

Comparing iron absorption from multiple micronutrient supplements and iron-folate supplements: a stable isotope study in Kenyan pregnant women

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Background

Sufficient intake of essential vitamins and minerals during pregnancy is crucial for maternal health and fetal development. Approximately 69% of nonpregnant women aged 15–49 y globally have a deficiency in at least one of three key micronutrients, and this rate is likely higher among pregnant women due to their increased nutritional needs (Stevens et al., 2022).

In 2016, the World Health Organization (WHO) recommended daily iron and folic acid (IFA) supplements during routine antenatal care (WHO, 2016c). In preparation for a review of the WHO guideline in 2020, a Cochrane review analyzed the effects of MMS and IFA on maternal anemia and other outcomes (Keats et al., 2019) in 19 studies from low- and middle-income countries (LMICs). The analysis of the overall effect of MMS versus IFA on third-trimester maternal anemia (defined as hemoglobin (Hb) <110 g/L) showed no significant difference between groups (RR 1.04, 95% CI 0.94–1.15). However, no subgroup analyses were performed based on varying doses of iron, which ranged from 20 to 60 mg.

In 2020, the WHO guidance regarding multiple micronutrient supplements (MMS) during pregnancy changed from “not recommended” to “recommended in the context of rigorous research” (WHO, 2020). The conditional recommendation on MMS in the 2020 WHO guidelines was based on several concerns, including that “...more evidence is needed on the effects of switching to a 30 mg dose of iron from a higher dose of iron (e.g. 60 mg), particularly in settings where higher doses of iron are routinely used due to a high anemia prevalence or other reasons.”

In a follow-up analysis of data from 11 trials, Gomes et al. (2022) compared IFA to MMS with either 30 mg or 60 mg of iron. This analysis focused on Hb, third-trimester anemia, and iron deficiency anemia (IDA), considering daily iron dosage, total supplemental iron intake, and the baseline prevalence of anemia. The results showed no significant differences between MMS and IFA regarding third-trimester Hb or the risks of anemia and IDA, regardless of the iron dosage or total supplemental iron consumed. Notably, MMS providing 30 mg of iron was found to be comparable to IFA with 60 mg of iron, with a mean hemoglobin difference of -0.26 g/L (95% CI: -1.41 to 0.89). The risk ratios were 0.99 (95% CI: 0.92–1.07) for anemia and 1.31 (95% CI: 0.66–2.60) for IDA.

It is surprising that the 30 mg dose of iron as ferrous fumarate in MMS (composition in **Table 1**) appears to have similar efficacy (on hemoglobin concentrations) to the 60 mg dose as ferrous sulfate in IFA (Gomes et al., 2022), because, in healthy nonpregnant women, iron absorption from ferrous fumarate and ferrous sulfate is comparable (Fidler et al 2003).

The equivalent efficacy of the two different doses may be due to several factors:

- Fractional absorption of iron decreases with increasing dose (Stoffel et al 2022).
- Other micronutrients in MMS, such as vitamins B12 and A, may correct deficiencies of these nutrients which can contribute to anemia (Gomes et al 2022).
- Other micronutrients in the MMS, such as vitamins A, C and riboflavin, may improve the absorption and/or utilization of iron, particularly in inhibitory food matrices. Vitamin C enhances iron absorption from ferrous fumarate in nonpregnant women (Fidler et al 2003; von Siebenthal et al 2023). Fidler et al. (2003) compared iron absorption from ferrous fumarate-fortified meals with and without ascorbic acid added at a 4:1 molar ratio (relative to 5 mg iron): ascorbic acid increased Fe absorption from 6.3 to 10.4%, ($P=0.02$). Similarly, von Siebenthal (2023) reported that iron absorption from a dose of 100 mg iron as ferrous fumarate was increased 30% by an 80 mg dose of ascorbic acid ($p < .001$).
- In nonpregnant women with iron deficiency or IDA, daily oral doses ≤ 40 mg may be preferred because iron doses ≥ 60 mg given daily can trigger a transient increase in circulating hepcidin that can inhibit iron absorption from the next day's dose, a phenomenon not seen with iron doses ≤ 40 mg (Stoffel et al 2022).

Thus, a key knowledge gap is whether there is a significant difference in total iron absorption from the 30 mg of iron in MMS compared to the 60 mg of iron in IFA, when given daily to pregnant women. To our knowledge: (i) iron absorption from MMS and IFA has not been directly quantified using iron stable isotopes in pregnant women; (ii) whether iron doses ≥ 60 mg given daily to pregnant women can trigger a transient increase in circulating hepcidin that can inhibit iron absorption from the next day's dose has not been studied, and (iii) the relative effect of an inhibitory meal matrix, characteristic of diets in LMIC, on iron absorption from the MMS and IFA formulations has not been tested.

Study Aims

Our study aims are, in pregnant women in the 2nd trimester:

1. Quantify and compare iron absorption (bioavailability) from MMS with 30 mg or 60 mg iron versus IFA with 60 mg iron, in the fasted state and when given with an inhibitory meal.
2. Determine whether the 30 or 60 mg iron dose in MMS, or the 60 mg iron dose in IFA, when given daily, can trigger a transient increase in circulating hepcidin that can inhibit iron absorption from the next day's dose.

Study participants

This study will be conducted in Q1-2 of 2025 at the ETH Zurich/Oxford/JKUAT research facility in Msambwemi Hospital in southern Kenya. We will recruit healthy pregnant women from the community and antenatal clinics. Inclusion criteria will be: female; pregnant at gestational age 12 (± 1) weeks (dated by ultrasound); age 18 to 35 y; Hb concentration ≥ 80 g/L; CRP < 10 mg/L (absence of significant inflammation); body weight < 80 kg; no major chronic diseases; no intake

of outside vitamin and mineral supplements in the 2 weeks before study start and during the study; no blood transfusion, blood donation, or significant blood loss over the past 4 mo. Exclusion criteria will be: severe anemia (defined as Hb <80 g/L), malaria, sickle cell disease (SS and SC) or hemoglobin C disease (CC). Exclusion criteria once enrolled will be inability to follow study procedures, miscarriage, a drop in Hb to below 80 g/L (will be referred to the antenatal care clinic) or major illness.

Ethical considerations

The study will be registered on www.clinicaltrials.gov before screening and enrolling participants. The study will be carried out according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human study participants will be reviewed and approved by two independent ethical committees, the ethical committee of ETH Zurich, Switzerland and the ethical committee of the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya. All subjects will provide written informed consent.

Study design

The study will be a controlled, open-label, randomized cross-over trial. During screening, 1 week before study start, we will assess women for eligibility. We will collect a venous blood sample for the determination of Hb concentration, Hb type and pregnancy status, and measure weight to the nearest 0.1 kg and height to the nearest 0.5 cm. Eligible and consented subjects will be enrolled in the iron absorption study.

All women will undergo four sets of absorption studies separated by 14-day isotope incorporation periods. The first set (Study A) will assess iron absorption from MMS with 30 mg or 60 mg iron versus IFA with 60 mg iron when given with an inhibitory meal. The second, third and fourth sets (Study B) will assess iron absorption from the three formulations when given fasted and when given daily and on alternate days.

Study A

Randomization and masking

All participants will receive a labeled oral iron supplement on three different days (Mon-Wed-Friday). Participants will be individually randomly assigned to a condition sequence, using a computer-generated list (Excel, Microsoft Office 2016). Assignment will not be masked. **Figure 1** shows the study design, with an example sequence.

Procedures

The iron doses will be administered on three days between 7:00 am and 9:00 am after an overnight fast. The IFA will contain 60 mg elemental iron as ferrous sulfate (FeSO_4) and 400 μg of folic acid (*producer*) and will be labeled with 3 mg of $^{58}\text{FeSO}_4$. $^{58}\text{FeSO}_4$ will be prepared at the ETH Zurich from ^{58}Fe -enriched elemental iron. The MMS will contain 30 or 60 mg elemental iron as ferrous fumarate (FeFum) (Table 1) (*producer*) and will be labeled with 3 mg of $^{57}\text{FeFum}$ or $^{54}\text{FeFum}$. $^{54}\text{FeFum}$ and $^{57}\text{FeFum}$ will be prepared by Dr. Paul Lohmann GmbH from ^{54}Fe - and ^{57}Fe -enriched elemental iron.

On days 1, 3 and 5 the three forms of iron will be given with whole maize porridge and 300 ml of bottled water. The maize porridge meal will consist of 200 g deionized water, 60 g of whole maize meal and a pinch of sugar, plus twice 20 ml rinsing water. The participants will consume the conditions under direct supervision of the investigators.

After consuming the iron, participants will remain at the study site for 2 hrs under the supervision of the study team and will be instructed not to eat or drink for 3 h, except for 500 mL of bottled water. The water will be provided to the women by the study team, and they can begin to drink it 1 h after the test condition. On each of the study days, participants will be instructed to consume only vegetarian meals low in iron. We will collect venous blood samples on days 1 (baseline) and 19 (after the 14-day incorporation period).

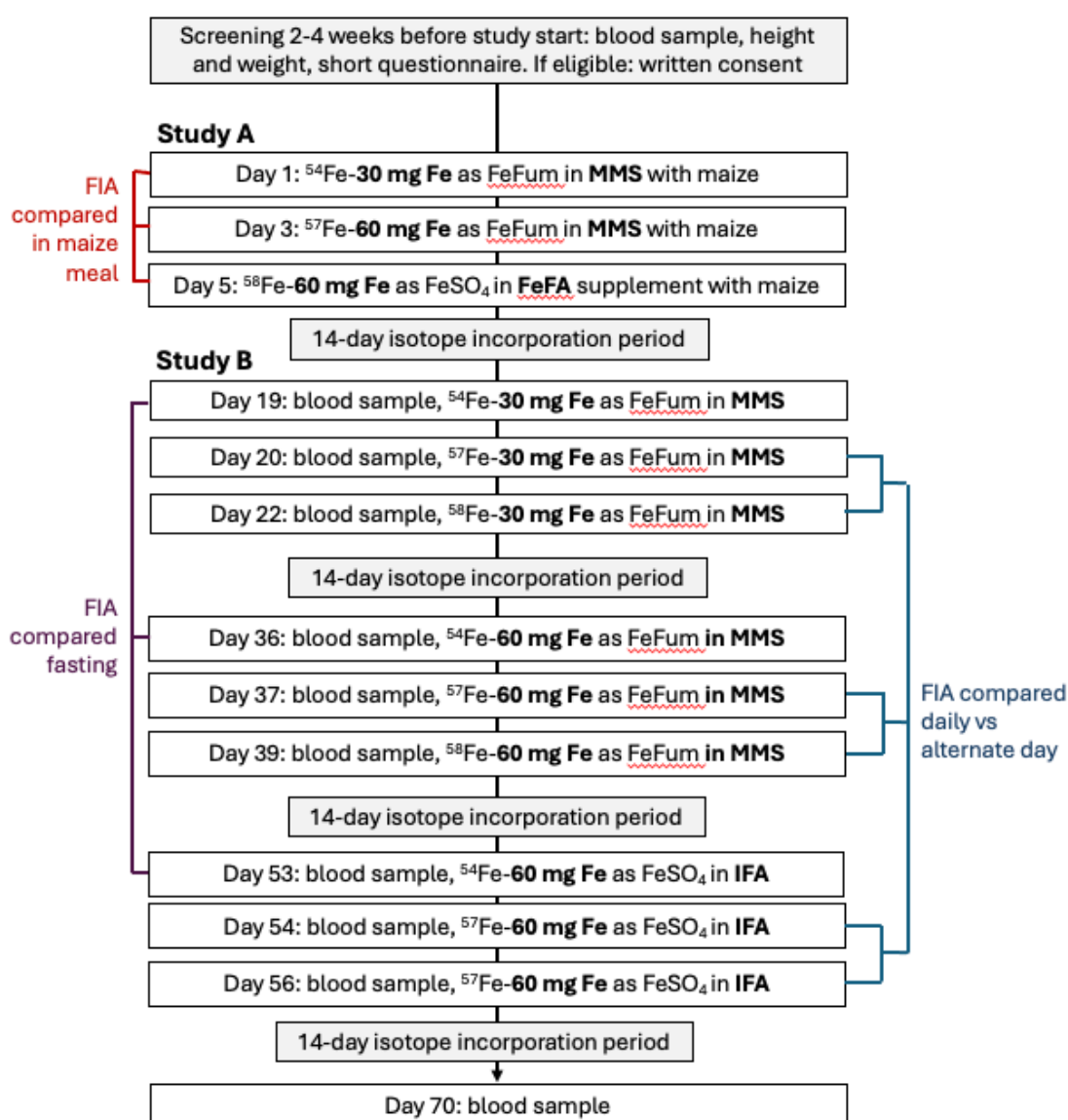


Figure 1: Study Design

Study B

Randomization and masking

Participants will be individually randomly assigned to a study sequence using a computer-generated list (Excel, Microsoft Office 2016). Assignment will not be masked. Figure 1 shows the study design, with an example sequence

Procedures

In this study, we will assess serum hepcidin profiles and fractional and total iron absorption from consecutive-day and alternate-day dosing of the MMS with 30 and 60 mg iron and the IFA with 60 mg of iron. Each participant will go through the three sets of conditions in randomized order. In each set of conditions, participants will receive three iron doses in MMS or IFA labelled with three different isotopes (^{54}Fe , ^{57}Fe , or ^{58}Fe) given with 200 mL bottled water. The iron doses will be administered between 07:00 am and 09:00 am after an overnight fast. We will collect a venous blood sample before the administration of each iron dose in the morning.

In the first set, three doses of MMS with 30 mg iron (as FeFum) will be given on consecutive- and alternate-days (e.g., days 19, 20 and 22). After a 14-day incorporation period, in the second set, three doses of MMS with 60 mg iron (as FeFum) will be given on consecutive- and alternate-days (e.g., days 36, 37 and 39). After a 14-day incorporation period, in the third set, three doses of IFA with 60 mg iron (as FeSO_4) will be given on consecutive- and alternate-days (days 53, 54 and 56). On day 70, 14 days after the last isotope administration, we will collect a final venous blood sample. After consuming the iron, participants will remain at the study site for 2 hrs under the supervision of the study team and will be instructed not to eat or drink for 3 h, except for 500 mL of bottled water. The water will be provided to the women by the study team, and they can begin to drink it 1 h after the test condition. On each of the study days, participants will be instructed to consume only vegetarian meals low in iron.

Laboratory analysis

We will analyze the native iron concentration in the maize flour, and the iron content of the IFA and MMS by inductively coupled plasma mass spectrometry (Q-ICP-MS iCap RQ, Thermo Scientific) after mineralization by microwave digestion (TurboWave, MLS) in HNO_3 . The phytic acid concentration in the maize flour will be measured according to a modified method from Makower, using cerium for the extraction of phytic acid (Makower, 1970). After hydrolyzation in sulfuric acid, inorganic phosphate will be determined (Van Veldhoven et al, 1987) and converted into phytic acid concentrations. We will measure ascorbic acid in the MMS after extraction in meta-phosphoric acid, with dehydroascorbic acid reduced to AA with dithiothreitol as reducing agent. The AA in the extract will be separated and quantified by HPLC (Acquity, Walters) on a C-18 reversed phase column.

We will measure Hb on the day of collection using a Sysmex XN-3000 analyzer (Sysmex Corporation) from EDTA whole-blood and then prepare whole-blood aliquots which will be stored at -80°C . Blood collected with serum tubes will be centrifuged at 3000 rpm for 10 min, and the serum stored at -20°C . We will measure serum ferritin, soluble transferrin receptor (sTfR), α -1-acid glycoprotein (AGP), and C-reactive protein (CRP) in serum using a multiplex

immunoassay (Erhardt et al 2004). We will measure serum hepcidin using the DRG Hepcidin 25 (bioactive) HS enzyme immunoassay (DRG Instruments GmbH, Marburg, Germany). Hemoglobiin A, S and C will be assessed by using HemoTypeSC (Silver Lake, USA). Serum hepcidin will be measured on relevant study days in both Study A and B.

Whole blood samples for isotopic analysis will be mineralized in duplicate using nitric acid and microwave digestion (TurboWave, MLS) followed by separation of the iron from the blood matrix by anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide (Hotz et al 2012). We will measure iron isotope ratios by using a multi-collector inductively coupled plasma mass spectrometry (Neptune, Thermo Fisher Scientific, Germany) (Hotz et al, 2011). The amounts of ^{54}Fe , ^{57}Fe , and ^{58}Fe isotopic tracers in whole blood collected 14 days after completion of each of the sets of absorption studies will be calculated on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. The enriched isotopic ratios resulting from each isotope administration will serve as the baseline for the next set of administrations. Circulating iron will be calculated based on blood volume and mean Hb concentrations for each participant (Brown et al, 1962). The calculations for fractional iron absorption (FIA) will be based on the principles of isotope dilution (Turnlund et al, 1993), taking into account that iron isotopic labels are not monoisotopic and assuming 80% incorporation of the absorbed iron into the erythrocytes (Hosain et al, 1961). Total iron absorption will be calculated based on FIA and the total amount of iron consumed per condition.

Statistical analysis

Power calculation

For both studies, we will power to detect a 20% within-subject difference in total iron absorption (TIA), based on the SD of the difference in TIA in nonpregnant women from 5 mg iron given with and without ascorbic acid (molar ratio to iron = 2:1) in a previous study conducted at the ETH Zurich (Cepeda-Lopez et al, 2017). Using the log SD of 0.1694, a power of 80% and a level of significance of 0.05 we estimate a sample size of 46. Anticipating a drop-out rate of 20%, we plan to enroll 50 participants.

Primary outcomes

The primary outcome will be TIA for each condition of iron administration, calculated as described above. The specified comparisons will be:

In Study A

- (i) TIA from MMS with 30 mg iron versus IFA given with maize porridge
- (ii) TIA from MMS with 60 mg iron versus IFA given with maize porridge

In Study B

- (i) TIA from MMS with 30 mg iron versus IFA given fasted with water
- (ii) TIA from MMS with 60 mg iron versus IFA given fasted with water
- (iii) TIA from MMS with 30 mg iron from the consecutive day versus alternate day dosing
- (iv) TIA from MMS with 60 mg iron from the consecutive day versus alternate day dosing
- (v) TIA from IFA with 60 mg iron from the consecutive day versus alternate day dosing

The SPSS statistical programming environment (IBM SPSS Software, Version 28) and Microsoft Office EXCEL 2016 (Microsoft, Redmond, WA) will be used for the data analysis. Per protocol analysis will be used to analyze primary outcomes. Data will be examined for normality by use of the Shapiro–Wilk test. Normally distributed data will be reported as mean (SD), and non-normally distributed data reported as median (IQR).

Linear mixed effects models will be used to assess the effect of the different conditions on iron absorption. Logarithmically transformed data will be used when model assumptions are not met. TIA will be the dependent variable, the iron administration condition and serum ferritin will be added to the models as fixed factors, and subject ID will be added as a random factor (intercept).

In Study A, TIA from the three formulations given with maize porridge will be compared in one model. In Study B, TIA from the three formulations given fasted will be compared in one model (first day of each sequence); and TIA from the three formulations given on the consecutive day versus alternate day will be compared in another model (second and fourth day of each sequence). We will not correct post hoc tests for multiple comparisons, as the comparisons are prespecified.

Separately, we will perform multiple linear regression analyses to determine predictors of TIA, including variables such as week of pregnancy, hepcidin, iron status (serum ferritin, Hb, sTfR) and inflammation markers (CRP, AGP).

Table 1. Ingredients in the MMS (UNIMMAP formulation)

Component	Chemical entity	Amount
Vitamin A	Retinyl acetate	800 mcg RAE
Vitamin C	Ascorbic acid	70 mg
Vitamin D	Cholecalciferol	5 mcg
Vitamin E	Alpha tocopheryl succinate	10 mg α-TE
Vitamin B1	Thiamine mononitrate	1.4 mg
Vitamin B2	Riboflavin	1.4 mg
Vitamin B3	Niacinamide	18 mg NE
Vitamin B6	Pyridoxine HCl	1.9 mg
Folic acid	Folic acid	400 mcg
Vitamin B12	Cyanocobalamin	2.6 mcg
Iron	Ferrous fumarate	30 or 60 mg
Iodine	Potassium iodide	150 mcg
Zinc	Zinc oxide	15 mg
Selenium	Sodium selenite	65 mcg
Copper	Cupric oxide	2 mg

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