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Hepcidin is low in children with moderate acute malnutrition and asymptomatic malaria: Secondary analysis of a 2x2x3 factorial randomized trial in Burkina Faso

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List of abbreviations:

AGP: Serum α-1-acid glycoprotein
CI: Confidence interval
CRP: Serum C-reactive protein
CSB: Corn-soy blend
DS: Dehulled soy
SI: Soy isolate
IQR: Interquartile range
LAZ: Length-for-age z-score
LNS: Lipid-based nutrient supplement
MAM: Moderate acute malnutrition
MUAC: Mid-upper arm circumference
RDT: Rapid diagnostic test
SAM: Severe acute malnutrition
SD: Standard deviation
s-FeCI: Serum inflammation-corrected ferritin
s-hepcidin: Serum concentration of hepcidin
sTfR: Soluble transferrin receptor
WHO: World Health Organization
WHZ: Weight-for-height z-score
WLZ: Weight-for-length z-score

ABSTRACT

Children with moderate acute malnutrition (MAM) have an increased risk of iron deficiency, anemia, and death from infectious diseases. The iron-regulating hormone hepcidin is increased in inflammation and may be important in regulating iron metabolism in children with MAM. Asymptomatic malaria has previously been associated with elevated s-hepcidin. We assessed the association between inflammation, iron status, anthropometry, and malaria and serum hepcidin (s-hepcidin) and evaluated the effect of food supplementation on s-hepcidin in a secondary analysis in 1019 children with MAM from a randomized intervention trial in Burkina Faso. Children received 12 weeks supplementation of 500 kcal/day as either corn-soy blend (CSB) or lipid-based nutritional supplements (LNS). S-hepcidin was measured at baseline and after 12 weeks. At baseline, correlates of s-hepcidin were determined using tobit regression. The effect of supplementation was determined using mixed effects tobit regression. Children with iron deficiency had 82% (95%CI 76; 87) lower s-hepcidin than those without, whereas children with acute infection and inflammation had elevated s-hepcidin. Children with symptomatic malaria had 103% (95%CI 32; 210) higher s-hepcidin than afebrile children without detectable malaria while children with recent or asymptomatic malaria had 51% (95%CI 35; 63) lower s-hepcidin. S-hepcidin increased 61% (95%CI 38; 87) after 12 weeks food supplementation with 22% higher (95% CI 2; 45) concentration in those who received LNS compared with CSB. Expectedly, morbidity and inflammation were associated with higher, and iron deficiency with lower, s-hepcidin. Further studies are needed to corroborate the finding of decreased s-hepcidin in malnourished children with asymptomatic malaria.

Key words: Hepcidin, malaria, moderate acute malnutrition, inflammation, iron deficiency, lipid-based nutrient supplement

INTRODUCTION

Moderate acute malnutrition (MAM) continues to be a global problem affecting an estimated 31 million children worldwide [1]. These children have an increased risk of infection, progression to severe acute malnutrition (SAM) and death and 5% of all deaths in children under the age of five can be attributed to MAM [1, 2]. Inadequate diets and infections can lead to iron deficiency and anemia and if energy intake is also low it may eventually result in MAM. This results in higher prevalence of iron deficiency and anemia in children with MAM [2]. In children under the age of five years, iron deficiency anemia is most frequent in sub-Saharan Africa with a prevalence of 32%. This is due to inadequate iron intake, the high phytate content of the diet resulting in low bioavailability of iron and reduced intestinal absorption because of infections [2, 3]. Iron metabolism is regulated by hepcidin, a peptide hormone produced by hepatocytes, and regulated through a negative feedback loop [4]. High iron concentration in the periportal blood increases the production of hepcidin, which in turn reduces the intestinal absorption of iron and reduces the release of recycled iron from both macrophages and hepatocyte storage [4]. Conversely, iron deficiency leads to down-regulation of hepcidin to increase intestinal iron absorption and macrophage and hepatocyte release.

Inflammation also regulates hepcidin as interleukin-6 stimulates hepcidin synthesis, which leads to reduced iron availability [4]. This increase in hepcidin during inflammation may serve as a host defense strategy to prevent growth of iron-dependent microbes such as malaria parasites [4]. According to the World Health Organization (WHO), the African region accounts for 94% of cases and 95% of the deaths from malaria and, of these, 78% were children under the age of five years [5]. In a general child cohort, malaria had a particularly strong effect on serum levels of hepcidin (s-hepcidin) as both symptomatic and asymptomatic infection with *Plasmodium falciparum* increased s-hepcidin [6]. There is a major overlap between malaria and MAM in sub-Saharan Africa, both of which may contribute to iron dysregulation. However, there is little knowledge about the interplay between malnutrition and malaria in the regulation of s-hepcidin, which could affect iron uptake from food. Furthermore, the effect of different food supplements in normalizing iron status is needed to optimally treat children with MAM. Therefore, the aim of our study was to assess the association between s-hepcidin and anthropometry, inflammation, malaria, and iron status in children aged 6-23 months in children with MAM and to evaluate the effect of food supplementation on s-hepcidin.

METHODS

Study settings, design, and participants

This study is based on data and cryopreserved samples from a randomized trial, Treatfood, which was carried out in 2013-2014. The trial had a 2x2x3 factorial design stratified on study site and was conducted in children with MAM evaluating the effects of 12 weeks of fortified food supplement providing 500 kcal/day and 12 mg/day of iron [(7)]. The children were randomized to 12 different food supplements with either corn–soy blend (CSB) or lipid-based nutrient supplement (LNS); with either dehulled soy (DS) or soy isolate (SI); and with either 0%, 20%, or 50% of protein from dried skimmed milk. The blinding and randomization have previously been described [(8)]. Treatfood was conducted in Burkina Faso at 5 different governmental health centers in Province du Passoré, Northern Region where the catchment area included 143 villages with a total population of approximately 258,000. The children in the area were screened by either community health workers or local screening teams using either mid-upper arm circumference (MUAC) or both MUAC and weight-for-length z-score (WLZ). Children could also be referred from a local health center or come on caretaker's initiative. The children were assessed on site and were eligible for inclusion if they were 6-23 months, had MAM (MUAC ≥ 115 mm and < 125 mm and/or WHZ ≥ –3 and < –2), were resident in the catchment area, and if the caretakers gave informed consent. They were excluded if they were enrolled in another nutritional program, if they needed hospitalization or had been hospitalized or treated for SAM in the last 2 months, if they had very low hemoglobin (<4 g/dL) or evidence of decompensated anemia, or if they had a suspected allergy to either milk, peanuts, CBS, or LNS.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and all caregivers gave written informed consent prior to inclusion using either signature or fingerprint. The study was approved by the Ethics Committee for Health Research of the Government of Burkina Faso (2012-8-059) and a consultative approval was given by the Danish National Committee on Biomedical Research Ethics (1,208,204). The trial registration was ISRCTN42569496. All treatment was free of charge and children who developed SAM were treated with ready-to-use therapeutic food and if that was not sufficient after 4 weeks, they were referred to the hospital.

Data collection

At baseline the study nurse collected information on sociodemographics, breastfeeding, and two-weeks history of illness using structured questionnaires and performed a clinical examination. Breastfeeding was defined as any current breastfeeding. Any illness in the last two weeks was based on both medical history and clinical examination. Fever was defined as axillary temperature ≥37.5˚C. If the children were not up to date with vaccinations in the current vaccine program, they were referred to a health center. A single dose of vitamin A (100,000 IU if 4 to 8 kg body weight; 200,000 IU if >8 kg body weight), albendazole (200 mg if <8 kg body weight; 400 mg if >8 kg body weight) or both was given if this had not been given in the last 6 months. The children were followed every two weeks with duplicate measures of anthropometrics. MUAC was measured to the nearest 1 mm on the left arm using a standard measuring tape, weight was measured to the nearest 100g using an electronic scale (Seca model 881 1021659, Hamburg, Germany) and length was measured to the nearest 1 mm using a wooden length board. STATA (Stata Corp, College Station, Texas) package "zscore06", which uses WHO anthropometric reference, was used to calculate weight-for-age z-score (WAZ), WLZ and length-for-age z-score (LAZ). Stunting was defined as LAZ <-2.

Blood collection and analyses

At baseline and after 12 weeks, a 2.5 mL venous blood sample was collected. One drop was used for a malaria rapid diagnostic test (RDT) based on *P. falciparum* histidine-rich protein 2 antigen (SD Bioline Malaria Ag Pf, Abbott Diagnostic Korea Inc, Yongin, South Korea) and one drop was used for determination of hemoglobin (HemoCue device, Hb 301, Ängelholm, Sweden). A clot activator sample tube was used for the remaining blood (Becton Dickinson, reference #268,392). The tube was stored at 2-8°C during transport and at the trial lab it was centrifuged at 700x*g* (EBA 20 S Hettich) after which serum was isolated and stored at -20°C during shipment to VitMin Lab in Willstaedt Germany and then at -80°C until analysis. At VitMin Lab a combined sandwich enzyme-linked immonosorbant was used to measure serum C-reactive protein (CRP), α_1-acid glycoprotein (AGP), and ferritin [9]. All samples were measured in duplicate, and the intra- and inter-assay coefficients of variation were <10%. Serum ferritin was adjusted for inflammation using regression models as previously described [10]. The cutoffs for categorical analyses of these variables were 110 g/L for hemoglobin [11]; 2, 5, 10, and 50 mg/L for CRP [9, 12]; 0.8, 1.0 and 1.2 g/L for AGP [13, 14]; 12 and 24 µg/L for serum inflammation-corrected ferritin (s-FeCI) [11, 14]; and 8.3 mg/L for

soluble transferrin receptor (sTfR) [9]. Symptomatic malaria was defined as positive malaria RDT in a child with fever, whereas positive malaria RDT without fever was interpreted as either asymptomatic or recently cured malaria. Serum hepcidin was measured at Copenhagen University Hospital, Denmark, by mass spectrometry as previously described [15]. The level of quantification was in the interval 0.6-13.9 nM with a coefficient of variation of < 11%. Samples with s-hepcidin >13.9 nM were diluted to obtain exact measurements.

Statistical analysis

Data was double entered into Epidata 3.1 software (Epidata Association, Odense, Denmark) and all statistical analyses were carried out using STATA 12 and STATA 18.

Baseline

Baseline characteristics were summarized as % (n) for categorical data, mean (SD) for normally distributed and median [interquartile range, IQR] for non-normally distributed quantitative variables based on visual inspection of histograms and probability plots. S-hepcidin as a function of sex and age was assessed using fractional polynomials with 95% confidence intervals. Log10 transformed tobit regression was used to assess the association between s-hepcidin and admission criteria, anthropometry, breastfeeding, clinical and paraclinical markers of inflammation, and markers of iron status both with and without age and sex adjustment. Tobit regression was used to take left censoring into account due to the high number of values (38%) under the limit of quantification [16]. The results were back transformed. Linear regression was used to assess the association between inflammation-adjusted ferritin and sTfR and admission criteria, anthropometry, breastfeeding, and clinical and paraclinical markers of inflammation both with and without age and sex adjustment. Linear regression was also used to assess the difference in CRP and AGP in those with and without malaria and/or fever. Interactions between anemia and depleted iron stores as well as malaria and fever were assessed using likelihood-ratio test. Differences between those who had s-hepcidin measured and those who did not and between those who were lost to follow-up and those who were followed up were investigated using chi-squared for categorical variables, independent t-test for normally distributed data and Wilcoxon rank-sum test for non-normally distributed data.

Follow-up

S-hepcidin at baseline and after 12 weeks for each supplementation group was summarized using median [IQR]. The change between the two visits were estimated using mixed effects tobit regression with log10 transformation and both site and ID as random effects and the results were back transformed. The effects of matrix, soy quality and amount of milk on s-hepcidin were assessed using mixed effects tobit regression models with log10 transformation. Interaction between the factors was tested for 3-way interactions using likelihood ratio tests, and where possible reduced to 2-way interactions or main effects. The models were adjusted for sex, age, admission month, baseline MUAC, WLZ, LAZ, and site as fixed effects and identification number as a random effect and the results were back transformed. Model assumptions were checked based on residual and normal probability plots and linearity of continuous variables.

RESULTS

Of the 1609 children included in the TreatFood trial, 1019 (63%) had s-hepcidin measured at baseline (Figure 1); insufficient serum was the main reason for missing s-hepcidin data. Their median [IQR] age was 11.5 months [8.3; 16.5] and 54.8% were females (Table 1). Low MUAC (≥ *115 mm and* < *125 mm*) was found in 78.8% (803), low WLZ (*WHZ* ≥ *–3 and* < *–2*) was found in 71.6% (730) and 36.4% (371) were stunted. Those who had s-hepcidin measured were slightly older (11.5 vs 10.8 months, p=0.008) and had higher s-FeCI compared with those who did not (16.8 vs 14.9 µg/L, p=0.02). They were also more likely to have had any illness in the last two weeks (80% vs 71%, p<0.001), to have had coughing in the last two weeks (32% vs 24%, p=0.001), and to have a positive malaria RDT (44% vs 35%, p<0.001). Other parameters were similar between those with and without s-hepcidin measurements.

Correlates of serum hepcidin

The median [IQR] s-hepcidin level was 1.3 nM [<0.6; 5.6] with 38% of the values under the lower quantification level. S-hepcidin was 43% (95% CI 11; 85, p=0.006) higher in females than in males, after adjustment for age (Table 2). From age 6 months, s-hepcidin decreased with nadir around 14 months after which it increased to a maximum at 24 months (Figure 2). Those who were breastfed had 55% (95% CI 21; 74, p=0.005) lower s-hepcidin than those who were not breastfed, after adjustment for age and sex. No associations were seen between

anthropometric indicators and s-hepcidin. (Table 2 with age and sex adjustment and Supplementary Table 1 without adjustments).

Febrile children had 169% (95% CI 97; 268, p<0.001) higher s-hepcidin than those with a normal body temperature. Children with a positive malaria RDT had 39% (95% CI 21; 53, p<0.001) lower s-hepcidin than those with a negative RDT (Table 3). There was an interaction between malaria and fever (p=0.03) as children with symptomatic malaria had elevated s-hepcidin (103%, 95% CI 32; 210, p=0.001), whereas the afebrile children with a positive malaria RDT had very low s-hepcidin, −51% (95% CI −63; −35, p<0.001) compared with children without fever and negative malaria RDT (Table 3). Additionally, s-hepcidin increased with higher serum CRP and AGP (Table 3 with age and sex adjustment and Supplementary Table 2 without adjustment). Those with positive malaria RDT had higher serum CRP (difference: 7 mg/L, 95% CI 5; 9, p<0.001) and AGP (difference: 0.3 g/L, 95% CI 0.2; 0.4, p<0.001) than those with negative malaria RDT. Furthermore, those with symptomatic malaria had higher serum CRP (difference: 19 mg/L, 95% CI 15; 23, p<0.001) and AGP (difference: 0.3g/L, 95% CI 0.2; 0.5, p<0.001) than those with asymptomatic or recent malaria.

Serum hepcidin and markers of iron status

S-hepcidin increased with increasing s-FeCI: children with s-FeCI <12 µg/L had 82% (95% CI 76; 87, p<0.001) lower s-hepcidin than those with s-FeCI ≥24 µg/L. S-hepcidin decreased with increasing serum sTfR and those with sTfR ≥8.3 mg/L had 83% (95% CI 76; 88, p<0.001) lower s-hepcidin than those with sTfR <8.3 mg/L (Table 4). S-hepcidin increased with increasing hemoglobin and those with anemia had 63% (95% CI 51; 73, p<0.001) lower s-hepcidin than those without anemia. However, no interaction was seen between anemia and s-FeCI <12 µg/L (p=0.98).

Effects of interventions

Of the 1019 children who had s-hepcidin measured at baseline, 903 had s-hepcidin measurement repeated after 12 weeks. The 116 who were not followed up had lower percentage of any illness in the last two weeks (73% (515) vs 80% (716), p=0.002); lower age (10.9 months [8.1; 15.0] vs 11.6 months [8.3; 16.7], p=0.006); and lower s-FeCI (14.9 [7.2; 29.2] vs 17.0 [8.9; 30.2], p=0.01) than those who were followed up at 12 weeks. Overall, s-hepcidin increased 61% (95% CI 38; 87, p<0.001) after 12 weeks food supplementation.

Those who received LNS had 22% (95% CI 2; 45, p=0.03) higher increase in s-hepcidin than those who received CSB (Table 5). There was no effect of soy quality (p=0.9) and milk content (20% vs. 0%, p=0.6 and 50% vs. 0%, p=0.9).

DISCUSSION

As expected, morbidity and inflammation were associated with elevated s-hepcidin in children with MAM. Interestingly, while symptomatic malaria was associated with high s-hepcidin, children with and asymptomatic or recent malaria had low s-hepcidin. Those with iron deficiency or anemia or both also had lower s-hepcidin. S-hepcidin increased with 12 weeks supplementation in children with MAM and it increased more in those who received LNS compared with CSB.

Serum hepcidin in children

We found an overall s-hepcidin of 1.3 nM which is lower than in healthy children aged 0-3 years from high income countries [17, 18]. Two studies from the Netherlands demonstrated that s-hepcidin in children measured with mass spectrometry ranged from 0.2 nM to 20.9 nM with a median between 1.8 nM and 3.7 nM whereas median s-hepcidin measured with immunochemical methods was 7.9 nM [17, 18]. They also found that s-hepcidin levels vary throughout childhood with lower levels in older children, but with no sex difference [17, 18]. The lower s-hepcidin in our study in children from Sub-Saharan Africa is in line with a review that found that s-hepcidin is lower in healthy children from low income countries compared with healthy children from high income countries [19]. Studies in healthy children in Sub-Saharan Africa showed that children aged six months from rural Kenya had a mean s-hepcidin of 2.3 nM (range 0.1; 18.1 nM) with a lower concentration in boys than in girls (1.5 vs. 3.2 nM) [20], which could be because boys are more likely to be undernourished [21]. Similarly, a study from Zimbabwe in children aged 3-12 months found that median (interquartile range) plasma hepcidin was 3.5 nM (0.9; 6.9) at 3 months, 1.6 nM (0.2; 2.6) at 6 months and 0.7 nM (0.3; 2.2) at 12 months, but with no sex difference [22]. A study in coastal Kenya found similar lower s-hepcidin at older ages with s-hepcidin in children aged 0-1 year of 3.0 nM (95% CI 2.1; 4.3) and in children aged 1-3 years of 0.9 nM (95% CI 0.7; 1.2) [6]. The relatively low levels of s-hepcidin in studies from Sub-Saharan Africa regardless of measurement method suggest that iron deficiency is widespread, which is consistent with previous studies [23, 24]. Iron deficiency in Sub-Saharan is caused by lower iron intake due to diets with low content or bioavailability of iron, reduced intestinal absorption due to

infections and the high burden of malnutrition among other things [2-4, 6, 25]. It also indicates that factors such as chronic inflammation, e.g. due to repeated infections that would tend to increase s-hepcidin are outweighed by the effects of iron deficiency [26]. Additional factors such as genetic differences may also play a role [3, 19].

No studies have looked at hepcidin in malnourished children in Sub-Saharan Africa. S-hepcidin was lower in our study than in healthy children without malnutrition from Kenya (1.3 nM vs 2.3 nM) which also measured s-hepcidin using mass spectroscopy. We had a high number of values (38%) under the quantification level. In comparison only 1.8% or less of healthy children in high-income countries and 15% of Kenyan children without malnutrition had values under the detection limit (0.5 nM) measured with mass spectroscopy [17, 18, 20]. Of those with values under the quantification level in our study, 91% had either anemia or depleted iron stores suggesting that the iron deficiency was the cause of the low values. Likewise, the study in Kenyan children found that 59% of those with iron deficiency and no inflammation had values under the detection limit [20]. Low s-hepcidin in children with iron deficiency is expected as lack of iron in tissues will downregulate s-hepcidin which will lead to higher absorption from the intestine [4].

Association of hepcidin changes with changes in iron status and inflammation

We showed that food supplementation for 12 weeks increased s-hepcidin in children with MAM. This increase in s-hepcidin could be due to an increase in iron stores as all food supplement contained iron. Oral iron therapy has previously been found to rapidly increase s-hepcidin in children with iron deficiency or anemia[27]. This is consistent with the decrease in anemia and iron deficiency after supplementation, previously shown in a study from the Treatfood project [28]. However, it also showed a decrease in inflammation after supplementation. Inflammation has previously been shown to increase s-hepcidin [26] and we also found positive associations between inflammation markers and s-hepcidin in our study, which suggest that s-hepcidin should decrease with supplementation. So, the effect of restoring iron is larger than the effect of reducing inflammation [20]. Children who got LNS had higher s-hepcidin than those who received CSB. This is most likely due to the better iron status previously demonstrated in those who received LNS compared with CSB and may be due to better acceptability of LNS [7, 28]. Also, inflammation levels were higher among those who received LNS [28]. Both factors may have contributed to the increase in s-hepcidin.

In our study we found no increase in s-hepcidin in children with MAM who had been ill in the last two weeks before inclusion. A study in Ghanian children found that s-hepcidin was already within the normal range 7 days after treatment of malaria [15]. This rapid change after infection can explain the lack of increase in s-hepcidin, as two weeks may be too long an interval to capture the rapid change in s-hepcidin following malaria treatment. This also aligns with the strong association with CRP as a relatively fast inflammation marker as opposed to AGP that is slower. Furthermore, not all diseases, e.g., gastro-intestinal infections, result in systemic inflammation and subsequent increase in s-hepcidin [6, 15, 20, 29].

Malaria

We found increased s-hepcidin, decreased sTfR and stable s-FeCI in children with symptomatic malaria and MAM. High s-hepcidin during symptomatic malaria with rapid decrease after infection has previously been found in other studies [6, 15]. A study in Burkinabé children showed that s-hepcidin was higher in children with parasitemia and the best predictor of parasitemia was malnutrition [30]. The parasite digests hemoglobin and releases hemozoin which inhibits erythropoiesis in bone marrow [25]. The bone marrow suppression is lifted rapidly after treatment [31]. These findings suggest that the low tissue iron could be due to either the use of iron by the parasite or that those with parasitemia also are the ones with insufficient nutritional intake including iron, which is the reason for the low tissue iron. The malaria RDT can be positive for up to a month after cure, so fever was used to distinguish between symptomatic malaria and recent or asymptomatic malaria. This also meant that we could not distinguish between recent and asymptomatic malaria.

We found that MAM children with recent or asymptomatic malaria had lower s-hepcidin and higher sTfR than those with negative malaria RDT, but similar s-FeCI. This differs from other studies in children without malnutrition where s-hepcidin was increased in asymptomatic malaria [6, 32]. The increase in s-hepcidin, caused by asymptomatic malaria, has been suggested to result in iron deficiency through reduced iron absorption [6], which is in line with the bone marrow suppression and anemia found in Ghanian children with persistent malaria and asymptomatic malaria [31, 33]. The lower s-hepcidin in our study and the decrease in iron deficiency with food supplementation shown in a previous study on the Treatfood project suggest that asymptomatic malaria does not block iron absorption in children with MAM [28]. This also suggests that s-hepcidin is differently regulated in children with malnutrition compared with well-nourished children. Further studies are needed to

corroborate this finding as we could not discriminate between asymptomatic and recently treated malaria.

Breastfeeding and anthropometry

We found that children with MAM who were currently breastfed had lower s-hepcidin compared with non-breastfed children when adjusted for age and sex. This is in line with a previous study in infants from the USA that showed that healthy children who were breastfed had lower s-hepcidin than those who were not breastfed even though ferritin levels were similar [34]. Breastmilk has a very low iron content and iron is poorly absorbed [35-37]. However, the non-breastfed group in our study was small and the reasons for not breastfeeding included factors that could influence iron status, e.g. age. Anthropometric measures before supplementation were not associated with s-hepcidin in our study. Results from previous studies vary showing positive, negative or no associations between hepcidin and anthropometric measures [19, 20, 38]. The fact that all children, included in our study had MAM at inclusion may have limited the range of anthropometric z-scores and thus the possibility to demonstrate an association. However, our previous study on the Treatfood project found an overall increase in anthropometry. The increase was larger in children who received LNS compared with CSB [8]. Studies comparing overweight and normal weight children have found higher hepcidin in overweight children and reduction in hepcidin with weight loss [39, 40]. This suggest that change in anthropometry can also influence hepcidin concentrations.

Limitations

There are some limitations in our study. Firstly, this was a secondary analysis of a randomized trial and this resulted in missing hepcidin values which may induce bias. Secondly, there was not enough blood to measure s-hepcidin for all children. Children without s-hepcidin assessment had smaller iron stores but fewer infections including malaria, both of which influence s-hepcidin. Thirdly, 38% of children had s-hepcidin under the quantification level of 0.6 nM so only 62% had s-hepcidin within the measurable interval. However, our measurements had precision and accuracy up to the expected standards and tobit regression was used to take left censoring into account when fitting the models. Fourthly, due to ethical concerns, no control group without supplementation was included to evaluate the s-hepcidin over time. Lastly, malaria parasitemia was not evaluated, which

would have given a clearer picture of the changes in s-hepcidin and iron stores especially in the group of children with positive malaria RDT but no fever.

Conclusion

Morbidity and inflammation were associated with high s-hepcidin and iron deficiency with low s-hepcidin in children with MAM. Unexpectedly, children with asymptomatic or recent malaria had low s-hepcidin. S-hepcidin increased with 12 weeks food supplementation in children with MAM with the highest increase in those who received LNS compared with CSB. Further studies confirming the downregulation of s-hepcidin in children with malnutrition and asymptomatic malaria are needed.

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Conflict of interests:

HF has received research grants from Arla Food for Health Centre and research grants from the Danish Dairy Research Foundation, KFM received funds from Arla Foods Amba and finally HF, KFM, SF and AB have had research collaboration with Nutriset, a producer of LNS. Other authors declare no financial relationships with any organisations that might have an interest in the submitted work in the previous five years, and declare no other relationships or activities that could appear to have influenced the submitted work.

Authorship

HF, KFM, SF, AB, VBC: designed the Treatfood trial, CWY, CF and AI-B: conducted the research, JK, KKL and BS conducted the laboratory work, TWH, CR: analyzed data, TWH: wrote the manuscript and had primary responsibility for final content, TWH, HF, JK, SF, VBC: contributed to data analysis, and all authors: read, edited and approved the final manuscript.

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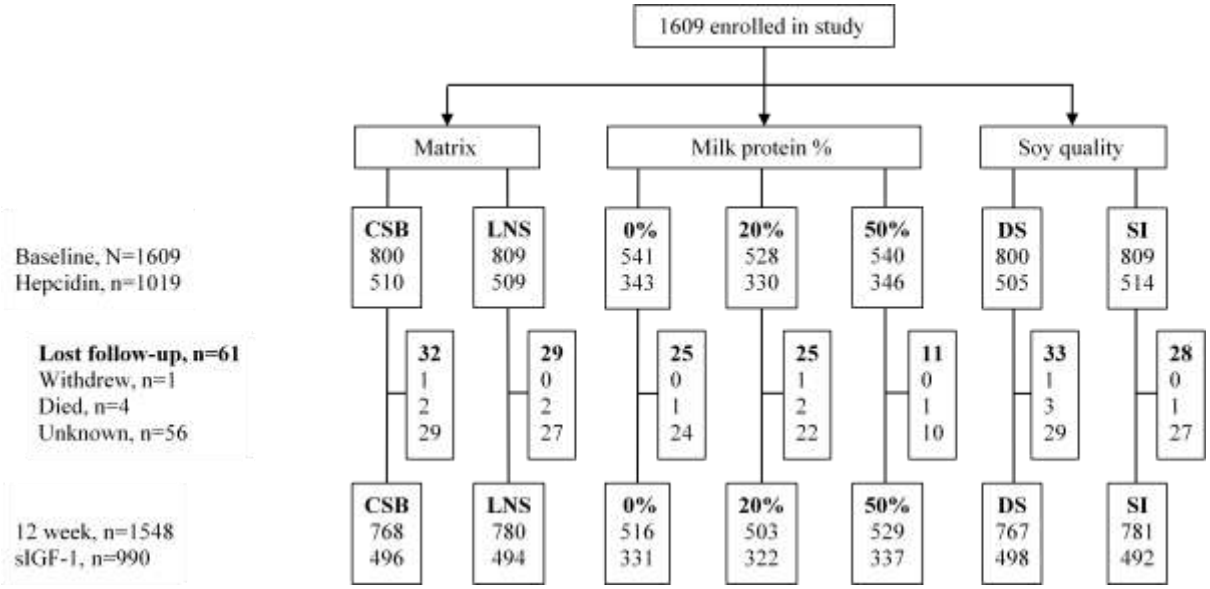

Figure 1: Trial profile. CSB, corn–soy blend; DS, dehulled soy; LNS, lipid-based nutrient supplement; SI, soy isolate

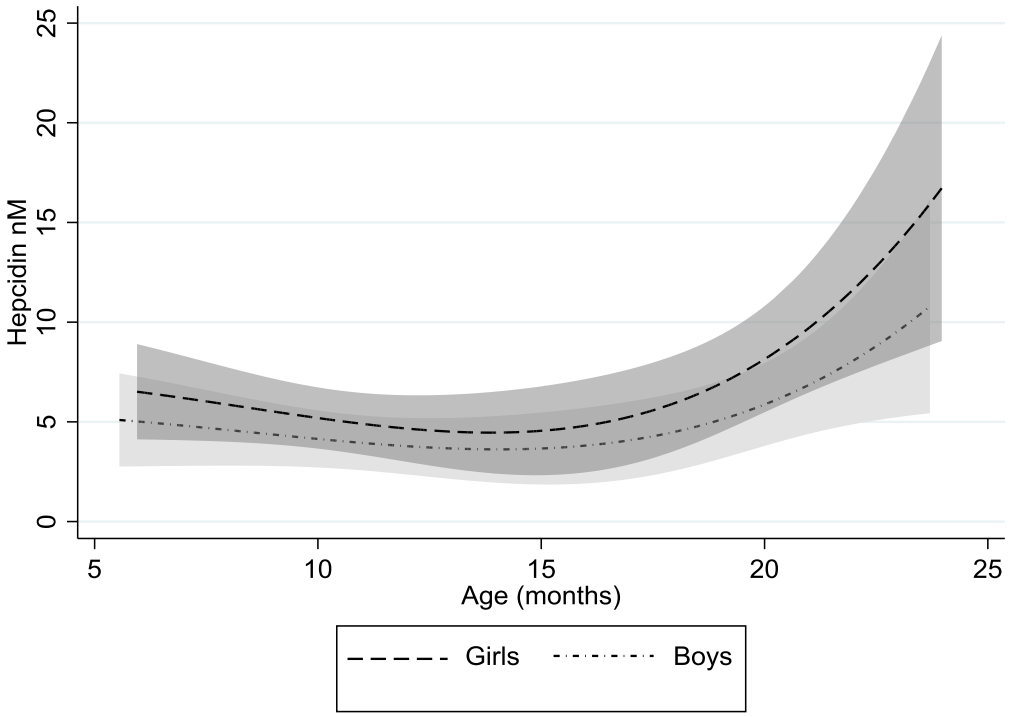

Figure 2: Serum hepcidin as a function of age for boys and girls with 95% CI

Table 1: Characteristics of 1019 children aged 6-23 months with moderate acute malnutrition

Sociodemographic characteristics	
Sex, girl	54.8% (558)
Age (months)	11.5 [8.3; 16.5]
Anthropometry	
Mid-upper arm circumference (mm)	123 (4)
Length-for-age (z-score)	-1.68 (1.13)
Weight-for-age (z-score)	-2.52 (0.64)
Weight-for-length (z-score)	-2.23 (0.50)
Breastfeeding	94.1% (957)
Morbidity and inflammation	
Any illness in the last 2 weeks[1]	80.4% (812)
Diarrhea[1]	19.0% (194)
Cough[1]	32.4% (329)
Fever[2]	18.1% (184)
Malaria Rapid-test positive	43.5% (441)
Serum CRP (mg/L)	2.5 [0.8; 9.8]
Serum AGP (g/L)	1.23 [0.88; 1.67]
Iron status	
Serum inflammation-corrected ferritin (µg/L)	16.8 [8.8; 30.5]
Serum soluble transferrin receptor (mg/L)	12.7 [9.1; 17.5]
Hemoglobin (g/L)	99.9 (16.2)

Values are presented as % (n), mean (SD) or median [interquartile range]

Abbreviations CRP = C-reactive protein, AGP = α_1-acid glycoprotein

[1]Based on maternal recall and physical examination at inclusion by trained study nurse

[2]Based on physical examination at inclusion by trained study nurse. Fever defined as temperature ≥37.5°C

[3]Detection level for hepcidin is 0.6 nM

Table 2: Admission criteria, anthropometry, and breastfeeding as correlates of serum hepcidin (nM) among 1019 children aged 6-23 months with moderate acute malnutrition

	Hepcidin (nM)				Inflammation-corrected ferritin (µg/L)				Soluble transferrin receptor (mg/L)			
			Age and sex adjusted				Age and sex adjusted				Age and sex adjusted	
	N	Median [IQR] hepcidin (nM)	% (95% CI)	p-value	*N*	Median [IQR]	% (95% CI)	p-value	*N*	Median [IQR]	% (95% CI)	p-value
All	1019	1.3 [<0.6; 5.6]	-	-	1007	16.8 [8.8; 30.5]	-	-	1015	12.7 [9.1; 17.5]	-	-
Sex												
Boys	461	1.0 [<0.6; 6.2]	Ref.		452	15.3 [8.0; 27.4]	Ref.		458	13.4 [9.8; 18.6]	Ref.	
Girls	558	1.7 [<0.6; 6.2]	43% (11; 85)	0.006	555	18.5 [9.5; 32.6]	18% (5; 32)	0.004	557	12.1 [8.6; 16.6]	-9% (-14; -4)	0.001
Age (month)												
6-11	533	1.4 [<0.6; 5.7]	Ref.		526	19.3 [10.4; 36.0]	Ref.		530	12.1 [9.0; 16.8]	Ref.	
12-17	294	1.2 [<0.6; 5.0]	-10% (-33; 21)	0.49	290	13.6 [7.0; 24.6]	-26% (-35; -15)	<0.001	294	14.0 [10.2; 18.6]	8% (1; 15)	0.02
18-24	192	1.4 [<0.6; 5.8]	14% (-18; 60)	0.43	191	16.1 [8.2; 26.2]	-21% (32; 8)	0.002	191	12.6 [8.5; 17.3]	0.4% (-7; 7)	0.90
Admission criteria												
MUAC and WLZ	514	1.4 [<0.6; 5.9]	Ref.		507	17.6 [9.1; 30.8]	Ref.		512	12.3 [9.0; 17.0]	Ref.	
WLZ only	216	1.3 [<0.6; 5.2]	-1% (-29; 39)	0.97	214	14.2 [7.5; 27.6]	-9% (-21; 6)	0.23	215	12.7 [9.6; 17.4]	1% (6; 8)	0.87
MUAC only	289	1.3 [<0.5; 5.6]	-17% (-39; 13)	0.23	286	17.8 [9.8; 31.1]	-6% (-18; 8)	0.39	288	13.7 [9.4; 18.6]	12% (5; 19)	0.001
Weight-for-length z-score												
≥-2	291	1.3 [<0.6; 5.7]	Ref.		288	17.8 [9.8; 31.2]	Ref.		290	13.6 [9.4; 18.6]	Ref.	
<-2	728	1.4 [<0.6; 5.5]	17% (-13; 57)	0.29	719	16.6 [8.5; 30.0]	4% (-9; 18)	0.59	725	12.5 [9.1; 17.2]	-10% (-16; -4)	0.001
Length-for-age z-score												
≥-2	648	1.5 [<0.6; 6.0]	Ref.		639	17.6 [9.7; 30.8]	Ref.		645	12.5 [9.0; 17.1]	Ref.	
<-2 and ≥-3	264	1.2 [<0.6; 4.8]	-17% (-39; 12)	0.22	263	15.7 [7.6; 30.5]	-5% (-17; 9)	0.42	264	12.7 [9.4; 17.8]	2% (-5; 8)	0.64
<-3	107	0.8 [<0.6; 3.4]	-32% (-56; 6)	0.09	105	16.8 [7.1; 27.2]	-4% (-21; 17)	0.72	106	14.1 [9.4; 19.1]	8% (-2; 18)	0.12
Weight-for-age z-score												
≥-2	213	1.3 [<0.6; 6.7]	Ref.		209	18.0 [9.9; 33.6]	Ref.		212	12.5 [9.5; 17.3]	Ref.	
<-2 and ≥-3	584	1.4 [<0.6; 5.2]	-5% (-32; 31)	0.74	578	16.5 [9.2; 28.9]	1% (-13; 17)	0.92	582	12.7 [9.1; 17.4]	-3% (-10; 4)	0.32
<-3	222	1.3 [<0.6; 5.4]	3% (-31; 54)	0.89	220	16.9 [7.1; 30.8]	1% (-16; 21)	0.91	221	12.8 [9.0; 17.5]	-4% (-12; 4)	0.32
Mid-upper arm circumference (mm)												
≥125	216	1.3 [<0.6; 5.2]	Ref.		214	14.2 [7.5; 27.6]	Ref.		215	12.7 [9.6; 17.4]	Ref.	
≥120 and <125	584	1.2 [<0.6; 5.1]	-8% (-34; 29)	0.64	575	17.0 [8.9; 29.7]	5% (-9; 21)	0.52	581	12.7 [9.2; 17.5]	3% (-4; 11)	0.42
>115 and <120	219	1.9 [<0.6; 6.6]	6% (-29; 58)	0.78	218	20.2 [10.6; 34.1]	17% (-2; 40)	0.09	219	11.7 [8.6; 18.1]	2% (-7; 11)	0.72
Breastfeeding												
No breastfeeding	60	2.9 [0.6; 9.6]	Ref.		60	16.0 [9.8; 27.0]	Ref.		60	11.5 [8.5; 15.1]	Ref.	
Breastfeeding	957	1.3 [<0.6; 5.4]	-55% (-74; -21)	0.005	945	17.0 [8.8; 30.5]	-18% (-36; 6)	0.13	953	12.8 [9.2; 17.9]	17% (4; 32)	0.01

Abbreviations: MUAC = Mid-upper arm circumference, WLZ = Weight-for-length Z-score

Detection level for hepcidin is 0.6 nM. Associations were analyzed by log10 transformed age- and sex adjusted tobit/linear regressions with back transformation, so the results are % differences.

Table 3: Clinical and paraclinical markers of inflammation as correlates of serum hepcidin (nM), inflammation-corrected ferritin (µg/L) and Soluble transferrin receptor (mg/L) among 1019 children aged 6-23 months with moderate acute malnutrition

	Hepcidin (nM)				Inflammation-corrected ferritin (µg/L)				Soluble transferrin receptor (mg/L)			
			Age and sex adjusted				Age and sex adjusted				Age and sex adjusted	
	N	Median [IQR] hepcidin (nM)	% (95% CI)	p-value	*N*	Median [IQR]	% (95% CI)	p-value	*N*	Median [IQR]	% (95% CI)	p-value
Any illness in the last 2 weeks[1]												
No	198	1.6 [<0.6; 4.6]	Ref.		196	16.5 [8.2; 31.2]	Ref.		197	10.4 [8.5; 14.0]	Ref.	
Yes	812	1.3 [<0.6; 5.8]	1% (-27; 38)	0.97	803	16.9 [8.8; 30.2]	4% (-10; 20)	0.61	809	13.5 [9.5; 18.6]	21% (13; 29)	<0.001
Diarrhea[1]												
No	825	1.3 [<0.6; 5.4]	Ref.		815	16.9 [8.9; 29.5]	Ref.		822	12.6 [9.2; 17.4]	Ref.	
Yes	194	1.5 [<0.6; 5.9]	6% (-23; 47)	0.71	192	16.3 [8.8; 34.2]	8% (-7; 24)	0.31	193	12.8 [9.0; 17.9]	1% (-6; 8)	0.76
Cough[1]												
No	687	1.2 [<0.6; 4.6]	Ref.		676	17.7 [9.4; 31.1]	Ref.		683	12.8 [9.1; 17.1]	Ref.	
Yes	329	1.7 [<0.6; 6.5]	41% (8; 85)	0.01	328	16.2 [7.5; 27.7]	-10% (-20; 2)	0.10	329	12.7 [9.2; 18.4]	2% (-4; 8)	0.61
Fever (≥37.5˚C)[2]												
No	833	1.2 [<0.6; 4.6]	Ref.		826	16.8 [9.2; 30.3]	Ref.		831	12.7 [9.2; 17.3]	Ref.	
Yes	184	3.1 [<0.6; 12.2]	169% (97; 268)	<0.001	179	16.4 [7.9; 30.5]	-5% (-18; 10)	0.46	182	13.0 [9.2; 18.3]	3% (-4; 11)	0.35
Malaria (Rapid test)												
Negative	572	1.8 [<0.6; 5.9]	Ref.		569	16.9 [8.7; 30.7]	Ref.		571	10.9 [8.4; 14.5]	Ref.	
Positive	441	0.8 [<0.6; 4.9]	-39% (-53; -21)	<0.001	436	16.8 [9.0; 29.9]	-2% (-12; 10)	0.78	438	15.4 [11.3; 20.5]	32% (26; 40)	<0.001
Malaria/fever				0.03[3]				0.68[3]				0.58[3]
No malaria + no fever	482	1.7 [<0.6; 5.6]	Ref.		480	16.8 [8.8; 30.2]	Ref.		481	10.9 [8.4; 14.5]	Ref.	
No malaria + fever	88	2.8 [0.8; 12.6]	103% (31; 214)	0.002	87	16.9 [8.0; 33.2]	-2% (-21; 21)	0.84	88	11.3 [8.7; 15.5]	2% (-7; 12)	0.64
Malaria + no fever	345	<0.6 [<0.6; 3.3]	-51% (-63; -35)	<0.001	344	16.8 [9.3; 30.5]	-0.1% (-12; 13)	0.98	344	15.4 [11.5; 20;5]	33% (26; 41)	<0.001
Malaria + fever	96	3.6 [<0.6; 11.8]	103% (32; 210)	0.001	92	15.9 [7.5; 28.7]	-8% (-25; 13)	0.41	94	15.5 [10.1; 20.1]	31% (20; 44)	<0.001
CRP mg/L				<0.001				0.04				0.009
≤2	456	0.9 [<0.6; 3.4]	Ref.		453	16.5 [8.8; 33.0]	Ref.		456	11.9 [8.8; 16.9]	Ref.	
>2 and ≤5	190	1.3 [<0.6; 4.9]	48% (7; 104)		188	17.7 [9.7; 32.6]	5% (-11; 22)		190	13.0 [9.3; 18.3]	6% (-1; 14)	
>5 and ≤10	118	1.0 [<0.6; 4.3]	14% (-23; 69)		117	17.0 [6.6; 27.1]	-17% (-31; 0.02)		118	14.2 [10.2; 19.6]	15% (5; 26)	
>10 and ≤50	185	2.6 [<0.6; 7.3]	149% (81; 243)		183	16.2 [9.2; 29.8]	-9% (-22; 6)		185	13.9 [10.4; 18.4]	10% (2; 18)	
>50	66	15.2 [4.6; 30.0]	1098% (647; 1821)		66	18.8 [9.2; 24.2]	-11% (-30; 13)		66	10.4 [7.7; 13.7]	-15% (-24; -5)	
AGP g/L				<0.001				<0.001				0.01
≤0.8	179	0.9 [<0.6; 3.6]	Ref.		179	16.9 [9.4; 34.8]	Ref.		179	10.6 [8.1; 14.7]	Ref.	
>0.8 and ≤1.0	157	1.1 [<0.6; 3.7]	7% (-31; 66)		157	20.2 [10.7; 52.5]	21% (-1; 47)		157	12.5 [9.0; 16.8]	15% (4; 26)	
>1.0 and ≤1.2	145	1.4 [<0.6; 4.4]	23% (-21; 93)		145	17.3 [8.0; 30.5]	-12% (-28; 7)		145	11.8 [8.5; 18.5]	14% (4; 25)	
>1.2	534	1.8 [<0.6; 6.8]	106% (45; 193)		526	15.9 [8.4; 27.0]	-15% (-28; -1)		534	13.4 [10.0; 18.4]	19% (11; 29)	

Abbreviations CRP = C-reactive protein, AGP = α_1-acid glycoprotein, B=beta coefficient, IQR=Interquartile range

[1]Based on maternal recall and physical examination at inclusion by trained study nurse

[2]Based on physical examination at inclusion by trained study nurse

[3]P for interaction

Detection level for hepcidin is 0.6 nM. Associations were analyzed by log10 transformed age- and sex adjusted tobit/linear regressions with back transformation, so the results are % differences.

Table 4: Markers of iron status as correlates of serum hepcidin (nM) among 1019 children aged 6-23 months with moderate acute malnutrition

			Unadjusted		Age and sex adjusted	
	N	Median [IQR]	% (95% CI)	p-value	% (95% CI)	p-value
Inflammation-corrected ferritin µg/L			2% (1; 2)	<0.001	2% (1%; 2%)	<0.001
≥24	352	3.9 [0.9; 9.1]	Ref.		Ref.	
≥12 and <24	297	1.4 [<0.6; 4.9]	-54% (-66; -39)	<0.001	-55% (-67; -40)	<0.001
<12	358	<0.6 [<0.6; 1.7]	-80% (-85; -73)	<0.001	-82% (-87; -76)	<0.001
Soluble transferrin receptor mg/L			-12% (-14; -10)	<0.001	-12% (-14; -10)	<0.001
<8.3	174	5.0 [2.1; 12.5]	Ref.		Ref.	
≥8.3	841	0.9 [<0.6; 4.0]	-79% (-84; -71)	<0.001	-78% (-84; -70)	<0.001
Hemoglobin g/L			2% (2; 3)	<0.001	2% (2; 3)	<0.001
≥110	304	3.1 [0.9; 6.6]	Ref.		Ref.	
<110	715	0.8 [<0.6; 4.3]	-59% (-69; -47)	<0.001	-59% (-69; -46)	<0.001
Anemia/depleted iron[1]						
No anemia + normal iron stores	228	3.8 [1.6; 7.6]	Ref.		Ref.	
No anemia + depleted iron stores	76	1.1 [<0.6; 3.2]	-69% (-81; -48)	<0.001	-69% (-81; -49)	<0.001
Anemia + normal iron stores	429	1.6 [<0.6; 6.4]	-52% (-64; -35)	<0.001	-52% (-65; -35)	<0.001
Anemia + depleted iron stores	282	<0.6 [<0.6; 1.4]	-85% (-89; -79)	<0.001	-85% (-89; -78)	<0.001

[1]Anemia defined as hemoglobin <110 g/L and depleted iron stores defined as inflammation-corrected ferritin <12 µg/L

Associations were analyzed by log10 transformed age- and sex adjusted tobit/linear regressions with back transformation, so the results are % differences. Detection level for hepcidin is 0.6 nM.

Table 5: The effect of matrix, soy quality and milk content of food supplements on hepcidin (nM) among children with moderate acute malnutrition

Type of supplements		n	Baseline Median [IQR] ng/ml	n	12 weeks Median [IQR] ng/ml	n	Change§ (95% CI)	Difference change* (95% CI)	in p
All supplements		1019	1.3 [<0.6; 5.6]	990	2.6 [<0.6; 7.2]	903	61% (38; 87)	-	
Matrix	CSB	510	1.3 [<0.6: 5.6]	496	1.9 [<0.6; 6.8]	455	22% (-2; 51)		
	LNS	509	1.4 [<0.6; 5.6]	494	3.4 [1.0; 7.7]	448	110% (70; 160)	22% (2; 45)	0.03
Soy quality	Dehulled	505	1.2 [<0.6; 5.0]	498	2.9 [0.6; 7.5]	454	95% (56; 143)		
	Isolate	514	1.6 [<0.6; 6.0]	492	2.3 [<0.6; 6.9]	449	33% (8; 64)	1% (-15; 21)	0.88
Milk content	0%	343	1.4 [<0.6; 5.5]	331	2.2 [<0.6; 7.2]	304	52% (18; 96)		
	20%	330	1.5 [<0.6; 5.6]	322	2.7 [0.7; 6.7]	290	66% (28; 114)	6% (-15; 31)	0.61
	50%	346	1.2 [<0.6; 5.7]	337	2.8 [<0.6; 7.7]	309	66% (26; 118)	2% (-18; 26)	0.86

Abbreviations: CSB, Corn-soy blend; CI, Confidence interval; LNS, lipid-based nutrient supplement; IQR, interquartile range

§Results are based on tobit random-effects models adjusted for sex, age, season, site, baseline MUAC, WLZ, and LAZ as fixed effects and ID as random effect. Hepcidin was log10 transformed and back transformed, so the results are % differences.

*Results are presented as the ratio of change based on tobit random-effects models adjusted for sex, age, season, site, baseline MUAC, WLZ, and LAZ as fixed effects and ID as random effect. Hepcidin was log10 transformed and back transformed, so the results are % differences. The 22% represents that those who received LNS had 22% higher increase in s-hepcidin than those who received CSB.

Detection level for hepcidin is 0.6 nM.