Title

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Publication Date

2016-11-29

Data Availability

The data associated with this publication are available upon request.

Peer reviewed

Effect of iron supplementation during lactation on maternal iron status and oxidative stress: a randomized controlled trial.

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Abstract

We examined the effect of iron-containing prenatal vitamin-mineral supplements (PNV) taken postpartum on biomarkers of iron status and oxidative stress. Lactating women (n=114) were randomly assigned to consume daily one iron-free PNV plus either 27 mg of iron or placebo for approximately 3.5 mo. The placebo group took the tablets between meals, while those given iron took the tablets either with (Fe-W) or between meals (Fe-B). Blood and urine samples were collected before and after the supplementation period to analyze hemoglobin (Hb), ferritin, hepcidin, transferrin saturation (TfSat), total plasma iron, and biomarkers of oxidative stress (isoprostane and 8-hydroxy-2-deoxyguanosine (8-OHdG)) and inflammation (C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP)). There was a trend toward a greater change in Hb among women in the Fe-B group compared to placebo (+2.5 vs. -3.7 g/L, respectively, *P*=0.063). When the iron groups were combined, there was a greater change in Hb (+1.4 g/L) compared to placebo (*P*=0.010). There were trends toward greater changes in TfSat (*P*=0.087) and total plasma iron (*P*=0.065) in the iron groups compared to placebo, yet no significant differences between the 3 groups in change in hepcidin (*P*=0.291), isoprostane (*P*=0.319) or 8-OHdG

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24(*P*=0.659), nor in change in ferritin among those with elevated CRP at baseline (60% of women;
25*P*=0.946); among those without elevated CRP (40% of women), ferritin increased more in the
26iron groups compared to placebo (*P*=0.001). Iron consumption during lactation moderately
27increased iron status, particularly among women without elevated CRP, and increased Hb, but
28did not significantly increase oxidative stress.

29Keywords: Iron, oxidative stress, inflammation, postpartum, breastfeeding, lactation

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31**Introduction**

32Both the Centers for Disease Control and Prevention and World Health Organization recommend
33iron supplementation during pregnancy to prevent iron-deficiency anemia in both mother and
34child (Prevention 1998, Moran et al. 2007). The Recommended Dietary Allowance (RDA) for
35iron is 27 mg/d during pregnancy and 18 mg/d for non-pregnant, non-lactating women (Trumbo
36et al. 2001). By contrast, the RDA for iron in lactation is only 9 mg/d because of the expectation
37that there will be no menstrual losses during the first 6 mo postpartum, and the iron accumulated
38during prenatal formation of maternal red blood cells can be recycled and used by the mother
39postpartum (Milman 2011). As a result, universal iron supplementation is generally not necessary
40for healthy lactating women. Despite this, many women are advised to continue taking prenatal
41vitamin-mineral supplements (usually containing at least 30 mg iron daily) for the duration of
42lactation. Data from the third National Health and Nutrition Examination Survey (NHANES)
43showed that, on average, lactating women took about 30 mg iron from supplements in addition to
44their daily dietary iron intake (~ 16 mg/d) (Heimbach 2001) – the combination of which exceeds
45the Tolerable Upper Intake Level (UL) of 45 mg/d specified for pregnant and lactating women.

46For women who have become iron deficient during pregnancy or experienced substantial blood
47loss during childbirth, continuation of iron supplements postpartum may be beneficial. However,
48for those who have adequate iron reserves after childbirth, iron supplements could pose some
49risk. Iron is able to exist in multiple valency states, which allows it to donate and accept
50electrons. Via the Fenton reaction, iron can donate an electron to hydrogen peroxide, forming a
51hydroxyl radical (Gutteridge 1986, Gutteridge et al. 1985, Burkitt and Mason 1991). The
52hydroxyl radical can initiate oxidation of various cellular components, including lipids and DNA,
53which can lead to cellular and tissue damage (Dargel 1992, Cooke et al. 2003). Reactive oxygen
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54species have been linked to hepatotoxicity (Videla et al. 2003, Uchiyama et al. 2008, Jaeschke et
55al. 2002), some types of cancer (Poulsen et al. 1998, Valavanidis et al. 2009), metabolic
56syndrome (Holvoet et al. 2008, Leiva et al. 2013), subsequent atherosclerosis (Hulsmans and
57Holvoet 2010, Holvoet 2004, Holvoet et al. 2004), and even periodontitis (Itabe 2012), although
58it is often difficult to discern whether reactive oxygen species are a cause or effect of such
59pathologies.

60Risks of excess iron consumption may be exacerbated by consuming iron-containing
61supplements between meals as is commonly advised to increase absorption. Plasma iron
62concentration peaks at a higher level when consumed between-meals compared to with-food
63(Kamp et al. 2003), which may increase the amount of unbound iron in circulation that is able to
64participate in oxidation reactions.

65The aim of this trial was to investigate the effects of daily consumption of prenatal vitamins with
66or without iron during lactation on hemoglobin (Hb), iron status, and two markers of oxidative
67stress. In addition to addressing the hypothesis that women supplemented with iron will have
68higher concentrations of Hb, iron status, and oxidative stress compared to those given placebo,
69we explored the hypothesis that those who consume iron between meals will have higher
70markers of oxidative stress than those who consume iron with meals.

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71**Methods**

72This study was a randomized, partially blind intervention trial of lactating women in Sacramento,
73California. Women less than 2 wk postpartum were recruited from the labor and delivery ward
74and the pediatric outpatient clinics at UC Davis Medical Center (UCDMC) from October 2008
75until April 2010. Women included in the study were at least 18 years of age, planned to return to
76UCDMC for future health care, consumed iron-containing prenatal vitamin mineral supplements
77(PNV) for at least 3 mo and 4 d/wk during pregnancy, and planned to breastfeed for at least 3
78mo. Women whose Hb concentration was less than 110 g/L were excluded from the study.

79Women were randomly assigned in blocks of 12 to 1 of 3 intervention groups by selecting a
80sealed envelope produced by study coordinators that contained within it the intervention group.
81Blocking was purely random and not based on any participant information, only to ensure equal
82sample sizes across the intervention groups over time. Women were assigned to a daily oral
83dose of 1 of 3 regimens for approximately 3.5 months: 1) PNV without iron (procured from
84GNC, Pittsburgh, PA) and 27 mg of iron as iron sulfate (procured from Rite Aid, Harrisburg, PA)
85consumed with meals (Fe-W); 2) PNV without iron and 27 mg of iron as iron sulfate consumed
86between meals (Fe-B); 3) PNV without iron and placebo (Placebo) consumed between meals.
87The PNV without iron and iron supplements were produced commercially and are safe for
88human consumption. The iron tablets were ground down and repacked by Investigational Drug
89Services (IDS) at UCDMC into capsules that were identical in size and appearance to the
90placebo capsules (methylcellulose powder) that were also produced by IDS. Women in the Fe-W
91group were instructed to consume the capsules with dinner, while women in the Fe-B and
92Placebo groups were instructed to consume the capsules at bedtime at least 2 h after consuming

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93any food. All participants agreed to not consume other vitamin or mineral supplements while
94participating in the trial. The baseline study visit was scheduled at the time of the infant's 2
95week well-child physician visit, and the follow up visit was scheduled at the infant's 4 month
96well-child visit. All women gave written consent to participate in the study. The study was
97approved by the Human Subjects Review Committee at UC Davis.

98Study coordinators were considered "partially blind", as they did not know group assignment for
99those in the "between-meals" groups (Fe-B and Placebo) until after the code was broken.
100Research assistants were unaware of the lack of a "with-meals" placebo group, and thus were
101assumed to be completely blinded to group assignment. Participants were followed through a
102phone call conducted by study coordinators one week after enrollment in the study and monthly
103phone calls thereafter. Study coordinators inquired about compliance with supplement
104consumption, breastfeeding status, and general health during the preceding period.

105Blood and urine samples were collected at the baseline study visit and after approximately 3.5
106mo of intervention. Urine was collected at home by the mother on the morning of her scheduled
107study visit. Women were instructed to collect a complete bladder evacuation of the first urine of
108the morning into an 800 mL urine collection receptacle and then transfer 50 mL into a sterile
109urine collection cup, which was stored in the refrigerator until leaving for her study appointment.
110Venous blood was collected from the antecubital vein by licensed UCDMC phlebotomists into a
111potassium-EDTA tube (BD Vacutainer, Franklin Lakes, NJ) and a trace mineral-free
112polypropylene syringe (Sarstedt Monovette, NH4-heparin, Sarstedt Inc., Newton, NC). Before
113blood draws on study visit days, participants were instructed to refrain from consuming foods
114high in iron (based on a pre-specified list of such foods) and to not consume any food or drink
115for an hour before each study visit. Those who failed to follow urine collection and dietary

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instructions were asked to continue consuming their assigned intervention and return for a later study visit.

Urine samples were put on ice upon arrival at the study site and aliquotted within 2 h into cryovials containing 0.005% BHT in methanol and stored at -80°C until analysis. Urine samples were used to analyze 8-isoprostane, 8-hydroxy-2-deoxyguanosine (8-OHdG), and creatinine. Isoprostanes are products of peroxidation of polyunsaturated fatty acids and are considered the best marker of lipid peroxidation (Halliwell and Whiteman 2004, Vincent et al. 2007). 8-OHdG is the most commonly analyzed marker of oxidized DNA damage due to its relative abundance (Cooke et al. 2002) and relative ease of detection (Chiou et al. 2003). The potassium-EDTA blood tube was sent to UCDMC Pathology within 1 h for Hb analysis. The heparinized tube was put on ice until centrifugation within 2 h at 2,500 rpm for 10 min. Plasma was aliquotted into cryovials and stored at -20°C or -80°C until analysis. Plasma samples used to analyze total antioxidant status (TAS) were stored at -80°C until analysis. TAS is the measurement of the quantity of a given free radical scavenged by a test solution, and therefore measures the antioxidant activity of the sum of all antioxidants in a sample, rather than measuring one or a limited number of specific antioxidant components (Ghiselli et al. 2000). Plasma samples stored at -20°C were used to analyze plasma iron, transferrin saturation (TfSat), ferritin, hepcidin, C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP), aspartate transaminase (AST), and alanine transaminase (ALT). TfSat and ferritin are commonly used as markers of iron status, whereas hepcidin is a more recently discovered protein that functions as a key regulator of iron homeostasis (Collins et al. 2008). Hepcidin has been shown to increase with iron loading and decrease with iron deficiency (Darshan and Anderson 2009, Ganz 2011), making it an additional marker of whole-body iron status. AST and ALT are transaminases that increase with liver

damage, and have been shown in animal models to be associated with iron-induced oxidative stress (Asare et al. 2009).

Hb was measured as part of a complete blood count by UCDMC Pathology (Beckman Coulter UniCel DxC 800, Brea, CA). Total plasma iron, TfSat, AST, ALT, and urinary creatinine were measured by an Autoanalyzer at UCDMC Pathology (Beckman Coulter LH-785). CRP, AGP, and TAS were measured by an automated procedure (Cobas Integra Autoanalyzer, Indianapolis, IN). Ferritin was measured by Coat-A-Count IRMA (Siemens, Los Angeles, CA). Hepcidin was measured by EIA kit (Peninsula Laboratories, San Carlos, CA). Isoprostane and 8-OHdG were measured by EIA kits (Cayman Chemical, Ann Arbor, MI). Isoprostane, 8-OHdG, ferritin, hepcidin, CRP, and AGP were analyzed in duplicate and any sample with a high coefficient of variation (CV > 10%), both baseline and final samples were reanalyzed in the same batch. Both isoprostane and 8-OHdG had high between-plate (29.1% and 25.7%, respectively) and within-plate (11.7% and 10.7%, respectively) coefficients of variation, with multiple attempts required for several samples in the isoprostane analysis to fall within the standard curve. To account for the large between-plate variation, baseline and final samples for each participant were analyzed on the same plate, and for any sample that had a CV > 10%, both baseline and final samples were reanalyzed on the same plate.

Iron intake was assessed at the baseline and final study visits by asking the frequency of consumption of iron-rich foods. The questionnaire was based on a validated food frequency questionnaire, from which the same response options and iron-rich food categories were used (Block et al. 1990).

Statistical methods:

We determined a sample size of 32 per group for each of the 3 groups, based on the ability to detect a difference of ≥ 7.2 g/L in Hb concentration (alpha = 0.05; 80% power; assuming a standard deviation of 10 g/L), similar to the difference found between iron and placebo groups of pregnant women (Hoa et al. 2005). The number enrolled was increased by 10% to allow for attrition, bringing the needed sample size to 35 per group, or a total of 105 enrolled. Since randomization was done in blocks of 12, the total enrollment goal was increased to 108, or 36 per group to attain a multiple of 12.

Data were tested for normality of distribution using the Shapiro-Wilk test. Ferritin, hepcidin, TfSat, plasma iron, CRP, AGP, AST, ALT, TAS, isoprostane, and 8-OHdG were found to be non-normally distributed and were log transformed before statistical analyses. Treatment effects were examined by using intention-to-treat analysis. The change from baseline to final study visit for each continuous outcome variable was analyzed by analysis of covariance (ANCOVA), with the main effect being treatment group and controlling for baseline status of each variable and chosen covariates. In randomized studies the ANCOVA method is generally recommended for being unaffected by correlation between baseline and endline measurements, being consistent regardless of chance baseline measurement group imbalances, and generally having more power to detect group differences than repeated measures approaches (Vickers and Altman 2001; Van Breukelen 2006). For the ANCOVA method with the baseline measurement as a covariate, modelling the final measurement and the change in measurements is equivalent. The covariates included in the ANCOVA models have been shown in prior work to influence the outcome and upon bivariate analysis were significantly associated ($p<0.10$) with the specific outcome of interest in that model. Differences in outcome variables were examined between each of the three groups, as well as between the placebo group and the combined iron groups. .

To examine effect modification, two-way interactions between group assignment and both ferritin and hepcidin at baseline were separately included in the ANCOVA model for change in isoprostane and 8-OHdG. Similarly, two-way interactions between group assignment and baseline CRP, AGP, and BMI were separately included in the ANCOVA model for change in ferritin, TfSat, total plasma iron, and hepcidin.

For all analyses, when the overall model was significant ($P<0.05$), groups were compared by using Tukey post hoc multicomparison test for ANOVA or with Bonferroni correction for ANCOVA. Logistic regression was used to determine the group-wise differences in proportion of participants that had values above or below chosen cut-offs at the final study visit after controlling for the baseline category (low or high) and covariates chosen as described above. Statistical analysis was carried out with the SAS 9.2 and 9.3 (SAS Institute Inc., Cary, NC) software packages.

Results

In total, 514 women were approached for recruitment into the study (**Figure 1**), Of the 174 women who were interested in participating at the time of recruitment, 57 could not be reached subsequently for the enrollment visit; thus Hb was assessed in 117 participants. Of those, 3 had Hb levels less than 110 g/L and were referred for care and excluded from the study.

Baseline demographic characteristics of all women who completed follow up in each of the intervention groups are displayed in **Table 1**. More women were lost to follow-up in the iron groups than in the placebo group (Figure 1; $P=0.048$ for the overall 3-group model; $P=0.014$ for Fe-B vs. Placebo; $P=0.047$ for Fe-W vs. Placebo; $P=0.017$ for the combined iron groups vs. Placebo), therefore, mean baseline demographic and laboratory values were compared between

women who completed follow up and those who were lost to follow up, and no significant differences were found (P>0.20 for all). Of those who completed the final study visit (Placebo: n=39; Fe-B: n=27; Fe-W: n=29), 2 women in the placebo group, and 3 in each of the Fe-B and Fe-W groups did not continue supplement consumption until the final study visit (*P*=0.640; **Figure 1**). Among women who continued supplementation until the final study visit, there were no significant differences among groups in the mean number of days women were enrolled in the study (Placebo: 107.4 d; Fe-B: 104.8; Fe-W: 109.9; *P*=0.166) or the mean number of reported days supplements were consumed (Placebo=98.3 d; Fe-B=96.9 d; Fe-W=99.4 d; *P*=0.763). Among all women who completed the final study visit, there were no differences in the frequency of iron-rich food consumption according to the modified food frequency questionnaire (Placebo=27.5 response points; Fe-B=27.1 response points; Fe-W=27.2 response points; *P*=0.951), percentage of women who continued to breastfeed throughout the intervention period (Placebo=89.3%, Fe-B=87.2%, Fe-W=83.3%; *P*=0.794), or percentage of women who resumed menstruation by the final study visit (Placebo=44.4%, Fe-B=52.6%, Fe-W=46.7%; *P*=0.788). There were no differences in the mean blood draw times between groups for either the baseline (11:50 AM; *P*=0.383) or final (11:57 AM; *P*=0.589) blood draws.

The mean (SD) changes from baseline to the final study visit in Hb, TfSat, total plasma iron, 8-OHdG, isoprostane, TAS, CRP, AGP, AST, and ALT are shown in **Table 2**. Hb increased in the Fe-B (+2.5 g/L) and Fe-W (+0.4 g/L) groups, and decreased in the placebo group (-3.7 g/L; adjusted *P*=0.034 for the overall model; Placebo vs. Fe-B, *P*=0.063; Placebo vs. Fe-W, *P*=0.114; adjusted *P*=0.010 for Placebo vs. the combined iron groups). There were marginally significant changes in ferritin between the baseline and final visits among the 3 intervention groups (*P*=0.091). Compared to the placebo group, the change in ferritin was marginally significantly

229greater in the Fe-W group (*P*=0.087). There were trends toward a greater decline in ferritin in 230the placebo group and compared to the combined iron groups (-9.2 ng/mL vs. -3.3 ng/mL; 231adjusted *P*=0.056). There were no differences between groups in change in hepcidin, TfSat, total 232plasma iron, 8-OHdG, isoprostane, TAS, CRP, AGP, AST, or ALT.

233In examining the differences between groups in change in ferritin, an interaction was found 234between treatment group and baseline CRP (*P*=0.028) when the iron groups were combined (2-235group analysis) (**Table 3**). Among those with CRP ≤ 5 mg/L (n=38), ferritin increased 236significantly more in the combined iron groups compared to placebo (*P*=0.001), while there were 237no differences between groups in change in ferritin among those with elevated CRP (> 5 mg/L; 238n=57). There were no other significant interactions.

239The percentages of women who had ferritin, hepcidin, or Hb below chosen cut-offs, or 8-OHdG, 240isoprostane, CRP or AGP above chosen cut-offs are shown in **Table 4**. At the final study visit, 241there was significant difference in the percentage of 8-OHdG greater than 1 SD above the mean 242among the 3 intervention groups (13% vs 11% vs 31%; adjusted *P*=0.043). There was a trend 243toward a greater percentage of women with higher 8OHdG in the Fe-W group compared to the 244Fe-B group (31% vs 11%; adjusted *P*=0.062 for Fe-W vs. Fe-B) and placebo group (31% vs 24513%; *P*=0.086 for Fe-W vs. Placebo). There were no differences among groups in the proportion 246of women with elevated isoprostane. At the final study visit there were no significant differences 247among groups in the percentage with ferritin < 15 ng/mL, which is a cut-off value commonly 248used to define iron deficiency. When the combined iron groups were compared to placebo, there 249was a trend toward a greater proportion of women with ferritin < 15 ng/mL in the placebo group 250(13% vs. 5%; *P*=0.070). When a cut-off of 30 ng/mL was used, a cut-off shown to have higher 251sensitivity (without compromising specificity) for detecting iron deficiency (Mast et al. 1998),

the percentage of women in the placebo group with low ferritin tended to be greater than in the Fe-B group (46% vs. 29% vs. 21%; overall adjusted P=0.019; Fe-B vs. Placebo, adjusted P=0.050). When the iron groups were combined, a greater proportion of women in the placebo group had ferritin < 30 ng/mL compared to the combined iron group (46% vs. 25%; P=0.006). After controlling for the baseline category, there were no significant differences in the proportion of women who had hepcidin below 8 ng/mL (P=0.997), which is the cut-off commonly used to detect iron deficiency. Similarly, there were no significant differences among groups when using a cut-off of 18 ng/mL (P=0.549), a value shown to score higher than 8 ng/mL on the Youden Index used to determine an optimal cut-off value (Pasricha et al. 2011, Fluss et al. 2005). There were no differences among groups in the percentage of women with low Hb or high CRP or AGP.

Discussion

To our knowledge, this is the first study to report the effects of oral iron supplementation on iron status, oxidative stress, and markers of inflammation in postpartum women. The results indicate that daily iron supplementation for approximately 3.5 mo at doses typically found in prenatal vitamins increased Hb and resulted in a lower percentage of women with a ferritin level indicative of iron deficiency. However, only one woman in the placebo group had a Hb value less than 120 g/L at the end of the intervention period, which indicates that the lower iron status among women in the placebo group was generally not severe enough to cause iron deficiency anemia. We did not find significant differences in the change from baseline in markers of iron status or oxidative stress between the group taking iron between meals and the group taking iron with meals; however, the proportion of women with elevated 8-OHdG tended to be greater in the Fe-W group than either the Fe-B or placebo groups.

Our primary hypothesis was that iron supplementation of lactating women would increase oxidative stress, as the excess iron consumed would cause free-radical oxidation via the Fenton reaction. Previous reports on the association between iron status and both 8-OHdG and isoprostane in humans are mixed. Cross-sectional studies have shown positive associations between iron status and these markers of oxidative stress (Nakano et al. 2003, Tuomainen et al. 2007, Hori et al. 2010, Crist et al. 2009, Signorini et al. 2008). However, intervention studies that have investigated the effect of iron supplementation on 8-OHdG and isoprostane in humans have yielded mixed results – either no change in 8-OHdG or isoprostane after relatively high-dose iron supplementation (Orozco et al. 2012, Braekke et al. 2007), or a two-fold increase in 8-OHdG and isoprostane after consuming 120 mg of iron for 7 days (Schumann et al. 2005). It is

important to note that the supplementation period of each of these studies was only 7 days. With such a short intervention period, it is possible that the antioxidant capacities in these individuals were not overwhelmed and were able to quench iron-induced free radicals before 8-OHdG or isoprostane levels were affected. However, in our study, we also found no difference among groups in the mean values of markers of oxidative stress between iron and placebo groups after 3.5 mo of supplementation. It is likely that the antioxidants in the PNVs consumed by the women (ascorbic acid, α-tocopherol, β-carotene, selenium), elevated the levels of TAS and prevented oxidative stress in all 3 groups. In fact, the levels of TAS at the end of the supplementation period in this study were much higher than levels reported elsewhere in healthy postpartum women who did not consume vitamin-mineral supplements (Hung et al. 2010, Schulpis et al. 2007, Schulpis et al. 2008, Zarban et al. 2009). We did, however, see a trend toward higher 8-OHdG in the Fe-W group compared to both Fe-B and placebo groups, which is contrary to our hypothesis – that those in the Fe-B group would have higher oxidative stress than either of the other two groups. Consuming iron between meals has been shown to be associated with elevated plasma iron (Kamp et al. 2003), which we hypothesized would be associated with an increase in oxidative stress, as plasma iron has been shown to be positively associated with isoprostane (Crist et al. 2013), thio-barbituric-acid-reacting-substances (TBARS) (Viteri et al. 2012), and lipid peroxidation (King et al. 2008). Conversely, consuming iron with meals has been shown to cause oxidation of dietary cholesterol and lipids (Lorrain et al. 2012), which once absorbed, become incorporated into chylomicrons and low-density lipoproteins (Staprans et al. 2003), leading to increased risk of cardiovascular disease (Staprans et al. 2005). To our knowledge, ours is the first study to find evidence that suggests that consuming iron supplements with food rather than between meals may increase systemic oxidative stress.

We also examined the effect of iron supplementation on iron status. While the finding of increased iron status among those who consumed iron was expected, a surprising number of studies have reported no differences in iron status between iron and placebo groups among lactating women (Baykan et al. 2006, Powers et al. 1985, Correia-Santos et al. 2011, Stuetz et al. 2012, Mello-Neto et al. 2012). The lack of increase in iron status in these studies, and the lack of significant change in TfSat and plasma iron in the current study may be explained by the dynamic state of iron status in postpartum women. During pregnancy, the requirement for dietary iron increases as maternal and fetal tissue synthesis increase. Much of the additional iron required is used to increase the mother's red cell mass. After giving birth, the red cells are broken down and the Hb iron contained within them is available for use or storage (Milman 2011). Even without iron supplements postpartum, an increase in Hb and serum ferritin from delivery up to 8 wk postpartum in women who consumed iron during pregnancy has been described (Milman 2006). It is likely that the iron stored in the expanded red cell mass in women in the placebo groups in these studies was sufficient to prevent significant declines in iron status during the intervention period relative to the iron groups.

Previous studies that have compared changes in iron status after consuming supplemental iron with or between meals are limited and have not been conducted with lactating women (Domellof et al. 2008, Cook and Reddy 1995, Kamp et al. 2003, Dawson et al. 1998, Dawson et al. 2000). Authors of all but one of the studies reported either increased ferritin or iron absorption when iron was consumed between meals compared to with meals. It is important to note that none of the studies, including the present study, had a group size greater than 35. Future studies would benefit from a larger sample size to determine if there are in fact differences in iron status after a lengthy intervention of iron supplementation with or between meals.

There were significant interactions between treatment group and baseline CRP with regard to the change in ferritin. For those without elevated CRP at the baseline visit (40% of those enrolled), the combined iron groups had a greater change in mean ferritin than the placebo group. However, among those with high CRP at baseline (60% of those enrolled), there was no discernible effect of iron supplementation on ferritin, suggesting that the acute phase reaction interfered with an effect of iron supplementation on iron status.

Greater proportions of women in both of the iron groups were lost to follow up than in the placebo group. The reasons for this are not known. However, among those who discontinued supplementation yet remained in the study, the main reason for discontinuing supplementation was gastrointestinal upset. Health care providers recommend continuation of PNVs postpartum because of the high requirements for many nutrients during lactation. The vast majority of PNVs on the market contain iron. Assuming our results are generalizable, gastrointestinal upset caused by iron-containing PNVs may be inhibiting some women from consuming PNVs.

Although this study was a randomized, placebo-controlled study, it was not without limitations. First, while the sample size was large enough to detect differences in Hb, it likely was not large enough to detect modest differences in other outcomes and between the two groups differing in the timing of iron supplementation (with vs. between meals). Secondly, participants were given relatively complicated instructions for in-home collection of urine samples, which could have led to variability in collection technique, timing, and storage. We attempted to limit the variability by asking details about the collection, and if instructions were not followed, the participant was asked to recollect and submit a sample on a later day. Diurnal variations in urinary markers of oxidative stress have been reported (Helmersson and Basu 1999, Kanabrocki et al. 2002, Kanabrocki et al. 2006), although no significant differences were found between markers

355collected from 24-h urine collections and complete bladder collections of the first urine of the
356morning (Miwa et al. 2004, Basu 2008). Third, for convenience of the new mothers, blood
357draws were scheduled one half hour before the child's doctor appointment. This meant that
358blood draws were non-fasting and varied as to the time of day samples were collected, although
359women were instructed to consume nothing but water for one hour before the blood draw, and to
360refrain from consuming iron-rich foods prior to the blood draw to prevent a postprandial spike in
361plasma iron. Those who consumed foods or beverages within the hour before the blood draw, or
362iron-rich foods on the day of the blood draw did not submit a blood sample and were asked to
363return at a later date for collection. Diurnal variation has been reported for plasma iron (Wiltink
364et al. 1973), hepcidin (Schaap et al. 2012), and TfSat (Wish 2006) but no diurnal variation has
365been shown in Hb or ferritin (Fleming et al. 2001). Moreover, there were no differences among
366groups in the average time of day of sample collection. Fourth, women who consumed iron
367between meals were instructed to do so at least 2 hours after dinner. It is possible that there was
368still food present in the stomach, particularly if the meal was a large one. This may have
369minimized the differences between the with- and between-meals groups. Fifth, the kits used for
370the isoprostane and 8-OHdG analyses had high between-plate (29.1% and 25.7%, respectively)
371and within plate (11.7% and 10.7%) coefficients of variation, with multiple attempts required for
372several samples in the isoprostane analysis to fall within the standard curve. To account for the
373large between-plate variation, baseline and final samples for each participant were analyzed on
374the same plate. Future analyses may benefit from using alternate methods of analyzing
375isoprostane and 8-OHdG.

376In conclusion, among this population of postpartum women, iron supplementation led to a
377moderate increase in iron status, particularly among women without elevated CRP, yet no

difference in markers of oxidative stress between intervention groups. These data suggest that the current practice of recommending that mothers continue to consume iron-containing PNV while lactating likely outweigh the potential harms of increased oxidative stress. More research is needed to examine whether consuming iron between meals is safer than consuming iron with meals in order to prevent oxidation of dietary lipids and subsequent oxidation of endogenous lipoproteins. Future intervention studies should also consider inflammatory status when examining the effect of iron supplementation on iron status among postpartum women. Additional research with larger sample sizes is needed to evaluate the effect of consuming iron supplements with or between meals on iron status, markers of oxidative stress and inflammation in postpartum women.

Key messages:

Breastfeeding women who consumed iron supplements from 2 to 17 weeks postpartum had a greater increase in Hb compared to women who consumed placebo, yet only one woman in the placebo group was mildly anemic at the end of the study (Hb < 120 g/L).

There were no differences in Hb or markers of iron status between women who consumed iron with vs. between meals.

Women who consumed iron with meals tended to have higher urinary 8-OHdG compared to women who consumed iron between meals or placebo.

The benefits of consuming iron-containing prenatal vitamin-mineral supplements while breastfeeding outweighed the potential harm of oxidative stress in this study population.

References:

Asare, G. A., Kew, M. C., Mossanda, K. S., Paterson, A. C., Siziba, K. and Kahler-Venter, C. P. (2009) 'Effects of exogenous antioxidants on dietary iron overload', *J Clin Biochem Nutr,* 44(1), 85-94.

Basu, S. (2008) 'F2-isoprostanes in human health and diseases: from molecular mechanisms to clinical implications', *Antioxid Redox Signal,* 10(8), 1405-34.

Baykan, A., Yalcin, S. S. and Yurdakok, K. (2006) 'Does maternal iron supplementation during the lactation period affect iron status of exclusively breast-fed infants?', *Turk J Pediatr,* 48(4), 301-7.

Block, G., Woods, M., Potosky, A. and Clifford, C. (1990) 'Validation of a self-administered diet history questionnaire using multiple diet records', *J Clin Epidemiol,* 43(12), 1327-35.

Braekke, K., Bechensteen, A. G., Halvorsen, B. L., Blomhoff, R., Haaland, K. and Staff, A. C. (2007) 'Oxidative stress markers and antioxidant status after oral iron supplementation to very low birth weight infants', *J Pediatr,* 151(1), 23-8.

Burkitt, M. J. and Mason, R. P. (1991) 'Direct evidence for in vivo hydroxyl-radical generation in experimental iron overload: an ESR spin-trapping investigation', *Proc Natl Acad Sci U S A,* 88(19), 8440-4.

Chiou, C. C., Chang, P. Y., Chan, E. C., Wu, T. L., Tsao, K. C. and Wu, J. T. (2003) 'Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers', *Clin Chim Acta,* 334(1-2), 87-94.

Collins, J. F., Wessling-Resnick, M. and Knutson, M. D. (2008) 'Hepcidin regulation of iron transport', *J Nutr,* 138(11), 2284-8.

Cook, J. D. and Reddy, M. B. (1995) 'Efficacy of weekly compared with daily iron supplementation', *Am J Clin Nutr,* 62(1), 117-20.

Cooke, M. S., Evans, M. D., Dizdaroglu, M. and Lunec, J. (2003) 'Oxidative DNA damage: mechanisms, mutation, and disease', *FASEB J,* 17(10), 1195-214.

Cooke, M. S., Lunec, J. and Evans, M. D. (2002) 'Progress in the analysis of urinary oxidative DNA damage', *Free Radic Biol Med,* 33(12), 1601-14.

Correia-Santos, A. M., Bolognini Pereira, K., Erthal Santelli, R., Teles Boaventura, G. and Blondet de Azeredo, V. (2011) 'Dietary supplements for the lactating adolescent mother: influence on plasma micronutrients', *Nutr Hosp,* 26(2), 392-8.

Crist, B. L., Alekel, D. L., Ritland, L. M., Hanson, L. N., Genschel, U. and Reddy, M. B. (2009) 'Association of oxidative stress, iron, and centralized fat mass in healthy postmenopausal women', *J Womens Health (Larchmt)*, 18(6), 795-801.

Crist, M. B., Melekhin, V. V., Bian, A., Shintani, A., Milne, G. L., Kallianpur, A. R., Dageforde, L. A., Haas, D. W. and Hulgan, T. (2013) 'Higher serum iron is associated with increased oxidant stress in HIV-infected men', *J Acquir Immune Defic Syndr*, 64(4), 367-73.

Dargel, R. (1992) 'Lipid peroxidation--a common pathogenetic mechanism?', *Exp Toxicol Pathol*, 44(4), 169-81.

Darshan, D. and Anderson, G. J. (2009) 'Interacting signals in the control of hepcidin expression', *Biometals*, 22(1), 77-87.

Dawson, E. B., Dawson, R., Behrens, J., DeVora, M. A. and McGanity, W. J. (1998) 'Iron in prenatal multivitamin/multimineral supplements. Bioavailability', *J Reprod Med*, 43(2), 133-40.

Dawson, E. B., Evans, D. R., McGanity, W. J., Conway, M. E., Harrison, D. D. and Torres-Cantu, F. M. (2000) 'Bioavailability of iron in two prenatal multivitamin/multimineral supplements', *J Reprod Med*, 45(5), 403-9.

Domellof, M., Lind, T., Lonnerdal, B., Persson, L. A., Dewey, K. G. and Hernell, O. (2008) 'Effects of mode of oral iron administration on serum ferritin and haemoglobin in infants', *Acta Paediatr*, 97(8), 1055-60.

Fleming, D. J., Jacques, P. F., Tucker, K. L., Massaro, J. M., D'Agostino, R. B., Sr., Wilson, P. W. and Wood, R. J. (2001) 'Iron status of the free-living, elderly Framingham Heart Study cohort: an iron-replete population with a high prevalence of elevated iron stores', *Am J Clin Nutr*, 73(3), 638-46.

Fluss, R., Faraggi, D. and Reiser, B. (2005) 'Estimation of the Youden Index and its associated cutoff point', *Biom J*, 47(4), 458-72.

Ganz, T. (2011) 'Hepcidin and iron regulation, 10 years later', *Blood*, 117(17), 4425-33.

Ghiselli, A., Serafini, M., Natella, F. and Scaccini, C. (2000) 'Total antioxidant capacity as a tool to assess redox status: critical view and experimental data', *Free Radic Biol Med*, 29(11), 1106-14.

Gutteridge, J. M. (1986) 'Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides', *FEBS Lett*, 201(2), 291-5.

Gutteridge, J. M., Rowley, D. A., Griffiths, E. and Halliwell, B. (1985) 'Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis', *Clin Sci (Lond)*, 68(4), 463-7.

Halliwell, B. and Whiteman, M. (2004) 'Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?', *Br J Pharmacol,* 142(2), 231-55.

Heimbach, J. T. (2001) 'Using the national nutrition monitoring system to profile dietary supplement use', *J Nutr,* 131(4 Suppl), 1335S-8S.

Helmersson, J. and Basu, S. (1999) 'F2-isoprostane excretion rate and diurnal variation in human urine', *Prostaglandins Leukot Essent Fatty Acids,* 61(3), 203-5.

Hoa, P. T., Khan, N. C., van Beusekom, C., Gross, R., Conde, W. L. and Khoi, H. D. (2005) 'Milk fortified with iron or iron supplementation to improve nutritional status of pregnant women: an intervention trial from rural Vietnam', *Food Nutr Bull,* 26(1), 32-8.

Holvoet, P. (2004) 'Oxidized LDL and coronary heart disease', *Acta Cardiol,* 59(5), 479-84.

Holvoet, P., Kritchevsky, S. B., Tracy, R. P., Mertens, A., Rubin, S. M., Butler, J., Goodpaster, B. and Harris, T. B. (2004) 'The metabolic syndrome, circulating oxidized LDL, and risk of myocardial infarction in well-functioning elderly people in the health, aging, and body composition cohort', *Diabetes,* 53(4), 1068-73.

Holvoet, P., Lee, D. H., Steffes, M., Gross, M. and Jacobs, D. R., Jr. (2008) 'Association between circulating oxidized low-density lipoprotein and incidence of the metabolic syndrome', *JAMA,* 299(19), 2287-93.

Hori, A., Mizoue, T., Kasai, H., Kawai, K., Matsushita, Y., Nanri, A., Sato, M. and Ohta, M. (2010) 'Body iron store as a predictor of oxidative DNA damage in healthy men and women', *Cancer Sci,* 101(2), 517-22.

Hulsmans, M. and Holvoet, P. (2010) 'The vicious circle between oxidative stress and inflammation in atherosclerosis', *J Cell Mol Med,* 14(1-2), 70-8.

Hung, T. H., Lo, L. M., Chiu, T. H., Li, M. J., Yeh, Y. L., Chen, S. F. and Hsieh, T. T. (2010) 'A longitudinal study of oxidative stress and antioxidant status in women with uncomplicated pregnancies throughout gestation', *Reprod Sci,* 17(4), 401-9.

Itabe, H. (2012) 'Oxidized low-density lipoprotein as a biomarker of in vivo oxidative stress: from atherosclerosis to periodontitis', *J Clin Biochem Nutr,* 51(1), 1-8.

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D. and Lemasters, J. J. (2002) 'Mechanisms of hepatotoxicity', *Toxicol Sci,* 65(2), 166-76.

Kamp, F., Jandel, D., Hoenicke, I., Pietrzk, K., Gross, R., Trugo, N. M. and Donangelo, C. M. (2003) 'Bioavailability of iron, zinc, folate, and vitamin C in the IRIS multi-micronutrient

supplement: effect of combination with a milk-based cornstarch porridge', *Food Nutr Bull*, 24(3 Suppl), S20-6.

Kanabrocki, E. L., Murray, D., Hermida, R. C., Scott, G. S., Bremner, W. F., Ryan, M. D., Ayala, D. E., Third, J. L., Shirazi, P., Nemchausky, B. A. and Hooper, D. C. (2002) 'Circadian variation in oxidative stress markers in healthy and type II diabetic men', *Chronobiol Int*, 19(2), 423-39.

Kanabrocki, E. L., Ryan, M. D., Murray, D., Jacobs, R. W., Wang, J., Hurder, A., Friedman, N. C., Siegel, G., Eladasari, B., Nemchausky, B. A., Cornelissen, G. and Halberg, F. (2006) 'Circadian variation in multiple sclerosis of oxidative stress marker of DNA damage. A potential cancer marker?', *Clin Ter*, 157(2), 117-22.

King, S. M., Donangelo, C. M., Knutson, M. D., Walter, P. B., Ames, B. N., Viteri, F. E. and King, J. C. (2008) 'Daily supplementation with iron increases lipid peroxidation in young women with low iron stores', *Exp Biol Med (Maywood)*, 233(6), 701-7.

Leiva, E., Mujica, V., Sepulveda, P., Guzman, L., Nunez, S., Orrego, R., Palomo, I., Andrews, M. and Arredondo, M. A. (2013) 'High levels of iron status and oxidative stress in patients with metabolic syndrome', *Biol Trace Elem Res*, 151(1), 1-8.

Lorrain, B., Dangles, O., Loonis, M., Armand, M. and Dufour, C. (2012) 'Dietary iron-initiated lipid oxidation and its inhibition by polyphenols in gastric conditions', *J Agric Food Chem*, 60(36), 9074-81.

Mast, A. E., Blinder, M. A., Gronowski, A. M., Chumley, C. and Scott, M. G. (1998) 'Clinical utility of the soluble transferrin receptor and comparison with serum ferritin in several populations', *Clin Chem*, 44(1), 45-51.

Mello-Neto, J., Rondo, P. H., Oshiiwa, M., Morgano, M. A., Zacari, C. Z. and Santos, M. L. (2012) 'Iron Supplementation in Pregnancy and Breastfeeding and Iron, Copper and Zinc Status of Lactating Women From a Human Milk Bank', *J Trop Pediatr*.

Milman, N. (2006) 'Iron and pregnancy--a delicate balance', *Ann Hematol*, 85(9), 559-65.

Milman, N. (2011) 'Iron in pregnancy: How do we secure an appropriate iron status in the mother and child?', *Ann Nutr Metab*, 59(1), 50-4.

Miwa, M., Matsumaru, H., Akimoto, Y., Naito, S. and Ochi, H. (2004) 'Quantitative determination of urinary 8-hydroxy-2'-deoxyguanosine level in healthy Japanese volunteers', *Biofactors*, 22(1-4), 249-53.

Moran, L. J., Noakes, M., Clifton, P. M., Wittert, G. A., Belobrajdic, D. P. and Norman, R. J. (2007) 'C-reactive protein before and after weight loss in overweight women with and without polycystic ovary syndrome', *J Clin Endocrinol Metab*, 92(8), 2944-51.

Nakano, M., Kawanishi, Y., Kamohara, S., Uchida, Y., Shiota, M., Inatomi, Y., Komori, T., Miyazawa, K., Gondo, K. and Yamasawa, I. (2003) 'Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people', *Free Radic Biol Med*, 35(7), 826-32.

Orozco, M. N., Solomons, N. W., Schumann, K. and Friel, J. K. (2012) 'Response of urinary biomarkers of systemic oxidation to oral iron supplementation in healthy men', *Food Nutr Bull*, 33(1), 53-62.

Pasricha, S. R., McQuilten, Z., Westerman, M., Keller, A., Nemeth, E., Ganz, T. and Wood, E. (2011) 'Serum hepcidin as a diagnostic test of iron deficiency in premenopausal female blood donors', *Haematologica*, 96(8), 1099-105.

Poulsen, H. E., Prieme, H. and Loft, S. (1998) 'Role of oxidative DNA damage in cancer initiation and promotion', *Eur J Cancer Prev*, 7(1), 9-16.

Powers, H. J., Bates, C. J. and Lamb, W. H. (1985) 'Haematological response to supplements of iron and riboflavin to pregnant and lactating women in rural Gambia', *Hum Nutr Clin Nutr*, 39(2), 117-29.

Prevention, C. f. D. C. a. (1998) *Recommendations to prevent and control iron deficiency in the United States. Centers for Disease Control and Prevention*, 47, Centers for Disease Control and Prevention.

Schaap, C. C., Hendriks, J. C., Kortman, G. A., Klaver, S. M., Kroot, J. J., Laarakkers, C. M., Wiegerinck, E. T., Tjalsma, H., Janssen, M. C. and Swinkels, D. W. (2012) 'Diurnal Rhythm Rather Than Dietary Iron Mediates Daily Hepcidin Variations', *Clin Chem*.

Schulpis, K. H., Lazaropoulou, C., Vlachos, G. D., Partsinevelos, G. A., Michalakakou, K., Gavrili, S., Gounaris, A., Antsaklis, A. and Papassotiriou, I. (2007) 'Maternal-neonatal 8-hydroxy-deoxyguanosine serum concentrations as an index of DNA oxidation in association with the mode of labour and delivery', *Acta Obstet Gynecol Scand*, 86(3), 320-6.

Schulpis, K. H., Papakonstantinou, E. D., Vlachos, G. D., Vlachos, D. G., Antsaklis, A., Papassotiriou, I. and Tsakiris, S. (2008) 'The effect of the mode of delivery on the maternal-neonatal carnitine blood levels and antioxidant status', *Clin Chem Lab Med*, 46(5), 680-6.

Schumann, K., Kroll, S., Weiss, G., Frank, J., Biesalski, H. K., Daniel, H., Friel, J. and Solomons, N. W. (2005) 'Monitoring of hematological, inflammatory and oxidative reactions to acute oral iron exposure in human volunteers: preliminary screening for selection of potentially-responsive biomarkers', *Toxicology*, 212(1), 10-23.

Signorini, C., Perrone, S., Sgherri, C., Ciccoli, L., Buonocore, G., Leoncini, S., Rossi, V., Vecchio, D. and Comporti, M. (2008) 'Plasma esterified F2-isoprostanes and oxidative stress in newborns: role of nonprotein-bound iron', *Pediatr Res,* 63(3), 287-91.

Staprans, I., Pan, X. M., Rapp, J. H. and Feingold, K. R. (2003) 'Oxidized cholesterol in the diet is a source of oxidized lipoproteins in human serum', *J Lipid Res,* 44(4), 705-15.

Staprans, I., Pan, X. M., Rapp, J. H. and Feingold, K. R. (2005) 'The role of dietary oxidized cholesterol and oxidized fatty acids in the development of atherosclerosis', *Mol Nutr Food Res,* 49(11), 1075-82.

Stuetz, W., Carrara, V. I., McGready, R., Lee, S. J., Erhardt, J. G., Breuer, J., Biesalski, H. K. and Nosten, F. H. (2012) 'Micronutrient status in lactating mothers before and after introduction of fortified flour: cross-sectional surveys in Maela refugee camp', *Eur J Nutr,* 51(4), 425-34.

Trumbo, P., Yates, A. A., Schlicker, S. and Poos, M. (2001) 'Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc', *J Am Diet Assoc,* 101(3), 294-301.

Tuomainen, T. P., Loft, S., Nyyssonen, K., Punnonen, K., Salonen, J. T. and Poulsen, H. E. (2007) 'Body iron is a contributor to oxidative damage of DNA', *Free Radic Res,* 41(3), 324-8.

Uchiyama, A., Kim, J. S., Kon, K., Jaeschke, H., Ikejima, K., Watanabe, S. and Lemasters, J. J. (2008) 'Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury', *Hepatology,* 48(5), 1644-54.

Valavanidis, A., Vlachogianni, T. and Fiotakis, C. (2009) '8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis', *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev,* 27(2), 120-39.

Van Brukelen, G.J.P. (2006) ' ANCOVA versus change from baseline had more power in randomized studies and more bias in nonrandomized studies', *J Clin Epi,* 59(9), 920-5.

Vickers, A.J., Altman, D.G. (2001) ' Analysing controlled trials with baseline and follow up measurements', *BMJ,* 323(7321):1123-4.

Videla, L. A., Fernandez, V., Tapia, G. and Varela, P. (2003) 'Oxidative stress-mediated hepatotoxicity of iron and copper: role of Kupffer cells', *Biometals,* 16(1), 103-11.

Vincent, H. K., Innes, K. E. and Vincent, K. R. (2007) 'Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity', *Diabetes Obes Metab,* 9(6), 813-39.

Viteri, F. E., Casanueva, E., Tolentino, M. C., Diaz-Frances, J. and Erazo, A. B. (2012) 'Antenatal iron supplements consumed daily produce oxidative stress in contrast to weekly supplementation in Mexican non-anemic women', *Reprod Toxicol,* 34(1), 125-32.

Wiltink, W. F., Kruithof, J., Mol, C., Bos, M. G. and van Eijk, H. G. (1973) 'Diurnal and nocturnal variations of the serum iron in normal subjects', *Clin Chim Acta,* 49(1), 99-104.

Wish, J. B. (2006) 'Assessing iron status: beyond serum ferritin and transferrin saturation', *Clin J Am Soc Nephrol,* 1 Suppl 1, S4-8.

Zarban, A., Taheri, F., Chahkandi, T., Sharifzadeh, G. and Khorashadizadeh, M. (2009) 'Antioxidant and radical scavenging activity of human colostrum, transitional and mature milk', *J Clin Biochem Nutr,* 45(2), 150-4.

Table 1 Baseline characteristics of the women by group for all women enrolled in the study separated into those who completed follow up and those who were lost to follow up.

	Placebo (n=39)	Fe-B (n=28)	Fe-W (n=29)	Fe groups combined (n=57)	Completed follow-up (n= 96)	Lost to follow-up (n=18)	*P*-value[1]
Age (y)	30.6 (5.2) [2]	29.4 (6.2)	31.0 (5.3)	30.2 (5.7)	30.3 (5.5)	28.7 (4.7)	0.256
Day postpartum	12.5 (5.3)	13.1 (6.0)	15.2 (8.1)	14.2 (7.2)	13.5 (6.5)	15.6 (6.2)	0.205
BMI (kg/m^2)	27.8 (5.7)	28.8 (4.7)	29.6 (4.8)	29.2 (4.8)	28.6 (5.2)	27.9 (5.1)	0.638
Total years of education	16.2 (2.7)	16.2 (2.1)	16.0 (1.6)	16.1 (1.8)	16.1 (2.2)	15.5 (2.7)	0.257
Number of children	1.8 (0.7)	1.9 (1.3)	1.7 (1.0)	1.8 (1.2)	1.8 (1.0)	1.7 (0.8)	0.685
% Married or living as married	79.5	64.3	79.3	71.9	75.0	66.7	0.294
MediCal[3] (% yes)	28.2	42.9	31.0	36.8	33.3	33.3	>0.999
WIC[4] (% yes)	33.3	46.4	20.7	33.3	33.3	38.9	0.787
Current smoker (% yes)	5.1	3.6	10.3	7.0	6.3	11.1	0.610

[1]P-value for the difference in baseline characteristics between those who completed follow-up and those who were lost to follow-up as analyzed by ANOVA for continuous variables and Fisher's Exact test for categorical variables.

[2] mean (SD) (all such values).

[3] California Medical Assistance Program - provides health coverage for people with low income.

[4] The Special Supplemental Nutrition Program for Women, Infants and Children - a federal assistance program for healthcare and nutrition of low-income pregnant women, breastfeeding women, and infants and children under the age of five.

Fe-B, iron supplement consumed between meals; Fe-W, iron supplement consumed with meals

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699**Table 2** Baseline and change from baseline Hb, transferrin saturation, and total plasma iron, 8-OHdG, isoprostane, TAS, CRP, AGP, AST and ALT.

	Time point	Placebo (n=38)	Fe-B (n=28)	Fe-W (n=29)	*P* Unadjusted [1]	*P* Adjusted[2]	Fe groups combined (n=57)	*P* Unadjusted [3]	*P* Adjusted[4]
Hb (g/L)	Baseline	136 (11)[5]	133 (11)	134 (11)			134 (11)		
	Change	-3.7 (12)	2.5 (9.6)	0.4 (11)	0.070	0.034[6]	1.4 (10)	0.028	0.010
Ferritin (ng/mL)[7]	Baseline	55.2 (38)	61.0 (47)	53.8 (38)			57.4 (42)		
	Change	-9.17 (34)	-13.1 (55)	6.12 (30)	0.088	0.091	-3.33 (44)	0.070	0.056
Hepcidin (ng/mL)	Baseline	47.6 (35)	65.0 (67)	46.2 (32)			55.8 (53)		
	Change	18.8 (51)	34.2 (88)	42.2 (73)	0.700	0.291	38.2 (80)	0.406	0.139
Transferrin Saturation (%)	Baseline	22.3 (11)	19.6 (8.0)	19.6 (11)			19.6 (9.7)		
	Change	-0.78 (9.7)	6.13 (11)	1.62 (9.2)	0.145	0.087	3.84 (10)	0.111	0.271
Total plasma iron (µg/dL)	Baseline	89.6 (44)	78.1 (31)	77.2 (42)			77.6 (36)		
	Change	-15.8 (38)	10.5 (38)	-4.03 (42)	0.158	0.065	3.09 (40)	0.109	0.314
8-OHdG (ng/mg creatinine)	Baseline	97.3 (37)	92.3 (36)	94.8 (39)			93.5 (37)		
	Change	-2.55 (43)	1.72 (51)	8.20 (57)	0.737	0.659	5.02 (54)	0.505	0.822
Isoprostane (pg/mg creatinine)	Baseline	366 (209)	406 (239)	377 (188)			392 (214)		
	Change	8.77 (126)	-3.80 (121)	-19.0 (131)	0.490	0.319	-11.5 (125)	0.403	0.226
TAS (mmol/L)	Baseline	1.69 (0.14)	1.69 (0.12)	1.65 (0.12)			1.67 (0.12)		
	Change	-0.01 (0.11)	0.01 (0.09)	0.02 (0.11)	0.504	0.754	0.02 (0.10)	0.273	0.571
CRP (mg/L)	Baseline	9.7 (9.8)	14.3 (25)	14.6 (19)			14.5 (22)		
	Change	-6.42 (11)	-8.89 (10)	-5.23 (12)	0.056	0.061	-7.0 (11)	0.610	0.642
AGP (g/L)	Baseline	1.2 (0.30)	1.3 (0.31)	1.3 (0.32)			1.3 (0.31)		
	Change	-0.33 (0.25)	-0.42 (0.23)	-0.28 (0.26)	0.237	0.094	-0.34 (0.25)	0.363	0.608
AST (IU/L)	Baseline	18.9 (6.0)	19.0 (7.1)	18.7 (6.9)			18.8 (7.0)		
	Change	0.37 (6.1)	2.07 (13)	0.69 (9.9)	0.656	0.489	1.37 (11)	0.458	0.367
ALT (IU/L)	Baseline	14.5 (8.9)	5.7 (1.4)	13.1 (6.2)			13.1 (5.9)		
	Change	-2.34 (10)	0.93 (7.9)	-0.52 (8.5)	0.312	0.332	0.19 (8.2)	0.210	0.224

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701Change in values from baseline to final study visit. Data for ferritin, hepcidin, TfSat, plasma iron, 8-OHdG, isoprostane, CRP, and
702AGP were log transformed before statistical analyses of group-wise differences were performed.
703[1] Unadjusted difference across three randomization groups as evaluated by using ANOVA.
704[2] Differences across three randomization groups as evaluated by using ANCOVA. The baseline marker and covariates found to be
705significantly associated ($P<0.10$) with the outcome variable were included in the ANCOVA models. In addition to the baseline

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value, hemoglobin was also adjusted for BMI and baseline ferritin, hepcidin, CRP, and TAS by including those variables in the model; hepcidin was also adjusted for total school years and baseline Hb and TAS; TfSat was adjusted for total school years and baseline Hb and CRP; total plasma iron was also adjusted for baseline Hb, CRP, and TAS; isoprostane was also adjusted for BMI, number of school years, and menstrual status at the final study visit; TAS was also adjusted for hemoglobin at baseline, CRP at baseline, and MediCal status (state-sponsored program that provides health coverage for people with low income); CRP was also adjusted for 8-OHdG at baseline, ferritin at baseline, BMI, number of school years, menstrual status at the final study visit; AST was also adjusted for ferritin at baseline; ALT was also adjusted for 8-OHdG at baseline and MediCal status.

[3] Unadjusted difference between the iron groups combined and the placebo group as evaluated by ANOVA.

[4] Difference between the iron groups combined and the placebo group as evaluated by ANCOVA. The same adjustments were made as with the 3-group analysis as described above.

[5] mean (SD) (all such values).

[6] Placebo vs Fe-B, *P*=0.063; Placebo vs Fe-W, *P*=0.114

[7] There was an interaction between group assignment and baseline CRP in the 2-group ferritin analyses. Interaction results are presented in Table 3.

Fe-B, iron supplement consumed between meals; Fe-W, iron supplement consumed with meals; Hb, hemoglobin; 8-OHdG, 8-hydroxy-2-deoxyguanosine; TAS, total antioxidant status; CRP, C-reactive protein; AGP, alpha-1-acid glycoprotein; AST, aspartate transaminase; ALT, alanine transaminase.

Table 3 Group-wise differences in change in ferritin from baseline as categorized by elevated and not elevated baseline CRP.

	CRP≤5 mg/L	
	Placebo (n=15)	Fe combined (n=23)
Ferritin (ng/mL)	-25.5 (29)	8.8 (26)

There was a significant interaction (P=0.03) between intervention group and baseline CRP for the change in ferritin. Data were log transformed before statistical analyses of comparisons between groups.

[1]Differences between the iron groups combined and the placebo group as evaluated by ANCOVA after adjusting for baseline ferritin by including it as a covariate in the model.

CRP, C-reactive protein.

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760**Table 4** Number and percentage of women with oxidative stress, ferritin, hepcidin, Hb, and inflammation values above or below
761cut-offs at the final study visit.
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	Cut-off value		Placebo (Baseline: *n*=41; Final: *n*=38)	Fe-B (Baseline: *n*=37; Final: *n*=28)	Fe-W (Baseline: *n*=36; Final: *n*=29)	*P* Adjusted [1]	Fe groups combined (Baseline: *n*=73; Final: *n*=57)	*P* Adjusted [2]
Hb (g/L)	<120	Baseline	3 (7) [3]	7 (19)	2 (6)		9 (12)	
		Final	1 (3)	0 (0)	0 (0)	0.996	0 (0)	0.954
Ferritin (ng/mL)	<15	Baseline	3 (7)	4 (11)	5 (14)		9 (12)	
		Final	5 (13)	1 (4)	2 (7)	0.194	3 (5)	0.070
	<30	Baseline	12 (29)	10 (27)	10 (28)		20 (27)	
		Final	18 (46)	8 (29)	6 (21)	0.019[4]	14 (25)	0.006
Hepcidin (ng/mL)	<8	Baseline	6 (15)	1 (3)	2 (6)		3 (4)	
		Final	3 (8)	0 (0)	1 (3)	0.997	1 (2)	0.960
	<18	Baseline	9 (22)	7 (19)	7 (19)		14 (19)	
		Final	4 (10)	1 (4)	2 (7)	0.549	3 (5)	0.409
8-OHdG (ng/mg creatinine)	> 1SD	Baseline	8 (20)	6 (16)	5 (14)		11 (15)	
		Final	5 (13)	3 (11)	9 (31)	0.043[5]	12 (21)	0.306
Isoprostane (pg/mg creatinine)	> 1SD	Baseline	4 (10)	5 (14)	4 (11)		9 (12)	
		Final	7 (18)	4 (14)	5 (17)	0.621	9 (16)	0.430
CRP (mg/L)	>5	Baseline	23 (56)	23 (62)	20 (56)		43 (59)	
		Final	8 (21)	6 (21)	11 (38)	0.330	17 (30)	0.346
AGP (g/L)	>1	Baseline	31 (76)	31 (84)	27 (75)		58 (79)	
		Final	12 (31)	7 (25)	9 (31)	0.511	16 (28)	0.251

763
764[1]Difference between three study groups in the proportion of women having values above or below the stated cut-off value adjusted
765for the baseline category (high or low) as determined by logistic regression modeling. 8-OHdG was also adjusted for high
766baseline CRP and AGP (categorical variables); isoprostane was also adjusted for BMI, total number of school years completed,
767high baseline CRP (categorical variable); ferritin was also adjusted for BMI; hepcidin was also adjusted for BMI and total number
768of school years completed; CRP was also adjusted for BMI and low baseline ferritin (categorical variable); AGP was also adjusted
769for BMI, menstrual status at the final study visit, and high baseline 8-OHdG (categorical variable). Adjustments were made by
770putting the covariates in the regression models.
771[2] Difference between the iron groups combined and the placebo group in the proportion of women having values above or below
772the stated cut-off value adjusted for the baseline category (high or low).

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773[3] *n* (%) (all such values).
774[4] Fe-W vs. Placebo, *P*=0.147; Fe-B vs. Placebo, *P*=0.050 as determined by Poisson regression.
775[5] Fe-W vs. Placebo, *P*=0.086; Fe-W vs. Fe-B, *P*=0.062 as determined by Poisson regression.
776Fe-B, iron supplement consumed between meals; Fe-W, iron supplement consumed with meals; 8-OHdG, 8-hydroxy-2-
777deoxyguanosine; Hb, hemoglobin; CRP, C-reactive protein; AGP, alpha-1-acid glycoprotein

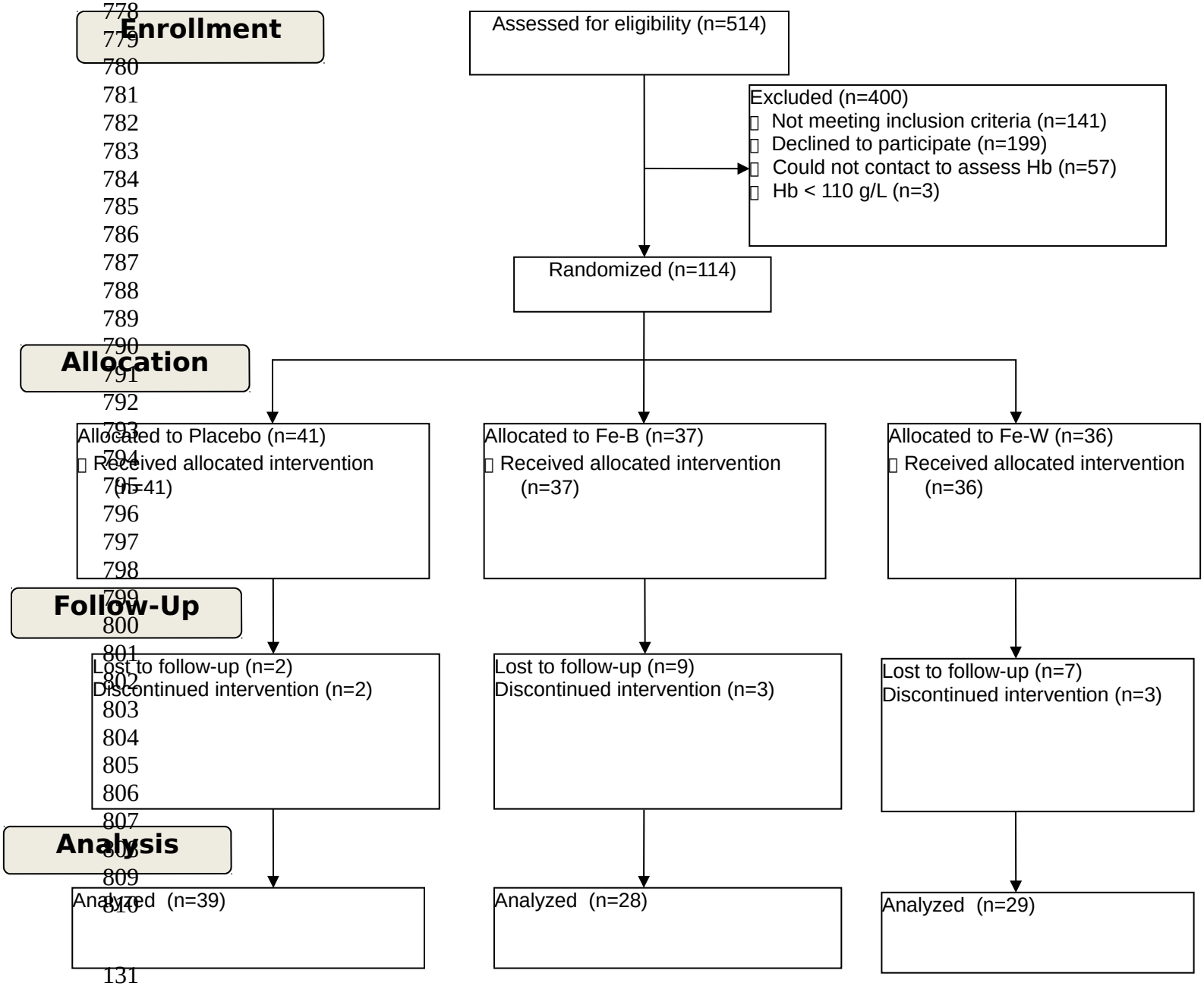
Enrollment
Assessed for eligibility (n=514)
Excluded (n=400)
Not meeting inclusion criteria (n=141)
Declined to participate (n=199)
Could not contact to assess Hb (n=57)
Hb < 110 g/L (n=3)
Randomized (n=114)
Allocation
Allocated to Placebo (n=41)
Received allocated intervention (n=41)
Allocated to Fe-B (n=37)
Received allocated intervention (n=37)
Allocated to Fe-W (n=36)
Received allocated intervention (n=36)
Follow-Up
Lost to follow-up (n=2)
Discontinued intervention (n=2)
Lost to follow-up (n=9)
Discontinued intervention (n=3)
Lost to follow-up (n=7)
Discontinued intervention (n=3)
Analysis
Analyzed (n=39)
Analyzed (n=28)
Analyzed (n=29)

Figure 1 Schematic representation of recruitment, enrollment, and follow-up. More women were lost to follow-up in the iron groups than in the placebo group (P=0.048 for the overall 3-group model; P=0.01 for Fe-B vs. Placebo; P=0.047 for Fe-W vs. Placebo; P=0.02 for the combined iron groups vs. Placebo). Fe-B, iron supplement consumed between meals; Fe-W, iron supplement consumed with meals.