

The double burden of malnutrition:

Obesity and Iron deficiency

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Abstract

Background: The world faces a “double burden” of malnutrition; this is true especially in transition countries like Mexico. The co-existence of obesity and iron deficiency (ID) within a person has been clearly demonstrated in several studies but the mechanisms linking them remain largely unknown.

Objectives: To investigate possible mechanisms that link obesity and iron status through the following specific objectives: a) reviewing the existing literature; b) investigating the coexistence of obesity and iron deficiency at the national level in Mexico; c) assessing and comparing iron absorption and blood volume (BV) in healthy, non-anemic women from different body mass index (BMI) categories, and evaluating if ascorbic acid improves iron absorption in overweight (OW) and obese (OB) women; d) evaluating if differences in BV explains reduced iron status in OW/OB women; and e) evaluating whether fat loss in obese subjects decreases inflammation and serum hepcidin and thereby improves iron absorption.

Methods: **a)** A literature review was conducted using Google Scholar and PubMed search engines; **b)** data from the 1999 Mexican Nutrition Survey, which included 1174 children (aged 5–12 y) and 621 nonpregnant women (aged 18–50 y), was used to assess the relationship between BMI, dietary iron, and dietary factors affecting iron bioavailability, iron status, and inflammation; **c & d)** healthy, non-anemic Swiss women (n=62) (BMI 18.5-39.9 kg/m²) consumed a stable-isotope labelled wheat-based test meal, without (-AA) and with (+AA) 31.4 mg ascorbic acid. We measured iron absorption, body composition by dual energy X-ray absorptiometry (DXA), blood volume by carbon monoxide (CO)-rebreathing method, iron status, inflammation and serum hepcidin; **e)** We performed a 6-month, prospective study in OB (BMI, $\geq 35 < 45$ kg/m²) adults from Mexico (n=..), who had recently undergone laparoscopic sleeve-gastrectomy (LSG). At 2 months and 8 months post-LSG, subjects consumed a test drink with 6mg ⁵⁷Fe as ferrous sulfate and were intravenously infused with 100 µg ⁵⁸Fe as iron citrate and we measured body composition by DXA, iron status, hepcidin and inflammation.

Results: **a)** Obesity-related subclinical inflammation and its effects on hepcidin levels seem to be the most plausible explanation for the link between ID and obesity; **b)** the risk of iron deficiency in OB women and children was 2-4 times that of normal-weight individuals at similar dietary iron intakes. In addition, we found that C-reactive protein but not iron intake was a strong negative predictor of iron status, independently of BMI ($P < 0.05$); **c)** dietary iron absorption was lower in OW/OB versus normal weight subjects (Geometric mean (95%CI): 12.9 (9.7, 16.9%) vs 19.0 (15.2, 23.5)%, $P=0.049$). Moreover, the enhancing effect of ascorbic acid on iron absorption in overweight/obese (28%) was only half that in normal weight women (56%); **d)** OW/OB women presented higher absolute blood volume and lower serum iron compared to the normal weight group. BV ($r^2=0.22$, $\beta=-0.29$, $P=0.02$) was a negative predictor for serum iron when adjusted for body iron stores. We developed an equation to calculate BV in OW and OB women considering weight, height and lean body mass; **e)** Fat loss lead to a reduction of inflammation (Interleukin-6) and hepcidin concentrations. In iron-deficient subjects ($n=17$), iron absorption significantly increased after fat loss (Geometric mean (95%CI): 9.7% (6.5-14.6) to 12.4% (7.7-20.1) ($P=0.03$), while in iron sufficient subjects ($n=21$), it did not change (Geometric mean (95%CI): 5.9% (4.0-8.6) and 5.6% (3.9-8.2)) ($P=0.81$)).

Conclusion: Increased hepcidin concentrations, along with subclinical inflammation, limits dietary iron absorption in subjects with excessive body fat, especially in iron deficient individuals. Due to a dilutional effect of blood volume, 'true' hypoferremia might be overestimated in populations with a high prevalence of obesity when using serum iron as an indicator. OW/OB individuals may require: higher dietary iron intake combined with iron absorption enhancers to keep their iron status in balance; and a reduction of the obesity-related inflammatory process in order to ensure adequate iron absorption.

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Chapter 1

General Introduction

1.1 The double burden of iron deficiency and obesity: general perspective

Overweight and iron deficiency are two of the most common health problems worldwide (1). The “double burden” of malnutrition at the individual level has been clearly demonstrated by the co-existence of obesity and iron deficiency in children and adults (2). Iron is a nutritionally essential trace element that is critical for optimal physical and cognitive performance (3). Iron deficiency is one of the most prevalent nutrition deficiencies in the world, affecting around one-fourth of the world’s women and children, and is one of the most common causes of anemia. Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (4). Currently the prevalence of overweight and obesity is increasing throughout the world’s population and it poses such a serious problem that the WHO has described it as a ‘global epidemic’ (1). In 2008, 35% of adults aged 20+ were overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$) (34% men and 35% of women) worldwide (5). Once considered a problem only in high-income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban settings. For example in Mexico, over the last years, the prevalence of overweight and obesity in women (12-49 years) increased from 62% in 2000 to 71% in 2012 (6). Moreover, the prevalence of iron deficiency in the same population (Mexican women) is still high (18%)(5).

Even though the nutritional transition is a global phenomenon there are several unique elements of the nutritional conditions in some of the low- and middle-income countries that must be considered. First, micronutrient malnutrition remains an important public health problem in these countries, undermining healthy growth and development in significant segments of the population (7). Second, contrary to high-income countries, food choices in low- and middle-income countries may remain limited, because of either market limitations or cost (3). And third, low income and limited access to education may pose constraints on people’s ability to seek and secure healthier foods and lifestyle (8). The urban settings are the most at risk of overweight and obesity because of the changes in the habitual diet towards one of high fat, energy-dense, micronutrient-poor foods, associated with a

sedentary lifestyle (9). Although higher socioeconomic status has been linked to higher risk of obesity in developing countries, the obesity epidemic is also shifting to the middle and lower classes (10, 11). As a result, a high prevalence of the two conditions, excess weight and micronutrient deficiencies, is common.

While obesity is clearly a major public health issue, iron-deficiency anemia is related to impaired cognitive development in preschool-aged children and to reduced work productivity, cognitive and behavioral problems in adults (12, 13). The combination of overweight and obesity with iron deficiency may have profound detrimental effects on health, especially in early childhood. Consequences may range from poor growth and cognitive development in childhood to increased risk for non-communicable diseases later in life. However there is no evidence for the health consequence of this combination and therefore it continues to be a subject of research.

1.2 Overweight, obesity and inflammation

Overweight and obesity results from an imbalance that develops between energy intake and expenditure when adipose tissue undergoes hypertrophic/hyperplastic expansion during energy oversupply (14). Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health (15). The body mass index (BMI) is considered a crude population measure of excess weight. BMI is calculated with a person's weight (in kilograms) divided by the square of his or her height (in meters). A person with a BMI equal to or more than 25 is considered overweight and a person with a BMI of 30 or more is generally considered obese. These standard definitions of overweight (BMI \geq 25 kg/m²) and obesity (BMI \geq 30 kg/m²) have been mainly derived from populations of European descent (9).

Adipose tissue has been traditionally subclassified into two distinct forms, white and brown, with different functions. Brown adipose tissue is a specialized thermoregulatory organ by nonshivering mechanisms, fuelled primarily by the oxidation of fatty acids, through the presence of a specific mitochondrial “uncoupling protein,” UCP1 (uncoupling protein-1), which dissipates the proton

gradient across the inner mitochondrial membrane. White adipose tissue has evolved to store excess energy and to mobilize free fatty acids when energy is needed (16). It has been suggested that white adipose tissue becomes hypoxic as adipocyte size and tissue mass expand in obesity, and that this underlies the development of inflammation and cellular dysfunction (17). Moreover, when hypertrophied adipose tissue cannot satisfy its storage function, excess free fatty acids are exposed to lipid-intolerant organs causing lipotoxicity and inducing low-grade inflammation in the adipose tissue (18). Therefore, adipocyte hypertrophy together with local tissue hypoxia, triggers adipokine overproduction enhancing macrophage infiltration in obesity.

Macrophages are regarded as being especially important in the inflammatory response that occurs in the tissue as obesity develops, and the obese state is associated with a major recruitment of these cells. However, increased infiltration of natural killer cells and T cells, as well as of macrophages, is evident in adipose tissue of obese subjects (5). Macrophages are the primary mediators of the innate immune response, and are important participants in adaptive immunity. They recognize and phagocytose foreign organisms, release antimicrobial peptides, secrete molecules that attract other immune cells to areas of infection, and present antigens to lymphocytes. Activated macrophages release stereotypical profiles of cytokines and biologically active molecules such as tumor necrosis factor (TNF)- α , and interleukines (IL)-6, and IL-1(19). Moreover, TNF- α promotes the release of IL-6, which in turn stimulates acute phase reactant production (C-reactive protein, CRP; and alpha-1-acid glycoprotein, AGP) by the hepatocytes in the liver (20). In recent years the associations between inflammation and obesity, including impaired insulin resistance, type II diabetes and coronary heart disease, have been extensively investigated. A particular focus of these investigations has been the association of the acute-phase protein, CRP, with obesity and cardiovascular outcomes (21). It has been shown that weight reduction improves the inflammatory profile of obese subjects through a decrease of proinflammatory factors and an increase of anti-inflammatory molecules (22).

In conclusion, obesity related inflammation impairs health in several ways and raises the risk of adult morbidity and mortality. It has been proposed that as a chronic inflammatory condition, obesity impairs iron homeostasis leading to iron

deficiency but the underlying pathophysiological mechanisms have not been fully understood yet (23).

1.3 Iron metabolism

Iron is an essential trace element for nearly every living organism. Dietary iron consists primarily of either nonheme iron or heme iron, and these are absorbed by different mechanisms (24). Nonheme iron is the largest component of dietary iron (approximately 85%) and is found mainly in plant-based foods. For humans the absorption of nonheme iron is influenced by individual iron status and by the balance between several potentially enhancing and inhibitory factors in the diet (25). For example, ascorbic acid (vitamin C), the most potent enhancer of nonheme iron absorption even in the presence of inhibitors such as phytates, tannates and calcium, can reduce ferric iron from food to the better-absorbed ferrous iron by 75-98%. Heme iron which comes mainly from hemoglobin and myoglobin in animal tissues (such as meat, poultry, and fish) is efficiently absorbed (about 25%, two to three times greater than that of nonheme iron) and this absorption is less affected by the overall composition of the diet (24).

Iron is an essential cofactor that for several biological processes, including oxygen and electron transport, gas sensing, DNA synthesis. However iron is also highly toxic due to its intrinsic ability to generate free radicals through Fenton reactions. Consequently, organisms have developed sophisticated pathways to import, chaperone, sequester, and export this metal ion (26). Most of the iron in the body is contained in the erythrocytes (about 1 mg of iron per milliliter of erythrocytes), whereas the total blood plasma volume contains only 2–4 mg of iron, bound to the iron-transport protein transferrin. The life span of human erythrocytes is around 120 days; subsequently erythrocytes are degraded by macrophages that recycle their iron content. The recycling of iron for erythrocyte production generates a stream of around 15–25 mg of iron a day (27). The physiological reserve of iron in the hepatocytes (approximately 1 g) further protects against imbalances in body iron homeostasis (28). Other cells contain and need less iron, and some are able to use non-transferrin bound iron as well. However, almost all serum iron is bound to transferrin (Tf). Plasma Tf delivers iron to tissues through surface receptors specific

for Tf called Tf receptor (TfR) that is the exclusive source of iron for erythropoiesis. The receptors bind to the Fe-Tf complex at the cell surface and carry the complex into the cell, where iron is released. The iron-Tf is mostly destined for hemoglobin synthesis by erythrocyte precursors in the bone marrow. This Tf cycle involves membrane trafficking along specific cellular compartments including plasma membrane, early endosomes, and recycling endosomes. Ferric iron released from the Tf-TfR complex is reduced within endosomes. The reduction of iron is an essential step in the Tf cycle, which is the dominant pathway for iron uptake by red blood cell precursors (29). Once reduced, the iron will be transported across the endosomal membrane and mobilized out of endosomes by divalent metal transporter DMT1 (30). The mechanism immediately following the endosomal iron release is not well understood. Within the cytoplasm, a proportion of iron is incorporated into the cytoplasmic iron-containing proteins and extramitochondrial iron-sulfur (Fe-S) clusters (31). Excess amount of iron is stored in cytoplasmic ferritin and is readily mobilized during periods of high iron demand (27, 32).

Dietary iron absorption in humans is regulated by physiological mechanisms that need to be flexible to handle variations in iron bioavailability, fluctuating from 5–12% in vegetarian diets to 14–18% in mixed diets (33). Despite this variation in bioavailability as well as differences in iron requirement due to growth or sporadic blood loss, iron stores are constant in most humans consuming an iron-adequate diet. Iron homeostasis in the body involves three key cell types: duodenal enterocytes that absorb dietary iron, macrophages which recycle iron from erythrocytes and other cells, and hepatocytes which store iron and can release it when needed. The first step takes place predominantly in the duodenum and upper jejunum. Iron crosses the apical membrane, followed by variable storage in cytoplasmic ferritin, it is then transferred across the enterocyte basolateral membrane into the blood. Two transporters appear to mediate the entry of dietary iron into the mucosal cells. Heme carrier protein 1 (HCP 1) transfers intact heme molecules and is upregulated by hypoxia and iron deficiency. Transport of nonheme iron from the intestinal lumen into the enterocytes is mediated by the divalent metal transporter 1 (DMT 1). DMT 1 transports only ferrous iron therefore ferric iron must be first reduced to ferrous iron by reducing agents, such as ascorbic acid and/or mucosal ferric reductases, of which duodenal cytochrome b (Dcytb) is being considered the most important. Efflux of iron across the basolateral membrane of

the duodenal enterocytes into plasma is mediated by the transport protein ferroportin 1, and the iron oxidase, hephaestin (34). Ferroportin is an iron exporter on the surface of absorptive intestinal enterocytes, hepatocytes, macrophages, and placental cells, all of which release iron into plasma (35). Therefore, the magnitude of the flux is relative to the concentration of ferroportin on the cell surface, which is largely determined by the circulating concentration of ferroportin's ligand, hepcidin (36).

Since the body misses a mechanism for the active excretion of iron, iron balance is predominantly regulated at the point of absorption (33). Hepcidin, a 25-amino acid peptide hormone synthesized primarily by hepatocytes, is recognized as a major regulator of body iron metabolism. Hepcidin regulates the major iron flows into plasma: dietary iron absorption in the duodenum (1–2 mg/day), iron recycling from erythrocytes (20 mg/day) and the recovery of iron from storage in hepatocytes and macrophages (a few mg/day depending on iron needs) (37). Though in quantitative terms the liver is the principal source of circulating hepcidin, macrophages, pancreatic islet cells (38) and adipose cells (39) can also express hepcidin. Hepcidin production is regulated by iron, erythropoietic activity, hypoxia and inflammation (28, 36). The cellular targets and the mode of action of hepcidin have been the subject of numerous recent studies. Functional data reveal that, once released, hepcidin acts as a negative regulator of both intestinal iron absorption and macrophage iron release suggesting that these tissues are the primary targets for hepcidin. A number of in vitro studies have demonstrated that hepcidin regulates cellular iron homeostasis by controlling ferroportin expression. Upon reaching its target tissues hepcidin binds to ferroportin and induces its internalization and degradation (40). This, in turn, impairs the release of iron from enterocytes, macrophages, and hepatocytes into the circulation consequently reducing iron availability (28, 36).

Plasma hepcidin levels are regulated by different stimuli, including cytokines, plasma iron, anemia, and hypoxia. Plasma iron and iron stores are the main regulators of hepcidin expression by the liver. Some studies suggest that body iron stores regulate hepcidin independently of the short-term effects of plasma iron concentration (37). A feedback mechanism exists that suppresses hepcidin in people who are iron deficient. In contrast, people with iron overload downregulate iron absorption via hepcidin. Dysregulation of hepcidin expression results in iron

disorders. Overexpression of hepcidin leads to the anemia of chronic disease, while low hepcidin production results in hereditary hemochromatosis (HFE) with consequent iron accumulation in vital organs (41).

Ferritin concentration and hemosiderin reflect the body iron stores. They store iron in an insoluble form and are present primarily in the liver, spleen, and bone marrow. Apart from iron losses due to menstruation, other bleeding or pregnancy, iron is highly conserved and not readily lost from the body (42). Three main variables determine erythropoiesis-associated demands for iron: tissue oxygenation, erythrocyte turnover, and erythrocyte loss from hemorrhage. Tissue oxygen supply and erythrocyte production usually remain constant throughout adulthood in the absence of hemorrhage, disease, or altered physical activity. Although the etiology of iron deficiency anemia is multifaceted, it generally arises when the balance of iron intake, iron stores, and the body's loss of iron are insufficient to fully support production of erythrocytes.

1.4 Obesity and iron deficiency: The connection

Although obesity and iron deficiency represent opposite ends of the spectrum of over- and under-nutrition, they appear to be linked: overweight individuals are at higher risk of iron deficiency than normal-weight individuals (23). Within an individual, obesity may occur in parallel with iron deficiency, due to shared underlying determinants or physiologic links. However, the mechanisms explaining reduced iron status in obese individuals have not been clearly determined. One of the potential hypotheses explaining this correlation is a reduced iron intake from poor quality diets, especially in developing countries undergoing the nutrition transition (43-46). However, it is likely that low-grade inflammation due to excess adiposity may negatively affect iron status due to hepcidin-mediated reduced iron absorption and/or increased iron sequestration rather than low dietary iron supply (23). Finally, low iron status in overweight and obese individuals might also be caused by greater iron requirements because of their larger blood volume (2). Considering the complexity and magnitude of the current worldwide prevalence of obesity, there is a need to provide more information about the possible impact of adiposity and inflammation on iron homeostasis. This is particularly important for

populations in low- and middle- income countries, where the rapid increase in overweight could impair efforts to control iron deficiency.

1.4 Rationale and research questions

The prevalence of overweight and obesity has especially increased in fast-growing developing countries where iron deficiency remains an important public health problem. A potential interaction between adiposity and iron deficiency could have important public health consequences. However most of the information regarding the co-occurrence of obesity and iron deficiency comes from cross-sectional studies in developed countries. Few studies have examined this association in transition countries, where iron deficiency and obesity are both prevalent. The cross-sectional studies have generally found higher rates of iron deficiency in overweight children and adults compared to normal weight individuals. However, it is unclear whether this association is due to 1) poor dietary iron intake, 2) dilution as a result of increased blood volume, 3) reduced iron absorption as a result of adiposity-related inflammation or a combination of the three scenarios. The main objective of this thesis was to understand the underlying mechanism for the association between obesity and iron status.

1.5 Research questions

The following research questions are addressed in this thesis:

1. What is the scientific evidence for the relationship between overweight/obesity and iron deficiency?
2. What is the relationship between BMI, dietary iron intake, dietary factors affecting iron bioavailability, inflammation [C-reactive protein (CRP)], and iron status in Mexico, a transition country where both obesity and ID are common?
3. How does BMI affect iron absorption? Does ascorbic acid improve iron absorption in overweight and obese subjects similar to normal weight subjects?
4. What is the relationship between blood volume and iron deficiency in obese subjects?
5. What is the effect of weight loss on iron absorption and hepcidin concentrations in obese subjects?

Chapter 2 comprises a narrative review of the literature addressing the association between obesity and iron deficiency, including dilutional hypoferrremia, poor dietary iron intake, increased iron requirements, and/or impaired iron absorption in obese individuals. In **Chapter 3** we examine the association between BMI, dietary iron intake, and dietary factors affecting iron bioavailability, iron status, and inflammation in two vulnerable populations in Mexico: school-age children and women of reproductive age. **Chapter 4** presents an iron absorption study investigating differences in iron absorption over a range of BMI values, i.e. normal weight, overweight or obese. In addition, we assess whether a luminal enhancer of dietary iron absorption (ascorbic acid) is also effective in overweight and obese individuals. The influence of blood volume on iron status in overweight and obese women and the need for a new formula to calculate blood volume in this population is investigated in **Chapter 5**. **Chapter 6** is focusing on the effect of weight loss on iron absorption in association with the modification of the inflammatory profile and hepcidin concentrations. In the General Discussion (**Chapter 7**), the main findings and conclusion of this thesis are summarized and discussed.

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Chapter 2

Does Obesity Increase Risk for Iron Deficiency? A Review of the Literature and the Potential Mechanisms.

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Abstract

Increasing obesity is a major global health concern while at the same time iron-deficiency anemia remains common worldwide. Although these two conditions represent opposite ends of the spectrum of over- and under-nutrition, they appear to be linked: overweight individuals are at higher risk of iron deficiency than normal-weight individuals. Potential explanations for this association include dilutional hypoferremia, poor dietary iron intake, increased iron requirements, and/or impaired iron absorption in obese individuals. Recent evidence suggests obesity-related inflammation may play a central role through its regulation of hepcidin. Hepcidin levels are higher in obese individuals and are linked to subclinical inflammation; this may reduce iron absorption and blunt the effects of iron fortification. Thus, low iron status in overweight individuals may result from a combination of nutritional (reduced absorption) and functional (increased sequestration) iron deficiency. In this review, we focus on subclinical inflammation in obesity, and its effect on hepcidin levels, as the most plausible explanation for the link between iron deficiency and obesity.

Introduction

Increasing obesity is currently a major health concern in both developed and developing countries. The World Health Organization's (WHO) latest global projections indicate that in 2005 approximately 1.6 billion adults (age 15+) were overweight and at least 400 million adults were obese (1). At the same time, iron-deficiency anemia remains common worldwide (2). Although these two conditions represent opposite ends of the spectrum of over- and under-nutrition, they appear to be linked: overweight individuals are at higher risk of iron deficiency than normal-weight individuals. Potential explanations for this association include dilutional hypoferrremia, poor dietary iron intake, increased iron requirements, and/or impaired iron absorption in obese individuals. Recent evidence suggests obesity-related inflammation may play a central role through its regulation of hepcidin, and thereby, iron metabolism. In this review, we focus on subclinical inflammation in obesity, and its effect on hepcidin levels, as the most plausible explanation for the link between iron deficiency and obesity.

Epidemiological studies

Cross-sectional studies have consistently shown an association between adiposity and poor iron status, defined in most studies as hypoferrremia (a low serum iron concentration). The inverse relationship between iron status and adiposity was first published in 1962, when Wenzel and colleagues (3) unexpectedly found a significantly lower serum iron concentration in obese adolescents compared with controls. One year later, Seltzer and colleagues (4) reported that serum iron levels and transferrin saturation index were significantly lower in obese male and female adolescents than in lean individuals, without differences in hemoglobin and hematocrit concentrations. Studies performed since then in children (5 – 10) and adults (11 – 18) have shown similar results. For example, in the U.S. National Health and Nutrition Examination Surveys ((NHANES) I, III, IV and 2003–2004), overweight toddlers, children, adolescents, and adults were more likely to be iron deficient than those who were not overweight in these age groups (7, 8, 10, 12, 19). In a cross-sectional study, overweight Israeli children and adolescents had lower iron status compared with normal-weight individuals (6). However, a study by

Ozata and colleagues reported no difference in iron status between obese and normal-weight individuals (20).

All of these studies were done in industrialized countries. A recent analysis of data from the 1999 Mexican Nutrition Survey, including 1,174 children aged 5 – 12 years and 621 non-pregnant women aged 18 – 50 years, has found a similar relationship between adiposity and iron status (21). In this study, body mass index (BMI) and BMI Z-scores were calculated, intakes of dietary iron, ascorbic acid (an enhancer of iron absorption), and calcium and fiber (inhibitors of iron absorption) were estimated, and iron deficiency (ID) was defined as either a low serum iron, or an elevated total iron-binding capacity (TIBC) and low transferrin saturation (TS). Regression analyses were done to determine relationships between hemoglobin, iron status, dietary nutrient intakes, inflammation (assessed by C-reactive protein, CRP), and BMI. The results showed that the prevalence of obesity was 3.5 % and 25.3 % in children and women, respectively. The prevalence of ID in obese women and children was significantly higher than in normal-weight subjects, (OR = 1.92 and 3.96, respectively, $p < 0.05$). Serum iron levels were lower in obese than in normal-weight women (62.57 $\mu\text{g/dL}$ vs. 72.35 $\mu\text{g/dL}$, $p = 0.014$) and TIBC was higher in obese than in normal-weight children (399.76 $\mu\text{g/dL}$ vs. 360.55 $\mu\text{g/dL}$, $p < 0.0001$). There were no significant associations between dietary intakes of iron or ascorbic acid and BMI. CRP concentrations were four times higher in obese women and children than in normal-weight counterparts ($p < 0.05$). Sub-clinical inflammation (elevated CRP), but not iron intake, was a strong negative predictor of iron status independent of BMI ($p < 0.05$). Thus, compared to normal-weight subjects, obese Mexican women and children have a two- to four-fold increased risk for ID despite comparable dietary iron intakes. Impaired iron metabolism due to obesity-related inflammation may play a role (further discussed below under ‘Mechanisms’).

Transition countries like Mexico are undergoing rapid dietary and lifestyle changes that produce a “double burden” of undernutrition and overweight (22, 23). Three transition countries in which this is occurring are Thailand, Morocco, and India (24 – 26). In Bangkok, Thailand, it is estimated nearly one-third of women are overweight and 24% are anemic (27, 28). In Morocco, 24% of children less than 5 years are stunted in growth and 30 % of school-age children are anemic (29); at the same time, 9 % of school-age children are overweight (30, 31). Similarly, in middle-

class Indian school children, anemia is present in 19 – 88 %, while overweight affects 9 – 29 % (32). In many lower income countries, the prevalence of overweight is increasing at two to four times the rate of the industrialized world [33]. The major adverse effects of iron deficiency are impaired physical and cognitive development in children (34) and poorer pregnancy outcome in women (2). A potential interaction between the “double burden” of adiposity and iron deficiency has not been examined in transition countries.

Thus, we decided to study the association between weight status, iron deficiency, and the response to iron fortification in children from transition countries. We analyzed data from baseline (n = 1688) and intervention (n=727) studies in children in Morocco and India to look for associations between BMI Z-scores and baseline hemoglobin, serum ferritin and transferrin receptor (TfR), whole blood zinc protoporphyrin (ZPP) and body iron stores, and changes in these measures after provision of iron. In this sample of children, 42 % were iron-deficient and 6.3 % were overweight. A higher BMI Z-score predicted poorer iron status (as judged by body iron calculated from the serum ferritin-to-transferrin receptor ratio)(35) at baseline ($p < 0.001$) and less improvement in body iron during the interventions ($p < 0.001$) [14]. The baseline data of this study are consistent with data on iron status and adiposity in studies discussed above from industrialized countries, for example in the U.S. from NHANES III (where 14 % were at risk for overweight, 10 % were over-weight, and 3 % were iron-deficient) (8), but demonstrate this same relationship exists in children in transition countries with high rates of iron deficiency and lower rates of adiposity. In the previous studies from industrialized countries, a limitation was the use of iron status indicators, such as serum ferritin (SF) and serum transferrin, that are acute-phase proteins (2) and may be confounded by the adipose-related inflammation. In the Moroccan and Indian children, we used multiple indicators of iron status, including TfR and ZPP, two measures less likely to be confounded by inflammation (36, 37). This study clearly demonstrates the negative impact of adiposity on response to iron fortification. It suggests the rapid increase in overweight in transition countries could impair their efforts to control iron deficiency in children. These findings need to be confirmed in other populations and settings, but imply that interactions of the “double burden” of malnutrition during the nutrition transition may have adverse consequences.

Absorption studies with stable iron isotopes

To investigate if adiposity was associated with dietary iron absorption, we studied (n=92) premenopausal Thai women (18–50 years of age) with a maximum body weight of 70 kg who were recruited in metropolitan Bangkok (14). About 20 % were iron-deficient and 22 % were overweight. They all received the same iron isotope-labeled reference meal of steamed white rice and vegetable soup. Each meal contained 4 mg of isotopically labeled fortification iron, as ($^{57}\text{Fe}/^{58}\text{Fe}$) ferrous sulfate. A second blood sample was drawn 14 days later and analyzed for isotopic composition by multicollector negative thermal ionization mass spectrometry to calculate fractional iron absorption [38]. We found that, independent of iron status, a higher BMI was associated with decreased iron absorption ($p < 0.030$). After correcting for differences in iron status among subjects using SF (39), in a multivariate regression including age, hemoglobin (Hb), CRP, and BMI, fractional iron absorption was negatively correlated with CRP (standardized $\beta = 0.422$; $p < 0.001$) and BMI (standardized $\beta = 0.106$; $P < 0.05$). The relationship between BMI and fractional iron absorption is shown in **Figure 2.1**

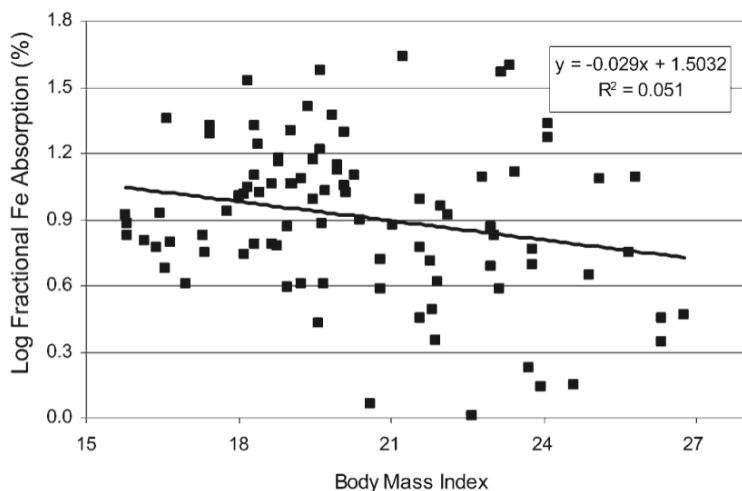


Figure 2.1 The relationship between log fractional iron absorption (%) and body mass index in healthy premenopausal Thai women (n=92) who consumed meals of rice and vegetables labeled with ≈ 4 mg of ($^{57}\text{Fe}/^{58}\text{Fe}$)-ferrous sulfate. To account for differences in iron status and their effect on dietary iron

absorption, iron absorption in each subject was corrected to a value corresponding to a serum ferritin of 40 µg/L (39).

Mechanisms

Several factors may explain why greater adiposity increases risk for iron deficiency. Overweight may be associated with poor quality or restricted diets low in iron, but when dietary iron intakes in overweight adults (13) and children (40) are estimated, they are not lower than in normal weight individuals. However, even if diets of overweight individuals are not lower in iron, the absorption of the iron may be reduced because increased circulating hepcidin in obesity may reduce iron absorption (see below). Iron requirements in overweight individuals may be increased due to larger blood volume and higher basal iron losses with higher body weight (41), but this has not been directly measured. In addition, overweight girls tend to mature and begin their menses at an earlier age, increasing their iron requirements (42).

The above findings in the Thai women suggest greater adiposity is associated with lower fractional iron absorption in humans, independent of iron status. Iron absorption occurs primarily in the proximal small intestine. Transport of non-heme iron from the intestinal lumen into the enterocytes is mediated by the divalent metal transporter (DMT) 1. Efflux of iron across the basolateral membrane of the duodenal enterocytes into plasma is mainly mediated by the transport protein ferroportin (2). Ferroportin is an iron exporter on the surface of absorptive intestinal enterocytes, hepatocytes, macrophages, and placental cells, all of which release iron into plasma (43). Therefore, the degree of the flux is proportional to ferroportin concentration, which is determined in large part by the circulating levels of hepcidin (44). Hepcidin, a 25-amino-acid peptide hormone with a key role in body iron regulation, is produced mainly by the liver but also, possibly, by adipose tissue (45, 46). Circulating hepcidin acts as a negative regulator of both intestinal iron absorption and macrophage iron release. When hepcidin binds to ferroportin on the target cell membrane, this induces ferroportin internalization and degradation, thus inhibiting both gastrointestinal iron absorption and iron release from the reticuloendothelial system (47, 48).

Hepatic hepcidin expression is modulated in response to body iron stores, hypoxia, and inflammation, and low-grade inflammation is a characteristic of obesity. Proinflammatory cytokines, such as interleukin-6 (IL-6), affect hepcidin gene transcription through JAK (Janus kinase)–STAT (signal transducer and activator of transcription)-3 interactions (49, 50). In addition, the adipokine leptin upregulates hepatic hepcidin expression through the JAK2–STAT3 signaling pathway (51). Thus, increased inflammation and/or leptin levels in obese individuals could reduce iron availability. Moreover, adipose tissue itself may also produce hepcidin (45). In severe obesity, while liver hepcidin expression is positively associated with increased body iron stores, adipose tissue hepcidin expression is positively correlated with BMI and may be negatively associated with transferrin saturation (45). Finally, lipocalin-2 is an iron-binding protein that is upregulated by inflammation and may help sequester iron during infections (52). It is produced by adipose tissue and its expression is increased in db/db (leptin receptor-deficient) obese mice (53). The link between adiposity-associated inflammation and iron deficiency has been suggested in cross-sectional studies, including the large Mexican study described above. Also, in a comparison of normal-weight to overweight and obese adults in the U.S. NHANES III study, serum ferritin and CRP were progressively higher with increasing BMI category, whereas serum iron and transferrin saturation were progressively lower (16).

Thus, low iron status in overweight individuals could result from a combination of nutritional (reduced absorption) and functional (increased sequestration) iron deficiency. In normal-weight individuals, SF concentrations are decreased and directly related to transferrin saturation if body iron stores are depleted (2). In contrast, in obese subjects, SF tends to be higher than in normal weight individuals and inversely related to transferrin saturation (17, 18, 54). This suggests SF, an acute-phase protein that can be elevated in inflammatory conditions even in the presence of iron deficiency, may be increased by adipose-mediated inflammation. However, this hypothesis is not supported by studies in genetically obese (ob/ob) mice (55, 56). When provided with an iron-sufficient diet, obese mice absorb twice as much iron as lean mice but have lower iron levels in the liver and small intestine (55, 56); these studies however, predate the discovery of hepcidin and hence there is no data on hepcidin concentrations available. Further, the studies were carried out in genetically obese mice (ob/ob mice), which are leptin-deficient

and this condition may differ from that of diet-induced obesity as it is usually found in humans.

To further clarify these relationships in humans, we recently compared iron status, dietary iron intake and bioavailability, as well as circulating levels of hepcidin, leptin, and IL-6 in overweight vs. normal-weight Swiss children (57). In 6- to 14-year-old normal and overweight children ($n = 121$), we measured dietary iron intake, estimated iron bioavailability, and determined body mass index standard deviation scores (BMI-SDS). In all children, we measured fasting serum ferritin, soluble transferrin receptor (sTfR), C- reactive protein (CRP), and leptin; in a subsample, we measured IL-6 ($n=68$) and serum hepcidin ($n=30$). The results showed that there were no significant differences in dietary iron intake or bioavailability comparing normal and overweight children. Although total dietary iron intake did not differ between weight groups, the intake of heme iron was significantly higher in the obese children because of their higher meat intake. These data agree with a recent adult study, in which total iron consumption and iron bioavailability did not differ between obese and non-obese subjects, whereas heme iron intake and the consumption of animal protein were higher in obese adults (11).

The prevalence of iron-deficient erythropoiesis (an increased sTfR concentration) was significantly higher in the overweight than in the normal-weight children (20 vs. 6%, $p<0.022$, with sTfR concentrations of 4.40 ± 0.77 and 3.94 ± 0.88 mg/L, respectively; $p < 0.010$). Serum hepcidin levels were significantly higher in the overweight children ($p < 0.001$). BMI- SDS significantly correlated with sTfR ($p < 0.009$) (**Figure 2.2**), serum hepcidin ($p<0.005$), and the three measures of subclinical inflammation, namely CRP ($p<0.001$), IL-6 ($p<0.001$), and leptin ($p<0.001$). In a multiple regression model, serum hepcidin was correlated with BMI-SDS ($p<0.020$) and body iron ($p < 0.029$), but not with the inflammatory markers. The findings are similar to those in a recent study in obese Italian children that found lower iron and transferrin saturation and higher circulating hepcidin levels compared with normal-weight controls (58). Thus, it appears there is reduced iron availability for erythropoiesis in overweight children and that this is unlikely due to low dietary iron supply but rather due to hepcidin-mediated reduced iron absorption and/or increased iron sequestration.

Given that obesity is associated with subclinical inflammation, and that SF is an acute-phase protein, sTfR is likely to be the best clinical measure of iron status in overweight individuals (14). In iron deficiency, sTfR is increased because cell expression of the transferrin receptor is upregulated to increase the up- take of circulating iron, primarily into marrow red cell precursors. sTfR concentrations are not significantly affected by inflammation, and are therefore useful in differentiating iron deficiency from inflammatory hypoferremia (2). Thus, in iron-deficient overweight children, sTfR and SF may be discrepant because of the confounding effect of obesity-associated inflammation on SF. In the study of Aeberli *et al.* (57), all three inflammatory markers (CRP, IL-6, and leptin) significantly increased with increasing adiposity, and CRP was a significant predictor of SF, but not TfR, independent of adiposity. These data emphasize the limitations of SF as an iron status indicator in over- weight individuals.

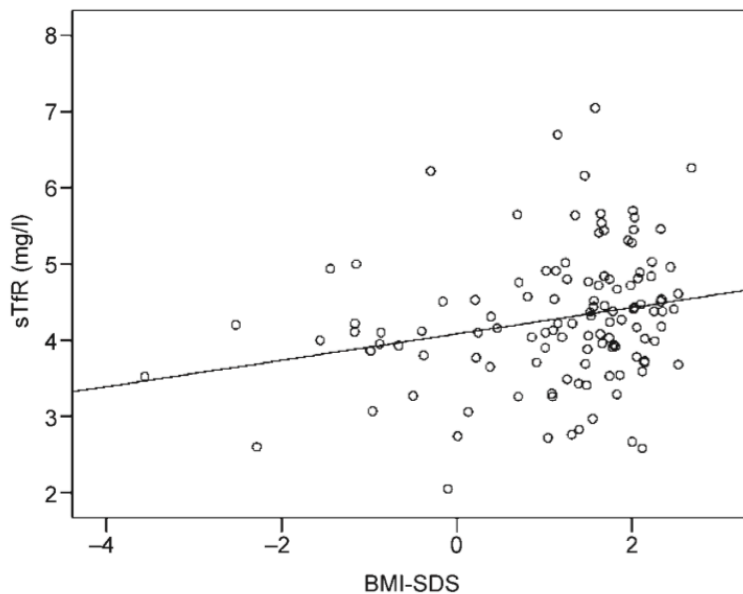


Figure 2.2 Associations between body mass index standard deviation scores (BMI-SDS) and soluble transferrin receptor (sTfR) in 6- to 14-year-old children in Switzerland ($n = 118$; $r^2=0.058$).

Prospective studies

The hypothesis that iron deficiency in obesity is due to hepcidin-mediated reduced iron absorption and/or increased iron sequestration needs confirmation by intervention studies that show that weight loss reduces circulating hepcidin levels, increases iron absorption, and improves iron status. An Italian study of fifteen obese children found that weight loss was associated with a significant decrease in circulating hepcidin levels and an increased response to iron supplementation (59). Two studies have been reported in bariatric surgery patients. In women who underwent bariatric surgery for morbid obesity (n=178), iron depletion was significantly correlated with increased markers of inflammation (CRP, orosomucoid, and haptoglobin) at baseline. Following significant weight loss six months after bariatric surgery, markers of inflammation decreased and were inversely correlated with an increase in transferrin saturation (60). In another study performed in premenopausal women, significant weight loss following restrictive bariatric surgery was associated with decreased serum hepcidin and improved iron status (sTfR, Hb, and Hct) (61).

Conclusions

The data discussed here suggest that increasing obesity may contribute to iron deficiency in high-risk populations. This is a cause for public health concern as both iron deficiency and obesity have adverse short- and long-term health effects. Overweight and obesity are major risk factors for chronic illnesses such as cardiovascular diseases. Iron deficiency affects growth and development during early life. Both are associated with impaired cognitive ability. Further research is needed to identify the specific mechanisms involved in the development of iron deficiency in obese children and reproductive-age women, and to clarify the adverse health effects. More studies on the modulation of obesity-related inflammation and the resulting effects on iron status would be welcome. Results from such studies may provide valuable information to ensure sufficient bioavailable iron for obese individuals.

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Chapter 3

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

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Abstract

Background: Obese individuals may be at increased risk of iron deficiency (ID), but it is unclear whether this is due to poor dietary iron intakes or to adiposity-related inflammation.

Objective: The aim of this study was to examine the relations between body mass index (BMI), dietary iron, and dietary factors affecting iron bioavailability, iron status, and inflammation (C-reactive protein (CRP)) in a transition country where obesity and ID are common.

Design: Data from the 1999 Mexican Nutrition Survey, which included 1174 children (aged 5–12 y) and 621 nonpregnant women (aged 18–50 y), were analyzed.

Results: The prevalence of obesity was 25.3% in women and 3.5% in children. The prevalence of ID was significantly ($P < 0.05$) higher in obese women and children compared with normal-weight subjects [odds ratios (95% CIs): 1.92 (1.23, 3.01) and 3.96 (1.34, 11.67) for women and children, respectively]. Despite similar dietary iron intakes in the 2 groups, serum iron concentrations were lower in obese women than in normal-weight women (62.6 ± 29.5 compared with 72.4 ± 34.6 $\mu\text{g/dL}$; $P = 0.014$), and total-iron-binding capacity was higher in obese children than in normal-weight children (399 ± 51 compared with 360 ± 48 $\mu\text{g/dL}$; $P < 0.001$). CRP concentrations in obese women and children were 4 times those of their normal-weight counterparts ($P < 0.05$). CRP but not iron intake was a strong negative predictor of iron status, independently of BMI ($P < 0.05$).

Conclusions: The risk of ID in obese Mexican women and children was 2–4 times that of normal-weight individuals at similar dietary iron intakes. This increased risk of ID may be due to the effects of obesity-related inflammation on dietary iron absorption. Thus, ID control efforts in Mexico may be hampered by increasing rates of adiposity in women and children.

Introduction

Cross-sectional studies in industrialized countries (1–15) have consistently shown that obese individuals are at increased risk of iron deficiency (ID). In the US National Health and Nutrition Examination Surveys (NHANES) I, III, IV, and 2003–2004, overweight toddlers, children, adolescents, and adults were more likely to be iron deficient than were those who were not overweight in these age groups (2, 3, 7, 11, 15); however, the overall prevalence of ID was relatively low in all groups. The underlying mechanisms of this association are unclear. The limited data available from small studies do not suggest that ID in obese subjects is due to lower dietary iron intake (12, 16) or increased iron requirements as a result of larger blood volume (5). A more likely explanation is that chronic adiposity-related inflammation increases hepcidin concentrations and thereby decreases intestinal iron absorption and/or increased reticuloendothelial iron sequestration (10, 15, 17).

As in many transition countries, evidence indicates that the current rapid dietary and lifestyle changes in Mexico have produced a “double burden of malnutrition” with under- and over- nutrition occurring in the same population (18). For example, in 1999 the combined prevalence of overweight and obesity was 19.5% in children and 59.6% in women, whereas for ID it was 36% and 40%, respectively (19). The combination of ID and obesity in vulnerable populations could be more detrimental to health than either of these conditions by themselves. For example, both, ID (20–23) and obesity (24) have been associated with decreased exercise capacity and impaired cognitive function.

Few studies have examined the association between ID and obesity in transition countries, where both conditions are prevalent. In addition, little is known about the mechanisms behind his observed association. Therefore, we examined the association between iron status and body mass index (BMI) in 2 vulnerable populations in Mexico: school-age children and women of reproductive age. We hypothesized that overweight and/or obese children and women have poorer iron status than do their normal-weight counterparts and that this would be associated with obesity-related inflammation rather than with differences in iron intake.

Subjects and methods

Data were obtained from the Second National Nutrition Survey (NNS-2, 1999), a large cross-sectional survey conducted by the National Institute of Public Health in Mexico between October 1998 and March 1999 (19, 25). The sampling methodology and response rates are published in detail elsewhere (25, 26). Briefly, the sample size was representative of the national level, urban and rural areas, and of 4 geographic regions: north, center, Mexico City, and south. A questionnaire was used to assess socio-demographic characteristics, and anthropometric measurements were taken. As defined by the National Household Sampling Frame of the National Institute of Statistics, Geography and Informatics, the subjects were categorized as rural if they lived in a locality with ,2500 residents; otherwise, they were categorized as urban (27). Education was assessed by completed years of schooling. For women, parity was defined as the number of live births. Height and weight were measured by using standardized equipment and protocols (25). From a subsample of children ($n = 1361$) and women ($n = 731$), venous blood and urine specimens for micronutrients status determinations were collected. Standard clinical assays were used for biochemical indicators of iron status, including hemoglobin, serum iron, and total-iron-binding capacity (TIBC). The percentage transferrin saturation (%TS) was calculated by dividing serum iron by TIBC (25, 28). High-sensitivity C-reactive protein (hs-CRP) was measured by using a high-sensitivity assay (25, 28). A previously validated 24-h dietary recall interview was administrated to a subsample of 2611 children and 2630 women (29). Trained staff conducted the recalls and converted each reported dish into grams or milliliters of individual foods. Nutrient intake was then estimated by using a nutrient composition database constructed from various references (30–34). The study was approved by the Research, Ethics, and Biosafety Committee of the Instituto Nacional de Salud Pública, Cuernavaca, Mexico. All participants gave informed consent, and all data were handled anonymously during analysis.

Data handling

The criteria for the present cross-sectional analysis included children aged 5–12 y and nonpregnant women aged 18–50 y who had complete information

available for the variables included in the analyses described below. This resulted in a sample size of 1174 children and 621 women. The region of Mexico City had a very small sample size ($n = 54$); therefore, data from Mexico City and the central region were grouped together because anthropometric, biochemical, and dietary intake variables were more similar between Mexico City and the central region than between Mexico City and the other 2 regions. This resulted in 3 main geographic region categories: north, south, and center. For women, 4 parity categories were created: 0, 1–2, 3–5, and >5 children. For assessing education status, 3 categories were created by using years of schooling: none (no schooling), primary and secondary (any primary and secondary schooling but nothing beyond), and more (any schooling beyond secondary schooling). For children, a new variable named caregiver education status was created by using information available from the woman's database. This was done by merging the data for women with those of their children and by matching cases by using the household variable. The older woman in the house was assumed to be the caregiver only when she was 18 y of age or older.

Body mass index (BMI) was calculated as $\text{weight (kg)} / \text{height}^2 \text{ (m}^2\text{)}$. Women were classified by BMI categories as normal weight ($\text{BMI} > 18.5 < 25$), overweight ($\text{BMI} \geq 25 < 30$), and obese ($\text{BMI} \geq 30$) according to the latest World Health Organization (WHO) criteria (35). For children, age- and sex- specific criteria from the Centers for Disease Control and Prevention (CDC) 2000 were used to calculate BMI z scores by using the Windows-based software Epi Info (Microsoft, Redmond, WA). Children were classified by BMI z score categories as normal weight ($\text{BMI } z \text{ score} > -2 < +1 \text{ SD}$), overweight ($\text{BMI } z \text{ score} \geq +1 \text{ SD} < +2 \text{ SD}$), and obese ($\text{BMI } z \text{ score} \geq +2 \text{ SD}$) also according to WHO criteria (36). Because there were too few underweight women ($\text{BMI} 18.5$; $n = 17$) and children ($\text{BMI } z \text{ score} \leq -2 \text{ SD}$; $n = 31$) in the sample to be analyzed as a separate group, they were excluded from the study. Values $>3 \text{ SD}$ above or below the median were considered outliers and were excluded from the analyses. ID was defined as either 1) low serum iron ($< 60 \mu\text{g/dL}$) or 2) elevated TIBC ($> 360 \mu\text{g/dL}$) and low %TS ($< 20\%$) values. Inflammation was defined as an hs-CRP $> 3 \text{ mg/dL}$ (28). Nutrient consumption data for the present study was limited to total energy intake and some selected micronutrients: iron, vitamin C, and calcium.

Statistical analysis

Normality of the distribution of variables was checked by Q-Q plot and histogram observation. Log transformation was done for variables that were not normally distributed. Data were expressed as means \pm SDs (normal distribution), medians and interquartile ranges (non-normal distribution), and frequency for categorical variables. Chi-square tests and analysis of variance were performed to assess the overall differences between BMI groups for demographic, biochemical, and dietary variables according to the Tukey multiple comparisons procedure. Linear regression models were used to assess the associations between BMI status, inflammation, and diet on iron-status indicators (eg, serum iron, TIBC, and %TS). Logistic regression analysis was used to determine the significant predictors of ID. Potential confounders were identified by using forward stepwise selection techniques. In women, the identified confounders were age, region, area, and parity, whereas in children these were age, sex, region, area, and caregiver education. All *P* values were 2-sided, and the statistical significance level was set at *P* < 0.05. All statistical procedures were performed by using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL).

Results

Women

Sociodemographic characteristics, anthropometric measurements, and biochemical and dietary intake indicators for women by BMI categories are shown in **Table 3.1**. The overall prevalences of overweight/obesity and ID were 61.2% and 52.7%, respectively. Obese women (34.9 ± 7.2 y) were significantly older than the normal-weight (29.1 ± 8.1 y) and overweight (31.9 ± 7.7 y) groups. The highest prevalence of obesity was in the north of Mexico (49.0%) and in urban areas (67.5%). No difference in hemoglobin concentrations was found between obese women and the other BMI groups. Obese women had significant lower mean serum iron concentrations than did the normal-weight group (62.6 ± 29.5 compared with 72.4 ± 34.6 $\mu\text{g/dL}$; *P* = 0.03). Low serum iron (<60 $\mu\text{g/dL}$) concentrations were present in 51.6% of the obese women compared with 39.7% and 38.8% in the overweight and normal-weight groups, respectively (*P* = 0.08). Although there was no significant difference, mean %TS tended to be lower in obese subjects than in the

normal-weight subjects ($16.7 \pm 8.7\%$ compared with $18.8 \pm 10.0\%$). Mean TIBC was not different between BMI groups. In obese women, the prevalence of ID was significantly higher than in normal-weight or overweight women ($P = 0.01$). Obese (4.4 ± 5.4 mg/dL) and overweight (2.1 ± 3.5 mg/dL) women had a significantly higher median hs-CRP concentration than did the normal-weight (1.2 ± 2.7 mg/dL) group ($P = 0.001$). Dietary energy and calcium intakes, but not iron or ascorbic acid intakes, were significantly lower in the obese women than in the normal-weight group. Other nutrient intakes were not significantly different between BMI categories. The average iron intake was 44% of the Recommended Dietary Allowance (RDA) in premenopausal women (37), and no differences were observed between BMI groups.

The relation between BMI and iron indicators in women is shown in **Figure 3.1 (A, B, and C)**. Serum iron concentrations showed a significant inverse correlation with BMI ($r = -0.09$, $P = 0.03$). No correlation was found between transferrin saturation and BMI ($r = -0.07$, $P = 0.13$) or between TIBC and BMI ($r = 0.04$, $P = 0.43$). The association between BMI and hs-CRP is shown in **Figure 3.1D**; a highly significant positive correlation was found between BMI and hs-CRP ($r = 0.40$, $P < 0.001$). Moreover, hs-CRP was negatively correlated with serum iron ($r = -0.09$, $P = 0.04$).

Predictors of iron status from the crude and adjusted linear regression model are shown in **Table 3.2**. BMI > 30 was a significant negative predictor of serum iron ($\beta = -9.56$, $P = 0.001$). Obesity tended to be positively associated with TIBC ($\beta = 17.3$, $P = 0.06$) and tended to be negatively associated with %TS ($\beta = -2.26$, $P = 0.07$). In addition, independent of BMI, log hs-CRP was a significant negative predictor of serum iron status ($\beta = -2.51$, $P = 0.04$). Dietary iron, vitamin C, and calcium intake were not significant predictors of iron status. Fiber intake was a significant positive predictor of TIBC ($\beta = 16.5$, $P = 0.03$). Results of logistic regression analysis of the prediction of ID by BMI categories are shown in **Table 3.3**. Consistent with the results of linear regression analyses, obesity was a significant independent predictor of ID with an odds ratio of 1.92 (95% CI: 1.23, 3.01).

Children

Sociodemographic characteristics, anthropometric measures, and biochemical and dietary intake indicators by BMI z score categories in children are shown in **Table 3.4**. The overall prevalences of overweight/obesity and ID were 45.8% and 58.5%, respectively. No significant differences were found between BMI z score categories in age or sex. Of all obese children ($n = 41$), 78.0% lived in the urban area and 43.9% in the north of Mexico. No difference in hemoglobin concentrations was found between obese children and the other BMI groups. Mean serum iron was not statistically different between BMI groups. Obese children had lower %TS ($P = 0.09$) and significantly higher mean TIBC ($P = 0.001$) than did the normal-weight group. Total energy and vitamin C intakes were significantly higher in the obese and overweight groups than in the normal-weight category ($P < 0.05$). Calcium and fiber intakes were not significantly different between BMI groups; however, they tended to be higher in the obese. Average iron intakes were $\approx 100\%$ of the RDA for school-age children (37), and no differences in intakes were found between BMI groups.

The relation between BMI z score and iron-status indicators is shown in **Figure 3.2 (A, B, and C)**. BMI z score showed no correlation with serum iron concentrations ($r = -0.001$, $P = 0.74$) or transferrin saturation ($r = -0.07$, $P = 0.07$). A significant positive correlation was found between BMI z score and TIBC ($r = 0.20$, $P = 0.001$). The association between BMI and hs-CRP is shown in **Figure 3.2 D**; a positive correlation was found between BMI and hs-CRP ($r = 0.22$, $P = 0.001$). Moreover, hs-CRP was negatively correlated with serum iron ($r = -0.30$, $P = 0.001$). Linear regression analyses for predictors of iron status in Mexican children are shown in **Table 3.5**. BMI z score was a significant positive predictor of TIBC ($\beta = 11.2$, $P = 0.001$), but not of serum iron or TS. In addition, independent of BMI, log hs-CRP was a significant negative predictor of iron status. Fiber, iron, vitamin C, and calcium intakes were not significant predictors of iron status. Logistic regression analysis showed that obesity was a significant predictor of ID (odds ratio: 3.96; 95% CI: 1.34, 11.67; **Table 3.3**)

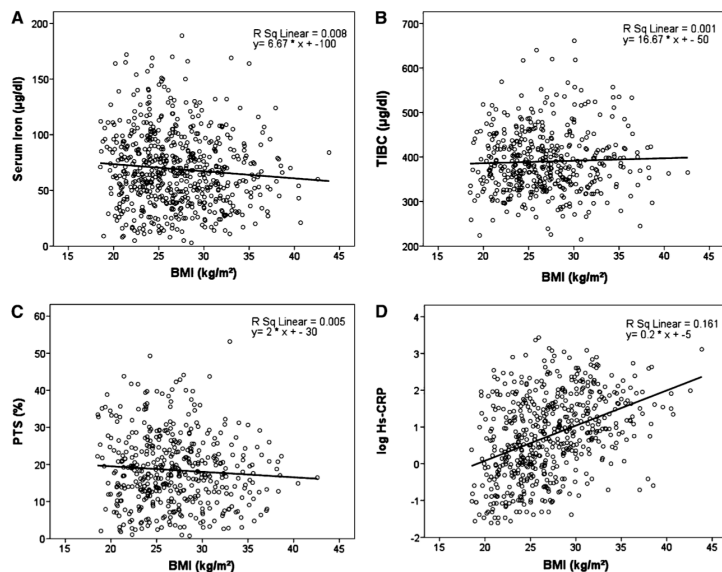


Figure 3.1 Correlations between BMI and serum iron ($r = -0.09$, $P = 0.03$) (A), total-iron-binding capacity (TIBC; $r = 0.04$, $P = 0.44$) (B), percentage transferrin saturation (PTS; $r = -0.07$, $P = 0.13$) (C), and log high-sensitivity C-reactive protein (Hs-CRP; $r = 0.40$, $P = 0.001$) (D) in nonpregnant Mexican women ($n = 621$). Hs-CRP was log transformed.

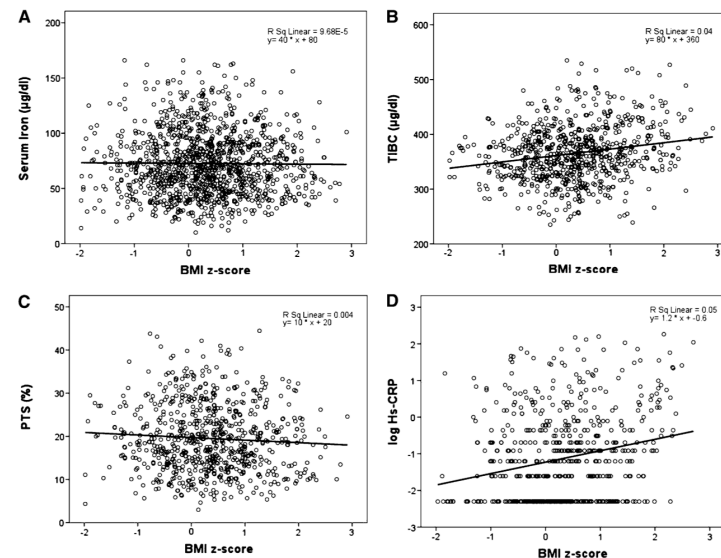


Figure 3.2 Correlations between BMI z scores and serum iron ($r = 0.01$, $P = 0.74$) (A), total-iron-binding capacity (TIBC; $r = 0.20$, $P = 0.001$) (B), percentage transferrin saturation (PTS; $r = -0.07$, $P = 0.07$) (C), and log high-sensitivity C-reactive protein (Hs-CRP; $r = 0.22$, $P = 0.001$) (D) in Mexican school-age children ($n = 1174$). Hs-CRP was log transformed.

Table 3.1 Data from 621 nonpregnant Mexican women in the second Mexican National Nutrition Survey (1999)¹

Variable	Normal weight (n = 240; 38.6%)	Overweight (n = 224; 36.1%)	Obese (n = 157; 25.3%)	Overall P value ²
Age (y)	29.1 ± 8.1 ³	31.9 ± 7.7*	34.9 ± 7.2*	0.001
Rural/urban (%) ^{4,5}	40.8/59.2	32.1/67.9	32.5/67.5	0.09
Height (cm)	154 ± 6	153 ± 6	152 ± 6*	0.02
Weight (kg)	53.1 ± 6.0	64.2 ± 6.2*	76.8 ± 8.0*	0.001
BMI (kg/m ²)	22.5 ± 1.6	27.3 ± 1.4*	33.3 ± 2.7*	0.001
Hemoglobin (mg/dL)	13.5 ± 1.6	13.5 ± 1.6	13.7 ± 1.6	0.52
Serum iron (µg/dL)	72.4 ± 34.6	70.2 ± 34.5	62.6 ± 29.5*	0.01
TIBC (µg/dL)	386 ± 64	388 ± 69	397 ± 80	0.43
TS (%)	18.8 ± 10.0	19.3 ± 9.6	16.7 ± 8.7	0.08
Iron deficiency (%) ^{4,6}	48.3	52.7*	61.2*	0.001
hs-CRP (mg/dL)	1.17 (2.67) ⁷	2.12 (3.47)*	4.44 (5.36)*	0.001
Energy (kcal/d)	1575 (871)	1462 (774)	1418 (833)*	0.05
Fiber (g/d)	19.5 (17.2)	18.0 (13.7)	17.1 (14.0)	0.06
Iron (mg/d)	8.92 (6.64)	9.01 (6.02)	8.74 (6.10)	0.74
Vitamin C (mg/d)	32.6 (53.8)	27.3 (53.2)	29.5 (58.4)	0.49
Calcium (mg/d)	716 (499)	649 (514)	597 (407)*	0.001

¹ TS, transferrin saturation; TIBC, total-iron-binding capacity; hs-CRP, high-sensitivity C-reactive protein. weight group, P<0.05 (Tukey's post hoc test).² Overall P values indicate significant differences between the groups by ANOVA. ³ Mean ± SD (all such values). ⁴ Values are frequencies.⁵ Rural subjects lived in a locality with 2500 residents; urban subjects lived in a locality with 2500 residents. ⁶ Iron deficiency was defined as either a low serum iron or an elevated TIBC and a low TS.⁷ Median; interquartile range in parentheses (all such values).

Table 3.4 Data from 1174 Mexican school-age children in the second Mexican National Nutrition Survey (1999)¹

Variable	Normal weight (n = 921; 78.4%)	Overweight (n = 212; 18.1%)	Obese (n = 41; 3.5%)	Overall P value ²
Age (y)	8.23 ± 1.88 ³	7.94 ± 1.97	8.34 ± 1.80	0.11
Female/male sex (%) ⁴	47.8/52.2	50.5/49.5	48.8/51.2	0.78
Rural/urban (%) ^{4,5}	50.3/49.7	36.3/63.7*	22.0/78.0*	0.001
Height (cm)	125 ± 12	127 ± 14	132 ± 13*	0.001
Weight (kg)	25.6 ± 6.1	33.1 ± 10.5*	44.9 ± 12.6*	0.001
BMI z score	0.08 ± 2.43	1.40 ± 0.48*	2.27 ± 0.33*	0.001
Hemoglobin (mg/dL)	13.3 ± 1.2	13.3 ± 1.3	13.5 ± 1.2	0.34
Serum iron (µg/dL)	72.8 ± 29.5	72.5 ± 26.9	65.0 ± 22.2	0.24
TIBC (µg/dL)	360 ± 48	379 ± 54	399 ± 51*	0.001
TS (%)	19.8 ± 8.0	19.1 ± 7.5	16.4 ± 5.6	0.09
Iron deficiency (%) ^{4,6}	45.0	45.8	58.5	0.23
hs-CRP (mg/dL)	0.20 (0.5) ⁷	0.40 (0.7)*	1.80 (3.1)*	0.001
Energy (kcal/d)	1384 (805)	1575 (972)	1630 (845)*	0.001
Fiber (g/d)	15.3 (14.3)	15.18 (11.00)	17.59 (12.39)	0.42
Iron (mg/d)	7.6 (5.9)	8.03 (5.95)*	8.74 (6.69)	0.02
Vitamin C (mg/d)	24.0 (53.7)	32.67 (73.65)	40.25 (72.55)*	0.001
Calcium (mg/d)	640 (449)	639 (549)	686 (446)	0.37

¹TS, transferrin saturation; TIBC, total-iron-binding capacity; hs-CRP, high-sensitivity C-reactive protein. *Significantly different from the normal- weight group, P < 0.05 (Tukey's post hoc test).²Overall P values indicate significant differences between the groups by ANOVA.³Mean ± 6 SD (all such values).⁴Values are frequencies.⁵Rural subjects lived in a locality with <2500 residents; urban subjects lived in a locality with >2500 residents. ⁶Iron deficiency was defined as either a low serum iron or an elevated TIBC and a low TS.

⁷Median; interquartile range in parentheses (all such values).

Table 3.2 Linear regression analyses for predictors of iron status in 621 non pregnant Mexican women¹.

Variable	β (95% CI)	
	Crude	Adjusted ²
Serum iron		
BMI (in kg/m ²)>30	-9.78 (-16.5,-3.06)*	-9.56 (-16.8, -2.35)*
BMI	-0.64 (-1.20, -0.07)*	-0.59 (-1.20, 0.02)
Log hs-CRP	-2.62 (-5.09, -0.15)*	-2.51 (-5.11, 0.09)*
Log fiber intake (mg/d)	-3.69 (-7.79, 0.39)	-5.22 (-11.2, 0.72)
Log iron intake (mg/d)	-0.34 (-5.18, 4.51)	0.26 (-4.68, 5.20)
Log vitamin C intake (mg/d)	1.74 (-0.32, 3.79)	1.48 (-0.73, 3.70)
Log calcium intake (mg/d)	-2.65 (-7.16, 1.86)	-3.56 (-9.21, 2.09)
Percentage TS		
BMI (in kg/m ²)>30	-2.09 (-4.40, 0.23)	-2.26 (-4.72, 0.21)
BMI	0.15 (-0.34, 0.45)	-0.18 (-0.39, 0.04)
Log hs-CRP	-0.64 (-1.48, 0.19)	-0.73 (-1.60, 0.13)
Log fiber intake (mg/d)	-1.22 (-2.59, 0.14)	-1.74 (-3.77, 0.29)
Log iron intake (mg/d)	0.01 (-1.60, 1.62)	0.26 (-1.38, 1.90)
Log vitamin C intake (mg/d)	0.56 (-0.11, 1.24)	0.58 (-0.15, 1.31)
Log calcium intake (mg/d)	-0.83 (-2.33, 0.66)	-0.91 (-2.83, 1.00)
TIBC		
BMI (in kg/m ²)>30	10.7 (-6.18, 27.6)	17.3 (-0.74, 35.3)
BMI	0.56 (-0.86, 1.97)	1.20 (-0.34, 2.75)
Log hs-CRP	-3.34 (-9.21, 2.52)	-2.47 (-8.60, 3.67)
Log fiber intake (mg/d)	11.5 (1.53, 21.4)*	16.5 (1.64, 31.3)*
Log iron intake (mg/d)	3.74 (-8.02, 15.5)	1.66 (-10.4, 13, 7)
Log vitamin C intake (mg/d)	-1.35 (-6.26, 3.56)	-1.68 (-6.99, 3.64)
Log calcium intake (mg/d)	4.89 (-6.06, 15.8)	1.25 (-12.8, 15.3)

¹TIBC, total-iron-binding capacity; TS, transferrin saturation; hs-CRP, high-sensitivity C-reactive protein.*P < 0.05. ²Adjusted for age, area (rural or urban), geographic region, parity, and iron intake.**Table 3.3** Crude and adjusted odds ratios (ORs) from logistic regression of iron deficiency as a function of BMI categories in nonpregnant Mexican women (n = 621) and school-age children (n = 1174)¹

Variable	OR (95% CI)	
	Crude	Adjusted ^b
Women		
Iron deficiency		
Normal weight	1.00	1.00
Overweight	1.19 (0.83, 1.71)	1.27 (0.87, 1.88)
Obese	1.68 (1.12, 2.53)*	1.92 (1.23, 3.01)*
Children		
Iron deficiency		
Normal weight	1.00	1.00
Overweight	1.03 (0.77, 1.39)	0.79 (0.51, 1.23)
Obese	1.73 (0.92, 3.26)	3.96 (1.34, 11.67)*

¹Values for women were adjusted for age, area (rural or urban), geographic region, parity, and iron intake; values for children were adjusted for age, sex, area (rural or urban), geographic region, caregiver education, and iron intake. *P < 0.05. ²Defined as either low serum iron or elevated total-iron-binding capacity (<360 µg/dL) and low percentage transferrin saturation (<20%).

Table 3.5 Linear regression analyses for predictors of iron status in 1174 Mexican children.

Variable	β (95% CI)	
	Crude	Adjusted ²
Serum iron		
BMI z-score >1SD	-1.42 (-5.47, 2.63)	2.67 (-3.09, 8.44)
BMI z-score	-0.32 (-2.19, 1.55)	-0.45 (-3.03, 2.14)
Log hs-CRP	-6.68 (-8.38, -4.99)*	-7.92 (-10.3, -5.57)*
Log fiber intake (mg/d)	-2.25 (-4.84, 0.33)	-3.07 (-7.71, 1.56)
Log iron intake (mg/d)	0.58 (-2.25, 3.41)	0.83 (-3.00, 4.65)
Log vitamin C intake (mg/d)	1.22 (0.59, 2.38)*	0.14 (-1.63, 1.91)
Log calcium intake (mg/d)	1.77 (-1.20, 4.74)	-0.39 (-5.11, 4.32)
Percentage TS		
BMI z-score >1SD	-1.00 (-2.38, 0.39)	0.20 (-1.70, 2.10)
BMI z-score	-0.59 (-1.24, 0.58)	-0.46 (-1.36, 0.43)
Log hs-CRP	-2.03 (-2.62, -1.45)*	-2.18 (-3.00, -1.36)*
Log fiber intake (mg/d)	-0.43 (-1.31, 0.45)	-0.53 (-2.07, 1.02)
Log iron intake (mg/d)	0.01 (-0.98, 1.00)	0.21 (-1.12, 1.54)
Log vitamin C intake (mg/d)	0.54 (0.13, 0.95)*	0.25 (-0.38, 0.87)
Log calcium intake (mg/d)	-0.05 (-1.04, 0.94)	-0.81 (-2.36, 0.74)
TIBC		
BMI z-score >1SD	21.7 (12.8, 30.6)*	20.4 (8.4, 32.4)*
BMI z-score	11.7 (7.55, 15.8)*	11.2 (5.7, 16.8)*
Log hs-CRP	4.89 (0.61, 9.17)*	5.16 (-1.06, 11.4)
Log fiber intake (mg/d)	1.89 (-3.76, 7.56)	0.35 (-9.98, 9.27)
Log iron intake (mg/d)	3.99 (-2.37, 10.3)	-1.39 (-9.71, 6.93)
Log vitamin C intake (mg/d)	-0.19 (-2.84, 2.46)	0.65 (-3.24, 4.55)
Log calcium intake (mg/d)	5.14 (-1.24, 11.5)	2.80 (-6.88, 12.5)

¹TS, transferrin saturation; TIBC, total-iron-binding capacity; hs-CRP, high-sensitivity C-reactive protein.

*P<0.05. ²Adjusted for age, sex, area (rural or urban), geographic region, caregiver education, and iron intake.

Discussion

The results of this study clearly show that in Mexican children and women, obesity is a predictor of low iron status. Obese women and children had an increased risk of ID: odds ratios of 1.92 and 3.96, respectively. Our results are consistent with most previous studies in children (1, 2, 5, 7, 11, 16, 17, 38) and women (3, 4, 10, 12, 13, 39). For example, in a cross-sectional study performed in 1688 school-age children from 2 transition countries (697 Indian and 991 Moroccan children), it was shown that BMI z score was a significant negative predictor of body iron stores (17). In adults as well, negative associations between iron status and obesity have been reported in developed (3, 4, 10, 12, 13, 39) and developing countries (17). However, most of these studies did not assess or compare dietary iron intakes between obese

and normal-weight individuals or assess obesity-related inflammation with an hs-CRP assay.

In our study, for both children and women, dietary iron intakes were similar in the obese and the normal-weight groups, and it was not an independent predictor of iron status. Other studies that estimated dietary iron intake in overweight children (40) and adults (10, 12) also found comparable intakes between obese and normal-weight individuals. Our results suggested that obese children had a higher intake of vitamin C, whereas no difference in vitamin C intake was found between BMI groups in women; calcium intake did not differ between BMI categories in children, whereas obese women reported less total energy and calcium intakes than did the normal-weight group.

Obese individuals tend to underestimate their dietary intakes compared with normal-weight individuals, and this also occurs in Mexico, where Barquera et al (41) showed that intakes of commonly reported energy-dense foods (such as tortillas, sugar, and oil) tend to be underestimated. A previous analysis of this data set suggested more under-reporting in obese (36%) than in nonobese women (20%) (41). This may explain the lower energy intake observed in obese women than in the other BMI groups. Moreover, this may have influenced our dietary intake estimates; however, more underreporting by obese subjects would have biased the data toward an underestimation of dietary iron in the obese group and thus is unlikely to have affected our conclusions. Overall, the data indicate that differences in dietary intake are unlikely to explain the low iron status of Mexican obese women and children.

In humans, hs-CRP is the main acute phase protein and it is also a sensitive marker of systemic inflammation. Recent studies have linked obesity with the presence of a low-grade systemic inflammation in these subjects. For example, Visser et al (42) investigated data from NHANES III and observed an elevated CRP concentration in obese women and men. Moreover, studies have shown that the CRP concentration decreases significantly after massive weight reduction. This decrease indicates that fat mass plays an important role in the production of CRP (43). In our study, CRP concentrations were higher in obese women and children and were positively associated with BMI and negatively associated with iron status. In addition, when CRP was included in the regression model, obesity was no longer an independent predictor of iron status, and the associations were weaker. Yanoff et

al (10) found that transferrin receptor, ferritin, and C-reactive protein were independent predictors of serum iron in obese adults. Bekri et al (44) proposed that adiposity-associated inflammation could have a direct effect on iron metabolism. In obese women, greater adiposity is associated with lower fractional iron absorption independently of iron status (17). Circulating hepcidin concentrations are elevated in overweight children, independent of iron status (15). Significant weight loss after restrictive bariatric surgery is associated with decreased serum hepcidin and improved iron status in adults (45). In a recent study, weight loss in obese children was associated with lower hepcidin concentrations and improvements in iron status and absorption (46). Overall, the data suggest that the link between iron depletion and obesity is likely to be due to inflammation-induced increases in circulating hepcidin.

In obesity, increased pro-inflammatory cytokines, such as leptin, interleukin-6, and CRP, may stimulate hepcidin production by the liver and adipose tissue (44, 47). Hepcidin excess has been suggested to decrease dietary iron absorption and increase iron sequestration from reticuloendothelial macrophages because of its inhibitory actions on the expression of ferroportin, which exports iron from cells into the plasma (10, 44, 48). Moreover, lipocalin-2, an iron-binding protein upregulated by inflammation, might also be responsible for iron sequestration within the adipocytes (49). Our findings are in line with these hypotheses and suggest that the adiposity low-grade inflammatory state might play a role in the hypoferrremia of obesity.

Our study had several limitations. First, because the study was cross-sectional, no conclusion regarding cause and effect could be made. Some variables that influence iron status, body weight, and inflammation—such as physical activity, common infections, and other micronutrient deficiencies—were not included in the analyses and could have attenuated our associations. Second, iron-status indicators that are not significantly affected by inflammation, such as serum transferrin receptor, were not available and could have helped to better assess iron status in obese subjects (17). Third, information on the heme and nonheme iron content in diet was not available, thus we could not accurately estimate iron bioavailability (50); however, intakes of ascorbic acid, calcium, and fiber—factors that modulate iron bioavailability—were assessed. Finally, an estimate of percentage body fat in addition to BMI would be a better indicator of adiposity than BMI alone. However,

BMI is a good indicator of adiposity in both adults and children in population studies, and, in our database, measures of body composition were not available. The strengths of our study included the large sample sizes and the use of an hs-CRP assay, which provided an estimate of systemic inflammation as a potential underlying mechanism. In addition, our study was conducted in a population with a high prevalence of both conditions: ID and overweight or obesity.

In conclusion, our findings support the hypothesis that obesity-related inflammation may lead to impairments in iron bioavailability and/or metabolism that increase the risk of ID. If true, national efforts to control ID in Mexico may be undermined by the increasing rates of obesity in women and children (51). Additional studies are needed to establish whether obese women and/or children have higher iron requirements than do their normal-weight counterparts. Such studies would be particularly valuable in countries, such as Mexico, undergoing the nutrition transition.

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Chapter 4

In overweight and obese women, dietary iron absorption is reduced and the enhancement of iron absorption by ascorbic acid is half of that in normal weight women

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Abstract

Background: Iron deficiency is common in overweight and obese subjects (OW/OB). This may be due to adiposity-related inflammation that increases serum hepcidin and decreases dietary iron absorption. Because hepcidin reduces iron efflux from the basolateral enterocyte, whether luminal enhancers of dietary iron absorption like ascorbic acid can be effective in OW/OB is uncertain.

Objective: In this study we compared iron absorption from a meal with and without ascorbic acid in normal weight (NW) women versus OW/OB women.

Design: Healthy, non-anemic women (n=62) (BMI 18.5-39.9 kg/m²) consumed a stable-isotope labelled wheat-based test meal without (-AA) and with (+AA) 31.4 mg ascorbic acid. We measured iron absorption, body composition by dual energy X-ray absorptiometry (DXA), blood volume by carbon monoxide (CO)-rebreathing method, iron status, inflammation and serum hepcidin.

Results: Inflammatory biomarkers (all $P < 0.05$) and hepcidin ($P = 0.08$) were lower in NW versus OW/OB women. Geometric mean (95%CI) iron absorption in NW and OW/OB from -AA meals were 19.0 (15.2, 23.5)% and 12.9 (9.7, 16.9)% ($P = 0.049$) and from +AA meals were 29.5 (23.3, 38.2)% and 16.6 (12.8, 21.7)% ($P = 0.004$), respectively. Median % increase in iron absorption comparing -AA to +AA was 56% in NW ($P < 0.001$) and 28% in OW/OB ($P = 0.006$). Serum ferritin ($R^2 = 0.22$, $\beta = -0.17$ (95%CI: -0.25, -0.09), TfR ($R^2 = 0.23$, $\beta = 2.79$ (95%CI: 1.47, 4.11) and hepcidin ($R^2 = 0.13$, $\beta = -0.85$ (95%CI: -1.41, -0.28) were significant predictors of iron absorption.

Conclusion: In OW/OB women, iron absorption is 2/3 and the enhancing effect of ascorbic acid on iron absorption is half of that in NW women. Recommending higher intakes of ascorbic acid (or other luminal enhancers of iron absorption) in obese individuals to improve iron status may have limited effect.

Introduction

Iron depletion is common in obese subjects (1-14). The hypoferremia of obesity may be caused by higher circulating hepcidin stimulated by sub-clinical inflammation; serum hepcidin may originate from both adipose tissue and liver (15, 16). Hepcidin, the master regulator of iron metabolism, is a 25-amino-acid protein produced mainly in the liver in response to iron stores and inflammation (17). Hepcidin interacts with ferroportin, the cellular iron exporter expressed on intestinal cells and macrophages, and regulates iron flow from cells into the circulation (18). Thus, in states of high hepcidin concentrations, intestinal iron absorption (through enterocytes) and recycling of iron from senescent red cells (through macrophages) is reduced, depriving developing erythroblasts of iron supply. An array of pro-inflammatory cytokines and adipokines are increased in the obese state including leptin, tumor necrosis factor alpha (TNF- α), interleukin-6 (IL6), and others (19). Leptin and IL6 directly mediate the hypoferremia of inflammation by inducing hepcidin synthesis (20). Consequently, obese subjects have increased hepcidin expression (9, 21-25), which may lead to reduced iron absorption and eventually to iron deficiency (26). We have previously shown associations between iron absorption and body weight in the normal weight to overweight range (up to BMI 27 kg/m²) (27) and also relations between overweight, hepcidin and iron status (22). Weight loss has further been associated with reductions in hepcidin concentrations that may improve functional iron status (28). However the direct influence of overweight and obesity on iron metabolism remains unclear.

The extent to which non-heme iron is absorbed from the diet is influenced by the composition of the diet (29). Ascorbic acid is a potent enhancer of non-heme iron absorption (30-33), likely through luminal reduction of dietary ferric iron (Fe³⁺) to more soluble ferrous iron (Fe²⁺) (29, 34, 35). However, since hepcidin reduces iron efflux at the basolateral membrane of the enterocyte, whether luminal enhancers of iron absorption would be effective in overweight and obese subjects is unclear. Data on the effects of ascorbic acid on iron absorption in obesity is important to be able to provide dietary recommendations to improve iron bioavailability in obese subjects. Thus, the purpose of the present study is to evaluate the influence of body

weight and body composition on iron absorption from a standardized test meal with and without added ascorbic acid.

Subjects and methods

Subjects

We recruited 64 women for the study: 24 with normal weight (BMI 18.5-24.9 kg/m²), 20 with overweight (BMI 25-29.9 kg/m²), and 20 obese women (BMI 30-39.9 kg/m²). No men were recruited since iron absorption is generally higher in women because of lower iron stores and higher losses during menstruation. In a screening visit we informed interested volunteers about the study procedure and written informed consent was obtained from all women after they were given a full oral and written description of the aims and procedures of the study and the associated risks. The study criteria were assessed using a questionnaire, weight and height were measured to determine BMI and a pregnancy test was carried out. Inclusion criteria for the study were: (1) female; (2) age 18-45 y; (3) pre-menopausal (no absence of a menstrual cycle in the past 12 months); (4) BMI 18.5-39.9 kg/m²; (5) no chronic illness and no significant medical conditions that could influence iron or inflammatory status other than obesity; (6) non-smoking (or at least no more than 1 cigarette/week and not smoking three days prior to the first measurement and during the study); (7) nonpregnant and not planning a pregnancy. The protocol was approved by the ethics committees of the Swiss Federal Institute of Technology (Zurich, Switzerland) and the Ethics Committee of the Canton of Zurich (Kantonale Ethikkommission Zürich), and registered at clinicaltrials.gov NCT01884506.

Sample size calculation

Sample size calculation was based on the expected differences between normal weight and obese subjects in iron absorption. Based on the differences in hepcidin concentrations observed in earlier studies (22, 24) a difference in iron absorption of 30-40% was expected. Using a standard deviation for the log of the difference of fractional iron absorption of 0.25 (this is a mean value calculated from a large series of Fe absorption studies carried out at the Human Nutrition Laboratory, ETH) and an expected difference in the absorption of 35%, with a power of 80% and an α -level of 0.05, resulted in a required sample size of 22 per group. Taking an

expected 10% non-completion rate into account, we aimed for a sample size of 25 per group.

Study design

Subjects reported fasting to the Human Nutrition Laboratory (no food for 12 hours, no drinks after midnight) on two consecutive mornings. On the first visit (d 1) anthropometric measurements were conducted. Following this, subjects consumed a first labeled test meal fed under standardized conditions and close supervision. The next day (d 2), subjects consumed the second labeled test meal under the same conditions. The order of the meals (-AA and +AA) was randomized in all subjects. Test meal -AA consisted of a standardized meal labeled with 5 mg ^{57}Fe as ferrous sulfate (FeSO_4) and served as the reference meal and test meal +AA consisted of the same meal but labeled with 5mg ^{58}Fe as FeSO_4 as well as ascorbic acid (AA) at a molar ratio of AA:Fe of 2:1 (31.4 mg) as an iron absorption enhancer. The ratio of ascorbic acid and iron used is suggested to effectively enhance iron absorption from meals with low to medium levels of iron absorption inhibitors (36). The isotopes (and AA) were added to the test meal just before consumption. The additional iron provided by the bread, butter and honey amounted to 1.4 mg per meal.

The standardized test meal consisting of white bread rolls with butter and honey was bought in a single large batch. The bread and butter were frozen and thawed the evening before test meal administration. A stock solution of food-grade L-ascorbic acid was prepared so that 1 mL of the stock solution corresponded to the 2:1 molar ratio of AA to added iron. One mL of the AA stock solution was pipetted onto the bread rolls with the isotope solution. After ingestion of the test meal subjects were asked not to eat or drink for 3 hours in order to minimize the impact of additional foodstuffs on iron absorption. Fourteen days after the second test meal was consumed (d 16), a fasting blood sample was obtained for analysis of hemoglobin and erythrocyte isotopic composition as well as for iron status, inflammation and hepcidin determination. Within 2 weeks before/after the administration of the test meal, all subjects were scheduled to undergo dual energy X-ray absorptiometry (DXA) for determination of body composition at University Zurich Irchel and a blood volume measurement using the carbon monoxide (CO)-rebreathing method at the University Clinic Balgrist in Zurich. To minimize fluctuations in blood volume

all measurements were, if possible, performed between 7 and 14 days after the beginning of the last menstrual cycle.

Anthropometric and body composition determinations

Body weight (kg) was measured to the nearest 0.1 kg on a digital scale, and height (m) was measured to the nearest 1.0 cm by using a stadiometer according to standardized procedures (37). Total body and segmental (arms, legs, trunk, gluteo-femoral and abdominal region) fat and lean mass were determined by using DXA (iDXA™; Lunar iDXA™, GE Healthcare, Madison, WI, USA) at the University of Zürich, Irchel. Visceral and subcutaneous adipose tissues at the level of the abdomen were estimated by using the iDXA™ CoreScan function.

Blood volume determination

The optimized CO rebreathing method (38) is a routinely applicable minimally invasive reliable method for assessing blood volume (39). This method has been described in detail elsewhere (38, 40, 41). Briefly, baseline venous samples were obtained (2 ml in an EDTA tube) 15 min after adopting a seated position for hemoglobin (Hb) and hematocrit (Hct) determination. All blood samples were immediately analyzed using a spectrophotometer for the blood gas determination (ABL 700 Serie, Radiometer A/S, Copenhagen, Denmark). Furthermore, capillary blood samples from the earlobe were obtained (35µl in pre-heparinized glass capillary tubes) in triplicate at baseline and at 6, 7 and 8 min after starting the rebreathing procedure. Percent carboxyhemoglobin saturation (HbCO%) was measured using a blood gas analyzer. The mean value of the measurements at 6 and 8 minutes was taken as the plateau value after CO-rebreathing with the sample at minute 7 as a backup. Total Hb mass was calculated as described previously (42), using a slightly different correction for loss of CO to myoglobin (0.3%/min of administered CO) (38). A bolus of chemically pure CO of 0.8 ml/kg body mass was administered during the first inspiration from a closed spirometric system (Blood tec GbR, Bayreuth, Germany) and was rebreathed for 1 min 50 seconds together with a small amount of oxygen (4 liters). To verify that no gas was leaking during the CO rebreathing procedure, the entire apparatus as well as the mouth piece and nose-clip were checked using a portable CO gas analyzer (Dräger PAC 7000; Dräger Safety;

Lübeck, Germany) with a parts-per-million sensitivity to monitor local CO levels. The analyzer was also used to calculate end-tidal CO concentration before the CO-rebreathing and after the onset of the rebreathing procedure with the subject wearing a nose-clip and then blowing into a mouthpiece until the maximal value of CO observed was recorded. The amount of CO remaining in the spirometer after rebreathing was also measured with the portable CO gas analyzer. The determined parameters were used first to calculate total hemoglobin mass and from this to derive blood, plasma and red blood cell volume using equations published previously (38, 40, 42).

Preparation of isotopically labeled iron

Isotopic labels ^{57}Fe -FeSO₄ and ^{58}Fe -FeSO₄ were prepared from isotopically enriched elemental iron by dissolution in diluted sulfuric acid. The solutions were stored in teflon containers, flushed with argon to keep the Fe in the +2 oxidation state. The preparation of the isotopic labels was done according to the method described by Walczyk et al. (43).

Laboratory analysis

Hemoglobin (Hb) concentration was assessed by using a Coulter counter (Beckman Coulter, Krefeld, Germany) with 3-level quality-control material (Liquichek; Bio-Rad, Irvine, CA) on the day of blood collection. Serum iron and total-iron binding capacity (TIBC) were determined by colorimetry as described previously (44, 45). Serum transferrin receptor (sTfR), serum ferritin (SF) and high sensitive C-reactive protein (CRP) and alpha 1 glycoprotein (AGP) were assessed by using an automated chemiluminescent immunoassay system (Immulite, Diagnostic Products Corporation, Los Angeles, USA). Interleukin 6 (IL6) was measured by a Quantikine ELISA kit (R&D systems, Minneapolis, MN) and hepcidin concentrations were determined using a commercial ELISA kit (Bachem, UK). Iron deficiency anemia was defined as Hb < 12 g/dl and SF < 15 $\mu\text{g/L}$ or TfR > 8.5 mg/L and iron deficiency without anemia as Hb > 12 g/dl and SF < 15 $\mu\text{g/L}$ or TfR > 8.5 mg/L (46). For isotope analysis, whole blood was mineralized by microwave digestion, and iron was separated by anion-exchange chromatography and a subsequent solvent-solvent extraction step into diethylether. The isotopic analysis of

^{58}Fe and ^{57}Fe were performed using inductively coupled plasma mass spectrometry with a high resolution double focusing mass spectrometer (Neptune; Thermo-Finnigan, Bremen, Germany) equipped with a multicollector system for simultaneous ion beam detection (43). The calculation of the amount of isotopic label present in the blood of the subject was based on the shift of the isotopic ratios in the blood after red cell incorporation of the absorbed isotopic label. When the circulating amount of isotopic label is known, the amount of label absorbed from the test meal and thus the fractional iron absorption can be calculated (43). The amount of natural iron circulating in the blood was calculated based on the blood volume determined by the CO-rebreathing method and the Hb concentration in the blood (38). Estimation of fractional iron absorption was based on the assumption of an 80% incorporation of absorbed iron into the erythrocytes. The observed shift in iron isotope ratios was converted to fractional iron absorption by using standard algorithms (43)

Statistical analysis

All statistical analysis was conducted using IBM SPSS Version 20 (IBM Company, Armonk, NY, USA). Data was checked for normal distribution (as assessed by Kolmogorov-Smirnov and Levene's normality testing) and for the presence of outliers (± 3 s.d. from the mean). Non-normally distributed data were logarithmically transformed for statistical analysis. Two subjects (1 overweight and 1 obese) did not complete the study measurements and were therefore excluded from all analyses. The mean (\pm s.d.) (for normally distributed data), geometric mean (95% confidence interval) (for data with normal distribution after log-transformation) or median (IQR) (for non-normally distributed data even after log-transformation) values for each parameter were determined. Nonparametric tests were applied for data that remained non-normally distributed after logarithmic transformation. Comparison between all BMI groups (normal weight (NW), overweight (OW), and obese (OB)) was conducted by analysis of variance (ANOVA) with post-hoc Bonferroni correction and Kruskal-Wallis test, followed by Mann-Whitney U test, as appropriate. Independent sample t-tests and nonparametric Mann-Whitney U tests were used to compare parameters between NW and OW/OB. Paired student's t tests or Wilcoxon Signed Ranks test were used to test differences in iron absorption from the two test meals (-AA and +AA) within the BMI groups. Linear mixed model

analysis was performed in order to confirm the effect of ascorbic acid (+AA) on iron absorption (-AA) among the different BMI groups (NW, OW and OB) with iron absorption from meal +AA as the dependent variable. BMI group, meal type (-AA and +AA) as well as the interaction between the two were defined as fixed factors. Subject ID was considered as the random factor, variance component as the covariance type and maximum likelihood as the estimation. One-way ANCOVA was conducted to determine the difference between BMI groups (NW, OW and OB) on iron absorption controlling for confounders (age and TfR). Since OW and OB subject showed to have similar fractional iron absorption rates, these groups were combined for some of the analyses. Bivariate Pearson's or Spearman correlations and multiple linear regression models including correction for confounding variables were used to study associations between continuous variables. Differences were considered significant at P-values <0.05.

Results

Anthropometric characteristics, body composition, inflammation and iron status indices by BMI groups are summarized in **Table 4.1**. Sixty two subjects completed the study (24 NW, 19 OW, and 19 OB). The age range was 20-44 years and the OW/OB women (27, 23-31 years) had significantly higher median age than the NW group (23, 22-26 years). Only 4 subjects presented with iron deficiency anemia (2 NW, 1 OW, and 1 OB). Hb, TfR, and serum ferritin were in the normal range and comparable between the three BMI groups, whereas serum iron ($P = 0.025$) and TIBC ($P = 0.04$) were significantly lower in the OB compared to the NW group. **Figure 4.1** shows differences in inflammation and serum hepcidin between BMI groups. As shown in **Table 4.1**, fractional iron absorption from the -AA meal was 19.0% in NW versus 12.2% in OW and 13.6% in the OB group. In the +AA meal iron absorption increased to 29.5% in the NW versus 14.9% in the OW and 18.4% in the OB group. Geometric mean (95%CI) iron absorption from the -AA meal in the OW/OB women was 12.9 (9.7, 16.9)% ($P=0.049$ compared to NW) and it was 16.6 (12.8, 21.7)% from the +AA meal ($P=0.004$ compared to NW) (**Figure 4.2**).

Thus, iron absorption in the -AA meal in the OW/OB group was 68% of that in the NW group ($P=0.049$). The median % change in iron absorption comparing

-AA to +AA was 56% in NW ($P < 0.001$) and 28% in OW/OB ($P = 0.006$) which represented a significantly higher increase with +AA in the NW women ($P = 0.004$). Moreover, meal type ($P < 0.001$) and the interaction of meal type and BMI group ($P = 0.008$), both significantly predicted iron absorption, but BMI group did not ($P = 0.08$) (data not shown). After controlling for age or TfR, there was a significant difference between the three BMI groups (NW, OW and OB) on iron absorption from test meal -AA ($P = 0.02$).

BMI was negatively correlated with serum iron ($r = -0.37$, $P = 0.003$) and TIBC ($r = -0.34$, $P = 0.007$), and positively correlated with all three inflammatory markers: CRP ($r = 0.39$, $P = 0.002$), IL6 ($r = 0.38$, $P = 0.003$), and AGP ($r = 0.43$, $P = 0.001$) (data not shown). There was no correlation between BMI and serum ferritin, TfR or hepcidin. Body fat % was negatively correlated with serum iron ($r = -0.38$, $P = 0.002$) and TIBC ($r = -0.33$, $P = 0.009$), and positively correlated with serum ferritin ($r = 0.26$, $P = 0.04$), all three inflammatory markers (CRP ($r = 0.42$, $P = 0.001$), IL6 ($r = 0.34$, $P = 0.006$) and AGP ($r = 0.47$, $P < 0.001$)) and hepcidin ($r = 0.27$, $P = 0.03$), but not with Hb and TfR (**Table 4.2**). CRP was positively correlated with AGP ($r = 0.26$, $P = 0.039$), IL6 ($r = 0.36$, $P = 0.004$) and ferritin ($r = 0.32$, $P = 0.01$) (data not shown). Moreover, serum hepcidin was significantly correlated with Hb ($r = 0.41$, $P = 0.001$), TIBC ($r = -0.37$, $P = 0.003$), serum ferritin ($r = 0.59$, $P < 0.001$), TfR ($r = -0.29$, $P = 0.02$) and AGP ($r = 0.29$, $P = 0.02$), but not with serum iron, CRP and IL6 (**Table 4.2**). In multiple linear regression analyses, (log) serum ferritin ($\beta = 0.59$, $P < 0.001$) and (log) Hb ($\beta = 0.27$, $P = 0.006$) were significant predictors of (log) serum hepcidin ($R^2 = 0.58$). When adding (log) AGP ($\beta = 0.09$, $P = 0.31$), (log) TIBC ($\beta = -0.11$, $P = 0.29$), and (log) TfR ($\beta = -0.03$, $P = 0.75$) to the regression model, they appeared not to be independent predictors of hepcidin concentration and did not change the predictive power of the model ($R^2 = 0.58$). ζ

Iron absorption (-AA) correlated significantly with Hb ($r = -0.27$, $P = 0.03$), serum ferritin ($r = -0.58$, $P < 0.001$), TfR ($r = 0.35$, $P = 0.006$), CRP ($r = -0.26$, $P = 0.04$) and hepcidin ($r = -0.36$, $P = 0.004$), but not with body fat, serum iron, IL6 and AGP (**Table 4.2**). There was also no significant correlation between iron absorption and age (-AA: $r = 0.125$, $P = 0.334$; +AA: $r = -0.044$, $P = 0.733$) (data not shown). **Figure 4.3** describes how higher hepcidin predicted lower iron absorption from both meals in univariate associations ($P < 0.05$). The prediction of iron absorption in both

test meals by hepcidin using a crude and adjusted (by age and TfR) linear regression analysis in the total study population are shown in **Table 4.3**. Serum ferritin ($R^2=0.22$, $\beta=-0.17$ (95%CI: -0.25, -0.09), TfR ($R^2=0.23$, $\beta=2.79$ (95%CI: 1.47, 4.11) and hepcidin ($R^2=0.13$, $\beta=-0.85$ (95%CI: -1.41, -0.28) were significant predictors of iron absorption. When either CRP or serum ferritin was included in the model, hepcidin was no longer an independent predictor of iron absorption.

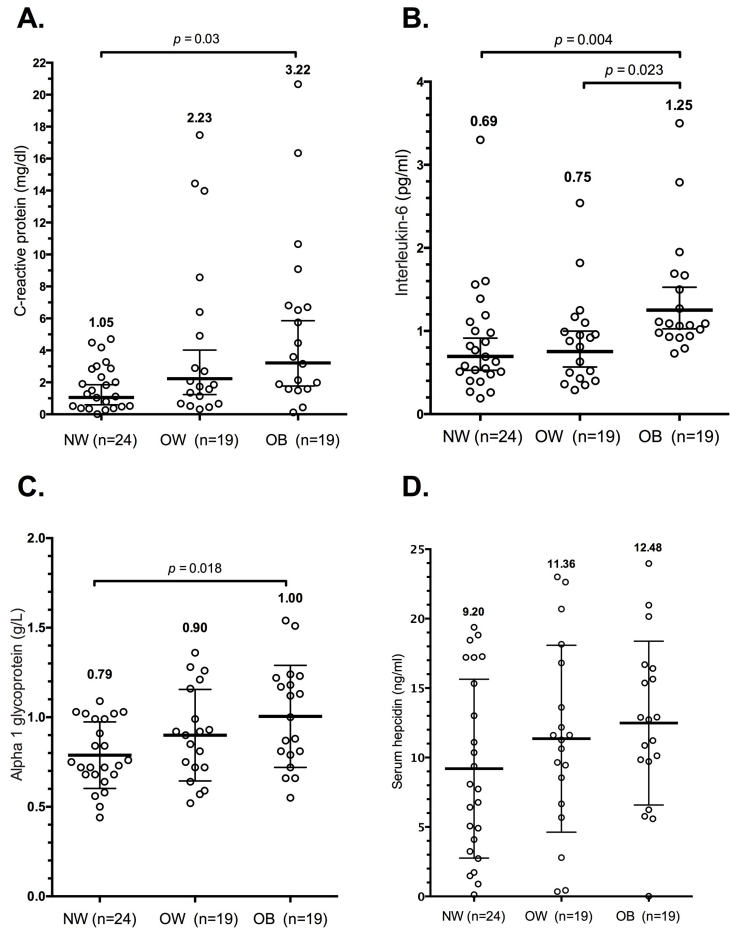


Figure 4.1 A) C-reactive protein (CRP) (mg/dl), B) interleukin-6 (pg/ml), C) alpha 1 glycoprotein (AGP) (g/L) and; D) serum hepcidin (ng/ml) concentration among the 3 BMI groups. Errors bars are mean and standard deviation (serum hepcidin and AGP) or geometric mean and 95% confidence interval (IL6 and CRP). Comparison between all BMI groups was conducted by one way ANOVA with post-hoc Bonferroni correction (n=62).

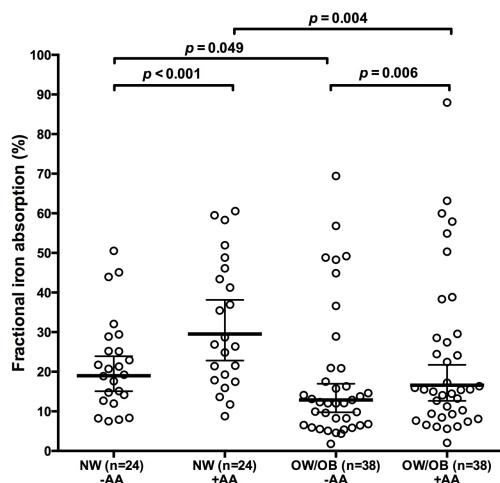


Figure 4.2 Fractional iron absorption from a wheat-based test meal without (-AA: ○) and with 31.4 mg ascorbic acid (AA) (+AA: Δ). Error bars represent geometric mean and 95% confidence intervals. Iron absorption in NW women: 19.0% (15.2 to 23.5) from the -AA meal and 29.5% (23.3 to 38.2) from the +AA meal ($P < 0.001$). Iron absorption in OW/OB: 12.9% (9.7 to 16.9) from the -AA meal and 16.6% (12.8 to 21.7) from the +AA meal ($P = 0.006$). Paired t -tests were used to assess differences in log-iron absorption between meals within BMI group. Independent t -tests were used to assess differences in log-iron absorption from each meal between NW and OW/OB.

Table 4.1 Baseline characteristics of the study population of apparently healthy normal weight (NW), overweight (OW) and obese (OB) women participating in an iron absorption study

	NW	OW	OB	OW/OB
n	24	19	19	38
Age (y) ¹	23 (22, 26) ^{b,c}	26 (22, 29)	27 (23, 33)	27 (23, 31)
Weight (kg) ²	61.8 ± 7.2	78.7 ± 7.3	89.0 ± 12.0	83.9 ± 11.1
BMI (kg/m ²) ²	21.9 ± 1.9	27.3 ± 1.5	32.8 ± 2.8	30.1 ± 3.6
Total body fat % ²	29.8 ± 4.8 ^{abc}	40.1 ± 4.4 ^b	45.8 ± 4.2	43.0 ± 5.1
Blood vol (ml/kg) ²	74.9 ± 5.8 ^{abc}	64.3 ± 5.3 ^b	59.4 ± 7.1	61.8 ± 6.7
Hb (g/dl) ²	13.5 ± 0.9	13.7 ± 1.2	13.7 ± 1.0	13.7 ± 1.1
Serum iron (μg/ml) ²	1.06 ± 0.44 ^b	1.01 ± 0.35	0.76 ± 0.27	0.89 ± 0.33
TIBC (μg/ml) ²	3.68 ± 0.66 ^b	3.62 ± 0.66	3.17 ± 0.66	3.39 ± 0.69
TfR (mg/l) ³	6.61 (5.86, 7.45)	6.69 (5.82, 7.68)	7.00 (5.90, 8.30)	6.84 (6.16, 7.59)
Serum ferritin (μg/l) ³	50.6 (40.2, 63.4)	59.9 (41.3, 86.8)	62.8 (45.4, 86.9)	61.35 (48.5, 77.6)
CRP (mg/dl) ³	1.05 (0.60, 1.85) ^{bc}	2.23 (1.23, 4.02)	3.22 (1.77, 5.86)	2.68 (1.78, 4.01)
IL6 (pg/ml) ³	0.69 (0.53, 0.91) ^{bc}	0.75 (0.57, 1.00) ^b	1.25 (1.03, 1.53)	0.97 (0.81, 1.17)
AGP (g/L) ²	0.79 ± 0.2 ^{bc}	0.90 ± 0.3	1.00 ± 0.3	0.95 ± 0.3
Hepcidin (ng/ml) ²	9.20 ± 6.44	11.36 ± 6.73	12.48 ± 5.90	11.92 ± 6.3
% IA -AA ³	19.0 (15.2, 23.5) ^c	12.2 (9.1, 17.0)	13.6 (8.8, 21.0)	12.9 (9.7, 16.9)
% IA +AA ³	29.5 (23.3, 38.2) ^{cd}	14.9 (11.3, 20.2)	18.4 (11.7, 28.4)	16.6 (12.8, 21.7) ^d

BMI, body mass index; Hb, Hemoglobin; TIBC, total iron binding capacity; sTfR, soluble transferrin receptor; CRP, C-reactive protein; IL6, interleukin-6; AGP, alpha 1 glycoprotein; IQR, % IA, fractional iron absorption; interquartile range; -AA: control meal; +AA: meal with ascorbic acid at a molar ratio of AA:Fe of 2:1. NW: normal weight BMI 18.5-24.9 kg/m²; OW: overweight BMI 25-29.9 kg/m²; OB: obese BMI 30-39.9 kg/m²; OW/OB, overweight and obese groups combined. Values are; ¹ median (IQR), ²mean (± s.d.) and ³geometric mean (95% confidence interval). Differences between NW, OW and OB were assessed using one-way ANOVA with post hoc Bonferroni correction and Kruskal-Wallis test followed by independent samples Mann-Whitney U test corrected for multiple comparisons. Difference between NW and OW/OB was assessed with independent samples t -test. ^a significantly different from overweight, ^b significantly different from obese, ^c significantly different from OW/OB, ^d significantly different from -AA meal in the same BMI group ($p < 0.05$).

Table 4.2 Correlations between body fat (%), hepcidin (ng/ml), iron absorption % (test meal without ascorbic acid (-AA)) with iron and inflammation markers in a group of women with BMI between 18.5 and 39.9 kg/m² (n=62).

	Body Fat (%)		Hepcidin		Iron Absorption (-AA)	
	r	P value	r	P value	r	P value
Body fat (%)	-	-	0.27	0.03	-0.23	0.07
Hb (g/dl)	0.17	0.18	0.41	0.001	-0.27	0.03
Serum iron (μg/ml)	-0.38	0.002	0.22	0.09	-0.11	0.38
TIBC (μg/ml)	-0.33	0.009	-0.37	0.003	0.18	0.15
TfR (mg/l)	-0.06	0.67	-0.29	0.02	0.35	0.006
Serum ferritin (μg/l)	0.26	0.04	0.59	<0.001	-0.58	<0.001
Hepcidin	0.27	0.03	-	-	-0.36	0.004
CRP (mg/dl)	0.42	0.001	0.16	0.23	-0.26	0.04
IL6 (pg/ml)	0.34	0.006	-0.01	0.94	-0.02	0.88
AGP (g/L)	0.47	<0.001	0.29	0.02	-0.08	0.53

Abbreviations: Hb, Hemoglobin; TIBC, total iron binding capacity; sTfR, soluble transferrin receptor; CRP, C-reactive protein; IL6, interleukin-6; AGP, alpha 1 glycoprotein. Iron absorption values from the control meal without ascorbic acid were used (5 mg ⁵⁷Fe as FeSO

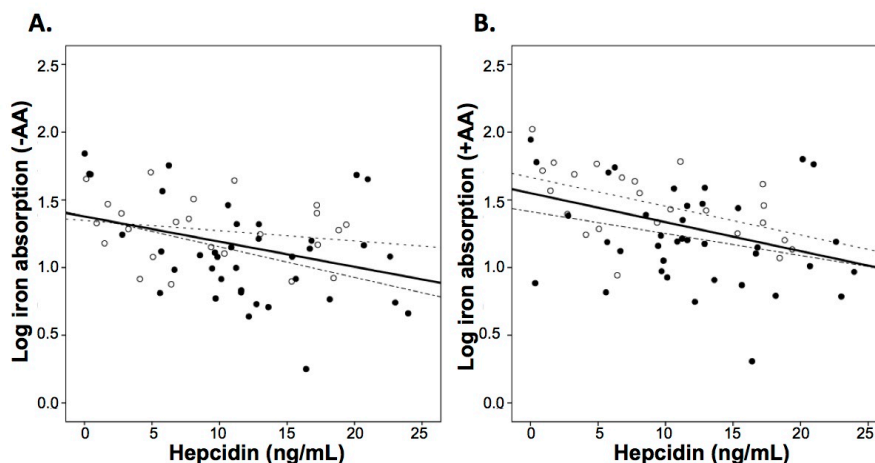


Figure 4.3 Associations between log-transformed iron absorption values and serum hepcidin from both meals for all women (n = 62) and BMI categories: NW (○, n=24) and OW/OB (●, n=38).
A. -AA: Total population: $r = -0.36$, $P = 0.004$; NW: $r = -0.21$, $P = 0.34$; OW/OB: $r = -0.39$, $P = 0.016$.
B. +AA: Total population: $r = -0.40$.

Table 4.3 Single and multiple linear regressions between % iron absorption from the two test meals (-AA/+AA) and hepcidin (ng/ml) in the total study population of women with a BMI between 18.5 and 39.9 kg/m² (n = 62)

	Crude			Adjusted		
	r²	β (95% CI)	P-value	r²	β (95% CI)	P-value
Iron absorption -AA	0.13	-0.85 (-1.41, -0.28)	0.004	0.30	-0.58 (-1.14, -0.03)	0.039
Iron absorption +AA	0.18	-1.41 (-2.18 -0.63)	0.001	0.25	-1.12 (-1.93, -0.32)	0.007

Adjusted for age and transferrin receptor (TfR).

-AA: 5 mg ⁵⁷Fe as FeSO₄

+AA: 5mg ⁵⁸Fe as FeSO₄ as well as 31.4 mg ascorbic acid at a molar ratio of AA:Fe of 2:1.

r² is the proportion of variance explained by the model. The β values represent the change in iron absorption associated with a change of 1 ng/mL in hepcidin concentration (for adjusted results it assumes that the effects of age and TfR are held constant).

Discussion

In this study we demonstrate that dietary iron absorption is lower in OW/OB versus normal weight women and the enhancing effect of ascorbic acid on iron absorption in OW/OB is only half that in normal weight women. There is expanding evidence that obesity and iron deficiency are linked (15, 47). It appears that the hypoferremia of obesity is not associated with dietary factors (2, 9, 22, 48) and it has been proposed that inflammation via hepcidin may be an important factor linking obesity with iron dysregulation (27). Our findings of lower serum iron and transferrin saturation are consistent with previous studies describing hypoferremia in obese subjects (6, 7). Moreover, our results show that TfR is a better predictor of iron absorption than SF or hepcidin in populations with different BMI. They are also consistent with the hypothesis that increasing hepcidin concentrations, along with subclinical inflammation, limit dietary iron absorption in subjects with excessive body fat. This is the first study assessing iron absorption using stable isotopes over a broad range of BMI that has measured serum hepcidin concentrations and several markers of inflammation.

Our results associating excess weight with reduced iron absorption are in line with previous studies: In Thai women, both adiposity and inflammation (assessed with CRP) were negatively correlated with iron absorption, independent of iron status, but hepcidin was not measured (27); similarly, in a recent cross-sectional study in Chilean women, obese women had lower fractional iron absorption compared with overweight and normal weight women ($P < 0.05$), but inflammatory biomarkers and serum hepcidin concentrations were not assessed (49). Numerous studies have reported serum hepcidin to be significantly elevated in obese compared to lean individuals but none of them have directly measured iron absorption (24, 28, 50, 51). In our study there was an increasing trend in hepcidin concentrations throughout the three BMI groups and hepcidin concentration was a negative predictor of iron absorption. One important result of the present study is that only a small portion of variability of iron absorption is associated with and thus potentially explained by hepcidin, with R^2 values ranging from 0.13 to 0.30 in simple and multivariate analyses. When we included serum ferritin together with serum hepcidin,

hepcidin was no longer a predictor of iron absorption. However, when TfR was included in the model both serum hepcidin and TfR were predictors of iron absorption.

This shows that when taking into account the separate effects of age and TfR on iron absorption, hepcidin still remained as a significant predictor. So hepcidin might play an important role regulating iron absorption regardless of iron status. We have previously reported that in 6-14 year-old children, overweight is associated with increased serum hepcidin compared to a normal weight group, and overweight children have poorer iron status, despite similar dietary iron intake (22). In that study, although serum hepcidin was positively correlated with BMI and body iron, there was no relationship between hepcidin and C-reactive protein, IL6 or leptin (22). Similarly, another study showed that elevated serum hepcidin in obese women was not correlated with IL6 or CRP (24). A recent study showed that overweight impairs efficacy of iron supplementation in iron deficient South African children. In that study, iron deficient children with higher BMI-for-age z-scores had a two-fold higher risk of remaining iron deficient after iron supplementation (52).

The mechanism of the up-regulation of hepcidin by adiposity and its influence on iron status is not fully understood. Hepcidin expression in the liver is regulated by a combination of factors including iron stores, erythropoiesis and inflammation, which control transcription (53). Bekri and colleagues (21) reported that hepcidin was produced by visceral and subcutaneous adipose tissue in vitro and that hepcidin expression was positively correlated with BMI and IL-6, suggesting that the liver is not the only source of increased hepcidin in obesity. However, Tussing-Humphreys and colleagues (54) found that there is no net release of hepcidin from abdominal subcutaneous adipose tissue depot in vivo in obese and lean women. Moreover, they reported that liver hepcidin mRNA expression was positively correlated with serum hepcidin concentrations, whereas adipose mRNA expression was not (24). Another study reported that leptin stimulates hepcidin mRNA production via the JAK (Janus kinase)-2 STAT (signal transducer and activator of transcription)-3 signaling pathway in human hepatoma cells (55), similar to IL6 (17, 20). However, it has also been demonstrated that adipose tissue may influence iron homeostasis in obese patients by expression of major iron-regulatory proteins, and the bone morphogenetic protein-hemojuvelin (BMP-HJV) signaling pathway could

be involved in regulating hepcidin expression in this tissue (16). Thus, the multimodal regulation of the increased hepcidin expression in obese subjects may explain the lack of correlation between hepcidin concentrations and the inflammatory markers CRP and IL6 in our study, and others. The other major factor that affects hepcidin secretion by the liver is iron status (53). It is difficult to define iron status in obesity because the associated inflammation increases SF. Although in our study there were no differences in TfR with weight status, both serum iron and transferrin saturation were lower in the obese subjects. If the obese subjects had slightly lower iron status, this would have been a trigger to lower serum hepcidin and increase iron absorption, but this signal was likely overridden by the inflammatory pathways described above.

The enhancing effects of ascorbic acid on non-heme iron absorption can be used to overcome the effect of food components that inhibit iron absorption, such as phytic acid (29) and to enhance iron absorption in iron depleted subjects (33). In this study we demonstrate for the first time that the enhancing effect of ascorbic acid on non-heme iron absorption in overweight and obese individuals is blunted. This is likely due to the different sites of action on the enterocyte of ascorbic acid and serum hepcidin in dietary iron absorption. Increased hepcidin reduces iron efflux into the circulation at the basolateral membrane of the enterocyte (18). In contrast, ascorbic acid improves transport of iron into enterocytes through their apical membrane (via the divalent metal transporter (DMT)-1), by reducing Fe^{3+} to Fe^{2+} (56). Thus, an explanation for our findings could be that although ascorbic acid allows more iron to enter the enterocyte, increased hepcidin reduces its efflux into the circulation, and when the enterocytes shed from the luminal surface the iron is lost. These findings argue against recommending increased intakes of ascorbic acid in obese individuals to improve iron status.

Strengths of the present study include: a) assessment of fractional iron absorption over a wide BMI range; b) direct measurement of blood volume that allows for a more precise calculation of iron absorption using stable isotopes, which is particularly valuable in obese subjects where estimation of blood volume is challenging; c) measurement of most factors known to influence iron absorption, including hepcidin, iron status, IL6, leptin and CRP. However, there are also some limitations to the study. As we have only used oral isotopic labels, we have not been

able to directly determine iron incorporation into erythrocytes but have assumed it to be the same for all subjects (80%). It was previously shown in a study in subjects with afebrile malaria, that even though iron absorption was reduced significantly before malaria treatment, incorporation was not affected (57). Based on this and other earlier studies in different subjects we concluded that iron incorporations seems to be relatively constant (58, 59). We have only studied women in Switzerland in this trial. Whether the results would have been the same in men and in other ethnicities remains to be demonstrated.

The widespread increase in overweight and obesity may limit current dietary strategies to improve iron absorption in iron deficient women, especially in certain low and middle-income countries where both conditions are prevalent. Further research is needed to better understand the underlying mechanisms that reduce iron absorption in overweight individuals in order to develop specific dietary recommendations to improve iron status in this population group.

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Chapter 5

In obese women, increased blood volume and reduced serum iron partially explain the higher risk for iron deficiency

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Abstract

Background: Excess weight, has been suggested to lead to increased blood volume (BV) which may explain lower serum iron (sFe) concentrations observed in obese individuals.

Objective: This observational study compares BV in normal-weight (NW) versus overweight (OW) and obese (OB) healthy, non-anemic women (n=62) (BMI 18.5-39.9 kg/m²). We examined if differences in BV might explain the reduced sFe in OW/OB women. Additionally we develop an equation to calculate BV in OW/OB women.

Design: We assessed body composition by dual energy X-ray absorptiometry (DXA), iron status, inflammation and BV by the carbon monoxide CO-rebreathing method.

Results: OW/OB women presented higher absolute BV, plasma and red blood cells, and lower sFe compared to NW ($P<0.05$). There were no significant differences in body iron stores comparing NW, OW and OB women. Weight ($r=0.74$), total lean mass ($r=0.82$) and body fat ($r=0.56$) were correlated with BV ($p<0.05$). BV ($r^2=0.22$, $\beta=-0.29$, $P=0.02$) was a negative predictor for sFe when adjusted for body iron stores. Equations utilizing combined height-weight and lean body mass were the most accurate for predicting BV in all BMI groups.

Conclusion: Due to the dilutional effect of BV, 'true' hypoferremia may be overestimated in populations with a high prevalence of obesity when using sFe as an indicator.

Keywords: Blood volume, obesity; iron deficiency; dilution

Introduction

Obesity has been shown to be a risk factor for low serum iron status in different settings (1). The hypoferrremia of obesity might be explained by a combination of biological factors such as inflammation, hemodilution and increased iron requirements. A likely mechanism is that adiposity-related inflammation increases hepatic hepcidin production impeding iron absorption in the duodenum (2). Moreover, obesity produces an increment in total blood volume and cardiac output that is caused, in part, by the increased metabolic demand induced by excess body weight (3). The blood volume enlargement encountered in obesity is explained by increased requirements caused by adipose tissue perfusion and by the concomitant increase in lean body mass (4). To what extent this larger blood volume could compromise plasma iron concentration in obese individuals has not been studied yet (2, 5-11).

Currently several equations exist for the determination of blood volume (12-14). These equations, however, were developed using data from normal weight subjects and it is unclear, whether they can also be used in overweight and obese women. Traditional methods for the exact determination of blood volume are invasive and often use radioisotopes (15, 16). The CO-rebreathing method is only minimally invasive and does not require the use of radioisotopes without losing validity or reliability (17). This optimized method is therefore an ideal tool to develop a new algorithm for the determination of blood volume in obese individuals.

The present study was undertaken to determine whether differences in blood volume between overweight/obese and normal-weight women might account for the hypoferrremia observed in the former group. Our hypotheses were that blood volume would be increased in overweight/obese subjects, and this would be associated with low serum iron, because of a dilutional effect. An additional goal was to develop a new equation to allow reliable calculation of blood volume based on simple measures such as weight and height in women of all weight categories.

Subjects and methods

Subjects

The subjects included in this study were reproductive-age women ($n = 64$): 24 with normal weight (NW: BMI 18.5-24.9 kg/m²); 20 with overweight (OW, BMI 25-29.9 kg/m²); and 20 with obesity (OB: BMI 30-39.9 kg/m²). This study was part of a study that investigated the influence of body weight and fat on iron absorption in NW, OW and OB women. No men were recruited since iron absorption is generally higher in women because of lower iron stores and higher losses during menstruation. Data on the relationship of body weight/fat to iron absorption has been published previously (18). In a screening visit we informed interested volunteers about the study aims, procedure and associated risks. Written informed consent was obtained from all the participants. Inclusion criteria for the study were: (1) female; (2) age 18-45 y; (3) pre-menopausal (no absence of a menstrual cycle in the past 12 months); (4) BMI 18.5-39.9 kg/m²; (5) no chronic illness and no significant medical conditions that could influence iron or inflammatory status other than obesity; (6) non-smoking (or at least no more than 1 cigarette/week and not smoking three days prior to the first measurement and during the study); (7) nonpregnant and not planning a pregnancy. The study criteria were assessed using a questionnaire, weight and height were measured to determine BMI and a pregnancy test was carried out. The study was approved by the ethics committees of the Swiss Federal Institute of Technology (Zurich, Switzerland) and the Ethics Committee of the Canton of Zurich (Kantonale Ethikkommission Zürich), and registered at clinicaltrials.gov NCT01884506.

Anthropometric measurements were conducted at the screening visit and a fasting blood sample was obtained for analysis of hemoglobin, iron status, inflammation and hepcidin concentration. Dual energy X-ray absorptiometry (DXA) was performed for determination of body composition at University of Zurich Irchel and a blood volume measurement using the carbon monoxide (CO)-rebreathing method at the University Clinic Balgrist in Zurich. To minimize fluctuations in blood volume all measurements were, if possible, performed between 7 and 14 days after the beginning of the last menstrual cycle.

The optimized CO rebreathing method (19) is a routinely applicable minimally invasive reliable method for assessing blood volume (20). This method has been described in detail elsewhere (17, 19, 21). Briefly, baseline venous samples were obtained (2 ml in an EDTA tube) 15 min after adopting a seated position for hemoglobin (Hb) and hematocrit (Hct) determination. All blood samples were immediately analyzed using a spectrophotometer for the blood gas determination (ABL 700 Serie, Radiometer A/S, Copenhagen, Denmark). Furthermore, capillary blood samples from the earlobe were obtained (35 μ l in pre-heparinized glass capillary tubes) in triplicate at baseline and at 6, 7 and 8 min after starting the rebreathing procedure. Percent carboxyhemoglobin saturation (HbCO%) was measured using a blood gas analyzer. The mean value of the measurements at 6 and 8 minutes was taken as the plateau value after CO-rebreathing with the sample at minute 7 as a backup. Total Hb mass was calculated as described previously (22), using a slightly different correction for loss of CO to myoglobin (0.3%/min of administered CO) (19). A bolus of chemically pure CO of 0.8 ml/kg body mass was administered during the first inspiration from a closed spirometric system (Blood tec GbR, Bayreuth, Germany) and was rebreathed for 2 min together with a small amount of oxygen (4 liters). To verify that no gas was leaking during the CO rebreathing procedure, the entire apparatus as well as the mouth piece and nose-clip were checked using a portable CO gas analyzer (Dräger PAC 7000; Dräger Safety; Lübeck, Germany) with a parts-per-million sensitivity to monitor local CO levels. The analyzer was also used to calculate end-tidal CO concentration before the CO-rebreathing and after the onset of the rebreathing procedure with the subject wearing a nose-clip and then blowing into a mouthpiece until the maximal value of CO observed was recorded. The amount of CO remaining in the spirometer after rebreathing was also measured with the portable CO gas analyzer. The determined parameters were used first to calculate total hemoglobin mass and from this to derive blood, plasma and red blood cell volume using equations published previously (17, 19, 22).

The reproducibility of the method was verified by a test-retest correlation analysis and quantified by the calculation of the typical error of measurement. For this assessment the same optimized CO rebreathing method was applied to the same respondents (n=8) on two separate occasions and the typical error (TE) was 1.2% with an R² 0.98 (23).

Laboratory analysis

Venous blood samples were drawn into EDTA-coated and into trace element-free Vacutainer tubes. Hemoglobin (Hb) concentration was measured using a Coulter counter (Beckman Coulter, Krefeld, Germany) with 3-level quality-control material (Liquichek; Bio-Rad, Irvine, CA) on the day of blood collection. Serum iron and total-iron binding capacity (TIBC) were determined using a colorimetric method as described previously (24, 25). Serum transferrin receptor (sTfR), serum ferritin (SF) and high sensitive C-reactive protein (CRP) and alpha 1 glycoprotein (AGP) were assessed by using enzyme-linked immunosorbent assay (ELISA) technique (26). Interleukin 6 (IL6) was measured by a Quantikine ELISA kit (R&D systems, Minneapolis, MN). The Thurnham correction factors were used in order to remove the effects of subclinical inflammation on serum ferritin concentrations (27). Body iron content was then calculated using the following equation proposed by Cook JD et al: body iron (mg/kg) = $-(\log(\text{TfR/SF ratio}) - 2.8229)/0.1207$ (28).

Statistical analysis

The statistical analyses were carried out using IBM SPSS Version 20 (IBM Company, Armonk, NY, USA). Data was checked for normality by visual observation and by using the Kolmogorov-Smirnov and Levene's normality tests. Non-normally distributed data were logarithmically transformed for statistical analysis. The mean (\pm s.d.) (for normally distributed data), geometric mean (95% confidence interval) (for data with normal distribution after log-transformation) or median (interquartile range) (for non-normally distributed data even after log-transformation) values for each parameter were determined. Nonparametric tests were applied for data that remained non-normally distributed after logarithmic transformation. Differences between NW, OW and OB were assessed using analysis of variance (ANOVA) with post hoc Bonferroni correction and Kruskal-Wallis test, followed by Mann-Whitney U test, as appropriate. Bivariate Pearson's or Spearman correlations and multiple linear regression models including correction for confounding variables were used to study associations between continuous variables. Differences were considered significant at p-values <0.05 .

To create a new equation for the calculation of blood volume, basic parameters such as height, weight, body surface area (BSA), BMI, total lean and fat mass were used in a regression analysis. Parameters were screened for those that best estimated blood volume. R square was used to determine the accuracy of this approximation.

Results

Of the 64 women enrolled, two subjects (1 OW and 1 OB) did not complete the study measurements and were therefore excluded from all analyses. Age, anthropometry, body composition, blood volume, iron status and inflammatory data are shown in **Table 5.1**. OW and OB individuals were older, heavier, had higher lean and adipose mass than NW participants. Hb, TfR, serum ferritin and body iron stores were in the normal range and comparable between the three BMI groups, whereas serum iron ($P = 0.025$) and TIBC ($P = 0.04$) were significantly lower in the OB compared to the NW group. OW and OB women had significant lower blood volume per unit of mass compared with those with NW ($P < 0.05$), however, absolute blood, plasma and red blood cell volume were higher in OW and OB than in NW women.

Correlations between weight, lean and fat mass and absolute blood volume are presented in **Figure 5.1**. Blood volume was more closely correlated with total lean mass ($r = 0.83$, $P < 0.001$), than fat mass ($r = 0.55$, $P < 0.001$), waist circumference ($r = 0.52$, $P < 0.001$), height ($r = 0.58$, $P < 0.001$) or BMI ($r = 0.50$, $P < 0.001$). Relations of blood volume with body weight ($r = 0.74$, $P < 0.001$), however, were similar to that with lean mass. Correlations between serum iron with anthropometry, body composition, blood volume and inflammation biomarkers are shown in **Table 5.2**. Serum iron was positively associated with hemoglobin ($r = 0.32$, $P = 0.01$), body iron stores ($r = 0.38$, $P = 0.002$), serum ferritin ($r = 0.34$, $P = 0.01$), and negatively associated with BMI ($r = -0.37$, $P = 0.003$), weight ($r = -0.36$, $P = 0.004$), fat mass ($r = -0.38$, $P = 0.002$), AGP ($r = -0.27$, $P = 0.04$), blood- ($r = -0.20$, $P = 0.11$) and plasma volume ($r = -0.25$, $P = 0.05$). In multiple linear regression analysis, total body fat was a significant negative predictor of serum iron ($r^2 = 0.32$, $\beta = -0.42$, $P < 0.001$) when corrected for body iron stores. Lean mass was not a predictor of serum iron ($\beta = 0.00$, $P = 0.98$). In a different model blood volume was a significant negative predictor for serum iron

($r^2=0.22$, $\beta=-0.29$, $P=0.02$) when corrected for body iron stores (**Table 5.2**).

The following equations for the prediction of blood volume by anthropometric and body composition measurements were created using stepwise regression with a forward selection of covariates (**Table 5.3**):

- (1) Blood volume (ml) = $4698.8 * \text{height (m)} + 32.5 * \text{weight (kg)} - 5342.1$ ($r^2=0.69$, $P<0.01$)
- (2) Blood volume (ml) = $2686.7 * \text{height (m)} + 18.9 * \text{weight (kg)} + 66 * \text{lean mass} - 3932.3$ ($r^2=0.76$, $P<0.01$)

Table 5.1. Baseline characteristics of the study population of apparently healthy normal weight (NW), overweight (OW) and obese (OB) women participating in a blood volume study (n=62)

n	NW (24)	OW (19)	OB (19)	OW/OB (38)
Age (y) ¹	23 (IQR 4) ^{b,c}	26 (IQR 6)	27 (IQR 10)	27 (IQR 8)
Weight (kg) ²	61.8 \pm 7.2 ^{abc}	78.7 \pm 7.3 ^b	89.0 \pm 12.0	83.9 \pm 11.1
BMI (kg/m ²) ²	21.9 \pm 1.9 ^{abc}	27.3 \pm 1.5 ^b	32.8 \pm 2.8	30.1 \pm 3.6
Total body fat % ²	29.8 \pm 4.8 ^{abc}	40.1 \pm 4.4 ^b	45.8 \pm 4.2	43.0 \pm 5.1
Fat mass (kg)	18.0 \pm 4.6 ^{abc}	30.9 \pm 5.1 ^b	40.1 \pm 8.1	35.5 \pm 8.1
Lean mass (kg)	42.9 \pm 4.9 ^{bc}	46.2 \pm 4.8	47.1 \pm 5.0	46.7 \pm 4.8
Blood volume (ml/kg) ²	74.9 \pm 5.8 ^{abc}	64.3 \pm 5.3 ^b	59.4 \pm 7.1	61.8 \pm 6.7
Blood volume (ml) ²	4593 \pm 463 ^{abc}	5098 \pm 583	5327 \pm 1012	5212 \pm 823
Plasma volume (ml/kg)	46.6 \pm 4.8 ^{abc}	40.1 \pm 4.1	36.9 \pm 4.9	38.5 \pm 4.7
Plasma volume (ml)	2855 \pm 301 ^{abc}	3172 \pm 401	3312 \pm 657	3242 \pm 541
RBC (ml/kg)	28.2 \pm 2.1 ^{abc}	24.3 \pm 2.6	22.4 \pm 2.8	23.4 \pm 2.8
RBC volume (ml)	1738 \pm 217 ^{abc}	1926 \pm 259	2015 \pm 388	1970 \pm 328
Hemoglobin Mass (g/kg)	9.2 \pm 0.7 ^{abc}	7.9 \pm 0.8	7.3 \pm 0.9	7.6 \pm 0.9
Hemoglobin Mass (g)	566 \pm 71 ^{abc}	627 \pm 85	656 \pm 126	641 \pm 107
Hb (g/dl) ²	13.5 \pm 0.9	13.7 \pm 1.2	13.7 \pm 1.0	13.7 \pm 1.1
Serum iron (μ g/ml) ²	1.06 \pm 0.44 ^b	1.01 \pm 0.35	0.76 \pm 0.27	0.89 \pm 0.33
TIBC (μ g/ml) ²	3.68 \pm 0.66 ^b	3.62 \pm 0.66	3.17 \pm 0.66	3.39 \pm 0.69
TfR (mg/l) ³	6.61 (5.86, 7.45)	6.69 (5.82, 7.68)	7.00 (5.90, 8.30)	6.84 (6.16, 7.59)
Serum ferritin (μ g/l) ³	50.6 (40.2, 63.4)	59.9 (41.3, 86.8)	62.8 (45.4, 86.9)	61.35 (48.5, 77.6)
Body iron (mg/kg)	5.64 \pm 2.70	5.92 \pm 3.31	5.46 \pm 3.10	5.70 \pm 3.17
CRP (mg/dl) ³	1.05 (0.60, 1.85) ^{bc}	2.23 (1.23, 4.02)	3.22 (1.77, 5.86)	2.68 (1.78, 4.01)
IL6 (pg/ml) ³	0.69 (0.53, 0.91) ^{bc}	0.75 (0.57, 1.0) ^b	1.25 (1.03, 1.53)	0.97 (0.81, 1.17)
AGP (g/L) ²	0.79 \pm 0.2 ^{bc}	0.90 \pm 0.3	1.00 \pm 0.3	0.95 \pm 0.3

Abbreviations: NW: normal weight (BMI 18.5-24.9 kg/m²); OW: overweight (BMI 25-29.9 kg/m²); OB: obese (BMI 30-39.9 kg/m²); OW/OB: overweight and obese (BMI 25-39.9 kg/m²). BMI, body mass index; Hb, Hemoglobin; TIBC, total iron binding capacity; TfR, soluble transferrin receptor; CRP, C-reactive protein; IL6, interleukin-6; AGP, alpha 1 glycoprotein; IQR, interquartile range; Values are; ¹ median (IQR), ² mean (\pm s.d.) and ³ geometric mean (95% confidence interval). Differences between NW, OW and OB were assessed using one-way ANOVA with post hoc Bonferroni correction and Kruskal-Wallis test followed by independent samples Mann-Whitney U test corrected for multiple comparisons. Difference between NW and OW/OB was assessed with independent samples *t*-test. ^a significantly different from overweight, ^b significantly different from obese, ^c significantly different from OW/OB. P values <0.05 were considered significant.

Discussion

In the present study we show that overweight and obese women had significantly larger blood volume and a lower serum iron concentration than the normal weight group. Moreover, blood volume was a significant negative predictor of serum iron concentration. Thus, our results confirm the hypothesis that combinations of biological factors including dilution play a role in the hypoferrremia of obesity. Similar to other studies we found that obese women present lower serum iron concentrations compared to normal-weight women (6-8, 11) but Hb and body iron stores were not significantly different between BMI groups. In our study the increase in blood volume encountered in obesity partly explained the decrease in serum iron. Moreover, serum iron was positively associated with serum ferritin and negatively associated with BMI, weight, fat mass and AGP but not with CRP. Therefore, the obesity related inflammation as measured with AGP could also explain the observed decrease in iron status in overweight and obese women. Our results are in agreement with previous studies showing that increased levels of inflammatory markers could contribute to diminished iron status despite elevated serum ferritin concentrations (11, 29). Typically, blood volume has been expressed in terms of ml per kg of total body weight (ml/kg), using fixed ratios for normal values, with different ratios for men and women. Calculation of the Estimated Blood Volume (EBV) is usually performed with the following formula: body weight (kg) x average blood volume (ml/kg). Blood volume is estimated to be 65-75 ml/kg (adult men, 75ml/kg; and women, 65ml/kg) (30). However, estimation of blood volume in overweight and obese individuals is challenging.

Obesity produces an increment in total blood volume and cardiac output that is caused in part by the increased metabolic demand induced by excess body weight (31). Therefore, with increasing body weight there is an increase in blood volume, but the relationship between blood volume and body weight is not linear. In overweight and obese individuals adipose tissue comprises a substantial proportion of total body weight. However, perfusion of the increased amount of adipose tissue alone does not explain the increase in blood volume since adipose tissue is oligemic compared with lean tissue. One explanation is that the enlarged vascular beds of adipose tissue are less vascularized than other tissues (3). Another explanation is that modulation of blood flow in adipose tissue typically prevents the redistribution of the extra volume present in the interstitial space of adipose tissue into circulation (32).

Finally, the simultaneous increase in lean body mass in obese subjects also account for some of the increased blood volume (4, 33). In a previous study made in 3107 American Indian overweight individuals, a comparison of echocardiographic left ventricular mass to fat free mass and adipose body mass determined by bioelectric impedance was performed. They found that left ventricular mass is more strongly related to fat free mass than to adipose mass, waist/hip ratio, BMI, or height-based surrogates for mean body weight (30). Therefore, body composition is an important factor determining blood volume in overweight and obese individuals. Currently existing formulas were developed between the 1950's and the 1970's (12, 16, 34-38). Each equation involves different parameters that could influence blood volume such as body size (height, weight and body surface area), body composition (fat or lean body mass) or combinations of these parameters as predictors. However the study populations used to derive the equations rarely included women with overweight and obesity and thus, the equations may not be suitable for this population. Therefore a distinct advantage of the formulas for calculating blood volume that we are proposing is that they were derived from a population of women with an extensive BMI range. Moreover, inclusion of lean mass in the formula contributes to a better estimation of blood volume in individuals with overweight and obesity.

Table 5.2 Univariate correlations between serum iron with anthropometry, body composition, blood volume and inflammation biomarkers in a group of women (n=62) with BMI between 18.5 and 39.9 kg/m²

Serum iron		
Variable	r *	P value
BMI (kg/m ²)	-0.37	0.003
Weight (kg)	-0.37	0.004
Lean mass (g)	-0.16	0.21
Fat mass (g)	-0.38	0.002
Blood volume (ml)	-0.20	0.11
Plasma Volume (ml)	-0.25	0.049
RBC (ml)	-0.10	0.43
Hb Mass (g)	-0.10	0.46
Hb (g/dl)	0.32	0.01
TfR (mg/L)	-0.18	0.16
Serum ferritin (μg/l)	0.34	0.01
Body iron (mg/kg)	0.38	0.002
CRP (mg/dl)	-0.09	0.48
IL6 (pg/ml)	-0.16	0.22
AGP (g/L)	-0.27	0.04

Abbreviations: BMI, body mass index; RBC, Red Blood Cell; Hb, Hemoglobin; TfR, soluble transferrin receptor; CRP, C-reactive protein; IL6, interleukin-6; AGP, alpha 1 glycoprotein.

* Spearman's rho coefficient or Pearson's coefficient. P values <0.05 were considered significant.

Table 5.3 Blood volume calculated with CO-rebreathing method and derived from linear regression equations with weight and height (Formula 1) or weight, height and lean mass (Formula 2) as predictor variables in women (n=62) with body mass index (BMI) 18.5 to 39.9 kg/m²

	Normal weight (n=24)			Overweight/Obese (n=38)			Total population (n=62)		
	Mean BV	SD	R ²	Mean BV	SD	R ²	Mean BV	SD	R ²
	ml	ml		ml	ml		ml	ml	
Calculated CO-rebreathing	4593	463	-	5212	823	-	4973	764	-
Formula 1: weight and height	4557	448	0.52	5235	593	0.69	4973	633	0.69
Formula 2: weight, height and lean mass	4575	531	0.75	5221	627	0.75	4971	668	0.76

Abbreviations: BV, Blood Volume; CO, Carbon Monoxide; Formula 1: (4698.8 X height) + (32.5 X weight) - 5342.1; Formula 2: (2686.7 X height) + (18.9 X weight) + (66.0 X lean mass) -3932.3. Height in centimeters and weight in kilograms. The accuracy of the regression equation was assessed using R², the coefficient of determination. R² presents the proportion of the observed variance that can be explained through the equation

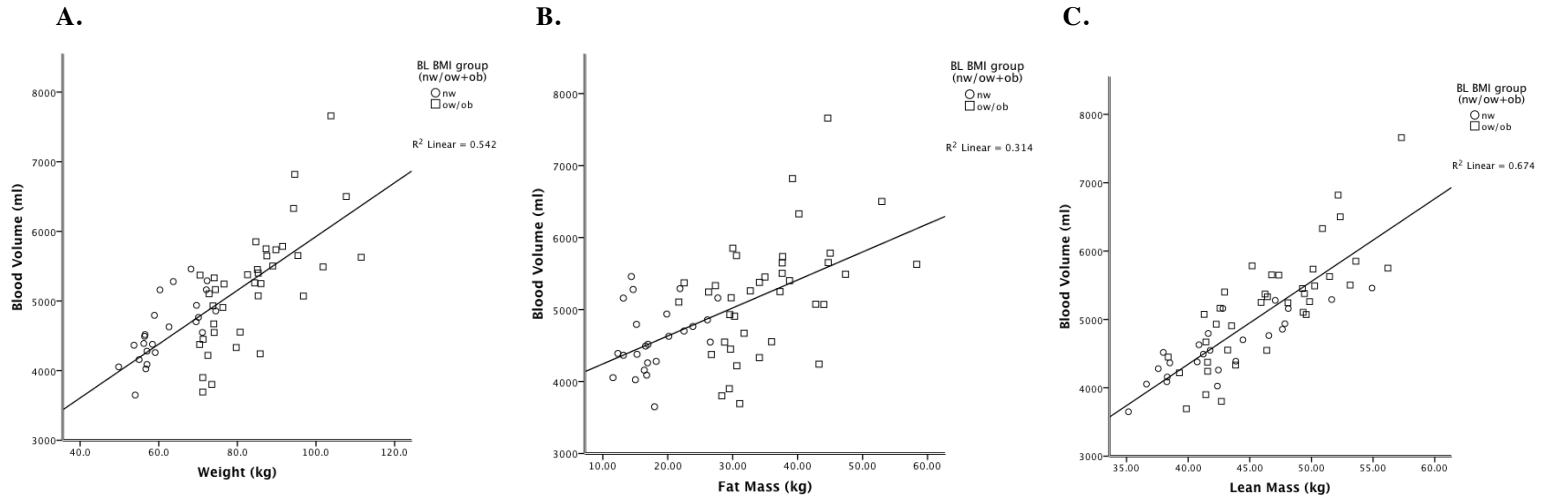


Figure 5.1. Correlations between absolute blood volume and **A.** weight; **B.** lean mass and; **C.** fat mass.

Our interest in calculating blood volume of overweight and obese subjects was to be able to correctly interpret absorption data from studies conducted in overweight and obese subjects, for which blood volume calculation is necessary. The assessment of blood volume is however also an important tool in clinical medicine for the evaluation of several disorders or diseases. Additionally, changes in blood volume affect the distribution of numerous drugs. For example, increases in total blood volume can affect peak plasma concentration, clearance and elimination half-life of many anesthetic agents (39). Thus, doses of drugs are scaled based on the individual patient characteristics including body composition and blood volume (40).

The strengths of the present study include the assessment of blood volume using the CO-rebreathing method over a wide BMI range; including an assessment of the reproducibility of the CO-rebreathing method; measurement of different iron status indicators such as serum iron, TfR and serum ferritin; the use of different inflammatory indicators such as AGP, IL6, and CRP. A limitation of the study is that we have only studied women in Switzerland in this trial. Whether the results would have been the same in men and in other ethnicities remains to be demonstrated. Moreover, different steps involved in blood volume measurements by CO-rebreathing method present potential opportunities for error. However, most of the parameters that could influence the measurements were standardized and therefore measurement errors were minimized. Moreover the reproducibility of the method was verified by a test-retest correlation analysis and the coefficient of variation was low (1.2%)

Our findings confirm that obese subjects have increased blood volume and that a dilutional effect, together with inflammation, seems to be playing a role in the hypoferrremia of obesity. Based on our results we can also conclude, that the iron requirements of obese women actually seem to be increased. Efforts for controlling iron deficiency in countries with high prevalence of obesity require selection of appropriate interventions that suit the population to ensure effectiveness.

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Chapter 6

The effects of fat loss after bariatric surgery on inflammation, serum hepcidin and iron absorption: a prospective 6-month iron stable isotope study

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Abstract

Background: Iron deficiency is common in obese subjects. This may be due to adiposity-related inflammation increasing serum hepcidin and decreasing iron absorption.

Aim: We evaluated whether weight and fat loss in obese subjects would decrease inflammation and serum hepcidin and thereby improve iron absorption.

Methods: We performed a 6-month, prospective study in obese (body mass index (BMI), $\geq 35 < 45$ kg/m²) adults who had recently undergone laparoscopic sleeve-gastrectomy. At 2 months and 8 months post-surgery, subjects consumed a test drink with 6mg ⁵⁷Fe as ferrous sulfate and were intravenously infused with 100 µg ⁵⁸Fe as iron citrate. We compared erythrocyte incorporation of iron isotopic labels, changes in body composition, iron status, hepcidin and inflammation at 2 and 8 months.

Results: 43 subjects were studied at baseline, 38 completed the protocol (32 women, 6 men). After 6 months, total body fat (TBF%), inflammation and hepcidin were significantly lower (all, $P < 0.005$). In iron-deficient subjects ($n=17$), geometric mean (95%CI) iron absorption significantly increased (from 9.7% (6.5-14.6) to 12.4% (7.7-20.1) ($P=0.03$), while in iron sufficient subjects ($n=21$), it did not change (5.9% (4.0-8.6) and 5.6% (3.9-8.2)) ($P=0.81$)). In all subjects, there was a non-significant increase in iron absorption (7.3% at 2 months vs. 8.0% at 8 months, $P=0.37$).

Conclusion: Our findings suggest adiposity-related inflammation reduces the normal up-regulation of iron absorption in iron-deficient obese subjects, and that this adverse effect is ameliorated by fat loss.

Introduction

Overweight and diet-related non-communicable diseases are major public health problems in many low and middle-income countries where micronutrient deficiencies remain common (1). This “dual burden” of undernutrition and obesity exists not only in countries and communities (2) but within households (3), and even within individuals, who may be obese and have micronutrient deficiencies. Data from several cross-sectional studies has shown an association between obesity and iron deficiency in children and adults (4-11). This poses a double health burden in high-risk populations, as both iron deficiency and obesity have adverse short and long-term effects (12).

In Mexico, the combined prevalence of overweight and obesity in adults is 71.3% (overweight 38.8% and obesity 32.4%). The northern cities of Mexico have the highest burden of the obesity epidemic (73%). At the same time, iron deficiency remains a public health problem in Mexico, where 23.1% of non-pregnant women of reproductive age (20 to 50 years) are iron deficient (13). The observed double burden in Mexico may be due to sedentary lifestyles and a high intake of energy-dense, micronutrient-poor foods (14). The co-existence of micronutrient deficiencies and overweight in low and middle-income countries, like Mexico, is projected to have exponentially increasing social and economic costs (15, 16).

Various explanations for the link between obesity and low iron status have been proposed including, low iron intake, increased iron requirements and impaired systemic iron homeostasis. The difference in iron status when comparing normal and overweight individuals does not appear to be explained by dietary iron intake (17-20). A likely mechanism is that adiposity-related inflammation increases hepatic hepcidin production, which blocks iron release from enterocytes and macrophages by degrading iron exporter ferroportin, impairing iron homeostasis in obese individuals (18, 21). Serum hepcidin concentrations are significantly higher in obese compared to lean individuals (19, 22-24). In obese women undergoing weight reduction after restrictive bariatric surgery, serum hepcidin concentrations normalized and iron status improved, however, iron absorption was not measured (24).

Thus, there is little available data on the direct effects of fat mass loss on iron absorption in obesity. Moreover, previous studies were done in populations without a high prevalence of iron deficiency, so it is unclear whether high serum hepcidin in obesity would persist even in the presence of iron deficiency. The purpose of this study was to examine if weight loss, particularly fat loss, in obese adults would affect iron absorption, as measured by iron stable isotopes. Our hypotheses were: a) adiposity-related inflammation would be reduced by fat loss, resulting in a decrease in serum hepcidin; and b) this would improve iron absorption, with a stronger effect in iron deficient individuals, because of the physiological drive to up-regulate iron absorption.

Subjects and methods

Study site

The study was conducted between January 2011 and December 2012 at the Hospital Conchita Christus Muguerza in Monterrey, an industrial city in the state of Nuevo Leon, located in northeast Mexico. The research protocol was approved by the Medical Ethical Committee of the WUR, the Netherlands; the Medical Ethical Committee of the ETH, Switzerland; the medical research and ethical committee of University of Monterrey; and the ethics committee of the Federal Commission for the Protection against Sanitary Risk (COFEPRIS). The followed procedures were in accordance with the Helsinki Declaration of 1975 as revised in 1983. The clinicaltrials.gov identifier for this study is NCT01347905.

Subjects

Forty-three obese (BMI $\geq 35 < 45$ kg/m²) individuals (36 premenopausal women and 7 men), who elected to undergo laparoscopic sleeve gastrectomy, (LSG) were recruited for this study in a pre-surgery assessment consult. This population was selected for the study because the LSG induces significant weight loss within a period of 6 months, while preserving the small intestine and not causing significant nutrient malabsorption. Thus, allowing us to evaluate the impact of fat loss on inflammation, hepcidin, iron status and absorption. Since the mechanism of iron absorption is the same in men and women, both genders were recruited. Subjects

were ineligible for the study if they had any disorder that could influence iron metabolism or inflammatory status (i.e., cancer, HIV/AIDS, gastrointestinal bleeding, rheumatoid arthritis, renal disease or hemochromatosis), had any serious complications during surgery (i.e. blood loss > 500ml or perforation of the gastrointestinal tract), or if they were using medication known to influence inflammation or iron metabolism, were pregnant or gave birth within the past 6 months, were lactating 6 weeks prior to the study, had full or partial hysterectomy, performed strenuous exercise (> 10 hours per week), or consumed large amounts of alcohol (> than 14 drinks for women or > 21 drinks for men in a typical week).

Sample size calculation for this study was based on changes in iron absorption observed in previous studies. The expected standard deviation of the difference (intra-subject) in log fractional iron absorption was 0.27 (25, 26). In order to detect a 30% difference in iron absorption with an alpha of 0.05 and a power of 80%, a sample size of 30 subjects would be sufficient. It was increased by 45%, final size of 43, to account for 30% of subjects not expected to successfully achieve a 10% weight loss (of the initial body weight) in 6 months (based on information provided by surgeon) and a potential 15% dropout rate.

Study procedures

Obese individuals, who elected and were cleared to undergo LSG, were invited to attend a screening meeting, where the study was explained and a brief questionnaire, including lifestyle and relevant medical history, was administered. Subjects fulfilling the inclusion criteria - with the exception of post-surgery complications, which were evaluated at the time of enrolment (3-4 weeks after surgery) - were asked to participate in the study. A pre-surgical blood sample was drawn to analyze inflammatory markers [high sensitive C-reactive protein (CRP) and alpha 1 acid glycoprotein (AGP)] and iron status [serum ferritin (SF) and soluble transferrin receptor (TfR)], which were later compared to post-surgical concentrations. Participants were asked not to consume any dietary supplements containing iron for the duration of the study.

Subjects underwent a baseline iron absorption test 2 months after LSG (when surgery-related inflammation was expected to be resolved) and a follow up

iron absorption test 6 months after baseline. A week before measurements, participants were asked if they were experiencing any common infectious diseases (common cold, flu, urinary infection, etc.) that could influence the inflammatory analyses. If this was the case, the appointment was rescheduled until resolution of the problem. During the testing period participants were asked not to consume nonsteroidal anti-inflammatory drugs to minimize possible acute effects on iron status, inflammation or hepcidin production. Subjects arrived around 0700-1000 h in a fasted state (>8 h). During the first visit all subjects were carefully instructed about the aims and procedures of the study and written informed consent was obtained. A well-trained professional administered a structured questionnaire to collect background information including age, education level, occupation, family size, reproductive history, menstrual status, current health status, disease prevalence, cigarette use, alcohol consumption and use of fortified foods. A baseline venous blood sample was drawn for determination of hemoglobin (Hb), hematocrit (HCT), iron status (SF and TfR), inflammation [CRP, AGP, interleukin-6 (IL6), tumor necrosis factor-alpha (TNF- α) and leptin] and hepcidin concentrations. Subsequently, subjects received a standardized test drink containing \approx 46 ml of distilled water labeled with 6 mg ^{57}Fe as ferrous sulfate followed by 100 ml of distilled water. One hour later, 2 ml of an aqueous solution containing 100 μg ^{58}Fe as iron citrate in \approx 260cc of normal saline was infused over 50 min. Immediately after the infusion, a blood sample was drawn to estimate blood volume by isotopic dilution techniques.

The use of the intravenous dose of ^{58}Fe along with the oral dose of ^{57}Fe allows for a precise determination of iron absorption, even in obese subjects in which blood volume cannot be estimated easily. Subjects were asked not to eat or drink for 4 hours after ingestion of the labeled test meal in order to minimize the impact of additional foodstuffs on iron absorption. During the same visit (plus two weeks) anthropometry and body composition was measured at the body composition laboratory of the Autonomous University of Nuevo Leon (UANL) as described below. This procedure was performed again at 6-months follow-up.

Anthropometric and body composition measurements

Subjects were weighed to the nearest 0.1 kg wearing a standardized non-woven medical coat on a digital scale. Height (cm) was measured to the nearest 0.1

cm using a fixed stadiometer, and waist circumference was measured using a flexible tape, to the nearest 0.1 mm according to standardized procedures (27). Measurements were done in triplicate. Body composition, especially body fat mass and lean body mass, was assessed by dual-energy X-ray absorptiometry (DXA) with a Lunar DPX-L densitometer (GE Lunar, Madison, WI).

Laboratory analysis

Venous blood samples were drawn in tubes containing EDTA as anticoagulant and in coagulation tubes. Hb concentration was measured on site in whole blood with an automated hematology analyzer (Sysmex Corporation, Kobe, Kansai, Japan). The samples from the coagulation tubes were centrifuged for 10 min at 3000 rev/min to separate the serum. The rest of the blood and serum samples were aliquoted and stored at -80°C until analysis. SF and CRP were measured using an automated Abbot analyzer (reagents from Abbott Laboratories, Wiesbaden, Germany). ELISA kits from different producers were used to measure TFR (Quantikine IVD ELISA kit, R&D systems, Minneapolis, MN), AGP, IL6 and TNF- α (Quantikine ELISA Kits R&D systems, Minneapolis, MN), leptin (sensitive ELISA kit, EMD Millipore Corporation, Billerica, MA) and hepcidin (hepcidin-25 ELISA kit, Bachem, UK). Baseline and follow-up samples of all subjects were assayed in the same batch to minimize inter-assay variability. Anemia was defined as $\text{Hb} \leq 12 \text{ g/dl}$ and subjects were classified as iron deficient ($\text{SF} < 30 \mu\text{g/L}$ or $\text{TfR} > 28.1 \text{ nmol/L}$) or iron sufficient ($\text{SF} \geq 30 \mu\text{g/L}$ or $\text{TfR} \leq 28.1 \text{ nmol/L}$) (28).

For isotope analysis, whole blood was mineralized by microwave digestion, and iron was separated by anion-exchange chromatography and a subsequent solvent-solvent extraction step into diethylether. The isotopic analysis of ^{58}Fe and ^{57}Fe were performed by inductively coupled plasma mass spectrometry with a high-resolution double focusing mass spectrometer (Neptune; Thermo-Finnigan, Bremen, Germany) equipped with a multicollector system for simultaneous ion beam detection.

The fractional absorption of iron was calculated using the oral to intravenous tracer ratio method (29, 30). The oral to intravenous tracer ratio was calculated from the isotopic ratios measured in the erythrocytes 14 days after

administration, using isotopic dilution principles and taking into account that the isotopic tracers were not mono-isotopic (31):

$$\left(\frac{n_{oral}}{n_{iv}}\right) = \frac{\delta - \beta}{\alpha - \gamma} \quad Abs_{oral} = \left(\frac{n_{oral}}{n_{iv}}\right) \cdot \frac{d_{iv}}{d_{oral}} \cdot 100 \quad \text{Where:}$$

$$\alpha = \frac{{}^{56}a_{oral} - R_{56/57} \cdot {}^{57}a_{oral}}{{}^{56}a_{nat} - R_{56/57} \cdot {}^{57}a_{nat}} \quad \beta = \frac{{}^{56}a_{iv} - R_{56/57} \cdot {}^{57}a_{iv}}{{}^{56}a_{nat} - R_{56/57} \cdot {}^{57}a_{nat}}$$

$$\gamma = \frac{{}^{56}a_{oral} - R_{56/58} \cdot {}^{58}a_{oral}}{{}^{56}a_{nat} - R_{56/58} \cdot {}^{58}a_{nat}} \quad \delta = \frac{{}^{56}a_{iv} - R_{56/58} \cdot {}^{58}a_{iv}}{{}^{56}a_{nat} - R_{56/58} \cdot {}^{58}a_{nat}}$$

With n_{oral} and n_{iv} representing the amounts in mol of respectively the oral and the intravenous labels in the erythrocytes; ${}^{56}a_{nat}$, ${}^{57}a_{nat}$, ${}^{58}a_{nat}$, ${}^{56}a_{oral}$, ${}^{57}a_{oral}$, ${}^{58}a_{oral}$ and ${}^{56}a_{iv}$, ${}^{57}a_{iv}$ and ${}^{58}a_{iv}$ are the isotopic abundances in % mol of ${}^{56}\text{Fe}$, ${}^{57}\text{Fe}$ and ${}^{58}\text{Fe}$ in natural iron, oral tracer and intravenous tracer, respectively. $R_{56/57}$ and $R_{56/58}$ represent the isotopic ratios measured in the erythrocytes, and d_{oral} and d_{iv} the administrated doses of tracers, in mol. Abs_{oral} is the fractional absorption of the orally administrated tracer, in %.

Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 (IBM Company, Armonk, NY, USA). Results are expressed as means and standard deviations (\pm SD). If not normally distributed, the results are presented as geometric mean and 95% confidence interval or median and interquartile range (IQR) and were log transformed to achieve normality prior to analysis. A change score percentage was created (e.g., (follow-up – baseline / baseline)*100) for all study parameters (anthropometry, body composition, iron status, inflammation, serum hepcidin and iron absorption). Paired t-tests and Wilcoxon signed-rank test (for non-normally distributed variables after log transformation) were used to examine if changes over time in body weight, waist circumference, body composition, iron absorption, and biochemical variables were significant. Pearson and Spearman correlation coefficients were determined to assess the relationship between body fat, iron status, inflammation, hepcidin and iron absorption. Potential confounders (age, gender, iron status, hemoglobin and inflammation) were examined by using forward stepwise selection techniques. Multiple linear regression models were used to evaluate the effect of change in weight, fat mass inflammation, leptin and serum hepcidin from

baseline on follow-up variables (change in iron status, inflammation, serum hepcidin and iron absorption as the dependent variable).

A sub-group analysis of anthropometric and biochemical changes over time was used to compare subjects classified according to baseline iron status as “iron deficient” or “iron sufficient”. Differences between baseline and follow-up within each of the 2 groups were analyzed by using paired samples t-test and Wilcoxon signed-rank test. Within group differences were analyzed by using independent sample t-test and Mann-Whitney U test. Considering that iron absorption is generally higher in women because of lower iron stores and periodic losses during menstruation, analyses were performed with data from men and women combined and women only. However, the results remained the same and data is presented for both genders combined. Moreover, there was no significant difference in age or BMI between sexes. A probability value <0.05 was considered statistically significant.

Results

Of the 43 subjects studied at baseline, 5 (4 women and 1 men) did not return for follow-up (4 relocated and 1 did not lose $>10\%$ of the initial body weight), which resulted in a study population of 38 (32 women and 6 men) that was used for analysis. Mean age was $34 (\pm 8)$ years.

Table 6.1 summarizes anthropometric characteristics, body composition and inflammatory biomarkers categorized by baseline iron status into iron deficient and iron sufficient groups. Baseline (2 months post-surgery) body composition, inflammatory markers and anthropometric characteristics except for weight were comparable between iron status groups. Weight was lower in the iron deficient compared to the iron sufficient group ($P = 0.03$). **Table 6.2** shows iron status biomarkers, hepcidin and fractional iron absorption (%) at baseline and after 6 months weight loss, categorized by baseline iron status. Anemia was seen in 16% ($n = 6$) of the subjects at baseline and 34% ($n = 13$) at follow-up. Likewise, iron deficiency was detected in 45% ($n = 17$) at baseline and 47% ($n = 18$) of the study population at follow-up. Anemia and iron deficiency were mainly present in women, except for one man who had iron deficiency at baseline.

The changes in inflammation and iron status indices from pre-surgery to baseline measures are illustrated in **Figure 6.1**. CRP concentration was significantly decreased ($P < 0.001$) after LSG surgery and no significant change was observed for AGP ($P = 0.78$). Additionally, SF concentration decreased ($P = 0.03$) and TfR increased ($P = 0.01$). At 6 months follow-up, weight, total body fat (TBF%), inflammation and hepcidin were significantly reduced (weight $P < 0.001$, TBF%, $P < 0.001$; CRP, $P < 0.001$; IL6, $P = 0.004$; leptin, $P < 0.001$; hepcidin, $P = 0.001$) in the entire study population (**Figure 6.2**). Also, Hb, HCT and iron status indices were reduced ($P < 0.05$). However, the geometric mean (95%CI) of iron absorption did not change from baseline to follow-up (7.3% vs. 8.0%, $P = 0.37$).

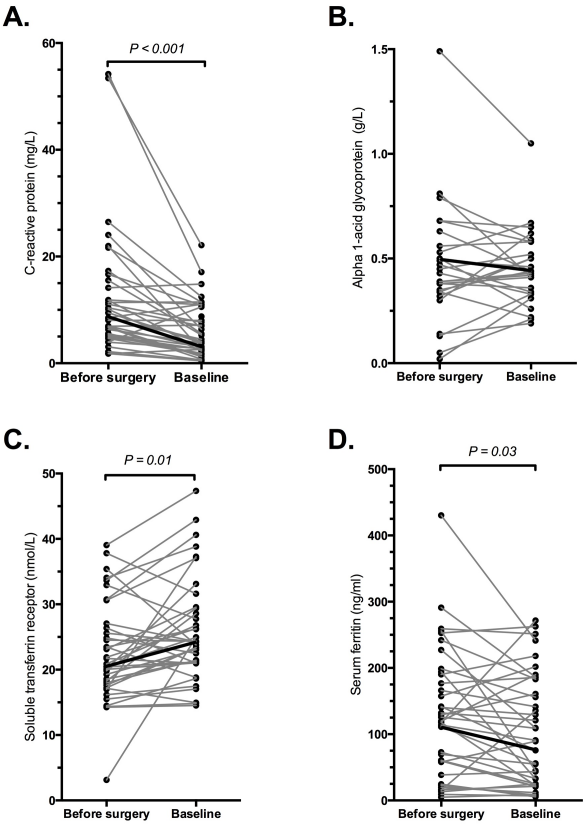


Figure 6.1 Change in inflammation and iron status from pre-surgery to baseline measures (2 months after surgery) in a group of adults ($n=38$) participating in an iron absorption study. **A)** C-reactive protein (CRP) was significantly decreased after LSG surgery [8.46 (1.80-228.5 mg/L) and 4.16 (0.45-22.13 mg/L, pre-surgery and at baseline, respectively ($P < 0.001$)]]; **B)** no significant change was observed for Alpha 1 glycoprotein (AGP) [0.50 (± 0.30 g/L) and 0.46 (± 0.18 g/L) pre-surgery and at baseline, respectively ($P = 0.78$)]]. Additionally, **C)** soluble transferrin receptor (TfR) increased [(20.7 (3.1-39.1 nmol/L) and 24.3 (14.5-47.3 nmol/L) pre-surgery and at baseline, respectively ($P = 0.01$)] and **D)** serum ferritin (SF) concentration decreased [118.8 (4.8-430.3 ng/mL) and 90.1 (5.5-271.7 ng/mL) pre-surgery and at baseline, respectively ($P = 0.03$)]]. Paired *t*-tests and Wilcoxon signed-rank test were used to assess differences in inflammation and iron status over the two time points (from pre-surgery to baseline) in the total study population

When differences in study parameters were assessed by baseline iron status; subjects with iron deficiency (n=17) had lower hepcidin concentrations and higher fractional iron absorption rates at baseline compared to those without iron deficiency (n = 21) (**Table 6.2**). Furthermore, iron deficient subjects had a significant reduction in IL6 (P = 0.001) and hepcidin (P = 0.006) concentrations, while in the iron sufficient group remained constant (IL6, P = 0.23 and hepcidin, P = 0.10) (**Table 6.1**). **Figure 6.3** shows that fractional iron absorption significantly improved in iron deficient subjects after 6 months of weight loss, with a geometric mean (95%CI) of 9.7% (6.5-14.6) at baseline and 12.4% (7.7-20.1) at follow-up (P = 0.03), while in iron sufficient subjects this did not change (5.9% (4.0-8.6) and 5.6% (3.9-8.2), P = 0.8)].

At baseline, weight showed a significant positive correlation with fat mass (r = 0.76, P < 0.001), CRP (r = 0.38, P = 0.02), leptin (r = 0.57, P < 0.001) and hepcidin (r = 0.33, P = 0.04). Further, hepcidin was positively correlated with hemoglobin (r = 0.57, P < 0.001) and SF (r = 0.86, P < 0.001), and negatively correlated with iron absorption (r = -0.69, P < 0.001). No relationship was observed between hepcidin and BMI, fat mass, sTfR or with any of the inflammatory markers. In subgroup analysis, SF was positively correlated with hepcidin in the iron deficient (r = 0.90, P < 0.001) and iron sufficient (r = 0.51, P = 0.02) groups, whereas, a positive correlation between hepcidin and CRP only existed in the iron sufficient group (r = 0.44, P = 0.04).

Univariate correlations between changes in fat mass, serum hepcidin and iron absorption with variations in weight, iron status and inflammation are presented in **Table 6.3**. Change in fat mass showed a significant positive correlation with change in weight, lean mass, IL6 and leptin. No significant correlation was seen between change in fat tissue and change in AGP, TNF- α , Hb, SF or TfR. Changes in CRP were significantly correlated with changes in SF (r = 0.58, P < 0.001) but not with changes in TfR (r = -0.11, P = 0.52). Change in SF showed a significant positive correlation with change in hepcidin. No significant correlation was seen between change in anthropometric indicators and inflammation and change in hepcidin. Change in iron absorption was negatively correlated with change in ferritin and positively correlated with change in TfR.

In sub-group analysis, changes in CRP and SF were significantly correlated with changes in hepcidin only in the iron sufficient group ($P < 0.05$). Changes in weight, leptin and SF were significantly correlated with changes in iron absorption only in the iron deficient group, whereas changes in TfR were significantly correlated with changes in iron absorption in both iron deficient and iron sufficient groups.

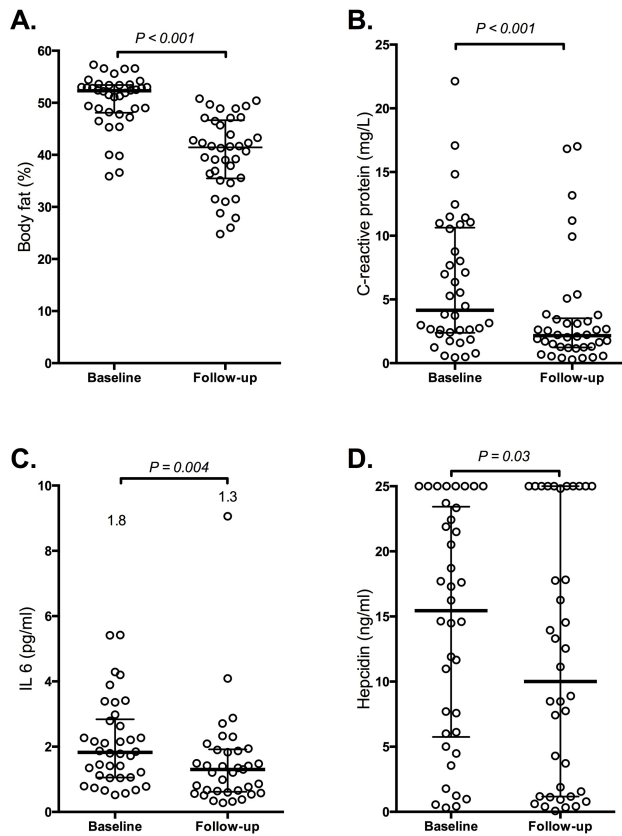


Figure 6.2 A) Body fat (%), B) C-reactive protein (CRP) (mg/L), C) interleukin-6 (pg/ml) and D) serum hepcidin (ng/ml) concentration of the entire study population ($n=38$) at baseline and at follow-up (after 6 months of weight loss). Error bars represent median and interquartile range. Paired t -tests and Wilcoxon signed-rank tests were used to assess differences over the two time points (baseline and follow-up).

Table 6.1 Anthropometric measurements and inflammatory biomarkers in a group of adults (n=38) participating in an iron absorption study at baseline and after 6 months weight loss, categorized by baseline iron status.

	All (n=38)		Iron deficient (n=17)		Iron sufficient (n=21)	
	Baseline (T0)	6-m follow-up (T1)	Baseline (T0)	6-m follow-up (T1)	Baseline (T0)	6-m follow-up (T1)
Weight (kg) ¹	86.2 (77.9; 96.9)	70.3 (63.3; 78.7) ^a	79.6 (75.8; 91.1) ^b	65.6 (62.5; 70.9) ^a	88.3 (85.3; 106.9)	73.4 (69.2; 83.8) ^a
Waist (cm) ²	104.0 (±11.9)	88.2 (±10.4) ^a	100.2 (±10.5)	86.4 (±10.3) ^a	107.3 (±12.3)	89.8 (±10.4) ^a
BMI (kg/m ²) ²	34.3 (±3.5)	28.1 (±3.8) ^a	33.3 (±3.1)	27.3 (±3.5) ^a	35.2 (±3.7)	28.7 (±4.0) ^a
Fat mass (kg)	44.0 (±7.4)	29.1 (±7.6) ^a	41.7 (±5.6)	27.7 (±7.2) ^a	45.8 (±8.2)	30.3 (±7.8) ^a
Total Fat (%) ¹	52.3 (48.1; 53.4)	41.5 (35.5; 46.7) ^a	52.4 (49.0; 52.9)	41.3 (36.3; 43.4) ^a	52.2 (46.6; 54.9)	41.6 (33.1; 47.2) ^a
Lean mass (kg) ²	40.7 (±8.8)	40.3 (±8.5)	38.5 (±7.8)	38.2 (±7.1)	42.5 (±9.3)	41.9 (±9.4)
CRP (mg/dl) ¹	4.16 (2.39; 10.63)	2.16 (1.23; 3.53) ^a	3.74 (2.36; 9.28)	2.03 (0.89; 3.27) ^a	4.48 (2.23; 10.77)	2.61 (1.37; 7.67) ^a
AGP (g/L) ²	0.45 (±0.18)	0.42 (±0.14)	0.47 (±0.22)	0.39 (±0.11)	0.43 (±0.14)	0.44 (±0.15)
IL6 (pg/ml) ¹	1.83 (1.06; 2.84)	1.30 (0.62; 1.92) ^a	1.85 (1.27; 3.38)	1.21 (0.63; 2.00) ^a	1.80 (1.04; 2.71)	1.38 (0.58; 1.92)
TNF-α (pg/ml) ¹	1.84 (1.02; 2.39)	1.79 (1.52; 2.56)	1.87 (1.63; 2.44)	1.99 (1.63; 2.66)	1.65 (0.77; 2.47)	1.73 (1.39; 2.41)
Leptin (ng/ml) ²	12.5 (±5.5)	8.50 (±4.8) ^a	12.1 (±5.39)	8.63 (±4.63) ^a	12.8 (±5.67)	8.44 (±5.03) ^a

Abbreviations: BMI, body mass index; CRP, C-reactive protein; AGP, alpha 1 glycoprotein; IL6, interleukin-6; TNF-alpha, tumor necrosis factor – alpha; Values are; ¹median (IQR 25 and 75 percentile), ²mean (± s.d.) and ³geometric mean (95% confidence interval); Iron status was classified as iron deficient (serum ferritin < 30 ng/ml or TfR > 28.1 nmol/L with our without anemia) or iron sufficient (serum ferritin ≥ 30 ng/ml or TfR ≤ 28.1 nmol/L). ^a Difference between baseline and follow-up within-group (paired T-test or Wilcoxon rank) ($P < 0.05$); ^b Difference between iron deficient or iron sufficient at baseline; ^c Difference between iron deficient or iron sufficient at follow-up (independent sample t-test or Wilcoxon rank) ($P < 0.05$); ^d Difference in changes (T1 – T0) between-group (independent sample t-test or Wilcoxon rank) ($P < 0.05$). Baseline measures were performed 2 months after surgery and follow-up 6 months after baseline.

Table 6.2 Iron status biomarkers, hepcidin and fractional iron absorption (%) in a group of adults (n=38) participating in an iron absorption study, at baseline and after 6 months weight loss, categorized by baseline iron status.

	All (n=38)		Iron deficient (n=17)		Iron sufficient (n=21)	
	Baseline (T0)	6-m follow-up (T1)	Baseline (T0)	6-m follow-up (T1)	Baseline (T0)	6-m follow-up (T1)
Hb (g/dl) ²	13.2 (±1.3)	12.7 (±1.9) ^a	12.6 (±1.2) ^b	12.0 (±2.0) ^{ac}	13.6 (±1.3)	13.2 (±1.7)
Hct (mmol/l) ²	40.1 (±3.4)	38.6 (±4.8) ^a	38.8 (±3.1) ^b	36.8 (±4.8) ^{ac}	41.2 (±3.4)	40.0 (±4.5) ^a
MCV (fL) ¹	87.6 (84.5; 91.6)	89.7 (86.0; 92.2) ^a	86.3 (78.7; 90.4)	88.8 (77.3; 90.4)	88.2 (86.1; 93.0)	90.1 (86.4; 94.7) ^a
Anemia n (%)	6 (16)	13 (34)	4 (24)	7 (41)	2 (10)	6 (29)
Ferritin (ng/ml) ¹	90.1 (24.3; 183.7)	50.3 (8.4; 133.1) ^a	22.6 (10.3; 118.2) ^b	8.6 (5.0; 89.4) ^{ac}	129.4 (72.7; 186.0)	90.7 (38.4; 134.8) ^a
sTfR (nmol/L) ¹	24.3 (21.0; 29.4)	17.6 (15.0; 27.1) ^a	29.6 (25.2; 38.1) ^b	27.0 (17.0; 35.0) ^c	21.5 (18.7; 24.3)	17.1 (13.6; 18.4) ^a
Hepcidin (ng/ml) ¹	15.4 (5.8; 23.4)	10.0 (1.2; 25.0) ^a	5.0 (1.11; 16.8) ^b	1.2 (0.51; 9.8) ^{ac}	18.7 (14.6; 24.4)	16.3 (8.7; 25.0)
Fe absorption (%) ³	7.34 (5.55; 9.72)	8.02 (5.89; 10.9)	9.73 (6.47; 14.6)	12.4 (7.67; 20.1) ^{ac}	5.85 (3.97; 8.60)	5.64 (3.90; 8.16)

Abbreviations: Hb, hemoglobin; Hct, hematocrit, MCV, mean cell volume; sTfR, soluble transferrin receptor; Values are: ¹median (IQR 25 and 75 percentile), ²mean (± s.d.) and ³geometric mean (95% confidence interval); Anemia was defined as Hb ≤ 12 g/dl. Subjects were classified as iron deficient (serum ferritin < 30 ng/ml or sTfR > 28.1 nmol/L) or iron sufficient (serum ferritin ≥ 30 ng/ml or sTfR ≤ 28.1 nmol/L). ^a Difference between baseline and follow-up within-group (paired T-test or Wilcoxon rank) (*P* < 0.05); ^b Difference between iron deficient or iron sufficient at baseline; ^c Difference between iron deficient or iron sufficient at follow-up (independent sample t-test or Wilcoxon rank) (*P* < 0.05); ^d Difference in changes (T1 – T0) between-group (independent sample t-test or Wilcoxon rank) (*P* < 0.05). Baseline measures were performed 2 months after surgery and follow-up 6 months after baseline.

Table 6.3 Correlations between change in fat tissue, hepcidin concentration and fractional iron absorption with the change in weight, lean mass and biochemical markers (indicators for assessing inflammation and iron status) in a group of adults participating in an iron absorption study (n=38).

Variable (%)	Total population			Iron deficient			Iron sufficient		
	Δ Fat mass (%)	Δ Hcpidin (%)	Δ Fe absorption (%)	Δ Fat mass (%)	Δ Hcpidin (%)	Δ Fe absorption(%)	Δ Fat mass (%)	Δ Hcpidin (%)	Δ Fe absorption (%)
Δ Weight	0.86**	-0.08	-0.00	0.88***	-0.28	0.53*	0.85***	0.13	-0.28
Δ Fat Mass	-	-0.17	-0.10	-	-0.31	0.50*	-	-0.05	-0.42
Δ Lean Mass	-0.37*	0.27	0.14	-0.52*	0.21	-0.04	-0.27	0.39	0.23
Δ CRP	-0.10	0.28	-0.20	-0.06	-0.04	-0.25	-0.13	0.44*	-0.18
Δ AGP	-0.15	-0.28	-0.15	0.22	-0.23	-0.13	-0.30	0.25	-0.13
Δ IL6	-0.39*	0.00	0.11	-0.11	-0.18	0.00	-0.54*	-0.04	0.19
Δ TNF-α	0.23	0.06	-0.14	-0.31	0.14	-0.22	0.34	0.03	-0.13
Δ Leptin	0.59**	0.08	0.09	0.46	0.07	0.65**	0.69**	0.14	-0.26
Δ Hemoglobin	-0.14	0.06	-0.10	-0.11	0.07	-0.39	-0.19	-0.04	0.24
Δ Ferritin	-0.21	0.43**	-0.39*	-0.42	0.27	-0.51*	-0.10	0.58**	0.58**
Δ sTfR	0.16	-0.15	0.49**	0.50*	-0.22	0.54*	-0.45*	0.26	0.26
Δ Hcpidin	-0.17	-	-0.13	-0.31	-	-0.31	-0.05	-	0.07

Abbreviations: CRP, C-reactive protein; AGP, alpha 1 glycoprotein; IL6, interleukin-6; TNF-alpha, tumor necrosis factor-alpha; sTfR, soluble transferrin receptor. * P < 0.05; ** P < 0.05; *** P < 0.00

A multiple stepwise regression analysis was performed to identify independent predictive factors for changes in hepcidin and iron absorption. At baseline, hepcidin concentration was associated with sTfR ($\beta = -0.38$, $P = 0.02$) and SF ($\beta = 0.85$, $P < 0.001$) when adjusted for age and gender. Independent predictors of changes in hepcidin included changes in CRP ($\beta = 0.33$, $P = 0.05$) and SF ($\beta = 0.43$, $P = 0.01$) when adjusted for age and gender. At baseline iron absorption was associated with hepcidin ($\beta = -0.59$, $P < 0.001$) and SF ($\beta = -0.65$, $P < 0.001$) when adjusted for age and gender. Change in weight or fat mass were not significant predictors of change in iron absorption. Changes in TfR were predictive for the changes in iron absorption ($\beta = 0.48$, $P = 0.002$) when corrected for inflammation, age and gender. Moreover, changes in SF were predictive for the changes in iron absorption ($\beta = 0.48$, $P = 0.003$) when corrected for baseline TfR, inflammation, age and gender. In sub-group analysis, changes in fat mass were predictive for the change in iron absorption only in the iron deficient group ($\beta = 0.50$, $P = 0.039$) (data not shown).

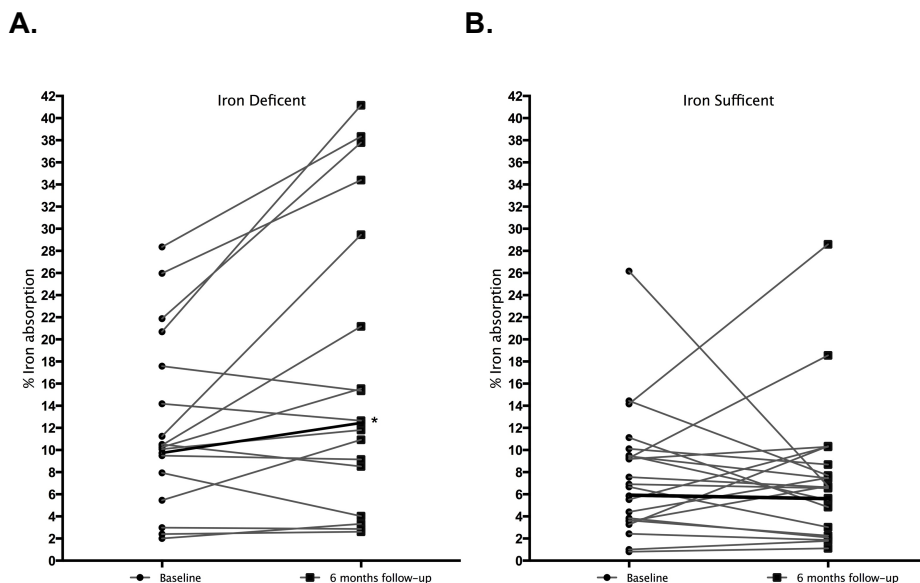


Figure 6.3 Fractional iron absorption from baseline to follow-up in **A)** iron deficient ($n=17$) with a geometric mean (95%CI) of 9.7% (6.5-14.6) at baseline and 12.4% (7.7-20.1) at follow-up ($P = 0.03$), and **B)** iron sufficient ($n=21$) individuals. * Overall geometric mean significantly differ between each other, $P < 0.05$. Fractional iron absorption significantly improved in iron deficient subjects after 6 months of weight loss, while in iron sufficient subjects this did not change (5.9% (4.0-8.6) and 5.6% (3.9-8.2), $P = 0.81$). Paired t -tests and Wilcoxon signed-rank tests were used to assess differences over the two time points (baseline and 6 months follow-up).

Discussion

This study demonstrates that fat loss in obese subjects leads to a reduction of inflammation (IL6) and hepcidin concentrations, and increases iron absorption only in iron deficient subjects. At baseline, obese iron deficient subjects had significantly lower hepcidin and higher iron absorption compared to those without iron depletion, and there was a direct association observed between hepcidin and weight, hemoglobin, serum ferritin and iron absorption. However, during the study, changes in serum ferritin and TfR predicted changes in iron absorption, but changes in hepcidin did not. Our findings suggest that, although low-grade inflammation associated with obesity has a clear effect on hepcidin and iron absorption, this signal can be at least partially overruled by the physiological drive to increase iron absorption during iron deficiency.

Our results are consistent with other studies showing that obese individuals absorb less iron than normal weight individuals. In Thai women, both adiposity and inflammation were negatively correlated with iron absorption, independent of iron status, but hepcidin was not measured (21). In a cross-sectional study in Chilean women, obese women had lower fractional iron absorption compared with overweight and normal weight women ($P < 0.05$), but inflammatory biomarkers and serum hepcidin concentrations were not assessed (32). Recently, we performed a study in which we demonstrated that dietary iron absorption is lower in OW/OB versus normal weight women. In that study there was an increasing trend in hepcidin concentrations throughout the three BMI groups, and hepcidin concentration was a negative predictor of iron absorption ($\beta = -0.85$ (95% CI: -1.41, -0.28), standardized $\beta = -0.36$, $P = 0.004$) (33). In the same way, earlier studies have shown a reduction in hepcidin concentrations as a result of weight loss, similar to our results. In a study performed by Amato and colleagues, after following a 6 month weight loss program, overweight children had significantly lower serum hepcidin and improved intestinal iron absorption (34). Intestinal absorption of iron in that study was measured using the iron-loading test, but body composition was not assessed. A study performed by Tussing-Humphreys and colleagues reported that weight loss 6 months post restrictive bariatric surgery was associated with significantly lower serum hepcidin, inflammatory markers and TfR, but iron absorption and body composition were not measured (24). In a study performed in children, iron absorption was assessed using

the iron-loading test. The obese group had higher serum hepcidin compared with lean controls ($P = 0.001$). Also, an inverse correlation between hepcidin and iron absorption ($P = 0.003$) and a direct correlation between leptin and hepcidin were shown ($P = 0.006$). After adjusting for BMI, gender, pubertal stage, and IL6 concentrations, the correlation between leptin and hepcidin remained significant indicating that weight loss decreased serum hepcidin (22).

Physiologically, hepatic hepcidin synthesis is decreased by iron deficiency and erythropoiesis, and increased in inflammation and iron repletion (35). Obesity is recognized as a metabolic inflammatory state characterized by abnormal production of several adipokines and cytokines (such as interleukin 6 and leptin) (36), that have been linked to hepcidin misregulation. Therefore, inflammation in obese individuals may lead to increased hepcidin production followed by sequestration of iron in macrophages, reduced iron absorption and a decrease in iron availability for erythropoiesis. Hepcidin misregulation is present in many diseased states where inflammation plays an important role, such as the anemia of chronic diseases (37). Thus, hepcidin regulation seems to be an important factor influencing iron status in obese individuals. In the present study hepcidin was only influenced by changes in serum ferritin, which reflects the concentration of stored iron in the liver but is also an acute-phase protein (38). Moreover, in sub-group analysis changes in hepcidin were correlated with changes in CRP only in the iron sufficient group but not in the iron deficient group. Therefore, further studies should assess the underlying mechanisms of hepcidin regulation by inflammation in overweight and obese individuals.

The strengths of this study include the use of stable isotopes to directly measure iron absorption and the use of an intravenous iron infusion to precisely estimate iron incorporation in the erythrocytes. Another strength includes the use of DXA, a robust and well-accepted measure to assess changes in percentage body fat. Different markers of chronic inflammation (IL6, TNF- α , AGP and CRP) and iron status (serum ferritin, soluble transferrin receptor) were assessed. Additionally, we measured hepcidin, a useful diagnostic test of iron deficiency in populations with chronic inflammation (39). Moreover, this study was performed in Mexico, a setting where obesity and iron deficiency normally co-exist. A limitation of this study is that subjects underwent a surgical procedure that could influence iron absorption and

inflammation. However, we were able to assess inflammatory markers before LSG surgery and compared them with baseline parameters. We observed a decrease in inflammation two months after surgery and therefore, it is unlikely that post-surgery inflammation significantly influenced our results. A study performed by Ruz et al. (2013) studying the effects of bariatric surgery (both, restrictive (LSG) and malabsorptive (roux-en-Y gastric bypass)) on iron absorption and status showed that iron absorption was reduced immediately after bariatric surgery (40). A major feature of LSG is the dramatic reduction in stomach size; therefore, an immediate consequence is a reduction of the interaction of food with gastric juice. Gastric juice is important in the release of nonheme iron from its protein matrix and in the solubilization and ionization of dietary iron. Therefore, it is possible that the reduction in stomach size negatively affected iron absorption. However, other studies in overweight/obese subjects that had not undergone bariatric surgery showed similar iron absorption levels as we found in this study (32). Moreover, in our study, both baseline and endpoint measurements of iron absorption took place after the surgery thus eliminating a potential effect of the surgery itself. Another limitation of our study is that we did not assess dietary iron intake in our participants, therefore, differences in iron intake could not be taken into account to explain differences or changes in iron status.

It is unclear whether current dietary guidelines for iron intake in overweight individuals are adequate to prevent a disturbance of iron balance. The development of interventions should consider the double-burden of malnutrition, especially in low and middle-income countries like Mexico where dietary intake of iron is low and overweight is highly prevalent (41, 42). In conclusion, elevated hepcidin concentration due to inflammation among overweight individuals might explain part of the burden of iron deficiency in low and middle-income countries. We found that iron absorption improved in individuals that were overweight or obese and iron deficient after weight loss. Our findings suggest that low iron stores fail to fully downregulate hepcidin secretion in obese and overweight subjects due to inflammatory processes, thereby blocking iron absorption to some degree. Therefore, overweight and obese individuals may require a higher dietary iron intake level to keep their iron status in balance as compared to normal weight individuals. Further research is needed to identify effective dietary methods to prevent iron deficiency in this setting.

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Chapter 7

General Discussion

Many countries have overlapping burdens of micronutrient deficiencies and overweight in children and adults. This thesis aimed to understand the underlying mechanism for the association between obesity and iron status. To achieve this objective different pathways that could lead to the co-occurrence of obesity and iron deficiency at the individual level have been investigated. We explored whether this association is due to 1) poor dietary iron intake, 2) dilution as a result of increased blood volume, 3) reduced iron absorption as a result of adiposity-related inflammation or a combination of the three scenarios. We examined this association in Mexico a transition country, where iron deficiency and obesity are both prevalent. This section summarizes the main findings of this thesis and discusses some methodological issues about the quality of the studies. Additionally, the main results are discussed in a broader perspective considering the context of existing literature. Finally, suggestions for future research and the main implications of this thesis for public health in Mexico and other low and middle-income countries are provided.

7.1 Main findings

The key findings of the studies presented in this thesis are summarized in **Table 6.1**. In a review of the literature and potential explanations for the association between obesity and iron deficiency we summarized the abundant data from epidemiological studies, dating back, as far as 1962, documenting the inverse association between adult and infant adiposity and poor iron status. We presented possible mechanisms describing data from iron absorption studies using stable isotopes and emphasizing the evidence from intervention studies. Together this information suggests that obesity-related inflammation may play a central role through its regulation of hepcidin (**chapter 2**).

In a cross-sectional study using data from the 1999 Mexican Nutrition Survey comprising 1174 children (aged 5-12 y) and 621 nonpregnant women (aged 18-50 y) we found that the risk of iron deficiency in obese women and children was 2-4 times that of normal-weight individuals at similar dietary iron intakes. In addition, we found that C-reactive protein (CRP) but not iron intake was a strong negative predictor of iron status, independent of body mass index (BMI) ($P < 0.05$). Therefore,

we concluded that the increased risk of iron deficiency might be due to the effects of obesity-related inflammation and iron absorption rather than differences in iron intake. This finding suggest that iron deficiency control efforts in Mexico may be hampered by increasing rates of adiposity in women and children (**chapter 3**).

In a study comparing iron absorption from a meal with and without ascorbic acid in healthy, non-anemic women (n=62) (BMI 18.5-39.9 kg/m²), dietary iron absorption was lower in the overweight/obese versus the normal weight subjects. Moreover, the enhancing effect of ascorbic acid on iron absorption in overweight/obese subjects was only half that in normal weight women. Confirming previous studies, poorer iron status was more common in heavier participants. Moreover, serum ferritin, soluble transferrin receptor (TfR) and hepcidin were significant predictors of iron absorption. Our results were also consistent with the hypothesis that increasing hepcidin concentrations, along with subclinical inflammation, limit dietary iron absorption in subjects with excessive body fat. These findings suggest that recommending higher intakes of ascorbic acid (or other luminal enhancers of iron absorption) in obese individuals to improve iron status may have limited effect (**chapter 4**).

The assessment of blood volume determined by the carbon monoxide (CO)-rebreathing method showed that overweight/obese women presented higher absolute blood volume and lower serum iron compared to the normal weight group. Our data indicate that the hypoferremia of obesity is partially explained by a dilutional effect due to increased blood volume and partially by inflammation. We concluded that due to the dilutional effect of blood volume, 'true' hypoferremia might be overestimated in populations with a high prevalence of obesity when using serum iron as an indicator. Moreover we proposed an equation utilizing combined height-weight and lean body mass for predicting blood volume in overweight/obese women (**chapter 5**).

In a 6-month prospective study in obese (BMI $\geq 35 < 45$ kg/m²) adults from Mexico who had recently undergone laparoscopic sleeve-gastrectomy we found that fat loss leads to a reduction of inflammation (IL6) and hepcidin concentrations, and increases iron absorption, but only in iron deficient subjects. After 6 months, total body fat (TBF%), inflammation and hepcidin were significantly lower (all, $P < 0.005$).

In iron-deficient subjects (n=17), geometric mean (95%CI) iron absorption significantly increased (from 9.7% (6.5-14.6) to 12.4% (7.7-20.1), $P=0.03$), while in iron sufficient subjects (n=21), it did not change (5.9% (4.0-8.6) and 5.6% (3.9-8.2), $P=0.81$). These findings suggest that low iron stores fail to fully downregulate hepcidin secretion in obese and overweight subjects due to inflammatory processes, thereby blocking iron absorption to some degree. We concluded that overweight and obese individuals may require a higher dietary iron intake level to keep their iron status in balance as compared to normal weight individuals (**chapter 6**).

7.2 Overall methodological considerations

To obtain more insight in the link between adiposity and iron status we performed a combination of study designs in this thesis: a literature review, cross-sectional observational studies and a prospective study. Methodological considerations were addressed in each chapter, however this section will elaborate on issues to take into account when interpreting the findings or applying the methods used in this thesis in a different setting.

7.2.1 Study design

A major disadvantage of cross-sectional studies is the inability to establish a causal relationship between exposure and outcome. Cross sectional studies are therefore mostly hypothesis generating. Although the cross-sectional evidence showing that obese individuals are at increased risk of iron deficiency seems abundant (**chapter 2**), few studies have examined the association in transition countries, where both conditions are prevalent. Moreover, most of these studies did not assess or compare dietary iron intakes between obese and normal-weight individuals or assess obesity-related inflammation with an hs-CRP assay as we did (**chapter 3**). Regarding the mechanisms behind the hypoferremia of obesity, there are only few studies showing associations between iron absorption and body weight in the normal weight to overweight range (up to BMI 27 kg/m²) and a few studies showing relations between overweight, hepcidin and iron status. Providing information on iron status (using different indicators such as serum iron, transferrin receptor, and ferritin), iron absorption, inflammation, hepcidin, blood volume and body composition contributes importantly to the knowledge base in this field of

research (**chapter 4 & 5**). In addition, the majority of evidence for the effect of weight loss on hepcidin concentrations and iron status emerges from studies lacking measurement of iron absorption or body composition. In our prospective study we compared erythrocyte incorporation of iron isotopic labels (iron absorption), changes in body composition, iron status, hepcidin and inflammation in both iron-deficient and iron-sufficient obese subjects, adding new evidence to this research field (**chapter 6**). Discrepancies in hepcidin and CRP concentration between **chapter 4 and 6** might be explained by differences in BMI and iron status. **Chapter 6** comprises a population with higher BMI and higher rates of iron deficiency, whereas in **chapter 4** BMI was lower (even in the obese population) and only 4 subjects presented iron deficiency (2 NW, 1 OW, and 1 OB).

7.2.2 Selection of study population

An important methodological consideration is the selection of the study population. Selection bias may occur during enrolment or due to decline to participate or loss to follow-up. Selection bias in a study affects the generalizability of the results, but does not compromise the internal validity. We have mainly studied women in a specific population groups (e.g., Mexico and Switzerland) in these trials (**chapter 3, 4 and 5**). Whether the results would have been the same in men and in other ethnicities remains to be demonstrated. In **chapter 3** we used national survey data that are representative for Mexico, thus results from this study might be also representative for women and children in similar transition countries such as China.

The study population of our prospective cohort underwent a laparoscopic sleeve gastrectomy (LSG) procedure that could influence iron absorption. LSG surgery is assumed to negatively influence iron status through reduced gastric acid secretion which is needed to convert dietary ferric iron to the absorbable ferrous state and reduced tolerance to red meat (81). So, it is possible that the reduction in stomach size negatively affected iron absorption. However, with LSG the small intestine is preserved, reducing the impact on nutrient malabsorption. Other studies in overweight/obese subjects that had not undergone bariatric surgery showed comparable iron absorption rates (at baseline and end measures) as we found in this study (1). Since we anticipated a minimal but possible impact of surgery on iron

absorption, both baseline and endpoint iron absorption measurements took place after surgery thus reducing a potential effect of the surgery itself.

To minimize a possible impact of the surgery on inflammatory parameters, baseline measurements were performed 2 months after LSG surgery when surgery-related inflammation was expected to be resolved. To avoid bias due to the surgery effect, we assessed inflammatory markers from routine blood sample taken before LSG surgery and compared them with baseline parameters. We observed a decrease in inflammation from the pre-surgery assessment to baseline measures (two months after surgery) and therefore, it is unlikely that post-surgery inflammation significantly influenced our results. The ideal setting of a prospective study examining the effects of weight reduction on iron absorption and status would have been by a dietary intervention instead of a surgical approach. However in a dietary intervention it is difficult to induce significant weight loss within a short period of time (6 months) and the success rate is low. Thus, the LSG allowed us to evaluate the impact of fat loss on inflammation, hepcidin, iron status and absorption in a realistic manner, while minimally compromising the accuracy of the results.

7.2.3 Errors in the assessment of exposure variables

Commonly used biochemical iron parameters (e.g., serum iron, transferrin receptor and ferritin), are influenced by adiposity related inflammation and therefore may not be adequately representative for overweight individuals (2). The main limitation of our cross-sectional study (**chapter 3**) was the use of serum iron as an indicator of iron status. Infection and inflammation can decrease serum iron concentration, and there may be day-to-day variations within individuals. In one of our studies (**chapter 5**) we found low serum iron and high serum ferritin concentration in obese women and they were negatively correlated. The later has been associated with a redistribution of iron into the liver and mononuclear phagocyte system, both mediated by cytokines (3). However, unlike anemia of chronic diseases, a previous study showed that obesity does not appear to be associated with iron sequestration or impaired mobilization of iron from stores but rather with iron depletion (4). Serum ferritin is also an acute phase protein that arises from damaged cells, and is thus a marker of cellular damage. It is unclear whether serum ferritin reflects or causes inflammation, or whether it is involved in an inflammatory cycle (5).

Table 7.1 Summary of the main findings of studies described in this thesis

Study population, setting, sample size and design	Main findings
Research question: Does overweight/obesity increase risk for iron deficiency? If yes, which are the potential mechanisms (Chapter 2)	
Literature Review of the association between adiposity and poor iron status. Published journal articles in PubMed, manual search for identified potential articles from reference list	<ul style="list-style-type: none"> • Evidence from cross-sectional studies shows an association between adiposity and poor iron status • Obesity related subclinical inflammation, and its effect on hepcidin, is the most plausible explanation for the link between iron deficiency and obesity
Research question: What is the relationship between BMI, dietary iron intake, dietary factors affecting iron bioavailability, inflammation [C-reactive protein (CRP)], and iron status in Mexico, a transition country where both obesity and ID are common? (Chapter 3)	
Children aged 5-12 y (n=1174) and non-pregnant women aged 18-50 y (n=621); Cross-sectional	<ul style="list-style-type: none"> • Iron deficiency in obese women and children was 2-4 times that of normal-weight individuals at similar dietary iron intakes • C reactive protein but not iron intake was a strong negative predictor of iron status independently of BMI (P < 0.05)
Research question: How does BMI affect iron absorption? Does ascorbic acid improve iron absorption in overweight and obese subjects similar to normal weight subjects? (Chapter 4)	
Healthy, non-anemic women (n=62): normal weight (n=24); overweight (n=19) and obese (n=19); Observational/experimental	<ul style="list-style-type: none"> • Iron absorption was lower in overweight/obese versus normal weight • The enhancing effect of ascorbic acid on iron absorption in overweight/obese was only half that in normal weight women
Research question: What is the relationship between blood volume and iron deficiency in obese subjects? (Chapter 5)	
Healthy, non-anemic women (n=62): normal weight (n=24); overweight (n=19) and obese (n=19); Observational	<ul style="list-style-type: none"> • Overweight/obese women presented higher absolute blood volume (BV) and lower serum iron compared to the normal weight group. • BV was a significant negative predictor for serum iron when adjusted for body iron stores. • Equations utilizing combined height-weight and lean body mass were found to be most accurate for predicting BV in all BMI groups.
Research question: What is the effect of weight loss on iron absorption and hepcidin concentrations in obese subjects? (Chapter 6)	
38 obese subjects BMI $\geq 35 < 45$ kg/m ² : non-pregnant women aged 18-50 y (n=32) and men (n=6); Prospective cohort	<ul style="list-style-type: none"> • After weight loss, iron absorption improved in overweight/obese individuals that were iron deficient at baseline while in iron sufficient subjects it did not change.

The use of other iron-status indicators that are not significantly affected or associated with inflammation (increased serum ferritin (SF)) and dilution (decreased serum iron), such as serum transferrin receptor (TfR), could have helped to better assess iron status in overweight and obese subjects. Unfortunately, these indicators (SF or TfR) were not assessed in the 1999 Mexican national survey (**chapter 3**). The adequate selection and interpretation of the biochemical and inflammatory assays is fundamental for assessing iron status in overweight and obese subjects.

Some variables that influence iron status, body weight, and inflammation—such as dietary iron intake, physical activity and other micronutrient deficiencies—were not evaluated in the studies and may have attenuated our associations. In the cross-sectional study presented in **chapter 3** we estimated dietary iron intake as well as other dietary factors that can affect iron absorption including vitamin C and calcium obtained from 24-h dietary recall interviews. Underreporting of energy intake has been shown to be more prevalent and severe among individuals with higher BMI. Therefore, the fact that estimates were based on 24-h dietary food recall interviews may have caused bias due to conscious or subconscious underreporting of specific energy-dense foods (such as tortillas, sugar, and oil). This may have influenced our dietary intake estimates; however, more underreporting by obese subjects would have biased the data toward an underestimation of dietary iron intake in the obese group and thus is unlikely to have affected our conclusions that differences in dietary intake are unlikely to explain the low iron status of obese individuals. Another inconvenience in this study was that information on phytate as well as the heme and nonheme iron content in diet was not available in the data-set and consequently we were not able to accurately estimate iron bioavailability from the diet. We concluded that differences in dietary intake are unlikely to explain the low iron status of Mexican obese women and children. Therefore, iron intake assessment was not considered in the other observational studies and prospective cohort described in this thesis (**chapter 4-6**). However in our prospective cohort study iron intake assessment would have been valuable especially considering that LSG is believed to reduce tolerance to red meat (81). In our prospective study we found that anemia was present in 16% (n = 6) of the subjects at baseline and 34% (n = 13) at follow-up. Likewise, iron deficiency was detected in 45% (n = 17) at baseline and 47% (n = 18) of the study population at follow-up. Due to a lack of

dietary intake data, differences in iron intake could not be taken into account to explain part of the variance in changes in iron status in this study (**chapter 5**).

Iron absorption in **Chapter 4 and 6** was calculated using different methodologies that might explain the discrepancy in our results. As we have only used oral and no intravenous isotopic labels in **chapter 4**, we have not been able to directly determine iron incorporation into erythrocytes but have assumed it to be the same (80%) for all subjects (6). It was previously shown in a study in subjects with afebrile malaria, that even though iron absorption was reduced significantly before malaria treatment, incorporation was not affected (7). Based on this and other earlier studies in different subjects we concluded that iron incorporation seems to be relatively constant, independent of inflammatory status (8, 9). Whereas, in **Chapter 6** the use of an intravenous iron infusion helped us to precisely estimate iron incorporation in the erythrocytes.

Finally, various steps involved in blood volume measurements by the CO-rebreathing method present potential sources of error (**chapter 5**). There are individual biological factors and measurement deviations that could have altered the results. However, most of the parameters that could influence the measurements were standardized and therefore measurement errors were minimized. For example, gender and age are biological factors influencing blood volume but our study only included women within a similar age range. As expected, statistical analysis of the data in **Chapter 4** showed that age had no significant correlation or influence on blood volume. Another biological factor that could have influence the result was the menstrual cycle but it was also anticipated in the study design and all measurements were made between the first and second week after the last menstruation. Further, the reproducibility of the method was verified by a test-retest correlation analysis. For this assessment the same optimized CO rebreathing method was applied to the same participants (n=8) on two separate occasions and the typical error (TE) was 1.2% and the R² was 0.98. On average, there were five days between the tests. Physical activity (fitness level) would have been a particularly interesting variable to control, but unfortunately the results from the self-administered physical activity questionnaire (PAQ-A) were neither valid nor reliable. The questionnaire was misunderstood and activity misreported by the subjects, and therefore was excluded from the analyses. The assessment of physical activity is particularly problematic

when using self-report instruments (10). However, for our studies practicing vigorous intensity physical activity was an exclusion criteria.

7.3 Public health implications of the findings

Raising awareness of the association between iron deficiency and obesity

Low and middle-income countries like Mexico are facing complex, overlapping, and interconnected burdens of malnutrition. A report documenting the coexistence of undernutrition and obesity at the individual, household, and country levels in 11 Latin American countries has been recently published (11). The percentage of households with a stunted child and an overweight or obese mother, referred to as the prevalence of the double burden at household level, ranged from 20% in Guatemala to 13.1% in Ecuador, 8.4% in Mexico, 6.3% in Uruguay, 5.1% in Colombia and 2.7% in Brazil (12-18). The prevalence of the double burden of overweight and anemia (or zinc deficiency in Ecuador) at the individual level in preschool- or school-aged children ranged from as low as 1.2% in Brazil to 8.4% in Ecuadorian children >5 y. Moreover, the prevalence of the double burden of overweight and anemia at the individual level in women ranged from 3.4% to 13.6%. Brazil presented the highest prevalence (13.6%) followed by Guatemala (11.7%). The authors concluded that contrary to expectations, the double burden is either lower or equal to expected values: undernutrition and excess body weight appear to be unrelated at the individual and household levels. However, the fact remains that both types of conditions are very common in Latin American countries: overweight and obesity coexist with undernutrition (either stunting, anemia, or zinc deficiency) at the national level (11).

A study analyzing data from a Chinese sub-national cross-sectional survey showed that women (aged 20 y and above) with overweight/obesity or central obesity were less likely to be anemic as compared to normal weight women (19). Similarly, the present thesis shows that BMI or obesity per se is not independently associated with lower hematological parameters. However, isolated measurement of the hemoglobin concentration is unsuitable as the sole indicator of iron status. The latter can be expected since the sensitivity of hemoglobin measurements for determining iron status is poor. There is an extensive overlap in hemoglobin values between

healthy and iron-deficient persons (**Figure 7**) (20). Discrepancies between studies that used hemoglobin as an outcome compared to studies that used iron status indicators might be explained by the amount of dietary iron absorbed which may be adequate to allow tissues that need iron to maintain normal physiologic function, as indicated by the lack of correlation with hemoglobin concentrations, but may not be adequate to maintain iron stores resulting in mild or moderate iron deficiency (21). So, when assessing iron status in overweight and obese subjects it is fundamental to adequately select the biochemical indicators and carefully interpret them. The next section will cover the diagnostic challenges encountered in the assessment of iron status in overweight and obese individuals.

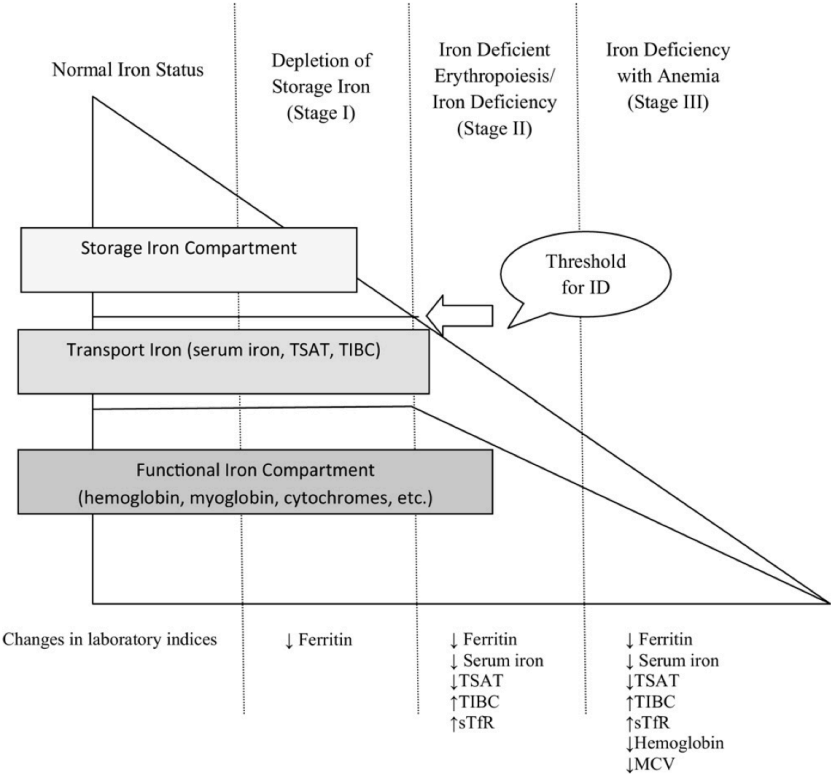


Figure 7 Stages of advancing iron deficiency (ID). The gradual reduction of different iron compartments and the concomitant changes seen in the laboratory parameters are presented schematically in relation to the three separate stages of advancing iron deficiency. Transferrin saturation (TSAT); total iron binding capacity (TIBC). Soluble transferrin receptor (TfR); mean corpuscular volume (MCV). Reproduced from the American Society of Hematology (ASH) and Dr. Tussing Humphreys L, reference (21, 49).

Assessment of iron status in overweight and obese individuals: biomarkers

Usually, iron status assessment in healthy populations includes evaluation of iron stores, circulating iron, and hematologic parameters. The “gold standard” for the assessment of storage iron, especially in hospitalized patients, is the examination of a bone marrow aspirate stained with Prussian blue to determine the presence or absence of iron (22). Unfortunately, this test is invasive, time consuming and expensive. Hematological indexes are also included but hemoglobin concentration or hematocrit levels are unsuitable as the sole indicator of iron status (23). The biochemical markers commonly used to assess iron stores and circulating iron in healthy populations normally comprises serum ferritin, serum iron concentration, total iron binding capacity (TIBC, an indirect estimate of transferrin concentration) and percent transferrin saturation [%TS = (serum iron/TIBC) × 100] (3). Moreover, soluble transferrin receptor (TfR) can be used to detect early changes in iron status only when iron stores are depleted and no underlying cause of abnormal erythropoiesis exists (24). However, the assessment of iron status in overweight and obese individuals with low-grade inflammation encounters diagnostic challenges. Based on our findings we can conclude that serum iron should be carefully interpreted when used as an indicator of iron status in overweight and obese individuals. It is possible that there is an overestimation of a ‘true’ hypoferremia due to the dilutional effect of increased blood volume in this population (**chapter 5**). Moreover, our results are consistent with other studies, which have shown that in overweight individuals, serum ferritin tends to be higher than in their normal weight counterparts, as well as positively related to CRP and inversely related to transferrin saturation (25-27). Thus, serum ferritin not always reflects iron stores, especially in situations where serum ferritin synthesis is primarily driven by inflammation such as in overweight/obesity. Therefore, the use of serum ferritin to assess iron status in overweight and obese individuals is inadequate and should be carefully interpreted.

Expression of TfR by cells of the reticuloendothelial system is directly proportional to cellular iron demand and is subsequently an indicator of erythropoietic activity. TfR is produced by the cleavage of the cell-bound transferrin receptor, which is essential for the uptake of transferrin, and is sensitive to the delivery of iron

to bone marrow and tissue. Serum concentration of soluble TfR is directly proportional to the total amount of cellular TfR (20). Therefore, TfR represents a good indicator of iron status in settings with high prevalence of overweight and obesity. Moreover, hepcidin concentrations could provide additional information to these measurements for detecting body iron status and requirements. The diagnostic value of hepcidin as an index of iron status is being intensively investigated (28). At this stage there are a number of research questions still to be resolved before this indicator can be used on a large scale like the costs and lack of standardization between various hepcidin assays, cut-offs and reference ranges. However, it is unlikely that hepcidin will be a useful indicator for iron status in obesity as it is also triggered by inflammation (**Chapter 4 & 6**).

Assessing blood volume in overweight and obese women

Excess weight, has been suggested to lead to increased blood volume which may explain lower serum iron concentrations observed in obese individuals, because of a dilutional effect. Therefore in **chapter 5** we investigated whether differences in blood volume between overweight/obese and normal-weight women might account for the hypoferrremia observed in the former group. In this study we found that absolute blood volume was higher in overweight and obese (5.2 ± 0.8 liters) than in normal weight women (4.6 ± 0.5 liters). An additional goal was to develop a new equation to allow reliable calculation of blood volume based on simple measures such as weight and height in women of all weight categories. Blood volume assessment was performed using the optimized carbon monoxide (CO) rebreathing method. The assessment of blood volume is an important tool in clinical medicine for the evaluation of several disorders or diseases. Methods such as radioactive labelling by ^{51}Cr , the Evans blue, hydroxyethyl-starch and indocyanine green dilution techniques were first used to measure total hemoglobin mass (tHbmass) and blood volume, however, these are harmful and invasive to use on regular population (29). The optimized CO rebreathing method (30) is a routinely applicable minimally invasive reliable method for assessing blood volume (31). This method has been described in detail elsewhere (30, 32, 33). Moreover, the precision of the CO-rebreathing method to determine blood volume has been compared to other methods and has shown to be reliable (31).

Several equations were developed using data from studies assessing blood volume using different methods but including mainly normal weight individuals (few included obese women) (34-40). So, the currently existing formulas may not be suitable for overweight and obese women. Moreover, in the clinical practice such as anesthesiology blood volume calculation is usually performed with the following formula: Estimated blood volume (EBV) = body weight (kg) x average blood volume (ml/kg). Blood volume is estimated to be 65-75 ml/kg (adult men, 75ml/kg; and women, 65ml/kg) (41). However, estimation of blood volume in overweight and obese individuals is challenging since the relationship between blood volume and body weight is not linear (42). The primary physiological determinant of blood volume is body composition, especially lean mass (41). We provided two new equations utilizing combined height-weight and lean mass for predicting blood volume in all BMI groups (**chapter 5**):

- 1) Blood volume (ml)= $4698.8 * \text{height (m)} + 32.5 * \text{weight (kg)} - 5342.1$
($r^2=0.69$, $P<0.01$).
- 2) Blood volume (ml)= $2686.7 * \text{height (m)} + 18.9 * \text{weight (kg)} + 66 * \text{lean mass} - 3932.3$
($r^2=0.76$, $P<0.01$)

The advantage of these equations is that they were developed using data from women with an extensive BMI range. It is important to consider that the blood volume values that were used to derive the formulas were higher compared with previously reported data using other assessment methods, however, the results ranged within a reasonable range (32, 38, 43).

7.4 Possible implications for public health

The findings of our literature review and cross-sectional study re-emphasized the importance of the public health issue of the double burden: overweight and iron deficiency. Moreover we provided background for several other studies investigating the association between iron deficiency and obesity. Our findings show that overweight and obese individuals present reduced iron absorption due to adipose related inflammation and increased hepcidin circulation. Because of the potentially harmful effects of iron deficiency, obese individuals, especially

children, should be routinely screened and treated. A recent study showed that overweight iron deficient South African children had a reduced response to iron supplementation, compared with their normal weight counterparts. In that study, iron deficient children with higher BMI-for-age z-scores had a two-fold higher risk of remaining iron deficient after iron supplementation (25). The combination of overweight and obesity with iron deficiency may have profound detrimental effects on health, especially in early childhood. Consequences may range from poor growth and cognitive development in childhood to increased risk for non-communicable diseases later in life (44-46). Iron deficiency may further contribute to a poor exercise capacity of the overweight and obese population who may already show difficulties performing even minor physical tasks. However there is no evidence for the health consequence of this combination and therefore it continues to be a subject of research.

Our results call for policy makers and health care professionals to recognize obesity as a nontraditional risk factor for iron deficiency. The hypoferrremia of obesity is an example of overlap in malnutrition and represent the complexity of exploring multiple burdens that requires a fresh look at malnutrition in any context. For example, obesity may complicate the interpretation of iron status biomarkers since some of the most commonly used are influenced by different biological conditions associated with excess weight, mainly inflammation. Serum ferritin, for example, is an acute inflammatory protein and therefore it tends to be increased in obese subjects due to the adiposity related inflammation. Moreover, due to the dilutional effect of blood volume on serum iron, 'true' hypoferrremia may be overestimated in populations with a high prevalence of obesity when using serum iron as an indicator. We can conclude from this thesis that the coexistence of undernutrition and obesity at the individual, household, and country levels might be underestimated. Especially considering that: 1) iron deficiency is the most common micronutrient deficiency in the world (3); 2) increasing obesity is currently a major health concern in both developed and developing countries (47); and 3) a high prevalence of the 2 conditions has been documented in Latin America and it is therefore clear that the double burden does exist (11).

The nutrition community must urgently develop tools and innovating strategies for prioritizing and sequencing nutrition-relevant actions in such complex contexts. For example, we showed a suboptimal response on iron absorption when

providing ascorbic acid supplementation to overweight and obese women. Although ascorbic acid allows more iron to enter the enterocyte, increased hepcidin in overweight and obese individuals reduces its efflux into the circulation, and when the enterocytes shed from the luminal surface the iron is lost. Moreover, we showed an increased iron absorption after fat loss and reduction of inflammation, only in obese iron deficient individuals. Therefore, commonly used strategies to control iron deficiency might not work in populations with high overweight and obesity prevalence.

7.5 Implications for future research

The widespread burden of overweight and obesity may limit current dietary strategies to improve iron absorption in iron deficient individuals, especially in certain low and middle-income countries where both conditions are prevalent. In this thesis we demonstrate that overweight and obese individuals absorb less iron and present increased blood volume compared to normal weight individuals. We show that ascorbic acid supplementation alone is not sufficient to enhance iron absorption in overweight and obese individuals. Moreover we provide evidence showing that losing fat increases iron absorption only in obese iron deficient subjects.

Based on our findings we recommend the following important areas for further research:

- 1) Identification of the optimal biomarkers and reference ranges for predicting iron deficiency in overweight/obesity. As previously mentioned one of the challenges in studies evaluating iron status in overweight and obese subjects is the interpretation of iron status indicators. Therefore, it is necessary to establish optimal biomarkers and reference ranges for defining iron status in overweight and obese subjects.
- 2) Establishment of specific dietary iron requirements based on BMI. It is important to determine if overweight and obese individuals may require higher recommendations for iron intake in order to prevent iron deficiency and maintain optimal body function as well as normal erythropoiesis.
- 3) Evaluation of the strategies and efficacy of obesity/chronic inflammation treatment with lifestyle/behavioral intervention and drug therapy to improve iron status in overweight and obese iron deficient individuals. In this thesis we showed that ascorbic acid has an enhancing effect on non-heme iron absorption in normal

weight individuals, but not in overweight and obese individuals. This finding may be explained by the fact that the site of action of ascorbic acid is on the luminal side of the enterocyte, whereas hepcidin reduces iron absorption on the basolateral side of the enterocyte. Therefore it is necessary to investigate if strategies aiming to reduce inflammation (e.g. omega 3 supplementation) could improve iron absorption in obese iron deficient subjects, by lowering hepcidin concentrations, and removing the basolateral inhibition of iron absorption.

4) A better understanding of how the interaction between iron deficiency and overweight/obesity could impair cognitive and physical development, especially in children. Although these two conditions, iron deficiency and obesity, may contribute to poor exercise capacity and impaired cognitive development, there is no evidence for the health consequence of this combination. Providing evidence for the adverse health consequences of the interaction of overweight and iron deficiency could contribute to a better understanding of the situation and will help to create awareness of the problem;

5) A better understanding of ethnic/racial/genetic differences in the development and progression of iron deficiency in overweight/obesity. There is evidence showing that populations with high prevalence of risk alleles (A and G rs855791 and rs4820268) are associated with reduction in hemoglobin and increased in transferrin receptor concentrations. It has been suggested that this population with elevated prevalence of risk alleles may require higher recommendations for iron intake in order to maintain normal body functions, especially those with unfavorable environmental conditions such as diets with low bioavailable iron and high inflammation burden (48). This could be of special importance for transition countries like Mexico, which present both conditions: a high prevalence of overweight and obesity and diets with low bioavailable iron.

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Summary

Obesity has become a major global health problem and at the same time iron-deficiency anemia remains common worldwide. At the individual level these two conditions appear to be linked: overweight persons are at higher risk of iron deficiency than normal-weight individuals. The main objective of this thesis was to understand the underlying mechanism for the association between obesity and iron status. **Chapter 1** provides background information on iron homeostasis, obesity and obesity related inflammation, as well as a detailed description of the research questions. In our literature review presented in **chapter 2** we set the scene by examining how iron deficiency and obesity are potentially linked and considering the underlying explanations. In that review we conclude that obesity related subclinical inflammation and its effect on hepcidin concentration is the most likely explanation for the link between iron deficiency and obesity.

In **chapter 3**, data from the 1999 Mexican Nutrition Survey were analyzed, which included 1174 children (aged 5–12 y) and 621 nonpregnant women (aged 18–50 y). We showed that obese children and women were at higher risk of iron deficiency than normal-weight counterparts at similar dietary iron intakes. Additionally, (C-reactive protein) CRP but not iron intake was a strong negative predictor of iron status, independently of (body mass index) BMI ($P < 0.05$). Our findings support the evidence showing that overweight individuals (women and children) are at higher risk of iron deficiency than normal-weight individuals at similar dietary iron intakes.

In **chapter 4**, sixty-two healthy women from different BMI categories (BMI 18.5–39.9 kg/m²) were recruited for two-test meal rounds labeled without (-AA) and with (+AA) 31.4 mg ascorbic acid. In this study we demonstrate that dietary iron absorption is reduced not only in obese but also in overweight women compared to the normal weight group. The enhancing effect of ascorbic acid on iron absorption in overweight/obese was only half that in normal weight women. Moreover, there was an increasing trend in hepcidin concentrations throughout the three BMI groups and hepcidin concentration was a negative predictor of iron absorption ($P < 0.05$).

Chapter 5 examines differences in blood volume measured by the carbon monoxide CO-rebreathing method among the three BMI categories and the

influence of blood volume on serum iron concentration. Overweight/obese women presented higher absolute blood volume, plasma and red blood cells, and lower serum iron compared to normal weight women ($P<0.05$). Blood volume was a negative predictor for serum iron when adjusted for body iron stores ($P=0.02$). Therefore, obese individuals might have increased iron needs because of their increased blood volume and body surface area. Our findings confirm that obese subjects have increased blood volume and that a dilutional effect, together with a reduced iron absorption due to chronic inflammation, seems to be playing a role in the hypoferremia of obesity. Additionally we developed two equations to calculate blood volume in overweight/obese women; equations utilizing combined height-weight and lean body mass were the most accurate for predicting blood volume in all BMI groups.

In **chapter 6** we evaluated whether weight and fat loss in obese subjects would decrease inflammation and serum hepcidin and thereby improve iron absorption. We performed a 6-month, prospective study in obese (body mass index (BMI), $\geq 35 < 45$ kg/m²) adults who had recently undergone laparoscopic sleeve-gastrectomy (LSG). Forty three subjects were studied at baseline, of whom 38 completed the protocol (32 women, 6 men). After 6 months, total body fat, inflammatory markers and hepcidin concentration were significantly lower (all, $P<0.005$). In iron-deficient subjects ($n=17$), geometric mean (95%CI) iron absorption significantly increased, while in iron sufficient subjects ($n=21$), it did not change. This study demonstrates that fat loss in obese subjects leads to a reduction of chronic inflammation (measured with interleukin-6) and hepcidin concentrations, and increases iron absorption, but only in iron deficient subjects.

The main findings and conclusions of this thesis are discussed and placed in a public health perspective in **chapter 7**. Moreover, recommendations for possible future research are provided. Our findings show that overweight and obese individuals present: 1) reduced iron absorption due to adipose related inflammation and increased hepcidin circulation; 2) increased blood volume, diluting serum iron and possibly increasing iron requirements; 3) a suboptimal response on iron absorption when providing ascorbic acid supplementation; and 4) increased iron absorption after fat loss, but only in iron deficient individuals.

Future research in this topic is necessary in order to: a) establish optimal biomarkers and reference ranges for defining iron status in overweight and obese subjects; b) determine if overweight and obese individuals may require higher recommendations for iron intake in order to prevent iron deficiency and maintain optimal body function; c) determine if lowering inflammation and hepcidin concentrations could remove the basolateral inhibition of iron absorption in obese iron deficient subjects and; d) provide a better understanding of ethnic/racial/genetic differences in the development and progression of iron deficiency in overweight/obesity. This could be of special importance for transition countries like Mexico, which present both of the situations: a high prevalence of overweight and obesity as well as iron deficiency.

Samenvatting

(Summary in Dutch)

Zwaarlijvigheid is uitgegroeid tot een belangrijk gezondheidsprobleem wereldwijd en tegelijkertijd blijft bloedarmoede door ijzertekort een veel voorkomend probleem. Op individueel niveau komen deze twee vaak samen voor: mensen met overgewicht hebben een hoger risico voor ijzertekort dan personen met een normaal gewicht. De belangrijkste doelstelling van dit proefschrift was het onderliggende mechanisme dat overgewicht en ijzertekort verbindt te begrijpen. **Hoofdstuk 1** geeft achtergrondinformatie met betrekking tot de regulatie van normale ijzergehaltes en de ontstekingsverschijnselen ten gevolge van overgewicht en zwaarlijvigheid, alsmede een gedetailleerde beschrijving van de vraagstelling.

De bevindingen van onze oriënterende literatuurstudie worden gepresenteerd in **hoofdstuk 2**. We introduceren hier niet alleen het concept van een samenhang tussen zwaarlijvigheid en een ijzertekort, maar ook de verschillende verklaringen voor dit verschijnsel. Als resultaat van dit onderzoek concluderen wij dat veranderde hepcidine concentraties ten gevolge van subklinische ontstekingsverschijnselen door zwaarlijvigheid de meest waarschijnlijke verklaring is voor het verband tussen ijzergebrek en zwaarlijvigheid.

In **hoofdstuk 3** analyseren we de gegevens van het Mexicaanse Voedingsonderzoek dat in 1999 werd uitgevoerd onder 1174 kinderen in de leeftijd van 5 tot 12 jaar en 621 niet-zwangere vrouwen in de leeftijd van 18 tot 50 jaar. De analyse van het onderzoek bevestigt dat zwaarlijvige kinderen en vrouwen een verhoogd risico hebben voor ijzertekort in vergelijking met kinderen en vrouwen met een normaal gewicht ondanks vergelijkbare ijzerinname. Bovendien bleek dat C-reactief proteïne (CRP), en niet ijzerinname, een belangrijke negatieve voorspeller was van het ijzergehalte, en het effect was onafhankelijk van de lichaamsgewichtindex (Engels: *Body Mass Index*, BMI) ($P < 0.05$). Onze bevindingen bevestigen dat mensen (vrouwen en kinderen) met overgewicht een hoger risico hebben voor ijzergebrek dan mensen met een normaal gewicht ondanks vergelijkbare inname van ijzer in de voeding.

In **hoofdstuk 4** beschrijven we een interventiestudie waarin 62 gezonde vrouwen, van verschillende BMI categorieën (BMI 18.5-39.9 kg/m²), twee testmaaltijden nuttigen: één zonder ascorbinezuur (-AA) en de ander met 31.4 mg ascorbinezuur (+AA). Deze studie toonde aan dat ijzeropname vermindert in zowel

zwaarlijvige vrouwen als vrouwen met overgewicht in tegenstelling tot vrouwen met een normaal gewicht. Na toevoeging van ascorbinezuur vertoonden vrouwen met overgewicht of zwaarlijvigheid een toename in ijzeropname die slechts de helft was van die van vrouwen met een normaal gewicht. Verder was de hepcidineconcentratie geassocieerd met de BMI en een negatieve voorspeller voor ijzeropname ($P < 0.05$).

Hoofdstuk 5 beschrijft een onderzoek naar de samenhang tussen BMI, bloedvolume (bepaald met de koolmonoxide-herademingsmethode), en serum ijzergehalte. Vrouwen met overgewicht of zwaarlijvigheid hadden een groter bloedvolume, meer rode bloedcellen en plasma, en een lager serum ijzergehalte dan vrouwen met een normaal gewicht (< 0.05). Na correctie van het totale lichaamsijzergehalte, was het bloedvolume een negatieve voorspeller voor het serum ijzergehalte ($P = 0.02$). Onze bevindingen bevestigen dat zwaarlijvige personen een groter bloedvolume hebben, wat wellicht een verdunnende werking heeft. Dit verdunningseffect van een vergroot bloedvolume tezamen met een verminderde ijzeropname ten gevolge van chronische ontsteking spelen waarschijnlijk een rol in het ijzergebrek geassocieerd aan overgewicht. Daarnaast ontwikkelden we twee formules om het bloedvolume in vrouwen met overgewicht/zwaarlijvigheid te berekenen. Het bleek dat formules die lichaamslengte-gewicht en vetvrij lichaamsgewicht combineerden het meest nauwkeurig waren om het bloedvolume in de verschillende bloedgroepen te voorspellen.

In **hoofdstuk 6** onderzochten we of het verlies van vetweefsel en gewicht zou leiden tot een vermindering van de chronische ontsteking, een daling van het hepcidinegehalte, en een verhoging van de ijzeropname. Daartoe voerden we een 6-maands prospectief onderzoek uit met zwaarlijvige volwassenen ($35 \leq \text{BMI} < 45 \text{ kg/m}^2$) die recentelijk een laparoscopische mouw gastrectomie (*Engels: LSG*) hadden ondergaan. De aanvangswaarden werden bepaald van drieënveertig personen, van wie 38 personen (32 vrouwen, 6 mannen) het onderzoek voltooiden. Na 6 maanden waren het totale lichaamsvet, de ontstekingswaarden (interleukine 6), en de hepcidineconcentraties significant verminderd (voor allen: $P < 0.005$). Bovendien verbeterde de ijzeropname significant in personen met ijzergebrek ($n = 17$), maar bleef ongewijzigd in personen met normale ijzergehaltenes ($n = 21$). Dit onderzoek toont aan dat vetverlies in zwaarlijvige personen niet alleen leidt tot een vermindering van

chronische ontsteking en van serum hepcidineconcentratie, maar ook tot een verhoogde ijzeropname in personen met een ijzergebrek.

De belangrijkste bevindingen en conclusies van deze dissertatie ten aanzien van de volksgezondheid worden bediscussieerd in **hoofdstuk 7**. Bovendien worden aanbevelingen voor toekomstige studies beschreven. Volgens onze bevindingen hebben mensen met overgewicht of zwaarlijvigheid de volgende, onderling gerelateerde gezondheidsproblemen: 1) verminderde ijzeropname ten gevolge van vet-geassocieerde ontsteking en verhoogde hepcidine concentratie in het bloed; 2) een lager ijzergehalte in het serum ten gevolge van een groter bloedvolume, en wellicht een toegenomen ijzerbehoefte; 3) een suboptimale toename in ijzeropname door toevoeging van ascorbinezuur aan een maaltijd; en 4) een toename in ijzeropname na vetverlies, in het geval dat er ijzertekorten waren.

Toekomstig onderzoek op dit onderwerp is nodig: a) voor het vaststellen van optimale biomarkers en referentiewaarden voor ijzergehalte in personen met overgewicht of zwaarlijvigheid; b) voor het opstellen van richtlijnen om een gewenste ijzeropname in personen met overgewicht of zwaarlijvigheid te garanderen, en zodoende ijzertekorten te voorkomen en een goed lichaamsfunctioneren te bevorderen; c) om te bepalen of een vermindering van chronische ontsteking en lagere hepcidineconcentraties de remming van ijzeropname in zwaarlijvige personen met ijzertekort kan tegengaan, en; d) om de rol van genetische en omgevingsfactoren in de ontwikkeling en progressie van ijzergebrek bij overgewicht beter te begrijpen. Dit laatste kan van bijzonder belang zijn voor overganglanden, zoals Mexico, met een bevolking gekenmerkt door een hoge prevalentie van zowel overgewicht/zwaarlijvigheid als ijzertekort.

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About the author

Curriculum Vitae

Ana Carla Cepeda López was born on May 21, 1979 in Torreon Mexico. She completed primary and secondary school at colegio Alpes in 1995 and high school at Tecnologico de Monterrey campus Saltillo in 1998. She spent 6 months at rural Oaxaca la Sierra Mixe performing voluntary work at a malnourish children shelter and teaching at the local school. Thereafter she started her Medical Doctor degree at the University of Monterrey (UDEM) where she had the opportunity to perform social work and medical assistance at Mangola's Clinic, Tanzania during the summer of 2002. After completing her social service as a health promoter and general physician of 16 rural communities in a mobile health care unit in General Cepeda and receiving her MD degree in 2006, Ana Carla started a MSc program at the Division of Human Nutrition of Wageningen University. She focused on nutritional public health and epidemiology and she obtained her MSc degree in 2009. During this period she obtained the MSc student award at Wageningen University Nutritional Sciences Forum for the presentation "Emergence of stunted obesity in developing countries".

After receiving her MSc degree, Ana Carla started her sandwich PhD project entitled "The double burden of malnutrition: obesity and iron deficiency" at the Division of Human Nutrition of Wageningen University. Under the supervision of Prof. Dr Michael B. Zimmermann, Dr Alida Melse-Boonstra, Dr Isabelle Herter-Aeberli and Dr Saskia Osendarp she performed iron absorption studies using stable isotopes in subjects with different BMI categories. During her PhD, Ana Carla has been novel professor at University of Monterrey (UDEM) where she has been involved in teaching, thesis supervision and leading nutrition education research for the Mexican Food Bank.

List of publications

Publications in peer-reviewed journals

Peer Review Publications

Does obesity increase risk for iron deficiency? A review of the literature and the potential mechanisms. **Cepeda-Lopez** AC, Aeberli I, Zimmermann MB. Int J Vitam Nutr Res. 2010 Oct;80(4-5):263-70.

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. **Cepeda-Lopez** AC, Osendarp SJ, Melse-Boonstra A, Aeberli I, Gonzalez-Salazar F, Feskens E, Villalpando S, Zimmermann MB. Am J Clin Nutr. 2011 May;93(5):975-83.

Published abstracts

In overweight and obese women, dietary iron absorption is reduced and the enhancement of iron absorption by ascorbic acid is diminished. **Ana Carla Cepeda-Lopez**, Sophia Wussler, Alida Melse-Boonstra, Michael Bruce Zimmermann, Isabelle Aeberli. 11-1 2014 - vol. 39 / n. Suplemento

Obesity increases risk for iron deficiency in children and women in Mexico. Cepeda-Lopez AC, Osendarp SJM, Melse-Boonstra A, Aeberli I, Zimmermann MB. Annals of nutrition and metabolism. 2009; 55, 76-76

Submitted publications

In overweight and obese women, dietary iron absorption is reduced and the enhancement of iron absorption by ascorbic acid is half of that in normal weight women. **Cepeda-Lopez** AC, Melse-Boonstra A, Zimmermann MB. Aeberli-Hearter I, Am J Clin Nutr.

In obese women, increased blood volume and reduced serum iron partially explain the higher risk for iron deficiency.

The effects of fat loss after bariatric surgery on inflammation, serum hepcidin and iron absorption: a prospective 6-month iron stable isotope study.

Cepeda-Lopez AC, Allende-Labastida J, Melse-Boonstra A, Aeberli-Hearter I, Osendarp SJ, Gonzalez-Salazar F, Villalpando S, Zimmermann MB. Am J Clin Nutr.

Overview of completed training activities

Discipline specific activities

Micronutrient Forum, Addis Ababa, Ethiopia 2014
Laboratory stable isotopes, ETH Zurich 2013
International Conference on Nutrition ICN, Granada, Spain 2013
Annual Scientific Meeting, San Antonio, USA 2012
2nd Congress of Medicine UDEM on Metabolic Syndrome, Monterrey 2011
8th International Congress of Nutrition and Obesity, Monterrey 2010
XLIX IC of the Mexican Society of Nutrition and Endocrinology, Monterrey 2009
International Conference on Nutrition ICN, Bangkok, Thailand 2009

General courses

Workshop on low-grade inflammation ILSI Granada, Spain 2013
Online lecture video recording using Tegrity UDEM, Monterrey 2012
Good Clinical Practices, Hospital Muguerza, Monterrey 2010
Scientific writing, Wageningen University, 2009
Working with EndNote X2, Wageningen University, 2009
EndNote X2 Advanced, Wageningen University, 2009

Optionals

Preparation PhD research proposal, 2015
ETH PhD Excursion, Zurich 2013
Understanding and Controlling Obesity Epidemics, Johns Hopkins University, 2012
WUR PhD Excursion Mexico, 2011
Organising PhD Excursion WUR Mexico 2011
HNE course Food and Nutrition Security, 2009
Staff seminars in WUR, 2009 and 2014
Staff seminars in ETH, 2013

Colophon

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