Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women

Running title: Hepcidin and absorption from iron supplements

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Key Points (max 140 characters each)

Iron supplements at doses of 60 mg Fe as FeSO₄ or higher increase hepcidin for up to 24h, and are associated with lower iron absorption on the following day.

The soluble transferrin receptor/ferritin ratio and hepcidin are equivalent predictors of iron absorption from supplements.
Abstract

Iron supplements acutely increase hepcidin, but the duration and magnitude of the increase, its dose dependence and effects on subsequent iron absorption have not been characterized in humans. Better understanding of these phenomena might improve oral iron dosing schedules.

We investigated whether the acute iron-induced increase in hepcidin influences iron absorption of successive daily iron doses and twice daily iron doses.

We recruited 54 non-anemic young women with plasma ferritin ≤20 µg/l and conducted: 1) a dose-finding investigation with 40, 60, 80, 160 and 240 mg labeled Fe as [57Fe]-, [58Fe]- or [54Fe]-FeSO4 given at 8.00 fasting on one or on two consecutive days (study 1, n=25; study 2, n=16); and 2) a study giving three 60 mg Fe doses (twice a day dosing) within 24h (study 3, n=13).

In studies 1 and 2, 24hr after doses of 60 mg or greater, serum hepcidin was increased (P<0.01) and fractional iron absorption was decreased by 35-45% (P<0.01). With increasing dose, fractional absorption decreased (P<0.001), while absolute absorption increased (P<0.001). A six-fold increase in iron dose (40 to 240 mg), resulted in only a 3-fold increase in iron absorbed (6.7 to 18.1 mg). In study 3, total iron absorbed from three doses (two mornings and afternoon) was not significantly greater than that from two morning doses.

Providing lower dosages (40-80 mg Fe) and avoiding twice a day dosing maximizes fractional absorption. The duration of the hepcidin response supports alternate day supplementation, but longer-term effects of these schedules require further investigation.

These clinical trials were registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT01785407 and NCT02050932.
Introduction

Anemia affects ≈33% of the world population, and accounts for 8.8% of global disability. In the US, ID is estimated to affect 9.2% of females aged 12-49 years. Oral iron supplementation with FeSO₄ is a primary approach for the treatment of iron deficiency anemia (IDA). While both daily and intermittent supplementation can replete iron stores and increase hemoglobin levels, iron supplements often cause gastric irritation, nausea, epigastric discomfort and constipation, which may decrease compliance and long term efficacy. The absorption of iron supplements ranges from 2 to 13% and 5 to 28% in subjects with low iron stores when consumed with and without food, respectively. Thus, a majority of the iron is unabsorbed. While its role in the emergence of side effects is uncertain, high iron doses can potentially adversely affect the composition of the gut microbiome and increase inflammation, as assessed by fecal calprotectin levels.

Hepcidin is the key regulator of systemic iron balance in mammals, acting in concert with intracellular iron metabolism. Iron supplementation acutely increases the circulating plasma hepcidin level, but the magnitude and duration of this increase has not been characterized in humans. Plasma hepcidin negatively correlates with iron bioavailability and has a circadian increase over the day, in association with a fall in transferrin saturation. Morning iron supplementation enhances this increase in plasma hepcidin, potentially affecting iron absorption from supplements given as divided doses in the morning and in the afternoon.

Iron supplementation recommendations typically advise provision of 60-120 mg Fe/day to treat IDA. Intermittent schedules are advised for primary prevention in young women while in
pregnant women, the WHO\textsuperscript{24} and CDC\textsuperscript{21} recommend 30-60 mg Fe per day. This guidance is not shared by all organizations\textsuperscript{22} and depends on anemia prevalence.\textsuperscript{25} In clinical practice, dose spacing and timing vary widely.

Our aim was to quantify the magnitude and duration of the acute iron-induced increase in hepcidin at different iron doses and to measure the effect of administration on consecutive days on hepcidin, iron absorption, and iron status markers. We measured the fractional and absolute amounts of iron incorporated in red blood cells from iron supplements with the use of stable iron isotopic labels.
Methods

Subjects

We recruited apparently healthy females aged between 18 and 45 years, with depleted iron stores (defined as PF ≤20 µg/L) but no anemia (Hb >117 g/L, the lower limit of the reference range at the University Hospital Zürich). Further inclusion criteria were no chronic medication (except oral contraceptives), no reported chronic disease, no pregnancy or lactation, no blood donation in the last 4 months, non-smoking, no intake of mineral, vitamin or herbal supplements within 2 weeks of study start and during the entire duration of the study, BMI between 18 and 25 kg/m² and body weight less than 68kg. We excluded subjects who had a C-reactive protein >5 mg/L at screening.

Design

We conducted three separate studies with the aim of measuring the acute iron-induced increase in hepcidin caused by FeSO₄ supplements while quantifying iron absorption using stable isotopic labels as tracers (Figure 1). We monitored plasma hepcidin (PHep) and iron status markers prior administration and up to 48h post administration at 8.00, 12.00 and 17.00. In study 1, using a cross over design, we administered two iron challenges either as 1) single dose or 2) as two doses given on consecutive days. Subjects were randomly assigned to start the study with one of the two treatments. Iron was administered at 8.00h (±1h), in four different iron concentrations (40, 80,160 and 240 mg as elemental Fe). In study 2, we administered two single doses of 60 mg elemental iron at 8.00h on two consecutive days and similarly assessed hepcidin response until 48h post administration. Both study 1 and 2 started with a control day where no supplements were administered. In study 3 we assessed the effect of administering 60 mg Fe twice daily during 24h (three doses in total) on hepcidin levels and iron absorption. In
all studies subjects acted as their own controls and iron absorption was assessed by measuring the amount stable isotopic tracers incorporated in red blood cells 14 days post administration (a detailed description of study procedures is available as online supplemental material).

Iron supplements and label administration

Each supplement consisted of 36, 56, 76, 156 or 236 mg Fe as pharmaceutical grade (Ph.Eur.7. Ed) anhydrous FeSO₄ (Lohmann GmbH, Emmertal, Germany) in gelatin capsules (Cantonal pharmacy, Canton of Zürich, Switzerland) administered with 100 ml of deionized high-purity water (resistivity 18 MΩ/cm; NANOpure system, Barnstead-Thermolyne, Dubuque, USA) containing 4 mg of labeled FeSO₄ in the form of [⁵⁷Fe]-FeSO₄, [⁵⁸Fe] -FeSO₄ or [⁵⁴Fe]-FeSO₄ (Chemgas, Boulogne, France) prepared as previously described. At administration, we rinsed the plastic cup with additional 100 ml water divided in 10 ml and 90 ml to guarantee quantitative administration.

Iron status and oral iron absorption

We characterized all samples collected during the study with the multiplex ELISA method described by Erhardt et al. simultaneously assessing plasma ferritin (PF), the soluble transferrin receptor (sTfR), C-reactive protein (CRP) and alpha acid glycoprotein (AGP) at each time point. We assessed plasma iron (PFe) and total iron binding capacity (TIBC) at all-time points in study 1 by using the methods recommended by the International Committee for Standardization in Hematology, and transferrin saturation (%TS) was calculated with the formula (SFe/TIBC) x 100. We calculated body iron stores (BIS) for study participants at each time point using the formula based on sTfR/PF ratio proposed by Cook et al (2003).
We used a c-ELISA method to quantify plasma hepcidin (PHep). This method has a lower limit of detection than weak cation exchange time of flight mass spectrometry (TOF-MS) and is therefore a preferred method in the present study because of the anticipated low hepcidin levels in healthy subjects with depleted iron stores.

We analyzed each isotopically enriched blood sample for its iron isotopic composition in duplicate under chemical blank monitoring, according to previously published methods from our laboratory.

Statistical analysis

We conducted the statistical analysis with SPSS (IBM SPSS statistics, Version 22) using linear mixed models (LMM) to assess the effect of iron supplementation on hepcidin and iron status markers. Body iron stores were calculated with the formula: Body Fe (mg/kg) = \[\log(\text{sTfR}/\text{PF}) - 2.8229\]/0.1207; where sTfR is the concentration in soluble transferrin receptor and PF the plasma ferritin level. To increase comparability of absorption data between studies, we present fractional and absolute iron absorption for a PF level of 15 µg/l, obtained with the formula: \[\log(A_{15}) = \log A_0 + \log F_0 - \log 15\]; where \(A_{15}\) is the standardized absorption at PF of 15 µg/l, \(A_0\) the measured absorption and \(F_0\) the measured PF level in the subject.

Time and dose were defined as fixed effects and subjects as random intercept-effects using a variance component structure matrix. To test the time x dose interaction, an ad hoc variable describing the time between each hepcidin measurement and iron administration was defined. Due to the generally improved models we used log transformed hepcidin data in the linear
mixed models, and estimates and confidence intervals were obtained by back transforming the obtained parameters.

For difference testing, normality was assessed by visualizing Q-Q plots and difference tests conducted with log-transformed data. When comparing specific control and post supplementation time points, Wilcoxon signed rank tests were used for comparisons in Study 1, where group sample sizes were low (n=6), and normality could not easily be assessed. Paired t-tests were used to test for within subject effects for normally distributed data (log-transformed data from study 2 and 3). For between subject effects, independent sample t-tests were used (study 2 vs. study 3). Predictors of iron absorption were assessed using linear mixed models and univariate general linear model with fractional iron absorption (in %) and absolute iron absorption (in mg) as dependent variables and time and dose as factors and iron status markers as covariates.

To investigate at which time point hepcidin concentration best predicted iron absorption, we fitted regression models on the combined datasets of study 1 and 2. The statistical difference between different $R^2$ in non-nested regression models was tested with Steiger’s Z test. Significance was defined as $P<0.05$.

Hepcidin and iron status parameters assessed in study 1 and 2 were analyzed using LMM against the concentration on a control day at 8.00 as the reference.
Results

Iron status

The baseline iron status of the women in studies 1 and 2 are shown in Tables 1 and 2, respectively. Subjects were iron-depleted but not anemic, and there was a low prevalence of iron deficient erythropoiesis as indicated by normal concentrations of sTfR, with the exception of the group receiving 40 mg Fe in study 1, which had an elevated mean sTfR of 8.4 mg/l. In each of the three studies, there was no systemic inflammation as defined by CRP >5 mg/l, or AGP (>1 g/l) at baseline.

Acute effect of iron supplements on iron status markers

With iron administration, %TS increased within 4 h at all doses examined (all P<0.001). The %TS reached a maximum at 4h post administration, with mean (SD) %TS at 61.5 (15.9), 72.1 (14.2), 72.2 (30.3), 64.7 (25.1) for 40, 80, 160, 240 mg Fe, respectively. These values were not statistically different between different dosage groups. Transferrin saturation remained elevated during the day of administration (17.00, P<0.001) and returned to baseline levels after 24h (not different from baseline levels, Figure 2). The sTfIR concentration decreased transiently within 4 to 24h after administration of doses of 160 and 240 mg (P<0.01). An increase in PF was detectable from 8 to 24h after administration for all doses, and the concentration remained significantly increased at 24h compared to baseline for the 40, 80 and 160 mg doses, and at 56h for the 240 mg dose (Figure 2). Plasma ferritin returned to baseline levels in all dose groups by 14 days after supplement administration. Inflammation, as assessed by CRP and AGP, was not affected by iron administration.

Acute effect of different Fe doses on hepcidin and iron absorption (40, 80, 160, 240 mg Fe)
In an overall linear mixed model including all data points in the study, time had a significant effect on PHep (P<0.001), and there was a significant time x dose interaction for PHep (P<0.05). For all iron doses tested, supplementation increased PHep at 24h by a factor of 2.7 (95% CI 1.6-4.6). The second dose of iron increased PHep by a factor 2.1 (95% CI 1.2-3.5). There was a significant increase in PHep at 24h after the doses of 60, 80, 160 and 240 mg Fe (P<0.05), but not at 40 mg Fe. PHep was not significantly elevated 48h post iron administration in the overall model or after any of the iron doses. The increase in PHep from 8.00 to 17.00 differed between control and iron administration days: PHep increased by a factor of 1.8 (95% CI 1.2-2.7) and 3.7 (95% CI 2.5-5.5), respectively. For the doses tested in study 1, fractional iron absorption decreased with increasing dose (P<0.001), while absolute absorption increased (P<0.001). Although the dose increased six-fold (from 40 to 240 mg), the absolute amount of iron absorbed increased by only about three-fold, e.g. a dose of 40 mg Fe given 24h after the first dose provided 6.7 mg of absorbed iron while the second 240 mg dose provided 18.1 mg of absorbed Fe, and the highest fractional absorption was achieved with dosages between 40-80 mg Fe (Table 1).

Iron absorption was significantly lower on the second day of administration (day 10) compared to the first day of administration (day 2 and day 9; P<0.001). There was no significant difference in absorption when the supplement was administered as the first dose on day 2 or day 9. The fractional absorption from the second dose of 80 mg, 160 mg and 240 mg iron was 37%, 35% and 45% lower, respectively, compared to the first iron dose (all P<0.01). Absorption of the second dose of 40 mg iron was 20% lower than when 40 mg was administered as a single dose on day 2 and day 9; fractional absorption did not differ between day 9 and 10 (P=0.19), but fractional absorption on day 9 was lower than on day 2 (P=0.040).
Taken together this data suggests that acute absorption is inhibited at dosages of 80, 160 and 240 mg within 24 h, and suggests a possible effect at 40 mg Fe.

*With 60 mg Fe PHep increases and iron absorption decreases at 24h.*

In study 2, there was a significant effect of time on PHep (P<0.001); at 24 h post administration PHep increased by a factor of 2.2 (95% CI 1.48-3.24). Two doses given on consecutive days at 8.00 resulted in a PHep at 8.00 on the third day that was 1.5 (95% CI 1.01-2.22) fold higher than the baseline value at the start of the study. From 8.00 to 17.00, PHep increased by a factor of 7.0 (95% CI 4.7-10.6) and 1.76 (95% CI 1.19-2.60) with and without iron, respectively. Fractional iron absorption decreased by 36% when 60 mg iron was administered on the second day compared to the first day (P<0.001) (*Figure 3*).

In study 3, twice daily administration of 60 mg iron at 10.00 and 16.00 resulted in a higher PHep at 8.00 am on the following day compared to once daily administration (independent sample t-test, P<0.01); PHep increased by a factor of 6.7 (95% CI: 4.1-10.8) compared to baseline (*Figure 4*). Iron absorption from the afternoon dose decreased by 26% compared to the first morning dose (P<0.01). Iron absorption from the successive morning doses decreased by 43% compared to the first dose 24 h earlier (P<0.01) and was 20% lower than the afternoon dose given on the preceding day (P<0.01). The absorption of the third dose given in the morning of day 2 in the twice daily administration study was >50% lower than the absorption measured on day 2 in study 2, when no afternoon dosage was given (P<0.05). Absolute iron absorption from a dose of 60 mg given at 08.00 was 13.8 mg when there was no preceding dose, 8.8 mg Fe when given after a single morning dose on the preceding day, and 5.9 mg Fe when given after
twice-daily dosing on the preceding day (Table 2). The total iron absorbed was 23.6 mg Fe if 3 doses were administered within 24h, compared to 22.6 mg Fe when only the two morning doses were given (P=0.79).

**Total iron absorbed**

The total amount of iron absorbed from the supplements was generally higher with increasing dose (P<0.001). For the first and second doses, respectively, the relationship between dose administered and dose absorbed was best predicted by the formulas (Figure 5):

\[
\text{Dose absorbed}_{(\text{first dose})} = 0.816 \times (\text{dose administered})^{0.678}; R^2=0.450; P<0.001
\]

\[
\text{Dose absorbed}_{(\text{second dose})} = 0.752 \times (\text{dose administered})^{0.596}; R^2=0.467, P<0.001
\]

Total iron absorbed from the 160 and 240 mg doses was significantly higher than that absorbed from the 40, 60, 80 mg doses (P<0.05), but were not significantly different from each other.

**Predictors of iron absorption**

The logarithm of fractional iron absorption was best predicted by a model including body iron stores (BIS), PHep, time of administration and dose (R^2=0.69; P<0.001). A simplified model without PHep resulted in a different model (P=0.011) with similar predictive power (R^2=0.67; P<0.001) Table 3. Including serum ferritin in the model instead of BIS resulted in slightly worse prediction (R^2=0.64). Using log PHep alone and time of administration and dose as independent variables resulted in a larger decrease in predictive power R^2 (R^2=0.54; P<0.001), and predictive power was lower than a model using BIS alone (P<0.05). Models including PHep and BIS measured at time of iron administration resulted in higher R^2 coefficients than when these measures were assessed only at the start of the study (Table 3).
Fractional iron absorption of the first dose was best predicted by a model including solely BIS measured at time of iron administration, explaining 65% of the variability in fractional iron absorption (Table 4), in this case PHep was not a significant predictor. This was in contrast to the models explaining fractional absorption of the second dose where BIS combined with PHep explained 79% of data variability (Table 4). PHep only significantly contributed to prediction of absorption of the second dose when either the PHep at 8.00h on the preceding day was used, or when the increase in PHep between 8.00h and 17.00h on the preceding day was used. In contrast, PHep concentration measured at the time of administration of the second iron dose did not contribute to the model beyond the effect of BIS.
Discussion

In iron depleted young women, oral iron doses of 60, 80, 160 and 240 mg Fe given in the morning acutely increased PHep on the same day and 24h later. This increase was strongly associated with decreased absorption from the second iron dose, given 24hr after the first. Providing 60 mg of iron twice daily amplified the PHep increase and decreased the fractional absorption of both the afternoon dose and the next morning dose, so that total iron absorbed from the three doses (two mornings and afternoon) was not different to that from two morning doses. While these results require confirmation in longer term supplementation schedules, the short term effects observed on hepcidin suggest that oral iron at doses of 60 mg or greater will result in higher fractional absorption when dosages are spaced 48h. For 40 mg iron we found borderline effects. Similarly, hepcidin profiles after supplementation indicate that increasing the interval between doses to more than 48hr would not result in higher absorption than dosing at 48hr intervals, although we did not test this directly. The WHO recommends intermittent iron supplementation in children and menstruating women, proposing as the rationale a mucosal block in enterocytes lasting for 5–6 days. Our data, based on the acute effect of supplements on hepcidin, suggest that 48 hours, not 5 or 6 days, is sufficient for iron absorption to return to baseline.

We investigated which iron status parameter best predicted iron absorption and the best overall model included time of administration, dose and both BIS and PHep. However, a simplified model only including time, dose and BIS without PHep had only a marginal decrease in predictive power. This may be due to the relatively low analytical and biological variation in PF and sTfR compared to PHep, the high correlation of BIS with PHep in healthy subjects and/or the possibility that BIS (PF/sTfR) reflect a different pathway of cellular iron regulation,
In the models predicting the first dose absorption, only BIS was a significant predictor and not hepcidin. By contrast, for absorption of the second dose, both BIS and PHeP are predictors of absorption, and overall prediction increased ($R^2=0.791$ vs $R^2=0.650$) relative to the first dose. Interestingly, the level of PHeP on the preceding day (8.00) and its increase from 8.00-17.00 significantly contributed to explaining Fe absorption, but PHeP at the time of administration did not. Both these observations are consistent with the concept that a PHeP surge results in ferroportin degradation, the re-synthesis of which would be inhibited in iron-deficient enterocytes. The variation in the absorption data is not fully explained by iron markers and PHeP: it is possible that the remaining variance – besides analytical variation- is explained by effects on absorption modulated via the iron regulatory protein/iron responsive elements (IRP/IRE) system, HIF2α, or H-ferritin related intra-enterocyte functions.

In our data, in absence of inflammation and infection, BIS appears to be the best predictor of iron absorption. These findings are consistent with those from a recent study in anemic patients, where after completion of treatment for malaria a measure of BIS (the TfR/PF index) was the strongest predictor of absorption, but during malaria and the 3 days of treatment, PHeP, together with CRP, were the best predictors.

Consistent with previous reports in humans, we show that the PHeP increase after acute oral iron doses parallels an increase in transferrin saturation, which is followed by a transient increase in PF that then returns to baseline after 14 days. The observed effect in iron-depleted subjects suggests that intracellular ferritin may be elevated by oral iron though a mechanism...
secondary to the increase in PHep and ferroportin degradation\textsuperscript{14} which would then be followed by an increase in circulating ferritin levels.

We show clear differences in absorption depending on dose spacing when doses are higher than 40 mg. Our results contrast with those from an earlier study comparing daily with weekly supplementation\textsuperscript{6} that found only a non-significant decrease in iron absorption (13\%) during daily supplementation with 50 mg Fe.\textsuperscript{6} The reasons for this difference may be linked to dose, but may be more likely due to greater inhibition immediately after beginning supplementation because short-term dietary changes appear to induce stronger inhibitory or enhancing effects on iron absorption.\textsuperscript{31,32} In animal models, it has been suggested that PHep response to an iron challenge is differentially regulated with chronic and acute iron administration.\textsuperscript{41} A follow up study to investigate longer-term alternate day iron supplementation is currently being planned in our laboratory.

Strengths of this study include: a) we tested a wide range of iron doses from 40 to 240 mg Fe; b) we studied young women, a target group for iron supplementation; c) each subject acted as her own control for the iron absorption measurements and PHep profiles; and d) iron absorption and PHep profiles were accurately quantified by using stable iron isotope techniques and a c-ELISA with high sensitivity.\textsuperscript{30} Limitations of our study include: a) we tested relatively small numbers of subjects because of the logistics and expense of employing stable iron isotopes; b) our studies were limited to a supplementation phase of 2 days; and c) we did not study subjects with anemia, who may respond differently to iron supplementation than our iron-depleted, non-anemic subjects.
In conclusion, our data show that fractional absorption in iron-depleted women is highest at low iron doses (40-80 mg) and that acute, consecutive day dosing results in decreased iron bioavailability. For total iron absorption, twice daily iron supplementation seems to have limited additional effect compared to daily administration. These findings emphasize the need to study longer term, alternate day schedules of iron supplementation and advocate the hypothesis that low dose iron given on alternate days may maximize fractional iron absorption, increase dosage efficacy, reduce gastrointestinal exposure to unabsorbed iron, and ultimately improve tolerance of iron supplements.
Acknowledgments

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Authorship Contributions

DM, MZ, DWS and JG and conceived the studies and obtained funding. All authors contributed to the design of the trials. DM, MJ, VK, JG and MZ conducted the studies. DM analyzed the data and wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

Conflict of Interest Disclosures: DS and HT are employees of Radboud University and Medical Centre that offers high quality hepcidin measurements to the scientific, medical and pharmaceutical community, at a fee for service basis. The other authors do not have conflict of interest to disclose.
References


### Table 1. Iron absorption and iron status markers with increasing oral doses of FeSO₄ in young women (Study 1).

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<th>Fe dose (mg)</th>
<th>Day</th>
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<th>Fe absorbed, mg&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Plasma Fe, µg/mL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Transferrin Saturation, %&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>160</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>0.93 (0.1-4.2)</td>
<td>5.4 (9.3)</td>
<td>29.5 (9.5)</td>
<td>21.4 (8.8-39.9)</td>
<td>4.6 (1.4)</td>
<td>4.1 (3.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.9 (11.1-26.8)</td>
<td>25.4 (17.8-42.9)</td>
<td>0.95 (0.35-3.8)</td>
<td>0.79 (1.4)</td>
<td>23.2 (12.3)</td>
<td>20.4 (5.8-63.1)</td>
<td>4.8 (1.4)</td>
<td>3.8 (3.0)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>14.2 (6.4-8.3)</td>
<td>22.7 (9.7-77.4)</td>
<td>0.50 (0.20-13)</td>
<td>0.84 (0.80)</td>
<td>24.3 (5.8)</td>
<td>16.6 (7.2-34.9)</td>
<td>4.8 (1.0)</td>
<td>3.1 (1.9)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.7 (7.1-22.4)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>15.6 (11.3-35.9)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.56 (0.90-5.8)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.89 (0.81)</td>
<td>25.9 (10.2)</td>
<td>42.1 (6.8-172)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4.6 (1.0)</td>
<td>5.8 (3.3)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>240</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>1.0 (0.1-2.4)</td>
<td>0.85 (0.41)</td>
<td>22.1 (12.6)</td>
<td>16.1 (10.5-23.3)</td>
<td>5.8 (1.7)</td>
<td>3.2 (2.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.0 (7.1-393)</td>
<td>3.11 (17.0-94.3)</td>
<td>0.95 (0.40-4.1)</td>
<td>0.57 (0.22)</td>
<td>15.2 (5.0)</td>
<td>16.1 (12.2-28.3)</td>
<td>6.0 (1.9)</td>
<td>2.3 (2.1)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>14.8 (7.4-42.6)</td>
<td>35.5 (17.9-102.3)</td>
<td>0.88 (0.23-5.6)</td>
<td>0.54 (0.13)</td>
<td>13.5 (3.0)</td>
<td>21.9 (16.5-20.5)</td>
<td>5.8 (1.7)</td>
<td>3.3 (1.9)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.5 (5.6-21.4)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>18.1 (8.6-51.5)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.4 (0.83-13.7)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.56 (0.3)</td>
<td>16.4 (12.4)</td>
<td>33.4 (19.3-73.0)</td>
<td>5.2 (1.6)</td>
<td>2.3 (1.7)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Doses are given on day 2, 9 and 10 at 08.00 am immediately after iron status assessment; for 40, 80 and 240 mg, n=6; for 160 mg, n=7. ND: not determined; NA: not applicable; PHeP, plasma Hepcidin; sTfR, soluble Transferrin Receptor; PF, plasma Ferritin, BW, Body weight

<sup>a</sup> Geometric means (range). <sup>b</sup> Means (SD). <sup>c</sup> Different from day 7 and day 2 (P<0.05); <sup>d</sup> Different from day 7 and day 2 (P<0.01); <sup>e</sup> Different from day 2 (P=0.08); <sup>f</sup> Different from day 7 and day 2 (P<0.001); <sup>g</sup> Not different to all other time points (P<0.05); <sup>i</sup> Different from day 2 (P<0.05)
Table 2. Daily and twice daily administration of 60 mg Fe (Studies 2\(^\wedge\) and 3\(#\)).

<table>
<thead>
<tr>
<th>Time and day of administration</th>
<th>Fractional Fe absorption, %(^a)</th>
<th>Fe absorbed, mg(^a)</th>
<th>PHep, nM(^a)</th>
<th>PF, µg/L(^a)</th>
<th>sTfR, mg/L(^b)</th>
<th>Body iron stores, mg/kg BW(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily (^\wedge)</td>
<td>8:00 am d1</td>
<td>NA</td>
<td>NA</td>
<td>0.6 (0.5-8.9)</td>
<td>16.2 (13.5-23.0)</td>
<td>4.4 (1.7)</td>
</tr>
<tr>
<td>8:00 am, d2</td>
<td>22.9 (10.5-49.4)</td>
<td>13.8 (6.3-29.6)</td>
<td>0.8 (0.4-6.1)</td>
<td>15.5 (7.2-30.0)</td>
<td>5.1 (1.3)</td>
<td>2.7 (2.1)</td>
</tr>
<tr>
<td>8:00 am, d3</td>
<td>14.6 (7.2-28.3)(^c)</td>
<td>8.8 (4.6-17.0)(^c)</td>
<td>1.5 (0.3-8.5)(^d)</td>
<td>26.7 (11.6-57.5)(^a)</td>
<td>5 (1.5)</td>
<td>4.7 (2.3)</td>
</tr>
<tr>
<td>8:00 am, d15</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>16.9 (7.3-34.0)</td>
<td>5.1 (1.4)</td>
<td>3.0 (1.7)</td>
</tr>
<tr>
<td>Twice daily (#)</td>
<td>10:00 am, d1</td>
<td>17.1 (8.5-37.3)</td>
<td>10.2 (5.1-22.4)</td>
<td>0.9 (0.3-3.7)</td>
<td>13.6 (7.1-32.0)</td>
<td>4.9 (1.1)</td>
</tr>
<tr>
<td>16:00 pm, d2</td>
<td>12.5 (6.3-19.2)(^e)</td>
<td>7.5 (3.8-11.5)(^e)</td>
<td>4.1 (0.5-10.7)(^h)</td>
<td>15.9 (6.1-37.5)(^i)</td>
<td>5.2 (1.3)</td>
<td>2.5 (2.4)</td>
</tr>
<tr>
<td>8:00 am, d3</td>
<td>9.9 (4.4-16.3)(^g)</td>
<td>5.9 (2.6-9.8)(^g)</td>
<td>6.3 (1.3-14.1)(^h,j)</td>
<td>32.2 (19.3-57.8)(^h)</td>
<td>5.1 (1.4)</td>
<td>5.2 (1.6)</td>
</tr>
<tr>
<td>8:00 am, d15</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>16.4 (8.4-53.1)</td>
<td>4.6 (0.9)</td>
<td>3.2 (2.2)</td>
</tr>
</tbody>
</table>

\(^{\wedge}\),\(^\#\), Studies 2 and 3 are two distinct studies conducted with either daily or twice daily administration of 60 mg supplements. \(^\wedge\)In study 2 doses are given at 08.00h; \(^\#\), in study 3 doses given at 10.00 and 17.00 to fasting subjects. All administrations were given to fasting subjects immediately after iron status determination.

\(^a\) Geometric means (range). \(^b\) Means (SD). All doses 60 mg Fe as FeSO\(_4\), daily study, n=16; twice daily study, n=13. ND: not determined; NA: not applicable; PHep, Plasma Hepcidin; PF, Plasma Ferritin; sTfR, soluble Transferrin Receptor; BW, body weight.

\(^c\) Different from d1 (paired t-test, P<0.01); \(^d\) Different from d1, d2 (P<0.05); \(^e\) Different from d1, d2 and d16 (P<0.01); \(^f\) Different from d1, d2 and d16 (P<0.05); \(^g\) Different from other time points (P<0.05); \(^h\) Different from 10.00 am d1 (P<0.01); \(^i\) Different from preceding time point (P<0.05); \(^j\) Different from daily study 8:00 am d2 (P<0.05); \(^k\) Different from all other time points (P<0.001)
Table 3. Regression models predicting fractional (% of dose) and absolute (mg) iron absorption from iron supplements in relation to timing of administration, dosage, body iron stores and hepcidin concentrations at administration or at start of the study. Differences between nested models was tested with change in F statistic; differences between non nested models was tested by comparing different R coefficients with Steiger’s Z test. Within one category of dependent variables, reported models differ significantly if superscript differs, P<0.01.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Log absorption (%), n=98</th>
<th>Log absolute absorption mg, n=98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Independent variables, standardized β</td>
<td>Independent variables, standardized β</td>
</tr>
<tr>
<td>Model R²</td>
<td>Time</td>
<td>Dose, mg</td>
</tr>
<tr>
<td>0.689&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.146&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.352&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.666&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.165&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.371&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.579&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.228&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.397&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.604&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.367&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.319&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.520&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.367&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.529&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.378&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.385&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.514&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All models are significant at P<0.01. Differences between nested models was tested with change in F statistic; differences between non nested models was tested by comparing different R coefficients with Steiger’s Z test. Within one category of dependent variables, reported models differ significantly if superscript differs, P<0.01; PHep, plasma hepcidin; BIS, body iron stores; BW, body weight

<sup>a</sup>Significant regression parameter P<0.05

<sup>b</sup>Significant regression parameter P<0.001

<sup>d</sup>Significant regression parameter P<0.01
Table 4. Prediction of iron absorption from the first and second dose of iron supplements, depending on the time point of plasma hepcidin (PHep) and body iron stores assessment (BIS). Differences between nested models were tested with change in F statistic; differences between non nested models were tested by comparing different R coefficients with Steiger’s Z test. Within one category of dependent variables, reported models differ significantly if superscript differs, \( P<0.01 \).

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>First dose fractional absorption (%), n=40</th>
<th>Second dose fractional absorption, %, n=40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent variables, standardized β</td>
<td>Independent variables, standardized β</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model ( R^2 )</th>
<th>Dose, mg</th>
<th>BIS mg/Kg BW</th>
<th>Log hepcidin, nM</th>
<th>Model ( R^2 )</th>
<th>Dose, mg</th>
<th>BIS mg/Kg BW, preceding day</th>
<th>BIS at administration mg/Kg BW</th>
<th>Log hepcidin, nM, preceding day</th>
<th>Log hepcidin nM at administration</th>
<th>Hepcidin increase on previous day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.650(^a)</td>
<td>-0.397(^b)</td>
<td>-0.565(^c)</td>
<td>n.s.</td>
<td>0.791(^a)</td>
<td>-0.480(^a)</td>
<td>-</td>
<td>-0.447(^a)</td>
<td>-0.330(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.642(^a)</td>
<td>-3.94(^a)</td>
<td>-0.628(^b)</td>
<td>-</td>
<td>0.780(^a)</td>
<td>-0.463(^a)</td>
<td>-0.535(^a)</td>
<td>-</td>
<td>-0.535(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.391(^b)</td>
<td>-0.509(^b)</td>
<td>-</td>
<td>-0.376(^a)</td>
<td>0.768(^b)</td>
<td>-0.419(^b)</td>
<td>-</td>
<td>-0.461(^b)</td>
<td>-</td>
<td>-</td>
<td>-0.283(^a)</td>
</tr>
<tr>
<td>0.248(^b)</td>
<td>-0.498(^b)</td>
<td>-</td>
<td>-</td>
<td>0.727(^a)</td>
<td>-0.365(^b)</td>
<td>-0.590(^b)</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.719(^a)</td>
<td>-0.406(^b)</td>
<td>-</td>
<td>-0.565(^b)</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.706(^a)</td>
<td>-0.407(^b)</td>
<td>-0.664(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.557(^b)</td>
<td>-0.55(^b)</td>
<td>-</td>
<td>-</td>
<td>-0.519(^b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.476(^b)</td>
<td>-0.391(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.452(^b)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.476(^b)</td>
<td>-0.463(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.421(^a)</td>
</tr>
</tbody>
</table>

All models are significant at \( P<0.01 \).

\(^b\)Significant regression parameter \( P<0.05 \)
Significant regression parameter $P<0.001$

Significant regression parameter $P<0.01$

n.s.: non-significant parameter in the model
Figure Legends

Figure 1. Study design for studies 1-3. Panel A: Study 1 (n=25), fasting subjects received 40, 80, 160, 240 mg Fe at 8h and were randomly allocated to start the study either with single or with consecutive day doses (cross over design). Subjects acted as their own controls. Hepcidin and iron status was assessed at 8h,12h, 17h (day1-2) and at 8h on days 3, 4 and 5 (single dose schedule) or at 8h,12h, 17h (day1-3) and 8h on days 4 and 5 (consecutive dose schedule). Panel B indicates study 2 (n=16) which foresaw only one week of supplementation and only two consecutive 60 mg Fe doses. Panel C pictures the study design of study 3 (n=13) where bi-daily supplementation was tested: For that aim, the diet of the subjects was controlled between subjects to maintain at least 3h of fasting between iron dosages, which were given at 10h and at 16h after a standardized breakfast and lunch, respectively. A full description and more detailed representation of the study design are available as online supplemental material). Numbers refer to consecutive study days. LFe: labeled iron supplement administration; L: determination of isotopic composition (iron absorption).

Figure 2. Iron status indices and Hepcidin profiles during control and supplementation days with 40 mg Fe and 240 mg Fe (Study 1). (A, D) Transferrin Saturation (%TS). (B, E) Plasma Hepcidin (nM) and (C, F) Plasma Ferritin (µg/l). * indicate significantly different concentration from reference concentration (control day, 8.00). Supplementation days are indicated with the symbol Fe in the x-axis. Data presented as geometric means with brackets indicating the interquartile range.

Figure 3. A supplemental iron doses of 60 mg Fe results in an increase in hepcidin after 24h and in a decreased iron absorption from the consecutive dose (n=16). Doses are given both at 8:00 in the morning on consecutive days 2, and 3, and compared to day 1 (control day); Study 2.. (A) hepcidin profiles during the observation period, boxes indicate median and interquartile ranges, whiskers describe the range of the data (min to max). Boxes with different subscript letter differ significantly (P<0.05). (B) Fractional iron absorption measured on day 2 and day 3 from the 60 mg Fe dose. D1, day 1.

Figure 4. Bi-daily iron administration at 10.00 and at 17.00 results in increased hepcidin on the consecutive day and decreased iron bioavailability (n=13). Subjects followed a controlled diet during the day of iron administration (Study 3). Boxes indicate median and interquartile ranges, whiskers describe the range of the data (min to max). (A) hepcidin profiles. (B) Fractional iron absorption at the three time points of the 60 mg Fe dose.
Figure 5. Absolute amount of iron absorbed in relation to the dose administered for the first administration (continuous line,* ) and the second administration (broken line,†). Each dose was consumed by different subjects (n=41). At doses of 60 mg and higher, first and second dose absorption differed significantly (P<0.01). Data with different superscripts differ significantly (capitals: first dose; minuscule: second dose) The first dose absorption was predicted by the following model (R²=0.450; P<0.001): (Dose absorbed) = 0.816 x (dose administered)⁰.⁶⁷⁸ ; while the second dose absorption (R²=0.467, P<0.001): (Dose absorbed)= 0.752 x (dose administered)⁰.⁵⁹⁶. Absorption data are standardized to a plasma ferritin level of 15µg/L.
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Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women

Diego Moretti, Jeroen S. Goede, Christophe Zeder, Markus Jiskra, Vaiya Chatzinakou, Harold Tjalsma, Alida Melse-Boonstra, Gary Brittenham, Dorine W. Swinkels and Michael B. Zimmermann