Effect of soy protein on endogenous hormones in postmenopausal women

Victoria W Persky, Mary E Turyk, Ling Wang, Sally Freels, Robert Chatterton Jr, Stephen Barnes, John Erdman Jr, Daniel W Sepkovic, H Leon Bradlow, and Susan Potter

ABSTRACT

Background: The long-term clinical effects of soy protein containing various concentrations of isoflavones on endogenous hormones are unknown.

Objective: We examined the effects of ingestion of soy protein containing various concentrations of isoflavones on hormone values in postmenopausal women.

Design: Seventy-three hypercholesterolemic, free-living, postmenopausal women participated in a 6-mo double-blind trial in which 40 g protein as part of a National Cholesterol Education Program Step I diet was provided as casein from nonfat dry milk (control), isolated soy protein (ISP) containing 56 mg isoflavones (ISP56), or ISP containing 90 mg isoflavones (ISP90). Endogenous hormone concentrations were measured at baseline and at 3 and 6 mo.

Results: The concentration of thyroxine and the free thyroxine index were higher in the ISP56 group, and the concentration of thyroid-stimulating hormone was higher in the ISP90 group than in the control group at 3 and 6 mo ($P < 0.05$). Triiodothyronine was significantly higher in the ISP90 group only at 6 mo. Thyroxine, free thyroxine index, and thyroid-stimulating hormone at 6 mo were inversely associated with measures of baseline estrogenicity. No significant differences were found for endogenous estrogens, cortisol, dehydroepiandrosterone sulfate, insulin, glucagon, or follicle-stimulating hormone after baseline hormone values were controlled for.

Conclusions: This study does not provide evidence that long-term ingestion of soy protein alters steroid hormone values, but it suggests that soy protein may have small effects on thyroid hormone values that are unlikely to be clinically important. The thyroid effects are, however, consistent with previous findings in animals and highlight the need for future research investigating possible mechanisms of action. Am J Clin Nutr 2002;75:145–53.

KEY WORDS Soy protein, hormones, postmenopausal women, isoflavones, National Cholesterol Education Program Step I diet

INTRODUCTION

Previous studies suggested that high soybean intakes are associated with lower concentrations of serum cholesterol (1) and may be related to decreased rates of coronary artery disease (2), cancer (3), and osteoporosis (2). One of the postulated mechanisms of these effects is that soy products, particularly the isoflavones in soy, act through changes in endogenous hormonal balance. Isoflavones were shown to have both estrogenic and antiestrogenic effects in animals (4–9). Although Divi et al (10) noted that genistein inhibits thyroid peroxidase and decreases thyroxine synthesis, most animal studies found that soy protein increases thyroxine ($T_4$) concentrations and has inconsistent effects on triiodothyronine ($T_3$) and thyroid-stimulating hormone (TSH) (11–18).

The results of human studies have been inconsistent. In men, Ham et al (19) found increases in $T_4$ and in the free thyroxine index (FTI) in hypercholesterolemic men after ingestion of soyprotein isolate for 4 wk, but not after ingestion of soy flour. In premenopausal women, one observational study noted positive correlations of sex hormone binding globulin (SHBG) with excretion of lignans and phytoestrogens (20), another study found no association of SHBG with intake of soy products (21) but an inverse association with serum estradiol (21), and a third study found a positive association of SHBG with dehydroepiandrosterone sulfate (DHEAS) (22). Trials in premenopausal women have also been inconsistent. One month of soy ingestion suppressed midcycle surges of follicle-stimulating hormone (FSH) and luteinizing hormone, increased follicular-phase estradiol, decreased cholesterol concentrations (23, 24), and increased menstrual cycle length and decreased serum estradiol, progesterone, and DHEAS (25).

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2 Supported by the Illinois Soybean Program and the National Cancer Institute (RO3CA64459-01A1). The mass spectrometer used in this study was purchased with funds from an NIH Instrument Grant (S10RR06487) and the University of Alabama at Birmingham (UAB). Operation of the Mass Spectrometry Shared Facility at UAB is supported in part by an NCI Core Research Support Grant (P30 CA13148) to the UAB Comprehensive Cancer Center. Soy protein products were provided by Protein Technologies International, St Louis.

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TABLE 1

Isoflavone content of the 3 test proteins in the 3 diet groups

<table>
<thead>
<tr>
<th></th>
<th>ISP56</th>
<th>ISP90</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzin</td>
<td>7.5</td>
<td>17.8</td>
<td>ND</td>
</tr>
<tr>
<td>Malonyl daidzin</td>
<td>14.0</td>
<td>24.2</td>
<td>ND</td>
</tr>
<tr>
<td>Acetyl daidzin</td>
<td>ND</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Daidzein</td>
<td>1.9</td>
<td>1.6</td>
<td>ND</td>
</tr>
<tr>
<td>Total daidzein</td>
<td>13.6 (0.44)</td>
<td>26.0 (0.82)</td>
<td>0</td>
</tr>
<tr>
<td>Genistin</td>
<td>15.7</td>
<td>29.5</td>
<td>ND</td>
</tr>
<tr>
<td>Malonyl genistin</td>
<td>26.2</td>
<td>35.7</td>
<td>ND</td>
</tr>
<tr>
<td>Acetyl genistin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Genistein</td>
<td>2.8</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>Total genistein</td>
<td>26.2 (0.84)</td>
<td>39.1 (1.23)</td>
<td>0</td>
</tr>
<tr>
<td>Glycitin</td>
<td>1.3</td>
<td>2.5</td>
<td>ND</td>
</tr>
<tr>
<td>Malonyl glycitin</td>
<td>2.4</td>
<td>3.6</td>
<td>ND</td>
</tr>
<tr>
<td>Glycitein</td>
<td>1.3</td>
<td>3.2</td>
<td>ND</td>
</tr>
<tr>
<td>Total glycitin</td>
<td>3.5 (0.11)</td>
<td>6.8 (0.21)</td>
<td>0</td>
</tr>
<tr>
<td>Total isoflavones</td>
<td>43.2 (1.39)</td>
<td>71.8 (2.25)</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Values are per 40 g protein. Data provided by Protein Technologies International (St Louis). ND, none detected. ISP56 and ISP90, isolated soy protein containing 56 and 90 mg isoflavones per 40 g protein, respectively. 2 Adjusted to represent the total isoflavones in aglycone units. Values in parentheses are per g protein.

Another 3-mo trial in premenopausal women found that ingestion of isolated soy protein (ISP) providing 129 mg isoflavones/d resulted in lower free T3, estrone, and DHEAS values; lower urinary 2-hydroxyestrone (16OHE1)/estradiol values, and a higher ratio of urinary 2-hydroxyestrone to 16α-hydroxyestrone (16OHE1) during the follicular phase of the women's cycles than did ingestion of 10 mg isoflavones/d (26, 27).

In postmenopausal women, a similar 3-mo trial with a high-isoflavone diet resulted in a small but significant decrease in estrone sulfate values, a trend toward a decrease in estradiol and estrone values, and a small but significant increase in SHBG values (28). There were no significant differences in thyroid hormones or in measures of estrogenicity in vaginal epithelium or endometrial biopsies (28). In contrast, also in postmenopausal women, Baird et al (29) found no changes in FSH, luteinizing hormone, SHBG, or estradiol, but did note a small estrogenic effect on vaginal cytology after soy ingestion; Petrakis et al (30) found no consistent changes in plasma prolactin, SHBG, estradiol, or progesterone after 5 mo of soy ingestion.

Many of these studies, especially in premenopausal women, were short term (22–25). Yet, chronic exposure to soy alters the kinetics of isoflavone metabolism (31). Several months of exposure may be required to delineate long-term endocrine effects. The present study was undertaken to determine the effects of ingestion of soy protein with different concentrations of isoflavones for 6 mo on endogenous hormones in postmenopausal women.

METHODS

Subjects

Postmenopausal women were recruited from the vicinity of Urbana-Champaign, IL, through flyers, letters, and physician referrals. The participants were studied during 3 separate 24-wk cohort periods: cohort 1 during the summer and fall of 1994 (n = 24), cohort 2 during the spring and summer of 1995 (n = 25), and cohort 3 during the summer and fall of 1995 (n = 25). Within each of these cohorts, the women were randomly assigned to each of the 3 diet groups described below. Inclusion criteria were the completion of menopause, ≥1 y since the last menstrual period, and a total plasma cholesterol concentration of 6.2–7.8 mmol/L. Women were excluded if they were receiving hormone replacement therapy, taking other medication known to lower cholesterol, had a history of diabetes mellitus or thyroid disease, had a chronic illness that might affect lipid measurements or limit their ability to participate in the study, or had an allergy to soybean protein.

Of a total of 134 women screened, 81 women aged 49–83 y gave informed consent and 74 women completed the study. Seven women withdrew from the study during weeks 1–4 for various reasons: 1 moved to another state, 3 withdrew because of medical problems unrelated to soy-product consumption, and 3 withdrew because of their inability to comply with the study protocol. One woman who was taking levothyroxine was inadvertently included, and her results were, therefore, excluded from all analyses. Thus, statistical analyses were conducted on data from a total of 73 women. The study was approved by the Institutional Review Boards of the University of Illinois at both Chicago and Urbana-Champaign.

Diet

Two weeks before the study, participants completed a 2-d dietary intake record and were interviewed by a registered dietitian to calculate their daily energy requirement for a baseline low-fat, low-cholesterol National Cholesterol Education Program Step I diet. Each participant received a booklet published by the American Heart Association containing a long list of foods, along with a calculated fat gram prescription. All participants followed the basal diet for ≥14 d. After this, baseline blood samples were drawn on 2 separate days, and participants were randomly assigned to 1 of 3 dietary treatment groups. Most of the participants were already consuming diets similar to the National Cholesterol Education Program Step I diet before enrolling in the study. All 3 groups continued to consume their basal diets to which 40 g test protein/d was incorporated as ISP (Supro 675; Protein Technologies International, St Louis) containing moderate (56 mg) concentrations of isoflavones (ISP56 group; n = 24), as ISP containing higher (90 mg) concentrations of isoflavones (ISP90 group; n = 23), or as casein (0 mg total isoflavones/g protein; New Zealand Milk Products, Wellington, New Zealand) from nonfat dry milk (control group; n = 26) (Table 1). Units of isoflavones presented in this paper reflect only the aglycone weight of the molecule. The ISPs were fortified to provide 800–900 mg Ca/d in the form of calcium bisphosphate. This amount was consistent with the amount of calcium provided through the dairy component for the control group. Participants were advised not to ingest other soy products during the study and were blinded to the source of test protein during the trial period. Investigators involved in the production of test protein–containing products were aware of dietary treatment codes; however, laboratory personnel were not.

The Department of Food Science and Human Nutrition at the University of Illinois developed recipes that incorporated the casein and ISPs into baked products. Ready-to-mix beverages and soups containing ISP or casein were also used. Participants were informed of the amount of protein, fat, and energy provided by each of the test protein items. All baked products were prepared by a professional baker in the University of Illinois Quantity...
Foods Laboratory and were individually packaged and distributed to participants during a breakfast held at the study site 3 d/wk. Cereals, fruit, tea, coffee, juice, and low-energy carbonated beverages were also offered during the breakfast. Throughout the study, all participants were instructed by a registered dietitian to maintain their body weights and physical activity levels and about dietary requirements and acceptable food and beverage intakes. Additionally, every 4 wk, participants were asked to complete a dietary intake record on 3 d (including 2 weekdays and 1 weekend day) chosen at random. Actual daily nutrient intakes were analyzed by using a nutritional analysis software program (NUTRITIONIST IV, version 3.0; N-Squared Computing, Salem Park, OR). A summary of the isoflavone content of the 3 diets is presented in Table 1.

**Blood and urine sample collection**

Blood samples for hormone measurements were collected at baseline and 3 and 6 mo after initiation of the diets. Blood was collected at 0600–0900 after a 12-h fast from either the antecubital or dorsal hand vein. Serum and plasma were separated by centrifugation at 1190 × g for 15 min at 4°C and stored at −70°C before shipment to the laboratories of 2 of the authors (RC and SB) for analysis of hormones and isoflavones, respectively. For estrogen metabolites, 24-h urine specimens (each bottle containing 100 mg ascorbic acid) were collected only from the second 2 cohorts at baseline, 3 mo, and 6 mo, and aliquots were stored at −70°C before shipment to Bradlow’s laboratory for analysis.

**Weight measurements**

Body weight was measured weekly by using a scale equipped with a beam balance (Detecto Physician’s scale; Detecto Scale Company, Webb City, MO); the scale was calibrated weekly.

**Hormones**

**Measurements**

The Delphia system (Walla, Gaithersburg, MD) was used for the assay of serum SHBG. This system is a solid-phase, two-site, time-resolved fluorimunometric assay that uses a sandwich technique. The intrassay and interassay CVs were 5.0% and 5.3%, respectively. DHEAS was measured in unextracted serum with the use of radioimmunoassay (RIA) as described previously (32). The intrassay and interassay CVs were 4.6% and 10.9%, respectively. Plasma cortisol was measured with a direct assay described by Seth and Brown (33) as used in other studies (34). The intrassay CV was 6% and the interassay CV was 10%. Values for the individual metabolites were divided by the total urine volume.

In the present study, 11.6% of duplicate urine samples were submitted blind as an additional quality control. The CVs from the split urine samples were 16.0% for 2OHE1 and 36.4% for 16aOHE1. One-half of the limit of detection for the assay (0.325 nmol/d) was imputed for 13 samples that had nondetectable amounts of 16aOHE1. Concentrations of urinary estrogen metabolites were multiplied by the total volume of 24-h urine samples.

**Plasma isoflavones and lipids**

Plasma total and unconjugated isoflavones were measured with the use of a modified version of an HPLC–mass spectrometric method described previously (45). For a 1-mL blood sample, the overall detection limit ranged from 4 to 10 nmol/L. The median value for the CV was determined for 2 ranges: from the limit of detection for each compound to 40 nmol/L (10.2%–21.5%) and from 40 nmol/L upward (3.1–7.7%). CVs from split samples were 20.1% for equol, 25.0% for daidzein, 23.4% for dihydridaidzein, 34.4% for O-desmethylangolensin (O-DMA), and 21.8% for genistin. Plasma lipids were analyzed as previously described (46, 47).

**Bone mineral density**

Bone mineral density of the lumbar spine (L1–L4), proximal femur (including the femoral neck and Ward’s triangle), and total

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**Quality control**

External quality-control standards for steroid hormones were obtained from the American College of Pathologists. For internal quality control, a single batch of each of the quality-control materials and antisera for each of the hormones analyzed were prepared and reserved for all assays during the 2 y of the study to minimize interassay variability. Blood specimens were analyzed by techni- cians unaware of the participants’ diet group, and 2.3% of the samples were submitted to the laboratory as blind duplicates as an additional quality control for steroid hormones. CVs from the 5 split serum samples were 10.6% for cortisol, 36.5% for DHEAS, 4.5% for SHBG, 2.4% for SHBG-bound estradiol, 11.1% for FSH, 24.0% for estradiol, and 29.2% for estrone sulfate. Serum was stored at −70°C in 3–4-ml aliquots for measurement of additional hormones if subsequent research indicates that these hormones may be affected by soy or isoflavone intake.
The effects of dietary supplementation on endocrine outcomes at 3 and 6 mo were further analyzed by using random-effects models (PROC MIXED) to evaluate the ISP56 diet (ISP56 compared with control) and the ISP90 diet (ISP90 compared with control), adjusting for clustering of hormone measures within participants at 3 and 6 mo, baseline hormone concentration, and time of sample (3 mo compared with 6 mo), and including interaction terms for diet group and time of sample. Group × time interaction terms indicated differences in effects between treatment groups at 3 and 6 mo. When a group × time interaction term was significant, the model was recoded to indicate differences in effects between treatment groups at 3 and 6 mo. When group × time interaction terms were not significant, they were removed from the model. Treatment group terms then indicated differences in the averages of 3- and 6-mo effects between treatment groups.

The outcomes analyzed included body mass index (wt/ht²); serum T₄, T₃, FTI, TSH, estradiol, estrone sulfate, cortisol, insulin, glucagon, insulin-glucagon, DHEAS, FSH, SHBG, and SHBG-bound estradiol; and urinary 2OHE₁, 16αOHE₁, and 2OHE₁:16αOHE₁.

Analyses of 2OHE₁ and 16αOHE₁ (included data presented in tables) and did not include (data not presented) data from 5 participants who had metabolic values or ratios of 2OHE₁ to 16αOHE₁ ≥ 3 SDs from the mean. The results were not altered by exclusion of these 5 women.

Spearman’s correlation coefficients were used to associate changes in isoflavone concentrations (6 mo concentration – baseline concentration) with changes in hormone values (6 mo concentration – baseline concentration). Partial Pearson’s correlation coefficients, after baseline thyroid hormone values and treatment group (as defined for the multiple regression analyses) were controlled for, were used to assess the relation of baseline estrogenicity with changes in thyroid hormones at 6 mo.

CVs for split samples of hormones were computed as the technical error per mean. Technical error was estimated as \((2d_i^2/2n)^{1/2}\), where \(d_i\) is the difference in values between the 2 identical samples and \(n\) is the number of pairs of split samples. SAS (version 8.0; SAS Institute, Inc, Cary, NC) was used for all analyses. \(P ≤ 0.05\) was considered statistically significant.

RESULTS

Daily energy intakes of the diet groups were not significantly different at baseline or during the study (46). There were also no significant differences in intakes of selected nutrients, except for total protein intake, which increased in all 3 groups during the study (46). The dietary intervention resulted in expected changes in serum isoflavone concentrations. After baseline concentrations were adjusted, women in both ISP groups had higher concentrations of isoflavones at 6 mo than did women in the control group (Table 2).

There were no significant differences at baseline in mean age or body mass index between the 3 diet groups (data not shown). The average ages were 59.3, 61.9, and 61.0 y and the average body mass indexes were 28.0, 26.9, and 29.6 in the ISP56, ISP90, and control groups, respectively. Similarly, there were no significant differences in body mass index after 3 or 6 mo of dietary intervention. Few women smoked (2–3 in each diet group) or drank any alcohol (4–7 in each diet group).

There were no significant differences in any of the estrogen values at baseline or over time (Table 3). Similarly, there were no significant differences in DHEAS, FSH, insulin, or glucagon between groups (Table 4). SHBG concentrations differed between the ISP90 and control groups averaging over time, but this difference was of borderline significance (\(P = 0.06\)). Cortisol concentrations at baseline were higher in the ISP56 group than in control subjects, but differences were no longer present averaging over 3 and 6 mo.

The effects of ISP ingestion on thyroid hormones are shown in Table 5. T₄ concentrations and the FTI were significantly

### Table 2

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>ISP56 (n = 23)</th>
<th>ISP90 (n = 21)</th>
<th>Control (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>14 (8, 25)</td>
<td>19 (14, 27)</td>
<td>31 (21, 46)</td>
</tr>
<tr>
<td>0 mo</td>
<td>14 (8, 25)</td>
<td>19 (14, 27)</td>
<td>31 (21, 46)</td>
</tr>
<tr>
<td>6 mo</td>
<td>426 (298, 609)</td>
<td>568 (443, 727)</td>
<td>21 (15, 31)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>8 (4, 15)</td>
<td>7 (4, 12)</td>
<td>10 (5, 21)</td>
</tr>
<tr>
<td>0 mo</td>
<td>8 (4, 15)</td>
<td>7 (4, 12)</td>
<td>10 (5, 21)</td>
</tr>
<tr>
<td>6 mo</td>
<td>174 (111, 272)</td>
<td>226 (169, 307)</td>
<td>10 (6, 18)</td>
</tr>
<tr>
<td>O-DMA</td>
<td>2 (1, 2)</td>
<td>1 (1, 2)</td>
<td>2 (1, 4)</td>
</tr>
<tr>
<td>0 mo</td>
<td>2 (1, 2)</td>
<td>1 (1, 2)</td>
<td>2 (1, 4)</td>
</tr>
<tr>
<td>6 mo</td>
<td>20 (10, 42)</td>
<td>55 (36, 84)</td>
<td>2 (1, 3)</td>
</tr>
<tr>
<td>Total daidzein</td>
<td>16 (5, 44)</td>
<td>11 (3, 39)</td>
<td>2 (1, 3)</td>
</tr>
<tr>
<td>0 mo</td>
<td>15 (8, 25)</td>
<td>8 (5, 15)</td>
<td>14 (7, 29)</td>
</tr>
<tr>
<td>6 mo</td>
<td>475 (333, 677)</td>
<td>656 (497, 867)</td>
<td>17 (10, 28)</td>
</tr>
<tr>
<td>Total isoflavones</td>
<td>32 (19, 54)</td>
<td>33 (25, 42)</td>
<td>53 (34, 81)</td>
</tr>
<tr>
<td>0 mo</td>
<td>32 (19, 54)</td>
<td>33 (25, 42)</td>
<td>53 (34, 81)</td>
</tr>
<tr>
<td>6 mo</td>
<td>925 (656, 1304)</td>
<td>1264 (994, 1607)</td>
<td>44 (33, 59)</td>
</tr>
</tbody>
</table>

1 Geometric mean; 95% CIs in parentheses. Means of natural log-transformed isoflavones at 0 mo were evaluated by Student’s t tests. Means of natural log-transformed isoflavones at 6 mo were evaluated by using regression models that compared the effects of the ISP56 and ISP90 diets with those of the control diet after adjustment for baseline isoflavone concentrations. DHD, dihydrodaidzein; O-DMA, O-desmethylangolensin; ISP56 and ISP90, isolated soy protein containing 56 and 90 mg isoflavones per 40 g protein, respectively.

2 Significantly different from control group, \(P < 0.05\).

3 Total daidzein = equol + daidzein + DHD + O-DMA.

4 Total isoflavones = total daidzein + genistein.
higher in the ISP56 group than in the control group when averaged across 3 and 6 mo. TSH was significantly higher only in the ISP90 group when averaged across time. T3 was also significantly higher only in the ISP90 group, but for T3, there was a significant group × time interaction, indicating that the difference was significant only at 6 mo.

Changes in T4, FTI, and TSH between baseline and 6 mo were inversely related to measures of estrogenicity at baseline (Table 6). Thus, the change in T4 was significantly and inversely associated with total bone mineral density, serum estradiol, and urinary 2OHE1 concentrations. The change in the FTI was significantly and inversely related to body mass index, total bone mineral density, serum estradiol, serum estrone sulfate, urinary 2OHE1, and urinary 16αOHE1. The change in TSH concentration was significantly and inversely related to body mass index, total bone mineral density, and lumbar spine bone mineral density at baseline.

Changes in isoflavone concentrations were also significantly related to changes in measures of estrogenicity, as reflected by changes in bone mineral density and HDL-cholesterol concentrations at 6 mo compared with baseline (data not shown). Changes in the concentrations of genistein, daidzein, dihydrodaidzein, O-DMA, total daidzein, and total isoflavones were significantly and positively associated with changes in HDL concentrations at 6 mo. Changes in T4 and the FTI at 6 mo, however, were significantly and positively associated only with changes in the concentrations of the daidzein metabolites dihydrodaidzein and O-DMA (data not shown).

DISCUSSION

This study provides no evidence that long-term ingestion of ISP containing moderate or high concentrations of isoflavones alters serum or urinary estrogen, cortisol, DHEAS, insulin, glucagon, or FSH in postmenopausal women. There is a suggestion that SHBG concentrations may be increased in the ISP90 group, but the relation was of only borderline significance. Petrakis et al (30) found no change in plasma estrogen concentrations in 10 postmenopausal women after they ingested 38 g soy protein for 6 mo. Duncan et al (28) noted small but significant decreases in estrone sulfate concentrations, a trend toward lower estradiol and estrone concentrations, and small but significant increases in SHBG concentrations in postmenopausal women who had consumed a high isoflavone diet for 3 mo. Several studies in premenopausal women found decreased estrogen concentrations and changes in estrogen metabolite concentra-

### TABLE 3

<table>
<thead>
<tr>
<th>Estrogen values at 0, 3, and 6 mo†</th>
<th>ISP56</th>
<th>ISP90</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum estradiol (pmol/L)‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>0 mo</td>
<td>17.5 (8.1, 37.9)</td>
<td>9.7 (3.0, 31.4)</td>
<td>15.6 (6.1, 40.0)</td>
</tr>
<tr>
<td>3 mo</td>
<td>12.6 (3.9, 41.0)</td>
<td>7.6 (2.4, 24.2)</td>
<td>11.0 (3.7, 32.5)</td>
</tr>
<tr>
<td>6 mo</td>
<td>24.5 (11.7, 51.5)</td>
<td>10.8 (3.9, 30.1)</td>
<td>12.7 (4.3, 37.3)</td>
</tr>
<tr>
<td><strong>Serum estrone sulfate (nmol/L)‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>0 mo</td>
<td>0.77 (0.53, 1.12)</td>
<td>0.42 (0.31, 0.57)</td>
<td>0.50 (0.36, 0.69)</td>
</tr>
<tr>
<td>3 mo</td>
<td>0.72 (0.54, 0.97)</td>
<td>0.58 (0.43, 0.78)</td>
<td>0.60 (0.46, 0.78)</td>
</tr>
<tr>
<td>6 mo</td>
<td>0.79 (0.57, 1.11)</td>
<td>0.66 (0.44, 1.00)</td>
<td>0.59 (0.43, 0.80)</td>
</tr>
<tr>
<td><strong>SHBG-bound estradiol (%)†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>0 mo</td>
<td>32.8 (28.9, 36.6)</td>
<td>35.7 (31.9, 39.4)</td>
<td>35.6 (32.1, 39.1)</td>
</tr>
<tr>
<td>3 mo</td>
<td>30.6 (27.1, 34.0)</td>
<td>34.2 (30.3, 38.1)</td>
<td>32.8 (29.1, 36.5)</td>
</tr>
<tr>
<td>6 mo</td>
<td>30.5 (27.1, 34.0)</td>
<td>33.7 (30.2, 37.2)</td>
<td>31.0 (27.5, 34.4)</td>
</tr>
<tr>
<td><strong>Urinary 2OHE1 (nmol/d)‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
<td>16–17</td>
</tr>
<tr>
<td>0 mo</td>
<td>30.2 (24.5, 37.2)</td>
<td>24.7 (19.2, 31.7)</td>
<td>28.0 (19.5, 40.2)</td>
</tr>
<tr>
<td>3 mo</td>
<td>29.6 (23.3, 37.6)</td>
<td>26.3 (20.0, 34.5)</td>
<td>33.4 (26.5, 42.1)</td>
</tr>
<tr>
<td>6 mo</td>
<td>28.7 (21.4, 38.5)</td>
<td>24.8 (17.0, 36.2)</td>
<td>36.5 (30.9, 43.2)</td>
</tr>
<tr>
<td><strong>Urinary 16αOHE1 (nmol/d)‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
<td>16–17</td>
</tr>
<tr>
<td>0 mo</td>
<td>11.2 (9.2, 13.6)</td>
<td>11.1 (7.9, 15.6)</td>
<td>9.3 (6.3, 13.8)</td>
</tr>
<tr>
<td>3 mo</td>
<td>11.2 (7.8, 16.1)</td>
<td>10.9 (7.9, 15.1)</td>
<td>12.3 (10.1, 15.0)</td>
</tr>
<tr>
<td>6 mo</td>
<td>8.7 (5.4, 13.9)</td>
<td>9.9 (6.2, 15.8)</td>
<td>11.6 (8.5, 15.7)</td>
</tr>
<tr>
<td><strong>Urinary 2OHE1:16αOHE1‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
<td>16–17</td>
</tr>
<tr>
<td>0 mo</td>
<td>2.7 (2.0, 3.6)</td>
<td>2.2 (1.6, 3.1)</td>
<td>3.0 (2.5, 3.6)</td>
</tr>
<tr>
<td>3 mo</td>
<td>2.6 (2.0, 3.5)</td>
<td>2.4 (1.6, 3.6)</td>
<td>2.7 (2.4, 3.1)</td>
</tr>
<tr>
<td>6 mo</td>
<td>3.3 (2.4, 4.6)</td>
<td>2.5 (1.8, 3.4)</td>
<td>3.2 (2.5, 4.0)</td>
</tr>
</tbody>
</table>

† There were no significant differences in values at baseline or over time. ISP56 and ISP90, isolated soy protein containing 56 and 90 mg isoflavones per 40 g protein, respectively; 2OHE1 and 16αOHE1, 2- and 16α-hydroxyestrone, respectively.

‡ Geometric mean; 95% CI in parentheses.

§ Arithmetic mean; 95% CI in parentheses.
tions (21, 25–27), and one study in premenopausal women found an increase in the concentration of follicular estradiol and a decrease in the midcycle surge in the concentrations of luteinizing hormone and FSH (23, 24); however, these studies used shorter intervention periods than those in the present study. Differences between those findings and the findings in the present study may be due to altered isoflavone metabolism after long-term ingestion of soy protein, as noted previously (31). It is also possible that soy may act as an estrogen antagonist in premenopausal women with high endogenous estrogen concentrations and as an estrogen agonist in postmenopausal women with lower endogenous hormone concentrations, with estrogenicity better reflected in end-organ effects than in serum hormone concentrations. This possibility is supported by the findings of Baird et al (29) that soy ingestion was associated with increased vaginal estrogenicity but was not associated with any alterations in the concentrations of serum estradiol or FSH. This possibility is also consistent with reductions in hot flushes (48), increased vaginal cell maturation (49), and perceived severity of vasomotor symptoms (50) noted in other studies, and with the increases in bone density found in our previous study (47) and in animal studies (51, 52).

Data on the effects of soy on DHEAS are also conflicting. In premenopausal women Lu et al (25) and Duncan et al (27) found decreases in DHEAS concentrations, whereas Persky and Van Horn (22) found increases in DHEAS concentrations. Doses of isoflavones, however, differed among the studies. The average dose of 129 mg/d in Duncan et al’s high-isoflavone group (27) was higher than that ingested by the women studied by Persky and Van Horn (22). DHEAS concentrations in the study by Duncan et al were insignificantly higher in the low-isoflavone group than in control subjects after 3 mo (27), suggesting that different doses may account for some of the discrepancies among the studies. In postmenopausal women, Duncan et al noted inconsistent effects on DHEAS of 129 mg soy isoflavones/d in the high-isoflavone group; again, concentrations were higher than in the present study. Alternatively,
TABLE 5
Thyroid hormone values in the 3 diet groups at 0, 3, and 6 mo

<table>
<thead>
<tr>
<th></th>
<th>ISP56</th>
<th>ISP90</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₄ (nmol/L)</td>
<td>24</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>0 mo</td>
<td>1.83 (1.70, 1.96)</td>
<td>1.75 (1.66, 1.85)</td>
<td>1.72 (1.64, 1.80)</td>
</tr>
<tr>
<td>3 mo</td>
<td>1.79 (1.65, 1.92)</td>
<td>1.66 (1.56, 1.76)</td>
<td>1.65 (1.55, 1.75)</td>
</tr>
<tr>
<td>6 mo</td>
<td>1.79 (1.66, 1.92)</td>
<td>1.80 (1.67, 1.93)</td>
<td>1.66 (1.55, 1.76)</td>
</tr>
<tr>
<td>T₃ (nmol/L)</td>
<td>24</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>0 mo</td>
<td>102.1 (94.7, 109.5)</td>
<td>97.2 (88.9, 105.5)</td>
<td>93.3 (87.5, 99.0)</td>
</tr>
<tr>
<td>3 mo</td>
<td>100.9 (93.0, 108.9)</td>
<td>92.9 (84.3, 101.5)</td>
<td>86.4 (79.3, 93.5)</td>
</tr>
<tr>
<td>6 mo</td>
<td>104.3 (97.2, 111.5)</td>
<td>98.4 (90.9, 105.9)</td>
<td>89.4 (82.3, 96.5)</td>
</tr>
<tr>
<td>FTI (%)</td>
<td>24</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>0 mo</td>
<td>2.44 (2.28, 2.60)</td>
<td>2.36 (2.19, 2.53)</td>
<td>2.29 (2.14, 2.44)</td>
</tr>
<tr>
<td>3 mo</td>
<td>2.40 (2.24, 2.57)</td>
<td>2.27 (2.07, 2.46)</td>
<td>2.13 (1.95, 2.31)</td>
</tr>
<tr>
<td>6 mo</td>
<td>2.52 (2.36, 2.67)</td>
<td>2.42 (2.25, 2.59)</td>
<td>2.24 (2.06, 2.42)</td>
</tr>
<tr>
<td>TSH (mU/mL)</td>
<td>24</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>0 mo</td>
<td>2.22 (1.94, 2.54)</td>
<td>2.04 (1.68, 2.49)</td>
<td>2.25 (1.82, 2.79)</td>
</tr>
<tr>
<td>3 mo</td>
<td>2.13 (1.88, 2.41)</td>
<td>2.39 (1.89, 3.03)</td>
<td>2.23 (1.87, 2.65)</td>
</tr>
<tr>
<td>6 mo</td>
<td>2.05 (1.83, 2.29)</td>
<td>2.22 (1.79, 2.75)</td>
<td>2.00 (1.65, 2.43)</td>
</tr>
</tbody>
</table>

1The effects of diet on hormones were evaluated for the ISP56 diet (ISP56 compared with control) and for the ISP90 diet (ISP90 compared with control) by random-effects regression models after adjustment for clustering of hormone measures within participants at 3 and 6 mo, for baseline hormone value, and for time of sample (3 mo compared with 6 mo). ISP56 and ISP90, isolated soy protein containing 56 and 90 mg isoflavones per 40 g protein, respectively.

2Arithmetic; 95% CI in parentheses.

3Significantly different from control group, P = 0.04.

4,5,7Averages of differences at 3 and 6 mo are significantly different from those of the control group: 4 P = 0.02, 5 P = 0.03, 7 P = 0.01.

6Geometric; 95% CI in parentheses.

differences could reflect the fact that the present study compared the effects of 2 concentrations of isoflavones in soy protein with milk protein, whereas the study by Duncan et al (28) was designed to compare the effects of isoflavone-rich protein with the effects of isoflavone-free protein.

The present study provides some evidence that soy protein increases measures of thyroid function. Differences reflect changes in both the experimental and control groups and could occur by chance. The differences are also small and unlikely to be clinically important. This is one of the few studies of the effects of soy on thyroid hormones in postmenopausal women.

The results are consistent with many previous studies in animals that showed elevations of T₄ and, less consistently, of T₃, FTI, and TSH after ingestion of diets containing ISP products (11–16, 53). The results are also consistent with those previously published in a study of men by Ham et al (19). In that study, however, thyroid concentrations of men who ingested soy protein were significantly higher than their concentrations at baseline but were not significantly higher than those of men who consumed control diets, suggesting a general response to dietary change. The results are not consistent with the decrease in free T₄ previously noted in premenopausal women (27) or with the lack of change in thyroid hormones noted before in postmenopausal women (28) who ingested higher amounts of isoflavones than consumed in the present study.

The fact that some of the thyroid effects were most apparent in the present study at 6 mo suggests that these may be an adaptation to long-term dietary changes. The dissociation of the effects on T₄ and the FTI compared with those on TSH, with changes in T₄ and FTI values manifested at moderate concentrations of isoflavones and changes in TSH concentration only at high concentrations of isoflavones, also suggests that there may be 2 mechanisms by which soy protein acts on thyroid function. One effect may result in an increase in the T₄ concentration, whereas another may result in a decrease in the T₄ concentration, from its binding proteins, similar to effects found previously with flavonoids (54). The other effect, at higher doses of isoflavones, may result in a decrease in the T₄ concentration, with a compensatory increase in TSH. The latter would be consistent with the decreased in vitro synthesis of thyroid hormones

TABLE 6
Pearson’s partial correlation coefficients (r) for the effects of baseline estrogenization on changes in thyroid hormones at 6 mo

<table>
<thead>
<tr>
<th>Estrogenization variable</th>
<th>T₄</th>
<th>FTI</th>
<th>TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>−0.20 [72]</td>
<td>−0.29 [72]</td>
<td>−0.24 [70]</td>
</tr>
<tr>
<td>Total BMD</td>
<td>−0.25 [65]</td>
<td>−0.29 [65]</td>
<td>−0.27 [65]</td>
</tr>
<tr>
<td>Lumbar spine BMD</td>
<td>0.04 [65]</td>
<td>0.02 [65]</td>
<td>−0.25 [65]</td>
</tr>
<tr>
<td>Serum estradiol</td>
<td>−0.29 [65]</td>
<td>−0.29 [65]</td>
<td>−0.10 [64]</td>
</tr>
<tr>
<td>SHBG-bound estradiol</td>
<td>0.06 [71]</td>
<td>0.10 [71]</td>
<td>0.002 [69]</td>
</tr>
<tr>
<td>Serum estrone</td>
<td>−0.15 [46]</td>
<td>−0.26 [46]</td>
<td>−0.06 [45]</td>
</tr>
<tr>
<td>Serum estrone sulfate</td>
<td>−0.20 [71]</td>
<td>−0.27 [71]</td>
<td>−0.02 [70]</td>
</tr>
<tr>
<td>Urinary 2OHE₁</td>
<td>−0.47 [42]</td>
<td>−0.47 [42]</td>
<td>0.06 [41]</td>
</tr>
<tr>
<td>Urinary 16αOHE₂</td>
<td>−0.20 [42]</td>
<td>−0.37 [42]</td>
<td>0.17 [41]</td>
</tr>
<tr>
<td>Urinary 2OHE₁:16αOHE₂</td>
<td>−0.16 [42]</td>
<td>−0.15 [42]</td>
<td>−0.12 [41]</td>
</tr>
</tbody>
</table>

1Values were controlled for baseline thyroid hormone values and diet group. n in brackets. BMD, bone mineral density; FTI, free thyroxine index; TSH, thyroid-stimulating hormone; SHBG, sex hormone binding globulin; 2OHE₁, and 16αOHE₂, 2- and 16α-hydroxyestrone, respectively.

2P < 0.05.
after ingestion of purified genistein and daidzein noted by Divi et al (10) and with the decreased thyroid hormone concentrations noted in a few reports of infants, most of which were in infants who were iodine deficient and which used relatively high doses of soy in formula (55–58).

The possibility that the effect of soy on thyroid hormones may be related to its estrogenic properties is suggested by the finding that changes in thyroid hormones were greatest in women with the lowest measures of estrogenicity at baseline. Estrogenic effects of soy are also supported by the associations of changes in isoflavone concentrations with changes in bone mineral density and HDL-cholesterol concentrations at 6 mo in the present study. It is possible that at different isoflavone doses soy may also act as an estrogen, as an antiestrogen, or through other mechanisms on thyroid balance. There is evidence that estrogens may increase the sensitivity of the pituitary or thyroid gland to normal feedback mechanisms (59, 60). The dissociation of changes in TSH and peripheral hormones, however, in the present study does not support this hypothesis. Previous studies also suggest that estrogens and thyroid hormones may interact in the regulation of gene expression in selected target tissues, but the effects of this interaction are not yet understood (61).

In summary, the present study does not show significant effects of soy protein on serum or urinary estrogens, SHBG, FSH, cortisol, DHEAS, insulin, or glucagon. The study does show small effects on thyroid hormones that are unlikely to be clinically important. Future animal and human studies are needed to confirm these findings and to address in more detail the mechanisms by which soy could affect thyroid hormones.

We are grateful to Lori Coward for performing the isoflavone assays and to Marion Kirk for performing the mass spectrometry analysis.

REFERENCES

Dietary treatment of iron deficiency?

Dear Sir:

We read with great interest the article by Patterson et al (1) published recently in the Journal. In the conclusion of their abstract, the authors purport to have shown that "in iron-deficient women of childbearing age, a high-iron diet produced smaller increases in SF [serum ferritin] than did iron supplementation but resulted in continued improvements in iron status during a 6-mo follow-up."

Certainly, the iron-deficient women in the diet group were advised to consume a diet high in absorbable iron for the first 12 wk of the study. However, they did not do this. Throughout the 12-wk intervention, there was no significant increase in either heme or nonheme iron intake; nor were there any significant changes in the intakes of vitamin C, meat, alcohol, phytate, calcium, or tea. Furthermore, 6 mo after the end of the formal intervention, bioavailable iron intake was, if anything, lower than at baseline; yet, the diet group’s serum ferritin concentration was slightly (2.1 g/L) higher at the end of the 12-wk intervention and moderately higher (4.2 g/L) 6 mo after the intervention. Given that any change in the intake of dietary iron or its absorption modifiers throughout the 9-mo study was negligible, what could account for the increase in serum ferritin concentration?

It is possible that at the end of the 12-wk intervention, women in the diet group chose to take or were prescribed an iron supplement because they knew that they were iron deficient and had not received an iron supplement during the study. It is also possible that because serum ferritin is an acute-phase reactant, the small increase in mean serum ferritin concentration at follow-up resulted from the inclusion of one or more individuals with a serum ferritin concentration that was elevated because of infection.

The absence of a true control group makes it particularly difficult to conclude that the changes in serum ferritin concentration were the result of an improvement in diet. The cornerstone of scientific research into the effects of diet on nutritional status is the randomized controlled trial in which participants are randomly assigned to treatment or control groups. Without an iron-deficient control group, it is difficult to quantify the effects on iron status of factors beyond the investigators’ control. For instance, it is well known that if a group of individuals is identified on the basis of a low biochemical index measured on one occasion, a subsequent measurement is likely, by chance, to be higher (ie, closer to the mean) even in the absence of any intervention effect. The only way to determine whether the increase in serum ferritin concentration in the iron-deficient diet group in this study was real, and not merely the result of a phenomenon such as regression to the mean, would be to compare it to changes in serum ferritin concentration in an iron-deficient control group. Concern about the ethics of not treating women with iron deficiency could have been minimized by recruiting women with low serum ferritin concentrations but normal hemoglobin concentrations.

In conclusion, the article does not show that dietary change is an effective treatment for iron deficiency. Rather, it strongly shows the difficulties that even motivated volunteers experience in attempting to modify their diets to increase iron absorption and suggests that iron supplementation is the most effective treatment for iron deficiency.

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REFERENCE

Extra dietary copper inhibits LDL oxidation

Wan et al (1) fed cocoa powder and dark chocolate to subjects for 4 wk and found that LDL oxidation was inhibited in vitro. This favorable effect on cardiovascular risk status was attributed to flavonoids, a group of polyphenolic compounds with antioxidant characteristics. An increase in dietary copper during this experiment may have contributed to their results.

The Western diet often is low in copper: approximately one-third of 849 diets that were analyzed provided 1 mg/d (2). The interquartile range was 0.91–1.86 mg/d. Milk chocolate and cocoa powder are in the upper quartile of 235 foods evaluated by Lurie et al (3), ranking 186 and 232, respectively, in copper concentration. Calculations using Lurie et al’s values for chocolate and cocoa powder of 2.86 and 50.0 µg/g, respectively, show that these supplements would have added nearly 1.15 mg Cu to the basal diet each day. Dark chocolate contains more copper than does light chocolate and would have increased this estimated amount even more. It seems likely that the total daily intake of copper might have been 3 times the daily estimated average requirement (0.7 mg) or twice the recommended dietary allowance (0.9 mg) for adults (4).

Although copper salts (at 10 µmol/L) and LDL are highly reactive in vitro, this phenomenon is probably irrelevant to...
human physiology because copper ions are virtually nonexistent (1 amol/L) in vivo (5–7). Indeed, after providing copper supplementation to middle-aged subjects, Rock et al (8) found that the subjects’ erythrocytes were more resistant to oxidation in vitro. Although this improvement occurred without an increase in the activity of superoxide dismutase (EC 1.15.1.1), an enzyme that provides defense against oxidative damage, the results may indicate that the subjects ordinarily ate too little copper and had other means of defense.

Perhaps the usual copper intakes of the subjects studied by Wan et al were too low also. Their basal diet probably was low in copper because it excluded beans and soybeans, 2 foods in the top quartile (see above). Control of the diets for copper intake as well as for intakes of caffeine, cholesterol, fat, and fiber would have been informative because the increased copper intake from chocolate seems smaller than some beneficial amounts given by Rock et al (8). Perhaps chocolate enhances the absorbability of copper.

Copper is an antioxidant nutrient for cardiovascular health (7) and has no prooxidant activity at a considerably higher intake (8) than that given by Wan et al. Diets low in copper are suggested as an explanation for much of the epidemiology and pathophysiology of ischemic heart disease (9). Chocolate is a pleasant dietary supplement.

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REFERENCES

Reply to LM Klevay

Dear Sir:

Klevay raises a very good question about whether or not copper could have contributed to the results of the lipid oxidation potential in the Cocoa and Dark Chocolate Study reported by researchers at Pennsylvania State University (1). Using nutrient database values, we calculated the copper content of the diets and report the following.

The base diet (averaging the 6 menus), at 2500 kcal/d, provided 0.844 mg Cu/d. (Note that not all foods in the menus have reported copper content; therefore, the total for the base diet and the experimental diet could be higher but would increase by the same amount.) The recommended dietary allowance for copper is 0.9 mg/d; therefore, the diets essentially provided the recommended dietary allowance for this micronutrient (94%). The addition of the dark chocolate (16 g/d) contributed an additional 0.128 mg Cu/d, and the cocoa powder (22 g/d) contributed, depending on the nutrient database used for values, between 0.17 and 0.83 mg Cu/d to the diets.

Therefore, the total copper content of the cocoa–dark chocolate diets was ≥ 1.14 mg Cu/d and could have been as much as 1.80 mg Cu/d. The difference between the control diet and the experimental diet was ≥ 0.298 and perhaps as much as 0.961 mg Cu/d.

Of note is that the base diet was adequate in copper. Whether the addition of the copper from the cocoa and dark chocolate was significant and whether it contributed to the effects on LDL oxidation would need to be tested. However, it is certainly possible that the copper contributed in some way to the overall antioxidant potential of the diets and the subsequent serum antioxidant capacity.

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REFERENCE
Population estimates of folate intake from food analyses

Dear Sir:

In their article on the folate intakes of the adult Dutch population, Konings et al (1) presented calculations of folate intake based on the folate contents of foods determined by liquid chromatography (LC). The dietary folate intake of the Dutch population was estimated to be 182 ± 119 μg/d. The limitations of their study, and hence possible biases in the final estimates of folate intake, require comment.

In comparison with other published data on folate intakes in European populations, the intakes presented by Konings et al (1) seem low. In the study by de Bree et al (2), average folate intakes in European populations were reported to be 291 and 247 μg/d for males and females, respectively. As part of the European Union’s fifth framework project, “Folate: From Food to Functionality and Optimal Health” (3), folate intakes for populations in 7 European countries (Finland, Germany, Italy, the Netherlands, Spain, Sweden, and the United Kingdom) were estimated on the basis of each country’s food consumption and food-composition data. Dietary folate intakes ranged from 217 to 310 μg/d, the average being 285 ± 30 μg/d for males and 230 ± 20 μg/d for females.

In addition, an intercomparison study of suitable methods of analysis for food folates was organized by using 4 certified reference materials (CRMs): CRM 121 (whole-meal flour), CRM 421 (milk powder), CRM 485 (mixed vegetables), and CRM 487 (pig’s liver). Nine project participants and the National Food Administration (Sweden) analyzed the samples. To increase the number of comparable results, the data from Konings previous publication (4) were included. The number of accepted results was 7–9 for each CRM, 3–4 of which were obtained by a microbiological assay (MA) and 4–5 by HPLC. One of the HPLC laboratories used microbiological detection (LC-MA). In addition, 2 HPLC laboratories determined 5-methyltetrahydrofolate only. The certified values for total folate in these CRMs were obtained by MA by a group of experienced laboratories (5).

Our findings indicate the current status of food folate analysis in Europe. The results obtained by HPLC were 27–40% lower than the microbiological results, except for pig’s liver. Indeed, Konings et al also reported a difference of the same magnitude (23–27%) between the results by LC and those by MA. The within-laboratory variation was generally small (3–11%), whereas the between-laboratory variation was large (7–51%). Generally, folic acid and 5-methyltetrahydrofolate could be determined with satisfactory accuracy but no agreement was found for the other vitamers.

The distribution of the folate vitamers and the total folate content of CRM 485 (mixed vegetables) are presented in Figure 1. The sum of folate vitamers was 73% of the microbiologically determined folate content for mixed vegetables but only 60% for whole-meal flour. The distribution patterns from CRMs containing several vitamers (whole-meal flour and pig’s liver) varied considerably from one laboratory to another.

There are several possible explanations for the observed discrepancy. Some of the peaks in HPLC chromatograms were often masked, which made identification and accurate quantification difficult. The existence of unidentified compounds, either folate or nonfolate, was common. It is known, for example, that 5-formyltetrahydrofolate and 10-formyltetrahydrofolate can convert to 5,10-methylenetetrahydrofolate under acidic conditions (pH < 2) used in the current HPLC mobile phases. 5,10-Methylenetetrahydrofolate does not have sufficient fluorescence to be quantified accurately. However, alternate detec-

**Figure 1.** The total folate content ( ), determined by microbiological assay (MA) in 4 laboratories and individual folate contents ( ), folic acid; ( ), 5-HCO-H4-folate; ( ), 5-CH3-H4-folate; ( ), H4-folate) determined by HPLC (LC) in 6 laboratories of certified reference material 485 (mixed vegetables) in the European intercomparison study. The SD of the certified value is indicated by the horizontal dashed lines. The results of Konings (4) are included in LC 3. LC-MA, HPLC with microbiological detection; DM, dry matter.
tion systems, such as electrochemical or mass spectrometry, have been developed (6).

There is also the possibility that the bacteria used in the MA gave unequal responses to the different vitamers or can be affected by some nonfolate compounds in the samples. However, the latter was evaluated on the basis of the combined LC-MA results. The data clearly show the absence of any nonfolate peaks with organism activity.

We conclude that the MA is preferred for the determination of the total folate content of foods, especially if data on individual folates is not considered necessary. Furthermore, we stress the need for careful validation of HPLC methods. Given the considerable, yet unidentified, difference between the microbiological and HPLC data, we advise caution when estimating dietary folate intakes at the population level.

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REFERENCES

Reply to Kariluoto et al

Dear Sir:

We appreciate the reaction from Kariluoto et al regarding our paper (1), particularly on the population intakes, which are based on newly established food folate data. We completely agree that this is an important issue. Kariluoto et al referred to an estimated intake of $182 \pm 119 \mu g/d$ for a population aged $1–92$ y and compared these data with data for adults. Our estimated intakes were $173$ and $215 \mu g/d$ for women and men, respectively.

Kariluoto et al reported that as a part of the European Union’s fifth framework project, baseline folate intakes for populations across 7 European countries were studied by using data based on each country’s food-composition data. An important problem with estimating folate intakes is the lack and the unreliability of the data on folate content in food-composition tables (2). Additionally, in 1996, a European working group, “COST 99,” compared folate data in food-composition tables for some vegetables, milk, bread, and cereals from 7 European countries. Total folate was measured by microbiological assay (MA). These food folate data showed a 2–3-fold variation (PM Finglas, unpublished observations, 1996), which indicates that folate intakes cannot be compared between countries.

In an intercomparison study that analyzed 4 certified reference materials (CRMs), estimated folic acid and 5-methyltetrahydrofolate contents were comparable in 4–5 laboratories, but no agreement in the other vitamers was found. Other than our previously published results (3), no other participating laboratory used affinity chromatography as a cleanup step before the final determination of all other vitamers with HPLC. Selhub (4) and Pfeiffer et al (5) used this technique successfully earlier. It must be stressed that only this cleanup procedure is suitable for determining the folate content in food-sample extracts when HPLC is used. Anion-exchange purification alone or solid-phase extraction leaves many interfering compounds in the chromatogram, which hampers interpretation and accurate quantitation (3, 6).

It is not correctly stated by Kariluoto et al that 5-formyltetrahydrofolate, 10-formyltetrahydrofolate, or both convert to 5,10-methylenetetrahydrofolate under acidic conditions. In particular, 10-formyltetrahydrofolate could be converted to 5,10-methylenetetrahydrofolate rapidly (ie, on column conversion) because of the low pH of the mobile phase (5).

5-Formyltetrahydrofolate also undergoes formation of 5,10-methylenetetrahydrofolate in acidic media, although at a much slower rate (5). Thus, the acidic mobile phase used is fully suitable for separation and quantification of 5-formyltetrahy-
HPLC should be the method of choice. Foods to ensure accurate quantification of total folate contents. The standardization of methods for the determination of folate in relevant folates in foods with reliable and reproducible results (3). This does not necessarily mean that the response of bacteria to nonfolate compounds is absent in all food matrices. According to Kariluoto et al., liquid chromatography–microbiological assay (LC-MA) showed the absence of any nonfolate peaks with organism activity, and all other peaks with microbiological activity add up to the same amount as found by HPLC in other analyses (Figure 1 of Kariluoto et al’s letter). This does not explain the discrepancy between the results by HPLC and MA. One would expect equal amounts between results of LC-MA and MA if MA is preferred as the standard method. According to these results, HPLC is preferred to MA as the standard method.

The determination of folates by MA is grounded on biological activity, whereas the determination by HPLC is grounded on the actual detection of separate folate vitamers. Problems with the response of Lactobacillus casei to different folate vitamers, as reported by Phillips and Wright (9), and the influence of nonfolate compounds on the bacterial growth response are still not refuted and might explain the difference between the HPLC and MA results.

New, accurate food-composition data for folates are needed. Our study (1) was the first comprehensive assessment of a large range of folate-containing foods. Furthermore, this study showed the effect of new analytic data for folates, including identification and quantification. In light of current knowledge, we determined all relevant folates in foods with reliable and reproducible results (3).

With regard to the relative importance of accurate food folate data, because they are frequently used in epidemiologic analyses in which intakes are related to disease endpoints, we recommend the standardization of methods for the determination of folate in foods to ensure accurate quantification of total folate contents. HPLC should be the method of choice.

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**REFERENCES**


**Lessons learned in iron intervention trials**

Dear Sir:

The December 2001 issue of the Journal featured the coincident publication of 3 articles related to anemia and its partition between iron deficiency and non-iron deficiency origins among African preschoolers living in areas of endemic malaria (1–3). Our attention was drawn to the article concerning an iron-supplementation intervention trial (3), which reported difficulties and caveats reminiscent of an experience of ours in Guatemala (4).

In the planning of their study, Zlotkin et al (3) approached their power and sample-size assumptions in the following manner: “On the assumption of 90% cure rates in the drops group and 80% in the sprinkles group and with a type I error set at 0.05 and a 0.9 probability of detecting a true difference, the final sample size estimate was 286 subjects per group.” They expected up to 90% of anemic Ghanaian infants and toddlers to have iron deficiency and iron-responsive anemias. This expectation raises 2 points in our minds. The first of these points is that such an expectation conflicts with the sense of the introductory section of their paper, where they state, “…we tested the hypothesis that the response to treatment of anemia would be better with 2 mo of treatment with microencapsulated ferrous fumarate sachets daily than with ferrous sulfate drops…” (3). Both of these statements use wording that seems to refer to a one-tailed test, but with the latter statement inclining toward superiority for the sprinkles and the former statement inclining toward a 10% better efficacy for the drops. Thus, it is important that the authors clarify whether the study was powered for a one-tailed or two-tailed test of the hypothesis of dif-
ference and that they specify how many degrees of freedom were assumed for the statistical comparison. This is not to question their conclusion of no difference between 58% and 56% recovery rates, but to address a conceptual point. If a one-tailed test were conducted as stated with the power assumptions, then no conclusions of superiority for sprinkles could have been possible; one would be testing an asymmetric hypothesis to find either 1) that the ferrous sulfate was 10% better than fumarate or 2) that it was not.

The second point relates to their assumptions about the magnitude of the response to anemia treatment. The actual findings in their study fell far short of their expectations for a cure rate: in the 2-mo trial, 80 mg microencapsulated ferrous fumarate added to porridge (sprinkles) cured 56.3% of the anemia, and 40 mg ferrous sulfate in elixir (iron drops) cured 57.7% of the anemia. The optimistic interpretation of this finding is that 40% of the children were compromised in their ability to respond to iron because their anemia was due to malaria, another infection, or some other micronutrient deficiency. Thus, in fact, 100% of the “truly responsive” candidates in both wings of the intervention trial were cured by the iron treatments. Such a direct and simple interpretation could be seen as being bolstered by the coincident findings in malarial areas of Kenya (1) and the Ivory Coast (2) that 22% and 44%, respectively, of the subjects with anemia did not have iron deficiency and by the authors’ own findings that malaria-infected children have a 23% lower chance of responding to iron treatments than do children without malaria (3).

Unfortunately, a more complex and pessimistic interpretation seems to be more logical; this interpretation even challenges the possibility of attributing any specific fraction of the anemia response to the iron treatments in the absence of concurrent observations on a third treatment wing, which serves as a placebo control. The authors address this issue in their statement: “Because it would be unethical to provide a placebo to a child with anemia, we did not include a placebo control” (3). Such a proclamation of ethical absolutism could be taken more seriously had it not come from 2 of the institutions that conducted one of the largest placebo-controlled trials of vitamin A and child morbidity and mortality ever conducted (5, 6). Are we to accept that 2 mo of observation of children with hemoglobin in the 70–99-g/L range is unethical, but it is acceptable to follow children at risk of hypovitaminosis A for an entire year in a placebo trial with mortality among the outcome variables? The consequence of this doctrine for anemia research in Ghana would mean that any field team in that country must surely bring liters of iron drops along with the HemoCue apparatus and lancets in their back packs, because teams would be obliged to treat almost all comers after any survey. This drawback is especially valid if one realizes that newer international standards for “anemia” in this age group have a cutoff of 110 g/L (7). If 65% of Ghanaian infants and toddlers in this region have a hemoglobin below the 100-g/L anemia definition used to enroll a presumably iron-responsive sample (3), over 90% might be classified as anemic with the more conventional 110-g/L criterion. It would be worth knowing what treatment, if any, was offered by Zlotkin et al to those children found on initial screening to be in the <70-g/L range of hemoglobin (ie, severely anemic) and those found to be in the 100–109.9-g/L range (ie, mildly anemic by international standards).

Without a placebo group, moreover, we are at a loss to assess the attributable efficacy of either form of iron in this study (3). Because all the children were chosen to be in the lower part of the hemoglobin distribution, mathematical regression to the mean would account for some of the test-retest increment in hemoglobin concentration. This regression-to-the-mean term, moreover, might be larger than anticipated, given the fact that capillary finger-stick samples were used. Morris et al (8) examined the reliability (within-individual variability) of measurements of hemoglobin concentrations in capillary blood. From samples taken from the same persons, either concurrently from different anatomical sites or on consecutive days, they showed that concentration measurements in capillary blood have lower reproducibility than do those in venous blood, which is attributable to larger biological variation with peripheral sampling than with venous sampling. Hence, those children admitted into the eligible study pool on the basis of a hemoglobin concentration just below 100 g/L at baseline might have a hemoglobin concentration just above the criterion level at the second measurement merely because of the inherent unreliability of capillary samples (8).

Another reason for the improved anemia status of the subjects may simply have been the development of the subjects during the 2 mo of the study; anemia rates tend to improve with age after infancy. Moreover, the effects of regression to the mean or asymmetrical diagnostic misclassification, in addition to developmental changes, could account for some of the observed improvements in the rates of anemia; thus, to determine the true degree of improvement in anemia attributable to the iron treatment, there would have to be a no-treatment (placebo) wing in the study design.

Aside from the issues of quantification with regard to efficacy, we also encountered a problem in the authors’ evaluation of safety. The authors comment on the similarity of the 14.5% rate of diarrhea with the ferrous sulfate treatment and the 12.8% rate with the ferrous fumarate treatment (3). Although we can readily accept the conclusion of no difference between treatments with regard to diarrhea experience, the design does not provide sufficient evidence to safeguard against intrinsic adverse effects of microencapsulated ferrous fumarate. Only by knowing that a no-treatment group also had diarrhea incidence in this range could we exonerate the iron treatments of causing the gastrointestinal symptoms. With regard to the assumptions of safety for a new treatment, moreover, its comparison with a placebo is procedurally indispensable. Even if the authors offer us “efficacy relativism” in the conclusion on the basis of comparative effects with the proven therapy as the standard for anemia cure (3), we simply cannot accept this logic of “safety relativism” with regard to adverse effects. It would not be sufficient to argue that sprinkles are safe on the basis of the fact that they do not have any more adverse effects than the elixir form of ferrous sulfate. The latter is an old medication that may have been grandfathered into the pharmacopeia with safety criteria that we may not now accept for a new agent or format. For new questions of safety, comparison against no exposure would still seem to be needed at some point.

The final conundrum for the ethical imperative to treat all anemias on sight comes from the findings of the 3 previously mentioned African studies in malarial areas. In Kenya, 22% of anemic children with a median age of 19 mo did not have iron deficiency anemia (1); in the Ivory Coast, 44% of anemic children with a mean age of 49 mo did not have iron deficiency anemia (2); in Ghana, 40% of anemic children with a mean age of 13 mo did not have iron-responsive anemia (3). Malaria is not the only possible confounding factor. In our 10-wk, placebo-controlled intervention with heme iron and ferrous sulfate in children with a mean age of 21 mo in the nonmalarial Guatemalan highlands, ≈43% of anemic children (hemoglobin concentration <115 g/L) had no evidence of iron deficiency (4). Thus, if we are to believe Zlotkin et
al that “it would be unethical to provide a placebo to a child with anemia,” the question is what do we treat the child with to cure the anemia? Iron will work for some children, although we do not know a priori which ones; what do we offer to the rest, and when?

This is not just an ethical question for investigators and their populations, who are screened for eligibility on the basis of anemia. It underlies a very important program and policy issue for micronutrient deficiency interventions in developing (and developed) countries: when is anemia a nutritional anemia, and when is a nutritional anemia an iron deficiency anemia? The results of all of the studies from Africa and Central America cited in this letter seem to converge toward a conclusion that “close to 100%” can no longer be the answer for either question. We may be obliged to do the “unthinkable,” namely, to screen and diagnose anemias before assigning treatments both in human research and in the public health domain. The experience reported in the articles from Kenya (1) and the Ivory Coast (2) provides a basis for such diagnostic screening.

Finally, the authors leave some numerical issues to be resolved. Although they reported screening 880 children and enrolling 557, in the side effects subsection of Results, they state, “Seventy-four percent (933 of 1277) of the mothers of children in the drops group” and “Diarrhea was reported in 76 of 523 ... subjects in the drops group and in 62 of 486 ... in the sprinkles group.” These latter figures must represent typographical errors that were not detected in editing.

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REFERENCES

Reply to NW Solomons and K Schümann

Dear Sir:

The major criticism by Solomons and Schümann of our recently published study (1) was our lack of a placebo control in the protocol, although their stance on this issue is equivocal, because they also criticize previous studies that included a placebo control. We stand firmly by our decision not to include a placebo control group. We recognize the extensive international debate on this issue (2, 3). In our study of infants in Ghana who had moderately severe anemia, subjects in a placebo group would have received no treatment, which is exactly what they would have received had they not been enrolled in the study. Yet, there is extensive literature on the adverse effects of moderately severe anemia (4). If the current local standard therapy is to do nothing, is it justifiable to include that standard therapy as a study arm? We agree with other ethicists that the standard of providing no less than people are able to get in their own country (which often is nothing) is a standard that is too low (S Benetar, unpublished observations, 2000). We strongly believe that, in clinical research, it is not ethical to offer a “treatment” of doing nothing just because that is what otherwise would be available to the population under study.

Solomons and Schümann make mention of earlier placebo-controlled vitamin A supplementation studies in Northern Ghana by members of our research group, which were conducted between 1988 and 1991 (5, 6). These studies formed part of a second generation of important field trials carried out in several countries that verified the landmark findings of Sommer et al (7). At that time, there was no global policy on universal vitamin A supplementation to children; nor was there a national program in Ghana. The protocols for these trials were reviewed and approved by the relevant ethics committees and the Ministry of Health of Ghana. We believe that it is unethical to withhold treatment of anemia today, and similarly, it would be unethical today to conduct a placebo-controlled trial of vitamin A supplementation in children. This issue begs the larger question of how to identify when the global scientific community has concluded that a specific research question has been adequately answered. In the case of treatment of iron deficiency anemia, we believe that the question has been answered and that further placebo-controlled confirmation studies are both unjustifiable and unethical.

Solomons and Schümann suggest that the biological impact of our not having included a placebo control group would place them at “a loss to assess the attributable efficacy of either form of iron in this study.” Statistically speaking, this is true, but is there any biological plausibility to their statistical contention? We believe there is very little. First, the study was of very short duration. What possible mechanisms would lead to significant improvement in anemia in a group receiving no intervention for 2 mo? Because we believe that the primary mechanism causing the anemia was a lack of bioavailable dietary iron, we would have to ask whether a no-intervention group could have rapidly improved their diet with more sources of bioavailable iron, such as meat or poultry. This could have happened if there had been a significant improvement...
in the economic status of families in the region—for example, that resulting from the sudden discovery of gold or oil deposits. That did not happen, and, to the best of our knowledge, the economic standards of the communities in the region remained static (and very poor). Thus, we doubt that anemia would have improved if a no-intervention group had been included.

Regression to the mean might have accounted for some of the test-retest increment in hemoglobin concentrations, but true regression to the mean is a time-dependent statistical measure. Over the relatively short intervention Solomons and Schümann describe, regression to the mean would have been negligible. They also suggested that the wide biological variation in capillary finger-stick samples (wider than that in venous samples) might have contributed to the regression to the mean. Again, we disagree. Although we acknowledge the wider variation in capillary samples, the variation may result in either an overestimation or an underestimation of hemoglobin concentration (compared with concentrations in venous samples) and thus would not contribute to a regression to the mean (which would imply a bias only to higher hemoglobin values).

There is nothing in the literature to support Solomons and Schümann’s contention that economically disadvantaged infants with anemia due to dietary inadequacy will rapidly improve with no intervention. Thus, although we cannot calculate the attributable change in incidence of anemia in the population we studied, we maintain that the argument against any treatment is fallacious.

Solomons and Schümann question our ability to comment on safety without having included a no-treatment group. They accuse us of “efficacy relativism.” We are guilty as charged, but we would argue that we did not include a no-treatment group for the reasons previously outlined and, perhaps more important, that neither ferrous fumarate or sulfate needs the rigorous scrutiny of a “new treatment.” Both have been used for decades with documented side effects such as dark stools, strong metallic taste (ferrous sulfate), staining of teeth (ferrous sulfate drops), and change in gut flora but little else. In sprinkles, ferrous fumarate is coated with a thin layer of soybean lipid to mask the taste of the iron and to prevent changes in the color or taste of the food to which the sprinkles are added. But the form of iron (ferrous fumarate) is not new, and, indeed, in our reported study, given that the fundamental comparison was between the 2 formulations (ferrous sulfate), staining of teeth (ferrous sulfate drops), and change in gut flora but little else. In sprinkles, ferrous fumarate is coated with a thin layer of soybean lipid to mask the taste of the iron and to prevent changes in the color or taste of the food to which the sprinkles are added. But the form of iron (ferrous fumarate) is not new, and, indeed, in our reported study, given that the fundamental comparison was between the 2 formulations (ferrous sulfate), staining of teeth (ferrous sulfate drops), and change in gut flora but little else. In sprinkles, ferrous fumarate is coated with a thin layer of soybean lipid to mask the taste of the iron and to prevent changes in the color or taste of the food to which the sprinkles are added. 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Erratum


It is incorrectly reported in the Abstract that the women gained 2.5 kg fat-free mass, rather than the 2.0 kg correctly reported in Table 2 and discussed in the text. In Table 3, the equation of Jackson et al (23) is incomplete, and the coefficients provided are wrong. Readers are unable to calculate a meaningful value from this equation and cannot determine which equation from the cited paper was used. Friedl et al actually used the first of the 18 equations published in Jackson et al’s article (see below), and this was their intention. Friedl et al have verified that the calculations were done correctly in the master data set and that their analyses were appropriately based on these calculations. The correct equation is

\[
\text{Body density} = 1.0970 - 0.00046971(\text{sum of 7 skinfold thicknesses}) \\
+ 0.00000056(\text{sum of 7 skinfold thicknesses})^2 - 0.00012828(\text{age})
\]

Friedl et al regret the inconvenience that these errors may have caused their readership.

Erratum


In Table 2, the units for plasma isoflavone concentrations should be nmol/L instead of μmol/L.