CLINICAL TRIALS AND OBSERVATIONS

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

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Key Points

- Iron supplements at doses of 60 mg Fe as $FeSO_4$ or higher increase hepcidin for up to 24 hours 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.

Iron supplements acutely increase hepcidin, but the duration and magnitude of the increase, its dose dependence, and its 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 nonanemic 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 [^{57}Fe]-, [^{58}Fe]-, or [^{54}Fe]-$FeSO_4$ given at 8:00 AM fasting on 1 or on 2 consecutive days (study 1, n = 25; study 2, n = 16); and (2) a study giving three 60-mg Fe doses (twice-daily dosing) within 24 hours (study 3, n = 13). In studies 1 and 2, 24 hours after doses ≥60 mg, serum hepcidin was increased ($P < .01$) and fractional iron absorption was decreased by 35% to 45% ($P < .01$). With increasing dose, fractional absorption decreased ($P < .001$), whereas absolute absorption increased ($P < .001$). A sixfold increase in iron dose (40-240 mg) resulted in only a threefold increase in iron absorbed (6.7-18.1 mg). In study 3, total iron absorbed from 3 doses (2 mornings and an afternoon) was not significantly greater than that from 2 morning doses. Providing lower dosages (40-80 mg Fe) and avoiding twice-daily dosing maximize 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 as #NCT01785407 and #NCT02050932. (*Blood*. 2015;126(17):1981-1989)

Introduction

Anemia affects ≈33% of the world population and accounts for 8.8% of global disability.[1] Although the etiology of anemia is multifactorial, iron deficiency (ID) is considered to be the most prevalent cause globally.[1] In the United States, ID is estimated to affect 9.2% of females aged 12 to 49 years.[2]

Oral iron supplementation with $FeSO_4$ is a primary approach for the treatment of iron deficiency anemia (IDA).[3] Although both daily and intermittent supplementation can replete iron stores and increase hemoglobin levels,[4] iron supplements often cause gastric irritation, nausea, epigastric discomfort, and constipation, which may decrease compliance and long-term efficacy.[5] The absorption of iron supplements ranges from 2% to 13% and 5% to 28% in subjects with low iron stores[6] when consumed with and without food, respectively. Thus, a majority of the iron is unabsorbed. Although 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.[7,8]

Hepcidin is the key regulator of systemic iron balance in mammals,[9] acting in concert with intracellular iron metabolism.[10-13] Iron supplementation acutely increases the circulating plasma hepcidin level,[14-16] but the magnitude and duration of this increase has not been characterized in humans. Plasma hepcidin negatively correlates with iron bioavailability[16,17] and has a circadian increase over the day, in association with a fall in transferrin saturation.[15,18,19] Morning iron supplementation enhances this increase in plasma hepcidin,[19] 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 to 120 mg Fe per day to treat IDA.[20-22] Intermittent schedules are advised for primary prevention in young women,[23] whereas in pregnant

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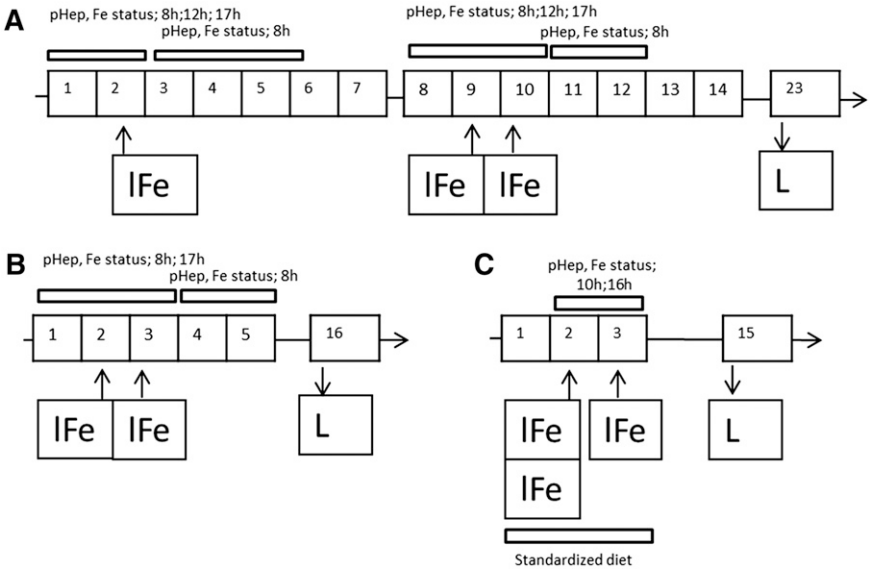

Figure 1. Study design for studies 1-3. (A) Study 1 (n = 25), fasting subjects received 40, 80, 160, and 240 mg Fe at 8:00 AM and were randomly allocated to start the study either with single or with consecutive day doses (crossover design). Subjects acted as their own controls. Hepcidin and iron status was assessed at 8:00 AM,12:00 PM, and 5:00 PM (days 1-2) and at 8:00 AM on days 3, 4, and 5 (single dose schedule) or at 8:00 AM,12:00 PM, and 5:00 PM (days 1-3) and 8:00 AM on days 4 and 5 (consecutive dose schedule). (B) Study 2 (n = 16) foresaw only 1 week of supplementation and only 2 consecutive 60-mg Fe doses. (C) Study design of study 3 (n = 13) where bi-daily supplementation was tested: the diet of the subjects was controlled between subjects to maintain at least 3 hours of fasting between iron dosages, which were given at 10 hours and at 16 hours 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).

women, the World Health Organization (WHO)[24] and the Centers for Disease Control and Prevention[21] recommend 30 to 60 mg Fe per day. This guidance is not shared by all organizations[22] and depends on anemia prevalence.[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 plasma ferritin [PF] $\leq$20 μg/L) but no anemia (hemoglobin >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 within the previous 4 months; nonsmoking; no intake of mineral, vitamin, or herbal supplements within 2 weeks of study start and during the entire duration of the study; body mass index between 18 and 25 kg/m^2; and body weight <68 kg. We excluded subjects who had a C-reactive protein >5 mg/L at screening.

Design

We conducted 3 separate studies with the aim of measuring the acute iron-induced increase in hepcidin caused by $FeSO_4$ supplements while quantifying iron absorption using stable isotopic labels as tracers (Figure 1). We monitored plasma hepcidin (PHep) and iron status markers before administration and up to 48 hours post-administration at 8:00 AM, 12:00 PM, and 5:00 PM. In study 1, using a crossover design, we administered 2 iron challenges either as a single dose or as 2 doses given on consecutive days. Subjects were randomly assigned to start the study with one of the 2 treatments. Iron was administered at 8:00 AM (± 1 hour) in 4 different iron concentrations (40, 80, 160, and 240 mg as elemental Fe). In study 2, we administered 2 single doses of 60 mg elemental iron at 8:00 AM on 2 consecutive days and similarly assessed hepcidin response until 48 hours 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 24 hours (3 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 of stable isotopic tracers incorporated in red blood cells 14 days post-administration (a detailed description of study procedures is available in the supplemental Material, found on the *Blood* Web site).

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 $FeSO_4$ (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, IA) containing 4 mg of labeled $FeSO_4$ in the form of [^{57}Fe]-$FeSO_4$, [^{58}Fe] -$FeSO_4$, or [^{54}Fe]-$FeSO_4$ (Chemgas, Boulogne, France) prepared as previously described.[26] At administration, we rinsed the plastic cup with an additional 100 mL of 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 enzyme-linked immunosorbent assay (ELISA) method described by Erhardt et al,[27] simultaneously assessing PF, the soluble transferrin receptor (sTfR), C-reactive protein (CRP), and α-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,[28] and transferrin saturation (%TS) was calculated with the formula (SFe/TIBC) × 100. We calculated body iron stores (BIS) for study participants at each time point using the formula based on the sTfR/PF ratio proposed by Cook et al.[29]

We used a c-ELISA method to quantify PHep.[30] This method has a lower limit of detection than weak cation exchange time-of-flight mass spectrometry and is therefore a preferred method in the present study because of the anticipated low hepcidin levels in healthy subjects with depleted iron stores.[16]

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.[31]

Statistical analysis

We conducted the statistical analysis with SPSS (SPSS statistics, Version 22, IBM) using linear mixed models (LMM) to assess the effect of iron supplementation

Table 1. Iron absorption and iron status markers with increasing oral doses of $FeSO_4$ in young women (study 1)

		Iron bioavailability		Iron status					
Fe dose (mg)	Day	Fractional Fe absorption (%)*	Fe absorbed (mg)*	PHep (nM)*	Plasma Fe (μg/mL)†	Transferrin saturation (%)†	PF (μg/L)*	sTfR (mg/L)†	Body iron stores (mg/kg BW)†
40	1	NA	NA	0.30 (0.12-0.48)	0.63 (0.34)	20.9 (15.1)	10.0 (3.4-21.8)	8.2 (4.2)	−0.05 (3.7)
	2	22.7 (14.7-57.1)	9.1 (5.8-22.8)	0.35 (0.11-0.77)	0.55 (0.35)	16.5 (11.7)	9.1 (4.9-25.4)	8.4 (3.8)	−0.8 (3.7)
	9	19.4 (15.8-22.9)	7.8 (6.3-9.2)	0.59 (0.19-4.6)	0.67 (0.61)	21.3 (24.6)	10.3 (5.1-40.8)	7.1 (3.2)	0.11 (4.1)
	10	16.7 (11.8-20.7)‡	6.7 (4.7-8.3)‡	0.45 (0.05-4.3)	0.60 (0.4)	18.6 (16.3)	15 (8.4-51.6)	7.8 (3.5)	1.2 (3.6)
	23	NA	NA	ND	ND	ND	7.7 (4.2-20.1)	5.6 (1.9)	−0.64 (2.4)
80	1	NA	NA	0.93 (0.1-3.7)	1.2 (1.1)	29.8 (12.8)	19.4 (6.0-38.4)	4.8 (1.7)	3.5 (3.5)
	2	19.0 (10.5-30.9)	15.2 (8.4-24.7)	0.90 (0.40-2.2)	0.80 (0.40)	21.3 (8.4)	17.7 (6.0-43.6)	4.8 (1.6)	3.5 (3.4)
	9	18.2 (8.5-26.0)	14.6 (8.5-26.0)	1.1 (0.62-2.1)	0.75 (0.41)	20.9 (9.6)	17.7 (6.5-51.1)	4.5 (2.5)	3.6 (3.4)
	10	11.7 (8.4-24.7)§	9.3 (4.8-12.4)§	2.1 (0.98-5.1)¶	0.96 (0.60)	23.5 (12.5)	33 (24.1-55.0)	3.9 (1.7)	5.3 (2.7)
	23	NA	NA	ND	ND	ND	15.2 (7.2-68.3)	2.9 (1.5)	4.8 (3.7)
160	1	NA	NA	0.93 (0.1-4.21)	5.4 (9.3)	29.5 (9.5)	21.4 (8.8-39.9)	4.6 (1.4)	4.1 (3.1)
	2	15.9 (11.1-26.8)	25.4 (17.8-42.9)	0.95 (0.15-3.8)	0.79 (1.4)	23.2 (12.3)	20.4 (5.8-63.1)	4.8 (1.4)	3.8 (3.0)
	9	14.2 (6.1-48.3)	22.7 (9.7-77.4)	0.50 (0.20-1.3)	0.84 (0.80)	24.3 (5.8)	16.6 (7.2-34.9)	4.8 (1.0)	3.1 (1.9)
	10	9.7 (7.1-22.4)‖	15.6 (11.3-35.9)‖	1.56 (0.90-5.8)#	0.89 (0.81)	25.9 (10.2)	42.1 (6.8-172)#	4.6 (1.0)	5.8 (3.3)**
	23	NA	NA	ND	ND	ND	17.5 (10.5-22.0)	3.5 (1.0)	4.4 (1.3)
240	1	NA	NA	1.0 (0.1-2.4)	0.85 (0.41)	22.1 (12.6)	16.1 (10.5-23.3)	5.8 (1.7)	2.3(2.1)
	2	13.0 (7.1-39.3)	31.1 (17.0-94.3)	0.95 (0.40-4.1)	0.57 (0.22)	15.2 (5.0)	16.1 (12.2-28.3)	6.0 (1.9)	2.3 (2.1)
	9	14.8 (7.4-42.6)	35.5 (17.9-102.3)	0.88 (0.23-5.6)	0.54 (0.13)	13.5 (3.0)	21.9 (16.5-30.5)	5.8 (1.7)	3.3 (1.9)
	10	7.5 (3.6-21.4)‡	18.1 (8.6-51.5)‡	3.4 (0.83-13.7)‡	0.56 (0.3)	16.4 (12.4)	33.4 (19.3-73.0)	5.2 (1.6)	2.3 (1.7)
	23	NA	NA	ND	ND	ND	14.7 (10-33.3)	3.7 (1.5)	3.8 (2.5)

BW, body weight; NA, not applicable; ND, not determined; PF, plasma ferritin; PHep, plasma hepcidin; STfR, soluble transferrin receptor.
Doses are given on days 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.
*Geometric means (range).
†Means (SD).
‡Different from day 7 and day 2 ($P < .05$).
§Different from day 7 and day 2 ($P < .01$).
¶Different from day 2 ($P = .08$).
‖Different from day 7 and day 2 ($P < .001$).
#Different to all other time points ($P < .05$).
**Different from day 2 ($P < .05$).

on hepcidin and iron status markers. Body iron stores were calculated with the formula[29]:

$$\text{Body Fe(mg/kg)} = [\log(\text{sTfR/PF}) - 2.8229]/0.1207,$$

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,[32] obtained with the formula:

$$\text{Log}(A_{15}) = \text{Log } A_0, + \text{Log } F_0 - \text{Log } 15,$$

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* × *dose* interaction, an ad hoc variable describing the time between each hepcidin measurement and iron administration was defined. Because of the generally improved models, we used log-transformed hepcidin data in the LMM, 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 be easily assessed. Paired Student *t* tests were used to test for within-subject effects for normally distributed data (log-transformed data from studies 2 and 3). For between-subject effects, independent sample Student *t* tests were used (study 2 vs study 3). Predictors of iron absorption were assessed using LMM 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 data sets of studies 1 and 2. The statistical difference between different R^2 in non-nested regression models was tested with the Steiger Z test. Significance was defined as $P < .05$.

Hepcidin and iron status parameters assessed in studies 1 and 2 were analyzed using LMM against the concentration on a control day at 8:00 AM 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 3 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 hours at all doses examined (all $P < .001$). The %TS reached a maximum at 4 hours post-administration, with mean (standard deviation [SD]) %TS at 61.5 (15.9), 72.1 (14.2), 72.2 (30.3), and 64.7 (25.1) for 40, 80, 160, and 240 mg Fe, respectively. These values were not statistically different

Table 2. Daily and twice-daily administration of 60 mg Fe (studies 2 and 3)

		Iron bioavailability		Iron status			
	Time and day of administration	Fractional Fe absorption (%)†	Fe absorbed (mg)†	PHep (nM)†	PF (μg/L)†	sTfR (mg/L)‡	Body iron stores (mg/kg BW) ‡
Study 2: Daily*	8:00 AM, d1	NA	NA	0.6 (0.5-8.9)	16.2 (13.5-23.0)	4.4 (1.7)	3.5 (2.7)
	8:00 AM, d2	22.9 (10.5-49.4)	13.8 (6.3-29.6)	0.8 (0.4-6.1)	15.5 (7.2-30.0)	5.1 (1.3)	2.7 (2.1)
	8:00 AM, d3	14.6 (7.2-28.3)§	8.8 (4.6-17.0)§	1.5 (0.3-8.5)‖	26.7 (11.6-57.5)¶	5 (1.5)	4.7 (2.3)#
	8:00 AM, d15	NA	NA	ND	16.9 (7.3-34.0)	5.1 (1.4)	3.0 (1.7)
Study 3: Twice daily*	10:00 AM, d1	17.1 (8.5-37.3)	10.2 (5.1-22.4)	0.9 (0.3-3.7)	13.6 (7.1-32.0)	4.9 (1.1)	2.3 (2.2)
	4:00 PM, d2	12.5 (6.3-19.2)**	7.5 (3.8-11.5)**	4.1 (0.5-10.7)††	15.9 (6.1-37.5)‡‡	5.2 (1.3)	2.5 (2.4)
	8:00 AM, d3	9.9 (4.4-16.3)**	5.9 (2.6-9.8)**	6.3 (1.3-14.1)††, §	32.2 (19.3-57.8)[a]	5.1 (1.4)	5.2 (1.6)††
	8:00 AM, d15	NA	NA	ND	16.4 (8.4-53.1)	4.6 (0.9)	3.2 (2.2)

BW, body weight; NA, not applicable; ND, not determined; PF, plasma ferritin; PHep, plasma hepcidin; sTfR, soluble transferrin receptor.

Studies 2 and 3 are two distinct studies conducted with either daily or twice-daily administration of 60-mg supplements.

*In study 2, doses are given at 08:00 PM; in study 3, doses are given at 10:00 AM and 5:00 PM to fasting subjects. All administrations were given to fasting subjects immediately after iron status determination.

†Geometric means (range).

‡Means (SD). All doses 60 mg Fe as $FeSO_4$, daily study, n = 16; twice-daily study, n = 13.

§Different from d1 (paired Student *t* test, $P < .01$).

‖Different from d1, d2 ($P < .05$).

¶Different from d1, d2, and d16 ($P < .01$).

#Different from d1, d2, and d16 ($P < .05$).

**Different from other time points ($P < .05$).

††Different from 10:00 AM d1 ($P < .01$).

‡‡Different from preceding time point ($P < .05$).

¶¶Different from daily study 8:00 AM d2 ($P < .05$).

[a]Different from all other time points ($P < .001$).

between different dosage groups. Transferrin saturation remained elevated during the day of administration (17.00, $P < .001$) and returned to baseline levels after 24 hours (not different from baseline levels; Figure 2). The sTfR concentration decreased transiently within 4 to 24 hours after administration of doses of 160 and 240 mg ($P < .01$). An increase in PF was detectable from 8 to 24 hours after administration for all doses, and the concentration remained significantly increased at 24 hours compared with baseline for the 40-, 80-, and 160-mg doses, and at 56 hours for the 240-mg dose (Figure 2). PF 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 LMM including all data points in the study, time had a significant effect on PHep ($P < .001$), and there was a significant *time* × *dose* interaction for PHep ($P < .05$). For all iron doses tested, supplementation increased PHep at 24 hours by a factor of 2.7 (95% confidence interval [CI], 1.6-4.6). The second dose of iron increased PHep by a factor of 2.1 (95% CI, 1.2-3.5). There was a significant increase in PHep at 24 hours after the doses of 60, 80, 160, and 240 mg Fe ($P < .05$), but not at 40 mg Fe. PHep was not significantly elevated 48 hours post–iron administration in the overall model or after any of the iron doses. The increase in PHep from 8:00 AM to 5:00 PM 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 ($P < .001$), whereas absolute absorption increased ($P < .001$). Although the dose increased sixfold (from 40 to 240 mg), the absolute amount of iron absorbed increased only ~threefold—eg, a dose of 40 mg Fe given 24 hours after the first dose provided 6.7 mg of absorbed iron, whereas the second 240-mg dose provided 18.1 mg of absorbed Fe, and the highest fractional absorption was achieved with dosages between 40 and 80 mg Fe (Table 1).

Iron absorption was significantly lower on the second day of administration (day 10) compared with the first day of administration (day 2 and day 9; $P < .001$). 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 Fe was 37%, 35%, and 45% lower, respectively, compared with the first iron dose (all $P < .01$). 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 day 10 ($P = .19$), but fractional absorption on day 9 was lower than that on day 2 ($P = .040$).

Taken together, these data suggest that acute absorption is inhibited at dosages of 80, 160, and 240 mg within 24 hours and suggest a possible effect at a dosage of 40 mg Fe.

PHep increases and iron absorption decreases at 24 hours with 60 mg Fe

In study 2, there was a significant effect of time on PHep ($P < .001$); at 24 hours 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 AM resulted in a PHep at 8:00 AM on the third day that was 1.5-fold (95% CI, 1.01-2.22) higher than the baseline value at the start of the study. From 8:00 AM to 5:00 PM, 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 with the first day ($P < .001$) (Figure 3).

In study 3, twice-daily administration of 60 mg iron at 10:00 AM and 4:00 PM resulted in a higher PHep at 8:00 AM on the following day compared with once-daily administration (independent sample Student *t* test, $P < .01$); PHep increased by a factor of 6.7 (95% CI, 4.1-10.8) compared with baseline (Figure 4). Iron absorption from the afternoon dose decreased by 26% compared with the first morning dose ($P < .01$). Iron absorption from the successive morning doses decreased by 43% compared with the

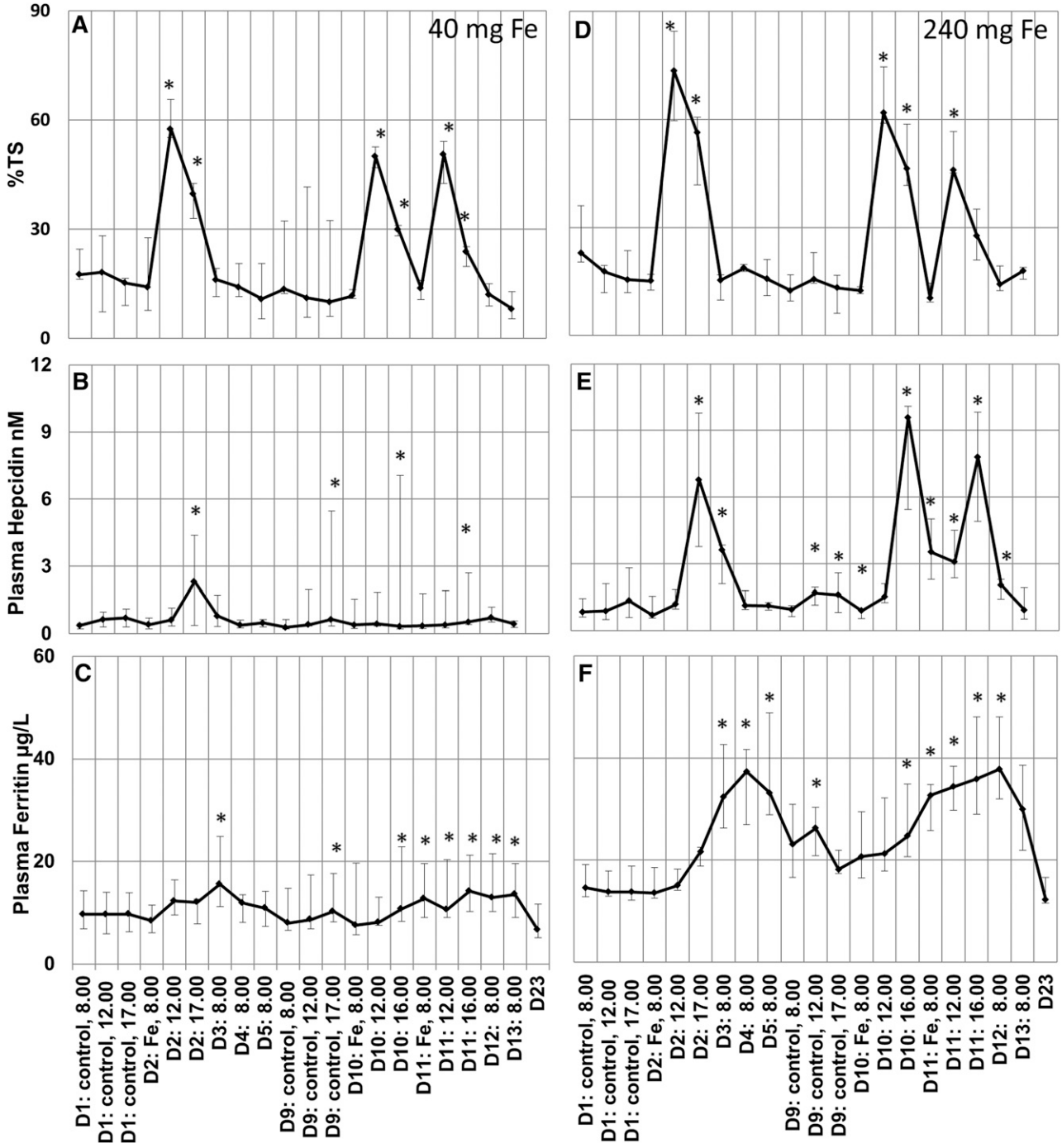

Figure 2. Iron status indices and hepcidin profiles during control and supplementation days with 40 mg Fe and 240 mg Fe (Study 1). (A,D) Transferrin saturation (%TS). (B,E) Plasma hepcidin (nM) and (C,F) PF (μg/L). *Significantly different concentration from reference concentration (control day, 8:00 AM). Supplementation days are indicated with the symbol "Fe" in the x-axis. Data are presented as geometric means with brackets indicating the interquartile range.

first dose 24 hours earlier ($P < .01$) and was 20% lower than the afternoon dose given on the preceding day ($P < .01$). 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 dose was given ($P < .05$). Absolute iron absorption from a dose of 60 mg given at 08:00 AM 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 24 hours compared with 22.6 mg Fe when only the 2 morning doses were given ($P = .79$).

Total iron absorbed

The total amount of iron absorbed from the supplements was generally higher with increasing dose ($P < .001$). For the first and second doses, respectively, the relationship between dose administered and dose absorbed was best predicted by the formulas (Figure 5):

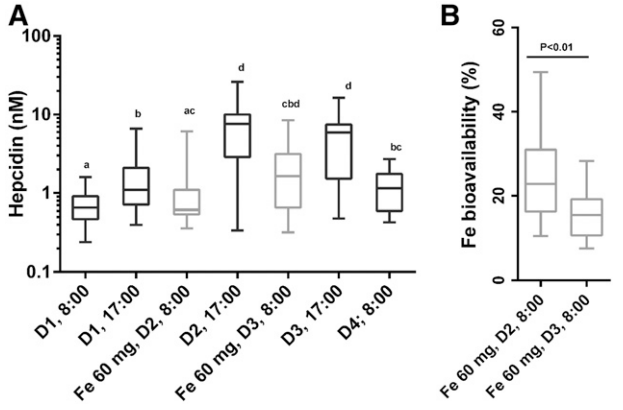

Figure 3. A supplemental iron dose of 60 mg Fe results in an increase in hepcidin after 24 hours and in a decreased iron absorption from the consecutive dose (n = 16). Doses are given both at 8:00 AM on consecutive days 2 and 3 and compared with day 1 (control day) (study 2). (A) Hepcidin profiles during 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 ($P < .05$). (B) Fractional iron absorption measured on days 2 and 3 from the 60-mg Fe dose. D1, day 1.

$$\text{Dose absorbed}_{(\text{first dose})} = 0.816 \times (\text{dose administered})^{0.678};$$
$$R^2 = 0.450; P < .001$$
$$\text{Dose absorbed}_{(\text{second dose})} = 0.752 \times (\text{dose administered})^{0.596};$$
$$R^2 = 0.467; P < .001$$

Total iron absorbed from the 160- and 240-mg doses was significantly higher than that absorbed from the 40-, 60-, and 80-mg doses ($P < .05$), 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 BIS, PHep, time of administration, and dose ($R^2 = 0.69$; $P < .001$). A simplified model without PHep resulted in a different model ($P = .011$) with similar predictive power ($R^2 = 0.67$; $P < .001$) (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 ($R^2 = 0.54$; $P < .001$), and predictive power was lower than a model using BIS alone ($P < .05$). Models including PHep and BIS measured at the 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 8:00 AM on the preceding day was used, or when the increase in PHep between 8:00 AM and 5:00 PM 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 24 hours later. This increase was strongly associated with decreased absorption from the second iron dose, given 24 hours 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 3 doses (2 mornings and afternoon) was not different from that of the 2 morning doses. Although these results require confirmation in longer-term supplementation schedules, the short-term effects observed on hepcidin suggest that oral iron at doses ≥60 mg greater will result in higher fractional absorption when dosages are spaced by 48 hours. For 40 mg iron, we found borderline effects. Similarly, hepcidin profiles after supplementation indicate that increasing the interval between doses to >48 hours would not result in higher absorption than dosing at 48-hour intervals, although we did not test this directly. The WHO recommends intermittent iron supplementation in children[33] and menstruating women,[34] proposing as the rationale a mucosal block in enterocytes lasting for 5 to 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 because of the relatively low analytical and biological variation in PF and sTfR compared with 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, independent from systemic signals.[35,36] 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

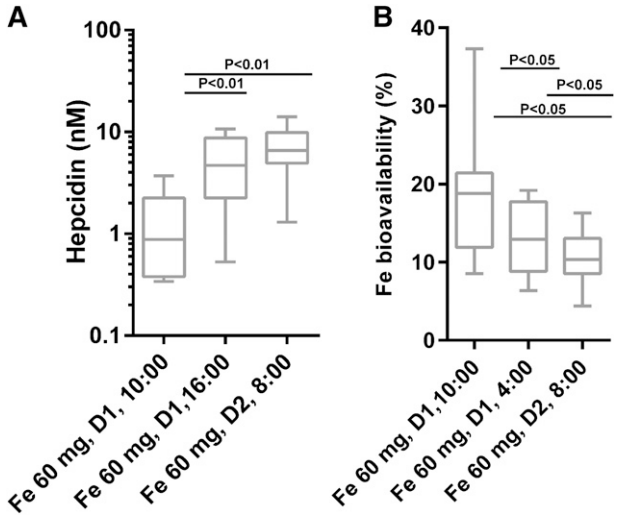

Figure 4. Bi-daily iron administration at 10:00 AM and at 5:00 PM results in increased hepcidin on the consecutive day and decreased iron bioavailability (n = 13). Subjects followed a controlled diet during the day of iron administration (study 3). Boxes indicate median and interquartile ranges, whiskers describe the range of the data (min to max). (A) Hepcidin profiles. (B) Fractional iron absorption at the 3 time points of the 60-mg Fe dose.

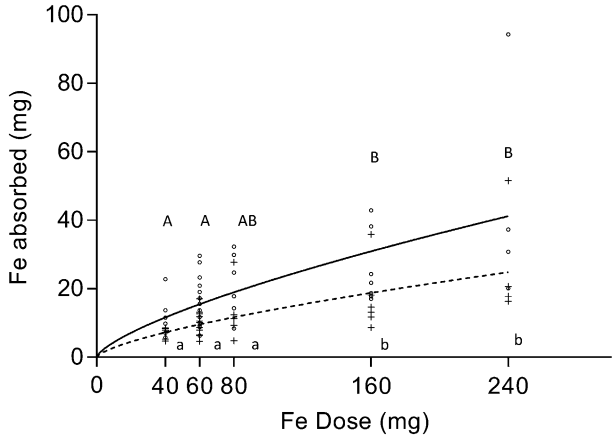

Figure 5. Absolute amount of iron absorbed in relation to the dose administered for the first administration (continuous line°) and the second administration (broken line⁺). Each dose was consumed by different subjects (n = 41). At doses of 60 mg and higher, the first and second dose absorptions differed significantly ($P < .01$). Data with different superscripts differ significantly (capitals: first dose; minuscule: second dose) The first dose absorption was predicted by the following model ($R^2 = 0.450$; $P < .001$): (Dose absorbed) = 0.816 × (dose administered)$^{0.678}$; whereas the second dose absorption ($R^2 = 0.467$, $P < .001$): (Dose absorbed) = 0.752 × (dose administered)$^{0.596}$. Absorption data are standardized to a plasma ferritin level of 15 μg/L.

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 AM) and its increase from 8:00 AM to 5:00 PM significantly contributed to explaining Fe absorption, but PHep at the time of administration did not. Both of these observations are consistent with the concept that a PHep surge results in ferroportin degradation,[37] the re-synthesis of which would be inhibited in iron-deficient enterocytes.[11] The variation in the absorption data are 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 system,[11] HIF2α,[10,12,13] or H-ferritin–related intra-enterocyte functions.[38]

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 of 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.[39]

Consistent with previous reports in humans, we show that the PHep increase after acute oral iron doses parallels an increase in transferrin saturation,[15,19] which is followed by a transient increase in PF that then returns to baseline after 14 days.[40] 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,[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[6] that found only a nonsignificant decrease in iron absorption (13%) during daily supplementation with 50 mg Fe.[6] The reasons for this difference may be linked to dose, but may be more likely caused by greater inhibition immediately after beginning supplementation, because short-term dietary changes appear to induce stronger inhibitory or enhancing effects on iron absorption.[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.[41] A follow-up study to investigate longer-term alternate-day iron supplementation is currently being planned in our laboratory.

The strengths of this study include: (1) we tested a wide range of iron doses from 40 to 240 mg Fe; (2) we studied young women, a target group for iron supplementation; (3) each subject acted as her own control for the iron absorption measurements and PHep profiles; and (4) iron absorption and PHep profiles were accurately quantified by using stable iron isotope techniques and a c-ELISA with high sensitivity.[30] Limitations of our study include: (1) we tested relatively small numbers of subjects because of the logistics and expense of using stable iron isotopes; (2) our studies were limited to a supplementation phase of 2 days; and (3) we did not study subjects with anemia, who

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

	Dependent variable												
	Log absorption (%), n = 98						Log absolute absorption mg, n = 98						
	Independent variables, standardized β						Independent variables, standardized β						
Model R^{2*}	Time	Dose (mg)	BIS mg/kg BW	Log PHep nM	BIS start mg/kg BW	Log PHep, start nM	Model R^2	Time	Dose (mg)	BIS mg/kg BW	Log PHep nM	BIS start mg/kg BW	Log PHep, start nM
0.689	−0.146†	−0.352‡	−0.463‡	−0.206§	—	—	0.659‖	−0.179§	0.656‡	−0.337‡	−0.222§	—	—
0.666	−0.165†	−0.371‡	−0.593‡	—	—	—	0.633¶	−0.199§	0.636‡	−0.477‡	—	—	—
0.579	−0.228§	−0.397†	—	−0.486†	—	—	0.601#	−0.238§	0.623‡	—	−0.426‡	—	—
0.604	−0.367‡	−0.319‡	—	—	−0.538‡	—	0.629¶	−0.346‡	0.679‡	—	—	−0.488‡	—
0.520	−0.367‡	−0.529‡	—	—	—	−0.423‡	0.599#	−0.341‡	0.516‡	—	—	—	−0.363‡
0.378	−0.385‡	−0.514‡	—	—	—	—	0.446**	−.376‡	0.521‡	—	—	—	—

BIS, body iron stores; BW, body weight; PHep, plasma hepcidin.

Differences between nested models were tested with change in F statistic; differences between non-nested models were tested by comparing different R coefficients with the Steiger Z test. Reported models differ significantly if superscript differs, $P < .01$. All models are significant at $P < .01$. Differences between nested models were tested with change in F statistic; Differences between non-nested models were tested by comparing different R coefficients with the Steiger Z test. Within one category of dependent variables, reported models differ significantly if superscript differs, $P < .01$.

*Different from other models of log absorption $P < .01$.

†Significant regression parameter $P < .05$.

‡Significant regression parameter $P < .001$.

§Significant regression parameter $P < .01$.

‖Different from other models $P < .01$.

¶Different from other models, $P < .01$.

#Different from other models, $P < .01$.

**Different from all models, $P < .01$.

Table 4. Prediction of iron absorption from the first and second dose of iron supplements, depending on the time point of PHep and BIS

	Dependent variable									
	First dose fractional absorption (%), n=40			Second dose fractional absorption, %, n=40						
	Independent variables, standardized β			Independent variables, standardized β						
Model R^2	Dose (mg)	BIS mg/kg BW	Log hepcidin (nM)	Model R^2	Dose (mg)	BIS mg/kg BW, preceding day	BIS at administration mg/kg BW	Log hepcidin (nM), preceding day	Log hepcidin (nM), at administration	Hepcidin increase on previous day
0.650*	−0.397†	−0.585‡	n.s.	0.791§	−0.480‡	—	−0.447‡	−0.330†	—	—
0.642*	−.394†	−0.628‡	—	0.780§	−0.463‡	−0.535‡	—	−0.535†	—	—
0.391‡	−0.509†	—	−0.376†	0.768§	−0.419‡	—	−0.461‡	—	—	−0.283†
0.248‡	−0.498†	—	—	0.727§	−0.365†	−0.590[2]	—	—	n.s.	—
				0.719§	−0.405‡	—	−0.565‡	—	n.s.	—
				0.706§	−0.407‡	−0.664‡	—	—	—	—
				0.557‖	−.550‡	—	—	−0.519‡	—	—
				0.476¶	−0.391†	—	—	—	−0.452†	—
				0.476¶	−0.463†	—	—	—	—	−0.421†

n.s., nonsignificant parameter in the model.

Differences between nested models were tested with change in F statistic; differences between non-nested models were tested by comparing different R coefficients with the Steiger Z test. Reported models differ significantly if superscript differs, $P < .01$.

All models are significant at $P < .01$.

*Different from models ‡, $P < .01$.

†Significant regression parameter $P < .05$.

‡Significant regression parameter $P < .001$.

§Different from all other models, $P < .01$.

‖Different from all other models; $P < .01$.

¶Different from all other models, $P < .01$.

may respond differently to iron supplementation than our iron-depleted, nonanemic 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 with 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.

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Authorship

Contribution: D.M., M.B.Z., D.W.S., and J.S.G. and conceived the studies and obtained funding; all authors contributed to the design of the trials; D.M., M.J., V.C., J.S.G., and M.B.Z. conducted the studies; D.M. analyzed the data and wrote the first draft of the manuscript; and all authors contributed to the final version of the manuscript.

Conflict-of-interest disclosure: D.W.S. and H.T. are employees of Radboud University and Medical Centre, which offers high-quality hepcidin measurements to the scientific, medical, and pharmaceutical community on a fee-for-service basis. The remaining authors declare no competing financial interests.

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