Iron absorption from oral iron supplements given on consecutive versus alternate days and as single morning doses versus twice-daily split dosing in iron-depleted women: two open-label, randomised controlled trials

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Summary

Background Current guidelines to treat iron deficiency recommend daily provision of ferrous iron divided through the day to increase absorption. However, daily dosing and split dosing might increase serum hepcidin and decrease iron absorption from subsequent doses. Our study aim was to compare iron absorption from oral iron supplements given on consecutive versus alternate days and given as single morning doses versus twice-daily split dosing.

Methods We did two prospective, open-label, randomised controlled trials assessing iron absorption using (^{54}Fe)-labelled, (^{57}Fe)-labelled, or (^{58}Fe)-labelled ferrous sulfate in iron-depleted (serum ferritin ≤25 µg/L) women aged 18–40 years recruited from ETH Zurich and the University of Zurich, Switzerland. In study 1, women were randomly assigned (1:1) to two groups. One group was given 60 mg iron at 0800 h (±1 h) on consecutive days for 14 days, and the other group was given the same doses on alternate days for 28 days. In study 2, women were assigned to two groups, stratified by serum ferritin so that two groups with similar iron statuses could be formed. One group was given 120 mg iron at 0800 h (±1 h) and the other was given the dose split into two divided doses of 60 mg at 0800 h (±1 h) and 1700 h (±1 h) for three consecutive days. 14 days after the final dose, the groups were each crossed over to the other regimen. Within-individual comparisons were done. The co-primary outcomes in both studies were iron bioavailability (total and fractional iron absorption), assessed by measuring the isotopic label abundance in erythrocytes 14 days after administration, and serum hepcidin. Group allocations in both studies were not masked and primary and safety analyses were done on an intention-to-treat basis. The studies were registered at ClinicalTrials.gov, numbers NCT02175888 (study 1) and NCT02177851 (study 2) and are complete.

Findings For study 1, 40 women were enrolled on Oct 15–29, 2015. 21 women were assigned to the consecutive-day group and 19 to the alternate-day group. At the end of treatment (14 days for the consecutive-day group and 28 days for the alternate-day group), geometric mean (–SD, +SD) cumulative fractional iron absorptions were 16·3% (9·3, 28·8) in the consecutive-day group versus 21·8% (13·7, 34·6) in the alternate-day group (p=0·0013), and cumulative total iron absorption was 131·0 mg (71·4, 240·5) versus 175·3 mg (110·3, 278·5; p=0·0010). During the first 14 days of supplementation in both groups, serum hepcidin was higher in the consecutive-day group than the alternate-day group (p=0·0031). In study 2, 20 women were enrolled between Aug 13 and 18, 2015. Ten women were assigned to receive once-daily dosing and ten were assigned to receive twice-daily divided dosing. No significant differences were seen in fractional (day 1–3 geometric mean: 11·8% [7·1, 19·4] once daily *vs* 13·1% [8·2, 20·7] twice daily; p=0·33) or total iron absorption (day 1–3: 44·3 mg [29·4, 66·7] once daily *vs* 49·4 [35·2, 69·4] twice daily; p=0·33) between the two dosing regimens. Twice-daily divided doses resulted in a higher serum hepcidin concentration than once-daily dosing (p=0·013). No grade 3 or 4 adverse events were reported in either study.

Interpretation In iron-depleted women, providing iron supplements daily as divided doses increases serum hepcidin and reduces iron absorption. Providing iron supplements on alternate days and in single doses optimises iron absorption and might be a preferable dosing regimen. These findings should be confirmed in iron-deficient anaemic patients.

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Introduction

Oral iron supplementation with ferrous sulfate ($FeSO_4$) is a recommended approach to treat iron deficiency anaemia.[1] However, iron absorption from supplements in iron-depleted patients is low: 2–13% when consumed with food and 5–28% when consumed without food.[2] To compensate for this low absorption, large iron doses are often administered, but large amounts of unabsorbed iron might worsen gastrointestinal symptoms and reduce compliance.[3] Hepcidin is the central regulatory molecule in iron metabolism in mammals,[4] and oral doses of supplemental iron acutely increase serum hepcidin.[5–7] We

Research in context

Evidence before this study

We initially searched PubMed using the search terms "iron supplementation" OR "iron supplements" OR "oral iron", with no language or date restrictions. The date of our first search was Jan 13, 2015, and our last search was on April 24, 2017. Many papers recommend oral iron supplementation to treat iron deficiency or iron deficiency anaemia, or both. Most guidelines and textbooks recommend daily provision of ferrous iron divided throughout the day to increase absorption. Iron absorption from oral supplements measured using stable isotopes or radioisotopes of iron is generally in the range of 5–30%. Several studies have reported that acute oral doses of iron increase serum hepcidin. One short-term study using stable iron isotopes showed that daily iron dosing and afternoon dosing might reduce iron absorption from subsequent doses. We could find no studies that assessed whether these short-term effects on fractional and total iron absorption persist during longer-term supplementation, and no studies that directly compared iron absorption using stable iron isotopes from single versus divided daily doses. Meta-analyses found daily iron supplementation to result in more side-effects than weekly or intermittent supplementation, but relative efficacy was uncertain. A previous study found no difference in fractional iron absorption with weekly versus daily iron supplementation in human beings.

Added value of this study

In this study, done in iron-depleted women using multiple iron stable isotope labels and serum hepcidin profiles, we quantitatively assessed iron absorption from different dosing regimens by measuring cumulative isotopic label abundance in red blood cells 14 days after administration. We assessed iron absorption, comparing consecutive-day versus alternate-day dosing over 28 days and single morning doses versus divided daily doses. We show that alternate-day oral supplementation with 60 mg iron results in 34% higher iron absorption than with consecutive-day supplementation. We also show that splitting a single oral dose of 120 mg iron into two daily doses of 60 mg iron does not improve iron absorption.

Implications of all the available evidence

By contrast with most current recommendations on iron supplementation, our findings suggest that providing oral iron on alternate days in single morning doses increases iron absorption in young women and is an effective regimen to optimise iron absorption. This regimen not only improves iron absorption but also, because of its simplicity, might increase compliance.

recently compared fractional absorption from a single oral dose of iron versus two consecutive day doses, and reported that fractional absorption from the second day's dose is decreased and associated with higher serum hepcidin;[5] both the decrease in fractional absorption and the increase in serum hepcidin were more pronounced at higher doses. However, whether or not these short-term effects occur and persist during long-term supplementation is uncertain. A previous study found no difference in fractional absorption between weekly and daily iron supplementation in women.[2]

To treat iron deficiency, splitting an iron dose over the day, either into two or three divided doses, is commonly recommended[8] and thought to increase tolerability and bioavailability; however, little evidence supports this practice. Because serum hepcidin follows a circadian rhythm and increases during the day,[9] and the acute hepcidin increase triggered by a morning dose augments this diurnal increase, afternoon doses might be particularly poorly absorbed.[5] We previously showed that administering three oral iron doses of 60 mg in the morning, afternoon, and the following morning resulted in total iron absorption similar to two consecutive daily morning doses of 60 mg.[5] However, whether iron absorption is higher from the same daily dose given entirely in the morning than that given in two divided doses is uncertain.

Adverse gastrointestinal side-effects might be less common with lower oral iron doses[10] and intermittent administration.[11] Large amounts of unabsorbed oral iron might cause gut inflammation, which might be due to irritation of the gut mucosa by high luminal concentrations of free iron, adverse changes in the gut microbiota, or both.[12] Faecal calprotectin is a non-specific marker of gut inflammation that reflects neutrophil infiltration of the mucosa.[13] Intestinal fatty acid binding protein (I-FABP) is released into the bloodstream by damaged enterocytes[14] and is a sensitive marker for enterocyte injury.[15] Therefore, we did two studies to assess the following hypotheses: (1) oral iron doses of 60 mg given on alternate days for 28 days would result in higher fractional and total absorption but lower faecal calprotectin and I-FABP than when given every day for 14 days (equal total doses); (2) during 3 days of supplementation, splitting doses of 120 mg iron into two daily divided doses of 60 mg would not result in higher fractional or total absorption than giving one 120-mg dose in the morning (equal total doses). In both studies, iron bioavailability was assessed by measuring the isotopic label abundance in red blood cells 14 days after administration.

Methods

Study design and participants

These open-label, randomised controlled trials were done at the Human Nutrition Laboratory of the ETH Zurich in Zurich, Switzerland.

Healthy women were recruited by a study author (NUS) from the students and staff of ETH Zurich and the University of Zurich, Switzerland, by email announcement. Inclusion criteria were as follows: age 18–45 years; depleted iron stores, defined as serum ferritin 25 µg/L or less; haemoglobin more than 8 g/dL (to exclude individuals with moderate-to-severe anaemia); C-reactive protein (CRP) less than 5 mg/L (to exclude individuals with inflammation); body-mass index 18·5–26·5 kg/m²; bodyweight less than 80 kg (to exclude overweight or obese patients because they often have subclinical inflammation); no chronic medication (except contraceptives); no major chronic disease; not pregnant or lactating; no blood donation in the previous 4 months (because recent blood donation might simulate erythropoiesis); non-smoking; and no intake of mineral and vitamin supplements within 2 weeks of the study start (appendix). Exclusion criteria were inability to follow study procedures or development of major illness. The studies were approved by the Zurich Cantonal Ethics Committee. All participants provided written informed consent.

Randomisation and masking

For study 1, participants were individually randomly assigned to the two study groups by a study author (NUS) using a computer program; this author was involved in the rest of the trial. The number of participants per group was defined, randomisation was done nine times, and of the nine randomisations, we chose the schedule with the most similar means and SDs for haemoglobin and serum ferritin in both groups.

For study 2, we assigned participants in a 1:1 ratio to two groups, stratified by serum ferritin, to have similar iron statuses in both groups. For group assignment, NUS ordered the participants from the lowest to the highest baseline serum ferritin value and participants were distributed equally to the two groups across the range of serum ferritin values. Group assignment was not masked in either study.

Procedures

In study 1, we administered 60 mg iron as $FeSO_4$ at 0800 h (±1 h) either on 14 consecutive days or on alternate days for 28 days. We labelled the first seven $FeSO_4$ doses with ^{57}Fe and the second seven doses with ^{58}Fe so that we could compare iron absorption from the first seven doses with that of the second seven doses. Participants were given the iron dose after an overnight fast and fasted for 3 h after the dose, except for a provided snack (yoghurt) at 1·5 h after the dose. The yoghurt was provided so the participants would not have to fast for the entire morning, and the small amount of calcium in the yoghurt was unlikely to affect iron absorption when consumed at 1·5 h after the iron dose. A venepuncture blood sample (about 8 mL) was collected just before administration of each iron dose. Before administration of each iron dose, a structured questionnaire was completed for each patient about adverse symptoms occurring since the preceding iron dose. We measured I-FABP in serum and calprotectin in a fecal sample at baseline and after the last supplement intake (at 14 days or 28 days).

In study 2, using a crossover design, patients received $FeSO_4$ on three consecutive days, either as a single dose or split into a morning and an afternoon dose. Half of the participants started with the single dosing, the other half started with the split dosing. Doses on the three different supplementation days were labelled with ^{54}Fe, ^{57}Fe, and ^{58}Fe in both groups. Participants received a standardised diet for the six study days. Patients were assigned to start with one of the two dosing regimens, and then crossed over to the other regimen 14 days after finishing the first regimen. For the single-dose regimen, 120 mg iron was administered at 0800 h (±1 h); for the split-dose regimen, 60 mg was administered at 0800 h (±1 h) and again at 1700 h (± 1 h). As in study 1, participants were given the iron dose after an overnight fast and fasted for 3 h after the dose, except a provided snack (yoghurt) at 1·5 h after the dose. Participants receiving the twice-daily dosing had lunch between 1100 h and 1300 h and dinner between 2000 h and 2100 h so that they were fasting when receiving the afternoon iron dose. Before each iron dose, a venepuncture blood sample (about 8 mL) was collected and the adverse symptoms questionnaire was completed for each patient.

See Online for appendix

Supplements consisted of 60 or 120 mg iron as pharmaceutical grade (Ph.Eur.7·2) anhydrous $FeSO_4$ (Lohmann GmbH, Emmerthal, Germany) in gelatin capsules administered with 200 mL of deionised high-purity water containing 0·5 mg (study 1) and 4 mg (study 2) of labelled $FeSO_4$ in the form of (^{57}Fe)-$FeSO_4$, (^{58}Fe)-$FeSO_4$, or (^{54}Fe)-$FeSO_4$ (Chemgas, Boulogne-Billancourt, France), prepared as previously described.[16]

Haemoglobin was measured with a Coulter counter. Blood was centrifuged at 3000 rpm for 10 min, and serum was stored at –20°C until the day of analysis. Serum was analysed for serum ferritin, soluble transferrin receptor (sTfR), CRP, and alpha-1-acid glycoprotein (AGP) using a multiplex ELISA.[17] CRP and AGP are complementary measures of systemic imflammation.[17] Anaemia was defined as haemoglobin less than 12 g/dL; iron deficiency was defined as serum ferritin less than 15 µg/L or sTfR more than 8·3 mg/L, or a combination of these.[18] Inflammation was defined as a CRP more than 5 mg/L or AGP more than 1 g/L. Serum iron and total iron binding capacity (TIBC) were measured using colourimetry, and transferrin saturation (%TS) was calculated using the formula (serum iron/TIBC)×100. Serum hepcidin was measured using c-ELISA.[19] Serum samples were analysed for I-FABP using ELISA (Hycult Biotech, Uden, Netherlands); the manufacturer does not supply a reference range, but a study in 20 healthy European adults reported a median value of 127 pg/mL (IQR 57–311).[20] Stool samples were analysed for calprotectin using ELISA

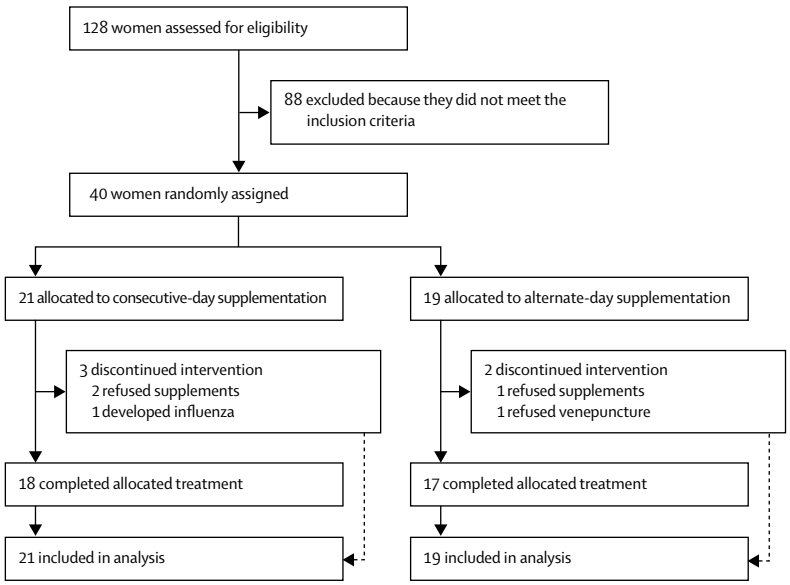

Figure 1: **Trial profile for study 1**

	Study 1		Study 2
	Consecutive-day dosing (n=21)	Alternate-day dosing (n=19)	All women (n=20)
Haemoglobin, g/dL	12·8 (11·7, 14·0)	13·2 (12·5, 14·0)	13·1 (0·9)
Serum ferritin, µg/L	13·8 (6·5, 29·3)	13·8 (8·1, 23·5)	20·0 (9·6)
Serum sTfR, mg/L	6·2 (3·6, 10·6)	5·6 (4·1, 7·5)	5·77 (3·82, 8·71)
Serum iron, µM	17·61 (6·72)	18·55 (6·13)	16·6 (6·6)
TIBC, µM	81·7 (75·3, 88·6)	83·4 (77·4, 89·9)	80·2 (9·3)
Transferrin saturation, %	21·5 (8·0)	22·1 (7·0)	21·1 (8·3)
Iron deficiency	10 (48%)	9 (47%)	6 (30%)
Serum hepcidin, nM	0·91 (0·49, 1·68)	0·63 (0·34, 1·14)	0·81 (0·38, 1·70)
C-reactive protein, mg/L	0·80 (0·23, 2·72)	0·31 (0·08, 1·15)	0·18 (0·09–0·54)
AGP, g/L	0·49 (0·25, 0·69)	0·44 (0·30, 0·65)	0·50 (0·13)
I-FABP, pg/mL	364 (241, 550)	311 (196, 492)	NA
Faecal calprotectin, µg/g faeces	3·65 (2·01, 6·61)	3·25 (1·59, 6·65)	NA

Data are geometric mean (–SD, +SD), mean (SD), or n (%). sTFR=soluble transferrin receptor. TIBC=total iron binding capacity. AGP=alpha-1-acid glycoprotein. I-FABP=intestinal fatty acid binding protein. NA=measurements not taken.

Table 1: **Baseline characteristics of participants in studies 1 and 2**

	Consecutive-day dosing for 14 days	Alternate-day dosing for 28 days	p value
Fractional iron absorption, %			
Week 1, first seven doses	16·1 (8·9, 28·9)	21·3 (13·2, 34·3)	0·13
Week 2, second seven doses	16·6 (9·4, 29·6)	22·3 (13·9, 35·8)	0·11
All 14 doses	16·3 (9·3, 28·8)	21·8 (13·7, 34·6)	0·0013
Total iron absorption, mg			
Weeks 1 and 2, first seven doses	66·9 (36·9, 121·1)	88·0 (54·8, 141·4)	0·13
Weeks 3 and 4, second seven doses	69·3 (39·3, 122·2)	92·7 (58·8, 146·2)	0·11
All 14 doses	131·0 (71·4, 240·5)	175·3 (110·3, 278·5)	0·0010

Data are geometric means (–SD, +SD). Analysed with mixed-effect models with group as fixed factor and participant as random factor (fixed-effect estimation obtained with bootstrapping).

Table 2: **Cumulative fractional and total iron absorption in study 1**

(Calprest, Eurospital, Trieste, Italy); the manufacturer's reference range is less than 50 µg/g faeces.

Iron absorption was assessed by measuring the cumulative isotopic label abundance in red blood cells 14 days after the final iron dose. Blood samples were analysed in duplicate for their iron isotopic composition by multicollector inductively coupled plasma mass spectrometry (Neptune, Thermo-Finnigan, Bremen, Germany) after microwave-assisted mineralisation with nitric acid and separation of the iron from its sample matrix by anionic chromatography followed by a precipitation step with ammonium hydroxide.[21] Fractional absorption was calculated from isotopic ratios measured in blood samples and the concentration of iron circulating in the blood, using the principles of isotopic dilution, and assuming 80% incorporation of the absorbed iron into the erythrocytes.[22] The total absorbed iron was calculated by multiplying the cumulative dose of iron administered over the study period by the fractional iron absorption.[23]

Outcomes

The coprimary outcomes in both studies were iron bioavailability (total and fractional iron absorption), assessed by measuring the isotopic label abundance in erythrocytes 14 days after administration of final supplement, and serum hepcidin concentration. The secondary outcomes in study 1 were serum haemoglobin concentration, iron status (defined by serum ferritin), and faecal calprotectin. Study 2 had no secondary outcomes. Safety was assessed using a forced-choice adverse symptoms questionnaire before each blood venepuncture, and adverse events were reported on a clinical report form.

Statistical analysis

The sample size for study 2 was estimated to be 18 participants on the basis of data from previous iron absorption studies providing $FeSO_4$ supplements to young women in our laboratory with an SD of 0·28 on log-transformed fractional iron absorption, a correlation of absorption within participants of r=0·82, a type I error rate of 5%, and 80% power;[5] this sample size would allow us to detect a clinically relevant difference of 30% with paired two-sided t tests. We estimated that 10% of participants would drop out so aimed to recruit 20 people.

The previous SD of 0·28 on log-transformed fractional iron absorption was derived from single labelled iron doses;[5] we had no previous data from multiple administrations on which to base our sample size calculation for study 1. We assumed that the cumulative measured absorption from seven labelled doses per

patient would reduce the SD; this, combined with the use of two isotopes per patient (resulting in a doubling of observations), would increase the power of the linear mixed-effects model. We estimated that these effects would allow us to distinguish a 30% difference in absorption between the consecutive-day and alternate-day regimens (two-sided, between-patient comparisons) in study 1 with 18 patients per group. We estimated that 10% of participants would drop out so aimed to recruit 20 people per group.

Primary and safety analyses were done on an intention-to-treat basis. Normally distributed data were reported as mean (SD), normally distributed data after log transformation were reported as geometric mean (–SD, +SD), and non-normally distributed data after log transformation were reported as median (IQR). In study 1, to assess the effects of consecutive and alternate dosing on variables, we fitted linear mixed models. Since iron status and inflammatory markers are correlated, we did not correct p values for multiple comparisons. Dosing regimen was defined as fixed effects, participants as random intercept effects using a variance component structure matrix, and the corresponding baseline values for each parameter as a covariate. To better estimate fixed-effects estimates for fractional absorption, we did bootstrapping (with resampling size of 1000). Log-transformed data was used and estimates and CIs were obtained by back-transforming the obtained parameters. To compare iron status and inflammation parameters between the groups in study 1 at baseline, independent sample student *t* tests were used. When assessing the effect of dosing regimen on serum hepcidin and iron status parameters, baseline values of the corresponding variable were included as covariates. Incidence of gastrointestinal side-effects was compared using the χ^2 test. To increase comparability of absorption data between the two study groups (study 1) and with previously published studies,[5] fractional and total absorption were adjusted for a serum ferritin level of 15 µg/L.[24] In study 2, repeated-measures ANOVA was used to assess the effect of once-daily and twice-daily divided dosing. Fractional absorption, serum hepcidin, serum ferritin, and TfR were the dependent variables for each separate model and the dosing regimen and the day were added to the models as independent variables. For within-patient effects on fractional absorption and serum hepcidin, dependent sample *t* tests were used. Statistical analyses were done using SPSS (IBM SPSS statistics, version 22.0). The trials are registered at ClinicalTrials.gov, numbers NCT02175888 (study 1) and NCT02177851 (study 2).

Role of the funding source

The funder of the study had no role in study design, the collection, analysis, or interpretation of the data, or in the writing of the report. NUS, DM, and MBZ had access to the raw data. MBZ had full access to all of the data and the final responsibility to submit for publication.

Results

Between Oct 15 and Oct 29, 2015, 40 women were enrolled in study 1: 21 in the consecutive-day group and 19 in the alternate-day group (figure 1). In the consecutive-day group, three women discontinued participation during the first week of supplementation (two refused to take the supplements and one developed influenza) and in the alternate-day group, two women discontinued participation during the first 2 weeks of supplementation (one refused to take the supplements and one refused venepuncture). The study was completed on Dec 14, 2015. Baseline characteristics of the women are shown in table 1. The median age of the women was 22 years

	Consecutive-day dosing for 14 days	Alternate-day dosing for 28 days	Group effect p value	Time effect p value	Time–group effect $p_{interaction}$ value
Haemoglobin, g/dL	13·2 (12·5, 14·0)	13·6 (12·9, 14·4)	0·16	0·90	0·70
Serum ferritin, µg/L	28·3 (15·7, 51·01)	23·6 (17·3, 32·1)	0·058	<0·0001	0·62
Serum sTfR, mg/L	6·3 (3·1, 12·6)	5·7 (4·1, 7·7)	0·99	0·0097	0·00028
Serum iron, µM	15·61 (5·40)	14·67 (6·22)	0·59	0·031	0·59
TIBC, µM	80·0 (74·6, 85·8)	83·4 (76·7, 90·6)	0·19	0·058	0·036
Transferrin saturation, %	18·4 (4·9)	18·5 (6·0)	0·40	0·081	0·43
Iron deficiency	3 (14%)	1 (5%)	0·56	<0·0001	0·59
Serum hepcidin, nM	1·09 (0·77, 1·54)	1·38 (0·78, 2·42)	0·23	0·024	<0·0001
C-reactive protein, mg/L	0·73 (0·26, 2·04)	0·65 (0·13, 3·19)	0·12	0·059	<0·0001
AGP (g/L)	0·39 (0·28, 0·56)	0·46 (0·31, 0·68)	0·086	0·80	0·0013
I-FABP, pg/mL	322 (119, 871)	251 (146, 433)	0·22	0·24	0·72
Faecal calprotectin, µg/g faeces	3·64 (2·14, 6·17)	3·97 (2·15, 7·34)	0·93	0·50	0·49

Data are geometric mean (–SD, +SD), mean (SD), or n (%). Group, time, and time by group effects are reported with no correction for multiple comparisons. sTFR=soluble transferrin receptor. TIBC=total iron binding capacity. AGP=alpha-1-acid glycoprotein. I-FABP=intestinal fatty acid binding protein.

Table 3: **Measurements after final supplement intake in study 1**

(IQR 21–25) in the consecutive-day group and 22 years (21–24) in the alternate-day group. Four of the women were mildly anaemic (three in the consecutive-day and one in the alternate-day group). No baseline differences were noted in any of the baseline variables.

At the end of treatment (day 14 for the consecutive-day group and day 28 for the alternate-day group), geometric mean (–SD, +SD) fractional iron absorptions were 16·3% (9·3, 28·8) in the consecutive-day group versus 21·8% (13·7, 34·6) in the alternate-day group (p=0·0013), and total iron absorption was 131·0 mg (71·4, 240·5) versus 175·3 mg (110·3, 278·5; p=0·0010; table 2). During the intervention, we noted significant time–group interactions on sTfR, TIBC, CRP, and AGP (table 3). An overall significant time–group interaction on serum hepcidin was present for the entire duration of the study (p<0·0001 at 14 days in the consecutive-day group *vs* 28 days in the alternate-day group); serum hepcidin was higher in the alternate-day group than the consecutive-day group at endpoint (table 3). By contrast, during the first 14 days of supplementation in both groups, we noted a significant time–group interaction on serum hepcidin (p=0·0031), with higher serum hepcidin in the consecutive-day group than the alternate-day group (figure 2A, B). No significant time–group interactions on faecal calprotectin or I-FABP were present (table 3). During the intervention, treatment group had a significant effect on cumulative fractional and total iron absorption (table 2, figure 2C). Within groups, no significant difference in fractional or total absorption was noted when comparing the first seven doses to the second seven doses (consecutive-day dosing, p=0·73; alternate-day dosing, p=0·33; table 2 and figure 2C).

All reported adverse events in study 1 were grade 1–2 (table 4). The total incidence of the two gastrointestinal side-effects that were assessed (nausea and abdominal pain) was 33% higher with consecutive-day dosing than with alternate-day dosing, although this difference was not statistically significant (p=0·57).

Between Aug 13 and 18, 2015, 20 women were enrolled in study 2: ten were assigned to the once-daily dosing group, and ten were assigned to the twice-daily dosing group. Two women dropped out, one after completing the once-daily dosing and one after completing the twice-daily dosing, because they refused to take the supplements (figure 3). The study was completed on Sept 25, 2015. Baseline characteristics of the women (median age 27 years [IQR 24–30]) are shown in table 1. Two of the women were mildly anaemic, and none had discernible inflammation (defined by increased CRP or AGP). We did not assess baseline differences between the two groups.

Group allocation (one whole dose *vs* two divided doses) did not significantly affect either fractional or total iron

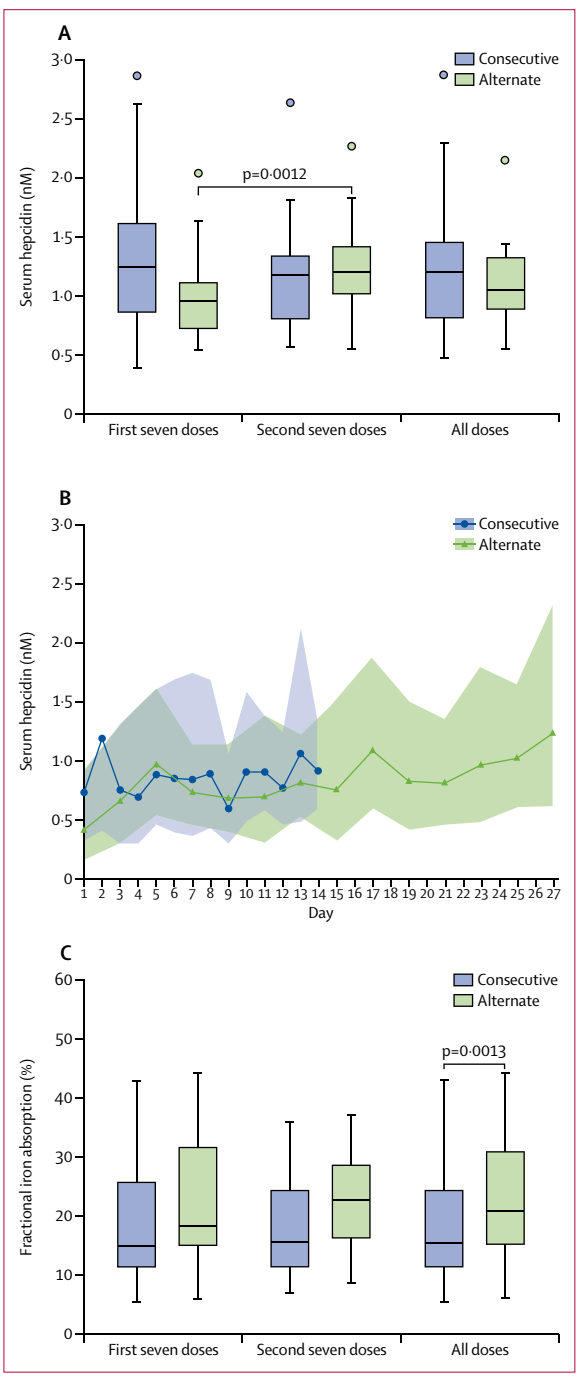

***Figure 2*: Serum hepcidin and fractional iron absorption in study 1**
(A) Serum hepcidin concentrations in samples taken during the first seven iron doses, the second seven doses, and during the entire supplementation phase, by group. Circles show statistical outliers. (B) Serum hepcidin profile over the study period presented as geometric means with shading indicating ±SD. (C) Fractional absorption (%) during the first seven iron doses, the second seven doses, and during the entire supplementation phase, by group. In (C) and (A), the horizontal black lines show the median, the boxes show IQRs, and whiskers show the range. A significant time–group interaction on serum hepcidin over the duration of the study (p<0·0001) and a significant time effect on serum hepcidin in both groups (p=0·024) was seen. Compared using paired *t* test (A) and unpaired *t* test (C).

	Study 1				Study 2			
	Consecutive-day dosing		Alternate-day dosing		Once-daily dosing		Twice-daily dosing	
	Number of events (n=24)	Number of people (n=21)	Number of events (n=25)	Number of people (n=19)	Number of events (n=12)	Number of people (n=10)	Number of events (n=14)	Number of people (n=10)
Nausea	11 (46%)	6 (29%)	6 (24%)	2 (11%)	1 (8%)	1 (10%)	2 (14%)	1 (10%)
Abdominal pain	5 (21%)	2 (10%)	3 (12%)	3 (16%)	2 (17%)	2 (26%)	2 (14%)	2 (20%)
Headache	4 (17%)	3 (14%)	11 (44%)	7 (37%)	9 (75%)	5 (50%)	10 (71%)	6 (60%)
Upper respiratory tract infection	4 (17%)	4 (19%)	5 (20%)	5 (26%)	0	0	0	0

Data are n (%). All events were grade 1–2; no grade 3–5 events were reported.

Table 4: **Adverse events in studies 1 and 2**

absorption (p=0·33 for both) and no significant time–group interaction on fractional or total iron absorption was seen (p=0·74 for both; table 5, figure 4A). However, in both dosing regimens, a significant effect of time on iron absorption (p<0·0001) was noted: absorption on day 1 was significantly higher than on day 2 (p<0·0001) or day 3 (p<0·0001), but did not differ significantly between day 2 and day 3 (p=0·77).

Group allocation (p=0·013) and time (p<0·0001) had significant effects on serum hepcidin, but no significant time–group interaction was seen (p=0·40; table 5 and figure 4B). During the 3 days, two daily divided doses resulted in a significantly higher serum hepcidin than once-daily dosing (p=0·013; table 5). In both dosing regimens, serum hepcidin was significantly higher on day 2 (p<0·0001) and day 3 (p<0·0001) than on day 1, and higher on day 2 versus day 3 (p=0·0066; table 5 and figure 4B). During once-daily dosing, fractional absorption and serum hepcidin were significantly correlated on all three administration days: on day 1 (r=–0·485; p=0·035); day 2 (r=0·700; p=0·0012); day 3 (r=–0·535; p=0·018). By contrast, during divided dosing, fractional absorption and serum hepcidin were significantly correlated on day 1 (r=–0·558; p=0·013), but not on day 2 (r=–0·329; p=0·17) or day 3 (r=–0·239; p=0·33).

All reported adverse events in study 2 were grade 1–2 (table 4). The most common adverse event was headache for both regimens, with nine in the once-daily dosing group and ten in the twice-daily dosing group.

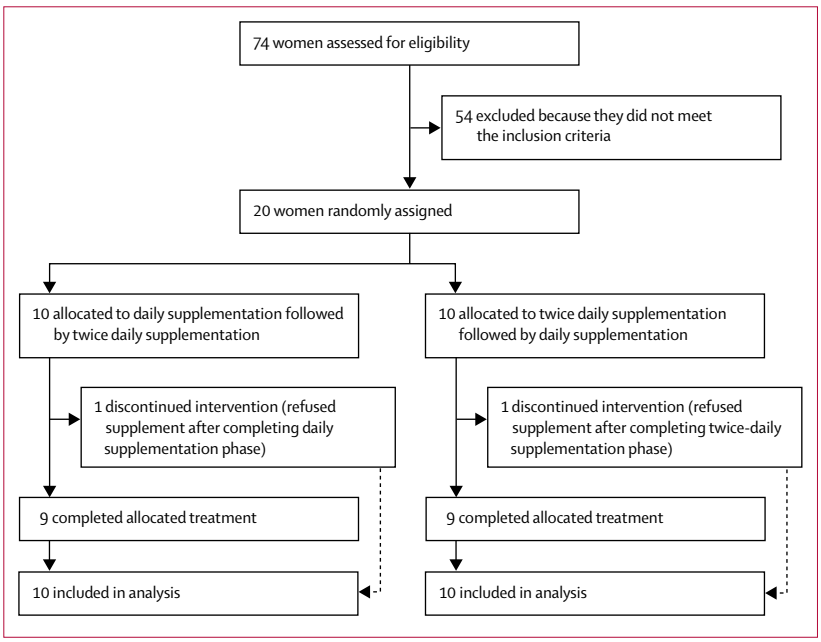

Figure 3: **Trial profile for study 2**

Discussion

The main findings of our studies are as follows: in iron-depleted young women, alternate-day dosing of 60 mg iron as $FeSO_4$ significantly increases fractional and total iron absorption compared with dosing iron every day; and fractional and total absorption are not increased by splitting a dose of 120 mg iron into two daily divided doses. These data confirm and extend our previous short-term studies.[5] In those studies, also done in non-anaemic, iron-depleted young women, we provided single morning doses of isotopically labelled iron as $FeSO_4$ (40–240 mg) given on one or two consecutive days. We showed that 24 h after doses of 60 mg or more, serum hepcidin was significantly increased and fractional absorption was decreased by 35–45%.[5] Our findings in study 1 show that this effect is maintained over a dosing schedule of 2 weeks, during which consecutive-day dosing results in significantly lower fractional and total absorption than alternate-day dosing. The difference in total iron absorption in study 1 between the two dosing regimens was approximately 44 mg. On the basis of these data, alternate-day dosing, compared with consecutive-day dosing, would translate to an estimated difference in haemoglobin of approximately 7 g/L during a dosing regimen providing 1800 mg of iron (eg, 30 doses of 60 mg).

We previously reported clear inverse associations between fractional absorption and serum hepcidin during two days of consecutive-day versus alternate-day iron supplementation.[5] In our study, the simultaneous effects on serum hepcidin of repeated oral iron doses against a

	Once-daily dosing				Twice-daily dosing			
	Day 1	Day 2	Day 3	Day 1–3	Day 1	Day 2	Day 3	Day 1–3
Fractional iron absorption, %	16·8 (11·0, 25·7)	10·1 (6·7, 15·1)*	9·7 (6·0, 15·6)*	11·8 (7·1, 19·4)	19·1 (13·7, 26·7)	11·0 (7·3, 16·4)*	10·6 (7·1, 15·9)*	13·1 (8·2, 20·7)
Total iron absorption, mg	17·5 (8·2, 37·3)	10·8 (5·6, 20·7)*	10·4 (5·2, 20·7)*	44·3 (29·4, 66·7)	19·8 (9·5, 41·3)	11·7 (6·0, 22·7)*	11·4 (5·9, 21·9)*	49·4 (35·2, 69·4)
Serum hepcidin, nM	0·75 (0·40, 1·41)	2·77 (0·88, 8·69)*	1·79 (0·77, 4·18)*†	1·53 (0·54, 4·32)‡	0·91 (0·40, 2·08)	4·69 (2·01, 10·98)*	2·77 (1·53, 5·02)*§	2·24 (0·80, 6·25)

Data are geometric means (–SD, +SD). Measurements were taken at 0800 h ±1 h each day before the iron dose. Fractional iron absorption and total iron absorption data are adjusted for a serum ferritin concentration of 15 µg/L. Analysed by repeated-measures ANOVA with Bonferroni corrected multiple comparisons. A significant time effect on fractional and total iron absorption was seen (p<0·0001 for both), but no group effect was seen. A significant time effect (p<0·0001) and group effect (p=0·013) was seen on serum hepcidin. *p<0·0001 *vs* day 1; †p=0·024 *vs* day 2; ‡p=0·013 *vs* twice-daily dosing; §p=0·0051 *vs* day 2.

Table 5: **Fractional and total iron absorption and serum hepcidin in study 2**

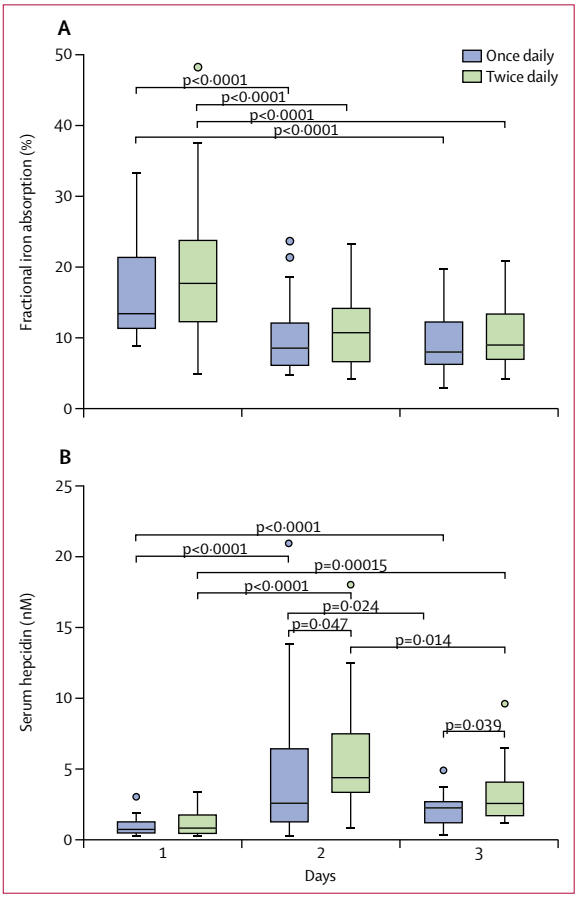

Figure 4: **Fractional iron absorption and serum hepcidin in study 2**
Fractional iron absorption (A) and serum hepcidin (B). Horizontal black lines show the median, boxes show IQRs, whiskers show ranges, and circles show statistical outliers. A significant time effect on fractional and total iron absorption (both p<0·0001), but no group effect was seen. A significant time effect (p<0·0001) and group effect (p<0·013) on serum hepcidin, but no significant time–group interaction was noted. Comparisons were done using paired *t* test.

backdrop of improving iron status resulted in a more complex pattern. Although serum hepcidin gradually increased in both groups over time, consecutive-day dosing resulted in higher serum hepcidin than alternate-day dosing during the initial 14-day phase of supplementation, possibly driven by the higher iron supplement frequency in the consecutive-day group. By contrast, serum hepcidin was higher in the second 14-day phase in the alternate-day group, possibly reflecting the longer supplementation period, but also improved iron status. Intracellular hepatic iron stores or hepatic endothelial cells might regulate hepcidin translation independently from the known signalling pathways, inducing hepcidin translation after an acute iron dose.[25–27] An earlier study comparing weekly to daily iron supplementation of 50 mg iron as radiolabelled $FeSO_4$ in 12 women found a non-significant 15% increase with weekly supplementation (mean fractional absorption was 9·8% for weekly *vs* 8·5% for daily supplementation);[2] the difference to our findings might be because of the smaller number of patients and lower dose in the previous study.

Our findings in study 2 suggest that the common practice of splitting an oral iron dose in two daily divided doses in an attempt to increase iron absorption is unnecessary; divided dosing does not significantly affect fractional or total absorption. This finding is consistent with our previous study[5] in which we gave three 60-mg iron doses (twice-daily dosing) within 24 h and reported that fractional and total absorption from three doses (two mornings and an afternoon) was not significantly greater than that from two morning doses. Because of the circadian regulation of iron metabolism, serum hepcidin increases during the day and supplemental oral iron enhances this effect.[7,9] Our findings in study 2 might be due to a combination of these effects: fractional absorption from the 120 mg morning dose was decreased compared with the 60 mg morning dose in the two daily divided dosing, but this difference was largely offset by the decreased fractional absorption of the 60 mg afternoon dose and the inhibiting effect of this afternoon dose on fractional absorption of the following 60 mg morning dose. However, because we labelled the different days of supplementation with different isotopes, rather than labelling the morning and afternoon doses with different isotopes, we could not distinguish this effect.

In study 2, whether iron was given once daily or in two daily divided doses, serum hepcidin was lower on day 3 of supplementation than day 2, but without a corresponding increase in iron absorption. These data are consistent with our previous data[5] showing that morning serum hepcidin on the day preceding oral iron administration is more predictive of iron absorption than is morning serum hepcidin on the day of administration. Additionally, although dosing regimen was a significant predictor of serum hepcidin, with higher serum hepcidin with two daily divided doses than with a single dose, iron absorption did not differ significantly between two daily divided doses and once-daily dosing.

A meta-analysis comparing daily iron supplementation to intermittent supplementation found an overall decrease in reported side-effects for the intermittent dose.[11] Our findings lend some support to this conclusion in that the cumulative incidence of gastrointestinal side-effects was higher with consecutive-day dosing than with alternate-day dosing, although this difference was not statistically significant. To assess adverse gastrointestinal effects from iron, we measured serum I-FABP and faecal calprotectin. I-FABP is a cytosolic protein located mainly in mature enterocytes of the small intestine, and is released into the bloodstream after enterocyte damage.[14] We are not aware of any previous studies that have measured I-FABP in response to oral iron supplementation. Faecal calprotectin is a biomarker for neutrophil infiltration of the gut wall, and is increased in infectious and inflammatory conditions.[28] Previous controlled studies in African infants and children have shown that faecal calprotectin increases during iron treatment.[12] In our study 1, we found no time or treatment effects of dosing regimen on these biomarkers, suggesting that oral iron supplementation in healthy young women does not cause significant enterocyte damage or inflammation.

The strengths of this study are as follows: we studied iron-depleted young women (six of whom were mildly anaemic), which is a target group for iron supplementation; in study 1, iron absorption and serum hepcidin profiles were accurately and repeatedly quantified by using stable iron isotope techniques and an immunoassay with high sensitivity over 14–28 days; tolerability and gastrointestinal effects were assessed by interview (studies 1 and 2) and measurement of intestinal biomarkers (study 1); and in study 2, the crossover design allowed within-patient comparisons of iron absorption measurements and serum hepcidin. The limitations of our study are as follows: we tested quite small numbers of women because of the logistics and expense of using stable iron isotopes, which could have resulted in a β error in the comparisons of clinical endpoints, such as side-effects; we did not study people with moderate-to-severe anaemia, who might respond differently to those with no anaemia or mild anaemia; we assessed iron absorption using erythrocyte iron incorporation, which is a measure not only of intestinal iron absorption but also erythropoietic response and the utilisation of iron during the formation of new erythrocytes; and subjective assessment of tolerability in study 1 might have been biased because the study was not masked.

In conclusion, our data show that providing 60–120 mg iron on alternate days in single morning doses increases iron absorption in young women. This regimen not only substantially improves iron absorption but also, because of its simplicity, might increase compliance.

Contributors

CIC, GB, DWS, DM, and MBZ conceived the studies and obtained funding. All authors contributed to the design of the trials. NUS, CIC, DM, and MBZ conducted the studies. NUS and DM analysed the data and wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

Declaration of interests

DWS and AJG-M are employees of Hepcidinanalysis.com at Radboud University and Medical Centre, a website that offers hepcidin measurements for a fee. All other authors declare no competing interests.

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