective study of 395 patients with Alzheimer disease, who had a mean age of 75.4 y. A standardized gerontologic evaluation was conducted at 6 mo and 1 y, including assessments of nutrition, neuropsychology, function, and caregiver burden.

Results: We investigated 2 modes of weight loss. The first, progressive loss (4% in 1 y), affected 33.4% of subjects. Disease severity was a risk factor [odds ratio (OR): 7.2; 95% CI: 1.4, 38.2 for a Reisberg score ≥ 5], whereas treatment with cholinesterase inhibitors at baseline decreased this risk (OR: 0.33; 95% CI: 0.14, 0.79).

The second mode of weight loss, a severe loss of ≥5 kg in 6 mo, affected 10.2% of subjects. The existence of an acute phase reaction was a risk factor (OR: 2.4; 95% CI: 1.2, 4.8), as was an intercurrent event, such as hospitalization, acute disease, institutionalization, and change of living arrangements (OR: 6.8; 95% CI: 1.2, 39.9).

Conclusion: During the follow-up of patients with Alzheimer disease, risk factors for these 2 modes of weight loss should be sought to identify patients who would benefit from a nutritional intervention. Our findings lead us to advocate follow-up, which involves an assessment of functional, nutritional, and neuropsychologic status every 6 mo. Am J Clin Nutr 2005;82:435–41.

KEY WORDS Weight loss, malnutrition, dementia, Alzheimer disease, aging, elderly, prospective study, malnutrition, nutritional assessment

INTRODUCTION

Alzheimer disease (AD) is a neurodegenerative condition that is characterized by a progressive loss of cognitive function. It is the most frequent type of dementia, affecting 30-50% of persons >85 y (1). An interventional strategy to prevent or delay the complications of this disease would have an important public health effect in industrialized societies (2).

Weight loss is a frequent complication of AD and occurs in ≈40% of patients at all stages, even in the early stages before diagnosis is possible (3). Malnutrition (namely undernutrition) contributes to the alteration of general health status, to the frequency and gravity of complications, especially infections, and to a faster loss of independence. These states of malnutrition can be prevented or at least improved if an early intervention strategy is set up, but management must be rapid and appropriate (4). In a geriatric population, a loss of >4% of body weight in 1 y is known to be an independent factor of morbidity and mortality (5).

In addition, weight loss is a phenomenon whose kinetics may vary; it can be a dramatic loss of several kilograms in a few months (severe weight loss) or a moderate but continuous loss as the disease progresses (progressive weight loss).

The aims of this prospective study were to identify patients with each of these 2 modes of weight loss in a large AD population with a standardized nutritional follow-up and to determine the risk factors for these patterns of weight loss.

SUBJECTS AND METHODS

Study design

This single-center, prospective cohort study was conducted in the hospitals of Toulouse, France, as part of the Etude Longitudinale de la Maladie d’Alzheimer (ELSA) study. The ELSA study has followed patients with AD since 1994. Follow-up included a clinical evaluation that was completed every 6 mo in the daycare hospital. The collation of intercurrent events by telephone was every 3 mo.

The diagnosis of AD was confirmed by initial evaluation through a detailed personal and family history and the administration of psychometric tests to confirm cognitive impairment. Conventional tests could also be used to exclude other causes of dementia (standard biological tests, thyroid function, vitamin measurements, brain computed tomography scan, and, in some cases, brain perfusion radioisotope scan and apolipoprotein E genotyping). All patients fulfilled the criteria for dementia as described in the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition (6) and had probable AD according to the criteria of the National Institute of Neurological

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and Communicative Disorders and Strokes—Alzheimer’s Disease and Related Disorders Association (7).

The presence of a caregiver able to ensure the quality of follow-up was an inclusion criterion. Exclusion criteria were diagnosis of AD >5 y before the study, an activities of daily living (ADL) score < 3, uncontrolled heart failure, severe or unstable angina, uncontrolled arterial hypertension, severe orthostatic hypotension, severe renal or liver failure, severe anemia, vascular disorders, systemic disease, clinically relevant hyperthyroidism or hypothyroidism, clinically relevant vitamin deficiency, concomitant malignancy, severe or total blindness, or deafness. The study was approved by the local research ethics board, CCPRPB Haute-Garonne.

Evaluation

The prestudy and 6-mo evaluations were multidimensional and multidisciplinary. The patients underwent clinical, neuropsychological, and biological investigations. The 6-mo evaluations included the following. A cognitive evaluation was conducted by a neuropsychologist who used Folstein’s Mini-Mental State Examination (8). Independence was evaluated by interviewing the family with the use of the ADL scale (9) and the Instrumental Activities of Daily Living (IADL) scale (10). Mood was scored with the use of Cornell’s depression scale (11). Behavior was assessed with the Cohen-Mansfield scale (12). A full nutritional evaluation included clinical [weight and body mass index (BMI; in kg/m²)] and biological variables [albumin, prealbumin, orosomucoid, C-reactive protein (CRP), prognostic inflammatory and nutritional index (PINI); (CRP × orosomucoid/albumin × prealbumin) (13)]. Nutritional status was quantified with the Mini-Nutritional Assessment (MNA) (14, 15). A dietitian also administered a dietary questionnaire to assess qualitative and quantitative food intakes. The caregivers were interviewed for assessment of their material and psychological burden (burden interview, 22 items) and their reactions to the patients’ behavioral problems and increased dependence (Memory and Behavior Problem Checklist, 17 items) or the Zarit scale (16).

The multiple components of the 6-mo evaluations were used to classify the patients according to the Reisberg scale (17), which

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**FIGURE 1.** Flow diagram of the study according to the Consolidated Standards of Reporting Trials (CONSORT) statement.
indicates the stage of AD. All intercurrent events, whether medical (hospitalization, visit to the treating physician, hospital consultation, and the reason thereof) or nonmedical (social in particular), were carefully recorded. Enrollment was completed in 2000; 395 patients were included.

Classification of weight loss

Two modes of weight loss were examined: progressive weight loss and severe weight loss. A progressive weight loss was determined by using a cutoff of 4% weight loss in 1 y in accordance with the works of Wallace et al (3), who found weight loss of this magnitude to be an independent risk factor of increased mortality in an elderly population. Severe weight loss was determined by using a cutoff of 5-kg weight loss in 6 mo. This pattern of weight loss was selected in accordance with the works of Guyonnet et al (18), who determined that there are 2 types of weight loss in AD patients: a slowly progressive and a severe weight loss of >10%/y (which was >5 kg for most subjects in this study). This loss of 5 kg was routinely observed as an important intercurrent event between 2 visits by the physicians who followed patients in the ELSA cohort, and it was always reported.

Statistical analysis

We individualized 2 groups of subjects: those who had lost ≥4% of body weight during the first year, and those who had lost ≥5 kg during the first 6 mo of follow-up. We also determined the factors at inclusion that were associated with each of these 2 modes of weight loss in the course of AD.

Student’s t tests were used to compare normally distributed quantitative variables across groups. When the hypothesis of normality was not met, nonparametric Kruskal-Wallis tests were used. For qualitative variables, chi-square or Fisher’s exact tests were performed as required in each category. Multivariate logistic regression was used to identify the factors independently associated with weight loss. The selection criteria for the variables introduced in the abovementioned multivariate models were a critical probability of $P < 0.20$ after bivariate analysis. When several strongly linked variables met this criterion, a single variable was selected to avoid the risk of multicolinearity. Statistical analyses of the data were carried out with the SAS statistical software package (version 8.0.2; SAS Institute, Cary, NC).

RESULTS

Population characteristics at inclusion

Of the 434 patients who fulfilled the inclusion criteria, 395 agreed to participate. Of these 395 patients, the weight of 341 subjects was known at 6 mo and that of 308 subjects at 1 y. During follow-up, 28 subjects left the study at the request of their family and 19 died. The flow diagram of the study is represented in Figure 1 (19). The characteristics of the population at inclusion in the ELSA study are represented in Table 1. Patients were on average in their 70s and had a normal nutritional status according to clinical and biological markers and to the MNA. More than one-half had normal ADL scores, indicating full autonomy, and their depression and behavioral disorders scores were low. Most had a moderate cognitive impairment, and the severity of their AD was also moderate. One of 3 patients received cholinesterase inhibitors for their AD.

### Table 1

<table>
<thead>
<tr>
<th>Variables at inclusion</th>
<th>No. of subjects</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>394</td>
<td>75.4 ± 6.7 (45.0–89.0)²</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>271</td>
<td>68.6</td>
</tr>
<tr>
<td>Men</td>
<td>124</td>
<td>31.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>392</td>
<td>60.8 ± 12.5 (30.0–118.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>386</td>
<td>24.3 ± 3.9 (13.9–38.1)</td>
</tr>
<tr>
<td>MNA score (points)</td>
<td>367</td>
<td>24.8 ± 2.5 (1–29)</td>
</tr>
<tr>
<td>MNA score category (%)</td>
<td>65</td>
<td>20.5</td>
</tr>
<tr>
<td>≥ 23.5</td>
<td>65</td>
<td>20.5</td>
</tr>
<tr>
<td>&lt; 23.5</td>
<td>252</td>
<td>79.5</td>
</tr>
<tr>
<td>C-reactive protein (mmol/L)</td>
<td>380</td>
<td>4.3 ± 4.2 (0–45.1)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>386</td>
<td>43.3 ± 4.5 (31.3–56.5)</td>
</tr>
<tr>
<td>Transhyretin (g/L)</td>
<td>380</td>
<td>0.27 ± 0.06 (0–0.90)</td>
</tr>
<tr>
<td>Orosomucoid (mmol/L)</td>
<td>378</td>
<td>0.88 ± 0.23 (0.10–1.80)</td>
</tr>
<tr>
<td>PINI</td>
<td>378</td>
<td>0.41 ± 0.79 (0–11.19)</td>
</tr>
<tr>
<td>Duration of follow-up (y)</td>
<td>395</td>
<td>2.5 ± 1.5 (0.5–7.0)</td>
</tr>
<tr>
<td>Mini-Mental State (points)</td>
<td>392</td>
<td>17.2 ± 5.9 (0–29)</td>
</tr>
<tr>
<td>Cohen score (points)</td>
<td>388</td>
<td>23.2 ± 7.7 (13–51)</td>
</tr>
<tr>
<td>ADL score (points)</td>
<td>392</td>
<td>5.3 ± 1.0 (0–6)</td>
</tr>
<tr>
<td>IADL score (points)</td>
<td>378</td>
<td>4.1 ± 2.3 (0–8)</td>
</tr>
<tr>
<td>Cornell score (points)</td>
<td>379</td>
<td>3 ± 3.2 (0–22)</td>
</tr>
<tr>
<td>Zarit score (points)</td>
<td>332</td>
<td>29.7 ± 17.2 (0–74)</td>
</tr>
<tr>
<td>Reisberg score (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 points</td>
<td>53</td>
<td>13.5</td>
</tr>
<tr>
<td>4 points</td>
<td>187</td>
<td>47.6</td>
</tr>
<tr>
<td>5 points</td>
<td>131</td>
<td>33.3</td>
</tr>
<tr>
<td>6 points</td>
<td>22</td>
<td>5.6</td>
</tr>
<tr>
<td>ADL score (%)</td>
<td></td>
<td></td>
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<tr>
<td>≥ 4.5 points</td>
<td>79</td>
<td>20.1</td>
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<tr>
<td>5 points</td>
<td>48</td>
<td>12.2</td>
</tr>
<tr>
<td>5.5 points</td>
<td>68</td>
<td>17.4</td>
</tr>
<tr>
<td>6 points</td>
<td>197</td>
<td>50.3</td>
</tr>
<tr>
<td>IADL score (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 points</td>
<td>19</td>
<td>6.8</td>
</tr>
<tr>
<td>1 points</td>
<td>21</td>
<td>7.6</td>
</tr>
<tr>
<td>2 points</td>
<td>40</td>
<td>14.4</td>
</tr>
<tr>
<td>3 points</td>
<td>32</td>
<td>11.5</td>
</tr>
<tr>
<td>4 points</td>
<td>34</td>
<td>12.2</td>
</tr>
<tr>
<td>5 points</td>
<td>55</td>
<td>19.8</td>
</tr>
<tr>
<td>6 points</td>
<td>25</td>
<td>9.0</td>
</tr>
<tr>
<td>7 points</td>
<td>25</td>
<td>9.0</td>
</tr>
<tr>
<td>8 points</td>
<td>27</td>
<td>9.7</td>
</tr>
<tr>
<td>Apolipoprotein e4 (%)</td>
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<td></td>
</tr>
<tr>
<td>Absent</td>
<td>131</td>
<td>47.1</td>
</tr>
<tr>
<td>Present</td>
<td>147</td>
<td>52.9</td>
</tr>
<tr>
<td>Specific treatments (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not treated</td>
<td>264</td>
<td>67</td>
</tr>
<tr>
<td>Treated (total)</td>
<td>130</td>
<td>33</td>
</tr>
<tr>
<td>Donepezil</td>
<td>72</td>
<td>18.3</td>
</tr>
<tr>
<td>Tacrine</td>
<td>41</td>
<td>10.4</td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>17</td>
<td>4.3</td>
</tr>
</tbody>
</table>

¹ MNA, Mini-Nutritional Assessment; PINI, Prognostic Inflammatory and Nutritional Index; ADL, Activities of Daily Living; IADL, Instrumental Activities of Daily Living.

² x ± SD; ranges in parentheses (all such values).

³ Range: 0–30; the lowest score is the most severe.

⁴ Range: 0–91; the highest score is the most severe.

⁵ Range: 0–6; the lowest score is the most severe.

⁶ Range: 0–8; the lowest score is the most severe.

⁷ Range: 0–38; the highest score is the most severe.

⁸ Range: 0–88; the highest score is the most severe.

⁹ Range: 3–6 in the study; the highest score is the most severe.
Progressive weight loss

Progressive weight loss (4% weight loss in the first year of follow-up) was observed in 33.4% of subjects \( (n/L115597) \) at 1 y.

Bivariate analysis

The variables that were different between this group of patients and the rest of the initial cohort are shown in Table 2. At baseline, patients with a progressive weight loss had a more severe AD (Reisberg scale) and a more pronounced cognitive impairment (Mini-Mental State Examination). The 1-point difference between groups in the mean IADL score for everyday activities is extremely relevant in clinical practice. The rate of admission to a hospital or institution was higher in these patients, as was the burden for their caregivers. Finally, treatment with cholinesterase inhibitors was inversely associated with progressive weight loss. The presence of an intercurrent event (besides hospitalization or institutionalization), depression, behavioral disorders, living arrangement, food intake, and nutritional status at inclusion was not different between patients with a progressive weight loss and the others.

Multivariate analysis

After multivariate analysis, 3 variables were independently associated with a progressive weight loss: a higher initial weight, the absence of a specific treatment of AD, and more severe AD (Reisberg score) (Table 3).

Severe weight loss

A loss of ≥5 kg body weight during the first 6 mo of follow-up occurred in 10.2% of the subjects \( (n/L32) \). Twenty (62.5%) of these patients were also part of the first group (progressive weight loss at 1 y).

Bivariate analysis

The variables that were different between this group of patients and the rest of the initial cohort are shown in Table 4. At baseline, patients with a severe weight loss had a higher initial weight and BMI and had lower prealbumin and higher CRP values. Their functional status was lower, and their educational level was higher. The rate of admission to a hospital or institution as a whole was lower, and their educational level was higher. The rate of admission to a hospital or institution

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group without 4% weight loss in 1 y ( (n/L193) )</th>
<th>Group with 4% weight loss in 1 y ( (n/L97) )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (kg)</td>
<td>60.25 ± 11.39</td>
<td>62.86 ± 13.61</td>
<td>0.14</td>
</tr>
<tr>
<td>Reisberg score (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 points</td>
<td>18.1</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>4 points</td>
<td>50.8</td>
<td>45.4</td>
<td></td>
</tr>
<tr>
<td>5 points</td>
<td>28.5</td>
<td>39.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>6 points</td>
<td>2.6</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>MMS (points)</td>
<td>18.3 ± 5.7</td>
<td>15.8 ± 6.0</td>
<td>0.001</td>
</tr>
<tr>
<td>MMS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 20 points</td>
<td>44</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td>≥ 12 to &lt; 20 points</td>
<td>39.4</td>
<td>38.1</td>
<td>0.01</td>
</tr>
<tr>
<td>&lt; 12 points</td>
<td>16.6</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td>IADL score (points)</td>
<td>4.6 ± 2.1</td>
<td>3.7 ± 2.5</td>
<td>0.0029</td>
</tr>
<tr>
<td>Cholinesterase inhibitor treatment (%)</td>
<td>36.3</td>
<td>21.6</td>
<td>0.011</td>
</tr>
<tr>
<td>Educational level (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>School</td>
<td>77.7</td>
<td>79.4</td>
<td></td>
</tr>
<tr>
<td>High school, baccalaureate</td>
<td>16.6</td>
<td>13.0</td>
<td>0.0184</td>
</tr>
<tr>
<td>College or university</td>
<td>5.7</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Zarit score (%)</td>
<td>27.5 ± 16.8</td>
<td>33.5 ± 17.6</td>
<td>0.015</td>
</tr>
<tr>
<td>&lt; 20 points</td>
<td>34.9</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>20 to &lt; 40 points</td>
<td>39.5</td>
<td>39.5</td>
<td>0.001</td>
</tr>
<tr>
<td>≥ 40 points</td>
<td>25.6</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>Hospitalization during the year (%)</td>
<td>10.7</td>
<td>32.3</td>
<td>0.0002</td>
</tr>
<tr>
<td>Institutionalization during the year (%)</td>
<td>3.8</td>
<td>15.6</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

1. MMS, Mini-Mental State; IADL, Instrumental Activities of Daily Living.
2. ± SD (all such values).
4. Fisher’s exact test.
5. Chi-square test.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher initial weight ( (n/L290) )</td>
<td>1.04 (1.00, 1.07)</td>
</tr>
<tr>
<td>Cholinesterase inhibitor treatment ( (n/L92) )</td>
<td>0.33 (0.14, 0.79)</td>
</tr>
<tr>
<td>Reisberg score</td>
<td></td>
</tr>
<tr>
<td>3 points ( (n/L42) )</td>
<td>1</td>
</tr>
<tr>
<td>4 points ( (n/L142) )</td>
<td>5.8 (1.1, 30.2)</td>
</tr>
<tr>
<td>5 or 6 points ( (n/L106) )</td>
<td>7.2 (1.4, 38.2)</td>
</tr>
</tbody>
</table>

1. Multivariate logistic regression.
TABLE 4
Comparison of variables at inclusion between groups with and without a 5-kg weight loss during the first 6 mo (bivariate analysis)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group without weight loss ≥ 5 kg</th>
<th>Group with weight loss ≥ 5 kg</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (kg)</td>
<td>60.3 ± 11.1\textsuperscript{2}</td>
<td>70.2 ± 16.8</td>
<td>0.0025\textsuperscript{5}</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>24.1 ± 3.5</td>
<td>27.9 ± 4.7</td>
<td>&lt;0.0001\textsuperscript{4}</td>
</tr>
<tr>
<td>C-reactive protein (mmol/L)</td>
<td>4 ± 3.3</td>
<td>5.6 ± 5.2</td>
<td>0.0172\textsuperscript{4}</td>
</tr>
<tr>
<td>Transthyretin (g/L)</td>
<td>0.27 ± 0.06</td>
<td>0.24 ± 0.07</td>
<td>0.035\textsuperscript{4}</td>
</tr>
<tr>
<td>PINI</td>
<td>0.33 ± 0.42</td>
<td>0.54 ± 0.86</td>
<td>0.089\textsuperscript{4}</td>
</tr>
<tr>
<td>IADL score (points)</td>
<td>4.4 ± 2.2</td>
<td>3.3 ± 2.5</td>
<td>0.05\textsuperscript{4}</td>
</tr>
<tr>
<td>Living alone at home (%)</td>
<td>16.3</td>
<td>21.9</td>
<td></td>
</tr>
<tr>
<td>Living at home with assistance (%)</td>
<td>79.1</td>
<td>68.7</td>
<td>0.022\textsuperscript{5}</td>
</tr>
<tr>
<td>Living in an institution (%)</td>
<td>4.6</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>Educational level (%)</td>
<td>77.3</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td>High school, baccalaureate</td>
<td>14.7</td>
<td>16.7</td>
<td>0.045\textsuperscript{5}</td>
</tr>
<tr>
<td>College or university</td>
<td>7.9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Zarit score (%)</td>
<td>29.7</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>&lt; 20 points</td>
<td>41.7</td>
<td>28.6</td>
<td>0.013\textsuperscript{5}</td>
</tr>
<tr>
<td>20 to &lt; 40 points</td>
<td>28.5</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>≥ 40 points</td>
<td>16.5</td>
<td>33.3</td>
<td>0.032\textsuperscript{5}</td>
</tr>
<tr>
<td>Hospitalization during the first 6 mo (%)</td>
<td>17</td>
<td>38.7</td>
<td>0.008\textsuperscript{6}</td>
</tr>
<tr>
<td>Change of living arrangements during the first 6 mo (%)</td>
<td>6</td>
<td>25.8</td>
<td>0.001\textsuperscript{5}</td>
</tr>
<tr>
<td>Institutionalization during the first 6 mo (%)</td>
<td>28.2</td>
<td>35.7</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} PINI, Prognostic Inflammatory and Nutritional Index; IADL, Instrumental Activities of Daily Living.
\textsuperscript{1} t ± SD (all such values).
\textsuperscript{2} Student’s t test.
\textsuperscript{3} Kruskal-Wallis test.
\textsuperscript{4} Fisher’s exact test.
\textsuperscript{5} Continuity-adjusted chi-square test.

was higher in these patients, as was the burden for their caregivers. Living arrangements differed significantly between the 2 groups: patients who lost weight generally lived at home alone or in an institution. The severity of AD, cognitive status, depression, and food intake at inclusion were not different between patients with and without severe weight loss.

**Multivariate analysis**

After multivariate analysis, 3 variables were independently associated with a severe weight loss at 6 mo (Table 5). These variables included a higher initial weight, a higher PINI score, and the presence of an intercurrent event (including hospitalization, acute disease, institutionalization, and change of living arrangements).

**DISCUSSION**

To our knowledge this was the first study to determine the effect of various nutritional variables (weight, MNA, albumin, prealbumin, orosomucoid, CRP, and PINI), as part of a standardized systematic follow-up procedure, on the progression of AD. Malnutrition is a feature of the original description of AD as reported by Aloïs Alzheimer in 1901. Afterward, in the early 1980s, Morgan and Hullin (20) reported that the weight of patients hospitalized for dementia was lower than that of healthy elderly subjects. White et al (21) explored the association between AD and weight changes in 362 subjects with AD and 317 control subjects for 2 y. The results showed that nearly twice as many subjects with AD experienced weight loss of ≥5% than did control subjects. Weight loss in AD can follow 2 patterns that are relevant to elderly people (22): progressive and severe.

In our cohort, more than one-third of subjects lost progressively (over 1 y) >4% of their body weight. The disease per se seemed to be the most important risk factor for this mode of weight loss, because patients with the most severe forms of AD were 6-7 times more likely to undergo a progressive weight loss. These results confirm the findings of White et al (23) who explored the association between progression of AD and weight loss in 666 subjects. They showed that changes in stage of AD correlated significantly with weight changes. Some patients with AD show a rapid cognitive decline (24) with risk factors (worse cognitive status, greater BMI, and less anxiety of burden) that are similar to risk factors for progressive weight loss (worse cognitive status and greater initial weight). Future studies are required to compare individuals with the different patterns of weight loss to identify common features. A higher initial body weight may confirm the relation that has been described between obesity and AD (25), maybe through the association of cardiovascular risk factors and AD, even though a small proportion of our patients...
were obese. A more surprising finding was the identification of treatment with cholinesterase inhibitor as a protective factor against progressive weight loss, even though we cannot attribute a cause-and-effect relation. Indeed, this study was not a controlled study of cholinesterase inhibitors, which have been proved effective against cognitive decline and behavioral disorders in patients with AD (26). Prevention of progressive weight loss may be part of the benefits of these drugs in AD. Unlike other investigators (27), we did not identify behavioral disorders (including anorexia) as risk factors for weight loss in AD. We observed no differences in food intake according to the pattern of weight loss. Similar findings have been reported, and those findings leave the question of anorexia and energy expenditure in AD intact (28). This disease-related weight loss can lead to cachexia at the end of the disease’s evolution, and at that stage nutritional support has been much debated and is generally not advocated (29). However, an earlier intervention might allow improvement of the nutritional status, thus justifying an early diagnosis of this progressive weight loss (4).

Ten percent of the cohort underwent a severe weight loss of >5 kg in 6 mo. This was the first time that such a weight loss had been prospectively quantified. The independent effect of a higher initial body weight on severe weight loss may be explained because we chose 5 kg as an absolute value rather than a percentage of weight. This severe weight loss was likely due to a concomitant disease, as shown by the frequency of an inflammatory syndrome or intercurrent event (health-related or social) in these subjects. Stress, especially in its medical meaning, is reflected here by a higher number of intercurrent events and by an elevated PINI, which takes into account at the same time the acute phase protein reaction (with a switch in liver protein synthesis as a result of the action of proinflammatory cytokines such as tumor necrosis factor α and interleukin 6) and the ensuing decrease in nutritional proteins. In elderly hospitalized patients, this prognostic index has proved to be helpful to predict both nearest lethality and chronic institutionalization (30). Cytokine-driven anorexia is a common symptom during an acute stress (such as infection, trauma, surgery) and is usually reversible within a few days after the course of the initial disease. However, this is only true for children and younger adults, and the concept of secondary anorexia is well known in the elderly who are unable to develop hyperphagia after a period of food restriction (31). The ensuing malnutrition-induced morbidity and mortality may superimpose those related to AD (32). Nutritional care is crucial in elderly patients with secondary anorexia, because it can result in a return to previous food intake and a subsequent regaining of the weight that was lost (33). Therefore, we believe that screening for this condition (acute weight loss) is important because it may lead to early and beneficial nutritional care. This needs to be confirmed by an interventional study in such patients.

Twenty patients (62.5%) with a severe weight loss were also those related to AD (32). Nutritional care is crucial in elderly patients with secondary anorexia, because it can result in a return to previous food intake and a subsequent regaining of the weight that was lost (33). Therefore, we believe that screening for this condition (acute weight loss) is important because it may lead to early and beneficial nutritional care. This needs to be confirmed by an interventional study in such patients.

In conclusion, 2 distinct modes of weight loss with different risk factors were identified in patients with AD. The first mode, which occurs quite frequently, is a progressive loss, which is related to the evolution and worsening of AD. The second mode, which occurs less frequently, is a severe loss, which is related to intercurrent medical or social events. Because the prognosis of these 2 modes of weight loss differ, with a nutritional support potentially being useless if provided too late in severe weight loss, we believe that a rigorous, early, and regular follow-up of nutritional variables is mandatory in patients with AD.

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SA and BV were responsible for the study concept and design. OG, SA, and BV were responsible for the analysis and interpretation of the data. OG, SA, SMS, and BV drafted the manuscript. All authors were responsible for the critical revision of the manuscript for important intellectual content. SA provided statistical expertise. BV obtained funding. MM, RB, and FB provided administrative, technical, or material support. BV supervised the conduct of the study. None of the authors had a conflict of interest.

REFERENCES

ABSTRACT

Background: Mandatory folic acid fortification of cereal-grain products was introduced in the United States in 1998 to decrease the risk that women will have children with neural tube defects.

Objective: The objective was to determine the effect of folic acid fortification on concentrations of serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma total homocysteine (tHcy) and methylmalonic acid (MMA) in the US population.

Design: Blood was collected from a nationally representative sample of ~7300 participants aged ≥3 y in the National Health and Nutrition Examination Survey (NHANES) during 1999–2000 and was analyzed for these B vitamin–status indicators. The results were compared with findings from the prefortification survey NHANES III (1988–1994).

Results: The reference ranges (5th–95th percentiles) were 13.1–74.3 nmol/L for serum folate, 347–1167 nmol/L for RBC folate, and 179–738 pmol/L for serum vitamin B-12. For plasma tHcy and MMA, the reference ranges for serum vitamin B-12–replete participants with normal serum creatinine concentrations were 3.2–10.7 μmol/L and 60–210 nmol/L, respectively. The prevalence of low serum folate concentrations (<6.8 nmol/mL) decreased from 16% before to 0.5% after fortification. In elderly persons, the prevalence of high serum folate concentrations (>45.3 nmol/L) increased from 7% before to 38% after fortification; 3% had marginally low serum vitamin B-12 concentrations (<148 pmol/L) and 7% had elevated plasma MMA concentrations (>370 nmol/L). Seventy-eight percent of the US population had plasma tHcy concentrations <9 μmol/L.

Conclusions: Every segment of the US population appears to benefit from folic acid fortification. Continued monitoring of B vitamin concentrations in the US population is warranted.

KEY WORDS: Nutrition survey, age, sex, race, ethnic groups, National Health and Nutrition Examination Survey, NHANES

INTRODUCTION

Folates act as one-carbon donors in the synthesis of the building blocks of DNA, thymidylate, and purines and of certain neurotransmitters, phospholipids, and hormones (1). Vitamin B-12, commonly referred to as cyanocobalamin, is required as a cofactor for 2 enzymes: methionine synthase, which catalyzes the conversion of homocysteine to methionine using 5-methyltetrahydrofolic acid as a methyl group donor, and L-methyl-malonyl-CoA mutase, which catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA (2). Although low serum vitamin B-12 concentrations are a sensitive indicator of vitamin B-12 deficiency and high vitamin B-12 concentrations generally indicate sufficiency, the risk of vitamin B-12 deficiency associated with an intermediate range of vitamin B-12 concentrations is unclear. Plasma methylmalonic acid (MMA) is a useful confirmatory diagnostic test in persons with a low or low normal serum vitamin B-12 concentration (3, 4). Plasma total homocysteine (tHcy) concentrations can be elevated because of low folate, vitamin B-12, or vitamin B-6 intakes or because of renal insufficiency, methylentetrahydrofolate reductase polymorphism, or the use of certain medications. The plasma MMA concentration can be elevated as a result of renal insufficiency.

Folate deficiency is already an established risk factor for the development of certain types of cancer in the general population (5). A chronic deficiency of folate in the diet can cause anemia (1), but low concentrations of serum folate and vitamin B-12 and elevated concentrations of plasma tHcy have also been associated with psychiatric disorders (6); the development of dementia, Alzheimer disease, and cognitive dysfunction (7–10); a decline in physical function (11); osteoporosis and hip fractures in the elderly (12, 13); and an increased risk of carotid artery stenosis (14). Two recent meta-analyses concluded that a 25% reduction of plasma tHcy is associated with an 11–16% decrease in risk of ischemic heart disease and a 19–22% decrease in risk of stroke (15, 16). Clinical trials have shown that folic acid supplementation decreases the risk of neural tube birth defects (17, 18). In 1998, the Food and Drug Administration required the fortification of enriched cereal-grain products with folic acid at a concentration of 140 μg/100 g cereal grain (19).

Although severe vitamin B-12 deficiency causes anemia, hematologic signs are not always present, and hematologic and neurologic abnormalities are inversely correlated in vitamin B-12 deficiency (4). Some evidence suggests that excess supplemental folate intake may precipitate or exacerbate the neurologic damage of vitamin B-12 deficiency; a summary of such human
case reports has been compiled by the US Institute of Medicine (20). Because elderly people have lower serum vitamin B-12 concentrations than do younger people (21–23), they may be particularly susceptible to the most serious sequelae because consequences may be irreversible when vitamin B-12 deficiency goes untreated.

Since 1998, a few studies have shown significant improvements in folate status in selected populations (24–27). The National Health and Nutrition Examination Survey (NHANES) 1999–2000 is the first and only source of nationally representative data on the B vitamins and their biochemically related products after the introduction of folic acid fortification. This report describes the concentrations of and presents the reference data for serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma tHcy and MMA in the US population aged ≥3 y. The changes in B vitamin concentrations between the prefortification and postfortification periods are discussed.

SUBJECTS AND METHODS

Survey design and subjects

NHANES, conducted by the National Center for Health Statistics (NCHS) at the Centers for Disease Control and Prevention (CDC), is a series of nationally representative cross-sectional examination surveys that uses a stratified, multistage design to provide a representative probability sample of the civilian non-institutionalized population in the United States. The NHANES program began in 1960 and is designed to provide periodic information about the health and nutritional status of the US population. NHANES III, conducted from 1988 to 1994, was the last survey that assessed the B vitamin status of the US population before the introduction of folic acid fortification. It was designed as two 3-y phases, each constituting a national probability sample in which there was an oversampling of Mexican American and non-Hispanic black persons, all children aged 2 mo through 5 y, and all persons aged ≥60 y (28). Blood for B vitamin measurements was collected from participants ≥4 y of age.

In 1999 NHANES became a continuous survey (29, 30). Although each year constitutes a national probability sample, ≥2 y of data are necessary to have adequate sample sizes for subgroup analyses. The procedures for selecting participants and for conducting interviews and examinations in NHANES 1999–2000 were similar to those used in NHANES III. The NHANES 1999–2000 survey sample comprised 9965 participants. Of these, 93% were interviewed in their homes and underwent physical examination in mobile examination centers. Mexican American and non-Hispanic black persons, all adolescents (ages 12–19 y), and all persons aged ≥60 y were oversampled to improve estimates in these groups. All respondents gave their informed consent, and the NHANES 1999–2000 protocol was reviewed and approved by the NCHS NHANES Institutional Review Board. Blood for B vitamin measurements was collected from participants aged ≥3 y.

Fasting status and supplement use

To assess the extent to which blood concentrations of folate and vitamin B-12 are affected after a meal is eaten, the NHANES 1999–2000 data were analyzed by fasting status. The respondents were asked to fast for either 10–16 h or for 6 h, depending on whether their appointment at the mobile examination center was in the morning or in the afternoon or evening. Before the phlebotomy was performed, the respondents were asked when they last ate or drank and the number of hours that they had fasted was calculated. Fasting status was categorized as fasting ≥9 h or <9 h (this category included those who reported having just eaten). The respondents were asked separately about their use of vitamin or mineral supplements in the past 24 h. Therefore, when analyzing fasting status, use of supplements in the past 24 h was controlled for by selecting only those persons who reported not taking a supplement. Approximately one-half of the participants (54.3%) had fasted ≥9 h before phlebotomy. These 2 fasting categories had no measurable effect on serum or RBC folate, serum vitamin B-12, or plasma tHcy and MMA concentrations. All further data analyses were conducted without the exclusion of supplement users or participants who fasted <9 h before phlebotomy.

Biochemical measurement of serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA concentrations

Depending on the age of the participant, data were collected on demographics, physical function, health condition, lifestyle behaviors, biochemical measurements of blood and urine, body measurements, and dietary intake. Blood was collected by venipuncture in mobile examination centers and processed under controlled, constant environmental conditions according to standard protocols (31).

The NHANES Laboratory of the CDC analyzed serum and RBC folate for both phases of NHANES III, serum vitamin B-12 for phase II of NHANES III (1991–1994), and all 5 indicators for NHANES 1999–2000 (32). Serum and RBC folate and serum vitamin B-12 were analyzed by using a commercially available radioprotein binding assay kit (Quanaphase I during phase I of NHANES III and Quanaphase II during phase II of NHANES III and during NHANES 1999–2000; Bio-Rad Laboratories, Hercules, CA). The serum and RBC folate assay measures primarily 5-methyltetrahydrofolinic acid and folic acid but is considered to estimate total folate in serum and whole blood. RBC folate was measured after lysis of 1 part of whole blood with 10 parts of 1% perchloric acid solution (performed in the mobile examination center) and one freeze-thaw cycle to ensure cleavage of polyglutamyl folates to monoglutamates by the action of the endogenous plasma deconjugase. The serum vitamin B-12 assay measures cyanocobalamin in its circulating bound and free forms. Long-term CVs for the NHANES 1999–2000 period were 4–7% for serum folate at 5.2–30.0 nmol/L, 4–6% for RBC folate at 134–1119 nmol/L, and 3–6% for serum vitamin B-12 at 281–1160 pmol/L.

Measurements of tHcy and MMA changed from NHANES III to NHANES 1999–2000 because different laboratories conducted the analyses, and different matrices and methods were used. Because optimally prepared EDTA-treated plasma was not available during NHANES III, tHcy and MMA were analyzed in surplus serum from phase II of NHANES III (1991–1994) at the US Department of Agriculture (USDA) Human Nutrition Research Center on Aging. tHcy was measured by HPLC with fluorometric detection (33); MMA was measured by gas chromatography–mass spectrometry by using solid-phase extraction and derivatization with cyclohexanol (34) for persons ≥60 y of age and for a subset of adults 30–39 y of age. For NHANES 1999–2000, EDTA-treated whole blood was processed within 30 min of collection to avoid an artificial increase in tHcy, and...
plasma was immediately frozen at −70 °C. Plasma tHcy was analyzed by using a commercially available fluorescence polarization immunoassay kit (Abbott Laboratories, Abbott Park, IL) on the Abbott IMx analyzer (35). Plasma MMA was measured by gas chromatography–mass spectrometry with cyclohexanol derivatization (36). A 10% subset of all samples was also analyzed for plasma tHcy by HPLC with fluorometric detection and cysteamine as internal standard (37, 38). Both assays measure tHcy in plasma, which is the sum of reduced, oxidized, and protein-bound homocysteine. The comparison of the 2 tHcy methods for the NHANES 1999–2000 period gave the following results: Pearson’s correlation coefficient, 0.98; Deming regression (Abbott), 0.97 × HPLC − 0.2; absolute bias (95% confidence limit), −0.4 μmol/L (−0.4, −0.3); and relative bias, −5%. Long-term CVs for the 2-y period were 3–6% for plasma tHcy by Abbott at 6.7–29.0 μmol/L, 4–7% for plasma tHcy by HPLC at 6.7–29.5 μmol/L, and 4–11% for plasma MMA at 120–10400 nmol/L. A formal tHcy method comparison was conducted between the HPLC methods used at the CDC NHANES and the USDA laboratories, and the results were as follows: Pearson’s correlation coefficient, 0.97; Deming regression, USDA HPLC = 0.82 × CDC HPLC + 0.9; absolute bias (95% confidence limit), −0.6 μmol/L (−0.5, −0.8); relative bias, −6% (39). We also determined that under the conditions applied to sample processing during NHANES III, tHcy in serum was overestimated compared with tHcy in optimally prepared EDTA-treated plasma by an average of 10% (39). Because of changes in matrix and methods, a direct comparison of the tHcy results obtained with the 2 surveys is inappropriate (39). No formal MMA method comparison was conducted between the gas chromatography–mass spectrometry methods used at the CDC NHANES and the USDA laboratory, which precludes direct comparison of the MMA results between these 2 surveys.

Statistical analysis

The statistical analyses were performed with SAS for Windows (version 8.0; SAS Institute Inc, Cary, NC) in conjunction with SUDAAN statistical software (version 8.0.2; 40). Data were weighted to account for survey design (unequal probability of selection, adjustments for oversampling of certain populations) and nonresponse. On the basis of questionnaire self-assignment, the participants were categorized into 3 racial-ethnic groups: Mexican Americans, non-Hispanic blacks, and non-Hispanic whites. No separate data analysis was performed for persons from other ethnic groups (n = 876). Because of significant metabolic changes in B vitamins and their biochemically related products during pregnancy, we excluded pregnant women from all data analysis. Supplement users were not evaluated separately in our data analysis. In the comparison of estimates, only statistically significant differences at a significance level of α = 0.01 are reported. We consistently used this more stringent significance level rather than using α = 0.05 and adjusting the level based on the number of comparisons made; the maximum number of groups that we compared was 5.

This report presents population means, SEMs, and selected percentiles for serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA that were representative of the noninstitutionalized civilian US population for NHANES 1999–2000. As recommended by NCHS, a Taylor series variance estimation method appropriate for complex survey data was used to estimate SEMs (29). Because the distributions of these biomarkers were skewed, logarithmic transformations of the distributions were used for the statistical analyses. We age-adjusted means by using SUDAAN PROC DESCRIPT standardization statements and the following 2000 Census population proportions: 20–39 y, 0.3966; 40–59 y, 0.3718; and ≥60 y, 0.2316 (30). We tested for sex-by-race (Table 1), sex-by-age (Figure 1), and race-by-age interactions using an analysis of variance model that included age (6 age groups), sex (males and females), racial-ethnic group (Mexican Americans, non-Hispanic blacks, and non-Hispanic whites) and the abovementioned interaction terms. If the interaction term was not significant (P ≥ 0.01) we only reported the means by subgroup without performing subgroup analysis to test for significant differences. We tested for main effects of sex, race, and age using the same analysis of variance model as for the interaction testing. We tested for significant differences between males and females or between racial-ethnic groups by using census age-adjusted geometric means and a 2-tailed, 2-group t test with 14 df (Table 1). We tested for significant differences between age groups (using age group 20–39 y as a reference) or between males and females (within one age group) by using geometric means and a 2-tailed 2-group t test with 14 df in a model that included age, sex, race, and sex-by-age interaction (Figure 1).

The average design effect, which is the ratio of the complex sampling design variance derived from SUDAAN to the simple random sample variance calculated by SAS, averaged over age categories, was used to determine the recommended minimum stratum sample size according to the NCHS analytic guidelines and recommendations to achieve stable estimates of means and percentiles (28). On the basis of an average design effect of ≈1.4 for our sample, strata with <220 individuals gave imprecise estimates of the 5th and 95th percentiles.

We used the 5th and 95th percentiles of the entire population, except for pregnant women, to estimate the population reference ranges for serum and RBC folate and serum vitamin B-12. To establish population reference ranges (5th and 95th percentiles) for plasma tHcy and MMA, we created a reference sample in which we included only persons who were vitamin B-12 replete (serum vitamin B-12 concentrations above the 50th percentile; 41) and excluded persons with serum creatinine concentrations indicative of impaired renal function (ie, >133 μmol/L for men and >115 μmol/L for women)—the principal cause of elevated plasma tHcy and MMA concentrations for reasons other than suboptimal B vitamin status. Serum and RBC folate concentrations were not considered exclusionary for preparing the tHcy and MMA reference sample because the entire population was considered to be folate replete (serum folate concentration >6.8 nmol/L).

To assess the effect of fortification on blood folate and vitamin B-12 concentrations, we compared NHANES III data with NHANES 1999–2000 data. We applied the same procedure as described above to calculate age-adjusted means, but, as recommended by the NCHS (30), we used different proportions derived from the 2000 census population: 20–39 y, 0.4332; 40–59 y, 0.4062; and ≥60 y, 0.1606. We tested for significant differences between prefortification and postfortification values for various subgroups by using age-specific or age-adjusted geometric means and a 2-tailed, 2-group t test. To compare the prevalence estimates from NHANES III with those from NHANES 1999–2000, we used the SE and df associated with each estimate to
compute the SE and df associated with the difference. We then performed a 2-tailed, 2-group t test.

RESULTS

Descriptive statistics for B vitamins and their biochemically related products for NHANES 1999–2000

Serum and RBC folate, serum vitamin B-12, and plasma tHcy and MMA concentrations were measured for 7235, 7321, 7233, and 7043 participants aged ≥20 y, respectively. The study population was 51% male and included =5% children aged 3–5 y, 41% children and adolescents (6–19 y), 34% adults (20–59 y), and 21% elderly persons (≥60 y), which reflected the oversampling in the survey as described earlier.

To allow a comparison with previous reports (23, 41–44), detailed tables containing age-specific means and distributions of B vitamin and metabolite concentrations for participants by sex are provided online; see Tables S1-S4 under “Supplemental data” in the current online issue at www.ajcn.org. Table S1 contains data for all racial-ethnic groups combined, whereas Tables S2-S4 contain data for individual racial-ethnic groups (Mexican Americans, non-Hispanic blacks, and non-Hispanic whites).

The reference ranges (5th–95th percentiles) for the B vitamins for the US population after the introduction of folic acid fortification were 13.1–74.3 nmol/L for serum folate, 347–1167 nmol/L for RBC folate, and 179–738 pmol/L for serum vitamin B-12. The references ranges for the metabolites for the part of the US population that was vitamin B-12 replete and did not exhibit elevated serum creatinine concentrations were 3.2–10.7 μmol/L for plasma tHcy and 60–210 nmol/L for MMA.

The age-adjusted geometric mean concentrations for adults aged ≥20 y were 30.5 nmol/L for serum folate, 646 nmol/L for RBC folate, 340 pmol/L for serum vitamin B-12, 7.7 μmol/L for plasma tHcy, and 137 nmol/L for MMA (Table 1). We found a significant sex-by-race interaction for serum vitamin B-12 but not for serum and RBC folate (P = 0.345 and P = 0.699, respectively) or plasma tHcy and MMA (P = 0.228 and P = 0.137, respectively). We found a significant main effect of sex for plasma MMA, and significant main effects of race for serum and RBC folate and for plasma tHcy and MMA. On the basis of census age-adjusted data, males had lower serum folate and higher plasma tHcy and MMA concentrations than did females, but RBC folate concentrations were not significantly different between the sexes (P = 0.043). Non-Hispanic whites had higher serum and RBC folate concentrations than did non-Hispanic blacks and Mexican Americans. Mexican Americans had lower plasma tHcy concentrations than did non-Hispanic whites and non-Hispanic blacks, but the difference between non-Hispanic whites and non-Hispanic blacks was not significant (P = 0.088). Non-Hispanic whites had higher plasma MMA concentrations than did non-Hispanic blacks and Mexican Americans, but the difference between non-Hispanic blacks and Mexican Americans was not significant (P = 0.444). The lack of a significant sex-by-race interaction for serum and RBC folate and plasma tHcy and MMA precluded further subgroup analysis. The presence of a significant sex-by-race interaction for serum vitamin

### Table 1

<table>
<thead>
<tr>
<th>Variable and sex</th>
<th>All racial-ethnic groups</th>
<th>Mexican Americans</th>
<th>Non-Hispanic blacks</th>
<th>Non-Hispanic whites</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>All</td>
<td>30.5 (28.9, 32.3)</td>
<td>27.9 (26.6, 29.3)</td>
<td>29.0 (27.5, 30.7)</td>
<td>32.0 (30.0, 34.2)</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>29.0 (27.5, 30.7)</td>
<td>27.0 (25.3, 28.7)</td>
<td>29.0 (27.5, 30.7)</td>
<td>32.0 (30.0, 34.2)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>32.0 (30.0, 34.2)</td>
<td>29.8 (27.8, 30.1)</td>
<td>28.9 (27.8, 30.1)</td>
<td>32.0 (30.0, 34.2)</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>All</td>
<td>646 (618, 676)</td>
<td>596 (575, 617)</td>
<td>634 (606, 665)</td>
<td>658 (625, 692)</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>642 (610, 672)</td>
<td>584 (553, 615)</td>
<td>634 (606, 665)</td>
<td>658 (625, 692)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>646 (618, 676)</td>
<td>596 (575, 617)</td>
<td>634 (606, 665)</td>
<td>658 (625, 692)</td>
</tr>
<tr>
<td>Plasma tHcy (µmol/L)</td>
<td>All</td>
<td>7.7 (7.6, 7.9)</td>
<td>7.3 (7.0, 7.6)</td>
<td>8.5 (8.3, 8.7)</td>
<td>7.1 (6.9, 7.5)</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>7.7 (7.6, 7.9)</td>
<td>7.3 (7.0, 7.6)</td>
<td>8.5 (8.3, 8.7)</td>
<td>7.1 (6.9, 7.5)</td>
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<td>8.5 (8.3, 8.7)</td>
<td>7.1 (6.9, 7.5)</td>
</tr>
<tr>
<td>Plasma MMA (nmol/L)</td>
<td>All</td>
<td>340 (335, 345)</td>
<td>383 (364, 403)</td>
<td>355 (341, 368)</td>
<td>344 (338, 350)</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>340 (335, 345)</td>
<td>383 (364, 403)</td>
<td>355 (341, 368)</td>
<td>344 (338, 350)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>340 (335, 345)</td>
<td>383 (364, 403)</td>
<td>355 (341, 368)</td>
<td>344 (338, 350)</td>
</tr>
</tbody>
</table>

*Age-adjusted geometric means are based on 2000 census data for adults aged ≥20 y. n in brackets.

*ANOVA (2 × 3) with 2-tailed, 2-group t test.

*Significantly different from non-Hispanic blacks, P < 0.01.

*Significantly different from non-Hispanic whites, P < 0.01.

*Significantly different from Mexican Americans, P < 0.01.

*Main effect of sex could not be determined because of a significant sex-by-age interaction.

*Significantly different from females, P < 0.01.

*Main effect of sex or race could not be determined because of a significant sex-by-race interaction.
B-12 precluded main-effect comparisons between males and females and between racial-ethnic groups. However, a subgroup analysis showed that non-Hispanic white males had lower serum vitamin B-12 concentrations than did non-Hispanic black and Mexican American males, but the difference between non-Hispanic black and Mexican American males was not significant ($P = 0.026$). The same pattern applied for females ($P = 0.597$).

We found a significant sex-by-age interaction for serum folate and plasma tHcy but not for RBC folate ($P = 0.021$), serum vitamin B-12 ($P = 0.020$), or plasma MMA ($P = 0.026$). We found a significant main effect of sex for plasma MMA but not for RBC folate ($P = 0.036$). The main effect of sex on serum folate and plasma tHcy could not be evaluated because of the significant sex-by-age interaction; that on serum vitamin B-12 could not be evaluated because of the significant sex-by-race interaction. A significant main effect of age was found for RBC folate, serum vitamin B-12, and plasma MMA. The main effect of age on serum folate and plasma tHcy could not be evaluated because of a significant sex-by-age interaction. If a sex-by-age interaction was found, a subgroup analysis was performed with a 2-tailed, 2-group $t$ test with 14 df. The solid black squares within each bar indicate significant differences between that age group and the reference age group (20–39 y) for that sex ($P < 0.01$; data controlled for race-ethnicity). *Significant difference between males and females within each age group, $P < 0.01$ (data controlled for race-ethnicity).

**FIGURE 1.** Age-specific geometric mean concentrations of serum and red blood cell (RBC) folate, serum vitamin B-12, and plasma total homocysteine (tHcy) and methylmalonic acid (MMA) for males (●) and females (□) aged ≥3 y in the United States (1999–2000). Error bars represent 95% CIs. Each bar represents a minimum of 164 subjects and a maximum of 1081 subjects. For the exact number of subjects represented by each bar, see Table S1 under “Supplemental data” in the current online issue at www.ajcn.org. We found significant sex-by-age interactions for serum folate and plasma tHcy but not for RBC folate ($P = 0.021$), serum vitamin B-12 ($P = 0.020$), or plasma MMA ($P = 0.026$). We found a significant main effect of sex for plasma MMA but not for RBC folate ($P = 0.036$). The main effect of sex on serum folate and plasma tHcy could not be evaluated because of the significant sex-by-age interaction; that on serum vitamin B-12 could not be evaluated because of the significant sex-by-race interaction. A significant main effect of age was found for RBC folate, serum vitamin B-12, and plasma MMA. The main effect of age on serum folate and plasma tHcy could not be evaluated because of a significant sex-by-age interaction. If a sex-by-age interaction was found, a subgroup analysis was performed with a 2-tailed, 2-group $t$ test with 14 df. The solid black squares within each bar indicate significant differences between that age group and the reference age group (20–39 y) for that sex ($P < 0.01$; data controlled for race-ethnicity). *Significant difference between males and females within each age group, $P < 0.01$ (data controlled for race-ethnicity).
for all age groups ≥12–19 y. Plasma MMA concentrations tended to be higher in elderly people (Figure 1).

Comparison between NHANES III and NHANES 1999–2000 for serum and RBC folate and serum vitamin B-12

From NHANES III to NHANES 1999–2000, the US population shifted to much higher serum folate [median (95% CI); from 12.5 (11.8, 12.9) to 32.2 (30.1, 33.8) nmol/L] and RBC folate [from 392 (381, 406) to 625 (600, 650) nmol/L] concentrations and to slightly higher serum vitamin B-12 concentrations [344 (336, 350) and 359 (352, 367) pmol/L] (Figure 2). Serum and RBC folate concentrations showed large increases and serum vitamin B-12 concentrations showed slight increases in each sex and racial-ethnic subgroup from before to after fortification, except for vitamin B-12 in non-Hispanic blacks (P = 0.181; Table 2). Serum and RBC folate concentrations also showed large increases in each age group. Serum vitamin B-12 increased only in elderly persons (P = 0.574 for the ≤5 y age group, P = 0.124 for the 6–11 y age group, P = 0.288 for the 12–19 y age group, P = 0.185 for the 20–39 y age group, and P = 0.015 for the 40–59 y age group; Table 2).

The most commonly used cutoff for defining low serum folate concentrations is 6.8 nmol/L (45). The prevalence of low serum folate concentrations decreased from 16% in NHANES III to 0.5% in NHANES 1999–2000 for the US population and from 20% to 0.8% for women of childbearing age (12–49 y).

The Life Sciences Research Office panel defined low RBC folate concentrations as <317 nmol/L (46). The prevalence of low RBC folate concentrations decreased from 31% in NHANES III to 3% in NHANES 1999–2000 for the US population and from 38% to 5% for women of childbearing age. Although the overall prevalence of low RBC folate concentrations decreased significantly after the introduction of folic acid fortification, pronounced ethnic differences remained in folate status: 2% of Mexican American, 4% of non-Hispanic white, and 11% of non-Hispanic black women of childbearing age had RBC folate concentrations <317 nmol/L.

We arbitrarily defined high serum folate concentrations as >45.3 nmol/L, which reflected the upper end of the Bio-Rad Quanaphase II calibration range, beyond which samples need to


| Table 2 |

<table>
<thead>
<tr>
<th>Geometric mean concentrations (and 95% CIs) of serum and red blood cell (RBC) folate and of serum vitamin B-12 in different subgroups of the US population: 1988–1994 compared with 1999–2000[^1^]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum folate (nmol/L)</strong></td>
</tr>
<tr>
<td>Non-Hispanic blacks[^6^]</td>
</tr>
<tr>
<td>Children aged ≤5 y</td>
</tr>
<tr>
<td>Children aged 6–11 y</td>
</tr>
<tr>
<td>Adolescents aged 12–19 y</td>
</tr>
<tr>
<td>Adults aged 20–39 y</td>
</tr>
<tr>
<td>Adults aged 40–59 y</td>
</tr>
<tr>
<td>Elderly aged ≥60 y</td>
</tr>
</tbody>
</table>

[^2^] Note that the measurements were made from 1991 to 1994.
[^3^] Values were age-adjusted on the basis of 2000 census data for adults aged 20–74 y.
be diluted and reanalyzed to obtain a valid result. The prevalence of high serum folate concentrations increased from 7% in NHANES III to 43% in NHANES 1999–2000 for children aged ≤5 y and from 7% to 38% for elderly persons.

Serum vitamin B-12 concentrations <74 pmol/L usually suggest vitamin B-12 deficiency (45); <1% of the entire population and of elderly persons had serum vitamin B-12 concentrations <74 pmol/L in NHANES III and in NHANES 1999–2000. Serum vitamin B-12 concentrations <148 pmol/L are considered moderately low, but do not necessarily indicate vitamin B-12 deficiency (45). The prevalence of moderately low serum vitamin B-12 concentrations in elderly persons was somewhat lower in NHANES 1999–2000 (3%) than in NHANES III (5%); the difference was significant. At a cutoff of 185 pmol/L, 7% of elderly persons had moderately low serum vitamin B-12 concentrations in NHANES 1999–2000 compared with 13% in NHANES III; this difference was also significant.

**Prevalence of elevated plasma tHcy and MMA concentrations**

No generally agreed on cutoff exists for elevated plasma tHcy, but 13 μmol/L has sometimes been used to define elevated tHcy concentrations (13). Five percent of the US population, 18% of elderly men and 11% of elderly women, had plasma tHcy concentrations >13 μmol/L in NHANES 1999–2000. Ubbink (47) suggested that a tHcy concentration of ≤9 μmol/L is desirable should the outcome of controlled clinical trials show that a lowering of plasma tHcy concentrations reduces the incidence of cardiovascular disease. In NHANES 1999–2000, 78% of the US population (72% of males and 85% of females) and 50% of elderly persons (40% of elderly males and 60% of elderly females) had plasma tHcy concentrations ≤9 μmol/L. A generally agreed on cutoff for elevated plasma MMA is 370 nmol/L (48, 49). Two percent of the US population and 7% of elderly persons had elevated MMA concentrations.

**DISCUSSION**

This study presents the first population-wide reference information for biochemical indicators of B vitamin status in a nationally representative sample of the United States after folic acid fortification began. B vitamin and metabolite concentrations in this population displayed age, sex, and racial-ethnic characteristics similar to those found earlier in NHANES III (23, 41-44, 50). The observed age-related reduction in serum and RBC folate from childhood through adulthood is consistent with other prefortification surveys (23). The difference in plasma tHcy concentration between males and females was of the same magnitude as found in NHANES III (42); however, it appears to be greater than can be explained by the difference in serum folate, which indicated that the sex difference in plasma tHcy may be attributed to factors other than folate status. The observed racial-ethnic differences are consistent with recent findings from a controlled feeding study that race-ethnicity is an important determinant of folate status (51). Non-Hispanic blacks displayed the lowest serum and RBC folate concentrations in our analysis. One of the National Health Objectives for 2010 is to increase the proportion of pregnancies for which RBC folate concentration is optimum by increasing the median RBC folate concentration to 499 nmol/L among women aged 15–44 y (objective 16.16b; 52). According to NHANES 1999–2000, this objective has been met differently by the 3 racial-ethnic groups: 26% of non-Hispanic white, 32% of Mexican American, and 50% of non-Hispanic black women of childbearing age had RBC folate concentrations <499 nmol/L.

Since folic acid fortification began in the United States in 1998, the US population shifted to significantly higher serum and RBC folate concentrations. We observed increases across all subgroups of age, sex, or race-ethnicity. Adolescents and adults have experienced the biggest relative increase, children aged ≤5 y the smallest increase, and elderly persons an intermediate increase. Without distinguishing between supplement and non-supplement users, serum and RBC folate increased by 18–23 and 204–272 nmol/L across all age groups.

These findings are consistent with earlier findings from NHANES 1999–2000 that folate status has improved significantly in women of childbearing age, the target group for the folic acid fortification (27), as well as reports of improved folate status in selected nonrepresentative subsets of the US population. In the Framingham population, mean serum folate concentrations among nonusers of B vitamin supplements increased from 11 nmol/L before to 23 nmol/L after fortification, and the prevalence of low serum folate concentrations (<6.8 nmol/L) decreased from 22% to 2% (13); RBC folate concentrations increased by 38% (25). Median serum folate concentrations in samples submitted to Kaiser Permanente’s Southern California Endocrinology Laboratory during 1994–1998 steadily increased from 28.6 nmol/L before to 42.4 nmol/L after fortification (26). After the introduction of folic acid fortification in Canada, Ray et al (53) reported from a retrospective cross-sectional study geometric mean serum and RBC folate concentrations of 34.5 and 957 nmol/L, respectively; these concentrations were higher than expected and even higher than concentrations found in the United States. In 2 later reports, the authors found increases of 64% in serum folate in a nonrepresentative sample of elderly women in Ontario and British Columbia (54) and 41% in RBC folate in a nonrepresentative sample of women of childbearing age in Ontario (55). In Chile, after the mandatory fortification of wheat flour with folic acid, targeted to increase daily folate consumption of women of childbearing age by 400 μg, serum and RBC folate concentrations in a representative population sample increased 284% and 144%, respectively (56). The increase in RBC folate concentrations in the US population after fortification also seems compatible with the increase shown by Daly et al (57) in a double-blind, randomized, placebo-controlled trial of women of childbearing age who were supplemented with 200 μg folic acid/d.

Although the increase in serum and RBC folate concentrations in the US population after fortification in our analysis is consistent with other reports on nonrepresentative subpopulations, the increase is still higher than was expected from the 70–130-μg/d increase predicted by the US fortification program (58). The discrepancy may be due to unknown sampling biases between NHANES III and NHANES 1999–2000, changes in vitamin supplementation, or an imprecise ability to predict folate intake from blood folate concentrations. Because of the stable results for long-term quality control of the Bio-Rad assay between the 2 surveys, changes in laboratory techniques are unlikely to account for the shift in folate concentrations. Another possible explanation for the higher than expected folate concentrations could be a higher than expected daily intake of folic acid due to either the underestimation of food quantities eaten or overage added to
cereal-grain products in the process of fortification. Rader et al (59) reported significant excess fortification in a survey of recently fortified products. Furthermore, the spectrum of voluntarily fortified snack foods seems to undergo constant growth and change.

The US Institute of Medicine has reviewed all potential adverse effects of high doses of folic acid and found that the only basis for tolerable upper intake levels (UL) was the potential masking of vitamin B-12 deficiency (20). The UL for adults was set to 1 mg folate/d from fortified food or supplements, the UL for children was set to 300–800 μg/d, depending on age. Although the serum folate concentration that should be considered excessively high and its health implications are not known, folic acid ingested in quantities >266 μg in one meal can appear unmetabolized in serum (60). In NHANES III, 3% of the US population, 7% of children aged ≤5 y, and 8% of elderly persons had high serum folate concentrations (>45.3 mmol/L). In NHANES 1999–2000, after folic acid fortification began, 23% of the US population, 43% of children aged ≤5 y, and 38% of elderly persons reached this putatively high serum folate concentration. The issue of potential adverse effects of excess folate in persons with untreated vitamin B-12 deficiency remains unsolved. Mills et al (61) reported that in persons with low serum vitamin B-12 concentrations (<258 pmol/L) examined during 1992–2000 at the Veterans Affairs Medical Center in Washington, DC, the proportion of persons without anemia did not increase significantly from the prefortification period (39.2%) to the period of optional fortification (45.5%) or in the postfortification period (37.6%). We are unaware of any epidemiologic evidence of an increased risk of masked vitamin B-12 insufficiency or related disease after folic acid fortification, but clinically recognized vitamin B-12 insufficiency may be uncommon. Indeed, <1% of the US population had low serum vitamin B-12 concentrations (<74 pmol/L) that suggested vitamin B-12 deficiency, and <5% had moderately low serum vitamin B-12 concentrations (<148 pmol/L). Interestingly, serum vitamin B-12 concentrations increased slightly but significantly in the US population between the prefortification and postfortification periods. This increase was limited mainly to elderly persons. Whether the increase may be due to unknown sampling biases between the 2 surveys, changes in vitamin supplementation, or a higher intake of vitamin B-12–rich foods is not known.

A direct comparison of plasma tHcy concentrations between NHANES 1999–2000 and NHANES III is not possible because of differences in the methods and matrices of the 2 surveys. However, tHcy concentrations after fortification appeared lower than in NHANES III (41, 42, 44). When we chose a cutoff of 13 μmol/L, we found that ≈14% of elderly persons had elevated plasma tHcy concentrations. This corresponds well with a 10% prevalence of elevated tHcy after folic acid fortification compared with a prevalence of ≈20% before fortification in the Framingham population (24). What seems even more important, though, is that nearly 80% of the US population had achieved a plasma tHcy concentration <9 μmol/L, which is considered a desirable concentration (47).

We have presented the first nationally representative US population reference ranges for the B vitamins serum and RBC folate, serum vitamin B-12, and their biochemically related products plasma tHcy and MMA after the mandatory folic acid fortification of cereal-grain products was introduced in the United States in 1998. The fortification program has increased concentrations of serum and RBC folate in the entire population and virtually eliminated folate deficiency. Vitamin B-12 concentrations in elderly persons appear to be slightly higher. Plasma tHcy concentrations appear to be lower after fortification, and ≈80% of the population has achieved desirable concentrations. Nutritional monitoring for folate status remains exceedingly important, not only for women of childbearing age but also for children and persons of all ages. Nutritional monitoring for vitamin B-12 status, particularly in elderly persons, is similarly important.

We thank Della Twite, Donna LaVoie, Sonya Strider, and Ming Zhang (CDC, National Center for Environmental Health) for performing the B vitamin and metabolite assays; Tracy Dearth-Wesley (CDC, National Center for Environmental Health) for technical assistance in compiling the data tables; and Clifford L. Johnson (CDC, National Center for Health Statistics) for his valuable input while reviewing the initial draft of the manuscript. EWG and EJS were involved in the design of NHANES. EWG and CMP supervised the laboratory work, SPC and CMP performed the data analysis. CMP wrote the initial draft of the manuscript. JO provided significant advice on the data analysis and interpretation. All authors contributed to the final manuscript. None of the authors declared any personal or financial conflict of interest.

REFERENCES


Dietary β-cryptoxanthin and inflammatory polyarthritis: results from a population-based prospective study1–3

Dorothy J Pattison, Deborah PM Symmons, Mark Lunt, Ailsa Welch, Sheila A Bingham, Nicholas E Day, and Alan J Silman

ABSTRACT
Background: Epidemiologic studies suggest that the antioxidant potential of dietary carotenoids may protect against the oxidative damage that can result in inflammation.

Objective: We investigated the hypothesis that some dietary carotenoids are associated with a reduced risk of developing inflammatory polyarthritis (IP).

Design: The European Prospective Investigation of Cancer Incidence (EPIC)-Norfolk study is a population-based, prospective study of >25 000 subjects who completed a baseline 7-d diet diary and were followed up to identify new cases of IP, which was defined as synovitis that affected ≥2 joint groups. Dietary carotenoid intakes were computed from the diet diaries of these subjects, and a nested, case-control analysis was undertaken to compare carotenoid intake between case subjects and age- and sex-matched control subjects.

Results: Eighty-eight incident cases of IP that occurred in the population surveyed were ascertained via the Norfolk Arthritis Register. The mean daily intakes of zeaxanthin and β-cryptoxanthin were 20% and 40% lower, respectively, in the cases than in the 176 controls, but there were no significant differences in the intakes of lutein or lycopene. Those subjects in the top one-third of intake of zeaxanthin and β-cryptoxanthin were at a lower risk of developing IP than were subjects in the lowest one-third [odds ratios (95% CI): 0.48 (0.24, 0.94) and 0.51 (0.25, 1.02) for zeaxanthin and β-cryptoxanthin, respectively]. The association with β-cryptoxanthin was significant after adjustments were made for total energy and protein intakes and for cigarette smoking.

Conclusion: These data are consistent with previous evidence showing that a modest increase in β-cryptoxanthin intake, equivalent to one glass of freshly squeezed orange juice per day, is associated with a reduced risk of developing inflammatory disorders such as rheumatoid arthritis. Am J Clin Nutr 2005;82:451–5.

KEY WORDS Prospective study, dietary β-cryptoxanthin, antioxidant, inflammatory polyarthritis

INTRODUCTION
The role of oxidative damage to the synovium in the pathogenesis of rheumatoid arthritis (RA) and other inflammatory disorders has been recognized for a long time. Circulating antioxidants have a role as scavengers of free radicals and may inhibit oxidative damage and lead to the suppression of inflammation (1, 2). It was recently postulated that this phenomenon might have a role in the prevention of cardiovascular disease. Specifically, reports from epidemiologic studies have shown that C-reactive protein and oxidized LDL-cholesterol concentrations, which have also been linked to the development of cardiovascular disease, are inversely related to serum concentrations of circulating antioxidants, including the carotenoids β-carotene, β-cryptoxanthin, and zeaxanthin (3–6). Although cross-sectional studies of serum antioxidant concentrations in patients with RA are consistent with inflammation that results in a relative depletion of antioxidants (7), the data are also consistent with the hypothesis that antioxidants may be protective against the development of RA. Two nested case-control studies, using serum samples collected before the onset of RA, suggested that serum concentrations of antioxidants are reduced before RA onset (8, 9). It is not known whether dietary carotenoid intake is also negatively related to RA onset.

The Iowa Women’s Health Study, a large, prospective population-based study of >29 000 women aged 55–69 y who completed a food-frequency questionnaire at baseline (1986) and were followed up by questionnaires to determine the onset of various diseases, recently reported a protective effect against the development of RA of a high dietary intake of β-cryptoxanthin but not of other carotenoids, including β-carotene, lutein, and zeaxanthin (10). Methodologic reasons may explain the lack of association with the other carotenoids, such as random misclassification of dietary intake, incomplete data on the carotenoid content of some foods, or lack of statistical power. Alternatively, the mechanisms of action of the carotenoids may vary, whereby

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2 The Norfolk Arthritis Register (NOAR) is supported by the United Kingdom Arthritis Research Campaign; the EPIC-Norfolk study is supported by Cancer Research United Kingdom and through additional support from the Medical Research Council, the British Heart Foundation, the Ministry of Agriculture Fisheries and Food, the Department of Health, and the Europe Against Cancer Programme of the Commission of the European Communities.
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the influence of β-cryptoxanthin on some markers of inflammatory activity may be greater than those of other carotenoids (4).

Robust epidemiologic studies of risk factors for RA require the ascertainment of incident cases. One problem that we have identified in such studies is that subjects with a recent onset of inflammatory polyarthritis (IP) are likely to eventually develop RA over a period of several years (11). Therefore, ideally, such studies should aim to recruit all eligible subjects with IP (12) and undertake subsequent subgroup analyses, where appropriate, of those subjects that ultimately satisfy the criteria for RA.

We recently showed that a low dietary intake of vitamin C is a risk factor for the development of IP (13). In that study we also found that high intakes of fruit, but not of vegetables, protect against IP, which would be consistent with a differential role for β-cryptoxanthin. We have also shown that obesity is associated with an increased risk of developing IP and RA (14), which is of particular interest because a recent report found that in the general population obesity is negatively related to serum concentrations of β-cryptoxanthin and positively related to C-reactive protein (15). Thus, we investigated the hypothesis that high dietary intakes of some carotenoids, specifically β-cryptoxanthin, zeaxanthin, lutein, β-carotene, and lycopene, are protective against the development of IP.

SUBJECTS AND METHODS
Study design
A nested, case-control study embedded within a population-based prospective study was undertaken. The participants in the prospective study had completed a 7-d diet diary at baseline. The baseline dietary intakes of carotenoids and other nutrients were compared between cases (new cases of IP arising within the study population between 1993 and 2001) and their matched controls.

Baseline survey
The subjects studied were recruited as part of the Norfolk arm of the European Prospective Investigation of Cancer Incidence study (EPIC-Norfolk). Details of the EPIC-Norfolk study have been published elsewhere (16). In brief, >25 000 subjects aged 45-74 y were identified from registers of primary care physicians and invited to participate in a major prospective study. At baseline, the subjects had their height (in m) and weight (in kg) measured with the use of standard techniques, and they completed a health and lifestyle questionnaire, which included details about current and past smoking habits.

Dietary assessment
The subjects were required to complete a 7-d diet diary in which they recorded all food and beverages consumed over 7 consecutive days. The subjects were encouraged to record the quantities consumed as accurately as possible by using household measures and a series of food portion photographs. This survey method was extensively validated within the EPIC-Norfolk study population, and repeated dietary assessments were shown to reflect dietary intake over a 1-y period. Pearson correlation coefficients between 2 repeat diaries over 1 y were as follows: energy, 0.71; protein, 0.67; vitamin C, 0.59; and β-carotene, 0.47 (17–20).

Ascertainment of inflammatory polyarthritis
Coincidentally, the population screened within EPIC-Norfolk was under continuous surveillance for the onset of all new cases of IP as part of the Norfolk Arthritis Register (NOAR). Details of NOAR have also been published elsewhere (21). In brief, all new adult attendees to primary care physicians, within the area served by the former Norwich Health Authority, with an onset of joint inflammation affecting ≥2 joints and persisting for >4 wk were alerted to NOAR. On notification, the affected subjects were interviewed and examined by a skilled research nurse and had blood samples taken and analyzed for the presence of rheumatoid factor. The American College of Rheumatology (ACR) classification criteria for RA (22) were applied at baseline and annually thereafter (11). Although there are 7 ACR criteria, it may take time from symptom onset to reach ≥4 criteria, which is the currently accepted cutoff applied in RA comparative studies.

Study participants
The database of subjects participating within EPIC-Norfolk was linked to the NOAR database, and those subjects who had participated in EPIC-Norfolk and who went on to develop IP after the baseline assessments were identified. The subjects were excluded if the inflammation affecting ≥2 joint groups could not be confirmed by the research nurses or if a diagnosis other than RA, psoriatic arthritis, viral arthritis, or undifferentiated polyarthritis was made either at baseline or at follow-up assessments. The remaining subjects were then selected as cases for the current analysis. For each case, 2 control subjects were selected from the EPIC-Norfolk database and were matched for age (to within 3 y), sex, and to within 3 mo of the recruitment to EPIC-Norfolk. All participants had consented to participation, and both the EPIC-Norfolk and the NOAR studies had been approved by the local medical research ethics committee.

Analysis
The dietary data were interpreted by one of us (DJP) and then analyzed for nutrient intake by using the Data Into Nutrients for Epidemiologic Research (DINER) software package, which is based on standard UK food-composition tables (23). Daily intakes of β-cryptoxanthin, zeaxanthin, lutein, lycopene, β-carotene, and vitamin C and total energy intake were estimated with the use of a recently extended and updated database of the carotenoid content of foods. For the analyses, nutrient intakes were stratified into tertiles of intake, and total energy intake was entered into the statistical models as a continuous variable. The referent group for each analysis was the group that was presumed the most at risk of developing IP; that is, those in the lowest tertile of intake for each of the carotenoids analyzed. A conditional logistic-regression analysis was undertaken to maintain the matched trios. The risk of developing IP in the 2 higher tertiles of intake for each of the carotenoids was investigated first by univariate analysis and then by multivariate analysis after adjustments for total energy intake, total protein intake [which was previously found to be associated with the risk of developing IP (13)], intake of each of the carotenoids that were not being investigated, vitamin C intake, and pack-years of smoking. Within individuals, carotenoid intakes were not highly correlated; hence, all carotenoid intakes were included in the multivariate analysis. All analyses were performed with STATA 8.0 (24).
TABLE 1
Characteristics of the cases and controls

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 88)</th>
<th>Controls (n = 176)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>61 ± 8.9</td>
<td>61 ± 8.9</td>
</tr>
<tr>
<td>Women [%]</td>
<td>61 (69)</td>
<td>122 (69)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27 ± 4.1</td>
<td>27 ± 3.9</td>
</tr>
<tr>
<td>Current smokers [%]</td>
<td>18 (21)</td>
<td>22 (13)</td>
</tr>
<tr>
<td>Pack-years of smoking in current smokers</td>
<td>28.1 (21.2–36.0)</td>
<td>19 (8.8–31.3)</td>
</tr>
</tbody>
</table>

1 ± SD (all such values).
2 Data available for 86 cases and 174 controls. Difference χ² = 3.4, P = 0.2.
3 Median; interquartile range in parentheses (all such values).
4 Significantly different from controls, P = 0.01.

RESULTS

In total, 88 cases that satisfied the entry criteria for this analysis were identified and successfully matched with 176 controls. The mean (±SD) interval between the EPIC-Norfolk survey and the onset of IP was 2.5 ± 1.8 y, and 35 of 78 cases (45%) with complete data satisfied the criteria for a diagnosis of RA at symptom onset. The baseline characteristics of these 88 cases and their matched controls are shown in Table 1. Obviously, the cases and controls were well matched for age and sex but, at the time of their baseline EPIC-Norfolk assessment, the cases were more likely to be current smokers than were the controls. There was no significant difference in body mass index, estimated as w/H², between the 2 groups.

Median dietary intakes of total energy (in kcal), protein (as a percentage of total energy intake), and carotenoids for cases and controls are shown in Table 2. There were no significant differences in the intakes of β-carotene, lutein, and lycopene between the 2 groups. The median zeaxanthin intake was 20% lower and the median β-carotoxanthin intake was almost 40% lower in cases than in controls and both differences were statistically significant.

β-Cryptoxanthin and zeaxanthin intakes in the highest tertiles of intake were associated with a reduced risk of developing IP (Table 3). Thus, cases were less likely to be in the highest third of intake of β-cryptoxanthin than were controls [odds ratio (OR): 0.51; 95% CI: 0.25, 1.02], but the association was not statistically significant. After adjustments were made for total energy intake, protein intake, and cigarette smoking, the association was significant (OR: 0.42; 95% CI: 0.20, 0.88). There was a strong correlation between the intakes of β-cryptoxanthin and vitamin C (r = 0.8); thus, we anticipated that adjusting for vitamin C intake would attenuate any association observed, and indeed this was the case. After adjustment for vitamin C intake, β-cryptoxanthin intake was no longer significantly correlated with the risk of developing IP, although the estimation changed only slightly (OR: 0.50; 95% CI: 0.22, 1.20). The analysis for zeaxanthin intake showed that cases were also significantly less likely to be in the highest tertile of intake (OR: 0.48; 95% CI: 0.24, 0.94), but after adjustments for the above covariates, the 95% CI included unity (OR 0.52; 95% CI: 0.26, 1.02). There was no association between β-carotene, lutein, or lycopene intakes and the risk of developing IP (Table 3). Thus, although there is a correlation between vitamin C intake and β-cryptoxanthin intake, adjustment for the former produced an effect estimate for β-cryptoxanthin that was little altered, although the CI widened.

DISCUSSION

In summary, we found that diets high in β-cryptoxanthin, and probably zeaxanthin, are associated with a reduced risk of developing IP. The extent of these associations, on the whole, persisted after adjustments for vitamin C intake, although the 95% CIs then included unity. Hence, these data are supportive, but not conclusive, of the fact that these carotenoids are independently associated with a reduced risk of IP. Interestingly, these data are also consistent with our previous observation that high-fruit diets may be protective against developing IP (13) and with one other study of diet and RA onset, which used a less intensive method of diet inquiry than the present study, and found that β-cryptoxanthin intake may have a stronger association with the onset of joint inflammation than do other dietary carotenoids (10).

The present study had several strengths. The study was population-based with regard to both the baseline survey (16) and the identification of subsequent cases of IP (21), which provides external validity to the findings. The cases were identified on the basis of detailed and standardized subject assessment (21). The study was prospective and, hence, free of both subject bias and recall error with relation to the dietary assessment. Additionally, the 7-d diet diary method may be considered, in general terms, to

TABLE 2
Daily intake of total energy and antioxidants by cases and controls

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Cases (n = 88)</th>
<th>Controls (n = 176)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>1799 (1529, 2093)</td>
<td>1763 (1448, 2168)</td>
<td>0.9</td>
</tr>
<tr>
<td>Total protein (% of energy)</td>
<td>15.9 (14.1, 17.8)</td>
<td>14.8 (13.7, 16.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>β-Carotene (µg)</td>
<td>1504 (897, 2251)</td>
<td>1505 (986, 2244)</td>
<td>0.9</td>
</tr>
<tr>
<td>β-Cryptoxanthin (µg)</td>
<td>87.0 (28.3, 332.8)</td>
<td>139.9 (39.3, 683.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Zeaxanthin (µg)</td>
<td>21.7 (6.7, 45.5)</td>
<td>27.2 (13.4, 53.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Lutein (µg)</td>
<td>645.4 (412.1, 1022.8)</td>
<td>638.9 (370.7, 965.1)</td>
<td>0.9</td>
</tr>
<tr>
<td>Lycopene (µg)</td>
<td>721.2 (350.6, 1479.4)</td>
<td>742.5 (299.3, 1265.7)</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>63.7 (44.0, 95.9)</td>
<td>77.7 (50.7, 107.2)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1 All values are median; interquartile range in parentheses.
2 Wilcoxon signed-rank test for difference between cases and controls.
TABLE 3

Associations between antioxidant nutrient intakes and risk of developing inflammatory polyarthritis

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 88)</th>
<th>Controls (n = 176)</th>
<th>OR (95% CI), energy adjusted</th>
<th>OR (95% CI), fully adjusted</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Carotene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1150 μg</td>
<td>31 (35)</td>
<td>57 (32)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>1150–1930 μg</td>
<td>30 (34)</td>
<td>58 (33)</td>
<td>0.97 (0.53, 1.78)</td>
<td>0.93 (0.49, 1.76)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;1930 μg</td>
<td>27 (31)</td>
<td>61 (35)</td>
<td>0.83 (0.44, 1.57)</td>
<td>0.82 (0.42, 1.61)</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>β-Cryptoxanthin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;56 μg</td>
<td>32 (36)</td>
<td>56 (32)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>56–365 μg</td>
<td>35 (40)</td>
<td>53 (30)</td>
<td>1.15 (0.61, 2.19)</td>
<td>1.09 (0.56, 2.09)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;365 μg</td>
<td>21 (24)</td>
<td>67 (38)</td>
<td>0.51 (0.25, 1.02)</td>
<td>0.42 (0.20, 0.88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;14.2 μg</td>
<td>36 (41)</td>
<td>52 (30)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>14.2–39.0 μg</td>
<td>29 (33)</td>
<td>59 (33)</td>
<td>0.67 (0.36, 1.26)</td>
<td>0.75 (0.39, 1.43)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;39.0 μg</td>
<td>23 (26)</td>
<td>65 (37)</td>
<td>0.48 (0.24, 0.94)</td>
<td>0.52 (0.26, 1.02)</td>
<td>0.06</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;466.9 μg</td>
<td>25 (28)</td>
<td>63 (36)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>466.9–835.0 μg</td>
<td>34 (39)</td>
<td>54 (31)</td>
<td>1.57 (0.84, 2.94)</td>
<td>1.66 (0.86, 3.19)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;835.0 μg</td>
<td>29 (33)</td>
<td>59 (33)</td>
<td>1.29 (0.65, 2.57)</td>
<td>1.34 (0.64, 2.83)</td>
<td>0.4</td>
</tr>
<tr>
<td>Lycopene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;456 μg</td>
<td>27 (30.5)</td>
<td>61 (34.5)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>456–1070 μg</td>
<td>27 (30.5)</td>
<td>61 (34.5)</td>
<td>1.00 (0.53, 1.90)</td>
<td>1.10 (0.56, 2.18)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;1070 μg</td>
<td>34 (39)</td>
<td>54 (31)</td>
<td>1.52 (0.80, 2.89)</td>
<td>1.42 (0.72, 2.79)</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55.9 mg</td>
<td>38 (43)</td>
<td>50 (28)</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>55.9–94.0 mg</td>
<td>25 (28.5)</td>
<td>63 (36)</td>
<td>0.45 (0.22, 0.92)</td>
<td>0.30 (0.14, 0.66)</td>
<td>—</td>
</tr>
<tr>
<td>&gt;94.0 mg</td>
<td>25 (28.5)</td>
<td>63 (36)</td>
<td>0.46 (0.23, 0.92)</td>
<td>0.34 (0.16, 0.72)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

1 P for trend applies to the fully adjusted model. Analysis was performed by using conditional logistic regression to calculate odds ratios (ORs) with 95% CIs.
2 Adjusted for energy, protein, and vitamin C intakes and for pack-years of smoking.
3 Adjusted for energy and protein intakes and for pack-years of smoking.

be the most robust method for dietary assessment (19, 25), although serum β-carotene, β-cryptoxanthin, and zeaxanthin concentrations also showed moderate correlations with dietary carotenoid intakes when measured by a food-frequency questionnaire (26).

Despite the large sample surveyed in the EPIC-Norfolk study, the number of incident cases of IP was modest, which likely limited both the power to detect a true dose-response relationship and the ability to identify independent associations with specific carotenoids in the present study. The diet diary method of assessment may also be less useful than other dietary assessment methods when the nutrients under investigation occur in foods that are consumed infrequently. In previous studies, we showed that the diet diary was repeatable when assessed through comparisons of one diary with another at different seasons over 1 y and with comparisons of biomarkers of dietary intake, urinary nitrogen, potassium, sodium, and plasma ascorbic acid (18). However, we were unable to assess the repeatability of the dietary intake of β-cryptoxanthin, which is present in significant quantities in only a few commonly eaten fruit (particularly oranges, bell peppers, and some tropical fruit). Hence, any relation between β-cryptoxanthin and IP shown in the present study is likely to be attenuated. Additionally, because the control subjects were matched for time of recruitment with the case subjects, seasonal variations would not have accounted for these findings. The intake of β-cryptoxanthin in this report is higher than those reported by others (10, 27). This is most likely explained by the recent update of the database that lists the carotenoid content of foods, which now gives a more comprehensive estimate of the consumption of β-cryptoxanthin from foods that are commonly consumed in the United Kingdom. In our subjects, β-cryptoxanthin intake was attributed mainly to the consumption of oranges, orange juice, and satsumas.

The outcome of interest in this study was the risk of developing IP and not RA specifically. First, our hypothesis was that some antioxidant nutrients protect against inflammation and, thus, we wanted to ensure that all cases of inflammatory arthritis were included. Second, the classification of individuals with a new onset of IP as having RA can be difficult (12). Briefly, the criteria for the diagnosis of RA were developed from observations of individuals with long-standing RA and thus are not easily applied at the time of disease onset. We have shown within NOAR that the cumulative rate of satisfying criteria for RA in individuals with IP continues to increase with time (11). A subgroup analysis showed no obvious differences between those who did and those who did not satisfy the diagnostic criteria by the time of our analysis.

A few reports agree with the suggestion that RA itself may result in reduced concentrations of serum antioxidants. Two small case-control studies found that RA was associated with a reduction in vitamin A (7) and other antioxidants (28). In the latter study, supplementation with β-carotene did not reduce inflammatory activity (28), although an uncontrolled study suggested that changing to a diet high in antioxidant nutrients might lead to an improvement in RA symptoms (29). However, the present study, which is based on the prospective assessment of
the dietary intake of carotenoids, is more supportive of a causal relation between diets that are low in such antioxidants and RA, rather than reduced concentrations of antioxidants being a consequence of RA.

In the present study, the strongest relation was observed between β-cryptoxanthin intake and IP onset, which supports the finding from the Iowa Women’s Health Study of an inverse association between high β-cryptoxanthin intakes and RA onset (10). Our finding also supports evidence from the Framingham Heart Study, which showed a stronger correlation between dietary intake and serum concentrations of β-cryptoxanthin than other carotenoids (26). Finally, some data suggests that a high BMI is linked to both low levels of β-cryptoxanthin and high levels of C-reactive protein in the general population (15), which is consistent with the findings in the present study and with our previous work that found that obesity was associated with a greater risk of developing IP (14).

In conclusion, these data add to a growing body of evidence that some dietary antioxidants, such as the carotenoids β-cryptoxanthin and zeaxanthin as well as vitamin C, may be protective against the development of IP. On the basis of the results of our study, supplementation of diets with just one glass of orange juice per day is sufficient to raise the intake of β-cryptoxanthin to the highest and most protective tertile of intake. Given the inherent difficulty of a randomized trial and the rarity of the disease, it may be difficult to prove the contribution of diet to the primary prevention of RA. However, there is perhaps sufficient evidence to suggest a trial to test whether an increase in the consumption of foods that are high in dietary carotenoids and other antioxidant nutrients during early inflammatory joint disease decreases the risk of persistent disease and possibly joint damage.

We acknowledge the collaboration of the rheumatologists in Norfolk led by David Scott and the major contribution by the NOAR and EPIC-Norfolk research nurses. We also acknowledge the work done by Subodha Shakya Shrestha, EPIC-Norfolk, who updated the carotenoid content of foods database.

AJS, DPMS, SAB, and NED were primarily responsible for the study design and contributed to the preparation of the manuscript. AW participated in the design of the study and assisted with the data acquisition and interpretation. DJP assisted with the data acquisition and was responsible for the statistical analysis and the preparation of the manuscript. ML advised on statistical analysis and assisted in interpreting the data. None of the authors had any conflicts of interest.

REFERENCES

Anthropometric indicators of body composition in young adults: relation to size at birth and serial measurements of body mass index in childhood in the New Delhi birth cohort

Harshpal S Sachdev, Caroline HD Fall, Clive Osmond, Ramakrishnan Lakshmy, Sushant K Dey Biswas, Samantha D Leary, Kolli Srinath Reddy, David JP Barker, and Santosh K Bhargava

ABSTRACT

Background: South Asians have a muscle-thin but adipose body phenotype and high rates of obesity-related disease. Adult body composition may be predictable in early life.

Objective: Anthropometric indexes of adult body composition were examined in relation to birth size and body mass index (BMI) during childhood.

Design: A population-based cohort of 1526 men and women aged 26–32 y in Delhi, India, who were measured sequentially from birth until 21 y of age were followed up. Adult weight, height, skinfold thicknesses, and waist and hip circumferences were measured. BMI and indexes of adiposity (sum of skinfold thicknesses), central adiposity (waist-hip ratio), and lean mass (residual values after adjustment of BMI for skinfold thicknesses and height) were derived.

Results: Mean birth weight was 2851 g. As children, many subjects were underweight-for-age (>2 SDs below the National Center for Health Statistics mean; 53% at 2 y), but as adults, 47% were overweight, 11% were obese, and 51% were centrally obese (according to World Health Organization criteria). Birth weight was positively related to adult lean mass (P < 0.001) and, in women only, to adiposity (P = 0.006) but was unrelated to central adiposity. BMI from birth to age 21 y was increasingly strongly positively correlated with all outcomes. BMI and BMI gain in infancy and early childhood were correlated more strongly with adult lean mass than with adiposity or central adiposity. Higher BMI and greater BMI gain in late childhood and adolescence were associated with increased adult adiposity and central adiposity.

Conclusions: Birth weight and BMI gain during infancy and early childhood predict adult lean mass more strongly than adult adiposity. Greater BMI gain in late childhood and adolescence predicts increased adult adiposity.


KEY WORDS Body composition, lean mass, obesity, developmental origins of adult disease, birth weight, childhood growth, nutritional transition, India

INTRODUCTION

Obesity is a strong risk factor for hypertension, type 2 diabetes, dyslipidemia, and ischemic heart disease (IHD). Although its harmful effects on health are thought to arise from an excess of body fat (adipose tissue), obesity is generally defined by using the body mass index (BMI; weight/height²), which does not distinguish between lean and fat components of body weight (1).

Body composition can vary widely at any given BMI, as highlighted recently by the debate concerning the appropriateness of BMI definitions of obesity in different ethnic groups (2, 3). South Asians have a low mean BMI, but this low BMI masks several adverse features of their body composition. South Asians have a lower muscle mass and a higher percentage body fat and are more centrally obese than are whites of comparable ages and BMIs (4–9). These characteristics are thought to partly explain South Asians’ high risk of developing type 2 diabetes and IHD (4, 9–12).

Considerable interest currently exists in the associations between growth in early life (fetal life, infancy, childhood, and adolescence) and the later development of obesity and obesity-related disease. Higher weight or BMI, or accelerated gain in weight or BMI, during childhood and adolescence is associated with a higher adult BMI (13, 14) and with an increased risk of adult hypertension (15), type 2 diabetes (16–18), and IHD (19–22). Paradoxically, however, although higher birth weight predicts higher adult BMI (14, 23, 24), it is associated with a lower risk of type 2 diabetes and IHD (18, 22, 25–29), an exception being high birth weight caused by maternal diabetes, which is associated with an increased risk of later type 2 diabetes (29, 30).

Higher weight or BMI in infancy is also associated with higher adult BMI (14) but a lower risk of type 2 diabetes and IHD (18, 22, 25–27). One possible explanation for these discrepancies is that weight gain at different periods of early life may have differential effects on the acquisition of fat and lean mass. There is good evidence, for example, that higher birth weight is associated...
more strongly with increased adult lean body mass than with adult adiposity (31–37).

We recently reported data from a cohort of young adults who were born in Delhi, India, and whose weight and height were measured at birth and throughout infancy, childhood, and adolescence (38). Lower BMI in infancy and accelerated BMI gain from 2 y onward were associated with an increased risk of adult type 2 diabetes and impaired glucose tolerance. Here we describe the relation of the early growth of this cohort with their adult body composition.

SUBJECTS AND METHODS

The methodology of the Delhi birth cohort study has been described (38). In brief, the cohort was established between 1969 and 1972 to study pregnancy outcomes and child growth. All families living in a 12-ka² area of South Delhi were identified, and 20,755 married women of reproductive age were followed up bimonthly to record menstrual dates. A total of 9169 pregnancies, resulting in 8181 live births (8030 singletons and 151 from twin pairs), were recorded. Trained personnel recorded the weight and length or height of the babies within 72 h of birth, at ages 6 and 12 mo, and every 6 mo thereafter up to the age of 14–21 y. At recruitment, 60% of the families had an income >50 rupees per month (national average = 28 rupees), and only 15% of parents were illiterate (national average = 66%). Nevertheless, 43% of families lived in only one room. Hindus were the majority religious group (84%), followed by Sikhs (12%), Christians (2%), Muslims (1%), and Jains (1%).

Current study

From August 1998 to August 2002, we retraced 2584 (32%) of the initial cohort. They were visited at home by a social worker who explained the study, obtained consent, and administered a questionnaire. Ethical approval for the study was granted by the All India Institute of Medical Sciences, and informed consent was obtained from each subject.

Questionnaire data

Education was recorded as 1 of 7 categories ranging from “no schooling” (category 1) to “professional degree” (eg, Master of Science degree, PhD, or medical qualification) and occupation as 1 of 6 categories ranging from “unemployed” (category 1) and “unskilled manual labor” (category 2) to “professional.” Housewives were categorized according to their husbands’ occupations. Information on material possessions was recorded as an indicator of socioeconomic status. Subjects were given a score of 1 for each of the following household items: electricity, fan, bicycle, radio, motorized 2-wheeled vehicle, gas stove, television, cable television, electric mixer, electric griller, electric air cooler, washing machine, car, air conditioner, computer, television antenna, and telephone. Alcohol consumption was recorded as the frequency of intake and volume of spirits, beer, and wine consumed per week. These data were converted into units of alcohol (1 unit = 25 mL spirits, 282 mL beer, or 125 mL wine) and were categorized as none, <7 units/wk, 7–14 units/wk, and >14 units/wk. Tobacco consumption was recorded as whether the subjects smoked (cigarettes, bidis, cigars, or hookah), chewed (raw tobacco or with pan), or inhaled (snuff). Subjects were categorized simply as current tobacco users or as nonusers. A score was derived as a summary estimate of daily physical activity. Work-related activity was classified on a 6-point scale ranging from “almost entirely sedentary” to “heavy physical work.” Additional time spent per day in domestic activities (eg, sweeping, washing clothes, and cooking) and leisure activities (eg, jogging, swimming, and yoga) was recorded. Distances walked and cycled each day, with and without a load, were recorded and converted into approximate periods of time spent in these activities. These were then multiplied by metabolic constants, which were derived from the relative energy expenditure of activities (39), and were summed to derive a score.

Clinic investigations

After the home visit, the subjects were asked to attend a clinic after fasting overnight. Their weight, height, waist and hip circumferences, midupper arm circumference, and skinfold thicknesses (triceps and subscapular) were measured by using standardized techniques. The upper measurable limit for skinfold thickness was 40 mm. BMI was calculated as weight/height² (in kg/m²). Subjects were categorized as obese if their BMI was ≥30 (40). Two definitions of overweight were used: the standard World Health Organization (WHO) cutoff of 25 (40) and that recently recommended for Asians of 23 (2). Central obesity was defined by using WHO criteria: waist-hip ratio >0.90 (males) or >0.85 (females) (41).

Two indexes of adiposity were derived from the skinfold-thickness measurements: I) the sum of subscapular and triceps skinfold thicknesses, and 2) percentage body fat (42, 43). Three indexes of lean mass were derived: I) arm muscle area, which was derived from midupper arm circumference and triceps skinfold thickness and was corrected for arm bone area (44); 2) lean body mass, which was derived as body weight minus fat mass and was adjusted for height, where fat mass = weight × percentage body fat; and 3) the residual value from a linear regression predicting BMI from the sum of skinfold thicknesses and height, adjusted for age and sex (designated the “lean residual”). The adjustments for height in these calculations were intended to derive proxies for lean tissue other than bone. Height adjustment was included in the derivation of the lean residual from BMI, even though BMI is often uncorrelated with height, because in Delhi there was a positive association between BMI and height (r = 0.09, P = 0.007 in men and r = 0.10, P = 0.009 in women).

Systolic and diastolic blood pressures were recorded by using an automated device (Omron 711; Omron Healthcare Europe, Hoosddorp, Netherlands) while the subjects were seated and after they had rested for 5 min. As described previously (38), plasma glucose and insulin concentrations were measured while the subjects were fasting and 120 min after a standard 75-g oral anhydrous glucose load. Plasma glucose, triacylglycerol, and cholesterol concentrations were analyzed by standard enzymatic methods by using Randox kits (Randox Laboratories Limited, Crumlin, United Kingdom) on a Beckman autoanalyzer (Beckman Instruments Inc, Brea, CA). HDL cholesterol was estimated by using the same method as for cholesterol measurement, after precipitation with phosphotungstate. Aliquots of plasma were stored at −70 °C for up to 8 mo and were analyzed for insulin concentrations in batches by radioimmunoassay (Coat-a-Count insulin kit; Diagnostic Products Corporation, Los Angeles, CA). The method had intraassay and interassay CVs of <5% and <7.5%, respectively. Insulin resistance was calculated by homeostasis model assessment. Biochemical measurements were
made in the biochemistry laboratory of the Department of Cardiology, All India Institute of Medical Sciences, New Delhi.

Statistical analyses

Variables with skewed distributions were log-transformed. Data were analyzed by using partial correlation coefficients and multiple linear regression. As previously described (38), we used all recorded data (not just the data for subjects recruited for this study) to derive SD scores for height and BMI for each subject at age 6 mo and at birthdays from age 1 to 21 y. The SD score is the number of SDs by which an observation differs from the mean for the cohort. Interpolated values were used if a measurement had been made within 6 mo (up to 1 y), 1 y (age of 2 y), 1.5 y (age of 3 y), and 2 y (all older ages). Back transformation provided estimates of the measurements at these ages. To measure the change in BMI in a time interval during childhood (for example between the ages of 2 and 5 y), we regressed the value at the end of the interval (age 5 y) on the value at the beginning of the interval (age 2 y) and at all preceding time points (birth, 6 mo, and 1 y) and expressed the residual as an SD score. This produces uncorrelated variables describing BMI change at specific time points in childhood, which we refer to as conditional SD scores. We calculated the age at adiposity rebound as the birthday between 2 and 9 y at which the lowest estimate of BMI occurred.

RESULTS

Of the 2584 men and women traced, 1583 agreed to participate. Of these, 57 were excluded (24 were pregnant, 2 left after recruitment, and 31 were unreliable linked to earlier data), leaving 1526 (59% of those traced, and 19% of the original cohort). Compared with the original cohort, among the recruited subjects, 7% more of the subjects were male, maternal literacy was 6% higher, mean birth weight was 32 g heavier, and birth length was 2 mm longer. Height and BMI in childhood and adolescence were 0.1 SD lower.

The characteristics of the 886 men and 640 women studied are shown in Table 1. At all ages from birth to adolescence, the subjects studied were short, light, and thin by international standards. For example, at 2 y, their mean SD scores for height, weight, and BMI relative to National Center for Health Statistics (NCHS) standards (45) were −1.54, −2.01, and −0.78 in boys and −1.55, −2.27, and −0.85 in girls. Percentages of children >2 SDs below the NCHS median for height, weight, and BMI at 2 y were 33%, 50%, and 12% (boys) and 30%, 57%, and 13% (girls). When they were reexamined as adults, most subjects were married, graduates (Bachelor’s degree or higher), and in nonmanual employment (Table 1). Few women drank alcohol or used tobacco. Approximately 1 in 10 subjects was obese by the WHO definition (BMI ≥ 30). Almost one-half of the subjects were overweight by the WHO definition (BMI ≥ 25), and nearly two-thirds were overweight by the Asian cutoff (BMI ≥ 23). Sixty-five percent of the men and 31% of the women were centrally obese.

To select the most appropriate indexes of body composition for the analysis, we correlated BMI, height, and the direct and derived measures of adiposity, central adiposity, and lean mass with the cardiovascular risk factor variables (Table 2). Skinfold thicknesses, percentage body fat, waist circumference, and waist-hip ratio were positively correlated with all the risk factors except HDL cholesterol, for which the correlation was negative. The subscapular-triceps ratio showed weak and mainly nonsignificant correlations with the risk factors. The indexes of lean mass were also positively correlated with the risk factors (negatively with HDL cholesterol), especially insulin resistance and blood pressure, although these correlations were less strong than those for the measures of adiposity. For subsequent analyses, we limited the outcome variables to BMI and 4 other body-composition variables: sum of skinfold thicknesses and waist-hip ratio as the measures of general and central adiposity, respectively, that were most strongly correlated with the risk factors; the lean residual as the measure of lean mass least correlated with the risk factors; and height as the measure of skeletal size. Waist-hip ratio was selected in preference to waist circumference because it was uncorrelated with height. The correlations of each of these 4 variables with the risk factors, adjusted for the other 3, were weaker than but generally in the same direction as those described above (bottom of Table 2).

Adult body composition in relation to age and adult lifestyle factors

BMI rose with increasing age (P < 0.001) and socioeconomic status (measured by each of the 3 indicators: education, occupation, and material possessions; P < 0.001 for all). It rose from 20.5 in those with fewer than 6 household possessions to 26.7 in those who owned 15 or 16 possessions. It was inversely related to physical activity (P = 0.01) and was higher in non-tobacco-users (men, P = 0.04; few women smoked) and in women of higher parity (P = 0.001 adjusted for age). Like BMI, the sum of skinfold thicknesses increased with age (P < 0.001), was inversely related to physical activity (P = 0.004), and was higher in men who were non-tobacco-users (P = 0.001) and in women of higher parity (P < 0.001). Waist-hip ratio rose with increasing alcohol consumption (men, P = 0.006; few women drank alcohol) and was inversely related to physical activity score (P = 0.001). The lean residual decreased with age (P < 0.001). All body-composition variables were strongly positively related to socioeconomic status as measured by occupation, education, and household possessions (P < 0.01 for all), except that waist-hip ratio and lean residual were not related to education status (P = 0.09 and P = 0.3 respectively). In further analyses, associations of early life variables with adult outcomes were adjusted for age, sex, socioeconomic status (all 3 measures), tobacco use, alcohol consumption, physical activity, and (in women) parity.

Adult body composition in relation to size at birth

In the sexes combined, higher birth weight was associated with higher adult lean residual and taller adult height (Table 3). In women, but not men, it was associated with higher adult BMI and sum of skinfold thicknesses (P values for the interaction between sex and birth weight were 0.006 for BMI and 0.01 for sum of skinfold thicknesses). Longer birth length was strongly associated with taller adult height. It was also associated with higher adult sum of skinfold thicknesses and lean residual and with higher BMI in women but not men (P for sex interaction 0.04). Ponderal index at birth was positively related to adult lean residual but to none of the other adult body-composition outcomes. None of the birth measurements was related to adult waist-hip ratio. Although it was not one of the selected variables, we examined adult subscapular-triceps ratio in relation to size at birth, because this has been reported frequently in the literature (24).

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>150.0 (0.7)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>45.0 (3.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.5 (1.5)</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.23</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.12</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.24</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.21</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.19</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.18</td>
</tr>
<tr>
<td>Lean residual</td>
<td>0.15</td>
</tr>
<tr>
<td>Skinfold thicknesses</td>
<td>0.14</td>
</tr>
<tr>
<td>Percentage body fat</td>
<td>0.13</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>0.12</td>
</tr>
<tr>
<td>Ponderal index</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (kg)</td>
<td>3.3 (0.5)</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>50.0 (0.7)</td>
</tr>
<tr>
<td>Ponderal index at birth (kg)</td>
<td>2.0 (1.5)</td>
</tr>
</tbody>
</table>

...
## TABLE 1
Characteristics of the study cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men</th>
<th>Women</th>
<th>P'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>2891 ± 436 [803]</td>
<td>2791 ± 383 [561]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>48.8 ± 2.1 [779]</td>
<td>48.3 ± 1.9 [558]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ponderal index (kg/m^3)</td>
<td>24.8 ± 2.6 [779]</td>
<td>24.7 ± 2.5 [558]</td>
<td>0.6</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>33.8 ± 1.3 [775]</td>
<td>33.3 ± 1.1 [555]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gestation (wk)</td>
<td>38.7 ± 2.6 [791]</td>
<td>39.1 ± 2.5 [588]</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Infancy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.0 ± 0.9 [836]</td>
<td>6.4 ± 0.9 [613]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>65.3 ± 2.4 [836]</td>
<td>63.7 ± 2.4 [613]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>16.4 ± 1.6 [836]</td>
<td>15.7 ± 1.6 [613]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>1 y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>8.5 ± 1.1 [706]</td>
<td>7.8 ± 1.1 [526]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>72.0 ± 2.7 [708]</td>
<td>70.1 ± 2.9 [526]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>16.4 ± 1.5 [706]</td>
<td>15.9 ± 1.5 [525]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>2 y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>10.3 ± 1.3 [834]</td>
<td>9.8 ± 1.2 [609]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>81.1 ± 3.6 [840]</td>
<td>79.6 ± 3.6 [609]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>15.8 ± 1.2 [833]</td>
<td>15.4 ± 1.2 [604]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Childhood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>15.5 ± 1.8 [866]</td>
<td>14.9 ± 1.7 [629]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>102.0 ± 4.5 [863]</td>
<td>100.6 ± 4.4 [628]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>14.9 ± 1.0 [863]</td>
<td>14.7 ± 1.1 [628]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>8 y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>20.8 ± 3.1 [863]</td>
<td>19.9 ± 2.7 [627]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>119.7 ± 5.4 [859]</td>
<td>118.1 ± 5.4 [625]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>14.5 ± 1.3 [859]</td>
<td>14.2 ± 1.2 [625]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Adolescence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>28.4 ± 4.7 [834]</td>
<td>27.6 ± 5.4 [608]</td>
<td>0.002</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>135.9 ± 5.7 [831]</td>
<td>134.2 ± 7.4 [607]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>15.3 ± 1.7 [830]</td>
<td>15.2 ± 1.8 [606]</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td><strong>14 y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>39.5 ± 8.8 [877]</td>
<td>42.1 ± 7.4 [636]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>153.0 ± 9.2 [876]</td>
<td>151.7 ± 5.7 [634]</td>
<td>0.002</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>16.7 ± 2.4 [876]</td>
<td>18.2 ± 2.7 [634]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Current demographic variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>29.2 ± 1.3 [886]</td>
<td>29.2 ± 1.4 [640]</td>
<td>0.8</td>
</tr>
<tr>
<td>Married (%)</td>
<td>69.8 [886]</td>
<td>81.1 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Educational status (%)</td>
<td>[886]</td>
<td>[640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Middle school or less</td>
<td>15.6</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>High school or diploma</td>
<td>32.5</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>Graduate</td>
<td>40.0</td>
<td>48.8</td>
<td></td>
</tr>
<tr>
<td>Postgraduate or professional</td>
<td>12.0</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>Employment status (%)</td>
<td>[886]</td>
<td>[639]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Housewife</td>
<td>—</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>2.1</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Unskilled or semiskilled manual labor</td>
<td>10.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Skilled manual labor</td>
<td>24.2</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Nonmanual labor, business, or professional</td>
<td>62.8</td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td>Current alcohol intake (%)</td>
<td>56.2 [886]</td>
<td>1.4 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tobacco use (%)</td>
<td>[886]</td>
<td>[640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Previous only</td>
<td>5.1</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>29.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>No. of household possessions</td>
<td>5.3 ± 2.8 [886]</td>
<td>5.4 ± 2.2 [640]</td>
<td>0.4</td>
</tr>
<tr>
<td>Physical activity score</td>
<td>876 ± 1.5 [886]</td>
<td>618 ± 1.8 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parity</td>
<td>-</td>
<td>1.7 ± 1.3 [640]</td>
<td></td>
</tr>
<tr>
<td>Current anthropometric indexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.8 ± 14.0 [886]</td>
<td>59.2 ± 13.4 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.70 ± 0.06 [886]</td>
<td>1.55 ± 0.06 [638]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>24.9 ± 4.3 [886]</td>
<td>24.6 ± 5.1 [638]</td>
<td>0.3</td>
</tr>
</tbody>
</table>

(Continued)
Means fell from 1.48 and 1.00 in men and women, respectively, were no significant interactions between birth size and sex.

The ratio was negatively associated with birth weight ($P < 0.001$), length ($P = 0.02$), and ponderal index ($P = 0.01$). There were no significant interactions between birth size and sex. Means fell from 1.48 and 1.00 in men and women, respectively, who weighed $\leq 2500$ g at birth to 1.38 and 0.95 in those who weighed $>3250$ g.

**Adult body composition in relation to BMI during infancy, childhood, and adolescence**

Correlations between adult BMI and BMI measured during infancy, childhood, and adolescence were positive and strengthened progressively with increasing age (6 mo: 0.19; 1 y: 0.21; 2 y: 0.24; 5 y: 0.32; 8 y: 0.47; 11 y: 0.58; 14 y: 0.65). The correlations between BMI from birth to 21 y and all the adult body-composition outcomes are shown in Figure 1. Like adult BMI, all outcomes except height were positively correlated with BMI measured at earlier ages and correlations strengthened with increasing age. In males, BMI at birth, childhood, and adolescence was more strongly correlated with adult lean residual than with adult sum of skinfold thicknesses (Figure 1). The differences between correlations with lean residual and the sum of skinfold thicknesses were significant ($P < 0.05$) for BMI at birth, 6 mo, 1 and 2 y, 5–13 y, and 16–18 y. In females, whereas BMI in early childhood was more strongly correlated with adult lean residual than with adult sum of skinfold thicknesses, differences between correlations were significant only at 2, 5, and 6 y, and BMI during adolescence was correlated more strongly with adult sum of skinfold thicknesses than with adult lean mass (statistically significant from 15 y onward).

The associations of changes in BMI SD scores during infancy, childhood, and adolescence with adult body composition were examined in the sexes combined by using conditional SD scores at birth–6 mo, 6 mo–1 y, and 1–2, 2–5, 5–8, 8–11, and 11–14 y (after the age of 14 y, the numbers available decreased rapidly). The analysis was limited to 957 men and women with values at all these time points. All the conditional SD scores were included in regression models simultaneously, together with age, sex, and additional confounders such as gestational age, birthweight, and early feeding practices.

**TABLE 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men</th>
<th>Women</th>
<th>$P^t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overweight (BMI ≥ 25) (%)</td>
<td>47.4 [886]</td>
<td>45.5 [638]</td>
<td>0.5</td>
</tr>
<tr>
<td>Overweight (BMI ≥ 23) (%)</td>
<td>66.0 [886]</td>
<td>61.8 [638]</td>
<td>0.09</td>
</tr>
<tr>
<td>Obese (BMI ≥ 30) (%)</td>
<td>9.5 [886]</td>
<td>13.0 [638]</td>
<td>0.03</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>90.2 ± 12.1 [886]</td>
<td>79.6 ± 12.4 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>97.5 ± 8.3 [886]</td>
<td>97.0 ± 10.4 [639]</td>
<td>0.3</td>
</tr>
<tr>
<td>Waist hip ratio</td>
<td>0.92 ± 0.06 [886]</td>
<td>0.82 ± 0.07 [639]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central obesity (%)</td>
<td>65.5 [886]</td>
<td>31.0 [639]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subscapular skinfold thickness (mm)$^d$</td>
<td>23.6 ± 9.6 [883]</td>
<td>25.5 ± 10.4 [637]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triceps skinfold thickness (mm)$^d$</td>
<td>16.7 ± 7.4 [885]</td>
<td>25.5 ± 9.5 [638]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sum of skinfold thicknesses (mm)</td>
<td>40.3 ± 16.2 [883]</td>
<td>51.0 ± 19.0 [635]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>24.2 ± 5.9 [883]</td>
<td>34.2 ± 7.1 [635]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subscapular-triceps ratio$^d$</td>
<td>1.43 ± 1.30 [883]</td>
<td>0.98 ± 1.29 [635]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Midupper arm circumference (cm)</td>
<td>29.6 ± 3.7 [885]</td>
<td>26.8 ± 4.2 [640]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lean residual (kg/m$^2$)</td>
<td>24.9 ± 2.7 [883]</td>
<td>24.6 ± 2.8 [633]</td>
<td>0.09</td>
</tr>
<tr>
<td>Arm muscle area (cm$^2$)</td>
<td>37.7 ± 10.3 [885]</td>
<td>21.9 ± 7.3 [638]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lean mass adjusted for height (kg)</td>
<td>53.9 ± 6.7 [883]</td>
<td>38.1 ± 5.0 [633]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Cardiovascular disease risk factors

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>$P^t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mmol/L)$^d$</td>
<td>5.4 ± 1.21 [869]</td>
<td>5.3 ± 1.17 [623]</td>
<td>0.09</td>
</tr>
<tr>
<td>2-h Glucose (mmol/L)$^d$</td>
<td>5.9 ± 1.34 [848]</td>
<td>6.1 ± 1.28 [591]</td>
<td>0.03</td>
</tr>
<tr>
<td>Insulin resistance (HOMA)$^d$</td>
<td>1.37 ± 2.73 [868]</td>
<td>1.13 ± 2.75 [623]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.2 ± 1.11 [870]</td>
<td>4.7 ± 1.0 [628]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)$^d$</td>
<td>1.57 ± 1.69 [869]</td>
<td>1.05 ± 1.51 [628]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)$^d$</td>
<td>1.13 ± 1.30 [827]</td>
<td>1.24 ± 1.28 [618]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>118.4 ± 11.4 [880]</td>
<td>106.6 ± 11.0 [631]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>77.9 ± 10.3 [880]</td>
<td>73.4 ± 9.2 [631]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^t$ The significance of the difference between the sexes was derived by using 2-sample t tests for continuous variables and chi-square tests for categorical variables.

$^d$ Geometric mean ± SD.

$^* $ Defined as a waist-hip ratio >0.90 for men and >0.85 for women.

$^6$ HOMA, homeostasis model assessment.

The significance of the difference between the sexes was derived by using 2-sample t tests for continuous variables and chi-square tests for categorical variables.

$^d$ Geometric mean ± SD.

$^* $ Defined as a waist-hip ratio >0.90 for men and >0.85 for women.

$^6$ HOMA, homeostasis model assessment.
and change in BMI between birth and 6 mo and between 1 and 2 y were significantly larger in subjects born prematurely than in those born at term \([P = 0.02\) for the interaction between the binary variable (preterm vs full-term) and change in BMI, for both time periods\]. There were no significant interactions at any age for the lean residual. Similar analyses were carried out in both time periods \([P = 0.02\) for the interaction between the binary variable \((<2500 \text{ g vs } \geq 2500 \text{ g})\) and BMI change\]. There were no significant interactions at any age for the other adult outcomes. These analyses show that the differences in the correlations between BMI gain in infancy and adult lean residual compared with adult sum of skinfold thicknesses were lower in subjects born prematurely or small for gestational age.

**Adapted body composition in relation to height during earlier life**

Length or height during infancy, childhood, and adolescence were increasingly strongly positively correlated with adult height (Figure 3). There were steep increases in the correlation coefficients during infancy and adolescence. Correlations between earlier height, or changes in height SD score, and adult sum of skinfold thicknesses, lean residual, and waist-hip ratio were weak and generally not significantly different from each other. There was no evidence of increased adiposity or central adiposity in subjects who were short or stunted \([-2 \text{ SDs shorter than the NCHS reference (45)}\]) at any time during childhood.

**DISCUSSION**

We studied 1526 men and women who grew up in the city of Delhi, India, at a time of rapid nutritional transition. According to international definitions, the subjects were underweight as children but as young adults had a high prevalence of overweight, obesity, and central obesity. Higher birth weight was associated with higher adult lean residual \((\text{an index of lean tissue mass})\) in both sexes and with higher adult BMI and sum of skinfold thicknesses in women. Higher BMI during infancy, childhood, and adolescence was associated with higher adult BMI, lean residual, sum of skinfold thicknesses, and waist-hip ratio. These associations strengthened with increasing age of earlier measurement. BMI and BMI gain during infancy and early childhood were more strongly correlated with adult lean residual than with adult...
TABLE 3
Adult body mass index, sum of skinfold thicknesses, waist-hip ratio, lean residual, and height according to birth weight, birth length, ponderal index at birth, and body mass index at ages 6 mo and 12 y

<table>
<thead>
<tr>
<th>n</th>
<th>BMI (kg/m²)</th>
<th>Sum of skinfold thicknesses (mm)</th>
<th>Waist-hip ratio</th>
<th>Lean residual (kg/m³)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Men</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²) at 6 mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤14.5</td>
<td>116</td>
<td>4.0</td>
<td>23.2 ± 4.1²</td>
<td>22.7 ± 4.9</td>
<td>38.0 ± 17.5</td>
</tr>
<tr>
<td>14.6–15.5</td>
<td>153</td>
<td>4.3</td>
<td>24.3 ± 4.2</td>
<td>24.6 ± 4.9</td>
<td>40.6 ± 16.0</td>
</tr>
<tr>
<td>15.6–16.5</td>
<td>221</td>
<td>4.4</td>
<td>24.4 ± 3.8</td>
<td>24.9 ± 5.4</td>
<td>39.0 ± 10.2</td>
</tr>
<tr>
<td>16.6–17.5</td>
<td>196</td>
<td>4.6</td>
<td>25.7 ± 4.1</td>
<td>25.9 ± 4.7</td>
<td>40.3 ± 16.2</td>
</tr>
<tr>
<td>&gt;17.5</td>
<td>148</td>
<td>4.8</td>
<td>26.3 ± 4.7</td>
<td>26.1 ± 4.8</td>
<td>42.1 ± 16.0</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

| BMI (kg/m²) at 12 y |
| ≤14.0 | 151 | 3.1 | 21.2 ± 3.1 | 20.2 ± 3.8 | 32.3 ± 15.0 | 37.2 ± 18.5 | 0.89 ± 0.06 | 0.80 ± 0.07 | 22.9 ± 2.0 | 23.1 ± 1.8 | 169.0 ± 6.4 | 154.5 ± 5.9 |
| 14.1–15.0 | 214 | 3.3 | 23.3 ± 3.2 | 22.5 ± 3.6 | 35.9 ± 14.8 | 46.8 ± 18.4 | 0.91 ± 0.06 | 0.81 ± 0.07 | 24.2 ± 2.1 | 23.5 ± 2.4 | 169.3 ± 6.4 | 155.1 ± 6.2 |
| 15.1–16.0 | 213 | 3.4 | 24.9 ± 3.0 | 24.0 ± 4.1 | 40.1 ± 14.7 | 49.4 ± 18.1 | 0.92 ± 0.05 | 0.82 ± 0.07 | 25.0 ± 2.2 | 24.4 ± 2.5 | 167.9 ± 6.4 | 155.0 ± 5.1 |
| 16.1–17.0 | 137 | 3.5 | 26.8 ± 4.3 | 25.4 ± 4.4 | 45.7 ± 15.6 | 52.7 ± 17.2 | 0.94 ± 0.06 | 0.82 ± 0.07 | 26.1 ± 2.7 | 25.0 ± 2.6 | 169.5 ± 6.4 | 154.7 ± 5.4 |
| >17.0 | 148 | 3.7 | 29.2 ± 3.8 | 28.7 ± 4.6 | 51.9 ± 14.6 | 62.0 ± 14.6 | 0.96 ± 0.05 | 0.84 ± 0.08 | 26.8 ± 2.8 | 26.3 ± 3.0 | 170.3 ± 5.9 | 155.2 ± 5.7 |
| P     | <0.001     | <0.001                          | <0.001          | <0.001                | <0.001     |

1 Birth weight was unknown for 162 subjects, birth length and ponderal index for 189, BMI at 6 mo for 77, and BMI at 12 y for 39. The largest number of values missing for calculating the group mean was 3. P values were derived by multiple linear regression analysis by using all variables as continuous variables and by adjusting for age, education, occupation, household possessions, alcohol intake, tobacco use, physical activity score, and (for women) parity. P values are given for the sexes combined, except when there were significant (P < 0.05) interactions between sex and the earlier measurement in relation to adult outcomes; in those cases, P values are shown for males and females separately.

2 ± SD (all such values).

adiposity or with central adiposity. This phenomenon was most prominent in males, in subjects born at full term, and in those who were not born small for gestational age. Higher BMI and greater BMI gain in late childhood and adolescence were strongly associated with adult adiposity and central adiposity.

The subjects came from an original population representing all live births within a defined area. The subjects’ families were affluent and well-educated compared with national averages. Only 19% of the original cohort participated, and the subjects are thus likely to be unrepresentative of the original sample. The differences in their mean size at birth and during infancy and childhood, however, although statistically significant, were trivial. Our analysis was based on internal comparisons within the study sample and would be biased only if the associations between early growth and adult body composition differed between those who were and those who were not traced. Adult body composition was assessed only anthropometrically. None of our indexes of adiposity or lean mass was ideal, although all have been used in population studies. We recognize that our findings need to be confirmed with the use of better methods of assessing body composition.

We found expected associations between measures of adult adiposity and cardiovascular disease risk factors. Insulin resistance and blood pressure were also positively related to indexes of lean mass. The most likely explanation is that the latter were inadequately adjusted for body fat, especially nonsubcutaneous fat. The associations may, however, reflect a biological relation; several studies have reported positive associations between muscularity and blood pressure, although the mechanisms are unclear (46, 47). The subscapular-triceps ratio was positively correlated with cardiovascular disease risk factors in other studies (48, 49), but the mainly nonsignificant relations observed in Delhi suggest that this ratio has limited clinical utility at this age in this population.

Several studies have reported higher adult BMI in individuals of higher birth weight (23, 24). In common with other studies (31–37, 50), our data showed that birth weight was more strongly related to the lean than to the fat component of adult BMI. This
difference was more marked in males than in females; higher birth weight in females was also associated with increased adiposity. There was a similar sex difference in one study (37), although others reporting data for both sexes showed no sex differences (31, 33, 34, 36); higher birth weight was significantly associated with muscle mass (31) or fat-free mass (33, 34, 36) but not fat mass in both sexes. Our data are consistent with the suggestion that fetal life is a critical period for the development

FIGURE 1. Correlations between BMI in early life and adult body-composition outcomes. The graphs show the adjusted correlation coefficients and 95% CIs at the age of 6 mo and at every birthday, with the points connected for ease of reading. The number of subjects at any age was always >600 (men) or >500 (women) up to the age of 17 y; thereafter, the minimum numbers at any age were 243 (men) and 178 (women). All analyses were adjusted for age, education, occupation, number of household possessions, tobacco use, alcohol consumption, physical activity, and (in women) parity. Differences between the lines were tested by using inverse hyperbolic tangent transformation and by assuming normality. There were significant interactions ($P < 0.05$) between sex and the difference in correlations with adult lean residual and adult sum of skinfold thicknesses for BMI measurements at 6 mo, 1 y, and 6–12 y.

FIGURE 2. Prediction of adult anthropometric variables from BMI changes in early life. The graphs show adjusted regression coefficients for each time period during childhood (indicated on the $x$ axis), with the points connected for ease of reading. The number of subjects included was 957. All analyses were adjusted for age, education, occupation, number of household possessions, tobacco use, alcohol consumption, physical activity, and (in women) parity by using multiple linear regression with the 5 indexes of adult body composition as outcomes and a standard set of $x$ variables included in all models (birth weight and BMI change in the 7 time intervals shown, conditional on BMI up to the start of the interval and expressed as an SD score). $m$, months.
This may explain the increased risk of insulin resistance and type 2 diabetes in persons of low birth weight (18, 27, 29) despite lower adult BMI. The positive correlations between adult BMI and BMI in earlier life, which strengthened with increasing age, have also been reported elsewhere (13). A novel finding of our study was the differing associations with fat and lean components of adult body mass depending on the age of BMI measurement in earlier life. Higher BMI gain in infancy and early childhood was associated with a greater increase in adult lean mass than in adult adiposity. Higher BMI gain in late childhood and adolescence was strongly associated with adult adiposity and central adiposity. As previously described in the Delhi cohort (38) and in Europeans (18, 27), lower BMI in infancy and greater BMI gain during childhood and adolescence are associated with an increased risk of adult impaired glucose tolerance and type 2 diabetes. Our data may provide an explanation for these findings. Low adult muscle mass could explain the association between adult glucose intolerance and low BMI in infancy, whereas increased generalized and central adiposity could explain its association with accelerated BMI gain in later childhood and adolescence. Skeletal muscle cells lose their ability to divide in early postnatal life (51, 52). In animals, undernutrition at this time permanently reduces muscle mass, and enhanced nutrition at later ages results in excessive fat deposition (52). We propose that infancy, like fetal life, is a critical period for the development of lean mass. The susceptibility of South Asians to type 2 diabetes and IHD may partly be caused by poor development of lean mass in fetal life and infancy, combined with increased adiposity in later childhood resulting from urban transition. Several other studies have examined adult body composition in relation to weight and BMI gain in infancy (50, 53, 54), but none compared the strength of associations for fat and lean measurements. In Guatemala, Li et al (37) studied anthropometric measurements of adult body composition in relation to birth length and changes in length during the first 2 postnatal years. In both sexes, a greater increase in length between birth and 2 y was associated with stronger effects on adult fat-free mass than on fat mass.

Debate currently exists about optimal infant weight gain. Randomized trials of different infant feeds in preterm and growth-restricted neonates have shown that rapid infant weight gain is associated with increased cardiovascular disease risk factors later in childhood (55), which suggests that rapid weight gain during infancy could be harmful. Our data indicate that associations between early postnatal growth and later body composition may vary according to gestational age at birth and fetal growth rates, and this needs to be investigated further. The findings for full-term infants and those who were not small for gestational age suggest that infancy may be a window of opportunity during which better nutrition and greater BMI gain could increase adult lean mass. Infant weight gain is an important issue in developing countries, where the emphasis is still on eradicating undernutrition and where it is routine practice to encourage weight gain in small infants because of evidence that this increases infant survival (56), prevents stunting (57), and enhances cognitive development (58).

In contrast with the findings in infancy, accelerated BMI gain in later childhood and adolescence was clearly associated with increased adult adiposity and central adiposity. As previously described, it was also associated with an increased risk of impaired glucose tolerance and type 2 diabetes (38). As a population, our subjects had low BMI values in childhood compared with international reference data. Those who went on to develop diabetes had accelerated BMI gain after infancy, becoming “obese relative to themselves,” but did not have a high BMI in absolute terms. Efforts to prevent obesity-related disease should start in childhood and should probably target not only children who are frankly overweight or obese but also those who are silently moving up the BMI percentiles. Serial BMI measurements and appropriate local reference standards would be needed to recognize this trajectory. Pediatricians would also need to develop effective ways of preventing children from acquiring
excessive adipose tissue without impairing lean mass and skeletal growth.

We thank the men and women and their families who took part in the study and the field and laboratory staff for their contribution. It is our privilege to acknowledge Shanti Ghosh, Former Professor and Head, Department of Pediatrics, Safdarjung Hospital, New Delhi, and IM Moriyama, former Director at the National Center for Health Statistics, United States, who initiated this study along with Santosh K Bhargava and provided valuable guidance and support throughout its completion up to 1990. We also thank Vinod Kapani for technical input and advice on data analysis. Rajeshwari Verma and Bhaskar Singh provided invaluable assistance in maintaining a liaison with the cohort and in completing the study.

SKB established the original cohort and designed the current study along with CHDF and DJPB. The data were analyzed and interpreted and the manuscript written by HSS, CHDF, and CO. Significant contributions were also made by SDL and SKDB (data collection and analysis), RL (laboratory analyses), and KSR (provision of advice on study design and data interpretation). None of the authors had a financial, commercial, or personal conflict of interest.

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45. Srinivasan SR, Myers L, Berenson GS. Predictability of childhood adiposity and insulin for developing insulin resistance syndrome (Syndrome X) in young adulthood, the Bogalusa Heart Study. Diabetes 2002;51:204–9.
Polymorphism of the 5-HT$_{2A}$ receptor gene and food intakes in children and adolescents: the Stanislas Family Study$^1$–$^3$

Bernard Herbeth, Eléonore Aubry, Frédéric Fumeron, Roberte Aubert, Frédéric Cailotto, Gérard Siest, and Sophie Visvikis-Siest

ABSTRACT

Background: Serotonin (5-hydroxytryptamine; 5-HT) is a key mediator in the control of food intake and is probably involved in the etiology of anorexia nervosa. An association between a polymorphism of the 5-HT receptor (5-HT$_{2A}$) gene promoter (−1438G/A) and anorexia nervosa has been reported.

Objective: We investigated the relation between the −1438G/A polymorphism of the 5-HT$_{2A}$ gene and the energy and macronutrient intakes of children and adolescents.

Design: This cross-sectional study included 370 children and adolescents aged 10–20 y (176 boys and 194 girls from 251 families) drawn from the Stanislas Family Study. Energy and macronutrient intakes were assessed by using 3-d food records. The −1438G/A polymorphism was analyzed by polymerase chain reaction and then by Hpa II digestion.

Results: In the overall group, after adjustment for age, sex, weight, height, and family correlation, the A allele was significantly associated with lower energy (P for trend = 0.045) and with total, monounsaturated, and saturated fat intakes expressed in g/d (P for trend = 0.007, 0.005, and 0.006, respectively). Subjects with the GA genotype had intermediate values. In addition, genotype × sex and genotype × age interactions were not significant.

Conclusions: The 5-HT$_{2A}$ gene polymorphism in the promoter region is associated with energy and fat intakes in young people. This could be explained by the role of the serotonergic system as a determinant of food intakes and eating behavior. Am J Clin Nutr 2005;82:467–70.

KEY WORDS Serotonin, 5-hydroxytryptamine, 5-HT receptor, 5-HT$_{2A}$, genetic polymorphism, food intake, children, adolescents

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a key mediator in the control of food intake. Serotonin reduces food intake and is probably involved in the etiology of anorexia nervosa and in weight regulation (1); moreover, drugs that increase serotonergic transmission exert anorectic activity. An association between a polymorphism of the 5-HT receptor (5-HT$_{2A}$) gene promoter (−1438G/A) and anorexia nervosa has been reported (2). In a previous study, we showed that this polymorphism could influence food and alcohol intakes in an adult overweight population because the A allele is associated with significantly lower energy and alcohol intakes and with a trend to lower intakes of proteins, fats, and carbohydrates (3). Because anorexia nervosa is almost exclusively found in young people and because we wanted to ascertain the validity of our previous results, we investigated in the current study whether the 5-HT$_{2A}$ polymorphism could influence energy and nutrient intakes in a sample of children and adolescents.

SUBJECTS AND METHODS

Subjects

This work is part of the Stanislas Family Study, a 10-y longitudinal follow-up study begun in 1994 in 1006 families selected at the Center for Preventive Medicine (Vandoeuvre-lès-Nancy, France) on the basis of being free of chronic or acute disease that could influence nutritional status (4). In this report, we present data obtained from a random subsample of 370 children and adolescents aged 10–20 y (176 boys and 194 girls from 251 families) at the first examination (1994–1995). All subjects underwent a complete medical examination including weight and height measurements. Body weight index was calculated as the ratio of weight to weight reference values for sex and age as delineated by Rolland-Cachera et al (5).

Written informed consent was obtained from each participant. The research protocol was approved by the Ethics Committee of Lorraine.

Methods

Dietary intake was assessed with the use of a 3-d dietary record (6), which was completed during 2 weekdays and 1 weekend day assigned at random for each family. All subjects received guidance from a dietician on the procedures for completing the dietary record and measuring food portions. For young children, the 3-d diary was filled in by the mother and the child together. One week later, the 3-d record was checked and completed by the dietician.

Food intake and other characteristics according to the −1438G/A polymorphism of the serotonin receptor (5-HT<sub>2A</sub>) gene in children and adolescents aged 10–20 y<sup>1</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
<th>P for trend&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>13.7 ± 2.7&lt;sup&gt;2&lt;/sup&gt;</td>
<td>13.6 ± 2.6</td>
<td>13.3 ± 2.6</td>
<td>0.293</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>49.4 ± 11.4</td>
<td>50.8 ± 13.5</td>
<td>48.9 ± 13.1</td>
<td>0.806</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>159.1 ± 11.4</td>
<td>159.9 ± 11.8</td>
<td>158.6 ± 11.7</td>
<td>0.749</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.2 ± 2.7</td>
<td>19.5 ± 3.1</td>
<td>19.1 ± 3.1</td>
<td>0.797</td>
</tr>
<tr>
<td>Body weight index (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>103.5 ± 13.2</td>
<td>105.4 ± 14.1</td>
<td>104.4 ± 16.0</td>
<td>0.695</td>
</tr>
<tr>
<td>Energy (MJ/d)</td>
<td>9.35 ± 2.70</td>
<td>9.02 ± 2.17</td>
<td>8.61 ± 2.17</td>
<td>0.045</td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (g/d)</td>
<td>84.5 ± 25.3</td>
<td>84.9 ± 23.3</td>
<td>80.9 ± 22.1</td>
<td>0.352</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>15.2 ± 2.5</td>
<td>15.7 ± 2.5</td>
<td>15.7 ± 2.7</td>
<td>0.171</td>
</tr>
<tr>
<td>Polynomialsaturated (g/d)</td>
<td>91.4 ± 30.0</td>
<td>86.2 ± 25.3</td>
<td>80.0 ± 23.1</td>
<td>0.007</td>
</tr>
<tr>
<td>(% of fat)</td>
<td>36.6 ± 5.1</td>
<td>35.7 ± 5.1</td>
<td>34.8 ± 4.9</td>
<td>0.027</td>
</tr>
<tr>
<td>Monounsaturated (g/d)</td>
<td>10.2 ± 3.8</td>
<td>9.8 ± 3.3</td>
<td>9.9 ± 4.6</td>
<td>0.728</td>
</tr>
<tr>
<td>(% of fat)</td>
<td>11.4 ± 3.3</td>
<td>11.6 ± 3.1</td>
<td>12.5 ± 4.7</td>
<td>0.114</td>
</tr>
<tr>
<td>Saturated (g/d)</td>
<td>35.5 ± 12.4</td>
<td>33.3 ± 10.4</td>
<td>30.5 ± 9.2</td>
<td>0.005</td>
</tr>
<tr>
<td>(% of fat)</td>
<td>38.7 ± 3.3</td>
<td>38.4 ± 2.9</td>
<td>38.1 ± 2.9</td>
<td>0.232</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (g/d)</td>
<td>270.5 ± 84.5</td>
<td>262.4 ± 69.3</td>
<td>256.0 ± 73.4</td>
<td>0.239</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>48.2 ± 5.3</td>
<td>48.5 ± 5.5</td>
<td>49.6 ± 5.7</td>
<td>0.185</td>
</tr>
<tr>
<td>Sucrose (g/d)</td>
<td>109.6 ± 46.0</td>
<td>102.8 ± 37.8</td>
<td>103.2 ± 34.7</td>
<td>0.335</td>
</tr>
</tbody>
</table>

<sup>1</sup>In multiple regression models adjusted for age, sex, weight, and height, the 3 genotypes were coded as a single variable and were assigned the following values: GG = 0, GA = 0.5, and AA = 1. For other characteristics, crude values were used.

<sup>2</sup>X ± SD (all such values).

<sup>3</sup>Body weight index = [weight/weight reference values for sex and age] × 100. Weight reference values are derived from Rolland-Cachera et al (5).

by using colored photographs of foods, each with 3 different portion sizes. Macronutrient and micronutrient intakes were estimated with a updated computerized version of the General Index of Foods (7).

Blood samples were collected after an overnight fast. Genomic DNA was extracted from peripheral blood samples by using the salting-out method (8). The presence of the −1438G/A polymorphism in the promoter region of the 5-HT<sub>2A</sub> gene was ascertained by polymerase chain reaction amplification with 2 primers: 5'-AACCTGCAAGGTAGCAACG-3' and 5'-AACCAACCTATTITCTACCAC-3'. The 468-base pair (bp) product was digested with Hpa II (which cuts the −1438 allele into 2 fragments of 244 and 224 bp), and the alleles were separated in an 8% polyacrylamide gel (9–11).

**Statistical analysis**

Statistical analyses were performed by using SAS software (version 8.01; SAS Institute Inc, Cary, NC). A chi-square test was used to determine whether the genotype frequency was in Hardy-Weinberg equilibrium. Multiple regression analysis was used to compare food intakes as a function of the 5-HT<sub>2A</sub> genotypes. To control for variables that could be related to energy and macronutrient intake, we used sex, age, height, and weight as covariates. In addition, sex × genotype and age × genotype interactions were evaluated. Because no interaction was significant and because we wanted to improve the statistical power, data from males and females were analyzed together. To calculate P for trend, the 3 genotypes were coded as a single variable and were assigned the following values: GG = 0, GA = 0.5, and AA = 1. Because persons within a family were not independent, regression analysis were conducted by using the SAS GENMOD procedure with a family factor as repeated statement. GENMOD was based on the generalized estimating equation, which provides a practical method with reasonable statistical efficiency to analyze correlated data such as familial data by modeling the covariance structure of the correlated measurements. Unlike other methods, the measurements must not be assumed to be multivariate normal (12). P ≤ 0.05 was accepted as significant.

**RESULTS**

In both males and females, the distribution of the −1438G/A polymorphism of the 5-HT<sub>2A</sub> gene did not significantly deviate from Hardy-Weinberg equilibrium. Food intakes and characteristics according to the 5-HT<sub>2A</sub> gene polymorphism in the entire
TABLE 1. Sex ratio, age, weight, height, body mass index, and body weight index did not differ significantly among the 3 genotypes. Because no significant sex × genotype interaction was found, only results in the overall sample of males and females were described. After adjustment for age, sex, weight, and height, the A allele was significantly associated with lower energy (P for trend = 0.045) and with total, monounsaturated, and saturated fat intakes expressed in g/d (P for trend = 0.007, 0.005, and 0.006, respectively); the values for subjects with the GA genotype were intermediate. Relative contributions of saturated, monounsaturated, and polyunsaturated fatty acids to fat intakes did not differ significantly between the 3 genotypes. When expressed as a percentage of daily energy levels, fat intakes were lower in A allele carriers than in G allele carriers (P for trend = 0.027).

DISCUSSION

In this study, we showed that the −1438G/A 5-HT2A polymorphism was associated with energy and fat intakes in a young population; A allele carriers had significantly lower intakes than did G allele carriers without significant relation to maturation indexes such as weight, height, and body mass index.

The genetic component of total energy and macronutrient intakes has been already documented by familial correlation and twin studies (13–15). Nevertheless, such investigations yielded a large range of heritability indexes, as reviewed by Faith and Keller (13). Moreover, in these studies, it can be difficult to separate true genetic effects from shared environment effects. For example, by using an extended family design including multiple family members with varied degrees of relatedness (ie, spouses, parent-child, siblings, and monozygotic and dizygotic twins), Pérusse et al (16) found significant genetic influences on macronutrient intakes (11% for protein and 20% for fat and carbohydrate) but not on total energy intake. One study showed that the familial effect could be adjusted for by more frequent intertwin contact among monozygotic twin pairs than among dizygotic twin pairs (17). Nevertheless, the results of a study of twins reared apart (18) yielded still higher correlations among monozygotic than dizygotic twins, which suggested that 20–30% of the variance in total energy and macronutrient intake could be determined by genes. In the San Antonio Family Heart Study (19), the familial relations accounted for 13–26% of the total population variation in total energy, fat, and carbohydrate intakes. In that study, familial influences were stronger when modeled as a genetic heritability than as a shared household effect. In a twin study in which nutrient intakes were measured directly instead of self-reported (20), the genetic component of total energy intake was estimated to be 24–33%, but the familial influences on specific macronutrient intakes could not be separated into genetic and shared environment components. In contrast, after quantifying eating behavior by using 7-d food diaries, de Castro (21, 22) observed significant genetic effects on daily intakes of total energy (65%), carbohydrate (8%), fat (10%), and protein (7%).

Including 2 publications from our group, only a few reports (3, 23, 24) have shown that a gene polymorphism may partly determine food intakes in humans. Two recent genome scans found chromosomal regions with some evidence of linkage for energy and nutrient intake, but not in the vicinity of the 5-HT2A receptor locus (25, 26). In our previous study in adults, the A allele was significantly associated with lower energy intakes, and associations with total, monounsaturated, and saturated fats were of borderline significance (3).

In the literature, the −1438A allele is significantly associated, according to a meta-analysis of case-control studies, with anorexia nervosa (2). This association is consistent with our data, which showed that the relation of genetic variation at the 5-HT2A receptor locus with food intakes can be evidenced also in a general population. A specific role of 5-HT2A in the regulation of food intake was shown in animal models, where specific agonists of 5-HT2A decreased the neuropeptide Y–stimulated food intake (27) and the 5-HT–induced hypophagia was antagonized by 5-HT2A receptor antagonists (28).

The 5-HT2A genotype was found to be significantly related to fat consumption. In human studies, there is evidence that some 5-HT drugs can readily reduce the intake of high-fat foods and data also suggest that 5-HT activation could lead to a selective avoidance of fat in the diet (29). Anorexia nervosa is prevalent mainly in young girls, and fat avoidance is a characteristic of the disease. Both these characteristics are in agreement with our results in young people of lower fat intakes associated with the −1438A allele. The associations of 5-HT2A genotype with food intakes were similar in boys and girls in our study, which could seem paradoxical, but, in a familial study of males with anorexia nervosa, it was shown that familial and genetic factors do not allow for distinction of the occurrence of anorexia nervosa between the sexes (30).

Conversely, the −1438G allele has been associated with abdominal obesity and with perturbations in the cortisol response to dexamethasone (31), and in the large National Heart, Lung, and Blood Institute’s Family Heart Study genome scan, evidence of linkage was found between body mass index and the D13S257 genetic marker located in the same chromosomal region (13q14) as the 5-HT2A gene (32). Because fat intake has also been related to obesity, these results are consistent with those of the current study.

Nevertheless, we cannot exclude the possibility that the association of the −1438A allele with low energy and fat intakes may be due to linkage disequilibrium with other genetic polymorphisms that could affect eating behavior. However, apart from the results of our previous study of the same polymorphism in adults (3), no candidate gene other than the 5-HT2A gene has been shown to be related to food intakes in human. Another restriction is that the −1438G/A polymorphism could be also associated with personality or behavior traits that were related to food intakes or to reliance on self-reported food ingestion. Despite the scarcity of data in the literature, it may be hypothesized that persons carrying the −1438G/A polymorphism have altered mood or other personality characteristics that could be associated with food intake (33–35).

In most preliminary studies, such as this one, very little information about the genetic effect size is available beforehand, and thus it is difficult to calculate a reasonable sample size. According to power analysis based on the results of Aubert et al (3) in obese adults, in population with allele frequencies of 0.50, the number of the participants in the current study was adequate to evaluate 2.3–6% of heritability (ie, the percentage of variance that is explained by the polymorphism) with an additive mode of inheritance, achieving a statistical power of >80% at 5% probability (P value). In the sample of 371 children and adolescents, values for fat intake heritability were between 2.3% and 2.6%. At this time, we cannot rule out the possibility that the absence of
association with intakes of the other macronutrients results from the low power due to the small sample size that was estimated on the basis of our former study with higher heritability. In addition, reliance on the 3-d dietary record could increase the random variability of diet intake and, consequently, the global variance of the studied traits. In overweight young subjects, for example, the food intake assessment could be less reliable (36, 37).

In conclusion, in spite of the limitations listed above, these data show that a gene polymorphism of the serotonergic system could be related to food intakes and eating behavior in humans. However, independent, confirmatory studies that involved larger samples with characteristics different from those in the children in the current study are necessary for definite conclusions about the role of this polymorphism in the physiology of eating behavior.

We are deeply grateful for the cooperation of the families participating in the Stanislas Family Study cohort. We acknowledge the management, reception, preclinical, laboratory, and medical staffs of the Center for Preventive Medicine, Vandoeuvre-lès-Nancy, France. We especially thank Sylvie Péchiné for collection of food intake data, Maryvonne Chaussard and Chantal Lafaurie for family recruitment, and Dominique Aguillon for technical assistance with the polymorphism determination. Frozen aliquots of DNA were stored in the tissue bank at the Center for Preventive Medicine and at the INSERM U525 (Nancy, France).

BH, FF, and RA designed the study, performed the statistical analysis, and wrote the manuscript. EA and FC were responsible for laboratory analyses and participated in writing the manuscript. GS and SV are the principal investigators of the Stanislas Family Study and participated in writing the manuscript. None of the authors had any financial or personal conflict of interest.

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A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents1–4

Steven A Abrams, Ian J Griffin, Keli M Hawthorne, Lily Liang, Sheila K Gunn, Gretchen Darlington, and Kenneth J Ellis

ABSTRACT
Background: Short-term studies in adolescents have generally shown an enhancement of calcium absorption by inulin-type fructans (prebiotics). Results have been inconsistent; however, and no studies have been conducted to determine whether this effect persists with long-term use.

Objective: The objective was to assess the effects on calcium absorption and bone mineral accretion after 8 wk and 1 y of supplementation with an inulin-type fructan.

Design: Pubertal adolescents were randomly assigned to receive 8 g/d of a mixed short and long degree of polymerization inulin-type fructan product (fructan group) or maltodextrin placebo (control group). Bone mineral content and bone mineral density were measured before randomization and after 1 y. Calcium absorption was measured with the use of stable isotopes at baseline and 8 wk and 1 y after supplementation. Polymorphisms of the Fok1 vitamin D receptor gene were determined.

Results: Calcium absorption was significantly greater in the fructan group than in the control group at 8 wk (difference: 8.5 ± 1.6%; P < 0.001) and at 1 y (difference: 5.9 ± 2.8%; P = 0.04). An interaction with Fok1 genotype was present such that subjects with an ff genotype had the least initial response to fructan. After 1 y, the fructan group had a greater increment in both whole-body bone mineral content (difference: 35 ± 16 g; P = 0.03) and whole-body bone mineral density (difference: 0.015 ± 0.004 g/cm²; P = 0.01) than did the control group.


KEY WORDS Calcium absorption, vitamin D receptor, stable isotopes, inulin, prebiotics, pubertal growth, bioavailability

INTRODUCTION
Absorption of an adequate amount of calcium is particularly important during early adolescence to help achieve peak bone mass. The current recommended daily intakes of calcium are largely based on dose-effect relations to maximize net calcium retention, which in adolescents is primarily determined by calcium absorption (1). In addition to dietary intake, intestinal absorption is a key factor that controls the retention of calcium. This is especially important, given the large disparity between recommended and typical intakes of calcium in adolescents.

Recent data have shown that prebiotic inulin-type fructans (ITFs) added to the daily diet significantly increase the absorption of both calcium and magnesium in growing animals and in adolescents. Numerous animal studies have shown that ITFs significantly increase calcium absorption (2) and bone mineralization (3). In humans, the most convincing data, up until now, have been obtained in adolescents (4–6) and in postmenopausal women (7, 8). These data suggest that a mixed short and long degree of polymerization (DP) fructan product is most effective for enhancing mineral absorption (2, 5, 6).

However, all of the reported studies in humans have been relatively short term and none have directly assessed the potential benefits of supplementation with ITFs on bone mineralization. It is important that such data be available in considering the inclusion of ITFs in the diet on a daily basis, as would occur with more widespread food fortification with ITF. We therefore evaluated the effects of a mixed short- and long-DP fructan on calcium absorption and bone mineralization in young adolescents. We further sought to evaluate the interactions of genetic factors in the response of calcium and bone mineral metabolism to ITFs.

1 From the US Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Department of Pediatrics (SAA, IJG, KMH, LL, and KJE); the Section of Endocrinology, Department of Pediatrics (SKG); and the Department of Pathology (GD), Baylor College of Medicine and Texas Children’s Hospital, Houston, TX.
2 This work is a publication of the USDA/ARS Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children’s Hospital, Houston, TX. The contents of this publication do not necessarily reflect the views or policies of the USDA, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.
3 Supported by the USDA/ARS (Cooperative Agreement no. 58-6250-6-001), the NIH, the NCRR General Clinical Research Center grant no. RR0188, NIH AR43740, NIDDK, and P30 DK56338. The orange juice used in the study was provided by Orafti, NV (Tienen, Belgium).
4 Reprints not available. Address correspondence to SA Abrams, USDA/ARS Children’s Nutrition Research Center, 1100 Bates Street, Houston TX 77030. E-mail: sabrams@bcm.edu.
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SUBJECTS AND METHODS

Subjects

Through public advertising, we identified 50 girls and 50 boys for this study. All subjects were between 9.0 and 13.0 y of age and had a body mass index between the 5th and 95th percentiles for age and sex. The subjects were selected to approximately match the ethnic distribution of the greater Houston area. All subjects received a screening physical examination, which included Tanner staging. To be enrolled in the study, the subjects had to be healthy and have a Tanner stage of 2 or 3 (breast stage for girls and pubic hair stage for boys). Girls had to be premenarcheal. Subjects with any chronic illnesses requiring them to take medications regularly were ineligible for the study.

Written informed consent was obtained from a parent or legal guardian for each subject; written assent was obtained from all of the study subjects. The Institutional Review Board of Baylor College of Medicine and Affiliated Hospitals approved this protocol.

Initial study visit

Within 8 wk of the screening visit described above, the subjects were admitted for 24 h to the General Clinical Research Center of Texas Children’s Hospital in Houston, TX. During this stay, calcium absorption and bone mineralization were measured as described below. Blood was collected for DNA analysis of vitamin D receptor polymorphisms.

At the end of the baseline study, the subjects were randomly assigned in a double-blinded fashion and stratified by sex to 1 of 2 carbohydrate supplement groups: fructan group (8 g/d oligosaccharides of an inulin-type fructan, Raftilose Synergy1; Orafti Frereres, Lestrem, France) was chosen as the placebo control because, contrary to the ITF, it is completely digested in the small intestine. Its sensory and other characteristics are consistent with their usual dietary practices. Subjects with higher usual intakes had other meal components (primarily dairy products) provide up to an additional 350 mg with their meal, depending on their usual intake. Toward the end of breakfast, the subjects were given 20 μg 46Ca, which had been mixed with 240 mL calcium-fortified orange juice. Different breakfast items were used to reflect the usual pattern of calcium intake of the subjects, but the calcium content of the isotope-containing meals was the same in each subject in all 3 studies.

After breakfast, 44Ca (1.2 mg) was infused over 2 min via a heparin-lock catheter. Beginning with breakfast, a complete 24-h urine collection was obtained. Subsequently, subjects collected a second 24-h urine collection at home after discharge from the General Clinical Research Center (6). Calcium absorption was calculated from the relative recovery of the oral and the intravenous tracers during the entire 48-h study period. A 48-h time period was chosen because of evidence that ITFs may increase the absorption of calcium in the large intestine. This would necessitate a longer collection period than the 24-h time period usually used in such studies to fully identify an effect (5, 11, 12).

The subjects were required to note any lost urine or failure to collect a urine sample at home.

Dietary methods

At the screening visit, the study dietitian asked the subjects what foods they usually ate on a normal day to determine food preferences. Inpatient menus for the overnight study visit were based on normal calcium intake (13). All foods and beverages used during the inpatient and outpatient visits were weighed before and after intake to accurately determine intake. The subjects were instructed to keep weighed food records for 6 d during the study: a 2-d period after the first overnight visit, a 2-d period 2 mo later, and a 2-d period after the 1-y visit. The subjects were called at home during the 1-y period to obtain a 24-h dietary recall of the previous day’s intake and to ensure that the subject maintained a relatively consistent calcium intake. To reflect the marketplace changes in dietary food contents during the study, dietary intake data were collected with the use of the NUTRITION DATA SYSTEM FOR RESEARCH software (versions 4.03 and 4.05; Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN).

To monitor compliance, subjects were provided with a calendar and instructed to put a sticker on the calendar for every day that they remembered to drink the juice with the supplement. They were instructed to keep all supplement packets after they had mixed them into the orange juice and return them to the research center every 3 mo along with their calendars and any unused supplement packets. The study staff counted compliant days and the number of supplement packets consumed. Subjects were mailed a small gift if they returned their calendars and packets in a timely fashion. If subjects chose to consume the supplement with milk, they were required to indicate this in writing on the calendar.

Analytic methods

Urine samples were prepared for thermal ionization mass spectrometric analysis as previously described by using an oxalate precipitation technique (9, 10). Samples were analyzed for...
isotopic enrichment with a magnetic sector thermal ionization mass spectrometer (model MAT 261; Finnigan Bremen, Germany). The accuracy and precision of this technique for natural-abundance samples compared with standard data are 0.15%.

**Dual-energy X-ray absorptiometry**

Bone mineralization measurements were performed by using a Hologic QDR-4500A dual-energy X-ray (DXA) absorptiometer (Hologic, Inc, Waltham, MA). The whole body was scanned in the fan-beam mode. Whole-body bone mineral content (BMC) and whole-body areal bone mineral density (BMD) were measured. Whole-body BMD precision was <1%, whereas whole-body BMC precision was <1.5% (14, 15).

**Genetic methods**

Genomic DNA was isolated from 3 mL whole blood collected in EDTA-coated tubes with the use of the Wizard TM Genomic DNA Purification Kit (Promega, Madison, WI). The DNA was analyzed for Fok1 genetic polymorphisms by the Gene Expression Core of the Texas Coast Digestive Disease Center. The VDR receptor phenotypes (Fok1) were analyzed as previously described (16). Primer sequences for Fok1 were obtained from Mark Johnson, Creighton University School of Medicine, Omaha, NE.

**Statistical methods**

Comparisons of carbohydrate supplement and genotype groups for fractional absorption of calcium were made by using analysis of variance and analysis of covariance (ANCOVA) techniques, in which changes over the time course of the study were determined by repeated-measures ANCOVA with subsequent determination of differences at specific measurement time points by ordinary ANCOVA. Covariate adjustments were based on the specific analysis conducted. Sex, ethnicity, and Tanner stage at enrollment were included as covariates in models of the general linear models (univariate and repeated-measures options) provided in SPSS 13.0 for WINDOWS (SPSS Inc, Chicago, IL). In addition, the proportion of responders with an increase in calcium absorption of ≥3% after 8 wk of the ITF treatment was determined by using the multiple logistic regression option of this program. All data are presented as means ± SEMs.

Sample size was determined on the basis of our earlier study, in which we found a 6% change (SD: 9%) in fractional calcium absorption in girls after adding an ITF to their diet for 3 wk. Therefore, enrollment of 80 subjects had a power >0.9 (P < 0.05) to identify this difference. We enrolled 100 subjects (50 of each sex) based on a 20% dropout rate by 1 y, whereas ultimately only 8% of the subjects failed to complete all aspects of the study.

We have separately reported the relation between vitamin D receptor polymorphisms, including the Fok1 gene, and calcium absorption and bone mineralization (17). Because we found a significant Fok1 genotype–related effect on calcium absorption and bone mineralization, Fok1 genotype was used as a covariate in evaluating the effects of the carbohydrate supplement on calcium absorption and bone mineralization.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Anthropometric characteristics of the children at baseline</strong></td>
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<tr>
<td><strong>Fructan group</strong> (n = 48)</td>
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<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Height (cm)</td>
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<tr>
<td>Weight (kg)</td>
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<tr>
<td>Tanner stage 2 (%)&lt;sup&gt;2&lt;/sup&gt;</td>
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<sup>1</sup> Children were randomly assigned to receive 8 g/d of either inulin-type fructan or maltodextrin placebo (control).

<sup>2</sup> ANOVA.

<sup>3</sup> ± ± SEM (all such values).

<sup>4</sup> All subjects were either Tanner stage 2 or Tanner stage 3.

**RESULTS**

**Subject and dietary description**

A total of 100 subjects met the study criteria and were randomly assigned to the fructan or the control group; 98 subjects completed the baseline and 8-wk absorption studies. Both drop-outs were from the fructan group, which left 48 subjects in the fructan group and 50 in the control group. One subject dropped out because of a failure to tolerate the ITF (increased stool frequency and diarrhea), and the other subject dropped out because of noncompliance with the study procedures, which was unrelated to the carbohydrate assignment. All other subjects tolerated the study protocol well. Three additional subjects (all in the control group) dropped out between 8 wk and 1 y for personal reasons that were unrelated to the group assignment. At 1 y, 3 additional subjects were unable to complete the absorption studies, but did complete the bone mineral measurements. Thus, the total sample number at 1 y was 95 for the bone mineral measurements and 92 for the calcium absorption measurements.

Anthropometric characteristics of the study subjects are shown in Table 1. The mean (=SEM) age of the subjects at the start of the study was 11.6 ± 0.1 y. The fructan group consisted of 24 whites, 5 African Americans, 11 Hispanics, and 8 Asians; the control group consisted of the 28 whites, 9 African Americans, 11 Hispanics, and 2 Asians. Compliance with daily carbohydrate supplementation was not significantly different between groups (84% in the fructan group and 81% in the control group). There was no significant relation between fractional absorption and compliance at any time period.

Total urinary calcium at the 3 time points was compared. Mean (=SEM) urinary calcium was 81 ± 7 mg/d at baseline, 78 ± 5 mg/d at 8 wk, and 87 ± 6 mg/d at 1 y (P = 0.10, repeated-measures analysis of variance). These results suggest no differences in the completeness of the urine samples collected at home and those collected while the subjects were inpatients. There were no differences in urinary calcium between the fructan and control groups at baseline or any time point (P > 0.2 at each time point after correction for ethnicity, sex, and Tanner stage).

Calcium intake was maintained throughout the study at the subject’s usual intake, and there were no significant differences in calcium intake between the carbohydrate supplement groups. The mean (=SEM) calcium intake at baseline was 907 ± 33 mg/d, at 8 wk was 959 ± 33 mg/d, and at 1 y was 906 ± 29 mg/d.
Calcium absorption at 8 wk and 1 y

The effects of the fructan and control groups on the fractional absorption of calcium during the study year were compared by repeated-measures ANCOVA at the baseline, 8-wk, and 1-y time points \((n = 92)\). After adjustment for ethnicity \((P = 0.04)\), sex \((P = 0.54)\), \(Fok1\) genotype \((P = 0.007)\), calcium intake at each visit \((P = 0.03\) at baseline; \(P > 0.1\) at 8 w and 1 y), and Tanner stage at enrollment \((P = 0.15)\), the effect of fructan on the fractional absorption of calcium was significant \((P = 0.02)\). The interaction of time point of measurement and carbohydrate group was significant \((P < 0.01)\).

We further evaluated the results for calcium absorption at 8 wk and 1 y relative to the baseline absorption values by ordinary ANCOVA (Table 2). After adjustment for baseline values and other covariates, calcium absorption was significantly greater at 8 wk \((8.5 \pm 1.6\%, P < 0.001)\) and 1 y \((difference 5.9 \pm 2.8\%, P = 0.04)\) in the fructan group than in the control group.

Inclusion of the 25-hydroxyvitamin D concentration in the model had no effect on the relation between carbohydrate groups and calcium absorption. The 25-hydroxyvitamin D concentration was not significantly related to calcium absorption at 8 wk \((P = 0.83)\) or at 1 y \((P = 0.51)\).

Effects of genotype on results at 8 wk and 1 y

We previously showed a significant effect of \(Fok1\) genotype on calcium absorption \((16, 17)\). Therefore, we sought to identify whether there was a nutrient-gene interaction by evaluating whether fractional calcium absorption at 8 wk and 1 y was related to an interaction of genotype with carbohydrate supplementation. The three-factor interaction of genotype with carbohydrate supplementation and time point of measurement was significant \((P = 0.04)\). Additionally, the interaction of genotype with carbohydrate supplementation was significant at 8 wk \((P = 0.03)\) but not at 1 y \((P = 0.43)\). We analyzed each genotype group at 8 wk and 1 y for the effects of the carbohydrate supplement, and the results indicate a preferential effect of fructan in the subjects with genotypes associated with higher calcium absorption \((FF\) and \(Ff)\) at 8 wk (Table 3).

Additionally, we determined the proportion of individuals who were “responders” to the carbohydrate supplement. In this analysis, we chose an increase of 3% to represent a responder a priori. This evaluation was only done at the 8-wk time period because there was a mean 2.3% increase in calcium absorption in the control group compared with baseline at 1 y, which would have made the 3% definition of a responder difficult to interpret. The interaction of carbohydrate supplement group with \(Fok1\) genotype was significant in determining responders at 8 wk \((P = 0.01)\) with sex, Tanner stage, and ethnicity as covariates. The percentage of responders by genotype is shown in Table 4. Overall, 67% (32/48) of the fructan group and 34% (17/50) of the control group were responders \((P = 0.004)\).

Bone mineralization results

Comparisons of groups for changes during the study year in whole-body BMC and BMD are shown in Table 5. After 1 y, fructan resulted in a greater increase in whole-body BMC \((35 \pm 16 \text{ g}; P = 0.03)\) and BMD \((0.015 \pm 0.004 \text{ g/cm}^2; P = 0.01)\) than did the control treatment. To calculate the approximate effect of this difference in whole body BMC on daily calcium accretion, we used a factor of 0.322 for the fraction of calcium per mg of BMC \((14)\). This leads to a calculation of an average net difference of 30 mg/d in calcium accretion between groups.

### Table 2

Calcium absorption in the children at baseline, 8 wk, and 1 y

<table>
<thead>
<tr>
<th>Time of study</th>
<th>Fructan group</th>
<th>Control group</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>29.9 ± 1.4 [48](^2)</td>
<td>29.4 ± 1.5 [50]</td>
<td>0.76</td>
</tr>
<tr>
<td>8 wk(^1)</td>
<td>38.5 ± 1.2 [48]</td>
<td>30.0 ± 1.3 [50]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 y(^1)</td>
<td>37.7 ± 2.1 [47]</td>
<td>31.7 ± 2.3 [45]</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(^1\) Children were randomly assigned to receive 8 g/d of either inulin-type fructan or maltodextrin placebo (control). All values were adjusted for genotype, ethnicity, Tanner stage at enrollment, sex, and calcium intake. Repeated-measures analysis of covariance showed a significant interaction of time of measurement and use of fructan \((P = 0.04)\). Subsequent comparisons were made by using ordinary analysis of covariance.

\(^2\) \(x \pm SEM; n\) in brackets (all such values).

### Table 3

Effect of genotype on calcium absorption in children at 8 wk and 1 y

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fructan group</th>
<th>Control group</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FF)</td>
<td>45.6 ± 1.7 [12](^2)</td>
<td>33.2 ± 1.3 [22]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(Ff)</td>
<td>37.3 ± 1.7 [26]</td>
<td>30.2 ± 2.0 [20]</td>
<td>0.02</td>
</tr>
<tr>
<td>(ff)</td>
<td>31.9 ± 1.6 [10]</td>
<td>28.5 ± 1.8 [8]</td>
<td>0.22</td>
</tr>
<tr>
<td>1 y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FF)</td>
<td>44.9 ± 4.5 [11]</td>
<td>35.8 ± 3.3 [20]</td>
<td>—</td>
</tr>
<tr>
<td>(Ff)</td>
<td>38.9 ± 2.5 [26]</td>
<td>30.6 ± 3.1 [18]</td>
<td>—</td>
</tr>
<tr>
<td>(ff)</td>
<td>31.0 ± 3.5 [10]</td>
<td>32.1 ± 4.2 [7]</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^1\) Children were randomly assigned to receive 8 g/d of either inulin-type fructan or maltodextrin placebo (control). All values were adjusted for baseline absorption, calcium intake at baseline and 8 wk or 1 y, Tanner stage, sex, and ethnicity. Repeated-measures analysis of covariance showed a significant genotype by carbohydrate treatment by time of measurement interaction \((P = 0.04)\). Subsequent pairwise comparisons of genotypes were made by using ordinary analysis of covariance. The interaction of genotype with carbohydrate supplementation was significant at 8 wk \((P = 0.03)\) but not at 1 y \((P = 0.43)\).

\(^2\) \(x \pm SEM; n\) in brackets (all such values).

### Table 4

Percentage of children with an increase in calcium absorption of ≥3% after 8 wk of treatment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fructan group</th>
<th>Control group</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(FF)</td>
<td>92 [12]</td>
<td>18 [22]</td>
<td>0.002</td>
</tr>
<tr>
<td>(Ff)</td>
<td>62 [26]</td>
<td>40 [20]</td>
<td>0.07</td>
</tr>
<tr>
<td>(ff)</td>
<td>50 [10]</td>
<td>63 [8]</td>
<td>0.53</td>
</tr>
</tbody>
</table>

\(^1\) \(n\) in brackets. Children were randomly assigned to receive 8 g/d of either inulin-type fructan or maltodextrin placebo (control). All values were adjusted for sex, Tanner stage, and ethnicity. In the initial 2-factor multiple logistic regression, the interaction of genotype by carbohydrate treatment was significant \((P = 0.01)\). Subsequent pairwise comparisons of genotypes were made by using a single-factor logistic regression.


**DISCUSSION**

We showed a greater enhancement in calcium absorption and in bone mineralization in young adolescents provided 8 g ITF/d than in those given the same amount of maltodextrin, a placebo control that itself appeared to have had a small effect on calcium absorption in some subjects. Our data further suggest that, at least initially, the magnitude of the effect was affected by genetic modifiers of calcium absorption, including polymorphisms of the Fok1 gene.

Although animal studies have uniformly shown enhancement of calcium (and magnesium) absorption by ITF (2, 3), results of investigations in humans have been mixed (12). Recent studies have tended to confirm such an effect in both adolescents (4–6) and in adults (7, 8, 18), but available data are limited by their short-term nature. Our finding of a benefit of ITF after 1 y of use is relevant because a failure to show a persistent change would suggest no clinical benefit of ITF. Indeed, the types of food in which ITF products are likely to be supplemented are products such as yogurts and juices, which are consumed over a long period of time.

We did not evaluate the relation between calcium intake and the magnitude of the effect of the ITF. We chose to give the ITF with a substantial calcium load at breakfast to reflect the equivalent calcium intake of a whole glass of milk or juice given with a typical breakfast for that subject. Future investigations, therefore, need to evaluate the specific effects of ITF on calcium absorption at very low intakes of calcium (eg, <450 mg/d) because such low intakes were not evaluated in the present study.

The mechanism of action of ITF responsible for the enhancement of calcium absorption remains unclear. It may be related to an increased absorption of calcium in the colon by scavenging unabsorbed calcium (11). ITFs typically are fermented in the proximal and distal colon. This fermentation results in the production of short-chain fatty acids (acetate, propionate, and butyrate) and lactate. These may increase the solubility of minerals, enhancing their absorption. Alternatively, it is possible that a trophic effect occurs throughout the intestines that enhances passive calcium absorption (2, 3, 11). In this regard, our finding of an interaction of ITF and Fok1 genotype suggests that a vitamin D–mediated enhancement of absorption may also be involved. However, this hypothesis is not supported by our failure to find any significant relation between vitamin D status at the start of the study and calcium absorption at 8 wk or vitamin D status at 1 y and calcium absorption at 1 y.

These genetic interactions indicate how difficult it is to determine the effects of a dietary intervention when it is short term or when the sample size is small, especially a dietary intervention that affects trophic gut function. Our study was larger than previous physiologic studies of ITFs. Although even larger population-based studies would be ideal, they are impractical because of the costs associated with the assessment of endpoints such as calcium absorption and bone mineralization in large populations.

The net benefit associated with ITF supplementation in the present study was the average increase in calcium accretion (~30 mg Ca/d) to the skeleton, an increase equivalent to ~11 g each year during pubertal growth. Although this increase is considerable, it is much less than the deficit of up to 80 g/y that we recently reported to be associated with extremely low-calcium diets providing an average of 450 mg Ca/d (13). Therefore, the addition of an ITF to the diet, although beneficial for net calcium balance, does not have the same magnitude of effect during puberty as do large changes in dietary calcium intake.

Nevertheless, an increase in calcium absorption and bone calcium deposition of 15–20% of the nonsupplemented amount may represent a substantial additional health benefit with negligible risks. Additionally, numerous other potential health benefits are associated with an increased intake of ITF (19). Supplementation with 8 g inulin/d appeared safe for virtually all of the study subjects; gastrointestinal symptoms were reported by only one subject (1%). These symptoms rapidly resolved after the ITF was discontinued.

It is important to consider the potential public health benefits of advocating enhanced calcium absorption from the diet through the use of more bioavailable calcium sources or, as in the present study, through the use of a nonabsorbable carbohydrate. This issue has been discussed extensively with regard to the relative merits of different sources of calcium in supplements and whether a potential small absorptive benefit of calcium citrate or calcium citrate malate over calcium carbonate is of enough clinical importance to affect the consumers choice of supplement (20, 21). In these cases, a cost-benefit analysis may be useful; however, consumers may choose what they consider to be the optimal supplement on the basis of scientific data, even if that supplement has a higher relative cost per absorbed milligram of calcium.

It is likely that ITFs are, and will likely remain, a premium food and beverage supplement. It is probable that calcium absorption can be increased with a calcium supplement (or increased food and beverage calcium intake) as readily as with an ITF-fortified product. However, these 2 decisions have different benefit and safety profiles. The mineral-absorptive benefit of ITF products is an important component of overall gastrointestinal health, even though this benefit is generally not the primary or sole reason for

**TABLE 5**

**Calcium accretion and bone mineral density (BMD) in the children after 1 y of treatment**

<table>
<thead>
<tr>
<th>Fructan group (n = 47)</th>
<th>Control group (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole-body BMC (g)</strong></td>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Whole-body BMC (g/cm²)</td>
<td>1338 ± 35</td>
</tr>
<tr>
<td>0.888 ± 0.009</td>
<td>0.047 ± 0.004</td>
</tr>
</tbody>
</table>

¹ All values are ±SEM and were adjusted for ethnicity, sex, Tanner stage at enrollment, and Fok1 genotype. Children were randomly assigned to receive 8 g/d of either inulin-type fructan or maltodextrin placebo (control). BMC, bone mineral content.

²,³ Significantly different from change after 1 y in the fructan group (analysis of covariance): F = 0.03, P = 0.01.
their being chosen by consumers. Furthermore, we have shown that a benefit of ITF on calcium absorption exists across a range of calcium intakes (5), which indicates that consumers have several strategies to choose from to enhance their calcium status.

We conclude that the daily inclusion of a modest amount of a commercially available nonabsorbable ITF with a mixture of short and long DP enhances calcium absorption and bone mineralization in pubertal adolescents. Genetic interactions, however, may modulate this affect.

We acknowledge the assistance of the nursing staff of the General Clinical Research Center of Texas Children’s Hospital for caring for the study subjects; Cynthia Edwards for study recruitment; Holly Endris, Angela Freeman, Melissa Knox, Courtney Edwards, Lora Plumlee, Yana Kriseman, Rachel Wolfson, Michelle Lopez, and Anh Mai for subject assistance; and E O’Brian Smith for statistical advice.

SAA was responsible for the overall conduct of the study. IJJ was responsible for the daily supervision of the study and Tanner staging of the boys. SKG was responsible for the medical management of the subjects during the study and the Tanner staging of the girls. KMH supervised all dietary aspects of the study. LL was responsible for the protocol design, the determination of the laboratory analysis in the stable-isotope studies. GD was responsible for the protocol design, the determination of the Fok1 genotype, and the interpretation of the genetic aspects of the study. KJE was responsible for all bone mineralization measurements. All authors were involved in the preparation of the manuscript for publication. SAA is a consultant for The Coca-Cola Company (member of the Beverage Institute for Health and Wellness). None of the other authors had a conflict of interest.

REFERENCES


Vitamin D and bone mineral density status of healthy schoolchildren in northern India\textsuperscript{1–3}


ABSTRACT

Background: Current data on the prevalence of vitamin D deficiency in India are scarce.

Objective: We assessed the calcium-vitamin D-parathyroid hormone axis in apparently healthy children from 2 different socioeconomic backgrounds in New Delhi, India.

Design: Clinical evaluation for evidence of vitamin D deficiency was carried out in 5137 apparently healthy schoolchildren, aged 10–18 y, attending lower (LSES) and upper (USES) socioeconomic status schools. Serum calcium, inorganic phosphorus, alkaline phosphatase, 25-hydroxyvitamin D [25(OH)D], and immunoreactive parathyroid hormone were measured in 760 children randomly selected from the larger cohort. Bone mineral density of the forearm and the calcaneum was measured in 555 children by using peripheral dual-energy X-ray absorptiometry.

Results: Clinical evidence of vitamin D deficiency was noted in 10.8\% of the children. Children in the LSES group had a significantly ($P < 0.01$) lower 25(OH)D concentration (10.4 ± 0.4 ng/mL) than did those in the USES group (13.7 ± 0.4 ng/mL). Concentrations of 25(OH)D <9 ng/mL were seen in 35.7\% of the children (42.3\% in LSES; 27\% in USES; $P < 0.01$). Boys had significantly ($P = 0.004$) higher 25(OH)D concentrations than did girls. There was a significant negative correlation between the mean serum immunoreactive parathyroid hormone and 25(OH) D concentrations ($r = -0.202, P < 0.001$). Mean forearm bone mineral density was significantly ($P < 0.01$) higher in the USES group than in the LSES group.

Conclusion: A high prevalence of clinical and biochemical hypovitaminosis D exists in apparently healthy schoolchildren in northern India. 


KEY WORDS Rickets, 25-hydroxyvitamin D, 25(OH)D, immunoreactive parathyroid hormone, hypovitaminosis D, bone mineral density, children, adolescents, India

INTRODUCTION

Vitamin D status has a profound effect on the growth and development of children and has major implications for adult bone health. Overt cases of vitamin D deficiency represent only the tip of an iceberg of vitamin D insufficiency (1). Whereas severe vitamin D deficiency, usually associated with 25-hydroxyvitamin D [25(OH)D] concentrations <5.0 ng/mL, results in rickets and osteomalacia (2), even less severe deficiency has been associated with numerous negative skeletal consequences, including secondary hyperparathyroidism, increased bone turnover, enhanced bone loss, and fracture risk (2, 3).

In assessing a person's vitamin D status, because 1,25-dihydroxyvitamin D [1,25(OH)\textsubscript{2}D] can be normal, high, or low in vitamin D deficiency (4), the most commonly used and most sensitive index is 25(OH)D. Age, sex, pubertal status, latitude, season, race, and ethnicity influence serum concentrations of 25(OH)D (5–7).

In developing countries such as India, data on clinical and subclinical vitamin D–deficiency status are scarce. There have been scattered epidemiologic studies, but few have provided detailed clinical and biochemical information on the prevalence of hypovitaminosis D in the population. Three hospital-based studies conducted in adults showed that the mean serum 25(OH)D concentration is significantly lower than that in Western countries (8–10); the latest of these studies (10) also correlated vitamin D status and bone mineral density (BMD) measurements.

The objectives of the current study were to assess the prevalence of clinical and biochemical vitamin D deficiency in healthy children and adolescents aged 10–18 y during their period of most rapid growth, to compare the biochemical variables of the calcium–vitamin D axis between 2 socioeconomic groups, and to study the effect of hypovitaminosis D on BMD.

SUBJECTS AND METHODS

The study was conducted in 5137 apparently healthy schoolchildren (aged 10–18 y) of both sexes in urban New Delhi, India, which is geographically located at 28°N. The children attended

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\textsuperscript{2} Supported by a Task Project Grant from the Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization, Ministry of Defence, Government of India.

\textsuperscript{3} Reprints not available. Address correspondence to RK Marwaha, Department of Endocrinology and Thyroid Research, Institute of Nuclear Medicine and Allied Sciences, Timarpur, New Delhi 110054, India. E-mail: marwaha_raman@hotmail.com.

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2 state-run schools that catered to children of lower socioeconomic status (LSES) and 2 private schools that enroll children of upper socioeconomic status (USES). Socioeconomic stratification of the subjects was based on the type of school attended. Of the above 5137 subjects, 3089 (1079 boys, 2010 girls) from state-run schools made up the LSES group, and 2048 (968 boys, 1080 girls) from private schools made up the USES group. The subjects were further divided into 3 age groups: 10–12, 13–15, and 16–18 y old.

This entire cohort of 5137 children and adolescents underwent clinical examination and anthropometric assessment, including a recording of the stigmata of vitamin D deficiency. A sunlight exposure questionnaire was administered to each child. Clinical vitamin D deficiency was diagnosed if a subject had either genu varum (bowlegs) or genu valgum (knock-knees). Genu varum and genu valgum were defined by intercondylar and intermalleolar distances >6 cm and >8 cm, respectively (11). We also looked for other clinical signs of vitamin D deficiency, eg, frontal bossing and epiphyseal enlargement of the wrists, but these signs were not considered because they are more subjective. We excluded from the study the subjects with clinical features that were suggestive of any concurrent systemic illness; intake of any drug that interferes with bone mineral metabolism, such as glucocorticoids, anticonvulsants, and antituberculous drugs; or any other skeletal disease.

Of this large cohort, 760 children (430 from LSES and 330 from USES groups) selected by randomization from each class of the school underwent further laboratory assessment. Each class was divided into 4 groups according to the number of sections in the class, and all children from one randomly selected section (cluster) were called for blood sampling the next day. Blood samples were collected from subjects in the fasting state at 0800 without venostasis under basal conditions for estimation of total calcium, phosphorus, alkaline phosphatase (AP) activity, 25(OH)D, and immunoreactive parathyroid hormone (iPTH). The serum was centrifuged at 4 °C for 15 min at 1200 × g and divided into 5 aliquots, which were refrigerated. Serum calcium, phosphorus, and AP were estimated on the same day, and the remaining aliquots were stored at −20 °C until 25(OH)D and iPTH were estimated. Serum calcium (Random Laboratory Ltd, Crumlin, UK) and inorganic phosphorus (Clontial; Ampli Medical SPA, Milan, Italy) were measured by colorimetric methods. Serum AP was measured by a liquid kinetic method (Clontial). The normal laboratory range in adults for total serum calcium is 2.02–2.60 mmol/L (8.10–10.04 mg/dL), and that for serum phosphorus is 0.81–1.55 mmol/L (2.5–4.8 mg/dL), according to the kit manufacturers. It is also known that the upper limit of serum phosphorous in mid-childhood is 5.8 mg/dL (12). The normal laboratory range for serum AP at 37 °C is 100–275 IU/L in adults and 180–1200 IU/L in children before epiphyseal closure.

Dietary assessment of total energy, protein, carbohydrate, fat, calcium, and phytate was done in 349 subjects randomly selected from the cohort of 760 (171 from the LSES and 178 from the USES groups) through a 24-h recall of their food intake. The serum concentrations of 25(OH)D (reference range: 9.0–37.6 ng/mL) and iPTH (reference range: 13–66 pg/mL) were measured by radioimmunoassay and immunoradiometric assay (DiaSorin, Stillwater, MN), respectively. The lowest concentration of vitamin D measurable by this kit, defined as the lowest quantity differentiated from zero at 2 SDs below the mean counts per min of the zero standard, is 1.5 ng/mL.

Distal forearm and calcaneal BMDs (g/cm²) were measured by using portable densitometry with dual-energy X-ray absorptiometry (DXA) in 555 children (PIXI-1.34; Lunar Corp, Madison, WI).

The parents of each participant were informed about the study protocol and gave written informed consent to their children’s participation. All subjects whose parents gave consent to blood sampling underwent the biochemical evaluation. The study protocol was approved by the institutional ethics committee of the Institute of Nuclear Medicine and Allied Sciences.

Definition of hypovitaminosis D

We classified hypovitaminosis D on the basis of the measurement of serum 25(OH)D concentrations, as recommended by Lips (3). Concentrations of 10–20, 5–10, and < 5 ng 25(OH)D/mL were classified as mild, moderate, and severe hypovitaminosis D, respectively.

Statistical analysis

Data are presented as means ± SDs. The independent-sample t test was used to compare differences between the 2 socioeconomic groups for continuous variables. Data were also analyzed with a 3-factor analysis of variance (ANOVA) with age, sex, and SES as factors. A chi-square test was performed for categorical variables. Pearson rank tests were used for correlation analysis when necessary. P values < 0.05 were considered significant.

We used SPSS statistical software (version 10.0; SPSS Inc, Chicago, IL) for the analysis. Because BMD is affected by body mass, the means used in 3-factor ANOVA were adjusted for height and weight for BMD only. The other biochemical and hormonal variables are unaffected by height, weight, and body mass, and thus the means of these variables were not adjusted for those factors.

RESULTS

A total of 5137 children were examined, and a 10.8% prevalence of clinical evidence of vitamin D deficiency was noted in 556 children. Boys had a prevalence of 10.4% and girls had a prevalence of 11.1%, and there was no significant difference between the 2 groups (P = 0.46). The prevalence of genu valgum was 3.3% (boys: 2.4%; girls: 3.9%; P = <0.01) and that of genu varum was 7.5% (boys: 8.0%; girls: 7.2%; P = 0.39). The prevalence of clinical evidence of vitamin D deficiency was 11.6% in the LSES group and 9.7% in the USES group, values that were not significantly different (P = 0.07). All of the children had a daily sunlight exposure of ≥30 min and had exposure of a minimum of 30% of the body surface area. There was a significant difference in the mean calcium intake between the 2 groups (314 ± 194 mg in LSES and 713 ± 241 mg in USES; P < 0.01), but there was no significant difference in the intake of phytate.

A total of 760 children were assessed biochemically—430 from the LSES group (167 males; 263 females) and 330 from the USES group (158 males; 172 females). The mean BMI of children in the 2 groups was significantly different (LSES group: 17.1 ± 2.9; USES group: 21.0 ± 4.7). The unadjusted mean concentrations of calcium, phosphorus, AP, iPTH, and 25(OH)D in children in the LSES and USES groups are shown in

<table>
<thead>
<tr>
<th>Variable</th>
<th>LSES Group</th>
<th>USES Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.5 ± 0.5</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>4.0 ± 0.2</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>AP (IU/L)</td>
<td>25.0 ± 5.0</td>
<td>27.0 ± 6.0</td>
</tr>
<tr>
<td>iPTH (pg/mL)</td>
<td>15.0 ± 3.0</td>
<td>16.0 ± 4.0</td>
</tr>
</tbody>
</table>

...
TABLE 1
Comparison of unadjusted means of variables in the 2 socioeconomic groups

<table>
<thead>
<tr>
<th>Variable and age category</th>
<th>LSES group</th>
<th>USES group</th>
<th>LSES group</th>
<th>USES group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>9.3 ± 1.3</td>
<td>9.7 ± 1.2</td>
<td>9.5 ± 0.6</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>13–15 y</td>
<td>9.5 ± 0.8</td>
<td>9.5 ± 1.2</td>
<td>9.4 ± 0.7</td>
<td>9.6 ± 1.9</td>
</tr>
<tr>
<td>16–18 y</td>
<td>9.3 ± 0.6</td>
<td>9.6 ± 0.7</td>
<td>9.2 ± 0.7</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>4.0 ± 0.5</td>
<td>4.7 ± 1.0</td>
<td>3.7 ± 0.6</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>13–15 y</td>
<td>4.0 ± 0.9</td>
<td>4.6 ± 0.9</td>
<td>3.8 ± 0.5</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>16–18 y</td>
<td>3.9 ± 0.5</td>
<td>3.6 ± 0.7</td>
<td>3.9 ± 0.5</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>509 ± 223</td>
<td>426 ± 162</td>
<td>481 ± 192</td>
<td>405 ± 118</td>
</tr>
<tr>
<td>13–15 y</td>
<td>469 ± 173</td>
<td>425 ± 147</td>
<td>347 ± 245</td>
<td>217 ± 108</td>
</tr>
<tr>
<td>16–18 y</td>
<td>333 ± 166</td>
<td>241 ± 114</td>
<td>192 ± 71</td>
<td>153 ± 81</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>35.4 ± 19.8</td>
<td>24.4 ± 13.6</td>
<td>44.3 ± 37.1</td>
<td>25.8 ± 12.0</td>
</tr>
<tr>
<td>13–15 y</td>
<td>42.3 ± 33.2</td>
<td>28.6 ± 15.5</td>
<td>46.7 ± 51.8</td>
<td>26.2 ± 18.4</td>
</tr>
<tr>
<td>16–18 y</td>
<td>37.9 ± 35.8</td>
<td>24.2 ± 14.4</td>
<td>32.1 ± 23.6</td>
<td>22.2 ± 10.4</td>
</tr>
<tr>
<td>25(OH)D (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>12.4 ± 5.5</td>
<td>19.3 ± 8.8</td>
<td>11.2 ± 6.5</td>
<td>12.5 ± 8.9</td>
</tr>
<tr>
<td>13–15 y</td>
<td>11.3 ± 5.8</td>
<td>13.1 ± 7.0</td>
<td>9.9 ± 6.2</td>
<td>10.2 ± 5.7</td>
</tr>
<tr>
<td>16–18 y</td>
<td>11.3 ± 5.3</td>
<td>13.5 ± 7.0</td>
<td>10.5 ± 5.7</td>
<td>12.9 ± 10.5</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; n in brackets. LSES, lower socioeconomic status; USES, upper socioeconomic status; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.
2 There were no significant interactions or main effects of sex, age, and socioeconomic status.
3 There was no significant 3-factor interaction. The interaction between age and socioeconomic status was significant, P < 0.001. The main effect of sex was significant, P < 0.001.
4 The 3-factor interaction was not significant. Age × sex interaction was significant, P < 0.001. The main effect of socioeconomic status was significant, P < 0.001.
5 None of the 3- or 2-factor interactions was significant. The only significant main effect was that of socioeconomic status, P < 0.001.
6 There was no significant 3-factor interaction. The age × sex interaction was marginal, P = 0.067. The main effects of socioeconomic status, age, and sex were significant, P < 0.004 for each.

Table 1. A sequential analysis of each of these variables by 3-factor ANOVA with interaction is given below.

**Calcium**

The overall unadjusted mean for serum calcium was 9.4 ± 0.97 mg/dL. There were no significant interactions or main effects of sex, age, and SES on mean serum calcium concentration.

**Phosphorus**

Adjusted mean ± SE values of serum phosphorus for LSES and USES were 3.9 ± 0.04 and 4.2 ± 0.5 mg/dL, respectively (P < 0.01). In the 2 younger categories, the mean phosphorus concentration was higher in the USES than in the LSES group. However, in the oldest category, the mean phosphorus concentration was lower in the USES than in the LSES group. Mean serum phosphorus concentration was significantly higher in the males than in the females in both socioeconomic groups.

**Alkaline phosphatase**

Adjusted mean ± SE values of serum AP in the LSES and USES groups were 387.1 ± 8.9 and 299.2 ± 10.3 IU/L, respectively (P < 0.01). Among males, a trend for higher AP was seen in the LSES group; this trend attained significance only in the 16–18-y-old category. There was a significant positive correlation of mean serum AP with iPTH (r = 0.330, P < 0.01).

**Immunoreactive parathyroid hormone**

Adjusted mean ± SE values of serum iPTH for LSES and USES were 42.8 ± 1.6 and 23.5 ± 1.9 pg/mL, respectively (P < 0.01). There was a significant negative correlation of iPTH with 25(OH)D (r = −0.202, P < 0.01) (Figure 1).

**25-Hydroxyvitamin D**

The unadjusted mean serum concentration of 25(OH)D for the entire group was 11.8 ± 7.2 ng/mL. Adjusted mean ± SE values of serum 25(OH)D for LSES and USES were 10.4 ± 0.4 and 13.7 ± 0.4 ng/mL, respectively (P < 0.01). Age, sex, and SES independently influenced the variations in 25(OH)D concentrations. Males had significantly higher mean serum concentrations than did females (P = 0.004). Two hundred seventy-one children (35.7%) had serum concentrations <9 ng/mL—ie, below the normal range given by the manufacturer. A value <9 ng/mL was found in children in the LSES group significantly more often (42.3%) than in those in the USES group (27%; P < 0.01) and among females more often (41.6%) than among males (27.4%; P = 0.01). According to the Lips classification (3), hypovitaminosis D was seen in 92.6% of the LSES group (severe: 11.2%; moderate: 39.5%; and mild: 42.1%) and in 84.9% of the USES group (severe: 4.9%; moderate: 25.5%; and mild: 57.6%).
hormonal variables in children who underwent BMD assessment did not differ significantly from those in children who did not. The mean BMD measurements (g/cm²) at forearm and calcaneum are reported in Table 2. The 3-factor interaction was significant for the forearm, but, at the calcaneum, only the main effect of age was significant ($P < 0.01$). There was no significant correlation between BMD measurements and the mean serum concentration of either iPTH or 25(OH)D.

### DISCUSSION

Metabolic bone disorders secondary to vitamin D deficiency continue to be prevalent in the Indian subcontinent, as documented by hospital-based studies. To the best of our knowledge, there are no recent, large, community-based studies that specifically evaluated the prevalence of hypovitaminosis D in children and adolescents in India. Hence, the current study was planned to

### TABLE 2
**Comparison of unadjusted means of bone mineral density (BMD) in the 2 socioeconomic groups**

<table>
<thead>
<tr>
<th>Variable and age category</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forearm BMD (g/cm²)$^1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>0.313 ± 0.044 [27]</td>
<td>0.387 ± 0.146 [24]</td>
</tr>
<tr>
<td>13–15 y</td>
<td>0.359 ± 0.067 [57]</td>
<td>0.397 ± 0.064 [56]</td>
</tr>
<tr>
<td>16–18 y</td>
<td>0.414 ± 0.059 [31]</td>
<td>0.408 ± 0.049 [30]</td>
</tr>
<tr>
<td>Calcaneum BMD (g/cm²)$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–12 y</td>
<td>0.424 ± 0.088 [27]</td>
<td>0.501 ± 0.073 [24]</td>
</tr>
<tr>
<td>13–15 y</td>
<td>0.464 ± 0.074 [57]</td>
<td>0.557 ± 0.095 [56]</td>
</tr>
<tr>
<td>16–18 y</td>
<td>0.505 ± 0.073 [31]</td>
<td>0.592 ± 0.089 [30]</td>
</tr>
</tbody>
</table>

$^1$ All values are $\bar{x} \pm SD$; $n$ in brackets. LSES, lower socioeconomic status; USES, upper socioeconomic status. The means reported in the table are adjusted for height and weight.

$^2$ Three-factor interaction was significant, $P < 0.01$ (ANOVA). The main effect of each factor was also significant, $P < 0.01$.

$^3$ Three-factor interaction was not significant. The main effect of age was significant, $P < 0.01$ (ANOVA).
investigate the extent of the problem in 2 socioeconomic groups by using clinical, biochemical, and hormonal variables and bone densitometry.

The earliest published description of adolescent rickets in India appeared in 1925 (13). In a study reported in 1997, the prevalence of clinical evidence of vitamin D deficiency in 5–15-y-old children was shown to be 0.19%; however, objective diagnostic criteria were not mentioned (14). In Indian immigrants in the United Kingdom during the 1960s and 1970s, the prevalence of clinical vitamin D deficiency in children and adolescents was shown to be 5–30% (15–18), whereas, in studies using biochemical and radiological variables, the prevalence was 12.5–66% (1, 19, 20). Later studies showed that the incidence of rickets had decreased in the immigrant population (21, 22), but so far no evidence of reduction in rickets in India has been reported. In children of Indian parentage who were born in and are residing in South Africa, the prevalence of knock knees and bow legs with gaps of ≥2.5 cm, reported in 1975, was 6.1–19.4% (23).

Adolescents are prone to vitamin D deficiency because of the greater mineral demands of their growing skeletons (16, 17, 20, 24, 25). Studies that provided biochemical documentation of vitamin D deficiency by estimating 25(OH)D in adolescents have renewed interest in this particular age group (26–29). The current study found clinical evidence of vitamin D deficiency in 10.8% of apparently healthy adolescents in New Delhi, India, and no significant difference between the upper and lower socioeconomic groups. In a similar age group, symptomatic rickets was observed in 68 per 100,000 child-years in Saudi Arabia (26) and in 9.4% of subjects in China (28).

A comparison of serum vitamin D data with other studies may not be entirely appropriate, given the fact that different studies were conducted in different seasons and using different assays. Nonetheless, according to the Lips classification (3), severe hypovitaminosis D (<5 ng/mL) was seen in 8.6% of our study population, in 23.5% of Finnish adolescents (25), and in 45.2% of Chinese adolescents in winter (28). In the latter study, severe hypovitaminosis D was present in only 6.7% of the subjects when they were evaluated in summer. In other studies from Finland, using cutoffs of 8–10 ng/mL, the prevalence of hypovitaminosis D was ≈13.5% (27, 29), which compares with 37% of children in the current study who had serum 25(OH)D concentrations <9 ng/mL (lower limit of manufacturer’s normal range).

The mean serum concentration of 25(OH)D in the current study is 11.8 ± 7.2 ng/mL. Other studies have also noted low serum 25(OH)D concentrations among adults of Indian origin in both India and the United Kingdom (8, 9, 30). The mean 25(OH)D concentration in adolescents in the current study is significantly lower than that reported in studies from Europe (6, 29, 31) and Brazil (32) and marginally higher than that reported from China (28).

Our study found that the LSES group adolescents had significantly lower mean 25(OH)D concentrations than did the USES group adolescents. The only other study that compared low and high socioeconomic status groups did not find any significance difference in mean vitamin D concentration between the 2 groups (32). However, the difference that we found is further supported by the observation that LSES group children also had higher iPTH, higher AP, and lower serum phosphorus concentrations than did USES group children. Because serum calcium concentration and sunlight exposure did not differ significantly between the 2 groups and because dietary calcium intake was significantly lower in the LSES group than in the USES group, nutrition may play an important role, as was reported earlier (33, 34). In addition, the lower serum phosphorus concentration could be correlated with the higher concentrations of iPTH in the LSES group.

There was a negative correlation between 25(OH)D and iPTH (r = −0.202, P < 0.001), which is in agreement with the findings of other studies (6, 35). Two hundred seventy-one children (35.7%) had vitamin D concentrations below the normal range given by the manufacturer. Only 28 (10.3%) of these 271 subjects had iPTH concentrations above normal. In another study, the serum iPTH concentration was elevated in 37.5% of adolescent girls with low vitamin D concentrations (29). Several studies in adults have investigated the threshold at which serum vitamin D induces an increase in iPTH concentrations, and values ranging from 20 to 38 ng/mL were reported (36–38). It is important to emphasize that the rise in iPTH seen in the current study was still within the normal range and that only when 25(OH)D concentrations fell below 5 ng/mL did iPTH values rise to or exceed the upper limit of normal. To the best of our knowledge, the current study is the first to investigate the relation between vitamin D and iPTH in apparently healthy children from different socioeconomic strata. The limited number of children with serum 25(OH)D concentrations >25 ng/mL prevents our arriving at a relation between vitamin D and PTH at higher values of vitamin D. The reasons for the lack of elevation of iPTH above the upper limit of normal, despite hypovitaminosis D, could be that, first, sufficient 25(OH)D is being converted to 1,25(OH)2D for maintenance of calcium homeostasis and, second, that prolonged exposure to low vitamin D concentrations and the poor nutritional status in these children may have lowered the threshold for iPTH release.

Children in the LSES group had significantly lower BMD values at the forearm than did those in the USES group. This difference could be due to poor overall nutrition, as evidenced by low BMIs, low dietary calcium intakes (39), low serum 25(OH)D concentrations, and secondary hyperparathyroidism (40). No significant correlation was seen between BMD and the 25(OH)D concentration and serum calcium concentrations in the current study, which is in agreement with other studies (29, 39, 41, 42). However, some studies in different age groups showed a significant correlation (10, 43).

We conclude that there is a high prevalence of clinical and biochemical hypovitaminosis D in apparently healthy schoolchildren in India. The observation that children from low socioeconomic backgrounds have significantly higher prevalences of vitamin D deficiency and low BMD suggests that nutrition plays an important role in the causation of hypovitaminosis D.

RKM and NT contributed equally to the study and can both be considered as first authors. RKM, NT, DHKR, RA, BS, and MG were responsible for the collection of data; RKM, NT, DHKR, and RS were responsible for the analysis of data; RKM, NT, and DHKR were responsible for writing the manuscript; RA, BS, and MG provided significant advice regarding conduct of study; and RCS and SS were responsible for the laboratory assays. None of the authors had any person or financial conflicts of interest.

REFERENCES

Dear Sir:

Cordain et al (1) are to be congratulated on a succinct and topical overview, recently published in the Journal, of the perils of a Westernized diet with respect to the risk of chronic degenerative diseases in humans. Indeed, there is widespread support for reinstitutioning several aspects of the so-called Paleolithic diet, especially higher fiber and lower content of refined, adulterated, or synthetic constituents. However, the authors do not seem to have made reference in their article to the effect of diet on a defining feature of modern humans—namely, the brain—whether that effect is related to brain development, advanced brain function, or risk of degenerative brain disease. Several micronutrients are discussed, but iodine seems to have been overlooked, despite the fact that it is 1 of the 2 nutrients (the other is iron) from which humans globally are considered to suffer the most common deficiency (2).

The most serious consequence of iodine deficiency is impaired neurologic development, a problem that most developed countries have avoided only by legislating the use of iodized table salt; commonly preferred food choices that exclude seafood simply do not provide enough iodine. Indeed, publications from Australia, the United States, and Europe during the past decade show that mild-to-moderate iodine deficiency is reemerging as people in developed countries consume less table salt, dairy products, meat, fish, and seafood (3–5).

Cordain et al gave an overview of the nutrient density in various major food groups in Table 4 of their article, but they made no mention of the relatively poor bioavailability of micronutrients, especially minerals, from some of these food groups. The concentration in plants of zinc or iron, without consideration of the phytate content of those plants, exaggerates the true value of plants as sources of these nutrients. Although iodine is not shown in Table 4, goitrogens in many cultivated plants greatly reduce the availability of iodine from plant-based diets. Hence, the overall ranking in Table 4 of seafood second to vegetables as a source of micronutrients underestimates the true value of seafood in protecting against the risk of the major nutrient deficiencies that affect humans. Among the food groups shown in the table, seafood actually has the highest available content of several minerals needed for brain development, including iodine, iron, zinc, copper, and selenium (6).

The focusing of some attention on the human health implications of declining iodine and seafood intakes is warranted because those declining intakes are directly linked to brain development. The modern-day vulnerability of the developing human brain to inadequate intakes of iodine, iron, docosahexaenoic acid, and several other brain-selective nutrients shows that, if seafood had not been a significant component of the Paleolithic diet, the modern human brain probably would not have evolved in the first place (6, 7).

The author had no conflicts of interest.

Origins and evolution of the Western diet: implications of iodine and seafood intakes for the human brain

Dear Sir:

Cordain et al (1) are to be congratulated on a succinct and topical overview, recently published in the Journal, of the perils of a Westernized diet with respect to the risk of chronic degenerative diseases in humans. Indeed, there is widespread support for reinstitutioning several aspects of the so-called Paleolithic diet, especially higher fiber and lower content of refined, adulterated, or synthetic constituents. However, the authors do not seem to have made reference in their article to the effect of diet on a defining feature of modern humans—namely, the brain—whether that effect is related to brain development, advanced brain function, or risk of degenerative brain disease. Several micronutrients are discussed, but iodine seems to have been overlooked, despite the fact that it is 1 of the 2 nutrients (the other is iron) from which humans globally are considered to suffer the most common deficiency (2).

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The author had no conflicts of interest.

References


Reply to SC Cunnane

Dear Sir:

We thank Dr Cunnane for his congratulatory words about our recent article in the Journal (1). Clearly, in a review article of a somewhat limited nature, it would not be possible to comprehensively document all diseases and maladies of civilization that have been linked to the typical Western diet. Instead, we referred interested readers to Cordain’s earlier, more exhaustive review of the numerous health problems and illnesses associated with the consumption of cereal grains, a food introduced in the relatively more recent Neolithic period (2). We agree with the notion that normal human brain development and function require a diet adequate in iron, iodine, and long-chain polyunsaturated fatty acids (PUFAs) of both the n–3 and n–6 families. Moreover, there is little doubt that animal foods, which were the dietary staples for historically studied hunter-gatherers (3, 4), are rich sources of these nutrients (1, 5, 6).

With respect to iodine and the brain’s development and function, it should be pointed out that a wide variety of staple foods domesticated during the Neolithic period and later (ie, millet, maize, soy,
cassava, sweet potatoes, lima beans, turnips, cabbage, cauliflower, rapeseed, mustard, onion, garlic, bamboo shoots, and palm tree fruit) contain a variety of goitrogens (7, 8) that may elicit symptoms of iodine deficiency despite adequate iodine intakes (7, 9). Hence, plant food–dominated diets containing goitrogens, which were adopted by humanity after the agricultural revolution, may play a significant role in impairing thyroid function and thereby adversely influencing human brain development (10). In contrast, iodine deficiency is rare among traditional societies that consume animal-based diets (11).

For reasons we outlined previously (6, 12), we respectfully disagree with Cunnane’s suggestion that seafood would have represented the primary source of long-chain PUFAs (22:6n−3 and 20:4n−6) and other micronutrients necessary to the relaxation of the selective pressure previously constraining encephalization in hominins. Exploitation of the marine environment is first documented in the archaeological record during the Middle Paleolithic period (≈110 000 y BP), and stable isotope data show that inland aquatic foods were not utilized by hominins living in Europe until the mid-Upper Paleolithic period (≈28 000–20 000 y BP) (13). Hence, aquatic animal foods, whether ocean- or inland-derived, would have played a minor role in providing nutrients that were crucial to the rapid hominin brain expansion that occurred during the Early Paleolithic period (≈2.5–2.0 million y BP). Rather, terrestrial animal foods (including muscle, brain, marrow, thyroid gland, and other organs) would have represented the primary source of long-chain PUFAs, iron, zinc, iodine, and other nutrients that were necessary for encephalization and normal brain development (6, 12, 14).

None of the authors had a conflict of interest.

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REFERENCES
Breastfeeding and risk of inflammatory bowel disease: results of a pediatric, population-based, case-control study

Dear Sir:

We read with great interest the meta-analysis recently published in the Journal by Klement et al (1). A protective effect of breastfeeding on the risk of inflammatory bowel disease (IBD) was shown; the risk of Crohn disease (CD) decreased by 33%, and the risk of ulcerative colitis (UC) decreased by 23%. Klement et al emphasized that out of a total of 17 published studies, only 4 studies of CD and 4 studies of UC were of high methodologic quality (ie, the diagnosis of IBD was confirmed by a physician, information on breastfeeding was confirmed by the patients’ mothers or another older close relative, and the response rate was ≥80% for both the case and control subjects). Klement et al suggested that additional research be conducted to strengthen the validity of their observations.

We recently conducted a pediatric, population-based, case-control study to examine the environmental risk factors associated with the development of IBD (2). The study area was the northern part of France, which has 5 790 526 inhabitants, 1 312 141 of whom are aged <17 y. Data on 222 incident cases of CD and 60 incident cases of UC occurring before 17 January 1988 and December 1997 were extracted from our IBD registry (EPIMAD), which has been in existence in northern France since 1988. The methods of the EPIMAD registry were published in detail previously (3). Briefly, interviewer practitioners collected the data for all patients who received a diagnosis of IBD between 1 January 1988 and 31 December 1997 from all of the gastroenterologists and pediatric gastroenterologists in the area. A final diagnosis of CD or UC was made by 2 expert gastroenterologists according to previously published criteria (3). The control subjects were randomly selected from telephone number lists (random digit dialing) and matched 1:1 to each case by age (±2 y), sex, and area of residence.

A questionnaire was devised that comprised 140 questions, including questions about the infants’ and children’s diets. Trained investigators personally interviewed both the study subjects and their mothers at home. The pediatric health booklet—which is mandatory in France for infants, children, and adolescents and contains information on pregnancy, delivery, childhood growth, vaccinations, and childhood infections—was also required to validate questions about the children’s health. The response rate was 100% for case and control subjects. In a multivariate model adjusted for the mother’s education level, breastfeeding (partial or exclusive) was a risk factor for CD (odds ratio: 2.1; 95% CI: 1.3, 3.4; P = 0.003). Children with CD were breastfed exclusively or nonexclusively for an average of 2 wk more than were the control subjects, but the difference was not significant (10 wk compared with 8 wk and 8 wk compared with 6 wk, respectively; P = 0.08). Familial history of IBD, Bacille Calmette-Guerin vaccination, and history of eczema were other significant risk factors for CD, whereas regular drinking of tap water was a protective factor. In the univariate analysis, history of breastfeeding had no effect on the risk of UC (OR: 1.07; 95% CI: 0.52, 2.22; P = 0.85). In a multivariate model, familial history of IBD, disease during pregnancy, and bedroom sharing were risk factors for UC, whereas appendectomy was a protective factor.

On the basis of Klement et al’s criteria, the quality of our study methods was high. The diagnosis of IBD was always confirmed by 2 experienced gastroenterologists; the information on breastfeeding was always confirmed by the patients’ mothers, and the response rate to the questions related to breastfeeding was very high (≥80% for both case and control subjects). Moreover, the availability of the pediatric health booklet enabled us to confirm the information given by the family. The association of breastfeeding with an increased risk of CD was an unexpected finding of our study. There is strong evidence that breastfeeding is associated with a decreased incidence and severity of a wide range of infectious diseases (4). Delayed infections occurring after weaning may lead to an inappropriate immune response and persistence of intestinal inflammation. The high level of pollution in our highly industrialized region may also have played a role, because industrial chemicals and environmental contaminants have been found in breast milk (5). Ultrafine and fine particles are potent adjuvants in antigen-mediated immune responses and cause inflammation in susceptible persons; a relation between microparticles and CD was recently considered (6, 7). Additional studies in other populations are needed to confirm or refute our results. From a public health perspective, the short and long-term benefits of breastfeeding overrule by far the increased risk of CD that we observed in our study (4).

None of the authors had a conflict of interest related to the topic of this letter.

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REFERENCES
6. Powell JJ, Harvey RS, Ashwood P, Wolsencroft R, Gershwin ME,
Dear Sir:

The recently published study by Baron et al (1) certainly sheds new light on the association between breastfeeding and inflammatory bowel disease (IBD). This study, which was conducted with the use of excellent methods, fulfills the criteria for the best-quality category in the meta-analysis we recently published (2). Incorporating the results of this study into the pooled estimate calculation would diminish the significant results of protective breastfeeding on Crohn disease (CD) [Mantel-Haenszel odds ratio (OR_MH): 0.62; 95% CI: 0.27, 1.43] and would not affect significantly the summary estimate of the protective association between breastfeeding and ulcerative colitis (OR_MH: 0.62; 95% CI: 0.43, 0.91). However, more important than its effect on the pooled estimate was the high heterogeneity that is implied from its inclusion in the CD studies (P < 0.001, chi-square heterogeneity test). In our study, the effects found by all of the studies had high heterogeneity, but this may have been partly attributed to the differences in studies quality, with heterogeneity in the highest-quality studies that showed no statistical significance.

Inclusion of the study by Baron et al as one of the highest-quality studies implies high heterogeneity in this group as well. Why some studies show a significant protective effect of breastfeeding while others show no effect or even suggest that breastfeeding is a risk factor for CD is an enigma that may have several possible explanations. One explanation relates to the different genetic characteristics of the studies’ populations. The highest-quality studies reviewed by us were all conducted in Sweden or North America; the study conducted by Baron et al was performed in northern France. It was previously shown that the genetic background of the population has a significant influence on the effect of some risk factors. A good example is the lack of effect of smoking on the development of CD in Jewish populations as opposed to other populations (3). The second explanation relates to the fact that CD may be regarded as a cluster of diseases that have the same manifestations but that are caused by different etiologies (4). Thus, the heterogeneous effect of breastfeeding on CD may relate to its different interactions with the yet unknown various etiologies of this disease. The third explanation suggested by Janchou et al may also account for the discrepancy between this study’s findings and those of previous studies; the components of breast milk in northern France may differ significantly from the components of breast milk in less industrialized areas.

Baron et al are the first investigators to implicate breastfeeding as a risk factor for CD. This, however, is not the only new finding of this study. The observed association between some vaccinations and CD in this study is also novel. This observation and the high rate of CD in this area suggest that the population of this study is unique either in its environmental exposure or in its genetic background. Thus, we agree with Janchou et al that breastfeeding should not be discouraged, especially on the basis of one study. On the contrary, on the basis of our meta-analysis (which showed a protective effect of breastfeeding on IBD), the biologic plausibility of this association, and the experimental evidence gathered in animal experiments (5), we still believe that breastfeeding should be encouraged. Baron et al’s study does, however, emphasize the need for further high-quality studies of other population types to fully understand the association between breastfeeding and IBD.

Neither author had a financial or personal conflict of interest related to any of the topics discussed in this letter.

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REFERENCES

Effects of theobromine should be considered in future studies

Dear Sir:

The report by Grassi et al (1) showing that “short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons” discusses flavanols as a potential component responsible for the phenomenon reported (acting through an increase in nitric oxide production and availability). The corresponding editorial in the same issue offers other possible mechanisms involving “oxidant-mediated cell signaling” and “the rennin-angiotensin system” (2). It should be noted that another factor, theobromine, may be partially responsible for the decrease in blood pressure reported.


Theobromine is “present in chocolate in uniquely high quantities” and it is cited that dark chocolate contains 237–519 mg theobromine per 50 g (3). Thus, the subjects studied by Grassi et al may have received 474–1038 mg theobromine in each chocolate bar consumed. Theobromine was once used to treat arteriosclerosis, some peripheral vascular diseases, angina pectoris, and hypertension (3). In one report, 5 of 7 subjects could discriminate the subjective effects of theobromine at doses as low as 100–560 mg (4). It was noted that “published work investigating the effects of theobromine is restricted to relatively few articles” (3). A MEDLINE (National Library of Medicine, Bethesda, MD) search using the keywords theobromine and insulin did not provide any results investigating the effects of theobromine on insulin sensitivity. Considering the high concentrations of theobromine in cocoa and its history of use in the treatment of hypertension, theobromine should be investigated or controlled for in future studies that seek to elucidate the components and mechanisms responsible for the recently reported effects of chocolate consumption in healthy persons.

The author had no personal or financial conflicts of interest.

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REFERENCES


Reply to CJ Kelly

Dear Sir:

In our recent paper (1), we observed a significant decrease in blood pressure and a significant amelioration of insulin sensitivity and insulin resistance indexes in healthy volunteers who consumed dark chocolate bars (100 g) over a period of 15 d. Throughout the study, total daily energy intake remained unchanged through appropriate diet changes. Therefore, no weight gain occurred in our study population. Because white chocolate bars did not modify blood pressure or insulin sensitivity, we suggested that the flavanols contained in the dark chocolate bars were responsible for the observed changes.

The above hypothesis is supported by in vitro data indicating that flavanols contained in cocoa can increase nitric oxide production (2) and decrease superoxide anion generation (2) and thereby have all the biological potential to positively influence blood pressure regulation and insulin-mediated glucose uptake (3). In vivo data also indicate that intake of dark chocolate reduces blood pressure and improves endothelium-dependent vasorelaxation in healthy humans (4) and elderly subjects with isolated systolic hypertension (5). In keeping with this, obesity, diabetes, and hypertension are relatively rare among Kuna Indians, a population of Amerinds living in the San Blas Island chain off the coast of Panama, who are known to ingest large amounts of cocoa (6).

Although convincing evidence supports our flavanol hypothesis, we think the additional explanation suggested by Kelly is of extreme interest. Kelly correctly emphasized that cocoa also contains methylxanthines, particularly theobromine and, to a lesser extent, caffeine (7). As is known, methylxanthines inhibit phosphodiesterase and thereby increase the intracellular concentration of cyclic AMP (cAMP) in pancreatic β cells, antagonizing P1 purinoreceptors (8). In contrast, methylxanthines are negatively coupled to adenylyl cyclase in hepatocytes and induce a decrement in cAMP concentration in these cells (8). Because both insulin secretion from pancreatic β cells and liver glucose output are directly dependent on the intracellular concentration of cAMP, we agree with Kelly’s hypothesis that it is reasonable to speculate that methylxanthines may positively influence glucose metabolism in humans. Kelly indicated that no data from the literature are available on his own intriguing hypothesis. However, we found that Cerasi and Luft (9) observed an amelioration of glucose-mediated insulin secretion after aminophylline administration. Similarly, Arias et al (10) reported a mild but significant decrease in serum glucose concentrations by stimulation of insulin secretion in patients with type 2 diabetes mellitus who received an intravenous infusion of the same drug. Furthermore, caffeine ingestion was recently reported to significantly reduce glucose disposal without variations in insulin concentrations during an euglycemic hyperinsulinemic clamp (11). Therefore, the hypothesis that theobromine contained in dark but not white chocolate bars may have influenced blood pressure and insulin sensitivity in our study population is not only intriguing but also supported by some data from the literature. Therefore, we agree with the extremely interesting suggestion from Kelly, ie, that future studies should focus not only on flavanols but also on theobromine to fully understand the potential benefits of cocoa.

It is intriguing to speculate that cocoa could help to counteract the pandemic explosion of cardiovascular disease risk factors and their sequelae. Nevertheless, the high energy density of cocoa must be taken into account. Because most vegetables are relatively low in calories, whereas each of the dark chocolate bars (100 g) that we used in our study (1) contained ≈500 kcal, we must ensure that each person consuming small amounts of chocolate per day simultaneously reduces his or her daily energy intake (particularly of calories derived from unhealthy foods). If not, the potential benefit of flavanols contained in cocoa would be contrasted, or even reversed, by increases in body weight.

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Safety of vitamin C

Dear Sir:

Hathcock et al (1) cite the 2000 Food and Nutrition Board statement on the lack of evidence that vitamin C increases oxalate formation (2) as confirmation that vitamin C intakes of ≤2000 mg/d do not increase the risk of kidney stone formation. However, research published since that panel concluded their literature review suggests otherwise. Chai et al (3) reported in 2004 that 2000 mg ascorbic acid (AA)/d increased both oxalate synthesis and absorption. Additional analysis of the responses of 29 calcium stone formers (SFs) and 19 non-stone formers (NSFs) found that consumption of 1000 mg AA twice daily resulted in 2 distinctly different oxaluric responses. Forty percent of the subjects, including both SFs and NSFs, experienced increases of ≥10% in 24-h urinary oxalate (4). The other 60% of the subjects had essentially no oxaluric response. Examination of individual responses from 3 published studies in which supplements of 1000–2000 mg AA/d were given (5–7) showed that 7 of the 19 total subjects (38%) had similar increases in urinary oxalate of >10%.

Genetic susceptibility in the study participants probably accounted for most of the discordance in response to AA that was previously reported; small sample sizes and the lack of a dietary control probably contributed as well.

Three other studies with less rigorous designs also came to similar conclusions about the risk of kidney stone formation related to AA supplements. Baxmann et al (8) reported an increase in urinary oxalate of 61% in SFs after 1000 mg AA/d, 41% in SFs after 2000 mg AA/d, and 56% in NSFs after 2000 mg AA/d. Chalmers et al (9) studied 17 SFs and 11 NSFs who consumed 2000 mg AA/d. They found that, compared with the NSFs, the SFs excreted 12% more oxalate with no AA supplementation and 22% more with AA supplementation. Traxer et al (10) conducted a similar study with 12 SFs and 12 NSFs who ingested 1000 mg AA with each morning and evening meal. Urinary oxalate excretion increased 33% (10 mg oxalate/d) in the SFs and 20% (6 mg oxalate/d) in the NSFs. As did we, Traxer et al (10) identified responders in both the SFs and the NSFs. Baxmann et al (8) identified increases in the Tiselius Risk Index and in urinary oxalate in 47 SFs and 20 NSFs who were randomly assigned to either 1000 mg AA/d (500 mg ingested twice/d) or 2000 mg AA/d (1000 mg ingested twice/d) for 3 d. Before day 1 and on day 3, a 24-h urine sample was obtained. The increase in the Tiselius Risk Index with 1000 mg AA/d (0.51) was similar to that with 2000 mg AA/d (0.56), which suggests that lower doses of AA may also be lithogenic in genetically susceptible individuals. Because hyperoxaluric responses have been shown at doses of both 1000 and 2000 mg AA/d, current evidence suggests that 500 mg AA/d is the maximum dose that can be considered safe, at least until additional testing at lower doses is done.

The author had no conflicts of interest.

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REFERENCES


Reply to L Massey

Dear Sir:

Massey cites 5 studies (one in press) on the relation between urinary oxalate and vitamin C published since the Food and Nutrition Board (FNB) revised the dietary reference intakes for vitamin C in 2000 (1). These studies reported that supplemental vitamin C (1000–2000 mg/d) was associated with increases in urinary oxalate ranging from 10% to 61%. Several studies identified 2 subpopulations in oxaluric response to vitamin C among stone formers and non-stone formers in the small groups examined. About 40% of the subjects showed an increase in urinary oxalate of ≥10%. The FNB reviewed earlier studies, which also found increases in urinary oxalate associated with supplemental vitamin C.

Although the increase in oxalate observed in some studies may be attributable to the ex vivo conversion of urinary vitamin C to its metabolic product oxalic acid (2), supplemental vitamin C may indeed increase the urinary excretion of oxalic acid, especially in genetically susceptible individuals. Several large-scale, long-term prospective studies have investigated the clinical significance of these findings. In the Health Professionals Follow-Up Study (HPFS), Curhan et al (3) followed 45 251 men with no history of nephrolithiasis. In the Nurses’ Health Study, a prospective study of 85 557 women followed for 14 y, a statistically significant difference in the age-adjusted RR for stone formation between subjects with vitamin C intakes of ≥1500 mg/d compared with men whose intake was <250 mg vitamin C/d. In the Nurses’ Health Study, a prospective study of 85 557 women followed for 14 y, Curhan et al (4) found no statistically significant difference in the age-adjusted RR for stone formation between subjects with vitamin C intakes of ≥1500 and subjects with vitamin C intakes of <250 mg/d. However, in a 14-y follow-up of 45 619 men from the HPFS, in which the referent intake of vitamin C was lowered, Taylor et al (5) found that the multivariate RR for stone formation was 1.41 (P for trend = 0.01) in men whose total intake (dietary and supplemental) of vitamin C was ≥1000 mg/d compared with men whose total intake was <90 mg/d (the recommended dietary allowance). The difference in age-adjusted RR between these quintiles was not statistically significant. The multivariate RR for stone formation between men with a total intake <90 mg vitamin C/d and men with a total intake <250 mg vitamin C/d was 1.22. The increased risk associated with vitamin C emerged only after dietary potassium, which was inversely associated with stone formation, was included in the multivariate analysis. The multivariate RR for stone formation in men who consumed ≥1000 mg supplemental vitamin C/d was 1.16 (P for trend = 0.01).

Routine restriction of vitamin C in the general population to prevent renal calculi is unwarranted based on the results of the few prospective studies that have been conducted (3–5). According to the recent follow-up to the HPFS (5), which is the only prospective study that has shown a positive association between total vitamin C intake and nephrolithiasis, prophylaxis in men would require restriction of vitamin C to intakes that are less than the recommended dietary allowance and may adversely affect health. As indicated above, the HPFS follow-up found a modestly increased risk at an intake of 90–249 mg vitamin C/d, which would easily be provided by following the recommendation of the US Department of Agriculture to consume 9 daily servings of fruit and vegetables to reduce the risk of cardiovascular disease, certain cancers, type 2 diabetes, and obesity. The observed risk associated with vitamin C in the HPFS follow-up largely occurred at dietary intakes, and high supplemental doses only slightly increased the risk. The Tolerable Upper Intake Level (2000 mg/d) is the highest intake of vitamin C that poses no risk of serious adverse effects for almost all individuals in the general population. The FNB concluded that restriction of vitamin C is warranted in certain subpopulations, such as those who have a glucose-6-phosphate dehydrogenase deficiency or renal disease. The limited data support the restriction of supplemental vitamin C to prevent stone formation only in men who have a propensity for oxalate nephrolithiasis.

JNH is employed by a dietary supplement trade association. None of the other responding authors had any conflicts of interest.

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Herbal and Traditional Medicine, edited by Lester Packer, Choon Nam Ong, and Barry Halliwell, 2004, 941 pages, hardcover, $199.95. Marcel Dekker, New York, NY.

This book is composed of 3 major sections. The first section, “Introduction,” comprises 5 chapters that mainly concern traditional medicine policy. The second section, “Herbal and Traditional Medicine,” comprises 33 chapters, each of which covers a specific traditional medicine. The third section, “Problems and Side Effects,” comprises 4 chapters. Ninety contributors, each an expert in his or her field and actively engaged in research on the topics covered, has successfully participated in the preparation of this book.

Each chapter presents a discussion of the biochemical, pharmacologic, and clinical (when pertinent) issues associated with plants, macroscopic fungi, or plant-derived constituents and includes both potential and real toxicologic problems for each issue. Of special interest in Section I are details on the challenges associated with the use of traditional medicines on a global scale, including criteria developed by the World Health Organization. Section II covers both widely used botanicals (eg, tea, echinacea, curcuma, ginkgo, ginger, ginseng, Crataegus, ephedra, and St John’s Wort), for which much clinical data are available, as well as lesser known (in the West) botanicals and fungi (eg, Cordyceps, Ganoderma, Lentinus, Chrysanthemum, Andrographis, rosemary, licorice, and Chromolaena).

This book is perhaps the best source of herbal and traditional medicines that I have seen, both in broad and specific terms. The book contains few errors and has an extensive reference list. I recommended this book for any library whose patrons are interested in medicinal plants, specifically the libraries of medical institutions, pharmacies, nursing schools, naturopathic and chiropractic colleges, and the pharmaceutical industry. However, the book is probably too technical for most lay persons.

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Where was this book when I was a nutrition graduate student? Replete with principles and practical examples, Public Health Nutrition provides good foundational knowledge for those who enter the public health nutrition field. After starting with an overview, the book proceeds to cover under- and overnutrition and then maternal and child health before finishing with chronic diseases. Each chapter is similarly organized to provide key messages, evidence-based content, case studies from peer-reviewed published research, future perspectives, suggestions for additional reading, and recommended websites.

The chapters on nutritional epidemiology and assessment alone are worth their weight in gold. In chapter 2, “Nutrition Epidemiology”, the student is walked through a sample-size calculation for testing a treatment of anemia. In the same chapter, a detailed table outlines the possible sources of error for 3 types of nutritional measurements (body weight, self-reported dietary intake, and blood analyte) for each major component of a simple regression equation (slope, constant, random error, and biased error). Chapter 3, “Assessment of Nutritional Status in Individuals and Populations”, offers recommendations on when to use the major measures of nutritional assessment, which include single or replicate daily measures, food-frequency measures, biomarkers, and anthropometric measures. Chapter 8, “Food Choice”, adds luster to the gold. This chapter touches on the human aspect of public health nutrition by spanning methodology, culture, media and advertising, access, sociodemographics (including religious, moral, and ethical influences), individual preferences, genetics and taste sensitivity, economics, beliefs and attitudes, satiety, and stress and mood.

Throughout the book, the authors strengthen certain information by focusing on principles—and in a sense add platinum to the already present gold to create a longer-lasting product. For example, in chapter 14, “Fear of Fatness and Fad Slimming Diets”, the authors present a checklist of sound slimming diets rather than a list of currently popular or fad diets. In chapter 21, “Cancer and Diet”, the authors present a figure that shows the dietary influences on carcinogenesis by using the principle-based column headings of cancer stage, dietary related factors, metabolic response, carcinogenesis-enhancing effect, and preventive response.

Some chapters may become outdated more quickly. Chapter 20, “Diabetes Mellitus”, is one such chapter. Any discussion of diabetes would be incomplete without presenting the criteria for the diagnosis of diabetes, which the authors provide; however, these criteria have been evolving rapidly as new evidence emerges.

Discussion questions at the end of each chapter would have been helpful additions to the book. However, this suggestion is directed more at the organizers and editors of the Nutrition Society Textbook Series, which Public Health Nutrition is a part of, than at the authors. A series of web pages that includes links to
teaching aids, project ideas, content updates, and sample test questions is promised to come. The framework exists already (www.nutritiontexts.com), but, as of May 2005, the links had not been activated.

This book grabbed my attention and held it. The content was trustworthy. As John Mathers wrote in the series forward, “Read, learn, and enjoy.”