**Lower hepatic iron storage associated with obesity in mice can be restored by
decreasing body fat mass through feeding a low-fat diet**

Hak Chung^a, Dayong Wu^b, Donald Smith^b, Simin Nikbin Meydani^b, and Sung Nim Han^{a,c,*}

^aDepartment of Food and Nutrition, College of Human Ecology,

Seoul National University, Seoul, Korea

^bJM USDA Human Nutrition Research Center on Aging,

Tufts University, Boston, MA, USA

^cResearch Institute of Human Ecology, College of Human Ecology,

Seoul National University, Seoul, Korea

*Corresponding Author:

Sung Nim Han

Department of Food and Nutrition

College of Human Ecology

Seoul National University

1 Gwanak-ro, Gwanak-Gu,

Seoul, Korea 151-742

Phone: 02-880-5011

Email: snhan@snu.ac.kr

- 23 **List of abbreviations**
- 24
- 25 BMP; bone morphogenetic protein
- 26 Dcytb; duodenal cytochrome *b*
- 27 p-Smad; phosphorylated Smad
- 28 HFD; high-fat diet
- 29 mRNA; messenger RNA
- 30 PCR; polymerase chain reaction

Abstract

High-fat diet (HFD)-induced obesity has been reported to result in low hepatic iron storage. In the current study, we tested the hypothesis that these obesity-related changes in hepatic iron status could be reversed by decreasing adiposity by feeding a low-fat diet. Five-week-old C57BL/6 mice were assigned to three groups: The LL group was fed a control diet for 31 weeks, the HH group was fed a HFD for 31 weeks, and the HL group was fed the HFD for 15 weeks and then switched to the control diet for 16 weeks. The fat mass of the HL group decreased by 3.2 g from the 14th to the 30th week. Fat mass was significantly different among the groups (11.4 g, 15.8 g, and 37.5 g in the LL, HH, and HL groups, respectively, $P<0.001$). The liver iron concentration of the HL group was similar to that of the LL group, which was about 30% higher than that of the HH group (74.2 $\mu\text{g/g}$, 72.7 $\mu\text{g/g}$, and 55.7 $\mu\text{g/g}$ in the LL, HL, and HH groups, respectively, $P<0.05$). Duodenal cytochrome *b* (Dcytb) messenger RNA (mRNA) levels were higher in the HL group than in the HH group. While bone morphogenetic protein 6 (*Bmp6*) mRNA levels showed no significant differences in the liver, duodenal *Bmp6* mRNA levels were significantly lower in the HH group compared with the LL and HL groups. Liver Smad1/5 proteins were differentially activated: The HH group had significantly less phosphorylated Smads (p-Smads) than the LL and HL groups. Our data demonstrate that hepatic iron storage levels are closely related to body adiposity, and reducing body fat mass through feeding a lower fat diet to HFD-induced obese mice restores liver iron storage.

Keywords: Obesity; Diet modulation; Fat loss; Iron status; Mouse

1. Introduction

Iron is stored primarily in the liver, spleen, and bone marrow, and it is an essential trace element required for a number of vital cell functions [1]. Iron deficiency is one of the most common nutritional deficiencies worldwide and a leading cause of anemia [2]. Obesity, a global health concern, has been shown to be associated with a higher prevalence of iron deficiency. The prevalence of iron deficiency among obese or overweight children and adolescents has been demonstrated to be higher than among those with normal weight [3, 4], and it has been shown that US children with body mass indexes (BMIs) over the 85th percentile have more than twice the prevalence of iron deficiency than children who are not overweight [5]. Further, obesity in both premenopausal and postmenopausal women has been associated with decreased iron status [6, 7].

In high-fat diet (HFD)-induced obese mice, significantly lower iron storage in the liver has been observed [8]. However, the mechanism of diminished iron status in obesity has not been clearly delineated. Studies have indicated that the increased prevalence of iron deficiency in the obese population is not attributed to different iron intake levels [9, 10]. It has been suggested that accumulated body fat and low-grade inflammation interferes with the normal regulatory mechanism of hepcidin on iron homeostasis [11–13]. In one study, hepatic *Hamp* expression was shown to be positively correlated with liver iron storage levels in the control group, but this positive correlation was not observed in the obese group [8]. These results suggest that the regulation of *Hamp* expression by body iron status may be abnormal in obesity, leading to disturbed iron homeostasis. Recently, Sonnweber et al. [14] showed that HFD consumption caused iron deficiency by reducing duodenal iron absorption, which points to a possible mechanism related to iron deficiency in obesity.

79

80 While further investigation is needed to delineate the exact mechanism(s), it is
81 evident that obesity leads to the depletion of body iron storage. However, an important
82 practical issue to be addressed is the fact that, to date, relevant findings have not been
83 consistent regarding whether these obesity-related changes to iron status could be reversed by
84 a reduction of body adiposity. In a review by Tussing-Humphreys et al. [15], studies that
85 observed the changes in iron status following weight loss were examined with conflicting
86 results: An increase [16], a decrease [17, 18], or no change [19] in serum iron indicators after
87 weight loss had been reported during the follow-up stage of bariatric surgery.

88

89 Several studies have investigated how weight loss, modulated by dietary intake,
90 impacts iron status, and the results remain inconclusive. In a study on obese children, Di Toro
91 et al. [20] observed no significant changes in serum iron indicators after weight loss
92 associated with a 13-week hypocaloric diet. A significant increase in hemoglobin
93 concentration was observed in obese women who lost weight by following a hypocaloric diet
94 for 6 weeks [21]. A significant decrease in hemoglobin and hematocrit levels was reported in
95 premenopausal women whose body weight decreased by 12.3 kg on average (10.4 kg as fat)
96 following 15 weeks of calorie restriction [22]. Amato et al. [23], however, reported increases
97 in iron absorption and serum iron concentration in children whose BMIs were decreased after
98 a 6-month weight loss program.

99

100 An explanation for the discrepant findings in these human studies has not been forthcoming,
101 but certainly, there are many possible contributing factors (including differences in
102 participant characteristics and study setting). Since mouse models are most commonly used
103 to study the effect of obesity on virtually all the body's systems, we conducted the current

104 animal study to test the hypothesis that the previously observed obesity-related iron
105 deficiency could be reversed by decreasing fat mass through reduced fat intake. To test this
106 hypothesis, we compared the hepatic iron levels of mice in which obesity was induced by
107 feeding a HFD for 31 weeks with those of obese mice fed a HFD for 15 weeks and then
108 switched to a control (low-fat) diet for 16 weeks and non-obese mice fed a control diet for 31
109 weeks.

2. Methods and materials

2.1. Animals and diets

Four-week-old C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed at the animal care facility at Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University. Mice were maintained in AAALAC-accredited facilities with an environmentally controlled atmosphere (22°C, 45% relative humidity, 15 air changes of 100% fresh hepa-filtered air per hour, and a 12/12-hour light/dark cycle- 07:00 on). Animals were observed daily for clinical signs of disease. After 5 days of acclimation, animals were assigned to one of three experimental groups and diets were fed *ad libitum*: The LL group was fed a control diet containing 10% kcal fat (Research Diets, New Brunswick, NJ, USA, #D12450B) for 31 weeks, the HH group was fed a HFD containing 60% kcal fat (#D12492) for 31 weeks, and the HL group was fed the HFD for the first 15 weeks and then switched to the control diet for the following 16 weeks. Mice in the HL group were fed the HFD for 15 weeks to ensure the induction of maximal obesity and subsequently placed on the control diet for 16 weeks to allow a significant reduction in body adiposity. The nutrient compositions of the diets are shown in **Table 1**. Body fat mass and lean mass were measured at the 14th and 30th weeks by EchoMRI™-700 (Houston, TX, USA), and body weight was measured weekly. At the 31st week, the animals were euthanized by CO₂ asphyxiation. Livers and small intestines were collected, frozen in liquid nitrogen, and stored at -80°C until use. All conditions and handling of the animals were approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA HNRCA at Tufts University.

2.2. Determination of liver iron concentration

Liver tissue was dried in a 55°C dry oven for 60 hours, and the dry weights for each 200-mg wet liver tissue sample were measured and recorded. Samples were acid-digested according to US Environmental Protection Agency (EPA) method 3051 [24]. Dried tissue was placed and sealed in perfluoroalkoxy (PFA) vessels with 70% nitric acid, and the vessels were heated in a MDS-2000 microwave sample digestion system (CEM Corp., Matthews, NC, USA). After cooling, the sample solution was filtered with a Whatman No.40 filter and diluted to a fixed volume. The ⁵⁶Fe isotope content of the digested solution was monitored using an inductively coupled plasma mass spectrometer (ICP-MS; Varian 820-MS, Mulgrave, Australia).

2.3. RNA preparation, reverse transcription, and real-time polymerase chain reaction (PCR)

RNA was isolated using RNAiso Plus (Takara Bio Inc., Shiga, Japan). Reverse transcription was performed using a PrimeScript 1st strand cDNA synthesis kit (Takara) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using the StepOne™ Real-time PCR System (Applied Biosystems, CA, USA). Specific primer sequences were 5'- GCA GCG GGC TCG AGT TTA -3' and 5'- TTC CAG GTC CAT GGC AGT CT -3' for duodenal cytochrome *b* (*Dcytb*); 5'- TTG TCT CAT GAA GAA CAT TAC AGC AC-3' and 5'- CAT ATG GCA ATC AAA GCA GAA GA -3' for *Heph*; 5'-TTG CGA TAC CAA TGC AGA AGA-3' and 5'-GAT GTG GCT CTA GGC TAT GTT-3' for *Hamp*; 5'-GGG GAG GCG AAC ATT AGG TA-3' and 5'-AAA CCA CGA ACA ACA GCA CA-3' for bone morphogenetic protein 6 (*Bmp6*); and 5'-GGA GAA ACC TGC CAA GTA-3' and 5'-AAG AGT GGG AGT TGC TGT TG-3' for *Gapdh* (Biomedic, Bucheon, Korea).

Amplification conditions were 30 seconds at 95°C followed by 40 cycles of 30 seconds at 95°C and 1 minute at 60°C. All assays were conducted in duplicate. Messenger RNA (mRNA) levels of all genes were calculated relative to *Gapdh* mRNA abundance.

2.4. Determination of ferritin and Smad protein levels by Western blot analysis

Protein was isolated from liver tissue using RIPA lysis buffer. Protein concentration was determined by the Bradford method using a protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Then, 20 µg of total protein for ferritin and 50 µg for Smad1 and phosphorylated Smad1/5 (p-Smad1/5) were diluted in Laemmli sample buffer, incubated at 95°C for 5 minutes, and run on a 10% SDS-polyacrylamide gel. Separated proteins were then blotted onto a PVDF membrane and blocked in tris-buffered saline/0.1% Tween-20 (TBS-T) containing either 5% skim milk for p-Smad1/5 and β-actin or 3% BSA for ferritin and Smad1. The membranes were probed with goat anti-ferritin heavy chain (Y-16) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho-Smad1(Ser463/465)/Smad5(Ser463/465) (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Smad1 (1:1000; Cell Signaling Technology), or mouse anti-β-actin (1:10000; Sigma-Aldrich, St. Louis, MO, USA). After washing with TBS-T, the membranes were incubated with anti-goat (1:8500; Abcam, Cambridge, UK), anti-rabbit (1:3000; Cell Signaling Technology), or anti-mouse (1:20000; Sigma-Aldrich) HRP-linked antibody. Membrane-bound antibody was visualized with enhanced chemiluminescence and exposed to X-ray film. Proteins were quantified using QuantityOne software (Bio-Rad Laboratories), using β-actin as a loading control.

2.5. Statistical analyses

Data were analyzed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test for individual group comparisons. Pearson correlation was used to determine the association between variables. Data are presented as means $\pm$ SEM. *P*-values below 0.05 were considered statistically significant. SAS 9.3 statistical software (SAS Institute Inc.) was used for all statistical analyses. Sample size was calculated to achieve 20% difference in iron levels with $\alpha=0.05$ and 80% power using mean and standard deviation from our previous study [8].

3. Results

3.1. Body weight and fat mass

At the 15th week, mice in the HL and HH groups gained significantly more weight and fat mass than those in the LL group. At the 31st week, body weights were significantly different between the groups. The body weight of the HL group was between that of the LL and HH groups. The HL group showed no significant change in body weight from the 15th week (40.9 ± 1.78 g) to the 31st week (40.6 ± 1.48 g), while the body weight of the HH group continued to increase (39.4 ± 1.98 g at the 15th week and 63.1 ± 1.48 g at the 31st week). After switching from a HFD to a control diet, the HL group showed a decrease in body fat mass (3.2 ± 0.65 g decrease) despite the maintenance of body weight, while fat mass continued to increase in the LL and HL groups (5.3 ± 1.94 g and 20.3 ± 1.03 g in the LL and HH groups, respectively) (**Table 2** and **Figure 1**).

3.2. Liver iron concentration and ferritin protein level

At the conclusion of the experiment, liver iron concentration was significantly lower in the HH group than in the LL and HL groups (74.2 ± 7.5 , 72.7 ± 2.6 , and 55.7 ± 3.8 $\mu\text{g/g}$ tissue in the LL, HL, and HH groups, respectively, $P < 0.05$). The HL group's final iron concentration was similar to that of the LL group. The liver iron concentration of the HH group was approximately 25% lower than those of the LL and HL groups (**Figure 2**). The HH group showed the lowest iron content (94.0 ± 6.5 μg , liver iron concentration multiplied by liver weight), while the HL group had a higher iron content (165.1 ± 12.0 μg) compared with the LL group (126.6 ± 11.4 μg) ($P < 0.001$, **Figure 3A**). Considering the body weight

difference, however, the LL and HL groups had similar levels of liver iron content per gram body weight (3.63 ± 0.34 and 4.02 ± 0.27 $\mu\text{g Fe/g body wt}$, respectively, $P>0.05$), which were significantly higher than that of the HH group (1.45 ± 0.07 $\mu\text{g Fe/g body wt}$, $P<0.0001$, **Figure 3B**). There was no significant difference in ferritin protein levels between the HL and LL groups. The HH group had significantly lower ferritin protein levels compared with the LL and HL groups ($P<0.001$, **Figure 4**). Body fat mass showed significantly negative correlations with liver iron concentration ($r=-0.627$, $P<0.01$, **Figure 5A**) and with liver ferritin protein levels ($r=-0.687$, $P<0.001$, **Figure 5B**).

3.3. Duodenal iron transporters, duodenal cytochrome b (*Dcytb*) and hephaestin (*Heph*) mRNA levels

Results of duodenal *Dcytb* and *Heph* mRNA levels are shown in **Figures 6A** and **6B**. *Dcytb* expression in the small intestine was significantly different between the groups ($P=0.05$). The mRNA level of *Dcytb* was higher in the HL group compared with the HH group. The LL group and the HH group showed no significant difference in *Dcytb* mRNA levels.

3.4. Liver hepcidin (*Hamp*) mRNA levels

Average liver *Hamp* mRNA levels are shown in **Figure 7A**. Liver *Hamp* mRNA levels were not statistically different between the groups ($P=0.117$). However, the average liver *Hamp* mRNA levels were 30% lower in the HH group compared with the LL and HL groups. Furthermore, liver iron concentration showed a statistically significant positive correlation with *Hamp* mRNA levels ($r=0.675$, $P<0.001$, **Figure 7B**).

3.5. Hepatic and duodenal bone morphogenetic protein 6 (*Bmp6*) mRNA levels

Hepatic *Bmp6* mRNA levels were not significantly different between the groups (Figure 8A). In the duodenum, however, *Bmp6* mRNA levels were significantly lower in the HH group (Figure 8B, $P<0.01$) compared with the LL and HL groups. Furthermore, duodenal *Bmp6* mRNA levels were found to be positively correlated with both liver iron concentrations (Figure 9A, $r=0.576$, $P<0.05$) and hepatic *Hamp* mRNA levels (Figure 9B, $r=0.517$, $P<0.05$).

3.6. Phosphorylation of liver *Smad1/5* proteins

To assess the activation or repression of the Smad signaling pathway through the phosphorylation of Smads, the ratio of p-Smad1/5 to unphosphorylated-Smad1 in the liver was determined by Western blot analysis. Smad phosphorylation levels were significantly different among the groups (Figure 10, $P<0.001$). The HL group and the LL group showed similar phosphorylation levels, whereas the HH group had an approximately 40% lower p-Smad/Smad ratio when compared to the LL and HL groups.

4. Discussion

In the current study, we confirmed previous studies' reports that lower hepatic iron status was associated with HFD-induced obesity and further demonstrated that this effect could be reversed by decreasing the body fat mass in female mice by switching to a lower fat diet. Obesity in humans is generally due to dietary and life-style factors rather than genetic defects. In this study, we investigated the relationship between obesity and iron status in an obesity model that is more relevant to the obesity associated with dietary changes by employing the diet-induced obesity model, which is commonly used. Mice in the HL group were fed the HFD for 15 weeks, which is within typical period of time for the induction of obesity. Subsequently they switched to the control diet for 16 weeks in order to allow a significant reduction in body adiposity. The HL group showed no significant change in body weight after switching from the HFD to the control diet; however, body fat mass decreased significantly. After 31 weeks, the body adiposity of the HL group was between those of the LL and HH groups but closer to that of the LL group. The HL group had a liver iron concentration similar to that of the LL group, whereas the HH group had an approximately 25% lower iron concentration compared to the LL group.

To investigate the mechanisms involved in the alteration of liver iron concentrations associated with body adiposity, we compared the mRNA levels of intestinal iron transporters. It is well known that the iron absorption rate in the small intestine varies depending on the expression levels of iron transporters (including *Dcytb* and hephaestin) that are modulated by the body's iron status [25]. Sonnweber et al. [14] reported that intestinal *Dcytb* and *Heph* mRNA levels were lower in mice fed a HFD. Another study found that obese Thai women had lower intestinal iron absorption compared with normal-weight subjects after consuming a

reference meal containing isotopically labeled fortification iron [26]. In this study, there was no difference in the mRNA levels of *Heph*, which is involved in iron transport at the intestinal basolateral membrane. The mRNA levels of *Dcytb*, which acts at the apical membrane, were higher in the HL group compared with the HH group; however, unexpectedly, we observed no difference in *Dcytb* mRNA levels between the LL and HH groups. Since higher *Dcytb* mRNA levels are expected to contribute to increased iron absorption, these results may provide an explanation for the higher hepatic iron concentration in the HL group compared with the HH group. However, the lack of difference in *Dcytb* mRNA expression between the LL and HH groups suggests that factors other than *Dcytb* may have contributed to the lower iron status associated with obesity in the HH group.

Liver *Hamp* mRNA levels were not statistically different between the groups. However, the average *Hamp* mRNA level was 30% lower in the HH group compared with the LL and HL groups, and there was a positive correlation between liver *Hamp* mRNA levels and measures of liver iron status. Hepcidin regulates the body's iron status by binding to ferroportin, which is a cellular iron exporter expressed on the surface of duodenal enterocytes, macrophages, and hepatocytes. Upon binding, hepcidin induces the internalization and lysosomal degradation of ferroportin. Thus, elevated hepcidin levels lead to decreased cellular iron efflux, which limits iron absorption by enterocytes and prevents the release of iron from hepatic stores [27]. The positive correlation between liver *Hamp* mRNA levels and liver iron status suggests that hepcidin may have played a role in regulating liver iron concentration, thus contributing to the observed differences in iron status in the current study.

We determined the changes in the BMP6-Smad signaling pathway to gain further insight into the regulatory mechanisms involved in the altered iron status associated with

obesity. BMP6, a member of the transforming growth factor (TGF)- β superfamily, binds to the cell-surface BMP receptor complex (BMPRI and BMPRII) and catalyzes the phosphorylation of receptor-regulated Smad proteins (Smad1, Smad5, and Smad9) [28, 29]. The phosphorylation of Smad1/5 promotes its association with Smad4 to form a heterodimer. The heterodimers translocate into the cell nucleus to act as transcription factors, leading to the expression of *Hamp* [30, 31]. In the present study, liver *Bmp6* mRNA levels were not significantly different between the groups. However, the phosphorylation level of Smad1/5 was significantly different between the groups. The ratio of p-Smad1/5 to Smad1 was significantly lower in the HH group compared with the LL and HL groups. These results indicate that although obesity did not induce changes in liver *Bmp6* mRNA expression, it did affect the signal transduction pathway that works in regulating hepcidin expression.

Controversy exists regarding the source of BMP6 that regulates liver *Hamp* mRNA expression [32]. Currently, non-parenchymal liver cells are suggested to be the primary source of BMP6 in response to elevated body iron levels. In one study, feeding mice an iron-enriched diet for 3 weeks resulted in significantly higher *Bmp6* mRNA expression in Kupffer cells and sinusoidal endothelial cells, but no significant difference in *Bmp6* mRNA levels was observed in hepatocytes [33]. However, Arndt et al. [34] observed no difference in *Bmp6* or *Hamp* mRNA expression when hepatocytes, hepatic stellate cells, and Kupffer cells were isolated from mice and treated *ex vivo* with FeSO₄ or holotransferrin. Isolated liver tissue treated with FeSO₄ or holotransferrin showed no significant change in *Bmp6* or *Hamp* expression, but *Bmp6* mRNA levels were higher in isolated small intestinal tissue treated with FeSO₄ or holotransferrin than untreated tissue. Moreover, when liver tissue isolated from *Bmp6*^{-/-} mice was co-cultured with small intestinal tissue from *Bmp6*^{+/+} mice, the *Hamp* mRNA level in the liver tissue was significantly elevated in response to FeSO₄. A co-culture

of liver tissue isolated from *Bmp6*^{+/+} mice and small intestinal tissue from *Bmp6*^{-/-} mice failed to induce such changes. Higher *Bmp6* mRNA levels in the small intestine as well as in the liver were observed after feeding mice an iron-enriched diet for 3 weeks. Based on these observations, it was proposed that iron induces BMP6 production in the small intestine and it then travels via the portal vein to the liver to activate the BMP6-Smad signaling cascade. However, these data are not consistent with a report by Kautz et al. [35], who reported higher *Bmp6* mRNA levels in the livers but not in the duodenums of mice fed an iron-enriched diet.

In the current study, *Bmp6* mRNA levels in the liver were not significantly different between experimental groups. In the duodenum, however, *Bmp6* mRNA levels were significantly lower in the HH group compared to the LL and HL groups. Since Smad1/5 phosphorylation levels differed without changes in *Bmp6* mRNA expression in the liver, these results suggest the possibility of crosstalk between the small intestine and the liver, as proposed by Arndt et al. [34]. Although the previously reported results were inconsistent with respect to whether excessive iron ingestion by non-obese mice could induce BMP6 production in the small intestine, our data suggest that HFD-induced obesity may have a negative effect on duodenal *Bmp6* mRNA expression. Since BMP6 produced by small intestinal tissue has been shown to have the potential to induce *Hamp* expression in liver tissue *in vitro* [34], the differentially expressed *Bmp6* mRNA in the duodenum might have influenced the hepatic BMP6-Smad signal activation. Further studies are needed to verify the possibility of duodenal BMP6 transport through portal circulation.

One of the limitations of this study is that the causal relationship between the expression of signaling molecules and the hepatic iron levels with changes in adiposity could not be established with the study design. That is, we could not determine whether changes in

the expression of signaling molecules associated with obesity directly affected the hepatic iron levels. However, we identified the associations among related markers in this study, and these findings will serve as a starting point to guide future studies. For example, direct evidence for the exact role of the BMP6-Smad signaling pathway in mediating the effects of obesity on hepatic iron status may be obtained by conducting experiments of targeted blockage or overexpression of related regulating molecules.

In conclusion, in this study, we demonstrated that obesity induced the depletion of hepatic iron. More importantly, the results from this study strongly support our hypothesis that reducing body fat mass by feeding a lower fat diet could restore liver iron storage in HFD-induced obese mice. These findings have expanded our knowledge of obesity-induced adverse changes in iron status and their reversal by dietary modulation, which may have significant implications for clinical practice.

Acknowledgements

This work was supported by research funds through the Research Institute of Human Ecology (RIHE) in Seoul National University and USDA contract #8050-15000-097-01S. The authors have no conflicts of interest to report.

References

- [1] Cairo G, Bernuzzi F, Recalcati S. A precious metal: Iron, an essential nutrient for all cells. *Genes & Nutrition*. 2006;1:25-40.
- [2] Iron deficiency—United States, 1999–2000. *MMWR morbidity and mortality weekly report*. 2002;51:897-9.
- [3] Moayeri H, Bidad K, Zadhoush S, Gholami N, Anari S. Increasing prevalence of iron deficiency in overweight and obese children and adolescents (Tehran Adolescent Obesity Study). *Eur J Pediatr*. 2006;165:813-4.
- [4] Pinhas-Hamiel O, Newfield RS, Koren I, Agmon A, Lilos P, Phillip M. Greater prevalence of iron deficiency in overweight and obese children and adolescents. *Int J Obes*. 2003;27:416-8.
- [5] Nead KG, Halterman JS, Kaczorowski JM, Auinger P, Weitzman M. Overweight children and adolescents: A risk group for iron deficiency. *Pediatrics*. 2004;114:104-8.
- [6] Lecube A, Carrera A, Losada E, Hernandez C, Simo R, Mesa J. Iron deficiency in obese postmenopausal women. *Obesity*. 2006;14:1724-30.
- [7] Tussing-Humphreys LM, Nemeth E, Fantuzzi G, Freels S, Guzman G, Holterman AX, et al. Elevated systemic hepcidin and iron depletion in obese premenopausal females. *Obesity*. 2010;18:1449-56.
- [8] Chung J, Kim MS, Han SN. Diet-induced obesity leads to decreased hepatic iron storage in mice. *Nutr Res*. 2011;31:915-21.
- [9] Menzie CM, Yanoff LB, Denkinger BI, McHugh T, Sebring NG, Calis KA, et al. Obesity-related hypoferremia is not explained by differences in reported intake of heme and nonheme iron or intake of dietary factors that can affect iron absorption. *J Am Diet Assoc*. 2008;108:145-8.
- [10] Cepeda-Lopez AC, Osendarp SJ, Melse-Boonstra A, Aeberli I, Gonzalez-Salazar F,

Feskens E, et al. Sharply higher rates of iron deficiency in obese Mexican women and children are predicted by obesity-related inflammation rather than by differences in dietary iron intake. *Am J Clin Nutr.* 2011;93:975-83.

[11] Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology.* 2006;131:788-96.

[12] Ausk KJ, Ioannou GN. Is obesity associated with anemia of chronic disease? A population-based study. *Obesity.* 2008;16:2356-61.

[13] Zafon C, Lecube A, Simo R. Iron in obesity. An ancient micronutrient for a modern disease. *Obes Rev.* 2010;11:322-8.

[14] Sonnweber T, Ress C, Nairz M, Theurl I, Schroll A, Murphy AT, et al. High-fat diet causes iron deficiency via hepcidin-independent reduction of duodenal iron absorption. *J Nutr Biochem.* 2012;23:1600-8.

[15] Tussing-Humphreys L, Pusatcioglu C, Nemeth E, Braunschweig C. Rethinking iron regulation and assessment in iron deficiency, anemia of chronic disease, and obesity: Introducing hepcidin. *J Acad Nutr Diet.* 2012;112:391-400.

[16] Anty R, Dahman M, Iannelli A, Gual P, Staccini-Myx A, Amor IB, et al. Bariatric surgery can correct iron depletion in morbidly obese women: A link with chronic inflammation. *Obes Surg.* 2008;18:709-14.

[17] Ruz M, Carrasco F, Rojas P, Codoceo J, Inostroza J, Rebolledo A, et al. Iron absorption and iron status are reduced after Roux-en-Y gastric bypass. *Am J Clin Nutr.* 2009;90:527-32.

[18] Toh SY, Zarshenas N, Jorgensen J. Prevalence of nutrient deficiencies in bariatric patients. *Nutrition.* 2009;25:1150-6.

[19] Gasteyger C, Suter M, Calmes JM, Gaillard RC, Glusti V. Changes in body composition, metabolic profile and nutritional status 24 months after gastric banding. *Obes Surg.*

2006;16:243-50.

[20] Di Toro A, Marotta A, Todisco N, Ponticiello E, Collini R, Di Lascio R, et al. Unchanged iron and copper and increased zinc in the blood of obese children after two hypocaloric diets. *Biol Trace Elem Res.* 1997;57:97-104.

[21] Rodríguez-Rodríguez E, López-Sobaler AM, Andrés P, Aparicio A, Navia B, Ortega RM. Modification of iron status in young overweight/mildly obese women by two dietary interventions designed to achieve weight loss. *Annals of Nutrition and Metabolism.* 2007;51:367-73.

[22] Kretsch MJ, Fong AKH, Green MW, Johnson HL. Cognitive function, iron status, and hemoglobin concentration in obese dieting women. *Eur J Clin Nutr.* 1998;52:512-8.

[23] Amato A, Santoro N, Calabro P, Grandone A, Swinkels DW, Perrone L, et al. Effect of body mass index reduction on serum hepcidin levels and iron status in obese children. *Int J Obes (Lond).* 2010;34:1772-4.

[24] Test methods for evaluating solid waste physical/chemical methods (SW-846). Version 1. Washington, D.C.: U.S. Environmental Protection Agency, Office of Solid Waste; Springfield, Va.: U.S. Dept. of Commerce, National Technical Information Service, 1996.

[25] Frazer DM, Wilkins SJ, Becker EM, Vulpe CD, McKie AT, Trinder D, et al. Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. *Gastroenterology.* 2002;123:835-44.

[26] Zimmermann MB, Zeder C, Muthayya S, Winichagoon P, Chaouki N, Aeberli I, et al. Adiposity in women and children from transition countries predicts decreased iron absorption, iron deficiency and a reduced response to iron fortification. *Int J Obes (Lond).* 2008;32:1098-104.

[27] Nemeth E, Tuttle MS, Powelson J, Vaughn MB, A. D, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization.

Science. 2004;306:2090-3.

[28] Andriopoulos B, Jr., Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, et al. *Bmp6* is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet. 2009;41:482-7.

[29] Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein *Bmp6* induces massive iron overload. Nat Genet. 2009;41:478-81.

[30] Wrana JL. Signaling by the TGFbeta superfamily. Cold Spring Harb Perspect Biol. 2013;5:a011197.

[31] Camaschella C. Genetics of cardiac repolarization. Nat Genet. 2009;41:386-8.

[32] Parrow NL, Fleming RE. Bone morphogenetic proteins as regulators of iron metabolism. Annu Rev Nutr. 2014;34:77-94.

[33] Enns CA, Ahmed R, Wang J, Ueno A, Worthen C, Tsukamoto H, et al. Increased iron loading induces *Bmp6* expression in the non-parenchymal cells of the liver independent of the BMP-signaling pathway. PLoS One. 2013;8:e60534.

[34] Arndt S, Maegdefrau U, Dorn C, Schardt K, Hellerbrand C, Bosserhoff AK. Iron-induced expression of bone morphogenic protein 6 in intestinal cells is the main regulator of hepatic hepcidin expression in vivo. Gastroenterology. 2010;138:372-82.

[35] Kautz L, Besson-Fournier C, Meynard D, Latour C, Roth MP, Coppin H. Iron overload induces *Bmp6* expression in the liver but not in the duodenum. Haematologica. 2011;96:199-203.

Figure Legends

Figure 1. Percentage of fat and lean body mass of the LL, HL, and HH groups at the 14th week (A) and the 30th week (B).

Figure 2. Liver iron concentration expressed as micrograms of iron per gram wet tissue in the LL, HL, and HH groups. Values are presented as means $\pm$ SEM, n=6 to 8 per group. Means without common letters differ significantly ($P<0.05$).

Figure 3. Total liver iron content calculated by multiplying liver iron concentration and liver weight (A), and liver iron content relative to body weight (B) in the LL, HL, and HH groups. Values are presented as means $\pm$ SEM, n=6 to 8 per group. Means without common letters differ significantly ($P<0.05$).

Figure 4. Hepatic ferritin protein levels in the LL, HL, and HH groups. A representative experiment is shown (A). The intensity of the ferritin band was densitometrically measured and normalized to the protein level of β -actin (B). Values are presented as means $\pm$ SEM, n=6 to 8 per group. Means without common letters differ significantly ($P<0.001$).

Figure 5. Correlations between liver iron status and body fat mass. Correlation between total liver iron concentration and fat mass at the 30th week (A), $r=-0.627$, $P<0.01$. Correlation between liver ferritin protein level and fat mass at the 30th week (B), $r=-0.687$, $P<0.001$.

Figure 6. mRNA levels of duodenal iron transporters. (A) Duodenal *Dcytb* expression, $P=0.05$. (B) Duodenal *Heph* expression, $P=0.553$. Values are presented as means $\pm$ SEM, n=6 to 8 per group. Means without common letters differ significantly ($P<0.05$).

Figure 7. (A) *Hamp* mRNA levels in liver tissues of the LL, HL, and HH groups, $P=0.117$.

Values are presented as means $\pm$ SEM, $n=6$ to 8 per group. (B) Correlation between *Hamp* mRNA expression and iron concentration in liver, $r=0.675$, $P<0.001$.

Figure 8. *Bmp6* mRNA levels in liver (A) and duodenum (B) of the LL, HL, and HH groups.

Values are presented as means $\pm$ SEM, $n=6$ to 8 per group. Means without common letters differ significantly ($P<0.01$).

Figure 9. (A) Correlation between duodenal *Bmp6* mRNA expression and liver iron concentration ($\mu\text{g/g}$ tissue), $r=0.576$, $P<0.05$. (B) Correlation between duodenal *Bmp6* mRNA expression and liver *Hamp* mRNA expression, $r=0.517$, $P<0.05$.

Figure 10. Hepatic p-Smad/Smad ratio in the LL, HL, and HH groups. Blots were stripped and reprobed in the order of p-Smad, Smad, and β -actin. A representative experiment is shown (A). The intensity of the bands was densitometrically measured to calculate the ratio of p-Smad1/5 relative to unphosphorylated-Smad1 (B). Values are presented as means $\pm$ SEM, $n=6$ to 8 per group. Means without common letters differ significantly ($P<0.001$).

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Table 1. Ingredient composition of the diets fed to mice

	Control diet (g) (10% kcal Fat)	High-fat diet (g) (60% kcal Fat)
Casein, 30 Mesh	189.6	258.4
L-Cystine	2.8	3.9
Corn Starch	298.6	0
Maltodextrin 10	33.2	161.5
Sucrose	331.7	88.9
Cellulose, BW200	47.4	64.6
Soybean Oil	23.7	32.3
Lard	19.0	316.6
Mineral Mix ^a	9.5	12.9
DiCalcium Phosphate	12.3	16.8
Calcium Carbonate	5.2	7.1
Potassium Citrate, 1 H ₂ O	15.6	21.3
Vitamin Mix ^b	9.5	12.9
Choline Bitartrate	1.9	2.6
FD&C Yellow Dye #5	0.05	0.06
Total	1000	1000
kcal/g diet	3.85	5.24

^a 10 g of mineral mix (Research Diets Inc., #S10026) provides 1.0 g of Na, 1.6 g of Cl, 0.5 g of Mg, 0.33 g of S, 59 mg of Mn, 37 mg of Fe, 29 mg of Zn, 6 mg of Cu, 2 mg of Cr, 1.6 mg of Mo, 0.16 mg of Se, 0.9 mg of Fl, 0.2 mg of I, and 3.99 g of sucrose.

^b 10 g of vitamin mix (Research Diets Inc., #V10001) provides 4000 IU of vitamin A, 1000 IU of vitamin D3, 50 IU of vitamin E, 0.5 mg of biotin, 10 µg of vitamin B₁₂, 2 mg of folic acid, 30 mg of niacin, 16 mg of pantothenic acid, 7 mg of vitamin B₆, 6 mg of vitamin B₂, 6 mg of vitamin B₁, and 978.42 mg of sucrose.

Table 2. Body weight, fat mass, and lean mass of LL, HL, and HH groups

	LL	HL	HH
Body weight at 0 week (g)	15.4 ± 0.3	15.9 ± 0.3	15.8 ± 0.2
Body weight at 15 th week (g)	26.8 ± 1.1 ^b	40.9 ± 1.8 ^a	39.4 ± 2.0 ^a
Body weight at 31 st week (g)	35.8 ± 1.9 ^c	40.6 ± 1.4 ^b	63.1 ± 1.5 ^a
Fat mass at 14 th week (g)	6.1 ± 1.1 ^b	19.0 ± 1.1 ^a	17.1 ± 1.4 ^a
Fat mass at 30 th week (g)	11.4 ± 2.0 ^c	15.8 ± 1.0 ^b	37.5 ± 1.1 ^a
Lean mass at 14 th week (g)	17.4 ± 1.8	18.4 ± 1.2	18.4 ± 1.5
Lean mass at 30 th week (g)	20.4 ± 0.6	20.7 ± 0.6	22.0 ± 0.4

Values are presented as means ± SEM. Means in a row without common letters differ significantly ($P<0.001$).