Correcting a marginal riboflavin deficiency improves hematologic status in young women in the United Kingdom (RIBOFEM)[1–3]

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ABSTRACT

Background: Moderate riboflavin deficiency is prevalent in certain population groups in affluent countries, but the functional significance of this deficiency is not clear. Studies have indicated a role for riboflavin in the absorption and use of iron.

Objective: We investigated the effect of riboflavin supplementation on hematologic status in a group of moderately riboflavin-deficient women aged 19–25 y in the United Kingdom.

Design: One hundred twenty-three women with biochemical evidence of riboflavin deficiency [erythrocyte glutathione reductase activation coefficient (EGRAC) >1.40] were randomly assigned to receive 2 or 4 mg riboflavin or a placebo for 8 wk. Measurements of hematologic status were made pre- and postsupplementation, and dietary intakes were also assessed; iron absorption was measured in a subgroup of women.

Results: One hundred nineteen women completed the intervention. The use of a riboflavin supplement for 8 wk elicited a significant improvement in riboflavin status with a dose response ($P < 0.0001$). For women who received supplemental riboflavin, an increase in hemoglobin status correlated with improved riboflavin status ($P < 0.02$). Women in the lowest tertile of riboflavin status at baseline (EGRAC >1.65) showed a significantly greater increase in hemoglobin status in response to the supplement than did women in the first and second tertiles ($P < 0.01$). Dietary iron intake and iron absorption did not change during the study.

Conclusions: Moderately poor riboflavin status can affect iron status: the lower the riboflavin status, the greater the hematologic benefits of improving status. The results also suggest that consideration should be given to raising the currently accepted EGRAC threshold for deficiency. This trial was registered at controlled-trials.com as ISRCTN35811298. *Am J Clin Nutr* 2011;93:1274–84.

INTRODUCTION

Riboflavin deficiency is endemic in many populations in which diets low in meat and dairy products are consumed, but more surprisingly, a high prevalence of riboflavin deficiency was reported in various population groups in affluent countries including the United States, France, and the United Kingdom (1–5). Recent National Diet and Nutrition Surveys (NDNSs) of the United Kingdom have revealed a high prevalence of biochemical riboflavin deficiency in 41% of free-living elderly people and in 95% of adolescent girls (1, 3, 4). The functional significance of riboflavin deficiency is not fully understood.

The most commonly used method for the determination of riboflavin status is the stimulation of the FAD-dependent enzyme glutathione reductase in erythrocytes, expressed as an activation coefficient [erythrocyte glutathione reductase activation coefficient (EGRAC)]. Although EGRAC is often referred to as a functional marker of riboflavin status, this is only true in the sense that changes in EGRAC can be related to the altered enzyme activity, but over a broad range of EGRAC values in human subjects, there is no well-characterized change in the physiologic function or flux through a metabolic pathway. Furthermore, although the conventional upper EGRAC threshold for normality is 1.30, this has not been established on strictly functional criteria, and further consideration should be given to the functional significance of EGRAC values above this threshold.

The majority of studies that examined the functional effects of riboflavin deficiency have been carried out in animal models of deficiency (6–8) or populations in which riboflavin intakes were very low, and riboflavin deficiency was endemic (9–12). Nevertheless, the findings were important and informed the debate regarding functional effects of more marginal deficiency states.

Of the various documented effects of poor riboflavin status in animals and humans, the evidence is strongest for effects that related to iron handling and hematologic status. Early studies in animals and human populations reported anemia associated with acute riboflavin deficiency (13, 14), and additional studies in riboflavin-deficient animals showed an impaired accumulation of hepatic iron, which was suggestive of lower absorption (15). Flavin-dependent enzymes that can mobilize iron from ferritin have been shown to be sensitive to riboflavin depletion in animal models (6, 16, 17). Through such a mechanism, riboflavin may influence iron handling at different sites, including absorption. Additional studies confirmed that riboflavin deficiency lowered iron absorption in animal models of deficiency (7, 18). A small number of riboflavin intervention studies in humans have indicated that an improved riboflavin status may have beneficial effects on indicators of iron status (9–11, 19).

In consideration of the recognized high prevalence of abnormally elevated EGRAC in young women in the United Kingdom

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and the lack of clarity with respect to the functional significance of this finding, a study was carried out to examine the hypothesis that the improvement of riboflavin status in young women with moderate riboflavin deficiency in the United Kingdom would lead to improvements in indicators of iron status.

SUBJECTS AND METHODS

Recruitment

Ethical approval for the study (RIBOFEM) was granted by the Sheffield University Research Ethics Committee (SMBRER15; Sheffield, United Kingdom).

Screening

The study was advertised through direct e-mails to Sheffield University student and staff volunteer lists, posters, and a website. Female volunteers were sought who self-reported a consumption of <250 mL milk/d (to exclude high milk consumers) and were healthy, aged between 19 and 25 y, and reported not using nutritional supplements. Suitable candidates were invited to a screening clinic at the Clinical Research Facility in the Royal Hallamshire Hospital (Sheffield, United Kingdom) where further information was given and consent to participate in the study was taken. Additional information was also collected, and 500 μL blood was removed by using a finger prick. The nonfasting blood sample was used to measure riboflavin status by EGRAC and plasma ferritin. Women with moderately low riboflavin status as assessed by an EGRAC value >1.40 were invited to take part in the study.

Exclusions

Women were excluded if they had any preexisting hematologic disorders, had given blood in the previous 3 mo, or were taking nutritional supplements. Women were also excluded if they had any diagnosed gastrointestinal problems such as Crohn's disease or celiac disease or were pregnant or breastfeeding.

Randomization to treatment

Eligible women were randomly assigned to receive 1 of 3 supplements as follows: a placebo, 2 mg riboflavin (2 mg group), or 4 mg riboflavin (4 mg group). Supplements were obtained from Research Products Ltd (Blackpool, United Kingdom), and the researchers and volunteers were blinded to the identity of the capsules. The riboflavin content in a random sample of each type of capsule was confirmed by HPLC with a Chromsystems kit (Chromsystems, Munich, Germany). A computer-generated randomization schedule was used in a 3-block randomization protocol that stratified for plasma ferritin concentrations <15, 15.1–59.9, and >60 ng/mL to ensure a comparable distribution of values across the 3 treatment arms. In addition, 1 in 4 women were allocated to the iron-absorption arm of the study by a random sampling from women in the placebo and 4 mg groups.

Power calculation

Main study

There was a lack of consistency in the design of studies that reported an improvement in hemoglobin status after riboflavin supplementation in terms of the size of the dose and duration of the intervention and baseline riboflavin and iron status. However, the study of Buzina et al (19) was the most relevant in that the study population was only moderately riboflavin deficient. This group showed an increase of 7.0 g hemoglobin/L over 12 wk of a modest supplement of 3 mg riboflavin/d. To achieve an improvement of 7.0 g hemoglobin/L in a population with an estimated baseline ($\pm$SD) hemoglobin concentration of 135 $\pm$ 10.5 g/L (4), it was calculated that we required 31 subjects/study arm. This would give a power of 90%. We aimed to recruit 40 volunteers to each arm and allowed close to a 15% dropout. On the basis of an expected level of riboflavin deficiency in this population of 66% (4), we anticipated needing to screen $\geq$200 young women.

Iron-absorption study

It was estimated that 15 subjects/group would be required to detect a 5% difference between the 4 mg and placebo groups in terms of iron incorporation into erythrocytes in response to the intervention. This estimate was based on a mean ($\pm$SD) absorption of 15 $\pm$ 5% (20) with a power of 90% and a significance level of 0.05.

Protocol for the intervention

Eligible women were invited to take part in the intervention study. As far as possible, the dates for baseline blood collections were arranged to coincide with the middle of the subject's menstrual cycle to reduce any effects of menstrual bleeding on measures of iron status. In practice, this proved quite challenging; 63% of women started the study in $\leq$7 d from the midpoint of their menstrual cycles. For other women, the timing was random. Once dates for all clinic visits had been agreed with the volunteer, an information pack that contained a 4-d food diary to complete, a food-portion booklet, and a guide to the study was sent by mail.

Subjects attended the Clinical Research Facility at the Royal Hallamshire Hospital for all study clinics, which were held between 0800 and 1000. Women were requested to fast from midnight of the previous night. Frequent contact was maintained by telephone or e-mail to remind volunteers of clinic dates, the requirement for a fasted blood sample, and the need to complete food diaries.

All women were expected to attend 4 clinic appointments (baseline, week 0; interim, week 4; postintervention, week 8; and follow-up, week 12). A fasted blood sample was collected for hematologic-status measurements and biomarkers of riboflavin status at the baseline and postintervention visits, and a nonfasted finger-prick blood sample was collected for plasma ferritin and EGRAC at the interim and follow-up visits. In addition, because changes in intakes of vitamins C, folate, B-6, and B-12 might also have influenced indicators of iron status, biochemical status measurements were made for these nutrients before and after the intervention to support the dietary information. At the first visit, height and weights of subjects were measured and a second food diary was given to subjects for completion by the interim visit. The diaries were discussed at subsequent clinics to ensure that all of the information was complete and accurate.

Women who took part in the iron-absorption study attended additional clinics. All clinic details are given below.

Protocols for the intervention and iron-absorption component are summarized in **Figure 1**.

Intervention-study clinics

Baseline clinic (week 0)

The clinic provided an opportunity to explain the study in more detail and answer any questions. The height and weight of each subject were measured. The completed food diary was returned by the volunteer, and the responses were discussed with the researcher. The next food diary was given, and dates were agreed upon for completion before the next visit. A fasted blood sample was taken (15 mL in an evacuated tube containing EDTA) for baseline biochemistry and hematologic measurements. Volunteers were given capsules and instructed to consume one capsule/d with water.

Interim clinic (week 4)

A finger-prick blood sample was taken (500 μL in a microvette containing EDTA) for measurements of EGRAC and ferritin to allow for the detection of an early response to the intervention. The second completed food diary was collected and discussed.

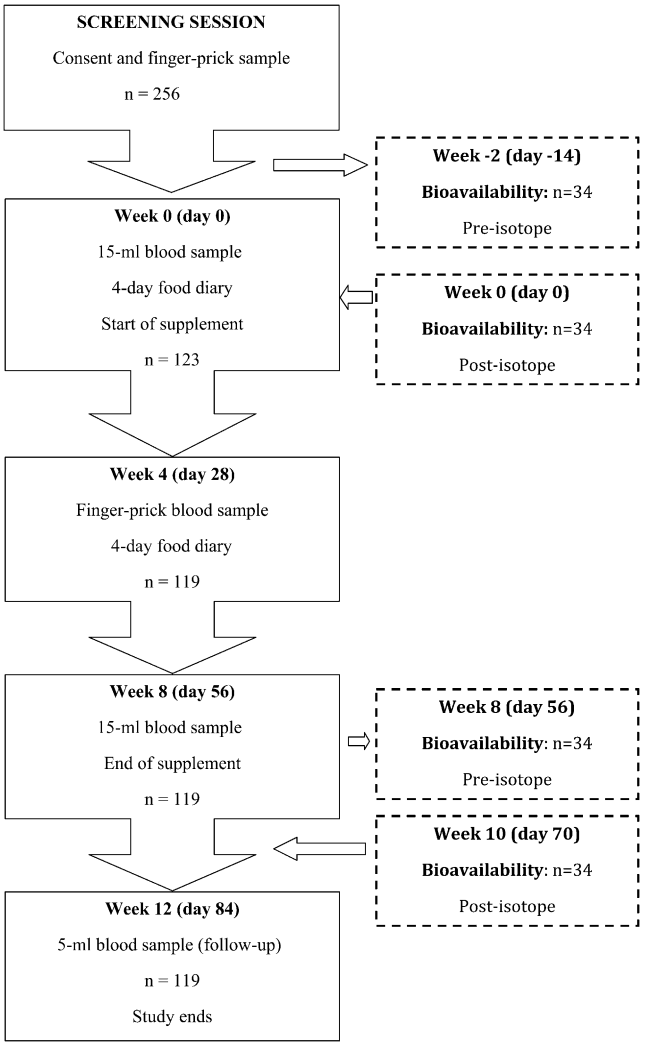

FIGURE 1. Flow diagram for participants in the screening and intervention.

Postintervention clinic (week 8)

A fasted blood sample was collected (15 mL in an evacuated tube containing EDTA) for postintervention biochemistry and hematologic measurements. Any remaining capsules were collected and counted.

Follow-up clinic (week 12)

An additional blood sample was taken (5 mL in an evacuated tube containing EDTA) for EGRAC and ferritin measurements to allow for the evaluation of the persistence of any response to the intervention.

Additional clinics for the iron-absorption study

Additional information regarding this aspect of the study was sent to the women before attending the first clinic. They were advised not to eat red meat for 24 h or drink alcohol for 12 h before the first clinic.

Iron absorption: first preisotope clinic (2 wk before baseline clinic)

Fasted volunteers attended the Clinical Research Facility, University of Sheffield, to receive the initial dose of the iron stable isotope (highly enriched with ^{58}Fe). Before receiving this first dose, 5 mL blood were collected into a trace element–free lithium-heparinized evacuated tube and transferred into acid-washed microcentrifuge tubes for background isotopic analysis. The isotope was given in 2 separate 2-mg doses, one with a specially prepared breakfast and the other with a specially prepared lunch. Just before administration, each dose was diluted with a lemonade drink and given to the volunteer within 10 min of preparation. The drink that contained the highly enriched ^{58}Fe was given with a simple breakfast of toast and jam and dairy-free spread that was consumed at 0900. Breakfast was followed, 4 h later, by a lunch of pasta and instant tomato sauce, a plain bread roll, and a second drink that contained the highly enriched ^{58}Fe isotope. Beverages and foods were carefully chosen so as not to contain any known enhancers or inhibitors of iron absorption. The beverage was free from phosphoric acid, tannin, and vitamin C according to the manufacturer's product description, and the flavor and fizzy nature of the drink masked any taste of the iron. In addition, meals were chosen on the basis of a negligible iron or riboflavin content. The pasta sauce was selected on the basis of its negligible vitamin C content, and this was confirmed by an enzymatic analysis by using the method described by Vuilleumier and Keck (21) with an automated centrifugal analyzer (COBAS BIO; Roche, United Kingdom). Volunteers were allowed to leave the facility between meals but were instructed not to eat, drink, or exercise between meals or for 2 h after the lunch. Water was permitted ad libitum.

Iron absorption: first postisotope clinic

This clinic coincided with the baseline clinic for the main study. In addition to the blood samples taken for the main study, an additional blood sample was collected (5 mL into a trace element–free heparinized evacuated tube).

Iron absorption: second preisotope clinic

This clinic coincided with the postintervention clinic of the main study. At this clinic, women repeated the isotope, meal, and blood sampling regimen as for the first isotope clinic.

Iron absorption: second postisotope clinic (2 wk after end of intervention)

Fasted blood samples (5 mL) were collected into trace element-free heparinized evacuated tube and transferred into acid-washed tubes for the postintervention isotopic analysis.

Blood handling and biochemical analyses

Finger-prick blood samples collected at the screening and interim visits were centrifuged to separate the plasma from red cells. Red cells were washed with PBS and lysed with an equal volume of water, and the hemolysates were stored at −20°C for the assessment of riboflavin status by using the EGRAC assay. Plasma was stored at −20°C for ferritin analyses.

At the baseline and postintervention visits, 10 mL fasted blood were collected by venipuncture into evacuated tubes containing EDTA and separated by centrifugation (10 min at 1000 × *g*) into plasma and red cells. Red cells were washed, hemolyzed, and stored at −20°C for EGRAC measurement as previously described. The plasma was divided into aliquots; one aliquot was stored at −20°C for ferritin, and the other aliquots were stored appropriately at −80°C for the measurement of other variables. For the measurement of plasma 5-methyltetrahydrofolate, plasma was stored with ascorbic acid (500 μL plasma with 10 mg ascorbic acid); for the measurement of plasma ascorbic acid, 50 μL plasma was stored with 450 μL metaphosphoric acid. Plasma was stored without preservative for transferrin receptor, C-reactive protein (CRP), flavins, pyridoxic acid (PA), and vitamin B-12. An aliquot of plasma was also stored for the measurement of total homocysteine as a putative functional biomarker of riboflavin status (22, 23). An additional 5 mL blood was collected into a lithium-heparinized evacuated tube and sent to the Hematology Department at the Royal Hallamshire Hospital for a full blood count and zinc protoporphyrin (ZPP) measurements.

At the follow-up visit, a single 5-mL fasted blood sample was collected into tubes containing EDTA for the measurement of EGRAC and plasma ferritin. All blood handling in the laboratory was carried out in subdued light to minimize effects of light on flavin concentrations.

Stable isotope oral dose preparation

Isotopically enriched iron-sulfate solutions were prepared from elemental Fe-58 (93.2% ^{58}Fe and 95.2% ^{58}Fe). Chemgas, Boulogne, France) on the basis of a previously described method (24). Briefly, the metal was dissolved in nitric acid (60% Ultrapur; VWR International Ltd, Lutterworth, United Kingdom) and the mixture was heated slowly in a silica crucible until almost dry before the addition of 15 mL 0.5M H_2SO_4 (made up from sulphuric acid, 96% Ultrapur; VWR International Ltd). This mixture was heated slowly until dryness, and the resulting powder was placed in a muffle furnace at 500°C for 30 min and redissolved in 0.2M H_2SO_4. The iron-sulfate solution was filtered through a 0.22-μmol/L filter (Millipore, Watford, United Kingdom) and divided into individual portions that were stored at −20°C under nitrogen gas. The isotope was transported to The University of Sheffield (United Kingdom) on dry ice and stored at −20°C until required.

Mass spectrometry measurements

Iron-isotope enrichment was measured in whole blood collected into trace element–free evacuated tubes containing EDTA (Sarstedt, Leicester, United Kingdom) and stored in acid-washed Eppendorf tubes at 20°C. The 0.5-mL aliquots were processed according to the method previously described (20). Samples were diluted to 0.5 μg Fe/mL with 2% HNO_3 before isotope-ratio analysis with a single focusing multicollector inductively coupled plasma mass spectrometer (Isoprobe; Micromass, Manchester, United Kingdom). The multicollector inductively coupled plasma mass spectrometer had a desolvating sample introduction system with a microconcentric nebulizer (Aridus and T1H; Cetac, Omaha, NE). Samples were randomly run in duplicate (90%) or triplicate (10%) throughout a run and were calibrated against IRMM014 (25), which was measured before and after each sample.

Iron-absorption calculations

Iron absorption was calculated from an equation that included the ratio of iron isotopes in red blood cells, the content of iron in whole blood, and the blood volume (26). An assumption was made that 80% of absorbed iron appears in red blood cells 14 d postabsorption (27).

Dietary analyses

To evaluate any dietary changes during the intervention, volunteers were asked to complete a 4-d food and beverage diary, including one weekend day, before the baseline visit and again before the interim visit, with photographs from the Ministry of Agriculture, Fisheries, and Food food atlas for the assessment of portion sizes (28). The diary was sent to the participant before the first visit and discussed with the researcher at the first visit for checking legibility, the correct entry of food types, and verification of portion sizes. The second diary was given at this visit for completion before the next interim visit. Data from the food diaries were analyzed for micronutrient and macronutrient contents with Windiets Research software (version 2005; Robert Gordon University, Aberdeen, United Kingdom).

Primary outcome variables

To assess hematologic variables, the following variables were measured in the hematology laboratory at the Royal Hallamshire Hospital: red blood cell numbers, packed cell volume, mean cell volume, hemoglobin, and ZPP. Enzyme-linked immunosorbent assay kits were used to measure plasma ferritin (Spectro Ferritin Kit; ATi Atlas Ltd, Chichester, United Kingdom) and plasma soluble transferrin receptor (Quantikine DTFR1 – Human sTfR Immunoassay; R&D Systems, Abingdon, United Kingdom). The intrabatch CV or the in-house quality control for ferritin was 11.1%, and the interbatch CV was 11.5%. The predetermined intrabatch CV for the transferrin receptor measurement was 7.1%, and the interbatch CV was 5.4%.

TABLE 1
Sample characteristics for women who completed the intervention according to randomly assigned group[1]

Characteristic	All (n = 117)	Placebo (n = 40)	2 mg riboflavin (n = 37)	4 mg riboflavin (n = 40)
Age (y)	21.5 ± 1.97[2]	21.3 ± 1.96	21.5 ± 2.00	21.7 ± 1.96
BMI (kg/m^2)	22.1 ± 2.96	21.7 ± 2.5	22.6 ± 3.6	22.4 ± 2.5
Smokers (n)	14	7	4	3
Contraceptive use (n)				
Oral	49	15	13	21
Implant, injection, or coil	10	6	3	1

[1] There were no significant differences between groups (ANOVA for continuous variables; chi-square test for categorical variables)
[2] Mean ± SD (all such values).

Riboflavin status was measured by using the EGRAC assay (16), and plasma flavin concentrations were measured by using an HPLC-kit method (Chromsystems). The intrabatch and interbatch CVs for EGRAC were 2.2% and 2.4%, respectively. The intrabatch CV was 4.1% for FAD, 18.7% for FMN, and 2.9% for riboflavin. The interbatch CV was 8.5% for FAD, 18.7% for FMN, and 4.8% for riboflavin.

Secondary outcome variables

To adjust for any confounding effects of changes in intakes of vitamins C, B-6, B-12, or folate that might have affected hematologic status, status measurements were made for each of these nutrients in addition to the estimation of dietary intakes. Plasma vitamin C was measured by using a fluorometric assay with a COBAS Bio centrifugal analyzer (21). The intrabatch CV was 2.1%, and the interbatch CV was 6.4%. Plasma pyridoxal phosphate (PLP) and PA, as measures of vitamin B-6 status, were measured with an HPLC kit (Chromsystems). The intrabatch CV was 2.4% for PLP and 8.3% for PA. The interbatch CV was 3.2% for PLP and 18.1% for PA. Plasma folate was measured as 5-methyltetrahydrofolate by isocratic reverse-phase HPLC (29). The intra-batch and interbatch CVs for 5-methyltetrahydrofolate were 5.4% and 6.0%, respectively. All plasma vitamin B-12 measurements were made in the Department of Clinical Chemistry, Royal Hallamshire Hospital, by using an immunoassay on a Bayer ADVIA Centaur Immunoassay System (Bayer Health Care Diagnostics, Germany); the average intrabatch CV was 3.5%, and the average between-batch CV was 4.8%.

Because of the documented influence that riboflavin status can have on plasma homocysteine concentrations (22, 23), plasma homocysteine was measured as an additional functional measure of riboflavin status with an IMX autoanalyzer (Abbott Diagnostics, Maidenhead, United Kingdom). Commercial quality controls provided with the kit were used to check the precision. For the low control (mean: 7.17 μmol/L) the intrabatch CV was 2.2%, and the interbatch CV was 1.9%. For the medium control (mean: 12.8 μmol/L), the intrabatch CV was 1.9%, and the interbatch CV was 1.9%.

Abnormally elevated plasma ferritin can be due to an underlying inflammation. To aid in the interpretation of such values, plasma CRP was measured with an enzyme-linked immunosorbent assay kit (Quantikine DCRP00 Human C-Reactive Protein Immunoassay; R&D Systems). The predetermined intrabatch CV was 3.8%, and the interbatch CV was 7.0%.

Statistical analyses

Data sets either did not deviate significantly from the normal distribution (Kolmogorov-Smirnov test) or did not deviate sufficiently from normality assumptions for parametric one-factor analysis of variance (ANOVA; F test) to generate results that were different from those generated by nonparametric ANOVA (Kruskal-Wallis test). Therefore, variables are presented as

TABLE 2
Values for biochemical variable at baseline[1]

Analyte	Placebo	2 mg riboflavin	4 mg riboflavin
EGRAC	1.55 ± 0.216 (40)	1.59 ± 0.179 (35)	1.61 ± 0.182 (40)
Plasma FMN (nmol/L)	13.11 ± 8.51 (40)	12.56 ± 11.46 (37)	12.63 ± 13.87 (39)
Plasma FAD (nmol/L)	100.57 ± 22.45 (40)	105.31 ± 14.99 (37)	100.36 ± 14.98 (39)
Plasma riboflavin (nmol/L)	21.97 ± 25.9 (40)	21.23 ± 26.53 (36)	17.43 ± 17.18 (39)
Plasma folate (nmol/L)	24.19 ± 10.67 (40)	31.84 ± 17.3 (36)	27.23 ± 16.39 (38)
Plasma homocysteine (μmol/L)	9.44 ± 2.44 (40)	9.39 ± 2.59 (37)	9.5 ± 2.68 (40)
Plasma PLP (nmol/L)	82 ± 49.7 (40)	79 ± 36.2 (37 (36)	77.7 ± 44.0 (39)
Plasma PA (nmol/L)[2]	27.42 ± 33.71 (40)	24.37 ± 9.32 (36)	19.44 ± 8.4 (39)
Plasma vitamin B-12 (pmol/L)	231.1 ± 73.2 (40)	233.3 ± 76.0 (37)	222.5 ± 77.2 (38)
Plasma vitamin C (μmol/L)	72.62 ± 65.64 (40)	65.81 ± 19.75 (37)	69.78 ± 16.47 (39)

[1] All values are means ± SDs; n in parentheses. EGRAC, erythrocyte glutathione reductase activation coefficient; PLP, pyridoxal phosphate; PA, pyridoxic acid.
[2] Significantly different between treatment groups at baseline, P = 0.040 (ANOVA).

TABLE 3
Hematologic variables at baseline according to random assignment[1]

Analyte	Placebo	2 mg riboflavin	4 mg riboflavin
Hemoglobin (g/L)	135.4 ± 8.2 (39)	134.3 ± 9.7 (36)	133.1 ± 9.4 (39)
Hematocrit (%)	0.39 ± 0.02 (30)	0.39 ± 0.03 (36)	0.39 ± 0.03 (39)
Red blood cell number (10^{12}/L)	4.43 ± 0.31 (39)	4.39 ± 0.30 (36)	4.37 ± 0.36 (39)
MCV (fL)	89.21 ± 3.3 (39)	88.91 ± 3.45 (36)	88.65 ± 4.42 (39)
MCH (pg)	30.76 ± 1.39 (39)	30.62 ± 1.51 (35)	30.55 ± 1.86 (39)
MCHC (g/dL)	34.34 ± 0.55 (39)	34.39 ± 0.66 (35)	34.44 ± 0.61 (39)
ZPP (μmol/mol hemoglobin)	58.13 ± 1.78 (38)	56.36 ± 18.02 (36)	58.89 ± 15.73 (38)
Plasma ferritin (ng/mL)	36.66 ± 21.11 (40)	35.91 ± 28.14 (37)	32.15 ± 25.28 (40)
Plasma sTfR (nmol/L)	18.63 ± 4.59 (40)	17.64 ± 4.1 (37)	18.65 ± 5.85 (40)

[1] All values are means ± SDs; *n* in parentheses. MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; ZPP, zinc protoporphyrin; sTfR, soluble transferrin receptor. There were no significant differences between groups for any variable (ANOVA).

means ± SDs, and results are given for parametric ANOVA. For descriptive statistics, baseline values were analyzed by using one-factor ANOVA (*F* test) according to the randomization group, and categorical variables were compared by using the chi-square test. The effect of treatment on the change in response to the intervention was analyzed by one-factor ANOVA (*F* test). When ANOVA indicated a significant difference across treatment groups, a Scheffe test or Kruskal-Wallis multiple-comparison *z* value test was carried out, as appropriate, to identify where the differences lay. Spearman rank correlations were conducted to examine possible associations between selected continuous variables. Data were analyzed with SPSS software (version 16.0; SPSS, Chicago, IL).

RESULTS

Subject recruitment, retention, and compliance

Over a period of 15 mo, 256 women were screened for moderate riboflavin deficiency (Figure 1). From this initial screening, 145 women were eligible for the intervention trial on the basis of a low riboflavin status (EGRAC >1.40), which represented 57% of our screened population compared with 66% of this age and sex group in the United Kingdom who had EGRAC >1.40 (4). Of these women, 123 women agreed to take part in the intervention study. In all, 4 women failed to complete the intervention study. In addition, 2 women were omitted from the postintervention analysis after a self-reported blood transfusion and diagnosis of β-thalassemia during the study. Therefore, analyses were carried out in 117 women, which satisfied the target sample size of 31 participants per group. Of these 117 women, 34 subjects completed the bioavailability study. Screening and recruitment to the intervention took place over 15 mo. Mean compliance of the volunteers, as judged by the number of capsules returned, was 96 ± 6% (range: 70–100%). All women who had a menstrual cycle started the intervention in ≤7 d on either side of the middle of the cycle.

Subject characteristics

Subject characteristics according to randomization groups are shown in **Table 1**. All women were European white women. Data were analyzed by using the chi-square test for categorical variables or ANOVA for continuous variables. There were no significant differences in any variable between randomly assigned groups.

Baseline biochemistry

Values for biochemical variables at baseline measurements, according to randomization group, are shown in **Table 2**. Values did not differ between randomization groups with the exception of plasma PA ($P = 0.040$; ANOVA), differences for which between groups were small and unlikely to be of any functional significance. Mean plasma total homocysteine and vitamin B-12 concentrations were comparable with means reported for women of this age group in the United Kingdom (9.3 μmol/L and 247 pmol/L, respectively). The mean plasma folate concentration was higher than the 20.6 μmol/L reported for this age group in the United Kingdom (4). Similarly, values for hematologic values at baseline did not differ between randomization groups (**Table 3**). Mean hemoglobin concentrations were comparable with the mean ± SD concentration of 13.50 ± 1.05 g/dL for this age group in the UK population (4). Mean values for hematocrit, MCV, and ferritin were lower than those reported for the same age group in the UK population (4). Values reported in the

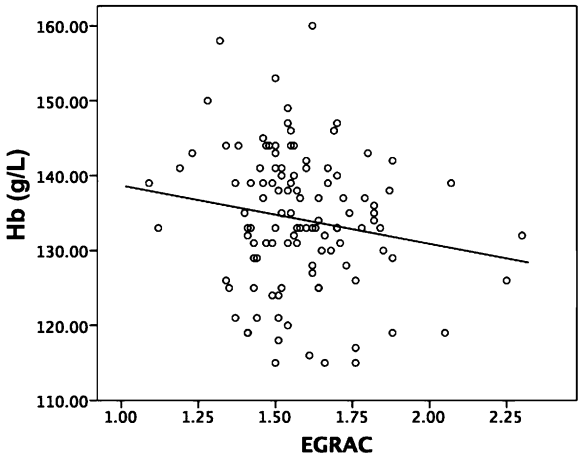

FIGURE 2. Correlations between hemoglobin (Hb) status and baseline erythrocyte glutathione reductase activation coefficient (EGRAC). The figure shows a negative correlation between hemoglobin and EGRAC at baseline ($n = 117$; $r = -0.220$, $P = 0.016$; Spearman's rank correlation).

TABLE 4
Daily intakes of energy and selected nutrients at baseline according to randomly assigned group[1]

Dietary intake	Placebo	2 mg riboflavin	4 mg riboflavin
Energy (MJ)	8.12 ± 1.54 (39)	7.97 ± 2.11 (36)	7.93 ± 1.58 (40)
Riboflavin (mg)	1.29 ± 0.37 (39)	1.14 ± 0.34 (36)	1.28 ± 0.47 (40)
Iron (mg)	10.95 ± 3.39 (39)	10.56 ± 3.61 (36)	10.73 ± 3.16 (40)
Vitamin C (mg)	119.1 ± 108.30 (39)	97.8 ± 53.98 (36)	121.9 ± 61.92 (40)

[1] All values are means ± SDs; *n* in parentheses. There were no significant differences between groups for any variable (ANOVA).

NDNSs were 41.6 ± 3.72%, 92.8 ± 6.77 fL, 41 ± 26.2 ng/mL for hematocrit, MCV, and plasma ferritin concentration, respectively. According to the World Health Organization cutoff value of 120 g hemoglobin/L (30), 11% of women had low hemoglobin amounts before the intervention. Twenty-one percent of women had a plasma ferritin concentration lower than the normally accepted cutoff of 15 ng/mL for women (4). Thirty-nine percent of women had a ZPP value above the upper threshold for normality (60 μmol ZPP/mol hemoglobin; Department of Clinical Chemistry, Sheffield Hospitals, National Health Service, Sheffield, United Kingdom) and 4.9% of women had a serum soluble transferrin receptor value above the normal threshold (28.1 nmol/L; Department of Clinical Chemistry, Sheffield Hospitals, National Health Service).

Baseline correlations

Baseline data were examined for correlations between riboflavin status, expressed as EGRAC, and measures of hematologic status. A modest but statistically significant ($P = 0.016$) negative correlation between hemoglobin and EGRAC for all subjects at baseline is shown in **Figure 2**. When the riboflavin status was better in these women with lower than average riboflavin status, the hemoglobin concentration was higher.

Dietary intakes

All dietary variables were examined for differences at baseline according to the intervention group; ANOVA revealed no significant differences. The mean dietary riboflavin intake for this population (**Table 4**) was lower than that reported for the same age group in the recent NDNS (4) (mean ± SD: 1.53 ± 0.806 mg). Means ± SDs for total energy, iron, and vitamin C intakes were higher than reported for this age group in the recent NDNS (4) (7.0 ± 1.93 MJ and 10.0 ± 4.88 and 96 ± 133.7 mg, respectively).

Effects of the intervention

Dietary intakes

Because there was no a priori reason for the effects of the intervention to be different between randomization groups, the effects of the intervention on dietary intakes were examined for all groups combined by means of a comparison of pre- and postintervention values. There were no differences in nutrient or energy intakes for any variable tested (data not shown).

Nondietary variables

Effects of treatment on changes in riboflavin status were compared by using ANOVA (**Table 5**). Both levels of supplement elicited a significantly greater decrease in EGRAC than that in the placebo group, with a significant dose-response effect ($P < 0.0001$). Increases in plasma FAD and FMN were significantly greater in the 4 mg group than in the placebo group ($P = 0.035$ and $P = 0.027$, respectively). EGRAC values showed a rapid response to riboflavin supplementation such that, by the midpoint of the intervention (4 wk), changes in the 2 and 4 mg groups were significantly greater than the change in the placebo group ($P < 0.001$), and values were not significantly different from values at 8 wk of supplementation (mean ± SD: 1.52 ± 0.193, 1.34 ± 0.106, and 1.25 ± 0.065 for the placebo and 2 and 4 mg groups, respectively). Four weeks after completion of riboflavin supplementation, EGRAC values had risen again and were no longer significantly different from preintervention values for the placebo and 2 and 4 mg groups (mean ± SD: 1.57 ± 0.222, 1.54 ± 0.183, and 1.56 ± 0.169, respectively). There were no significant changes in any other biochemical variables in response to the intervention. Changes in values for hematologic variables over 8 wk are shown in **Table 6**. There was no significant effect of treatment on the change observed for any hematologic variable when data for all participants were analyzed, although that for red blood cell numbers just failed to reach statistical significance ($P = 0.055$; ANOVA). At the end of the intervention, the percentage of women with values for red

TABLE 5
Changes in measures of riboflavin status after 8 wk of treatment[1]

Analyte	Placebo	2 mg riboflavin	4 mg riboflavin
EGRAC	−0.03 ± 0.19 (40)	−0.25 ± 0.20 (34)[2]	−0.37 ± 0.18 (40)[2,3]
Plasma riboflavin (nmol/L)	0.56 ± 9.53 (40)	13.86 ± 18.82 (35)	29.49 ± 28.07 (39)
Plasma FAD (nmol/L)	−5.73 ± 24.7 (40)	−5.78 ± 18.66 (36)	5.75 ± 22.53 (39)[2]
Plasma FMN (nmol/L)	0.05 ± 9.94 (40)	3.23 ± 7.69 (36)	5.18 ± 7.38 (39)[2]

[1] All values are means ± SDs; *n* in parentheses. EGRAC, erythrocyte glutathione reductase activation coefficient.
[2] Change significantly different from placebo, $P < 0.05$ (Scheffe multiple-comparison test).
[3] Change significantly different from 2-mg dose, $P < 0.05$ (Scheffe multiple-comparison test).

TABLE 6
Changes in measures of hematologic status after 8 wk of treatment[1]

Analyte	Placebo	2 mg riboflavin	4 mg riboflavin
Hemoglobin (g/L)	−1.2 ± 5.7 (38)	−0.3 ± 6.1 (36)	1.9 ± 6.5 (39)
Hematocrit (%)	−0.004 ± 0.02 (38)	−0.001 ± 0.02 (36)	0.01 ± 0.02 (39)
Red blood cell number (10^{12}/L)	−0.05 ± 0.20 (38)	−0.01 ± 0.20 (36)	0.06 ± 0.21 (39)
MCV (fL)	0.07 ± 1.27 (38)	−0.09 ± 1.14 (36)	0.14 ± 0.91 (39)
ZPP (μmol/mol hemoglobin)	0.14 ± 11.49 (36)	0.97 ± 15.1 (35)	0.16 ± 8.29 (38)
Plasma ferritin (ng/mL)	3.1 ± 20.36 (40)	4.21 ± 20.69 (37)	1.15 ± 19.79 (40)
Plasma sTfR (nmol/L)	0.31 ± 3.81 (40)	0.04 ± 2.92 (36)	−0.21 ± 4.15 (40)

[1] All values are means ± SDs; *n* in parentheses. MCV, mean cell volume; ZPP, zinc protoporphyrin; sTfR, soluble transferrin receptor. There were no significant effects of treatment (ANOVA).

blood cell ZPP above the normal threshold was 41%, and 3.4% of women had a plasma sTfR concentration above the threshold for normality, with both values being very close to pre-intervention values. Postintervention, 3.4% of all women had a hemoglobin concentration below the normal threshold compared with 11% of women at baseline, and 16% of women had a plasma ferritin concentration below the threshold compared with 21% of women at baseline. Three women presented with moderately elevated CRP concentrations (>10 mg/L) post-intervention, but each woman came from a different treatment arm, and none had a plasma ferritin concentration outside the normal range (>150 ng/mL).

When changes in hemoglobin were examined relative to EGRAC values at baseline, a clear pattern emerged, such that women with the highest EGRAC at baseline showed the greatest increase in hemoglobin in response to riboflavin. No such relation was seen in the placebo arm. The effects of the baseline riboflavin status on the change in hemoglobin in response to riboflavin supplements were clearly seen when each treatment arm was considered separately. Correlations between the change in hemoglobin compared with baseline EGRAC for each of the arms separately and the supplement arms combined are shown in **Figure 3**. There was no relation between baseline EGRAC and change in hemoglobin in either the placebo arm (Figure 3A) or

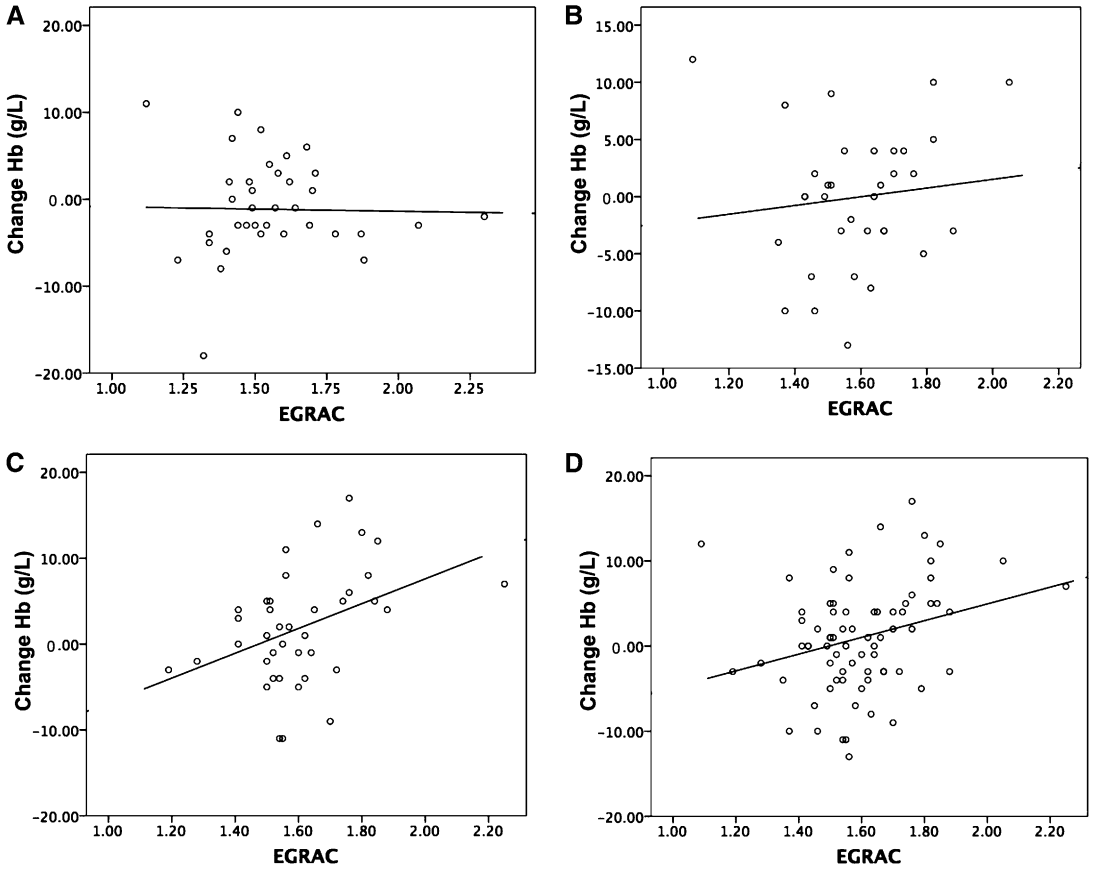

FIGURE 3. Correlations between changes in hemoglobin (Hb) concentration and erythrocyte glutathione reductase activation coefficient (EGRAC) values at baseline by using Spearman's rank correlation for women in the placebo group (n = 38; r = –0.020, P = 0.907) (A), women who received 2 mg riboflavin (n = 34; r = 0.114, P = 0.521) (B), women who received 4 mg riboflavin (n = 39; r = 0.406, P = 0.010) (C), and women who received either of the riboflavin doses (n = 73; r = 0.281, P = 0.016) (D).

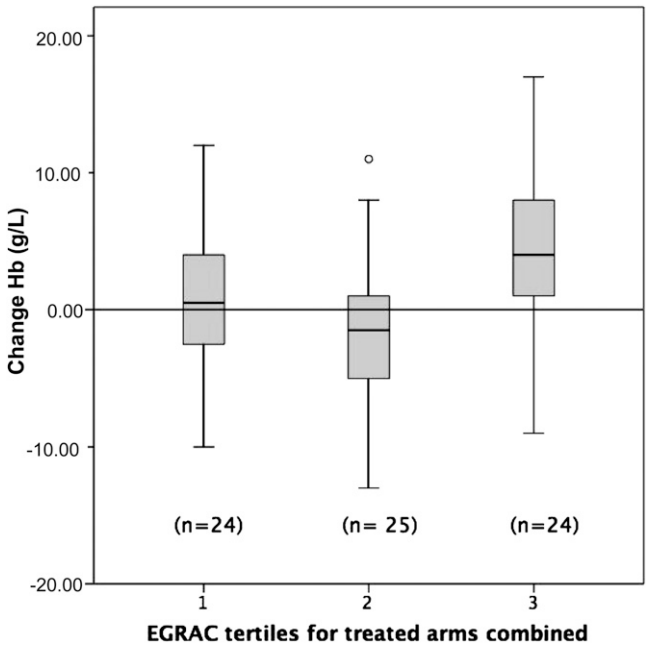

FIGURE 4. Median (interquartile range) changes in hemoglobin (Hb) concentrations (g/dL) in response to riboflavin supplementation according to baseline riboflavin status by using ANOVA. Box-and-whisker plot of changes in Hb concentrations for women with erythrocyte glutathione reductase activation coefficient (EGRAC) in tertiles 1 (<1.51), 2 (1.51–1.65), and 3 (>1.65) at baseline. Changes in Hb concentrations for women in tertile 3 were significantly greater than for women in tertiles 2 ($P = 0.034$) and 1 ($P = 0.001$).

2-mg supplement arm (Figure 3B), but a significant relation was evident between baseline EGRAC and the change in hemoglobin for the 4-mg supplement arm ($P = 0.010$; Figure 3C) and for the data from the 2- and 4 mg-supplements combined ($P = 0.016$; Figure 3D), which indicated a greater improvement in hemoglobin in those women with the poorest riboflavin status at the outset.

The effect of baseline EGRAC on the response to intervention was also analyzed by using a multiple linear regression analysis for both treatment arms combined but with the inclusion of the treatment as a factor, which addressed the fact that there were 2 treatment arms. The analysis confirmed that the baseline riboflavin status (as measured by EGRAC) predicted the change in hemoglobin ($P = 0.019$) in response to riboflavin supplementation, but the dose was not a predictor of this change ($P = 0.214$). A similar result was obtained for red blood cell numbers, for which baseline EGRAC was a significant predictor of the increase in red blood cell numbers ($P = 0.035$) in response to riboflavin supplementation, but the size of the dose was not predictive ($P = 0.215$).

To more fully understand the effect of the baseline riboflavin status on the response to supplementation, this effect was examined according to tertiles of baseline EGRAC, which were lowest (<1.51); middle (1.51–1.65), and upper (>1.65) tertiles. A box-and-whisker plot of the change in hemoglobin in response to a riboflavin supplement of 2 or 4 mg according to tertiles for EGRAC is shown in **Figure 4**. ANOVA showed a significant difference in response to riboflavin supplements according to tertiles for EGRAC at baseline ($P = 0.003$). There was a median increase in the hemoglobin concentration of 4.5 g/L in response to riboflavin in women in whom EGRAC at baseline was ≥1.65. This increase was significantly greater than that for women in the lowest tertile for EGRAC at baseline ($P = 0.001$) and for those in the middle tertile ($P = 0.034$).

Similarly, the baseline EGRAC value was a predictor of the magnitude of the change in red blood cell number (**Figure 5**). ANOVA revealed a significant effect of the baseline tertile for EGRAC on the change in red blood cell number ($P = 0.002$). There was a mean increase in red blood cell numbers of 0.145×10^{12} cells/ L in women in the highest tertile for EGRAC at baseline, which was significantly greater than in women in the lowest tertile ($P = 0.0005$) and in women in the middle tertile ($P = 0.036$).

The results for the subgroup of volunteers that underwent the iron-absorption study are shown in **Table 7**. There was a significant change in EGRAC for those volunteers who were allocated to the 4-mg riboflavin supplement/d group($P < 0.0001$) than for the placebo group. The change relative to baseline for all other variables was not significantly different from that for the placebo.

DISCUSSION

Riboflavin supplements at 2 doses achievable through diet led to a highly significant improvement in riboflavin status, which was determined as a lowering of EGRAC with a clear dose response. In turn, the improvement of riboflavin status led to an increase in the number of circulating red blood cells and hemoglobin concentrations; the poorer the riboflavin status was at entry into the study, the greater was the increase in red blood cell number or hemoglobin concentration. Women in the highest tertile of EGRAC at baseline (>1.65) showed significantly greater increases in hemoglobin and red blood cell numbers than did women in the first or second tertiles. These findings suggested that consideration should be given to raising the deficiency threshold for EGRAC.

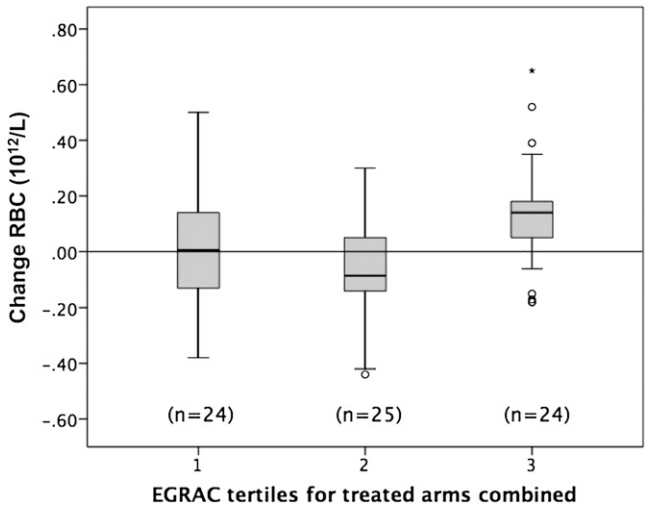

FIGURE 5. Median (interquartile range) changes in red blood cell (RBC) numbers (10^{12}/L) in response to riboflavin supplementation according to baseline riboflavin status by using ANOVA. Box-and-whisker plot of changes in RBC numbers for women with erythrocyte glutathione reductase activation coefficient (EGRAC) in tertiles 1 (<1.51), 2 (1.51–1.65), and 3 (>1.65) at baseline. Increases in red blood cell numbers for women in EGRAC tertile 3 were significantly greater than for women in tertile 2 ($P = 0.036$) and 1 ($P = 0.0005$).

TABLE 7
Pre- and postintervention values for hemoglobin, red blood cell number, plasma ferritin concentration, erythrocyte glutathione reductase activation coefficient (EGRAC), and iron absorption according to random assignment for the subgroup who underwent the iron-absorption study[1]

	Placebo		4 mg riboflavin	
Analyte	Preintervention	Postintervention	Preintervention	Postintervention
Hemoglobin (g/L)	136.5 ± 9.5 (15)	135.2 ± 9.5 (16)	135.6 ± 10.0 (18)	136.8 ± 118 (18)
Red blood cell number (10^{12}/L)	4.53 ± 0.34 (15)	4.49 ± 0.36 (16)	4.45 ± 0.40 (18)	4.50 ± 0.48 (18)
Plasma ferritin (ng/mL)	38.1 ± 24.3 (16)	44.2 ± 28.4 (16)	30.4 ± 19.3 (18)	30.2 ± 17.4 (18)
EGRAC	1.58 ± 0.21 (16)	1.54 ± 0.25 (16)	1.64 ± 0.14 (18)	1.22 ± 0.11 (18)[2]
Iron absorption (% of dose)	7.6 ± 5.0 (16)	9.7 ± 4.4 (18)	10.6 ± 5.8 (18)	8.4 ± 7.6 (18)

[1] All values are means ± SDs; *n* in parentheses.
[2] Change from baseline was significantly different from the change in the placebo group, $P < 0.0001$ (Scheffe multiple-comparison test).

EGRAC responded rapidly to changes in riboflavin intakes; 4 wk of supplementation lowered EGRAC values to the same extent as at 8 wk of supplementation. Similarly, EGRAC values rapidly reverted to baseline values after cessation of supplementation, which indicated the importance of an adequate daily intake.

About 15% of women aged 19–24 y in the United Kingdom had an EGRAC ≥1.65 (4). Results of our study strongly suggested that poor riboflavin status in this group of women impaired iron handling, and an improvement in their riboflavin status would lead to an increase in hemoglobin independent of any change in dietary iron. These findings were concordant with findings in other studies, predominantly in animal models of riboflavin deficiency, but with some human data too. Beneficial effects of riboflavin supplements on various measures of hematologic status have been reported in populations with a high prevalence of riboflavin deficiency. Studies in The Gambia have indicated that biomarkers of iron status improved in response to riboflavin supplements given either alone or in association with iron (10, 11, 31). Hematologic status has also been reported to improve in response to a supplement of 5 mg riboflavin for 8 wk in a small study of Nigerian adults (32). Pregnant women in rural China with low hemoglobin status (80–105 g/L) showed an enhanced hematologic response to a combined supplement of iron, folic acid, retinol, and riboflavin for 2 mo than to a supplement of iron and folic acid alone (33). Studies in mildly anemic populations in Indonesia showed that a supplement of riboflavin enhanced the increase in hemoglobin seen in response to conventional hematinics alone (9, 34).

The mechanisms by which riboflavin status influences hematologic status are uncertain. Animal models of riboflavin deficiency have suggested a role for riboflavin as FMN, in the reductive mobilization of iron from ferritin, via FMN oxidoreductase (Enzyme Commission 1.6.8.1) (6, 7, 17), but the tissue sites at which such an effect may be physiologically relevant in humans are not known. In the current study, we reported that there was no change in dietary iron over the period of the intervention, and we showed no measurable improvement in iron absorption in response to the supplement, which suggested that the increase in circulating red blood cell numbers and hemoglobin concentrations might have been driven by the mobilization of endogenous iron. The mean change in hemoglobin concentrations observed in volunteers with the lowest baseline riboflavin status (third tertile in Figure 4; EGRAC >1.65) equated to an increase in circulating iron of ≈33 mg by the end of the supplementation period. The plasma ferritin concentration is considered to reflect iron stores, and the mean change in ferritin amounts in those volunteers with the lowest baseline riboflavin status (EGRAC >1.65) equated to a decrease in iron stores of 21 mg as calculated by using a previously published equation (35). In a previous study conducted in Gambian men with low iron status and biochemical riboflavin deficiency a 10-mg supplement of riboflavin for 4 wk corrected the riboflavin deficiency, had no measurable effect on iron absorption, and led to a significant increase in hemoglobin (31). We suggested that the improvement riboflavin status increased the mobilization of endogenous iron stores.

In conclusion, the RIBOFEM study showed that an improvement of riboflavin status had a beneficial effect on some measures of hematologic status in young women. These findings are also relevant to other age groups in whom a high prevalence of riboflavin deficiency has been reported, including in adolescents and the elderly. The results also suggest that consideration should be given to raising the deficiency threshold for EGRAC to better reflect functional impairment.

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The authors' responsibilities were as follows—HJP and EAW: conceived the study; HJP: drafted the manuscript; MHH and SM: conducted the trial and carried out laboratory analyses; JRD: contributed to the experimental design of the bioavailability study; and JRD and GM-N: carried out the stable isotope work. All authors contributed to the final manuscript. None of the authors had a conflict of interest.

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