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Impact of Oral Iron Challenges on Circulating Non-Transferrin-Bound Iron in Healthy Guatemalan Males

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Key Words
Iron status · Serum iron · Non-transferrin-bound iron · Ferritin · Iron absorption · Guatemala

Abstract
Introduction: Oral iron as a supplement has been associated with adverse health consequences, especially in the context of young children with active malaria. A potential aggravating role of non-transferrin-bound iron (NTBI) has been proposed. ***Material and Methods:*** NTBI responses in both a fasting and post-oral iron dosing situation were related to serum iron concentration and ferritin status. Fasting and 1, 2, and 3 h postdose serum samples were obtained in conjunction with oral ferrous sulfate supplementation in aqueous solution of 0, 15, 30, 60, 120 and 240 mg Fe in a cohort of 8 healthy Guatemalan men over a 9-week metabolic protocol. Hemoglobin, serum ferritin, percent transferrin saturation, serum iron and NTBI were all measured. ***Results:*** Circulating levels of serum iron and NTBI increased in a graded fashion in response to oral iron, with the relative increment for NTBI slightly greater than that of iron. Detectable NTBI was occasionally measured in fasting specimens, more frequently in subjects with high ferritin status. Post-iron NTBI responses, by contrast, were higher in normal-ferritin subjects in absolute terms, and rose with increasing postabsorptive serum iron responses. ***Discussion:*** The appearance and response of circulating NTBI were consistent with recognized principles of iron regulation.

Introduction

Non-transferrin-bound iron (NTBI), first described in 1978 by Hershko et al. [1], is a self-explanatory term; it defines the fraction of serum iron not bound to transferrin in spite of the high affinity constant of the iron-transferrin complex [2]. NTBI is not a clearly defined chemical entity, consisting instead of iron aggregates and complexes with low molecular ligands or proteins, the concentrations of which may be in dynamic flux. Different approaches are used for NTBI determination and lead to different results when applied to aliquots of the same serum pool [3]. None of the methods thus far available have shown to be clearly superior to the rest. As one of the Cabantchik methods showed a very small standard deviation in a round robin test [3] and seemed to be most reproducible, it was chosen here.

NTBI has been described in situations of high transferrin saturation, as in hypotransferrinemic mice [4], in human serum after parenteral iron administration [5], in

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β-thalassemia [1, 6] and in patients with hereditary hemochromatosis [7]. However, NTBI has also been observed with only partially saturated transferrin [8] and after the intake of oral iron medication at different dosages [9, 10]. Several pathophysiological consequences have been attributed to high NTBI concentrations, such as increased risk of atherosclerosis [11, 12], neutrophil impairment [13], increased incidence of bacterial growth [14, 15] or transit of *Plasmodium falciparum* into cerebral tissue due to activated adhesion molecules in the vascular intima [16, 17], as well as impaired stability of platelet concentrates [18].

From this point of view, NTBI serves as a concept to integrate a not clearly defined mix of transient low-molecular iron complexes in the serum to a number of pathophysiological event chains. Many of these concepts are still controversial but take on relevance in the context of developing country settings and situations. Iron deficiency anemia is common in most low-income societies [19] and routine prophylactic administration of iron to young children and pregnant women is widely recommended [20]. Safety concerns from such iron exposures are valid, especially in the context of malaria-endemic regions [21, 22]. In this context, postabsorptive increments of NTBI are of particular interest. NTBI was shown to increase ICAM-1 activity in the vascular intima. ICAM-1 is suspected to support the transit of *P. falciparum* into the tissue and, thus, to lead to more severe clinical courses of the disease [16].

Stimulated by this contextual situation for developing countries and seeking variables to assess the safety of prophylactic iron supplementation, we have studied several aspects of NTBI in Guatemala. Our analysis for the present study applied a highly reproducible method of NTBI measurement. Among our aims was to explore whether one could establish a predictable, consistent and graded response of NTBI appearance by manipulating circulating iron with single-dose oral supplementation. Moreover, the short-term intraindividual and interindividual reproducibility of any NTBI responses with induced changes in serum iron, and any association with underlying iron status, were topical points of interest to us. We present here the experiences of 8 Guatemalan volunteers from whom we sampled blood, following oral iron supplementation at 6 graded dosage levels ranging from 0 to 240 mg. The results permit assessment of the intraindividual stability of serum ferritin in 25 samples taken over 9 weeks and provide the opportunity to advance our understanding of the queries of interest.

Subjects and Methods

Subjects

A group of 8 healthy Guatemalan males, aged between 23 and 54 years, were enrolled in the metabolic study. None had a history of chronic or acute disease or reported consumption of nutritional supplements during the previous 6 months. The Human Subjects Committee of the Center for Studies of Sensory Impairment, Aging and Metabolism, Guatemala City, approved the protocol. Each subject signed written informed consent after the nature, purpose, inconveniences, risks and benefits of the study had been explained, and each was compensated for their participation.

Study Design

The protocol of this 9-week longitudinal study called for administering a single dose of oral iron at weekly or biweekly intervals and collecting baseline and postdose samples for chemical analyses. The doses increased progressively from 0 (control) during the 1st week through 15, 30, 60 and 120 mg Fe for the 2nd through to the 5th week, and 240 mg in the 7th week. A commercial ferrous sulfate solution was diluted with enough water to reach each weekly concentration and a final one-time sample collection was conducted on the first day of the 9th week. A flow chart of the sampling points is given in figure 1.

On each experimental day, the first blood sample was drawn at 8.00 a.m. Circadian changes over the next 3 h were expected to show a net decreasing tendency and be small compared to day-to-day variations of 35–50 μg/dl [23] and particularly when compared to postabsorptive increments after the intake of oral iron preparations (fig. 2). Correspondingly, changes in serum iron over time without iron intake showed no consistent increments during this period.

Sample Collection and Transport Procedures

The patients arrived at the clinic in the morning, in a fasting state. Venous blood was sampled (Angiocath™; BD Medical, Sandy, Utah, USA) prior to administration of ferrous sulfate syrup (Fer-In-Sol®; Mead Johnson, Evansville, Ind., USA) and at 1, 2 and 3 h thereafter. On the supplementation days (the first day of each study week), the indwelling catheter was used to facilitate the hourly blood extractions. Against the extrapolation from the literature data for the time course of NTBI increments after ingestion of iron in a soluble form on an empty stomach [9, 10], the peak of NTBI concentrations seemed not to have been reached at the highest dose level (240 mg Fe). Extending the sampling period might have led to a slightly higher maximum. Approximately 5 ml of blood was required in each extraction for the assays. Blood was allowed to clot for about 10 min at room temperature, and then centrifuged at 10,000 rpm for 5 min. Serum aliquots were frozen at –20°C. No meals were allowed during the sampling period, but water was allowed ad libitum. No alcoholic beverages were permitted for at least 24 h before each study day and smoking was not allowed during the blood extraction days.

The samples were collected in Guatemala City. Since the relevant analysis facilities were not available, specialized, experienced labs in Europe were involved for the assay determinations, beyond hemoglobin, which was done locally. Serum was separated and the samples were frozen immediately and shipped to Innsbruck (Austria) and Utrecht (The Netherlands) on dry ice. The frozen-sample cold chain was maintained without interrup-

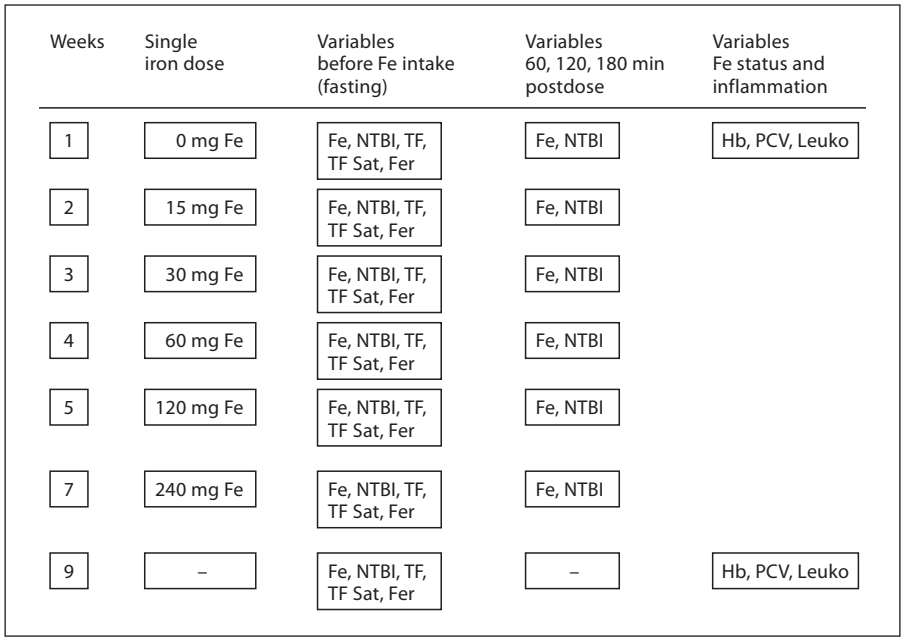

Fig. 1. Flow chart of sample collection. Fe = Serum iron; TF = transferrin; TF Sat = transferrin saturation; Fer = ferritin; Hb = hemoglobin; PCV = packed cell volume; Leuko = leukocytes.

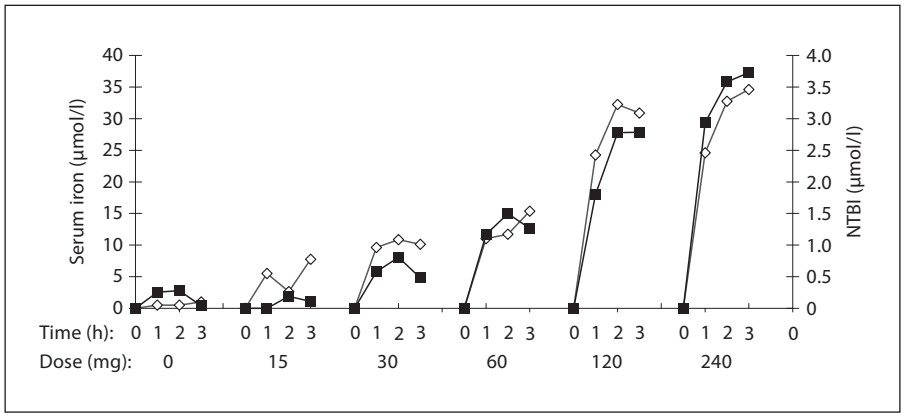

Fig. 2. Serum iron and NTBI responses over the 3 h following oral iron doses from 0 to 240 mg. The diamonds represent the mean of the changes with respect to the zero-time value for serum iron. The squares indicate the mean of the changes with respect to the zero-time value for NTBI. Indications of variance have been purposely omitted for visual clarity of the figure.

tion (the contract shipper, 'World Courier', guarantees adequate change of dry ice during transport, if necessary).

Laboratory Assays

Hemoglobin concentration, leukocytes and packed cell volume were determined at baseline and study end by use of a Coulter counter in the course of routine determinations in the Nuestra Señora de Pilar hospital in Guatemala City. Serum ferritin and transferrin concentrations were determined in the laboratory (laboratory pathway; Roche; P-unit) in the routine automated process in the central laboratory of the University Hospital in Innsbruck. Percent transferrin saturation (%TfSat) was calculated from plasma transferrin and serum iron concentrations.

The NTBI was determined using a fluorescence-based one-step assay [24]. The serum sample was mixed with reagent A, a HEPES-buffered saline containing 10 mM sodium oxalate (BDH Chemicals, Toronto, Ont., Canada), 0.1 mM gallium chloride (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 0.6 μM 5-(4,6-dichlorotriazinyl)-aminofluorescein (Molecular Probes, Eugene, Oreg., USA) – apotransferrin or reagent B, the same as reagent A but containing 25 μM apotransferrin (Kamada, Haifa, Israel). In reagent A, the accessible iron binds to the fluorescein-apotransferrin and quenches its fluorescence, whereas in reagent B the iron binds to the excess nonfluorescent apotransferrin rather than to fluorescein-apotransferrin, resulting in higher fluorescence. After 1 h of incubation, fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, Mass., USA). The ratio of the fluorescence readings (A/B) is inversely proportional to the concentration of NTBI in the original sample, in which the amount was determined from an Fe (III) nitrilotriacetate (Sigma-Aldrich, Zwijndrecht, The Netherlands) calibration curve, in a range of 0.4–25 μM. The arbitrary zero value [24] of the calibration curve was set to the highest value of A/B.

In addition to the white blood cell counts, IL-4 and TNF-α concentrations were quantified in the serum using the Quanti-

Table 1. Age, baseline BMI and serial hemoglobin and ferritin concentration in 8 healthy Guatemalan subjects

Subject	Age years	BMI	Hemoglobin, g/dl		Ferritin, μg/l	
			baseline	final	baseline	final
A	25	19.4	14.9	–	74	36
B	42	19.6	14.3	15.4	317	264
C	19	20.0	17.6	17.3	166	116
D	35	20.0	15.7	16.7	74	39
E	33	21.9	15.8	16.7	111	31
F	54	24.7	15.3	16.2	292	93
G	30	27.4	15.8	16.4	166	220
H	28	22.0	15.8	15.6	143	185
Mean	33	22.6	15.7	16.3	168	123
SD	11	3.4	1.0	0.7	92	91

No systematic changes in hemoglobin and serum ferritin concentrations were observed over the experimental period of 9 weeks.

kine® Human IL-4 Immunoassay and Quantikine® No. DTA00C assay, respectively (R&D Systems GmbH, No. D4050, Wiesbaden-Nordenstadt, Germany).

Data Handling and Statistical Analysis

Areas under the curve (AUC) were calculated using the triangle-trapezoid method for iron and NTBI [25–30]. For comparative purposes, in some instances, the 8 subjects were separated into two groups, one comprised of the B and F results and the other of the rest of the subjects. The objective was to compare the subjects with high basal iron indicators against subjects with normal values.

Descriptive statistics were computed for each variable using Excel version 5. Comparisons across 8 subjects and 6 iron dosages were tested using ANOVA and Student's t test. Pearson regressions among variables were calculated. A p value of <0.05 was considered to be statistically significant.

Results

Demographic and Descriptive Findings

The age, body composition and hematological characteristics of the 8 apparently healthy male volunteers are shown in table 1. Only 1 participant, subject G, had a BMI outside of the normal range; no subject was anemic (altitude-adjusted hemoglobin norms for the altitude of Guatemala City at 1,500 m above sea level [31]). Initial ferritin concentrations at baseline ranged from 74 to 317 μg/l.

All mean values for TNF-α were within the normal range (<20 pg/ml; mean ± SD: 2.4 ± 1.43 pg/ml). The median for IL-4 was extremely low in all subjects (mean ± SD: 0.001 ± 0.002 pg/ml; normal range: <13 pg/ml) and white blood cell count was marginally elevated above 10,000 mm^3 in only 1 subject (mean ± SD: 6,650 ± 1,800 cells/ml). These values assure that inflammation was low or nonexistent in these subjects and, thus, did not confound ferritin concentration as an indicator of iron status.

Table 2 provides the descriptive statistics data, organized by individual subjects, on NTBI, transferrin saturation and serum iron for all determinations when no oral iron was provided (n = 10). For serum ferritin, all specimens' values (n = 25) were grouped by individuals. We justify this because no short-term effect of iron intake would be expected; this biomarker of iron status showed high stability over repeated measurements. Subjects B and F, with median ferritins of 302 and 265 μg/l, were considered as having 'high' iron status. The remaining 6 subjects had normal ferritin concentrations. However, ANOVA analysis of the data in table 2 showed no significant differences among the mean subjects' values for any biomarker, including ferritin.

Table 3 shows the mean, standard deviation, median, and minimum and maximum concentrations for serum iron, grouped by iron dosing (including placebo) plus final blood sampling for all 8 volunteers. The same descriptive statistics are given for all ferritin concentrations grouped by iron dosage (basal and follow-up determinations). A far greater 'within-individual variance' than shown for ferritin across time is evident in the standard deviation values for serum iron. Means for %TfSat in first-morning fasting blood samples, collected through the eight collection opportunities in the protocol, ranged from a low of 24 ± 4% on the day of administering the 120-mg oral iron dose to a high of 40 ± 29% at final evaluation (table 3).

Response of Variables to Graded Dosages of Oral Iron

Figure 2 shows the serial collections of 4 serum samples over a 3-hour period, during which ascending dosages of oral iron from 0 to 240 mg were ingested as a single oral dose in a fasting state. The mean hourly increments of serum iron and of the correspondent NTBI over the baseline value on the corresponding sampling day are illustrated in a superimposed format. The μmol/l axis for serum iron is provided to the left and that for NTBI is shown to the right side of the panel. The data are presented without expression of interindividual variance for purposes of clarity in the illustration.

The expected, essentially flat, response in circulating levels to both analytes is seen in the control period, with no iron treatment. Thereafter, a dose-dependent progression in circulating iron species was observed with increasing dosages of iron. Three alternative formats have been

Table 2. Descriptive statistics for median, mean, SD, minimum and maximum values of 8 healthy Guatemalan subjects for fasting samples across the entire study, organized by individuals

	A	B	C	D	E	F	G	H
NTBI, μmol/l								
Median	0.00	0.34	0.00	0.00	0.00	0.34	0.18	0.15
Mean	0.36	0.48	0.49	0.03	0.08	0.53	0.26	0.25
SD	0.91	0.45	1.36	0.06	0.14	0.50	0.28	0.28
Minimum	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Maximum	2.92	1.29	4.34	0.19	0.36	1.67	0.87	0.71
n[1]	10	10	10	10	10	10	10	10
Transferrin saturation, %								
Median	15.0	37.5	33.0	19.0	27.0	27.5	27.0	27.5
Mean	20.4	34.8	37.0	19.3	25.4	36.8	28.1	29.2
SD	12.2	9.1	15.8	4.3	7.2	21.4	5.3	5.8
Minimum	8.0	21.0	19.0	11.0	13.0	21.0	23.0	23.0
Maximum	51.0	45.0	76.0	26.0	33.0	89.0	41.0	43.0
n[1]	10	10	10	10	10	10	10	10
Serum iron, μmol/l								
Median	14.5	18.0	21.0	14.5	21.5	17.0	19.0	18.5
Mean	17.5	18.1	23.7	14.7	19.0	23.1	19.8	19.3
SD	9.9	4.2	9.9	3.2	5.2	14.2	3.6	3.0
Minimum	7.0	12.0	11.0	9.0	10.0	14.0	16.0	15.0
Maximum	42.0	24.0	48.0	20.0	24.0	60.0	28.0	25.0
n[1]	10	10	10	10	10	10	10	10
Ferritin, μg/l								
Median	53	302	117	65	78	265	153	139
Mean	59	295	122	63	75	260	149	128
SD	18	43	31	12	19	49	29	30
Minimum	36	209	57	38	31	93	103	82
Maximum	110	360	175	91	111	334	220	185
n[2]	25	25	25	25	25	25	24	23

The ranges of nonpathological values are: NTBI: no universally accepted values available; serum iron: 4–24 μmol/l; transferrin saturation: 15–50%, and serum ferritin: 10–300 μg/l.

[1] This represents the number of samples collected after no iron had been ingested, i.e. at the 4 time points of the first (0 mg) test, the baselines at each of the following 5 dosage levels, and at the final 9-week follow-up. Missing values exist in some instances.

[2] This represents the number of all samples for all times in which assays for ferritin were performed (6 doses × 4 collection periods, plus 1 sample taken 2 weeks after the last iron dose). Missing values exist in some instances.

calculated: (1) mean of maximal postiron increments; (2) mean of the sum of the three postiron increments, and (3) AUC using the triangle-trapezoid method. The three conventions of expression had intermethod correlation coefficients of the highest order ($r > 0.90$, data not shown). As an illustrative example, only quantitative data for the AUC are given (fig. 2). The stepwise progression of AUC for NTBI (in μmol·h units) was: 0.6 ± 0.8 (0 mg); 0.2 ± 1.5 (15 mg); 1.9 ± 2.1 (30 mg); 3.9 ± 3.4 (60 mg); 7.4 ± 4.2 (120 mg), and 10.2 ± 4.2 (240 mg). These dose-dependent data are expressed as mean AUC produced by hourly progressions of the 8 volunteers (data not shown).

After iron administration, the NTBI fraction of circulating iron increases disproportionately in relation to the overall circulating serum iron as the dose. To document this pattern of changes in iron status over NTBI response, values were categorized as either low or high serum iron. The slope for the regression across the 77 values in which the serum iron remained in a low range (>40 μmol/l) was shallow, $y = 0.09x - 1.53$ ($r = 0.57$, $p = 0.007$, $n = 77$). A steeper progression of NTBI with increasing serum iron was seen for 37 values equal to or in excess of 40 μmol/l: $y = 0.12x - 3.0$ ($r = 0.69$, $p = 0.01$, $n = 37$). In this instance the slope was 33% steeper than in the former (fig. 3).

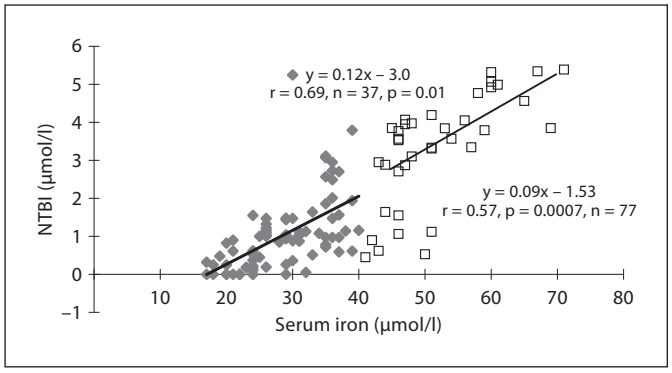

Fig. 3. Partial Pearson regressions between NTBI and serum iron, using the break-point criteria of <40 and >40 µmol/l. The diamonds represent the cross-coordinate points within the <40 µmol/l range of serum iron concentrations, with the partial regression shown as the thick line. The squares indicate the cross-coordinate points within the >40 µmol/l range of serum iron concentrations, with the partial regression shown as the thin line. The regression equation for the lower range has a shallower slope of 0.09, whereas it is steeper at 0.12 for the higher range.

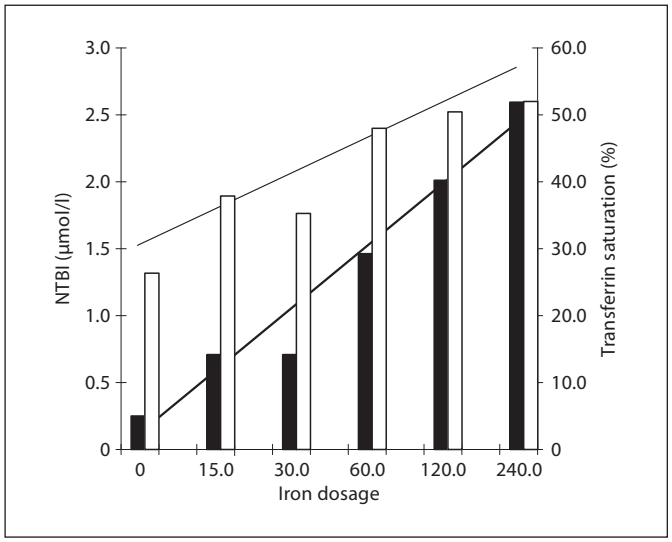

Fig. 4. Responses of NTBI and %TfSat at graded dosages of oral iron from 0 to 240 mg. The black bars represent the mean changes in NTBI and the white bars indicate the mean changes in %TfSat. The thick regression line represents the linear tendency for NTBI with ascending dose and the thin regression line represents the serial tendency for %TfSat.

Table 3. Descriptive statistics for median, mean, SD, minimum and maximum values of %TfSat, serum iron and ferritin among 8 healthy Guatemalan subjects for fasting samples across the entire study, organized by dosage level

	Iron dose (mg)/week						
	0	15	30	60	120	240	final
Transferrin saturation, %							
Median	27	33	30	25	25	22	27
Mean	27	34	29	28	24	25	40
SD	9	8	7	10	4	15	29
Minimum	15	25	21	14	14	8	11
Maximum	43	45	41	43	29	59	89
n[1]	32	8	8	8	8	8	8
Serum iron, µmol/l							
Median	17.9	23.5	18.5	19.5	17.5	15.0	20.0
Mean	18.0	22.5	19.4	19.1	16.9	16.3	27.9
SD	3.5	4.0	4.5	4.7	3.6	8.2	19.5
Minimum	13.5	16.0	15.0	11.0	10.0	7.0	9.0
Maximum	22.5	28.0	28.0	26.0	21.0	34.0	60.0
n[1]	32	8	8	8	8	8	8
Ferritin, µg/l							
Median	154	146	114	116	90	103	116
Mean	168	164	145	144	123	116	136
SD	92	943	90	96	89	66	89
Minimum	62	55	42	53	33	40	36
Maximum	333	360	340	355	292	229	264
n[2]	32	32	32	30	32	31	7

The ranges of nonpathological values are: serum iron: 4–24 µmol/l; transferrin saturation: 15–50%; serum ferritin: 10–300 µg/l.

[1] This represents the number of samples collected after no iron had been ingested, i.e. at the 4 time points of the first (0 mg) test in the 8 subjects, the baselines at each of the following 5 dosage levels and at the final 9-week follow-up. Missing values exist in some instances.

[2] This represents the number of all samples for all times in which assays for ferritin were performed (8 persons × 4 samples taken at baseline, 1, 2, 3 h after iron ingestion). Missing values exist in some instances.

Association of Iron Status and Detectible NTBI Levels in the Fasting State

There were 4 fasting samples with the 0-mg iron dose, 5 at baseline for the remaining iron doses and a single sample at the end of the protocol. Along with 5 values determined before iron intake at 5 different concentrations, this provides a total of 10 fasting samples per subject. In general in the remaining no-iron samples, NTBI was not detectible in the fasting state.

We noted a tendency, however, towards detectable NTBI in fasting samples of those 2 subjects with high-ferritin status (subjects B and F). The four-by-four contingency table analysis showed the partition of NTBI-positive and NTBI-negative fasting samples in the 2 ferritin status groups. Of 20 fasting analyses for the

high-ferritin men, 8 (40%) had detectable NTBI as compared to 12 (20%) in the 60 samples from the remaining 6 volunteers (χ^2 = 4.68 with 1 degree of freedom, p = 0.015, contingency table not shown).

Interaction of Iron Status with Serum Iron and NTBI Responses

As part of normal homeostatic regulation, individuals with high body iron stores are expected to have lower fractional iron absorption than those with less body iron [32]. To examine the consistency of our present data with this principle, we compared the uptake of iron from the series of oral supplement dosing in the 2 high-ferritin subjects with that of the 6 normal individuals. The mean of the maximal changes in postdose serum iron for subjects B and F was 20 ± 1 μmol/l as compared to 48 ± 17 μmol/l in the remaining 6 volunteers (p = 0.04). With respect to NTBI concentrations, the respective comparison was 2 ± 1 versus 7 ± 2 μmol/l (p = 0.05).

Discussion

The physiology of iron absorption and disposition has been extensively studied [32–34], and keys to the genetic and functional bases of its regulation have been emerging in recent years [36]. A clearly more practical consideration necessary regarding the absorption of iron in supplemental forms is its concerning tendency to aggravate tropical infections, notably malaria, in persons in the high-risk age groups for iron deficiency. This derives from the adverse consequences of routine supplementation with 12.5 mg of iron and 50 ng of folic acid seen in young Tanzanian children living in a holoendemic malaria area of the Zanzibar Islands [21]. Part of the interim recommendations to protect malarial populations from iron-induced risk is to give oral iron supplements along with food and to provide iron-fortified complementary foods spread across the day. These measures are to avoid bolus exposure to iron on an empty stomach, a situation that putatively causes large surges in serum iron concentrations [35], associated with NTBI labile iron species coursing through the systemic circulation. There is an urgent need for human model systems to study the phenomenon called 'non-transferrin-bound iron' generation. On the one hand this would mimic the setting of oral iron supplementation, while on the other hand it would allow us to examine the many safety issues arising in the context of supplementing in areas of malaria endemicity, as well as of intracellular pathogens such as those of HIV, tuberculosis and leprosy.

Limitations and Strengths of the Applied Approach

Among the limitations of our study are the uncertainties regarding the exact chemical nature of NTBI and the diverging results obtained by NTBI determinations when using different methods. The NTBI assay used here was chosen as it provided the highest consistency in detecting putatively labile iron in comparative, analytic studies [3]. Eight subjects is a small number for definitive description of the interrelationship between iron-related variables, particularly when the group is heterogeneous in its iron status. This could be an argument for the existence of greater variance in the fully aggregated averages, but served our purpose in terms of allowing exploratory contrasts between 2 individuals with above average ferritin concentrations and the remaining subjects with normal concentrations. Subjects B and F were at the upper margin of normal ferritin values, if not beyond. Both subjects did not know this when they entered the study and were advised to have a health check-up. Hereditary hemochromatosis was not likely to be the underlying cause, as they were both non-Caucasian and both had significantly lower postabsorptive increments in serum iron when compared to their fellow volunteers with lower ferritin values. This argues for adequate homoeostatic regulation of intestinal iron absorption, which is not commonly seen with hereditary hemochromatosis. However, both these candidates consumed alcohol on a regular basis. With just those 2 high-iron-status individuals who were fortuitously included in the tests, we must be cautious when generalizing about the nature of putative iron status influences until a larger group of individuals with high ferritin levels can be enrolled and studied. Iron-adequate individuals were chosen to model postabsortive NTBI increments in those iron-adequate children who had gone through more severe clinical courses of malaria after iron supplementation in the Pemba trial. The postabsorptive behavior of serum iron and NTBI in iron-deficient individuals is the subject of a subsequent study.

Among the strengths of the current study is that the dose range started from that close to the content of home-fortification powders (15 mg) [37], rising to that found in western prenatal supplements (30 mg) [38], then to that currently recommended for developing countries (60 mg) [19, 20], on to the time-honored dosage for prophylaxis in pregnancy (120 mg) [39] and finally to a dose approaching that of intensive, therapeutic treatment for iron deficiency anemia (240 mg) [40]. Thus, commercial iron supplements of constant composition at realistic dose levels applied in the field were used to investigate the relationship between iron status variables and NTBI. Moreover, the

study design permitted intraindividual comparison of the consistency of iron status parameters over 9 weeks.

Relationship of Iron Status Parameters and Estimates of Iron Absorption

The study population was not anemic and serum ferritin concentrations remained in the normal range, though they showed considerable interindividual variation with 2 individuals at the upper limit of normality. There were 5 courses of iron intake plus 1 placebo group (6 courses in total) and blood was sampled at baseline as well as 1, 2, and 3 h after ingestion (4 blood samples/course). Including 1 final sample taken 2 weeks after the last iron dose, 25 blood samples were taken from each individual over a period of 9 weeks. The ranking of the 8 subjects remained constant during the whole trial, underlining the consistency of serum ferritin [and in the 10 blood samples taken without previous iron intake (4 placebo samples, plus 5 baseline samples on iron dosing days, plus 1 final sample 2 weeks after the last dose), also of serum iron] to define iron status in the context of developing country field trials.

The 'iron tolerance test', i.e. the assessment of intestinal iron absorption by determination of the AUC of increments in serum iron concentration [25–30], was used as a venerable index of iron absorption, which can provide valid relative iron absorption estimates without providing quantitative uptake estimation. The 3-hour sampling frame used here did not exploit the full time course of serum iron excursions to the return of the equilibrium state, which would extend to over 8 h [29]. However, it covers the peak rise in serum iron for iron-sulfate-based preparations, as was shown again recently [10]. The constant reciprocal relationship between the iron status as defined by serum ferritin and the estimates of intestinal iron absorption suggests the applied 'short course' of 3 h provided a useful estimate of intestinal iron absorption and did not distort the quantitative AUC comparisons across the applied iron dosages.

Association of Iron Status and Detectable NTBI Levels

Esposito et al. [6] propose that iron of both endogenous and exogenous origin contributes to the NTBI pool, with differential implications for health. NTBI seems to represent a multicomponent pool of unknown chemical nature in which iron may constantly change its form between monomeric, dimeric and oligomeric iron(III) species [41]. At the same time, it may be bound to abundantly available low-molecular ligands such as citrate or lactate, or bind nonspecifically to plasma proteins, such as albumin [41]. Therefore, chemical speciation of NTBI is an unresolved issue. However, the different kinetic behavior of the NTBI pool in response to increasing doses and in volunteers with an adequate or high-iron status invites one to speculate on its nature.

The relation of the NTBI response to the degree of saturation of circulating transferrin has been a matter of discussion and speculation in the literature [42]. Earlier work proposed the NTBI would appear when the iron-binding capacity of transferrin is exceeded or at least highly saturated [42]. NTBI, however, was also seen in patients with normal transferrin saturation [8]. For some authors, the concept of NTBI being the consequence of an 'overflow' from its primary transport protein in the serum, transferrin, has seemed logical. Since %TfSat was measured in all samples, our present experiences can cast some insight into that discussion.

Postabsorptive NTBI concentrations as determined after the oral intake of highly bioavailable iron preparations have been observed before [9, 10]. The apparent response kinetics from those experiences showed comparable increases reaching their maximum between 2 and 4 h, depending on the galenic form (liquid, capsule or retarded release form [10]), or on whether the iron was ingested with or without food [9]. So, with such variation among studies to date, it is not unusual that our peaking of NTBI concentration is at variance with other findings. However, our data confirm some earlier observations of NTBI in nonhemochromatotic, normal subjects, i.e. with transferrin saturation levels below 60%. It was postulated by Sahlstedt et al. [42] that NTBI release is a 'threshold-driven' phenomenon, in which circulating NTBI would appear only after the transferrin iron binding had been exceeded. With the good fortune of having measured %TfSat in all samples in this study, we could test this hypothesis. If anything, the relationship between NTBI and %TfSat across our samples appeared to be roughly linear in nature (data not shown), with the correlation coefficient for the Pearson regression reaching 0.83. This provides important evidence against NTBI appearance being determined by an 'overflow' after the circulating iron binding capacity has been saturated.

In figure 4, we provide additional post hoc analysis of our data for the mean NTBI change with each of the six dosage levels of iron and the corresponding mean %TfSat achieved in the 8 subjects at the same oral iron dose. The regression line for the serial values has been superimposed. It is again obvious that no sharp threshold of unsaturated transferrin constrains the appearance of NTBI in the circulation of our subjects.

NTBI concentrations increased slightly faster than total serum iron, particularly in the upper dose range, as shown by the steeper regression line of the correlation between both analytes at higher oral iron intake and higher serum iron levels (fig. 3). In the upper dose range a smaller fraction of newly absorbed iron seems to bind to transferrin and larger fractions of iron join the NTBI pool. Iron from highly bioavailable preparations may form complexes with low-molecular food ligands, such as citrate, EDTA or ascorbate [43]. Such complexes have markedly lower stability constants than the Fe-transferrin complex (e.g. Fe-citrate = 10^{-9}; Fe-transferrin = 10^{-20}) [44]. However, the time for transferrin to reach a thermodynamic equilibrium with low-molecular iron(III) species and their nonspecific complex partners may be considerable. Thus, the exchange of iron between Fe-citrate or Fe-EDTA complexes and transferrin was estimated to take approximately 8 and 48 h, respectively [45, 46], to reach equilibrium. This could generate the transient pool of NTBI as observed during the first hours after iron ingestion (NTBI was gone after 7 days); unfortunately, we determined no data at shorter intervals.

Impact of Iron Status and Iron Absorption Rates on Serum Iron and NTBI Levels

Intestinal iron absorption in the high-ferritin-status volunteers B and F was lower and, consequently, their serum iron concentration increased much less after iron ingestion. However, measurable NTBI concentrations in the fasting state were significantly more frequent in these 2 individuals than in those with less replete iron stores. Absence of anemia and high serum ferritin values, which are not related to obvious signs of inflammation, indicate adequacy of iron stores and saturation of the functional iron pool [47]. In this situation the erythropoietic bone marrow expresses less transferrin receptor and takes up iron less rapidly [48]. Thus, a permanent low level of NTBI can be conceptually related to a decreased erythropoietic iron demand. Table 2 shows that particularly the maximum values for transferrin saturation are much higher in subjects B and F than in the other volunteers with lower ferritin values, which reconciles with this hypothesis. Thus, the NTBI observed without iron ingestion seems to be related to a decreased drain of transferrin-bound iron to the erythropoietic bone marrow and/or other functional and storage compartments, which has also induced the high observed serum ferritin values. The high transferrin saturation may be consistent with either reduced iron binding from the NTBI pool to transferrin, or increased NTBI and transferrin saturation, which may both derive from saturation of the 'mopping-up capacity' of functional and storage compartments for circulating iron of all kinds. This finding needs further investigation.

Conclusions

Plausible findings for NTBI status in relation to iron stores and responses to graded doses of oral iron were obtained in this small but exhaustively studied group of volunteers. High-ferritin-status individuals had more commonly circulating NTBI in the fasting state. Oral iron produces NTBI responses slightly disproportionate to the corresponding rises in serum iron, and NTBI appears well before the advanced saturation of circulating transferrin. Both these findings have plausibility and provide validity and credibility for the notion that incompletely bound iron may enter the circulation of malaria-infected and iron-supplemented infants and toddlers.

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Disclosure Statement

The authors declare no conflicts of interest of competing financial interests.

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