Direct assessment of body iron balance in women with and without iron supplementation using a long-term isotope dilution method in Benin and Switzerland

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ABSTRACT

Background: Long-term isotopic dilution measurements of body iron may allow quantification of basal body iron balance and iron gains during an iron intervention with higher precision and accuracy than conventional iron indices.

Objectives: We compared body iron balance before, during, and after oral iron supplementation in women in Benin and in Switzerland.

Methods: In prospective studies, Beninese ($n = 11$) and Swiss ($n = 10$) women previously labeled with stable iron isotopes were followed preintervention for 90–120 d, then received 50-mg iron daily for 90–120 d and were followed postintervention for 90–120 d. We used changes in blood isotopic composition to calculate iron absorption (Fe_{abs}), iron loss (Fe_{loss}), and net iron balance (Fe_{gain}).

Results: Compliance with supplementation was >90%. In Benin, during the preintervention, intervention, and postintervention periods, Fe means ± SDs were as follows: *1*) Fe_{abs}: 0.92 ± 1.05, 3.75 ± 2.07, and 0.90 ± 0.93 mg/d; *2*) Fe_{loss}: 1.46 ± 1.95, 1.58 ± 1.57, and 1.84 ± 1.61 mg/d; and *3*) Fe_{gain}: −0.55 ± 1.56 mg/d, 2.17 ± 1.81 mg/d, and −0.94 ± 1.13 mg/d. In Switzerland, the corresponding values were: *1*) 1.51 ± 0.37, 4.09 ± 1.52, and 0.97 ± 0.41 mg/d; *2*) 0.76 ± 1.37, 2.54 ± 1.43, and 2.08 ± 1.05 mg/d; and *3*) 0.75 ± 1.37, 1.55 ± 1.75, and −1.11 ± 1.06 mg/d. Inflammation was low in both settings, and isotopically calculated iron balance was comparable to that calculated from changes in conventional iron indices.

Conclusion: Without iron supplementation, Beninese women had lower long-term dietary iron absorption and higher iron losses in the preintervention period than Swiss women. During iron supplementation, both groups had high iron absorption and similar iron gains. However, there was a 3-fold increase in iron losses in the Swiss women during the supplementation and postintervention period compared with the preintervention period. Body iron isotope dilution is a promising new method for quantifying long-term body iron balance and for assessing the impact of iron interventions. The studies were registered at clinicaltrials.gov as NCT02979080 and NCT02979132, respectively. *Am J Clin Nutr* 2021;113:1657–1669.

Keywords: iron absorption, iron loss, iron balance, iron supplementation, women, iron stable isotopes, Benin, Switzerland

Introduction

Quantitative data in humans on iron absorption, iron loss, and iron balance are scarce. After equilibration of an iron isotope tracer in all body compartments, a decrease in the concentration of an iron isotope tracer in circulation can occur only by addition of iron of natural isotopic composition (1–3). Thus, in the absence of intravenous iron administration, any decrease in the concentration of an iron isotope tracer in the circulation is proportional to the quantity of iron absorbed. Similarly, a

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Abbreviations used: AE, adverse event; AGP, α-1-acid glycoprotein; BIS, Cook body iron stores; BRINDA, Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia; CRP, C-reactive protein; Fe_{abs}, daily amount of iron absorbed (mg/d); Fe_{gain}, net iron balance per day (mg/d); Fe_{loss}, daily amount of iron lost (mg/d); Fe_{total}, total body iron (mg); ^{57}Fe, stable isotope tracer enriched in iron 57; ^{58}Fe, stable isotope tracer enriched in iron 58; Hb, hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; k_{abs}, rate of change of body iron or rate of decrease in body iron composition, proportional to iron absorption (mmol/d); k_{loss}, rate of decrease in body iron, proportional to iron loss (mmol/d); RBC, red blood cell; REML, restricted maximum likelihood; SF, serum ferritin; SF_a, serum ferritin adjusted according to recommendations by the BRINDA initiative; sTfR, soluble transferrin receptor; URTI, upper respiratory tract infection.

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decrease in the quantity of an iron isotope tracer in the circulation can only occur through loss of body iron, with identical isotopic composition in total body iron and in the iron fraction lost. Iron isotopic dilution allows the direct determination of long-term iron absorption and loss resulting in an accurate estimation of iron balance.

Iron deficiency (ID) and iron deficiency anemia (IDA) are particularly common in women in low-income countries, due to several factors: *1*) dietary iron bioavailability from monotonous plant-based diets is generally low (4); *2*) inflammation is common and can reduce iron absorption by inducing hepcidin (5); *3*) many women have multiple pregnancies (6); and *4*) iron losses may be high due to frequent gastrointestinal infections and/or heavy menstrual bleeding because use of oral contraceptives is low (6, 7). The impact of oral iron supplementation programs in women in Sub-Saharan Africa has often been disappointing (8, 9), but accurate assessment of the efficacy of iron supplementation programs is challenging (6). Conventional iron indices such as hemoglobin (Hb), serum ferritin (SF), or soluble transferrin receptor (sTfR) often have poor specificity, sensitivity, or both (10, 11), are only indirect qualitative measures of changes in body iron, and are often confounded by infection, inflammation, and a variety of chronic disorders (6).

For these reasons, the use of isotopic dilution techniques to determine iron turnover, iron balance, and response to iron interventions may have advantages. To our knowledge, methodology for the dilution of stable iron isotopes has not been applied previously to evaluate the impact of iron interventions in women of reproductive age. Therefore, we performed prospective, exploratory long-term studies in women of reproductive age living in Benin and Switzerland to directly measure and compare iron absorption, iron losses, and iron balance before, during, and after iron supplementation between time periods and sites as related outcomes of primary interest. Secondary outcomes were changes in conventional iron indices during the supplementation period between sites. Our hypotheses were the following: *1*) pre- and postintervention, in Benin daily iron absorption would be lower and iron losses would be higher than in Switzerland, and *2*) during iron supplementation, higher iron gains would be achieved in Switzerland.

Methods

Study site and subjects

To comply with the prerequisite of adequately equilibrated isotope composition in all body compartments and to recruit 2 contrasting cohorts of young women, we contacted 44 young women living in Benin and 75 young women living in Switzerland. Our laboratory has done multiple stable iron isotope studies in young women in these 2 countries and thus a large pool of recently labeled young women was available. We recruited our participants from 3 previous stable iron isotope studies in Benin (12–14) and 7 in Switzerland (15–21). Inclusion criteria for the present study were the following: *1*) participation in a stable iron isotope absorption study in which isotope administration occurred ≥1 y prior to the current study [to allow uniform isotopic labeling of body iron (1–3)], *2*) written informed consent, and *3*) planned residence in the study area for the next year (urban areas of Natitingou and Cotonou and their surroundings in Benin and the Zurich urban area in Switzerland). To comply with requirements of the ethical committee in Switzerland to enroll only iron-depleted but nonanemic women, additional inclusion criteria only for Switzerland were the following: *4*) Hb concentration ≥11.7 g/dL and *5*) SF concentration <25 μg/L. We anticipated that women in Benin would have low iron status. Exclusion criteria in both countries were the following: *1*) major medical disorders or illnesses, *2*) chronic use of medications (except for oral contraceptives and antiacne medications), *3*) consumption of mineral and vitamin supplements during the study or within 2 wk prior to study start, *4*) known intolerance to iron supplements, and *5*) blood transfusion, blood donation, or significant blood loss during study or in the prior 6 mo. For the Swiss cohort we report data from a nonrandomized oral supplementation arm of a study assessing iron fortification as well as oral and intravenous iron supplementation in Switzerland (NCT02979132) that will be reported elsewhere. Ethical approval for the study in Benin was given by the ethics committees of the Biomedical Sciences Institute of the University of Abomey-Calavi (CER-ISBA) in Benin and ETH Zurich, Switzerland. Ethical approval for the Swiss study was given by the ethics committee of the canton of Zurich. The studies were registered at clinicaltrials.gov as NCT02979080 and NCT02979132, respectively.

Study design

As shown in **Figure 1**, both of our prospective studies included an intervention with an oral iron supplement. In Benin, women enrolled in the study were followed without intervention for 120 d [the duration of the average erythrocyte lifespan (22)], with venipuncture (~8 mL) at baseline (day 0), midpoint (day 60), and endpoint (day 120); this period constituted the preintervention control period. The women then received 50 mg iron as ferrous sulfate ($FeSO_4$) daily from day 120 to day 240 (Eisensulfat LOMAPHARM 50 mg; Rudolf Lohmann GmbH KG) under close supervision by weekly monitoring of adverse events (AEs) and monthly study visits including venipuncture (see below). Then, from day 240 to day 360, the women were again followed without intervention for 120 d, with venipunctures on days 300 and 360; this period constituted the postintervention control period. Women in Switzerland followed the same study design, with the exception that the 2 control periods and the intervention period [also with 50 mg iron as ferrous sulfate ($FeSO_4$) given daily] were each 90 d in length, consistent with the Swiss standard of care of providing iron supplements for a period of 3 mo (Figure 1). All participants were advised to consume iron supplements in the morning and without food. Both studies took place between June 2017 and July 2018. We measured body height and weight at the time of each venipuncture. We performed a urine pregnancy test (EVIAL, Core Technology) in Benin at each visit and in Switzerland at days 0, 90, and 180.

In Benin, members of the study team visited the women at home or called them by phone every second week during the 2 control periods and weekly during the iron supplementation period for the monitoring of AEs, medication, and supplemental iron intake. In Switzerland, AEs and medication intake were recorded in standardized health diaries by the women themselves, and the health diaries were checked and collected at each visit.

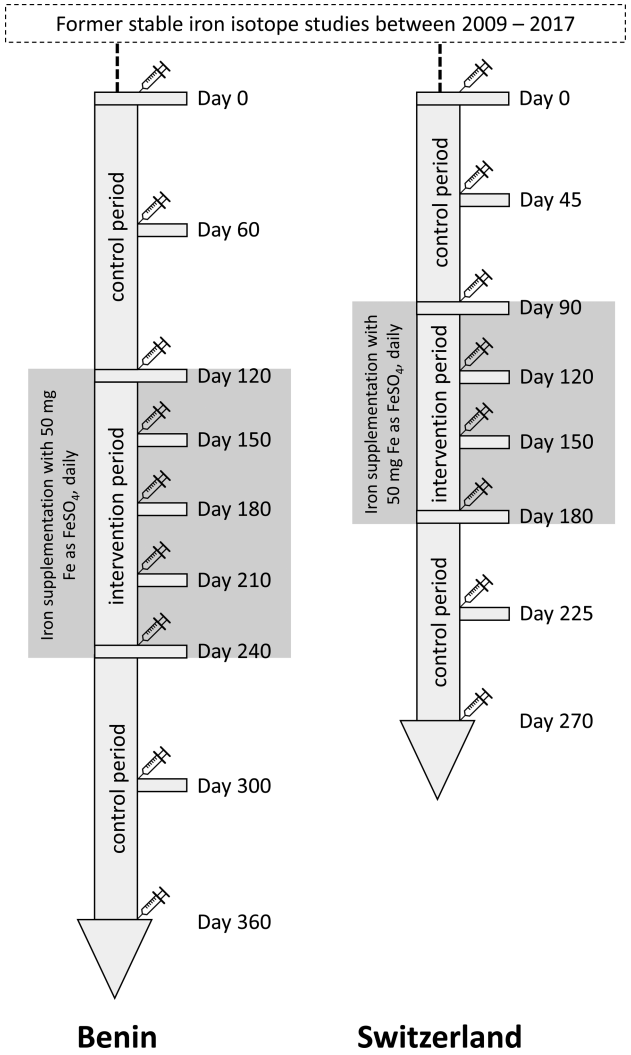

FIGURE 1 Study design. Overview of the study designs of the 2 long-term stable iron isotope dilution studies in Benin and Switzerland. The study in Benin consisted of a 4-mo intervention with 50 mg Fe as $FeSO_4$ framed by 4-mo pre- and postintervention control periods without iron supplementation. The study in Switzerland consisted of the same design with each of the 3 periods lasting for 3 mo. There were monthly study visits during intervention periods and study visits at the beginning, midpoint, and endpoint of both control periods.

At enrollment, women in Benin received anthelmintic treatment (albendazole 400 mg, Square Pharmaceuticals; and Bermoxel 600 mg Praziquantel, Medochemie). Any women in Benin who tested positive for malaria received antimalarial treatment (artemether 20 mg and lumefantrine 120 mg tablets, International Pharmacopoeia, S Kant HEALTHCARE) according to local guidelines.

During the supplementation period we dispensed iron tablets in blister packs containing only enough tablets to last until the next venipuncture. To determine compliance, women were instructed to always cross out the corresponding date printed on the blister pack after tablet consumption and to bring back all the blister packs after use so that the number of remaining tablets was recorded at each visit during the supplementation period. For women in Benin ($n = 8$) living close to the study clinic, we delivered the tablets daily to the women at home.

We assessed dietary iron intakes once during each study period, conducting 3-d weighed food records on 2 consecutive weekdays and 1 weekend day. Food portions during meal preparation and consumption were weighed and recorded. In Benin, 3-d weighed food records were conducted by trained local field workers familiar with the local languages and culture who visited the participants' households as described in (23). In Switzerland, 3-d weighed food records were self reported. We analyzed the Beninese dietary data using Nutrisurvey software (24) after insertion of the West African Food Composition Table (25), the Food Composition Table for Mali (26), and food composition data directly measured in our laboratories (27, 28). Phytic acid values were added from previous reports (29, 30). We analyzed Swiss dietary data using EBISpro software (31) based on Swiss (32) and German (33) food composition databases, with phytic acid values added from previous reports (29, 30). Based on intake data we defined estimated dietary iron bioavailability as isotopically determined iron absorption of each specific period (Fe_{abs}) divided by the mean iron intake of the whole study (i.e., mean iron intake calculated from three 3-d weighed food records, not including supplemental iron intake during the iron supplementation period).

Laboratory analysis

We measured Hb using a Sysmex XN-350 coulter counter (Sysmex Corporation), using controls supplied by the manufacturer. For samples collected in Switzerland, this was done in fresh venous blood samples on the collection day; for samples collected in Benin, this was done in samples that were divided into aliquots and frozen within 6 h after collection. We measured SF, sTfR, C-reactive protein (CRP), α-1-acid glycoprotein (AGP), and serum hepcidin concentrations using immunoassays [(34) and DRG Instruments GmbH]. All samples were measured with the same devices. Samples from Benin were measured in 1 batch (except samples of visit 1 measured in a separate batch), and samples from Switzerland were measured in several batches. Limits of detection always varied slightly due to different CVs of the according measurements but can be summarized as $\leq$5 μg/L for SF, $\leq$1 mg/L for sTfR, $\leq$0.5 mg/L for CRP, $\leq$0.1 g/L for AGP, and $\leq$0.3 μg/L for hepcidin (J Erhardt, VitMin Lab, personal communication, 2020). Values below the limit of detection were treated as random values. We adjusted SF for inflammation using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) approach (35): $SF_a = SF_{unadjusted} - [\beta_1 \times (CRP_{obs} - CRP_{ref})] - [\beta_2 \times (AGP_{obs} - AGP_{ref})]$, where CRP_{obs}/AGP_{obs} are observed/reported CRP/AGP values, CRP_{ref} is the BRINDA reference CRP value of 0.16 mg/L, AGP_{ref} is the BRINDA reference AGP value of 0.54 mg/L, and β_1 and β_2 are the regression coefficients of a linear regression with $SF_{unadjusted}$ as dependent and CRP_{obs} and AGP_{obs} as independent variables. We did not include the malaria term in our BRINDA adjustment. The current recommendation for sTfR adjustment is to use a within-study internal regression coefficient rather than an external AGP_{ref} (36), but we felt our sample size was too small to derive this coefficient, so we did not adjust sTfR concentration. We calculated body iron stores (BIS) from SF and sTfR concentrations using the equation $-[\log_{10}(\text{sTfR [μg/L]/SF [μg/L]}) - 2.8229]/0.1207$ (37). Unadjusted SF and sTfR concentrations were generally used for BIS calculation,

except if explicitly stating BIS_a, for which we included BRINDA-adjusted SF in the BIS calculation. We defined ID as SF_a <15 μg/L and/or sTfR >8.3 mg/L, and IDA as Hb <12.0 g/dL and SF_a <15 μg/L and/or sTfR >8.3 mg/L (38). CRP and AGP concentrations >5 mg/L and >1 g/L, respectively, were considered indicative of inflammation (39). We tested malarial parasitemia in full blood smears using the Giemsa staining method in all venipuncture samples in Benin. We performed an HIV rapid test (One-step anti-HIV, InTec Products) at baseline in Benin only if women explicitly consented for the test. All women explicitly consented.

We determined the isotopic composition of blood in duplicate and also repeatedly measured blanks to ensure quality control. Whole blood was mineralized by microwave digestion, and iron was separated by anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide. Iron isotope ratios were determined with a multicollector inductively coupled plasma MS instrument (NEPTUNE, Thermo Finnigan, Fisher Scientific) as previously described (40, 41).

Iron isotope tracer administration causes an initial isotopic enrichment in red blood cells (RBCs), followed by a sharp decrease due to redistribution of the iron to other tissues after RBC senescence (**Supplementary Figure 1**). After ~12 mo (2), a steady state of isotopic equilibration is reached, where the concentration of isotope tracer can be considered equal in all body compartments (42). From this point onward, a decrease in the isotope tracer concentration in circulation can occur only by addition of iron of natural isotopic composition. Thus, in the absence of intravenous iron administration, any decrease in the concentration of the iron isotope tracer in the circulation is proportional to the quantity of iron absorbed, expressed as the fraction of total body iron absorbed per unit of time, and the factor describing this relation is the slope of the regression of the natural logarithm of the iron isotope tracer concentration over time, k_{abs}. Similarly, a decrease in the absolute quantity of iron isotope tracer in the circulation can occur only with loss of isotope tracer and a corresponding loss of body iron. The factor describing this relation is the slope of the regression of the natural logarithm of the isotope tracer amount over time, k_{loss}. The concentration of the iron isotope tracer in circulation as well as the amount of iron isotope tracer were calculated as described by Fomon et al. (1) with slight modifications to account for the nonmonoisotopic tracer composition and the previous administration of >1 isotope tracer (**Supplementary Methods**, Section 1).

Mean iron absorption (Fe_{abs}) over the period of interest was calculated as follows:

$$Fe_{abs} = -k_{abs} \times \overline{Fe_{total}}, \quad (1)$$

where Fe_{total} is total body iron calculated as the sum of circulating iron, tissue iron, and storage iron, with circulating iron calculated as blood volume × Hb × 3.47, with blood volume estimated using the Brown equation (43), and 3.47 being the concentration of iron in Hb (mg/g); tissue iron estimated as 6 mg/kg body weight (44) and storage iron calculated as 9380 × log(SF) − 11,260 (45; Supplementary Methods, Section 2). $\overline{Fe_{total}}$ is the total body iron at the period midpoint calculated from the linear regression of Fe_{total} against time individually for each observation period, and k_{abs} is the slope of the linear regression of the natural logarithm of the iron isotope tracer concentration plotted against time.

Correspondingly, mean iron loss (Fe_{loss}) over the period of interest was calculated as

$$Fe_{loss} = -k_{loss} \times \overline{Fe_{total}} \quad (2)$$

where $\overline{Fe_{total}}$ is the average total body iron over the period, determined as above, and k_{loss} the slope of the linear regression of the natural logarithm of the iron isotope tracer amount, obtained by multiplying the iron isotope tracer concentration with total body iron (Fe_{total}), plotted against time.

Net iron balance (Fe_{gain}) over the period of interest was calculated isotopically by subtracting iron lost from iron absorbed ($Fe_{gain} = Fe_{abs} - Fe_{loss}$). Fe_{gain} was compared with the changes in total body iron (ΔFe_{total}) calculated conventionally by subtracting the baseline Fe_{total} from the Fe_{total} at the end of the corresponding period ($\Delta Fe_{total} = Fe_{total,endpoint} - Fe_{total,baseline}$).

Statistical analysis

For our power calculation, we aimed to detect a difference in daily iron absorption of 30% from 1.46 mg/d, which is the WHO daily iron requirement for menstruating adult women (46). We judged that an increment in iron absorption of >0.44 mg/d would be clinically relevant. In the absence of data on intrasubject variability in long-term iron absorption, we used the between-subject SD of 0.35 mg/d (24%) determined using stable iron isotope dilution in Chinese women (47) as our expected within-subject SD. We calculated that 9 women would be needed for within-subject comparisons using paired-sample *t*-tests, with a type I error rate of 5% and 80% power and a correlation of absorption within participants of $r = 0.5$. We anticipated high attrition due to the long study duration and many women having isotopic enrichment close to or below the detection limit. Because of these uncertainties, we enrolled all subjects who met the inclusion criteria and consented.

We conducted the statistical analysis with SPSS (version 24, IBM) and the lme4 package (48) in R for linear mixed-effects models. Normality was assessed with the Shapiro–Wilk test and accepting a significance level of 0.05 and visualizing Q-Q plots. Nonnormally distributed data were log transformed and used as log-transformed data if a Shapiro-Wilk test of log-transformed data revealed nonsignificance on a significance level of 0.05 and residuals of the transformed data were normally distributed. Data not normally distributed after log transformation are reported as untransformed data with median (IQR). We examined between-country differences in baseline characteristics and slope ratios using independent-samples *t*-tests and independent-samples Mann-Whitney *U*-tests (as appropriate). Changes in each indicator throughout the study were assessed using period-specific linear mixed models with subject identification number as a random factor, and time (continuous study day), country (0 = Benin, 1 = Switzerland), and time × country interaction as fixed factors. Differences between periods were assessed based on country-specific linear mixed models with subject identification number as random factor and period (ordinal variable) as fixed factor. Differences between the 2 countries were assessed using an overall linear mixed model with subject identification number as a random factor, and time (ordinal variable), country (0 = Benin, 1 = Switzerland), and time × country interaction as fixed factors. All linear mixed models were performed using restricted maximum

likelihood-fitting (REML) and Satterthwaite approximations. In case of significant effects of the fixed factor, we applied post hoc independent-samples *t*-tests and independent-samples Mann–Whitney *U*-tests (as appropriate) for country differences and post hoc paired-samples t-test and paired-samples Mann–Whitney *U*-tests (as appropriate) for periods. Due to the exploratory nature of the analysis, because our primary aim was not to determine efficacy of the iron intervention (49), and due to the expectation that our predefined outcomes of primary interest would be highly correlated (1, 50), we did not perform type I error adjustments on the *P* values of post hoc tests in our models. A pseudo R^2 for each model was calculated as described by Nakagawa et al. (51).

Differences between our data and WHO recommendations were analyzed using 1-sample *t*-tests. *P* values <0.05 were considered significant for all analyses (including Shapiro–Wilk tests).

Results

Subjects

We enrolled 34 women in Benin and 13 women in Switzerland (**Supplementary Figure 2**). Ten women in Benin dropped out of analysis for the following reasons: *1*) menopause onset ($n = 1$); *2*) pregnancy onset prior to completion of the iron supplementation period ($n = 4$); *3*) chronic infection ($n = 1$); *4*) SF_a >100 μg/L, because no iron supplements could be given ethically ($n = 2$); *5*) HIV positive ($n = 1$); and *6*) no supplements given for logistical reasons ($n = 1$). For 13 women in Benin the isotopic signature became indistinguishable from natural iron isotopic composition during the study year, so these women were also excluded from further analyses. In 2 women in Switzerland the isotopic signature became indistinguishable from the natural iron isotopic composition during the study, so these women had to be excluded from analysis. Data from another woman were excluded because no intervention endpoint blood sample was collected. Therefore, we included 11 women in Benin and 10 women in Switzerland in the final data analysis (Supplementary Figure 2); this number of women met the requirements of our power calculations of 9 women per group.

Characteristics at entry into the study by country (group) are shown in **Table 1**. No women in Benin and 5 women (50%) in Switzerland were using oral contraceptives. All women in Benin had given birth to ≥1 child since their initial isotope administration, while none of the women in Switzerland had ever given birth. The Beninese diet is characterized by a high intake of solid mashes or porridges (often fermented) of maize flour consumed with a sauce of green leafy vegetables (fresh or dried) or okra and condiments, or a spicy sauce of tomatoes or peanuts (27). Pounded yam or a mixture of rice and cowpeas are other frequently consumed dishes (27). Fruit consumption was low in Benin, but there were higher intakes during the mango season in April to June, increasing mean ascorbic acid intakes. Meat was rarely eaten by our participants in Benin, but because of their consumption of Beninese cheese (fromage peulh), animal products contributed reasonably to energy intake [see (23)]. Participants in Switzerland generally followed a Western diet of the type consumed by young women. None of the participants followed a strictly vegetarian diet, but participants reported a total of 34 occasions of meat intake and a total of 22 occasions of fish intake during the 3 × 3 d analyzed via 3-d weighed food records (range: 2–13 occasions of meat or fish intake). **Table 2** summarizes the determined dietary intake characteristics in the 2 countries. Iron intake and density were higher in Benin than in Switzerland ($P < 0.001$ for both) and phytic acid intakes were comparable. Thus, phytic acid to iron molar ratios were significantly lower in Benin than in Switzerland ($P = 0.001$), but close to 10 in both countries (Table 2). Ascorbic acid intakes were higher in Switzerland than in Benin ($P = 0.004$), and calculated iron bioavailability was significantly higher in Switzerland than in Benin ($P = 0.010$; Table 2). While participants in Switzerland were all former university students, the majority of participants in Benin were housewives. There was 1 participant in Benin with a university degree and 1 participant with a high-school degree, but all the other participants in Benin left school during secondary education, except 1 who had already left school during primary education and 2 participants never having attended school.

There were no significant between-country differences in age, anthropometric data, inflammation status, Hb, sTfR, or serum hepcidin concentrations. Women in Benin had higher SF_a concentrations than anticipated based on former studies (13, 14) and due to differences in inclusion criteria for SF owing to considerations regarding ethical approval (see above); women in Switzerland had significantly lower SF and SF_a concentrations than women in Benin ($P = 0.004$ and $P = 0.003$, respectively). At study entry, 3 women in Benin were anemic and 2 of these women had IDA. In Switzerland, 5 women were ID, but none had anemia (Table 1). Generally, the Beninese cohort was more heterogeneous for all characteristics, based on a larger SD (Table 1 and **Supplementary Figure 3**). Isotopic characteristics for both countries are summarized in **Supplementary Table 1**.

Compliance and morbidity

The median (IQR) compliance with supplementation was 96 (93–97)% in Benin and 90 (78–96)% in Switzerland. The median (IQR) total iron dose administered during the intervention in Benin was 5650 (5650–5800) mg over 120 d and 4300 (4175–4375) mg over 90 d; in Switzerland it was 4025 (3500–4300) mg over 90 d. The 11 women in Benin identified 35 infection-related AEs during the 1 study year and the 10 women in Switzerland identified 20 infection-related AEs during the 9 study months in Switzerland. Fever thought to be unrelated to malaria was the most common infection-related AE in Benin: self-reported 10 times, followed by diarrhea (reported 7 times), and upper respiratory tract infections (URTIs; cough and/or sore throat without fever; reported 5 times). During study visits in Benin, we detected 5 malaria cases (in 3 women), all during control periods without iron supplementation and in the rainy season. Women self-reported 8 more malaria cases (4 women; with 1 woman reporting 4 malaria cases). The most common infection-related AE in Switzerland was URTI, reported 17 times and affecting all but 2 women. Forty percent of all infection-related AEs in Switzerland occurred during the 3 mo of supplementation that coincided with the winter months when URTI prevalence generally peaks in Switzerland, while in Benin only 23% of all infection-related AEs occurred during the first 3 mo of iron supplementation, which was in the dry season. Use of medication was high in Benin, with 74 sessions of medication intake throughout the whole study year (7 medication intake sessions

TABLE 1 Baseline characteristics of young women in Benin and Switzerland ($n = 21$)[1]

	Benin ($n = 11$)	Switzerland ($n = 10$)
Age, y	27.1 ± 4.4	27.1 ± 6.4
Weight, kg	59.6 ± 10.1	57.3 ± 4.2
Height, cm	165.5 (160.0; 168.0)	166.8 (161.0; 168.0)
BMI, kg/m^2	23.0 ± 4.1	21.0 ± 1.7
Oral contraceptive users, *n* (%)	0 (0)	5 (50)
Number of children	1.7 ± 1.5	0
Hb, g/L	12.4 ± 1.4	13.1 ± 0.6
Anemia[2], *n* (%)	3 (27)	0 (0)
Hepcidin, μg/L	5.8 [1.9; 17.2]	4.6 [2.6; 7.9]
$SF_{unadjusted}$, μg/L	45.1 [19.2; 106.2][a]	14.3 [10.3; 19.8][b]
SF_a,[3] μg/L	34.7 [16.3; 74.0][a]	14.1 [10.2; 19.6][b]
sTfR, mg/L	6.3 [3.7; 10.6]	5.5 [4.2; 7.2]
ID,[2] *n* (%)	2 (18)	5 (50)
IDA,[2] *n* (%)	2 (18)	0 (0)
CRP,[4] mg/L	0.7 (0.3: 2.9)	0.8 (0.3: 2.6)
>5 mg/L, *n* (%)	2 (18%)	0 (0)
AGP,[5] g/L	0.6 [0.4; 0.9]	0.6 [0.4; 0.7]
>1 g/L, *n* (%)	2 (18%)	0 (0)
BIS,[6] mg Fe/kg body wt	5.6 ± 4.7	2.0 ± 2.1
Total body iron,[7] mg	2106.4 ± 320.1	1990.4 ± 131.2
Circulating iron, mg	1549.3 ± 245.6	1629.3 ± 117.3
Tissue iron, mg	360.2 ± 62.0	345.1 ± 24.7
Storage iron, mg	221.0 (89.4; 302.9)[a]	0.0 (0.0; 16.57)[b]

[1] All values are arithmetic mean ± SD for normally distributed data or geometric mean [–SD; +SD] for skewed data, except for height, CRP concentration and storage iron, where median (IQR) is reported. Values in a row having different superscript letters differ between the 2 groups based on independent-samples t-test and independent-samples Mann-Whitney U-test, $P < 0.05$. No superscript letters indicate no statistically significant differences between the countries, except for prevalence data where no statistical tests were applied. AGP, α-1-acid glycoprotein; BIS, body iron stores; BMI, body mass index; bw body weight; ID, iron deficiency, IDA, iron deficiency anemia; CRP, C-reactive protein; Hb hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor.

[2] Anemia defined as Hb <12 g/dL, ID defined as either SF_a <15 μg/L and/or sTfR >8.3 mg/L and IDA defined as Hb <12.0 g/dL and either SF_a <15 μg/L and/or sTfR >8.3 mg/L.

[3] BRINDA correction according to $SF_a = SF_{unadjusted} - \beta_1 \times (CRP_{obs} - CRP_{ref}) - \beta_2 \times (AGP_{obs} - AGP_{ref})$; CRP_{obs}/AGP_{obs} observed/reported CRP value, CRP_{ref} BRINDA reference CRP value, 0.16 mg/L, AGP_{ref} BRINDA reference AGP value, 0.54 mg/L (35). We did not consider malaria for BRINDA adjustment.

[4] Expected CRP concentrations for healthy individuals are ≤5 mg/L.

[5] Expected AGP concentrations for healthy individuals are ≤1 g/L.

[6] Body iron calculated as -[$\log_{10}$(sTfR [μg/L]/SF [μg/L])−2.8229]/0.1207 (37), with unadjusted SF values.

[7] Total body iron as sum of circulating iron, tissue iron, and storage iron.

per woman on average, range 1–15 sessions), with analgesics being the most often consumed type of medication (36 sessions), followed by antibiotics and antimalarials with 12 started sessions each. In Switzerland, 32 medication sessions were reported within the 9 study mo (3 medication intake sessions per woman on average, range 0–10 sessions). Analgesics accounted for 22 of these sessions in Switzerland. Although frequency of infectious morbidity appeared to be higher in Benin than in Switzerland, CRP concentrations during iron supplementation were significantly higher in Switzerland than in Benin ($P = 0.004$; **Table 3**); we assumed no relevant country, time or time × country interaction effects on CRP or AGP concentrations, based on results shown in Supplementary Figures 2 and 3.

Iron balance during the preintervention and postintervention periods

During the preintervention period, there was no time effect on any of the conventional iron status parameters, except for the inflammation unadjusted SF concentration and inflammation unadjusted BIS ($P = 0.013$ and $P = 0.022$, respectively; **Supplementary Table 2**). In contrast, the isotope tracer concentration significantly decreased with time ($P < 0.001$) due to dilution of the isotope tracer by absorbed dietary iron of natural isotopic composition. Significant country effects were found on isotope tracer concentration ($P < 0.001$), SF and SF_a concentrations ($P < 0.001$ and $P = 0.002$, respectively), and BIS and BIS_a ($P = 0.011$ and $P = 0.039$, respectively; Supplementary Table 2). Baseline isotope tracer concentration was lower in Benin, likely due to a greater time since original isotope administration in Benin than in Switzerland and the multiple pregnancies since isotope administration in the Benin women, which were responsible for the quantitative loss of a higher proportion of the originally administered isotope tracer by naturally occurring iron losses. SF and SF_a concentrations were higher in Benin due to differences in inclusion criteria, and this resulted in a country effect on BIS and BIS_a. A significant time × country interaction on unadjusted SF concentration ($P = 0.017$), BIS ($P = 0.018$), and BIS_a

TABLE 2 Mean ± SD dietary intake data assessed via 3-day weighed food records in both Benin and Switzerland ($n = 20$)[1]

	Benin $n = 10$[2]	Switzerland $n = 10$[3]
Energy intake, kcal/d	1683.5 ± 307.1	2006.6 ± 542.2
Iron intake, mg/d	17.2 ± 3.0[a]	11.5 ± 3.6[b]
Iron density, mg Fe/1000 kcal	10.4 ± 2.3[a]	5.8 ± 1.5[b]
Phytic acid intake, mg/d	1612.7 ± 484.3	1698.1 ± 680.1
Phytic acid density, mg PA/1000 kcal	962.1 ± 254.9	828.5 ± 221.9
PA:Fe molar ratio	9.1 ± 1.6[a]	12.8 ± 2.6[b]
Ascorbic acid intake,[4] mg/d	60.5 ± 30.8[a]	131.7 ± 57.6[b]
Ascorbic acid density,[4] mg AA/1000 kcal	34.9 ± 14.1[a]	65.1 ± 17.3[b]
AA:Fe molar ratio[4]	1.3 ± 0.6[a]	4.0 ± 1.3[b]
Estimated dietary iron bioavailability, %		
Preintervention period	3.8 [−0.2; 13.5][a]	13.4 [7.2; 23.3][b]
Postintervention period	2.7 [−1.0; 13.2][a]	9.3 [4.9; 15.6][b]

[1]Data assessed via 3-d weighed food records repeated 3 times in each subject's household, once during each study period. Estimated dietary iron bioavailability (%) calculated as the ratio of mean dietary iron intakes in all 3 periods (data of three 3-d weighed food records, not including supplemental iron intake during iron supplementation) and the isotopically measured daily iron absorption of the specified period (Fe_{abs}; mg/d). All values are arithmetic mean ± SD or geometric mean [−SD; +SD]. Means in a row having different superscript letters differ between the 2 groups based on independent-samples *t*-test. No superscript letters indicate no statistically significant differences between the countries. P values <0.05 considered significant.

PA, phytic acid; AA, ascorbic acid.

[2]One woman in Benin declined participation in the assessment of dietary intake data and no dietary data is available for this subject.

[3]No dietary data were available for the preintervention control period for 1 woman in Switzerland.

[4]Ascorbic acid has not been recorded during the intervention period in the Swiss study. Thus, the mean of only 2 records was used to calculate the mean ascorbic acid intake of women in Switzerland.

($P = 0.038$) was found, with unadjusted SF concentration significantly decreasing in Benin ($P = 0.024$). During the postintervention period, there was a decrease in SF_a concentration ($P = 0.011$), but no time effect on the other iron indices (data not shown). Isotope tracer concentration also decreased during the postintervention period ($P < 0.001$).

During the preintervention period, calculated iron absorption (**Figure 2**A) and iron losses (Figure 2B) were 0.92 ± 1.05 mg/d and 1.46 ± 1.95 mg/d in Benin and 1.51 ± 0.37 mg/d and 0.76 ± 1.37 in Switzerland, respectively. This resulted in a negative iron balance (−0.55 ± 1.56 mg/d) in Benin and a positive iron balance (0.75 ± 1.37 mg/d) in Switzerland (Figure 2C). Conventionally calculated changes in total body iron were −0.53 ± 1.53 mg/d in Benin and 0.75 ± 1.36 mg/d in Switzerland (Figure 2D). During the postintervention period, iron absorption was not significantly different from preintervention levels in both countries (0.90 ± 0.93 mg/d in Benin and 0.97 ± 0.41 mg/d in Switzerland; Figure 2A). In Switzerland, iron losses were significantly higher during the postintervention control period compared with the preintervention control period (2.08 ± 1.05 mg/d; $P = 0.031$; Figure 2B), resulting in a negative iron balance, significantly lower than during the preintervention control period (−1.11 ± 1.06 mg/d; $P = 0.006$, Figure 2C). In Benin, postintervention iron losses (1.84 ± 1.61 mg/d; Figure 2B) as well as postintervention iron balance (−0.94 ± 1.13 mg/d; Figure 2C) did not significantly differ from preintervention levels ($P = 0.606$, and $P = 0.549$, respectively). Conventionally (nonisotopically) calculated changes in total body iron during the postintervention period were −0.92 ± 1.12 mg/d in Benin and −1.11 ± 1.08 mg/d in Switzerland (Figure 2D) and were comparable to isotopically calculated iron balance during both periods ($P = 0.989$, and $P = 0.988$, respectively). There were no country effects on iron absorption and iron losses in the overall linear mixed models. However, the country effect was significant in the overall linear mixed model for iron balance ($P = 0.038$), but no significant differences between countries could be detected anymore in the period-specific *t*-tests during post hoc analysis [mean (95% CI) difference preintervention period: −1.30 (−2.64, 0.05) mg/d, mean (95% CI) difference postintervention period: 0.17 (−0.83, 1.17) mg/d; Figure 2C].

Effects of iron supplementation

During iron supplementation, there were significant time effects on Hb ($P = 0.005$) and SF and SF_a concentrations ($P < 0.001$ and $P = 0.001$, respectively), as well as on BIS and BIS_a (both $P < 0.001$; Table 3), all increasing during supplementation. The geometric mean [−SD; +SD] increase in Hb was 0.8 [0; 1.7] g/dL in Benin ($P = 0.011$), and 0.2 [−0.8; 1.4] g/dL in Switzerland ($P = 0.465$). There were country effects on Hb ($P = 0.035$), with higher values in Switzerland, and on SF and SF_a ($P = 0.049$ and $P = 0.006$, respectively), with lower values in Switzerland. There were no significant time × country interactions. When fitting the statistical models to the data, standardized residuals were smaller for the model predicting isotope tracer concentration than for the models predicting conventional iron indices (**Supplementary Figure 4**). Similarly, time, country and time × country interactions explained more of the variance in isotope tracer concentration than in the conventional iron indices, especially during supplementation (**Table 4**); indicating the higher accuracy of the

TABLE 3 Women's iron status at intervention baseline and intervention end, and multiple regression models predicting isotope tracer concentration and conventional iron indices in relation to time (intervention day), country, and time × country interaction ($n = 11$ in Benin and $n = 10$ in Switzerland)

	Baseline concentration	Endpoint concentration	Time		Country		Time × country	
			β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
Isotope Tracer Concentration (either ^{57}Fe or ^{58}Fe), ppm								
Benin	40.9 [20.2; 82.8]	34.8 [16.9; 71.3]	−0.07 (−0.10, −0.05)	<0.001	0.76 (0.49, 1.04)	<0.001	0.00 (−0.03, 0.03)	0.842
Switzerland	148.2 [98.9; 222.0]	125.7 [84.7; 186.7]						
Hb, g/dL								
Benin	12.2 ± 1.2	12.9 ± 0.8	0.26 (0.08, 0.43)	0.005	0.41 (0.04, 0.78)	0.035	−0.13 (−0.37, 0.11)	0.305
Switzerland	13.2 ± 1.0	13.4 ± 0.8						
SF, μg/L								
Benin	31.7 [13.1; 76.8]	61.3 [32.3; 116.4]	0.33 (0.16, 0.51)	<0.001	−0.36 (−0.71, −0.01)	0.049	−0.01 (−0.25, 0.23)	0.926
Switzerland	19.1 [10.1; 36.1]	34.7 [25.6; 47.0]						
SF_a, μg/L								
Benin	28.2 [12.0; 65.9]	56.2 [32.9; 96.0]	0.25 (0.11, 0.40)	0.001	−0.52 (−0.86, −0.17)	0.006	0.13 (−0.07, 0.33)	0.209
Switzerland	18.8 [10.1; 35.2]	34.0 [25.2; 45.9]						
sTfR, mg/L								
Benin	5.9 [4.1; 8.4]	5.3 [4.2; 6.8]	−0.16 (−0.32, −0.00)	0.053	−0.27 (−0.68, 0.14)	0.213	0.02 (−0.20, 0.24)	0.867
Switzerland	5.0 [3.7; 6.7]	4.4 [3.5; 5.4]						
Hepcidin, μg/L								
Benin[1]	3.7 [1.1; 12.4]	10.6 [4.2; 27.1]	0.54 (0.18, 0.89)	0.005	−0.11 (−0.48, 0.26)	0.562	−0.21 (−0.66, 0.23)	0.354
Switzerland	2.3 [1.1; 5.0]	5.4 [3.2; 9.1]						
BIS, mg/kg								
Benin	4.6 ± 3.9	7.4 ± 1. 8	0.37 (0.19, 0.54)	<0.001	−0.22 (−0.59, 0.14)	0.235	−0.02 (−0.26, 0.23)	0.888
Switzerland	3.4 ± 2.2	6.0 ± 1.3						
BIS_a, mg/kg								
Benin	4.2 ± 3.7	6.5 ± 2.6	0.31 (0.15, 0.48)	<0.001	−0.19 (−0.57, 0.19)	0.329	0.06 (−0.17, 0.28)	0.637
Switzerland	3.3 ± 2.1	5.9 ± 1.3						
AGP, g/L								
Benin	0.4 [0.3; 0.6]	0.5 [0.3; 0.7]	0.29 (0.04, 0.54)	0.034	0.21 (−0.13, 0.55)	0.230	−0.44 (−0.79, −0.08)	0.023
Switzerland	0.5 [0.4; 0.8]	0.4 [0.4; 0.5]						
CRP, mg/L								
Benin	0.6 (0.4; 0.6)	0.5 (0.3; 0.7)	0.21 (−0.10, 0.53)	0.186	0.43 (0.14, 0.71)	0.004	−0.46 (−0.89, −0.02)	0.005
Switzerland	1.2 (0.5; 2.8)	0.6 (0.3; 1.1)						

Baseline and endpoint concentrations are reported as arithmetic mean ± SD for normally distributed data or geometric mean, with [−SD; +SD] indicated in brackets for skewed data, except for CRP concentrations where median (IQR) is reported. Standardized regression coefficients (95% CI) assessed by linear mixed models: random factor: subject identification number, fixed factors: time (intervention day; continuous), country (0 = Benin, 1 = Switzerland), and time × country interaction. P values <0.05 were considered significant. AGP, alpha-1-acid glycoprotein; BIS, body iron stores ($-[\log_{10}(\text{sTfR}\ [\mu g/L]/\text{SF}\ [\mu g/L]) - 2.8229]/0.1207$); BIS_a, body iron stores with BRINDA-adjusted SF concentrations used for calculations; CRP, C-reactive protein; Hb, hemoglobin; SF, serum ferritin; SF_a, BRINDA adjusted serum ferritin [$SF_a = SF_{unadjusted} - \beta_1 \times (CRP_{obs} - CRP_{ref}) - \beta_2 \times (AGP_{obs} - AGP_{ref})$; CRP_{obs}/AGP_{obs} observed/reported CRP value; CRP_{ref} BRINDA reference CRP value, 0.16 mg/L; AGP_{ref} BRINDA reference AGP value, 0.54 mg/L (35)]; sTfR, soluble transferrin receptor.

[1]Intervention endpoint hepcidin concentration assessed on study day 120.

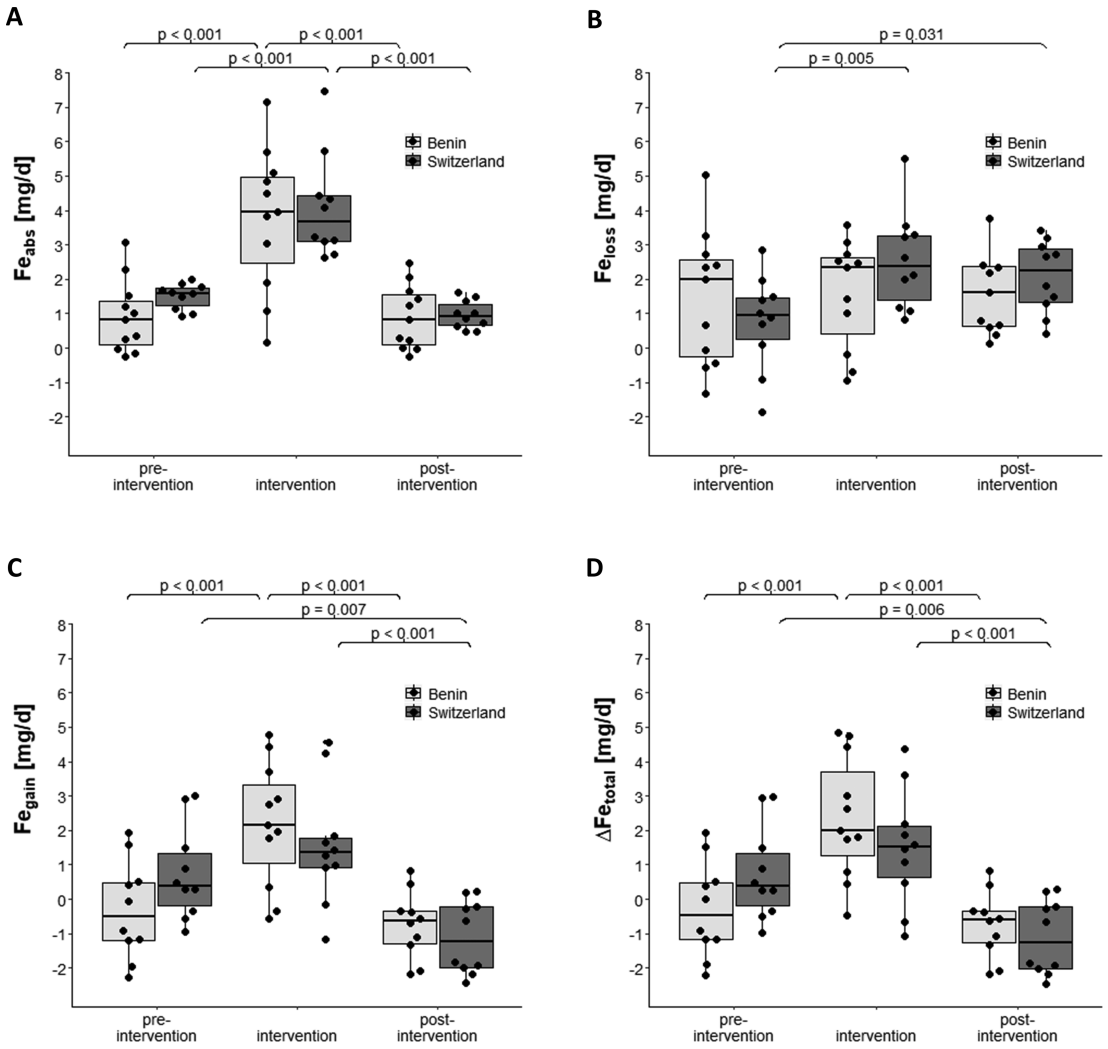

FIGURE 2 Median daily amounts of absorbed iron (Fe_{abs}, A), iron loss (Fe_{loss}, B), net iron gains determined isotopically (Fe_{gain}, C), and changes in conventionally calculated total body iron (ΔFe_{total}, D) in young women in Benin ($n = 11$) and in Switzerland ($n = 10$). Boxplots indicating median with IQR, with vertical lines indicating the data range, and dots representing individual data. Differences between periods assessed based on country-specific linear mixed models with subject as random factor and period (ordinal variable) as fixed factor. Differences between the 2 countries assessed based on overall linear mixed model with subject as random factor and period and country (ordinal variables) as fixed factors and post hoc period-specific independent-samples *t*-tests in case of significant country-effect in the overall model. In Benin, the iron supplementation period differed from the control periods for all parameters, except for iron losses which did not significantly differ between the study periods. In Switzerland, the iron supplementation period significantly differed from the postintervention control period in all parameters except for iron losses which significantly differed between the preintervention control period and the iron supplementation period, but not between the postintervention control period and the supplementation period. The pre- and postintervention control period significantly differed for all parameters in Switzerland, except for iron absorption, but no differences could be detected in Benin. Only *P* values considered significant ($P < 0.05$) are shown in the figure.

fraction of total body iron absorbed per unit of time, k_{abs}, in predicting iron absorption than changes in conventional iron indices.

During iron supplementation, isotope tracer concentration was diluted significantly more (resulting in significantly more negative slope k_{abs}) than during the control periods (for both, $P < 0.001$; **Figure 3**), while there were no country differences ($P = 0.230$; Figure 3). The median (IQR) slope ratios comparing k_{abs} during supplementation to k_{abs} during pre- and postintervention periods, respectively, were 1.9 (−3.8 to 4.1) and 2.0 (−11.3 to 3.4) in Benin and 2.7 (1.8–3.2) and 4.0 (2.6–4.5) in Switzerland. The slope ratio comparing the preintervention control period to the intervention period was significantly lower in Benin than in Switzerland ($P = 0.003$), indicating the beneficial impact of iron supplements was stronger in Switzerland than in Benin. Due to high variance, the difference in slope ratios comparing the postintervention control period to the intervention period between the 2 countries was no longer significant ($P = 0.053$). During the first 90 d of iron supplementation in Benin, iron absorption was 3.75 ± 2.07 mg/d (Figure 2A). In Switzerland, iron absorption during iron supplementation was 4.09 ± 1.52 mg/d (Figure 2A). In both countries daily iron absorption exceeded WHO daily iron absorption recommendations of 1.46 mg/d ($P < 0.001$ for both). During the first 90 d of iron supplementation in Benin, iron loss was 1.58 ± 1.57 mg/d (Figure 2B). In Switzerland, iron loss during iron supplementation was 2.54 ± 1.43 mg/d (Figure 2B).

TABLE 4 Overview of linear mixed models for isotope tracer abundance or conventional iron indices as explained by the fixed factors time (intervention day), country, and time × country interaction and participant identification number as random effects. Variance explained by the fixed effects (R^2_m) and the fixed and random effects (R^2_c) according to Nakagawa is presented along with Steiger's Z test comparing the models from conventional iron indices to isotope tracer abundance ($n = 21$)

Variance	Isotope tracer abundance	Hb	*P* value	SF	*P* value	SF_a	*P* value	sTfR	*P* value	Hepcidin	*P* value	BIS	*P* value	BIS_a	*P* value
Preintervention control period															
R^2_m	0.584	0.150	0.071	0.298	0.220	0.220	0.157	0.035	0.017	0.099	0.065	0.150	0.119	0.108	0.087
R^2_c	0.999	0.892		0.774		0.762		0.880		0.805		0.808		0.812	
Intervention period															
R^2_m	0.582	0.153	<0.001	0.238	0.180	0.284	<0.001	0.080	0.055	0.158[1]	<0.001	0.180	<0.001	0.313	0.100
R^2_c	0.999	0.657		0.660		0.770		0.885		0.522[1]		0.662		0.701	
Postintervention control period															
R^2_m	0.593	0.116	0.017	0.303	0.086	0.246	0.134	0.095	<0.001	0.129	0.081	0.211	0.132	0.162	0.140
R^2_c	0.999	0.658		0.792		0.843		0.759		0.399		0.827		0.891	

Linear mixed models with random factor: subject identification number, and fixed factors: time (intervention day; continuous), country (0 = Benin, 1 = Switzerland), and time × country interaction. *P* values <0.05 are considered significant.

BIS, body iron stores (−[$\log_{10}$(sTfR [μg/L]/SF [μg/L])−2.8229]/0.1207); BIS_a, body iron stores with BRINDA-adjusted SF concentrations used for calculations; Hb, hemoglobin; SF, serum ferritin; SF_a, BRINDA adjusted serum ferritin [$SF_a = SF_{unadjusted} - \beta_1 \times (CRP_{obs} - CRP_{ref}) - \beta_2 \times (AGP_{obs} - AGP_{ref})$; CRP_{obs}/AGP_{obs} observed/reported CRP value; CRP_{ref} BRINDA reference CRP value, 0.16 mg/L; AGP_{ref} BRINDA reference AGP value, 0.54 mg/L (35)]; sTfR, soluble transferrin receptor.

[1]Intervention endpoint hepcidin concentration assessed on study day 120.

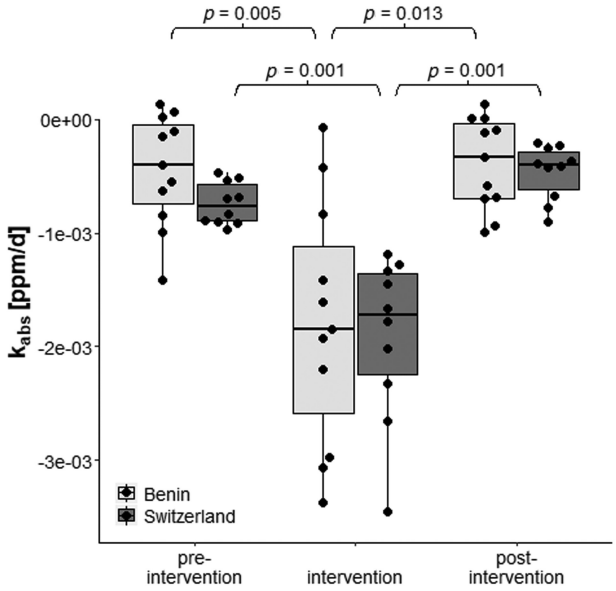

FIGURE 3 Median isotopic dilution rates in young women in Benin ($n = 11$) and in Switzerland ($n = 10$). Boxplots indicating median with interquartile range, with vertical lines indicating the data range, and dots representing individual data. Differences between periods assessed based on country-specific linear mixed models with subject as random factor and period (ordinal variable) as fixed factor. Differences between the 2 countries assessed based on overall linear mixed model with subject as random factor and period and country (ordinal variables) as fixed factors and post hoc period-specific independent-samples t-tests in case of significant country-effect in the overall model. No significant time × country interaction effects. Only p values considered significant ($P < 0.05$) are shown in the figure.

Iron balance during the first 90 d of iron supplementation in Benin was 2.17 ± 1.81 mg/d (Figure 2C). In Switzerland, iron balance during iron supplementation was 1.55 ± 1.75 mg/d (Figure 2C). Even though there was a significant country effect on iron balance in the overall model ($P = 0.038$), differences in iron balance between the 2 countries were no longer significant during the iron intervention period in the 2 countries; similarly as for the pre- and postintervention periods (Figure 2C). Conventionally (nonisotopically) calculated changes in total body iron were 2.37 ± 1.77 mg/d during the first 90 d of iron supplementation in Benin and 1.49 ± 1.70 mg/d during the 90-d iron supplementation in Switzerland (Figure 2D).

Discussion

To our knowledge, this study is the first to use long-term changes in blood iron-isotopic composition in young women in Europe and Africa: *1*) to determine basal iron absorption, iron losses, and iron balance; and *2*) to quantify iron balance during oral iron supplementation and to compare them between populations.

During the preintervention period, daily iron absorption in Benin was 0.92 ± 1.05 mg/d, which was lower ($P = 0.119$) than the estimated iron requirement of 1.46 mg/d for young women derived through factorial modelling by WHO and used for definition of iron intake recommendations (46). In Switzerland, daily iron absorption was 1.51 ± 0.37 mg/d during

the preintervention period, much closer to the WHO estimated iron requirement of 1.46 mg/d (46). The lower absorption in Benin was not due to lower overall iron intakes (Table 2), but instead to lower iron bioavailability. Low iron bioavailability in Benin might principally result from *1*) ascorbic acid (an iron absorption enhancer) intakes lower than in Switzerland ($P < 0.001$; Table 2), and *2*) the contribution of soil contamination iron (with poor bioavailability) to the iron values in the African food composition databases used to determine iron intake in Benin. Phytic acid contents were generally high also in the Swiss diet and the phytic acid to iron molar ratio was lower in Benin than in Switzerland. Another likely contributing factor to lower iron bioavailability in Benin was slightly elevated serum hepcidin concentrations; this may have been due to higher iron stores (SF_a) and/or a higher burden of low-grade infections that was not detected by AGP and CRP measurements.

Daily iron losses of 1.46 ± 1.95 mg/d during the preintervention period in Benin closely match estimates of iron loss in menstruating women derived from extrapolations from measured iron excretion in men (2) based on body weight and estimated menstrual blood losses, used by the WHO (46). Our data do not support the common assumption that iron losses are higher in individuals in low-income countries (4, 6). Daily iron loss in Benin is consistent with measurements of geometric mean [−SD; +SD] iron excretion using isotopic techniques in US women of 1.69 [0.98; 2.92] mg/d (mean body weight 74 kg) (3) and in Chinese women of 1.36 ± 0.18 mg/d (mean body weight of 59 kg) (47). Women in Switzerland had mean iron losses of only 0.76 ± 1.37 mg/d during the preintervention control period and reported a total of 28 menstruations during this preintervention control period [27 menstruations during intervention period, 29 menstruations during postintervention control period (data not shown)]. The generally low iron losses in women in Switzerland might thus mainly result from the more frequent use of oral contraceptives in women in Switzerland compared with women in Benin and also in the US women reported above (3). Use of oral contraceptives reduces menstrual blood losses in women by up to half (7). We observed mean ± SD iron losses of 1.38 ± 1.37 and 2.20 ± 1.48 mg/d (combining all 3 study periods) in the women using ($n = 5$) and not using ($n = 5$) oral contraception in Switzerland, respectively.

A baseline SF_a concentration of 34.7 [16.3; 74.0] μg/L suggested that women in Benin had adequate iron status on entry into the study. Their iron balance during the preintervention control period was negative (−0.55 ± 1.56 mg/d), possibly due to low iron absorption, and SF_a concentrations decreased to 28.2 [12.0; 65.9] μg/L at intervention start. Women in Benin benefited from iron supplements (as shown by their increase in Hb concentrations during intervention), so their true iron status might have been poorer than that suggested by the iron indices. Even though approaches to correct SF concentrations for inflammation are recommended (52), accurate determination of iron status in Sub-Saharan Africa, where infections are often common and genetic factors are different (53), remains difficult. Women in Switzerland were in positive iron balance (+0.75 ± 1.37 mg/d) during the preintervention period, possibly due to their low iron losses, and had marginal iron stores (46). However, they did not improve their Hb concentrations during the iron supplementation period.

The impact of oral iron supplementation programs in women in Sub-Saharan Africa often has been disappointing in the past (8, 9); this finding is thought to be due to a combination of limited distribution, poor compliance (54), and high background inflammation increasing serum hepcidin and reducing bioavailability (5). Yet, during iron supplementation, both groups absorbed similar amounts from the iron supplements in this study and our experimental data suggest that with good compliance, oral iron can be well-absorbed even in Sub-Saharan African populations with apparent near normal SF and slightly elevated hepcidin concentrations.

Compared with the preintervention period, iron supplementation was associated with a significant increase in protracted iron losses in the women in Switzerland, but not in the women in Benin (Figure 2). Thus, with similar iron absorption and lower iron losses, iron gains during iron supplementation in Benin are not lower than what they are in Switzerland. Higher iron saturation in desquamated cells might have led to slightly increased iron losses at normal iron status compared with ID status in the women in Switzerland. We could identify no methodological reasons for our observation of increased iron losses: the dilution of stable iron isotope method we employed relies on the same principles as the radioiron techniques previously used to assess iron losses (2, 3). We have also recently found increased iron losses during iron supplementation in Gambian toddlers with very poor iron status (55). Increased iron excretion during iron supplementation is an unlikely explanation for these increased iron losses as the intestine cannot regulate the amount of iron excreted in the feces (56, 57). Iron losses in sweat are negligible (58) as are urinary iron losses in the absence of kidney or bladder disease. Several endoscopy studies have suggested iron supplementation may cause irritation and inflammation of the gut mucosa leading to gastrointestinal blood loss (59–62). We recently detected significant increases in fecal hemoglobin losses during iron supplementation in Kenyan toddlers (A Giorgetti, ETH Zurich, personal communication, 2020), but the reason(s) why this effect was only seen in women in Switzerland, but not in Benin, remain unknown.

Our study has limitations. Although determination of the fraction of total body iron absorbed per unit of time, k_{abs}, involves only isotopic measurements and is highly accurate, estimates of the amount of total body iron absorbed or lost per unit of time, Fe_{abs} and Fe_{loss} respectively, require determination of Fe_{total}, which, in turn, depends on measurements of conventional iron indices with generally high variances (63, 64). Prevalence and severity of inflammation in Benin were less than anticipated. Thus, we could not demonstrate the advantage of the dilution of stable iron isotopes methodology in assessing iron interventions in settings with a high burden of infections compared with conventional iron indices. Morbidity and dietary data in Switzerland were self-reported which may have decreased reliability. Furthermore, we did not use a randomized cross-over design and cannot rule out effects of season on long-term measurements. Due to differences in inclusion criteria and study design, the 2 cohorts were not fully comparable. We anticipated lower SF_a concentrations in Benin based on previous data (13, 14). Especially in Benin, the participants were highly heterogeneous, which likely increased the variability of our findings. Our study populations may also not be representative of young women in the 2 countries as all participants had already participated in iron

absorption studies and there was a high participant exclusion rate. Testing several study outcomes by the application of different statistical tests within only 1 study increased the risk of type I errors, especially as the sample size was small. However, to better explore the potential of the novel dilution of stable iron isotopes methodology we decided against *P* value correction for type I error, as our outcomes leading to iron balance will be highly correlated, and as the primary aim of this study was not to assess the efficacy of a defined intervention. Even though being small, the sample size met the requirements of our power calculations.

Compared with conventional iron indices, isotopic dilution appears to have greater robustness and less confounding as demonstrated by: *1*) the smaller standardized residuals (Supplementary Figure 4); *2*) the higher percentages of variance explained by time, country, and time × country interaction; and *3*) the higher variance explained by the model in individual women through the random effect term (Table 4) using the isotope tracer concentration. The isotopic dilution's ability to accurately and directly quantify long-term changes in body iron may prove useful in several areas of iron nutrition research. In this study, it validated the use of conventional iron indices to assess iron interventions in populations with little or no inflammation. It also shows promise as a new reference method for better understanding iron balance and measuring iron bioavailability in free-living individuals. In addition, it may be useful for determining the impact of iron interventions in settings of high infection and inflammation, such as in HIV or tuberculosis patients. However, due to the unexpectedly low burden of infection and inflammation in our cohort in Benin, this needs to be confirmed in future research.

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The authors' responsibilities were as follows—CS, CIC, GB, DM, MBZ: designed the studies; CS, CIC, CEM: conducted the experiments; CS, CZ, GB, DM, MBZ: analyzed the data; CS, DM, MBZ: wrote the first draft: CS, DM, MBZ: had primary responsibility for the final content; and all authors read and approved the final version of the paper. The authors report no conflicts of interest.

Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

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