Iron absorption by heterozygous carriers of the HFE C282Y mutation associated with hemochromatosis

Janet R Hunt and Huawei Zeng

ABSTRACT
Background: Research conducted before genotyping was possible suggested that subjects heterozygous for the genetic mutation associated with hemochromatosis absorbed nonheme iron more efficiently than did control subjects when tested with a fortified meal. Heme-iron absorption in these subjects has not been reported.

Objective: We compared the absorption of heme and nonheme iron from minimally or highly fortified test meals between HFE C282Y-heterozygous and wild-type control subjects.

Design: After prospective genotyping of 256 healthy volunteers, 11 C282Y-heterozygous and 12 wild-type control subjects were recruited, and their iron absorption was compared by using a hamburger test meal with or without added iron and ascorbic acid. After retrospective genotyping of 103 participants in previous iron-absorption studies, 5 C282Y-heterozygous subjects were compared with 72 wild-type control subjects.

Results: HFE C282Y-heterozygous subjects did not differ significantly from wild-type control subjects in their absorption of either heme or nonheme iron from minimally or highly fortified test meals. No differences were detected in blood indexes of iron status (including serum ferritin, transferrin saturation, and non-transferrin-bound iron) or in blood lipids or transaminases, but heterozygotes had significantly greater, although normal, fasting glucose concentrations than did wild-type control subjects. Compound heterozygotes (those who had both HFE C282Y and H63D mutations) absorbed more nonheme (but not heme) iron from meals with high (but not low) iron bioavailability.

Conclusions: HFE C282Y-heterozygous subjects did not absorb dietary iron more efficiently, even when foods were highly fortified with iron from ferrous sulfate and ascorbic acid, than did control subjects. Iron fortification of foods should not pose an additional health risk to HFE C282Y heterozygotes.


KEY WORDS Nutrient-gene interactions, nonheme-iron absorption, heme-iron absorption, diet, iron bioavailability, iron fortification, hemochromatosis, serum ferritin, non-transferrin-bound iron, glucose

INTRODUCTION
Hemochromatosis is a disorder of excessive iron accumulation characterized by increased heme- and nonheme-iron absorption (1). In 1996, specific genetic mutations in the HFE gene were identified (2) in hemochromatosis patients of Northern European origin. Although it is not known how the HFE protein influences iron absorption, it may influence the affinity of plasma transferrin for its receptor, as part of an HFE, β₂-microglobulin, and transferrin receptor complex in the basolateral membrane of developing enterocytes (3). More than 80% of hemochromatosis patients are homozygous for a C282Y mutation in this gene, and a smaller proportion are compound heterozygous for both the C282Y mutation and an H63D mutation (2).

Identification of the C282Y mutation raised questions about the possible health effects of this common mutation for heterozygous carriers, who include 9.54% of non-Hispanic whites in the United States (4). Heterozygosity has been associated with shorter life expectancy in a Danish study (5), but not in other studies (6–8), and it has even been associated with longevity in some Italian women (9, 10). Increased risk of type 2 diabetes in C282Y heterozygotes found in one study (16) was unconfirmed in another (17). Genetic studies in healthy populations have identified many symptom-free C282Y homozygotes, which shows that the clinical phenotypic penetrance of the mutation is quite low (18–20). Heterozygosity has been inconsistently associated with slightly higher transferrin saturation and serum ferritin concentrations (18, 20–26). Non-transferrin-bound iron (NTBI) was reported in one small study to be greater in heterozygous subjects than in wild-type control subjects, but there was no difference in serum ferritin or transferrin saturation between these 2 groups (27).

Before C282Y genotyping was available, iron absorption by heterozygous carriers of hemochromatosis was tested by identifying as heterozygotes the children of patients with hemochromatosis or two siblings who shared a single HLA haplotype (1). No difference was found between heterozygous and control subjects in the absorption of nonheme iron from a hamburger meal (1). However, when the meal was fortified with 20 mg Fe (as...
ferrous sulfate) and 100 mg ascorbic acid, the heterozygous sub-
jects absorbed 2.5 times as much nonheme iron as the control
subjects absorbed. Because of the high frequency of the HFE
C282Y mutation, such an increase in absorption may have public
health implications for food fortification policies. The objective
of the current study was to compare iron absorption in heterozy-
gotes identified by the C282Y mutation with that in wild-type
control subjects by feeding test meals similar to those used by
Lynch et al (1). Secondary objectives were to examine iron ab-
sorption related to other HFE C282Y and H63D polymorphisms
and to test for differences in clinical chemistry, including an
increase in NTBI in subjects who were heterozygous for the
C282Y mutation.

SUBJECTS AND METHODS

General protocol

The absorption of dietary iron in healthy subjects who are
heterozygous for the HFE C282Y mutation and in wild-type
control subjects was compared by using 2 sets of data. First, 256
healthy volunteers were prospectively genotyped (tested for both
the HFE C282Y and H63D mutations), which led to the enroll-
ment of 11 HFE C282Y-heterozygous and 12 wild-type control
subjects for tests of heme- and nonheme-iron absorption from
test meals prepared with and without extra fortification with iron
(as ferrous sulfate) and ascorbic acid. Second, 103 participants in
previous iron-absorption studies were retrospectively geno-
typed, which allowed the comparison of 5 subjects who were
heterozygous for the C282Y mutation, 2 compound-
heterozygous subjects, and subjects with other HFE genotypes
with wild-type control subjects who had neither mutation.

Subjects

Healthy men and nonpregnant women were recruited through
public advertising and selected after an interview and blood
analysis helped ascertain that they had no apparent underlying
disease and had blood hemoglobin concentrations of \(\geq 120 \text{ g/L}\).
Although race was not designated as a selection criterion, all
volunteers were white and non-Hispanic. The participants gave
written informed consent. The University of North Dakota’s
Radioactive Drug Research Committee and Institutional Review
Board and the US Department of Agriculture’s Human Studies
Review and Radiological Safety committees reviewed and
approved this human study.

Dietary treatments

Prospective genotyping study

Iron absorption was tested from weighed standard or fortified
test meals similar to those used by Lynch et al (1). The standard
meal consisted of ground beef (113 g), a refined-wheat bun (53
g), French-fried potatoes (68 g), tomato ketchup (20 g), and a
vanilla milkshake (150 g). The refined-wheat bun was commer-
cially enriched with iron according to common US practice for
refined-wheat products (20 mg iron/460 g flour). The standard
meal contained 906 kcal and 5.1 mg Fe, of which 1.2 mg was in
the heme form. The fortified meal was similar, but it omitted the
French-fried potatoes and milkshake and added 200 g orange
juice supplemented with 100 mg ascorbic acid and 20 mg Fe as
ferrous sulfate. The orange juice was prepared and mixed with
supplements on the same morning that the meals were served.
The fortified meal contained 590 kcal and 25.1 mg iron, of which
1.2 mg was in the heme form.

Retrospective genotyping study

Subjects with mutations of interest had previously participated
in iron-absorption studies with 3 different sets of dietary treat-
ments. The subjects’ iron absorption was tested I from a 2-d
menu low in dietary iron bioavailability (no meat, small amounts
of poultry and fish, and \(\approx 20 \text{ mg ascorbic acid at each meal, with
considerable black tea and phytic acid–containing foods}) before
and after 10 wk of consumption of the same diet (28, 29); 2 from
a higher-bioavailability test meal containing beef, refined-wheat
roll, French-fried potatoes, and milkshake before and after 12 wk
of daily supplementation with a placebo (30); and 3 from 2 test
meals of beef, refined-wheat roll, French-fried potatoes, and
milkshake, with and without cheese, which were followed by a
complete gastrointestinal lavage procedure 8 h after each meal
(31). No C282Y mutations were identified in subjects formerly
treated with a high-iron-bioavailability diet (28, 29) or with iron
supplements (30). Subjects fasted for 8 h before and 4 h after
single test meals and ate only the food provided when tested with
complete menus for 2 d.

Iron-absorption measurements

Heme- and nonheme-iron absorption was measured by extrin-
sically labeling the foods (in the final stages of preparation) with
18.5 or 37 kBq \(^{55}\text{Fe}\)-labeled hemoglobin and 37 kBq \(^{59}\text{FeCl}_3\).
Radiolabeled hemoglobin was obtained by intravenously inject-
ing 74 MBq \(^{55}\text{Fe}\) into an iron-deficient, pathogen-free rabbit,
bleeding the animal 2 wk later, isolating the red cells by centrif-
ugation, and removing the stroma by lysing and further centrif-
ugation (32). For the low-bioavailability diet involving 2 d of
meals, the isotopes were added to the diet in proportion to the
heme- and nonheme-iron contents of the meals, which yielded
constant specific activities (ratios of \(^{55}\text{Fe}\) to dietary heme iron
and of \(^{59}\text{Fe}\) to nonheme iron) for all 6 meals. The tracers were
added to the foods that were the best sources of that form of iron
in each meal. Meat, poultry, and fish dishes were cooked in
advance, cooled, radiolabeled, and then minimally reheated in
the microwave just before service. All labeled meals were con-
sumed at the research center.

Nonheme-iron absorption was determined by whole-body
scintillation counting that detected only the gamma-emitting
\(^{59}\text{Fe}\) radioisotope. The custom-made whole-body counter used
32 crystal NaI(Tl) detectors, each 10 cm
\(\times\) 10 cm \(\times\) 41 cm, arranged in 2 planes above and below the participant, who lay
supine. The initial total-body activity was determined before any
unabsorbed isotope was excreted but \(\geq 1 h\) after the subjects
finished the single meals (or 2 meals of the 2-d menu, with
calculated adjustments for the fraction of the total activity ad-
ministered). The percentage of nonheme-iron absorption was
measured as the portion of initial whole-body activity that re-
mained after 2 wk. Heme-iron absorption was ascertained by
measuring the blood retention of \(^{55}\text{Fe}\) (together with \(^{59}\text{Fe}\); 33)
after 2 wk and by estimating total blood volume on the bases of
body height, sex, and weight (34, 35) and of the assumption that
80% of the absorbed heme iron was incorporated into blood (36).
All isotope determinations included corrections for physical de-
cay and background activity measured 1–2 d before isotope ad-
iministration.
Genotyping

Genomic DNA was extracted from buccal smears (for retrospective genotyping) or blood (prospective genotyping study) by using a DNA isolation kit (Qiagen, Valencia, CA). HFE C282Y and H63D mutations were ascertained by using a two-round polymerase chain reaction (PCR; semi-nested primers) approach (37, 38) and enzymatic digestion of the products similar to that in previous reports (2, 39). Briefly, genomic DNA was used as a first-round PCR DNA template, and then 0.5 µL of the first-round PCR product was used as the second-round PCR DNA template. As described by Feder et al (2), for the C282Y mutation, the first-round PCR primers were 5'-TGGCAAGGG-TAACAGATCC-3' and 5'-CCAATGAAACAGATGACGACAA-3', and the second-round PCR primers were 5'-TGGCAAGGTTAACAGATCC-3' and 5'-CTCAGGCAA-CTCCTCTCAACC-3'. For the H63D mutation, the first-round PCR primers were 5'-ACATGGTTAAAGCGCTTGC-3' and 5'-CCTTGGCTGTTGTGATTTC-3', and the second-round PCR primers were 5'-ACATGGTTAAGGCGCTTGC-3' and 5'-GCCACATGCTGTTGAAATT-3' (2). DNA spanning C282Y (560 base pairs) and H63D (295 base pairs) mutations was amplified in the first round of PCR. The C282Y and H63D mutations were detected by amplification of 389 and 208 base pairs, respectively, in the second-round PCR.

Negative controls were included in each PCR. The PCR conditions were as follows: 94°C for 2 min; then 25 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min; and, finally, 72°C for 2 min. Aliquots of second-round PCR products were digested with Rsa I and Mbo I endonuclease restriction enzymes to identify the C282Y and H63D mutations, respectively, as described previously (39). The enzymatic digestion product was subsequently separated by electrophoresis in 3% agarose 2000 (Gibco-BRL, Carlsbad, CA) gels. Each subject’s genotype was determined independently by 2 staff members.

Clinical chemistry

Hemoglobin was measured by using a Celldyne 3500 System (Abbott Laboratories, Abbott Park, IL). Serum iron was determined colorimetrically by using a Cobas Fara Chemistry Analyzer (Hoffman-LaRoche Inc, Nutley, NJ) with a commercial source and because several months had elapsed between measurements were later judged to be from an unreliable commercial source and because several months had elapsed between.

Statistical analysis

Iron-absorption and serum ferritin data were logarithmically transformed, and geometric means are reported. Power curves were used to plot relations between iron absorption and serum ferritin concentrations (these relations are linear when the data are logarithmically transformed).

For statistical tests of the effect of genotype, the known inverse relation between iron absorption and serum ferritin was used to normalize iron-absorption values to an arbitrary serum ferritin concentration of 40 µg/L, as follows:

\[ \ln(\text{normalized percentage nonheme-iron absorption}) = \ln(\text{percentage nonheme iron absorbed}) + \ln(\text{ferritin in } \mu\text{g/L}) - \ln(40 \ \mu\text{g/L}) \] (1)

\[ \ln(\text{normalized percentage heme-iron absorption}) = \ln(\text{percentage heme iron absorbed}) + 0.3 \times \ln(\text{ferritin in } \mu\text{g/L}) - 0.3 \times \ln(40 \ \mu\text{g/L}) \] (2)

This normalization uses a slope of −1.0 for the regression of ln(nonheme-iron absorption) on ln(serum ferritin) (44) and a slope of −0.3 for a similar regression with heme-iron absorption (1, 30, 45). [The slope from Lynch et al (1) was estimated from calculations by Cook (46).] All statistical tests were conducted by using PC/SAS software (version 8.02; SAS Institute Inc, Cary, NC; 47). For the prospective genotype experiment, a possible interaction between genotype and meal fortification was evaluated by using repeated-measures analysis of variance (ANOVA; 47). For retrospective data, t tests were used to compare selected individual absorption measurements with those of wild-type control subjects in the same experiment (47). The chi-square test was used to compare national genotyping results with those of this project (47). \( P < 0.05 \) (with two-tailed testing) was considered significant.

RESULTS

Prospective genotyping data set

The genotyping results from this convenience sampling in Grand Forks, ND, did not differ significantly (by chi-square analysis) from a representative sampling of the non-Hispanic white US population (4; Table 1). Of 256 subjects prospectively screened, 20 (8%) were C282Y heterozygous, without the H63D mutation. Of these 20 subjects, 12 agreed to participate in the iron-absorption study. Reasons for nonparticipation included anemia (blood hemoglobin < 120 g/L; \( n = 2 \)); pregnancy (\( n = 1 \)); lack of willingness to follow experimental protocol (\( n = 2 \)); and loss to follow-up contact (\( n = 3 \)). One subject was subsequently eliminated because of elevated hematocrit and referral for possible polycythemia, which left 11 subjects (8 females and 3 males) who were C282Y heterozygous. Twelve control subjects (wild-type, with neither the HFE C282Y nor H63D mutation; 9 females and 3 males) of roughly similar serum ferritin concentrations, age, sex, and body mass index (BMI; in kg/m²) were selected. These control subjects were not paired with heterozygous subjects for analysis because the initial serum ferritin measurements were later judged to be from an unreliable commercial source and because several months had elapsed between...
the initial serum ferritin measurements and the iron-absorption experiment. When it was concluded that the initial serum ferritin analyses were unreliable, this measurement was repeated with a different commercial kit, but only in those subjects who had participated in the iron-absorption measurements.

Iron absorption was not significantly affected by HFE C282Y heterozygosity. As expected, absorption of both forms of iron was inversely related to serum ferritin concentrations (Figure 1). After normalization of iron absorption to a serum ferritin concentration of 40 μg/L, there was no significant difference (by analysis of variance) in the absorption of either heme or nonheme iron by subjects with the 2 different genotypes, whether absorption was tested with the standard meal or with the meal fortified with additional iron (as ferrous sulfate) and ascorbic acid (Figure 2). Normalized absorption of nonheme iron in the heterozygous and wild-type subjects was, respectively, 10.9% and 7.5% from the standard meal and 7.7% and 7.5% from the fortified test meal. Normalized absorption of heme iron in the 2 groups was, respectively, 36% and 33% from the standard meal and 29% and 32% from the fortified test meal.

Several clinical chemistry measurements were compared between the C282Y-heterozygous (n = 11) and wild-type control (n = 12) subjects (Table 2). The control subjects had been selected for serum ferritin measurements, age, sex, and BMI that were similar to those of the C282Y-heterozygous subjects at the time of initial genotyping (several months before). At the time of the iron-absorption measurements, the 2 groups did not differ significantly, according to t tests, in any of the iron status measurements (Table 2). These included NTBI concentration, which was 0.65 ± 0.12 and 0.68 ± 0.08 μmol/L for the heterozygous and control subjects, respectively. No differences were found in serum lipid measurements or transaminases (AST or GGT). Of the clinical chemistry measurements conducted, only fasting serum glucose concentrations differed between the 2 groups (4.6 ± 0.1 and 4.1 ± 0.1 mmol/L for heterozygous and control subjects, respectively; P < 0.02 by t test; Table 2).

### Retrospective genotyping data set

Only 5 subjects who were heterozygous for the C282Y mutation were identified from the retrospective genotyping (Table 1). These subjects did not absorb either heme or nonheme iron more efficiently than did the wild-type control subjects who had neither mutation. This conclusion was based on t tests (C282Y-heterozygous compared with wild-type control subjects) when the absorption data within a single experiment were normalized to a serum ferritin concentration of 40 μg/L. Iron absorption had been measured twice in each subject, and the data were highly reproducible between measurements (eg, the absorption results at week 10, shown in Figure 3, were similar to those at week 0).

Two subjects were compound heterozygous for both the C282Y and H63D mutations, and their nonheme-iron absorption depended on the bioavailability of the test diet. The nonheme-iron absorption in one subject was substantially inhibited by the low-iron-bioavailability diet, which is similar to the results in the wild-type subjects (Figure 3). When the absorption data were normalized to a serum ferritin concentration of 40 μg/L (not shown), nonheme-iron absorption from the low-bioavailability diet in this compound-heterozygous subject did not differ significantly from that in the wild-type control subjects. The other

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**TABLE 1**

Comparison of genotyping results in the Grand Forks, ND, area with results representative of the non-Hispanic white US population (NHANES III)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NHANES III (n = 2016)</th>
<th>Prospective (n = 256)</th>
<th>Retrospective (n = 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>n (%)</td>
<td>n</td>
</tr>
<tr>
<td>C282Y-homozygous</td>
<td>0.30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C282Y-heterozygous</td>
<td>9.54</td>
<td>20 (8)</td>
<td>5</td>
</tr>
<tr>
<td>H63D-homozygous</td>
<td>2.15</td>
<td>6 (2)</td>
<td>1</td>
</tr>
<tr>
<td>H63D-heterozygous</td>
<td>23.55</td>
<td>62 (24)</td>
<td>23</td>
</tr>
<tr>
<td>C282Y/H63D-compound-heterozygous</td>
<td>2.35</td>
<td>3 (1)</td>
<td>2</td>
</tr>
<tr>
<td>Wild-type (control)</td>
<td>62</td>
<td>165 (64)</td>
<td>72</td>
</tr>
</tbody>
</table>

1 NHANES III, the third National Health and Nutrition Examination Survey. Neither the prospective nor the retrospective samples from Grand Forks differed significantly from the genotypic distribution in the nationally representative NHANES III (chi-square analysis).

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**FIGURE 1** Heme- and nonheme-iron absorption from a hamburger meal fortified with additional iron as ferrous sulfate and ascorbic acid in subjects who were heterozygous for the C282Y mutation (△; n = 11) and wild-type control subjects (○; n = 12). The 2 groups of subjects absorbed nonheme and heme iron similarly from the hamburger meal. For nonheme iron, regression coefficients were R² = 0.85 (P < 0.01) in the C282Y-heterozygous subjects and R² = 0.43 (P < 0.05) in the wild-type control subjects. For heme iron, regression coefficients were R² = 0.32 (P < 0.05) in the C282Y-heterozygous subjects and R² = 0.12 (NS) in the wild-type control subjects.
compound-heterozygous subject was tested with high-bioavailability meals and appeared to absorb nonheme iron from both a cheeseburger meal (Figure 4) and a similar hamburger meal (not shown) more efficiently than did the wild-type control subjects. When absorption measurements were normalized to a serum ferritin concentration of 40 µg/L, the compound-heterozygous subject absorbed significantly more of the nonheme iron from these high-bioavailability meals than did the 6 wild-type control subjects: 24% compared with 7.5 ± 4.7%, respectively, for the cheeseburger meal (P < 0.01; t test) and 22% compared with 7.9 ± 4.8%, respectively, for the hamburger meal (P < 0.02; t test). Heme-iron absorption was not affected by the compound-heterozygous condition (Figures 3 and 4). There was no indication of any difference in iron absorption between the wild-type control subjects and the single subject who was homozygous for the H63D mutation (Figure 4) or the subjects who were heterozygous for that mutation (Figures 3 and 4).

As indicated in Subjects and Methods, heme-iron absorption in this study was determined by measuring blood 55Fe 2 wk after isotope administration and assuming 80% erythrocyte incorporation of the absorbed isotope. Similar overall results were obtained if heme-iron absorption was determined without an assumption of 80% erythrocyte incorporation but with the assumption that the erythrocyte incorporation of 55Fe from administered heme iron was identical to that of the nonheme 59Fe, which was ascertained from blood and whole-body measurements. However, this latter calculation method resulted in unusually high estimations of heme-iron absorption (78% and 58% at 0 and 10 wk, respectively; calculated by dividing the fractional blood retention by the fractional whole-body retention of 59Fe). The use of this low erythrocyte incorporation of the nonheme radiotracer resulted in unusually high estimations of heme-iron absorption (78% and 58% at 0 and 10 wk, respectively). If erythrocyte incorporation of 80% was used in the absorption calculation, the estimated heme-iron absorption in this female subject

![Graph showing absorption of heme and nonheme iron](image)

**FIGURE 2.** Absorption of heme and nonheme iron as tested with the standard meal and the fortified meal in C282Y-heterozygous (△; n = 11) or wild-type control (○; n = 12) subjects. When absorption was normalized to a serum ferritin of 40 µg/L (see text), there was no significant difference (P > 0.05) in nonheme- and heme-iron absorption between the 2 groups of subjects, whether tested with the standard or the fortified meal.

### TABLE 2

Comparison of age, BMI, and clinical chemistry results between prospectively genotyped C282Y-heterozygous and wild-type control subjects

<table>
<thead>
<tr>
<th></th>
<th>C282Y-heterozygous subjects (n = 11)</th>
<th>Wild-type control subjects (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>49 ± 4 (26–76)</td>
<td>46 ± 10 (25–57)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 1.3 (20.6–34.9)</td>
<td>26.5 ± 1.0 (21.6–32.2)</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>37 [25, 55] (3–254)</td>
<td>52 [43, 63] (14–199)</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>29 ± 4 (4–43)</td>
<td>30 ± 2 (19–45)</td>
</tr>
<tr>
<td>Serum iron (µmol/L)</td>
<td>15 ± 2 (2–23)</td>
<td>16 ± 1 (10–26)</td>
</tr>
<tr>
<td>Non-transferrin-bound iron (µmol/L)</td>
<td>0.65 ± 0.12 (0.14–1.4)</td>
<td>0.68 ± 0.08 (0.29–1.1)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.66 ± 0.42 (4.34–7.96)</td>
<td>5.66 ± 0.46 (3.05–7.94)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.16 ± 0.14 (0.75–2.30)</td>
<td>1.29 ± 0.11 (0.75–1.94)</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.11 ± 0.13 (0.58–1.93)</td>
<td>1.09 ± 0.18 (0.37–2.73)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.6 ± 0.1 (3.9–5.3)</td>
<td>4.1 ± 0.1 (3.7–4.9)</td>
</tr>
<tr>
<td>AST (µkat/L)</td>
<td>0.35 ± 0.04 (0.22–0.60)</td>
<td>0.29 ± 0.02 (0.20–0.43)</td>
</tr>
<tr>
<td>GGT (µkat/L)</td>
<td>0.27 ± 0.02 (0.18–0.37)</td>
<td>0.30 ± 0.05 (0.13–0.82)</td>
</tr>
</tbody>
</table>

1 AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase.
2 ± SE; range in parentheses (all such values). Results are untransformed.
3 Geometric x̄ ± 1 SE, ± 1 SE in brackets; range in parentheses (all such values). Results are log transformed.
4 Only fasting glucose differed significantly between genotype groups, P < 0.02 (t test).
was not unusually high (55% and 42% at 0 and 10 wk, respectively; the latter value is shown in Figure 3). Although this subject’s erythrocyte 59Fe incorporation was unusually but reproducibly low for a woman with low serum ferritin, her blood retention of 55Fe was not unusually high. Accordingly, the heme-iron absorption results presented are based on an assumed 80% erythrocyte incorporation. In the prospective genotyping data set, the erythrocyte incorporation of 59Fe (percentage of ingested isotope) did not significantly differ between the 11 heterozygotes and the 12 control subjects (nonheme iron: $R^2 = 0.61$, $P < 0.01$; heme iron: $R^2 = 0.48$, $P < 0.01$). Similar measurements at week 0 gave similar results.

**DISCUSSION**

The results indicate that subjects who are heterozygous for the HFE C282Y mutation do not absorb dietary iron any more efficiently than do wild-type control subjects. These results confirm the finding of Lynch et al (1) that there is no difference between these groups in nonheme-iron absorption from a standard hamburger meal, but they are in contrast to the same group’s finding that heterozygous subjects absorb more nonheme iron from a meal fortified with additional iron (from ferrous sulfate) and ascorbic acid than do wild-type control subjects. Prospective power analyses had predicted that 10–15 subjects from each genotype would provide 90% power to detect an interaction (genotype × level of fortification) of the magnitude observed by Lynch et al (1)—i.e., similar nonheme-iron absorption from the minimally fortified meal, but more than twice as much nonheme-iron absorption from the fortified meal. However, the present data gave no indication of this interaction (Figure 2). This difference in results is likely related to the method of genotyping. Because the heterozygous subjects of Lynch et al were identified by their relation to hemochromatosis patients and by HLA assessment, they were more likely to have additional unidentified genetic, ethnic, or lifestyle characteristics that resulted in a phenotypic expression of excessive iron accumulation, or 2 to be mistakenly typed as heterozygous when some may have been compound heterozygous or C282Y homozygous. Moirand et al
(48) found that, of subjects whose heterozygous status was inferred by HLA assessment, 3% were C282Y homozygous, 20% were compound heterozygous, and only 70% were C282Y heterozygous. Such misclassification when more sensitive genotyping was not possible may have accounted for the positive results of Lynch et al (1).

This is the first report of heme-iron absorption by heterozygous subjects. None of the tested C282Y and H63D polymorphisms influenced heme-iron absorption in this study, including the 2 measurements in each of 2 subjects who were compound heterozygous.

The consistently elevated nonheme-iron absorption in the compound-heterozygous subject whose iron absorption was tested with a high-bioavailability meal is consistent with the increased risk of clinical hemochromatosis experienced by compound-heterozygous subjects (2). However, the consistent lack of elevation in nonheme-iron absorption in another compound-heterozygous subject tested with a low-iron-bioavailability diet suggests that, even in those genetically prone to absorb iron more efficiently, the iron must be available to the intestinal mucosa in a reduced, soluble form. Although limited conclusions can be drawn from these 2 subjects, our findings are consistent with an interaction between genotype and diet, which suggests that compound-heterozygous subjects absorb nonheme iron more efficiently than do wild-type subjects, but only if the dietary iron is at least moderately bioavailable.

The present negative results for heme- and nonheme-iron absorption by C282Y-heterozygous subjects are consistent with the minimal differences in iron status indexes such as serum ferritin and transferrin saturation observed in this study (Table 2) and other, much larger studies (20–24). The iron status indexes of the C282Y-heterozygous subjects we identified by recruitment from the general population covered a normal range and even included 2 women with iron deficiency anemia, although that condition eliminated them from the absorption testing. The present data do not confirm the finding in a report by de Valk et al (27) of more NTBI in 22 heterozygous subjects (0.51 μmol/L; range: −0.75 to 2.75 μmol/L) than in 17 control subjects (−0.30 μmol/L; range: −1.50 to 0.60 μmol/L). Both our study and that of Valk et al included a small number of subjects. The range of measurements was narrower in the present study (Table 2) and, unlike the measurements reported by de Valk et al (who did not report sample variation), did not include negative values. On the basis of the variation observed in the present study, we had 99% statistical power to detect the difference of 0.8 μmol/L in NTBI that was reported by de Valk et al (27), but we did not detect a difference (Table 2). The difference observed by de Valk et al may have been affected by the method of identification of heterozygous subjects. If subjects were identified from among the relatives of patients with hemochromatosis, rather than from the general population, they may have had additional, predisposing genetic factors that influenced the penetrance of the HFE C282Y mutation and the resulting control of iron metabolism.

The finding that the C282Y-heterozygous subjects had significantly higher fasting serum glucose concentrations than did control subjects (Table 2) was unexpected. Although heterozygous subjects have been reported to have a greater risk of type 2 diabetes than do control subjects (16), this was refuted by a recent meta-analysis of HFE mutation frequencies in subjects with type 2 diabetes and in control subjects (17). Beutler et al (18) reported no greater incidence of high fasting blood sugar (>6.7 mmol/L) among either C282Y-homozygous, C282Y-heterozygous, or wild-type control subjects, which is consistent with the results of the present study (Table 2). The fasting glucose concentrations that we found should be interpreted with caution, by recognizing that this one minor difference in blood glucose arose from multiple t test comparisons (Table 2).

In conclusion, these results indicate no significant difference in heme- or nonheme-iron absorption by heterozygous carriers of the HFE C282Y genetic mutation associated with hemochromatosis, whether tested by using common meals or meals highly fortified with iron as ferrous sulfate and ascorbic acid. The results are consistent with greater absorption of nonheme than heme iron from meals with high iron bioavailability in subjects who are compound heterozygous for the C282Y and H63D mutations. Although a substantial proportion of the US population is heterozygous for the C282Y mutation, the present results do not suggest any greater risk to these subjects due to consumption of foods that are highly fortified with this highly bioavailable form of iron or with ascorbic acid.

We gratefully acknowledge the contributions of members of our human studies research team, particularly Carol Anni Zito, who conducted the blood radioiron analyses and, together with Melissa Phelps and Cheryl Stjern, performed the genotyping assays. In addition, Emily J. Nielsen managed the volunteer recruitment and scheduling; Bonita Hoverson supervised the planning and service of the controlled diets; Sandy K Gallagher supervised the clinical laboratory analyses; Glenn I. Lykken designed and consulted in the use of the whole body counter; and LuAnn K Johnson performed the statistical analyses. We are especially grateful to the volunteers for their dedication and service.

JRH conceived and designed the study, supervised the iron-absorption measurements, and wrote the manuscript. HZ supervised genotyping measurements and contributed to the manuscript. Both were directly funded by the US Department of Agriculture Agricultural Research Service and had no conflict of interest.

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Letters to the Editor

Glycemic index and body weight

Dear Sir:

Some readers of the Journal have expressed surprise at the decision to use the glycemic index as a tool to control body weight. This appears to be related to the observed difference in LDL cholesterol. For example, Bouché et al. (2) found that an intake of a low-glycemic-index (GI) diet and during consumption of a macronutrient-matched high-GI diet. The authors reported a 2.5-fold difference in total body fat mass change (1.0 compared with 0.4 kg; $P = 0.20$) and significant difference in LDL cholesterol favoring the low-GI diet ($P < 0.05$). Although the difference in fat mass change was not significant, one could argue that the 0.6 kg difference might be directly related to the observed difference in LDL cholesterol. For example, Bouché et al. (2) found that an 700 g decrease in total fat mass even in the absence of changes in body weight was associated with an improved lipid profile and changes in the enzymes and genes controlling fat metabolism.

With 22–23 subjects per group, the study by Sloth et al. may have been underpowered to detect differences in body fat. With the use of the SD data reported, sample size calculations suggest that 90 subjects in each group would be necessary to lower the probability of a type 2 error to acceptable levels (ie, to achieve acceptable power). Alternatively, had the study continued for longer than 10 wk, the difference might continue to widen, as suggested by the trends in body weight loss in Figure 1 of the article. It may be possible for Sloth and her colleagues to reanalyze their dual-energy X-ray absorptiometry measurements to estimate changes in trunk fat mass specifically. Similarly, changes in waist circumference may be more indicative of visceral fat changes than is the waist-to-hip ratio.

A major limitation of the study design is the failure to demonstrate differences in glycemic and insulin day profiles during the low-GI compared with the high-GI diet. Without that evidence, a negative result may be subject to doubt that a real difference in overall GI was actually achieved. Although Sloth et al. offer in vitro assays of the rate of carbohydrate digestion [rapidly available glucose (RAG) and slowly available glucose (SAG)] as evidence of the low- or high-GI nature of their diets, in vitro assays are at best approximate and at worst clearly wrong. We have reported several instances of large discrepancies in the results of in vivo versus in vitro estimates of GI values (3). Readers should bear in mind that the GI is fundamentally defined by its in vivo nature. Studies that cannot show actual differences in day profiles or other measure of glycemia (eg, hyperglycemic events in persons with diabetes) can be challenged. Moreover, because in vitro RAG estimates were the same for the high- and low-GI diet, it is conceivable that Sloth et al. did not achieve a marked difference in glycemic or insulin profile. RAG has been found to be a stronger predictor of postprandial glycaemia than SAG (4–6). According to Englyst et al. (4), the SAG fraction does not contribute to the glycemic response over and above that which can already be accounted for by the RAG fraction. Although Sloth et al. found a difference in RAG after adjustment for dry matter, this is not normal practice and can be questioned.

Last, the hypothesis that low-GI diets may aid weight control because they are more satiating and reduce overall energy intake cannot be realistically assessed in a study in which the subjects are compelled to eat a specified amount of the starchy foods provided and to minimize the amount of sugar and other highly palatable foods consumed. Indeed, more than one-half the subjects complained that the quantity of starchy foods was far too high and was associated with nausea and discomfort in the first half of the study. Hence, Sloth et al.’s study could be criticized on the grounds that it is not a truly ad libitum and realistic comparison of high-GI versus low-GI diets. In this context, it may be informative to undertake an intention-to-treat analysis in which all subjects recruited to the study are included and the last reported data point is carried forward.

Sloth et al.’s article is undoubtedly a useful addition to the literature on GI and obesity. However, the limitations outlined above suggest that the conclusions should be tempered. In particular, the title of the paper should not have been as definitive.

JB-M serves on the board of directors of Glycemic Index Limited, a not-for-profit company that administers the Glycemic Index Symbol food labeling program in Australia (Internet: www.glycemicindex.com.au). She is also the director of a not-for-profit glycemic index testing service at the University of Sydney (Internet: www.glycemicindex.com) and the co-author of a series of books under the general title The New Glucose Revolution (published by Marlowe and Co, NL America) that explains the theory and practice of the glycemic index to the lay public.

Jennie Brand-Miller

School of Molecular and Microbial Biosciences
University of Sydney
Sydney
NSW 2006
Australia
E-mail: j.brandmiller@mmb.usyd.edu.au

REFERENCES

Reply to J Brand-Miller

Dear Sir:

We were surprised to read that Brand-Miller finds our title and conclusion too decisive, because we merely stated the findings of our study (both the pros and the cons of low-glycemic-index diets) and specifically pointed out the time frame of 10 wk. Furthermore, we stated that more studies are needed to substantiate our findings.

We deal with the questions raised by Brand-Miller as follows:

We found a significant 10% decrease in LDL cholesterol in the low-glycemic-index group compared with a 2% increase in the high-glycemic-index group. This difference cannot be explained by changes in fat mass, as suggested by Brand-Miller, because we observed no correlation between changes in fat mass and changes in LDL cholesterol (total fat mass: \( r = 0.17, P = 0.27 \); trunk fat: \( r = 0.09, P = 0.56 \)). Nor does inclusion of changes in total or trunk fat mass in the covariance analysis change the significance of the diet effect on LDL cholesterol.

Regarding the power in the study, these calculations were made before the study on the basis of body weight. A postexperimental calculation of power as suggested by Brand-Miller is not appropriate according to an article published in American Statistician (1). As stated in our discussion, we agree with Brand-Miller that a longer study period or inclusion of more subjects might have resulted in a significant difference in body weight. Nevertheless, we maintain that our finding of no significant difference in body weight loss after 10 wk with 22–23 subjects per diet group does at least question the clinical relevance of the glycemic index in body weight control. Furthermore, existing evidence that low-glycemic-index diets are an effective tool for achieving weight reduction is, at present, contradictory (2).

In response to Brand-Miller’s request for data more indicative of visceral fat changes, we reanalyzed our dual-energy X-ray absorptiometry measurements on trunk fat. We found no significant difference between the high-glycemic-index and low-glycemic-index group in trunk fat mass decreases from week 0 to week 10 (low-glycemic-index: \(-0.45 \pm 0.18 \) kg; high-glycemic-index: \(-0.18 \pm 0.20 \) kg; \( P = 0.65 \)). Nor did we find any significant difference between groups in waist circumference changes (low-glycemic-index: \(-1.9 \pm 0.7 \) cm; high-glycemic-index: \(-1.1 \pm 0.7 \) cm; \( P = 0.47 \)).

Brand-Miller also advocates the use of in vivo measurements of glycemic index, but if the glycemic index concept is to make any sense as a tool for consumers, in vivo analysis cannot be a prerequisite for composing a low-glycemic-index diet. Moreover, this requirement is not consistent with Brand-Miller’s own practice. For example, in vivo analysis was not an inclusion criteria in her previously published meta-analysis (3), in which not even in vitro measurements of glycemic index were a requirement. A hydrolysis index of the test foods was the basis for the glycemic index calculation in our study. This method correlates well with in vivo glycemic index determination, especially for starch-rich products such as those used in our study (4, 5). Another laboratory also analyzed the carbohydrate quality of our test foods by using the Englyst method, and the results from this analysis support earlier findings with use of the hydrolysis index method. We also calculated the mean glycemic index value of the 2 groups of test foods with the use of the latest international tables of glycemic index and glycemic load values and found a mean difference of 34.5 units. However, we do agree that it is difficult to predict the glycemic index of a mixed diet from the glycemic index values of the individual food items from table values. Last, we argue that the significant difference in LDL cholesterol between the 2 groups is a good marker for a true difference between the 2 groups of test foods, because the study was designed so that everything other than the glycemic index was similar in the 2 groups.

As reported in our paper, some subjects found the amounts of test food too high (not “far too high” as stated by Brand-Miller). This was not a complaint that continued throughout the study period, but a statement made mostly during the first week of the study period, and for most subjects, it was only addressed when specifically requested. It is not unusual for overweight subjects to complain about quantities of food when they are served low-fat, high-carbohydrate diets that have a high dietary fiber content because these diets are probably more satiating than the subjects’ habitual diets (6).

The dietary instructions were necessarily strict because we wanted to control the glycemic index and macronutrient composition of the subjects’ diets to effectively compare the effects on energy intake of a low-fat, high-carbohydrate diet with either a low or a high glycemic index. However, energy intake was not restricted; we merely supplied the subjects with carbohydrate-rich test foods comprising \( \approx49% \) of their estimated total energy intake. Dietary record data from weeks 5 and 10 clearly show that the test foods provided were only part of the subjects’ diets, as intended.

Finally, Brand-Miller suggests performing an intention-to-treat analysis on body weight changes. As could be expected, this analysis only reduces the difference between the 2 groups and increases the \( P \) value (low-glycemic-index, \(-1.55 \pm 0.44 \); high-glycemic-index, \(-1.19 \pm 0.29 \); \( P = 0.57 \)).

The original study was supported by Danone Vitapole, France. Rice was donated by Masterfoods as, Denmark, and Euryza GmbH, Germany, and rye bread by Cerealia R&D, Schulstad Brød A/S, Denmark. None of the authors had any conflicts of interest.

Birgitte Sloth
Inger Krog-Mikkelsen
Anne Flint
Inge Tetens
Arne Astrup
Anne Raben

Department of Human Nutrition
Centre for Advanced Food Studies
The Royal Veterinary and Agricultural University
30 Rolighedsvej
DK-1958 Frederiksberg C
Denmark
E-mail: bsl@kvl.dk

Inger Björck
Helena Elmståhl

Department of Applied Nutrition & Food Chemistry
Lund University
Sweden

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More support for dietary patterns involving high-fiber, high-complex carbohydrates

Dear Sir:

The experimental design of Gerhard et al (1) was refreshingly respectful of normal patterns of eating by examining the effects of varying macronutrient composition on serum lipids under ad libitum eating conditions and by varying the content of dietary fiber proportionally to the amount of carbohydrate in each diet. In keeping with what one would expect from a dietary pattern similar to the high-fiber, low-fat diets consumed for millennia by Mediterranean and Asiatic peoples, the results of adopting a high-carbohydrate (65% of energy) diet yielded more desirable glucose and triacylglycerol concentrations. These findings appeared to be in contrast with previous studies in which the effects of adopting a high-carbohydrate diet were examined only under isocaloric eating conditions and were found to yield less desirable serum glucose and triacylglycerol concentrations.

The authors might not have been as surprised by their results had they cited a couple of pertinent studies. One of these studies reported the clinical experience of 652 diabetic inpatients consuming a complex carbohydrate (70–75% of energy), low-fat diet ad libitum continuously for 3 wk. Barnard et al (2) reported a 33% decrease in serum triacylglycerol concentrations with this diet. A second, less obviously pertinent, study (3) was conducted, ironically, by the authors of one of the isocaloric macronutrient comparisons tested in diabetic patients. Garg et al (4) contrasted a high-carbohydrate (55% of energy) diet with a high-monounsaturated-fat diet (40% of energy from carbohydrate) under isocaloric conditions and purported to show that diabetic patients who adopted a high-carbohydrate diet experienced an undesirable 24% increase in serum triacylglycerol concentrations. A critique of this study (5) pointed out that the observed rise in serum triacylglycerol concentrations with their high-carbohydrate diet could have been an artifact of the isocaloric design of the study and a consequence of the authors failing to increase the dietary fiber intake commensurate with the increase in carbohydrate intake. In a study designed to test this critique, Chandalia et al (3) reported that the adoption by diabetics of a diet providing 50 g fiber/d (and 55% of energy from carbohydrate) for 6 wk was associated with a desirable 10.2% decrease in plasma triacylglycerol concentrations.

Despite these supportive studies validating the results of Gerhard et al, readers might still wonder about the long-term clinical applicability of their 6-wk findings to community-living diabetics. Fortuitously, Esposito et al (6) recently reported that Europeans diagnosed with the metabolic syndrome and randomly assigned to a high-carbohydrate (50–60% of energy), Mediterranean-style diet were able to adhere to it for 2 y, with salubrious consequences for markers of vascular function, a 12% decrease in serum triacylglycerol concentrations, and a 5.4% decrease in body weight. The consumption of complex carbohydrates with high contents of intact fiber and water, as is characteristic of classic Mediterranean and Asian diets, appears to yield salubrious benefits with respect to serum triacylglycerol concentrations and long-term weight control that appear to be sustainable long-term by adults at risk of diabetes.

The author consults occasionally for the Pritikin Longevity Center, a residential lifestyle change rehabilitation center that features a high-carbohydrate, low-fat diet.

William J McCarthy

University of California, Los Angeles
Division of Cancer Prevention and Control Research
Los Angeles, CA 90095-6900
E-mail: wmccarth@ucla.edu

REFERENCES

Reply to WJ McCarthy

Dear Sir:

We thank McCarthy for his comments regarding our study “Effects of a low-fat diet compared with those of a high–monounsaturated fat diet on body weight, plasma lipids and lipoproteins, and glycemic control in type 2 diabetes” (1). We agree that the results from the studies of Barnard et al (2, 3) are consistent with our findings. In those studies, the amount of dietary fat was limited to 10% of total energy, and the amount of carbohydrate was very high—75% of energy. In our study, dietary fat was restricted to 20% of total energy, and carbohydrate provided 65% of energy. This contrasted with the high–monounsaturated fat group of our study, who consumed 40% of energy as fat. There are at least 2 important effects of a high dietary fat consumption: one direct and one indirect. Unlike carbohydrate, except for fructose, dietary fat does not induce satiety; thus, the hormonal cues to stop eating are not operative (4). In our study, the diabetic subjects on the low-fat diet consumed 212 kcal less energy than they did on the high–monounsaturated fat diet. They then lost 1.53 kg of weight from the low-fat diet because of this energy deficit. Second, if less dietary fat is consumed, carbohydrate-containing foods rich in fiber and water can be increased. High-carbohydrate diets do induce satiety (4). The high-fiber content of the high-carbohydrate diet promotes weight loss and better lipid and...
diabetic control (5). All of these factors, we believe, led to more weight loss from the low-fat diet (20% of energy as fat and 65% as carbohydrate) than from the high–monounsaturated fat diet in the diabetic patients in our study. Contrary to the recommendations of the American Diabetes Association (6), we suggest that the amount of fat in the diabetic diet should be low and not flexible even if the source of fat is monounsaturated. Weight loss, the major goal in the treatment of type 2 diabetes, would be much more likely to occur if the diet is low in fat and high in complex carbohydrate and fiber, as in our study. Noteworthy was that the ad libitum low-fat, high-carbohydrate diet in our study was not associated with an increase in plasma triacylglycerol concentrations or with any impairment of diabetic control.

In view of the current interest in low-carbohydrate diets (eg, the Atkins diet), which are extremely high in fat (66% of energy), the long-term effects may not be salutary, given the lack of effect of fat on the satiety centers in the brain. What needs to be considered are the long-term effects of any dietary change.

Low-fat, high-carbohydrate, high-fiber diets may be useful not only in the treatment of but also in the prevention of type 2 diabetes mellitus. In 2 large prospective studies, the Finnish Diabetes Prevention Study (7) and the US Diabetes Prevention Program (8), persons with impaired glucose tolerance or elevated fasting plasma glucose concentrations but without frank diabetes, who received counseling to decrease dietary fat and increase fiber intake and physical activity, lost more weight and had a 58% decreased progression to diabetes compared with control subjects. From an epidemiologic viewpoint, human populations consuming diets low in fat and high in fiber and complex carbohydrates have low rates of type 2 diabetes and obesity. This suggests that such diets may protect against the development of diabetes. Other lifestyle factors, such as increased physical activity, may also be operative in these populations.

An important question is whether the results of short-term dietary studies such as our own have long-term applicability. Critical issues include whether low-fat, high-fiber diets can be adhered to long-term and whether or not weight loss would continue beyond the relatively brief intervention periods studied. Extrapolation from unpublished data from our study suggests that the study been continued for longer than 6 wk, subjects on the low-fat diet may have continued to lose weight, while subjects on the high–monounsaturated fat diet would have stabilized. Astrup et al (9), in a meta-analysis of 16 intervention trials of 2-12 mo duration in nondiabetic subjects, showed that ad libitum consumption of a low-fat diet resulted in weight loss (3.2 kg more than in the control group) and decreased energy intake compared with control groups. The National Weight Control Registry has compiled a national roster of persons who had lost an average of 30 kg for an average of 5.5 y. Persons who successfully maintain weight loss long term share several common lifestyle factors, such as increased physical activity, which may also be operative in these populations.

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In conclusion, the available evidence suggests that low-fat, high-fiber, high-complex-carbohydrate diets can be adhered to over the long term and will result in a modest, but significant, weight loss in healthy and diabetic persons. The results from our study and others suggest that such diets may be useful in the treatment and the prevention of type 2 diabetes, obesity, and the metabolic syndrome.

The authors had no financial or personal interest in any company or organization connected in any way with the research represented in this article, including serving as an expert witness or public advocate, grantee, shareholder, option holder, advisor, consultant, employee, or officer.

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Low plasma pyridoxal-5’phosphate and cardiovascular disease risk in women: results from the Coronary Risk Factors for Atherosclerosis in Women Study

Dear Sir:

Low plasma concentrations of pyridoxal-5’phosphate (PLP), the active metabolite of vitamin B-6, have been discussed as a cardiovascular disease (CVD) risk factor (1–3). However, this association may be caused by an influence of inflammation on plasma PLP concentrations (4) that was not corrected for in the early studies (1–3). Recently, Friso et al (5) reported in this Journal that low plasma PLP concentrations were a significant risk factor for coronary artery disease (CAD) in a case-control study in Italy. Plasma PLP was significantly and inversely associated with both angiographically defined CAD and high-sensitivity C-reactive protein (hs-CRP). However, adjustment for markers of inflammation did not change the association of plasma PLP with CAD.
The results of a retrospective case-control study of CVD risk factors in women in Germany, the Coronary Risk Factors for Atherosclerosis in Women (CORAS) Study, do not confirm the results of Friso et al. The German study is a case-control study including 200 case and 255 control subjects (all women). Details of the study design were reported elsewhere (6). Cases were selected after admittance to the Department of Internal Medicine, University Hospital Hamburg–Eppendorf, for incident CAD, which was usually verified by angiography. Age-matched control subjects from the same district were invited by mailing. Control subjects with any symptoms suggesting CAD were excluded. Fasting blood samples were collected within 24 h of admittance and were analyzed for CVD risk factors including hs-CRP, homocysteine, folate, vitamin B-12, and vitamin B-6. Vitamin B-6 was measured as PLP with the use of an HPLC method with fluorescence detection.

The CORAS Study used the same type of statistical analysis as was used in the study of Friso et al to ensure comparability between the 2 studies. Median PLP concentrations in the control subjects (44 nmol/L) were used to define low vitamin B-6 status. Univariate and multivariate logistic regression with log-transformed variables was performed, which allowed the analysis to be controlled for age, smoking status, hypertension, diabetes, total cholesterol, triacylglycerol, BMI, and serum creatinine.

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TABLE 1

Odds ratios (95% CIs) for coronary artery disease according to low pyridoxal-5’-phosphate (PLP) concentrations in the Coronary Risk Factors for Atherosclerosis in Women Study, 1997–2000

<table>
<thead>
<tr>
<th>Quintile of PLP</th>
<th>1 (reference)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not adjusted</td>
<td>1.0</td>
<td>0.54 (0.27, 1.08)</td>
<td>0.40 (0.21, 0.80)</td>
<td>0.18 (0.09, 0.39)</td>
<td>0.17 (0.08, 0.37)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.0</td>
<td>0.75 (0.24, 2.3)</td>
<td>0.57 (0.20, 1.7)</td>
<td>0.41 (0.15, 1.1)</td>
<td>0.14 (0.04, 0.51)</td>
<td>0.0014</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.0</td>
<td>0.78 (0.18, 3.5)</td>
<td>2.1 (0.43, 10.0)</td>
<td>1.14 (0.30, 4.3)</td>
<td>0.81 (0.14, 4.6)</td>
<td>0.97</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.0</td>
<td>0.64 (0.11, 3.9)</td>
<td>1.43 (0.22, 9.2)</td>
<td>0.89 (0.17, 4.7)</td>
<td>0.77 (0.10, 5.9)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1 n = 455.
2 Adjusted for age, smoking, hypertension, diabetes, total cholesterol, triacylglycerol, BMI, and serum creatinine.
3 Adjusted for the abovementioned risk factors plus high-sensitivity C-reactive protein.
4 Adjusted for homocysteine, vitamin B-12, and folate, as well as the same factors as in model 2.

Median PLP and hs-CRP concentrations differed significantly between case and control subjects (PLP: 30.5 and 44 nmol/L, respectively; hs-CRP: 12 and 2.2 mg/L, respectively) and were significantly associated with CAD (r = −0.37, P < 0.001). In the univariate logistic regression analysis, low PLP was significantly associated with CAD (odds ratio: 3.38; 95% CI: 2.23, 5.11). Adjustment for classic risk factors did not substantially change the association between PLP and CAD, but that association became nonsignificant after the inclusion of hs-CRP in the model (odds ratio: 1.77; 95% CI: 0.96, 3.28). This effect was seen in the analysis of both the dichotomized PLP concentrations (Table 1) and PLP quintiles (Table 2).

These results contrast with those of the case-control study of Friso et al. In our study, differences in PLP and hs-CRP concentrations between case and control subjects were more pronounced, probably because of the inclusion of acute coronary syndromes, which Friso et al did not include in their study.

In the study of Friso et al, the magnitude of the association of PLP with CAD was low (odds ratio: 1.89), but it remained significant after adjustment for markers of inflammation. However, we wonder whether the results of Friso et al would have been nonsignificant, because of substantial residual confounding in their model, if they had also made an analysis by quintiles.

We suggest that the association of low PLP with CVD risk is mainly due to the effect of inflammation on plasma PLP concentrations. It is not yet known whether the measurement of whole-blood PLP concentrations would confirm this association (7). However, the results of the CORAS Study and other studies of stroke (8) suggest that low plasma PLP concentrations should be evaluated in connection with markers of inflammation. Low plasma PLP concentrations in CAD patients obviously do not indicate vitamin B-6 deficiency, but they do appear to reflect systemic inflammation. A more definitive answer as to whether low PLP is associated with CVD risk independent of the inflammatory response will be obtained by studies with a prospective design.

None of the authors had any personal or financial conflicts of interest with the study by Friso et al or their article in the Journal.

Jutta Dierkes
Institute of Clinical Chemistry and Biochemistry
University Hospital Magdeburg
Leipziger Strasse 44
D-39120 Magdeburg
Germany
E-mail: jutta.dierkes@medizin.uni-magdeburg.de

TABLE 2

Odds ratios (95% CIs) for coronary artery disease according to quintiles of pyridoxal-5’-phosphate concentrations in the Coronary Risk Factors for Atherosclerosis in Women Study, 1997–2000

<table>
<thead>
<tr>
<th>Quintile of PLP</th>
<th>1 (reference)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not adjusted</td>
<td>1.0</td>
<td>0.54 (0.27, 1.08)</td>
<td>0.40 (0.21, 0.80)</td>
<td>0.18 (0.09, 0.39)</td>
<td>0.17 (0.08, 0.37)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.0</td>
<td>0.75 (0.24, 2.3)</td>
<td>0.57 (0.20, 1.7)</td>
<td>0.41 (0.15, 1.1)</td>
<td>0.14 (0.04, 0.51)</td>
<td>0.0014</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.0</td>
<td>0.78 (0.18, 3.5)</td>
<td>2.1 (0.43, 10.0)</td>
<td>1.14 (0.30, 4.3)</td>
<td>0.81 (0.14, 4.6)</td>
<td>0.97</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.0</td>
<td>0.64 (0.11, 3.9)</td>
<td>1.43 (0.22, 9.2)</td>
<td>0.89 (0.17, 4.7)</td>
<td>0.77 (0.10, 5.9)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1 n = 455.
2 Adjusted for age, smoking, hypertension, diabetes, total cholesterol, triacylglycerol, BMI, and serum creatinine.
3 Adjusted for the abovementioned risk factors plus high-sensitivity C-reactive protein.
4 Adjusted for homocysteine, vitamin B-12, and folate, as well as the factors in model 2.
Dear Sir:

Reply to J Dierkes et al

We thank Dierkes et al for their interest in our recent report on low vitamin B-6 concentrations and coronary artery disease (CAD) risk (1). In an earlier case-control study of angiographically defined CAD patients, we extended findings from a previous population-based study (2) and observed that low plasma pyridoxal-5′phosphate (PLP) concentrations were inversely related to major markers of inflammation and independently associated with increased CAD risk (1). Such an association of low PLP concentrations with higher risk of CAD remained significant even after the inclusion, in a multivariate logistic regression model, of high-sensitivity C-reactive protein (hs-CRP), fibrinogen, and variables related to homocysteine metabolism, such as total plasma homocysteine, vitamin B-12, and folate (1).

Several differences between our study and that of Dierkes et al may account for the dissimilar results. Their study, the Coronary Risk Factors for Atherosclerosis in Women (CORA) Study (3), was conducted only among women, whereas both sexes were represented in our study, and, in fact, there was a greater number of men than of women in our study. The participants in the CORA Study were selected for recent acute coronary symptoms, but that condition was an exclusion criterion in our study. Precisely because of the potential confounding role of inflammatory markers in the risk related to low PLP in CAD patients, the enrollment of women in acute phase may account not only for the higher hs-CRP in cases than in control subjects in the CORA Study but also for the lack of a significant association between low PLP and CAD after adjustment for hs-CRP, which was at a borderline concentration (odds ratio: 1.77; 95% CI: 0.96, 3.28). Moreover, female control subjects were enrolled only on the basis of the absence of clinical symptoms and not on that of angiography documentation. The inclusion of asymptomatic subjects who potentially had coronary atherosclerosis may also have influenced the significance of the association in the CORA Study. Indeed, it should be taken into account that the influence of low PLP on the estimate of risk in a multifactorial disease such as CAD is slight but significant [odds ratio: 1.89; 95% CI: 1.18, 3.03; \( P = 0.008 \) (multivariate logistic regression)] (1). This observation is substantiated in our study by a threshold effect for CAD risk-related PLP concentrations corresponding to the 50th percentile, an effect that is most likely due to the influence of PLP on CAD risk at the lowest concentrations.

Dierkes et al also performed a dichotomized analysis for the association with low PLP that took into account the median concentrations of PLP among control subjects in their study; this followed our model of analysis. The value that they observed, however, was higher than that in our study (44 and 36.3 nmol/L, respectively). Certainly, neither of those values can be regarded as a clear vitamin deficiency, but, considering that even a moderate vitamin B-6 impairment may influence the estimate of CAD risk, as shown by the results of our study, such a difference is likely to explain the dissimilar findings between the CORA Study and ours. Furthermore, Dierkes et al observed that the association of PLP with CAD risk became nonsignificant after the inclusion of hs-CRP in an analysis of PLP divided into quintiles, and they speculated that the association between low PLP and CAD is mainly due to the effect of inflammation on plasma PLP concentrations. As stated in our report (1), preliminary analyses by likelihood-ratio tests excluded any significant interaction between PLP and hs-CRP in our study, whether evaluated on the basis of the 50th percentile (chi-square test: 0.03, \( P = 0.86605 \)) or of quintiles (chi-square test: 0.13, \( P = 0.7190 \)). This finding further defined the independent association of low PLP with CAD risk and showed an additive effect of low PLP to that conferred by high hs-CRP, which was seen in the progressive increase in the estimate of CAD risk across increasing hs-CRP quintiles (1). Unfortunately, the data so far available from the literature do not allow a definite conclusion as to whether low PLP reflects systemic inflammation. In a study by Kelly et al (4), low PLP status was associated with stroke and only partially mediated via inflammation, as expressed by the major marker hs-CRP (4). Moreover, we can assume that, considering the median value of PLP in the CORA Study, even the lowest

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Kurt Hoffmann
Kerstin Klipstein-Grobusch
Cornelia Weikert
Heiner Boeing

Birgit-Christiane Zyriax
Eberhard Windler

Jürgen Kratzsch

German Institute of Human Nutrition
Potsdam-Rehbrücke
Germany

University Hospital Hamburg-Eppendorf
Hamburg
Germany

Institute of Laboratory Medicine
Clinical Chemistry and Molecular Diagnosis
University Hospital Leipzig
Leipzig
Germany

REFERENCES

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PLP quintile may be still within a range of values that may not allow detection of an influence on the risk of disease. We expect that the analysis by quintiles among the 200 cases and 255 controls of the CORA Study did not provide enough statistical power.

We certainly concur with Dierkes et al that further studies, particularly those with a prospective design, will better clarify the role of low PLP in CAD. It will be important to assess more precisely both the relation between PLP and inflammation and their specific contribution to the mechanisms underlying atherogenesis so that the role of this vitamin in the modulation of CAD risk can be elucidated.

None of the authors had any personal or financial conflict of interest with regard to this work.

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Simonetta Friso
Domenico Girelli
Nicola Martinelli
Oliviero Olivieri
Roberto Corrocher

REFERENCES

Erratum


On page 929, column 1, line 8, the data for erythrocyte incorporation of $^{59}$Fe are mistakenly listed as being given as percentages of ingested isotope; however, the data actually represent the percentages of absorbed isotope.
Erratum


On page 1737S, Table 2, the values for “Additional vitamin D intake to achieve 80 nmol/L” are incorrect for the following subgroups: white females aged 30-59 y and black females aged ≥60 y. The correct values are given in the revised version of the table given below (Table 2).

**TABLE 2**
Mean vitamin D status of the US population (winter, low latitude) and vitamin D intakes required to achieve 80 nmol/L serum 25-hydroxyvitamin D

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>No.</th>
<th>Serum 25-hydroxyvitamin D</th>
<th>Additional vitamin D intake to achieve 80 nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/L</td>
<td>µg/d</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–19 y</td>
<td>625</td>
<td>78.6</td>
<td>2</td>
</tr>
<tr>
<td>20–39 y</td>
<td>1289</td>
<td>69.1</td>
<td>16</td>
</tr>
<tr>
<td>40–59 y</td>
<td>864</td>
<td>70.6</td>
<td>13</td>
</tr>
<tr>
<td>60–70 y</td>
<td>827</td>
<td>72.5</td>
<td>11</td>
</tr>
<tr>
<td>≥80</td>
<td>204</td>
<td>68.7</td>
<td>16</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–19 y</td>
<td>699</td>
<td>64.9</td>
<td>22</td>
</tr>
<tr>
<td>20–39 y</td>
<td>1459</td>
<td>62.7</td>
<td>25</td>
</tr>
<tr>
<td>40–59 y</td>
<td>959</td>
<td>61.6</td>
<td>26</td>
</tr>
<tr>
<td>60–70 y</td>
<td>757</td>
<td>63.5</td>
<td>24</td>
</tr>
<tr>
<td>≥80</td>
<td>208</td>
<td>59.6</td>
<td>29</td>
</tr>
</tbody>
</table>

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