Cardiometabolic risk factors, iron status and hepcidin in obese individuals undergoing weight loss

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Dedication

To my parents, Edgar and Leonor, who have always believed in me and given me unconditional love and support. You both have taught me so much and my goal in life is to follow the example of your kindness and compassion.

To my husband, Ian, mi amor, who has stood by me through the good and the bad. Completing a PhD is not an easy task for the candidate, or for her partner. Your faith in me has meant so much.

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Abstract

Introduction: Increased risk of cardiovascular disease, iron deficiency and immune impairment are some of the complications of obesity and aging. There is strong evidence suggesting that chronic inflammation, a cardiometabolic risk factor, is a primary cause for these co-morbidities. However, the effect of obesity and inflammation in elderly individuals on iron status and immune response is not known. Hepcidin is a peptide hormone primarily synthesized by the liver, but also by peripheral blood mononuclear cells (PBMC) and adipose tissue, that regulates iron levels in circulation by binding to the iron exporter ferroportin and inducing intracellular iron sequestration. In this way, with higher hepcidin levels, there is lower iron in circulation. Hepcidin has an important role in immune response, which consists of shifting iron pools in the body to prevent iron acquisition by pathogens. Adaptive immunity appears to be dependent on hepcidin but there is limited evidence in this regard. The effect of weight loss (WL) through calorie restriction (CR) in young and older adults on inflammation concurrently with iron status and immune response has not been determined. The central hypothesis of this project is that obesity-induced inflammation and hepcidin dysregulation in young and older adults causes iron homeostasis and immune response impairment that can be corrected with WL through CR.

Methods: To this end, we have conducted two CR studies in obese individuals. The first study investigated the effect of CR on iron homeostasis and immune response in young (18-45 y) and older (>60 y) obese women. The second study determined the impact of WL on systemic and adipose tissue (AT) hepcidin and inflammation in obese adults.

Results: With the CR intervention in young and older women we found that, accounting for diet, moderate WL was sufficient to enhance iron status in the older, but not the younger group. There was a trend for inflammation to decrease in the older group but greater WL may be required to achieve a clinically and statistically significant change in hepcidin. We also found that interleukin (IL)-1β, a pro-inflammatory cytokine, decreased with WL and may be involved in obesity-associated hepcidin dysregulation. Hepcidin expression in lymphocytes was strongly correlated with their ability to proliferate in the older group, suggesting a role for hepcidin in lymphoproliferation. In the second study we found that decreases in hepcidin and inflammation were associated with WL and decrease in waist circumference in subjects with at least 5% WL. In addition we found that AT hepcidin was inversely correlated with iron status at baseline, and AT macrophage markers were strongly correlated with hepcidin before and after WL.

Conclusions: Our results suggest that WL through CR may be an effective therapy to enhance iron status and decrease inflammation in young and older adults. We identified IL-1β as a potential mediator of hepcidin dysregulation in obesity, and for hepcidin to be involved in lymphocyte proliferation of obese older adults. In addition our data support the hypothesis that AT hepcidin may contribute to obesity-associated iron deficiency. Overall, these results expand current knowledge on the mechanism of iron homeostasis and immune impairment with obesity and aging.

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Introduction

Statement of the Problem and Significance

Statement of Hypothesis

Statement of the Problem and Significance

The World Health Organization predicts that there will be over 2 billion overweight adults worldwide by 2015, 700 million of whom will be obese.[1] This obesity epidemic is happening simultaneously with a demographic shift, leading to an unprecedented growth of the elderly population worldwide.[2] Older individuals experience sarcopenia, or loss of lean body mass, and a gain in fat mass. In addition, they are being affected by the obesity epidemic at increasing rates. According to a publication using data from NHANES (2005-2006), almost 70% of those older than 60 years are overweight or obese,[3] and this number is expected to increase.

Iron deficiency has been identified as a complication of obesity in adults[4-8] even in the presence of adequate or supplemental iron consumption,[9, 10] as well as a complication of advanced age.[11] Little is known about the impact of obesity on iron homeostasis in the elderly. Obesity and aging are associated with impairment of immune function;[1, 12] however, the impact of obesity on the declining immune response of the elderly has not been studied. Together, iron deficiency and immune response impairment resulting from obesity and aging present heavy burdens to healthcare systems worldwide.

There is strong evidence for low-grade chronic inflammation of obesity and aging to be a primary underlying mechanism of iron and immune deficiency. Hepcidin is the main regulator of iron homeostasis. Its over expression, induced by chronic inflammation, is believed to be central to the association between obesity and iron deficiency. Studies have shown significantly higher hepcidin and inflammation, and lower iron status, in obese versus lean groups.[4, 6, 13] The role of hepcidin in elderly populations needs to be studied further.

Weight loss interventions are a good approach to study the underlying mechanisms of iron and immune deficiency in the obese. To date, two weight loss interventions have shown a significant decrease in inflammation and serum hepcidin, together with an improvement in iron status. The first was a bariatric surgery intervention with a 6-month follow up.[7] The second was a calorie restriction study in obese children.[14] No calorie restriction studies measuring iron status, immune response, inflammation and hepcidin have been conducted in obese adults or the elderly. Thus, further investigation is needed to determine the association between hepcidin, iron homeostasis and immune function in young and older obese individuals.

The overall objective of this dissertation is to determine the impact of weight loss through calorie restriction on iron homeostasis and immune response in young and older obese adults. Through this novel research we will further determine the mechanism of obesity-induced iron anemia and identify whether weight loss through calorie restriction is an appropriate strategy to address iron status and immune response impairment in these populations. This project is a contribution toward the design of approaches for population-specific iron status and immune response enhancement, leading to the improvement in health and quality of life of individuals burdened by the complications of aging and obesity.

Statement of Hypothesis

The central hypothesis of this project is that obesity-induced inflammation in young and, to a greater extent, older adults causes iron homeostasis and immune response impairment, where hepcidin plays an important role, and that can be corrected with weight loss through calorie restriction. This hypothesis will be tested through the following specific aims:

Specific Aim 1: Identify changes in inflammation, hepcidin, and iron status in young and older obese individuals undergoing weight loss.

Specific Aim 2: Determine changes in ferroportin expression and iron content of PBMC in young and older obese individuals undergoing weight loss.

Specific Aim 3: Establish how the inflammatory environment of obesity affects hepcidin production by peripheral blood mononuclear cells (PBMC) upon stimulation in young and older obese individuals undergoing weight loss.

Specific Aim 4: Measure the effect of weight loss through calorie restriction on adipose tissue expression of hepcidin and macrophage markers in obese individuals.

Chapter I

Literature Review

Iron biology, immunology, aging and obesity: Four fields connected by the small peptide hormone, hepcidin

Introduction

Over the past few decades, research on iron has spanned different disciplines of the biological sciences. This nutrient is essential for red blood cell (RBC) formation, immune function, fetal development, and physical and mental wellbeing. At the same time, however, anemia affects over one quarter of the world's population,[15] with even a greater prevalence of iron deficiency. Iron supplementation has at times proven ineffective and even detrimental to health. Several studies have demonstrated that iron supplementation in populations that are at risk of certain infectious diseases may increase the risk of morbidity and mortality. Furthermore, excessive iron intake may cause iron toxicity because free iron is a potent pro-oxidant that may damage cells and tissues. Also, certain conditions, such as obesity, chronic disease and aging are associated with iron deficiency. Both aging and obesity are also associated with impaired immune response, in which iron plays an important role.

Hepcidin is a small peptide hormone essential for iron homeostasis and immune response. In this review we will summarize the existing evidence and identify gaps in knowledge regarding why obese and elderly individuals are at a higher risk of iron deficiency and immune response impairment. First, we will review the role that hepcidin plays in iron homeostasis and immune response. We will then summarize the existing evidence regarding chronic inflammation of obesity and its impact on iron status, immune response and hepcidin. The final sections of this review are dedicated to aging, immunosenescense and iron deficiency, and how increasing rates of obesity in the elderly may be placing this age group at an even higher risk of impaired health.

Iron homeostasis and hepcidin

Maintenance of iron homeostasis is essential for proper cellular function. Adequate iron levels must meet the needs of different organs and tissues, but excess of iron causes cellular damage. Iron is excreted in an unregulated manner, mainly through enterocyte sloughing and loss of bodily fluids and skin cells.[16] Iron balance is primarily regulated by absorption through the intestine and iron export from cells. When ingested, iron is absorbed through the enterocyte in several stages. Approximately 1-2mg per day can be absorbed through the intestinal epithelium.[16] Iron generated from animal sources is absorbed as heme iron, which is the most bioavailable form of dietary iron, but the mechanism of absorption is yet unknown.[16, 17] Once in the enterocyte, heme iron is released by hemoxygenase 1 (HOX1). Non-heme iron is available from non-animal sources, and it is most frequently encountered in the ferric state (Fe^{3+}). In order to be absorbed, non-heme iron first needs to be reduced to the ferrous state (Fe^{2+}) by the ferrireductase duodenal cytochrome B (DcytB), which uses ascorbic acid as a cofactor. In this way vitamin C ingestion enhances iron absorption in the gut. Ferrous iron is absorbed through the apical side of the enterocyte by divalent metal transporter 1 (DMT1) through a proton cotransport mechanism (**Figure 1**). Other mechanisms of iron absorption may exist, but they have not been thoroughly elucidated.

Once in the enterocyte, iron can be stored in ferritin, an iron storage protein that can store up to 4,500 iron atoms.[17] If iron stored in enterocytes is not utilized it is lost when the enterocyte is shed off the intestinal lumen. To be absorbed into the bloodstream, iron is exported through ferroportin, the only known iron exporter located in the basolateral side of the enterocyte, with the aid of ceruloplasmin (soluble) or

hephaestin (membrane-associated), which oxidize iron back to its ferric state. To avoid oxidative damage, iron in circulation is bound by the carrier protein transferrin and transported to target cells. Transferrin binds to the transferrin receptor (TfR), and this complex is internalized through endocytosis. Iron is then released into the endosome and reduced by the metalloreductase STEAP3 to be exported into the intracellular environment through DMT1 or its homolog natural resistance-associated macrophage protein 2 (Nramp2, also known as SLC11A1),[17, 18] where it can be stored or used in cellular processes. The TfR-transferrin complex then returns to the membrane where holo-transferrin is released into the plasma.[19] This is known as the transferrin cycle[17] and it occurs mainly in erythroid precursor cells which have the highest iron demands, and to a lesser extent in other cell types.[16] Iron can be transported to other target tissues, such as liver which is the main site for iron storage, bone marrow, and peripheral blood mononuclear cells (PBMC), among others, to be used in important processes such as synthesis of hemoglobin or Fe-S clusters in mitochondria. About 70% of iron in the body is used for hemoglobin synthesis.[20] When erythrocytes have reached the end of their lifespan, they are recycled by reticuloendothelial macrophages. The amount of iron recycled through this system approximates 25 mg/day.[16] Almost all iron needed for erythropoiesis is provided by RBC recycling.[20] Regulation of iron recycling and storage, together with iron absorption, is essential for iron homeostasis.

Given the damage that iron may cause through oxidative damage, but also its importance for cellular processes, iron levels must be tightly regulated. Iron homeostasis is regulated at different levels, either post-translationally (**Figure 1**), or post-transcriptionally. Ferritin and transferrin levels, among other proteins involved in iron

homeostasis, are regulated through post-transcriptional modification in response to abundance or lack of iron. When iron is scarce, iron regulatory proteins (IRPs) bind to iron response elements (IREs) in the 5' untranslated region (5'-UTR) of ferritin mRNA, inhibiting translation and preventing iron storage. In the opposite scenario, when iron is abundant, IRPs are either degraded or unable to bind the 5'-UTR, ferritin mRNA is available for ribosome binding and translation occurs. Ferroportin is regulated in this manner as well.[16] On the other hand, TfR is regulated through IREs located in the 3'-UTR of the TfR mRNA. When iron levels are low the TfR mRNA is stabilized by IRPs binding to 3'-UTR IREs, allowing its translation. When iron levels are high and there is need for iron storage, the TfR mRNA transcript is not bound by IRPs, becomes unstable and degraded by nucleases.[17]

Post-translational regulation of iron homeostasis occurs primarily through hepcidin, a small peptide hormone central to iron homeostasis that regulates cellular iron export.[17] Hepcidin acts by binding the membrane-associated iron exporter ferroportin (**Figure 1**), which is expressed on enterocytes and macrophages, as well as other cells such as PBMC. Upon binding, hepcidin induces internalization and degradation of ferroportin through tyrosine phosphorylation, ubiquitination, and lysosomal degradation. In this way, iron remains in intracellular stores and export into the plasma is reduced.[16, 17] Iron export through ferroportin from enterocytes, macrophages and PBMCs has been shown to be prevented by hepcidin.[21-23]

Hepcidin is transcribed from the *Hamp* gene into an 84 amino acid preprohepcidin, which is then cleaved into a 60 amino acid pro-hepcidin, detectable in serum.[24, 25] This precursor is further processed into the mature bioactive form, a 25

amino acid peptide[24] containing four disulfide bonds. Hepcidin is expressed mainly by the liver,[17] although more recent studies have shown that other cell types such as adipocytes[13] and PBMC[23] also express hepcidin.

Hamp expression is induced through different signaling pathways. Primarily, hepcidin expression is induced through the bone morphogenetic protein (BMP)/Smad pathway, which is activated by interaction between the BMP receptor (BMPR) and the co-receptor hemojuvelin (HJV). Matriptase-2 (also called TMPRSS6), inhibits hepcidin upregulation by cleaving HJV into soluble HJV (sHJV) and preventing activation of the BMP/Smad pathway.[21] In addition, sHJV inhibits hepcidin expression by competing with membrane-bound HJV for the BMPR.

Hepcidin gene expression is also regulated through TfR signaling. Recent evidence suggests that the co-receptor HFE competes with iron-bound transferrin for TfR1 binding. When there is low transferrin-bound iron, HFE binds TfR1 and hepcidin expression is not induced. When iron levels are high, transferrin binds TfR1 and HFE is displaced. It is believed HFE is then sequestered by TfR2 which has both HFE and transferrin allosteric binding sites. In this way, HFE binding to TfR2 may be the signal that induces the ERK/MAPK signaling cascade that leads to *Hamp* expression, but more research is needed.[26] This system is intimately related to the BMP/Smad pathway but the details have not been fully elucidated.

Chronic or acute inflammation also induces hepcidin expression.[16, 17, 27] Studies in hepatocyte cell lines,[28] mice,[29] and humans[27] have shown that interleukin-6 (IL-6) upregulates hepcidin through the JAK/STAT3 pathway and STAT3 binding motif in the *Hamp* promoter. A study in humans showed that IL-6 or lipopolysaccharide (LPS)

injections induced hypoferremia and were directly correlated with an increase in serum hepcidin.[27]

Signals that inhibit hepcidin expression include erythropoiesis potentially through inactivation of the BMP/Smad pathway by BMPR binding protein TWSG1, and hypoxia through stimulation of erythropoietin (Epo) production and perhaps also of hypoxia-inducible factor 1 and 2 (HIF1 and HIF2).[16, 17] Genetic deficiency of hepcidin leads to hemochromatosis, an iron overload disorder. Mutations both in hepcidin and its regulators lead to this disease. Class I hemochromatosis is characterized by mutations in the *Hamp* gene; Class II hemochromatosis is due to defects in *HFE*, *HJV*, or *TfR2*; and Class III hemochromatosis occurs due to defects in ferroportin, preventing binding of hepcidin and regulation of cellular iron export.[17] *HJV* mutations are the most common cause of juvenile hemochromatosis, accounting for 95% of the cases.[21] Hemochromatosis is characterized by low and even undetectable hepcidin levels, iron overload, and iron deposition in liver, heart and endocrine organs.

It has been suggested that there is no cross-talk between the signals that regulate hepcidin.[16, 30] Iron status, inflammation and erythropoiesis seem to influence hepcidin expression independently from each other, with the strongest signal outweighing the others and determining hepcidin levels. This is particularly important for certain populations, such as the elderly, that are at risk of both low iron status (a signal that downregulates hepcidin) and chronic inflammation (a signal that induces hepcidin).

Innate and adaptive immune function are iron-dependent and influenced by hepcidin

The immune system can be divided into two types of responses. The first line of defense that occurs upon detection of antigen is innate immunity. During innate immunity cells such as macrophages and neutrophils attack and defend the host against microbes. Depending on the nature of the microbe, innate immune cells will take action to destroy it and to involve cells from the adaptive immune system, including T and B lymphocytes. These cells create a more targeted response and acquire memory of the antigen in case the infection recurs.

Excess or deficit of iron affects the immune response

Both iron deficiency and iron overload affect immune function. To begin with, as reviewed in Oppenheimer et al., iron deficiency impacts neutrophil function, lymphoproliferation, cytokine production and natural killer (NK) cell activity.[31] On the other hand, numerous studies have shown the negative effect of iron overload due to supplementation, disease, or blood transfusions on vulnerability to infection.[20, 32-34] The most notorious study showing this evidence was conducted by Sazawal et al. in Pemba, Tanzania. This study was discontinued before completion due to a significant increase in mortality or the likelihood for children to be hospitalized in the group supplemented with iron and folic acid.[35] Iron supplementation in malaria-endemic regions may increase the risk of malaria infection, but findings from different studies have not been uniform, with some studies showing increased malaria risk and others no increased risk together with a decrease in anemia.[36] Conversely, iron deficiency has been associated with protection against malaria.[20] This topic remains controversial, and evidence suggests that the

severity of malaria after iron supplementation depends on the host's health and nutritional status prior to supplementation. HIV viral replication and risk of secondary opportunistic infections, such as tuberculosis, has also been shown to increase with iron supplementation.[20, 37] Similarly, patients with iron overload as seen in hemochromatosis have increased susceptibility to infection.[38] Therefore, iron supplementation not only enhances the host immune function but it also provides an environment for pathogens to thrive and this is why there is controversy regarding iron supplementation in regions with high risk of iron-deficiency and infection. It is important to note that iron supplementation may have beneficial effects on immunity, and it has been shown to improve resistance to respiratory infection in children.[31]

Iron and hepcidin in innate immunity

Because iron is needed by most living organisms, one of the first lines of defense by the human body as a host is to deprive invading pathogens of the iron required for them to survive. In response to this, certain pathogens have evolved to invade iron-rich pools in the host. For example, the malaria parasite, *Plasmodium falciparum*, manipulates hepatocyte iron content through upregulation of DMT1 and downregulation of ferroportin in its liver-infection stage, but when it switches to the blood-infection stage, the parasite influences regulatory proteins to deplete hepatocyte iron stores so that the iron pool shifts from the liver to RBCs.[33] Other modes of attack by invading pathogens include production of siderophores, molecules produced by bacteria to chelate any available free and transferrin-bound iron and deliver it to the bacteria.[19] In fact, there are more than 500 known siderophores that accomplish this function.[33]

The host's immune system has developed different equally ingenious strategies of defense, which mainly involve depriving pathogens of iron. One mechanism of defense is the production of lipocalin-2, which is expressed in neutrophils and epithelial cells and binds to siderophores, inhibiting their action.[39] Lactoferrin, another protein employed in immune defense, has high affinity for free iron during infection. In addition, hepcidin plays a central role in host defense against extracellular pathogens. Hepcidin was simultaneously identified as an antimicrobial peptide and a regulator of iron status.[33] As mentioned previously, hepcidin is upregulated by pro-inflammatory cytokines, such as IL-6 and IL-1, through signaling pathways involving JAK/STAT3 and toll-like receptor 4 (TLR4).[40] Hepcidin upregulation upon infection by extracellular pathogens leads to decreased circulating iron levels and iron sequestration within the host's cells. Theurl et al. showed that hepcidin regulated ferroportin expression and intracellular iron content in an autocrine fashion in primary monocytes from patients with anemia of chronic disease and in the human monocyte cell line THP-1.[41] In addition, they showed how hepcidin knockdown with RNAi reversed this process and resulted in decreased iron sequestration and increased ferroportin in the monocytes. It is important to note that ferroportin expression is also downregulated at the gene expression level in monocytes by inflammatory cytokines independently of hepcidin.[42]

Immune system defense against intracellular pathogens requires a different approach. Interferon gamma (IFNγ), a macrophage-activating cytokine expressed by Th1 lymphocytes and NK cells, downregulates expression of TfR1 in macrophage phagosome membranes, thereby decreasing iron pools and preventing iron acquisition by intracellular bacteria.[19] Nairz et al. showed how iron deprivation through this mechanism could

prevent *Salmonella typhimurium* infection.[43] IFNγ also acts on macrophages to induce maturation and acidification of phagosomes.[20] Furthermore, evidence has shown that IFNγ induces hepcidin expression in lung epithelial cells.[44] Another method of defense is through Nramp2 (also known as SLC11A1, a homolog to DMT1), which is expressed in the phagosomes of macrophages and neutrophils and exports divalent metal protons from the phagosome to the cytosol. Ferroportin is also expressed in macrophage phagosomes.[45] In this way intracellular bacteria, such as *Mycobacteria*, are unable to acquire iron within the invaded cells.[19] Decreasing iron levels and manipulating iron pools in the host as a defense mechanism against iron-dependent pathogens has also been illustrated in studies showing that over expression of hepcidin and iron supplementation can increase the risk of intracellular pathogen infection, such as *Legionella pneumophila*,[46] and this could be reversed by localized iron depletion.

Besides manipulating iron pools, a crucial aspect of innate immunity in which iron is involved is the oxidative burst, which is the production of reactive oxygen species (ROS) to damage invading pathogens. These ROS created within immune cells, mainly neutrophils and macrophages, are released into phagosomes containing the engulfed or intracellular pathogen.[47] Iron is essential for the production of ROS, and it may be through hepcidin's action that iron is accumulated within cells involved in oxidative burst, but the role of hepcidin in this process has not been studied in depth. Iron status and intracellular macrophage iron levels impact the efficiency of the oxidative burst.[47] *In summary*, the literature reviewed above indicates the importance of maintaining an optimal iron balance specific to the type of infection, and the crucial role hepcidin plays

in regulation of the immune response, starting with innate immunity, but also as more recently has been demonstrated, for adaptive immunity.

Iron and hepcidin in adaptive immunity

An optimal iron status is essential for adaptive immune response. Iron and expression of TfR are essential for lymphocyte activation and proliferation.[48] Both iron deficiency and iron overload have been associated with impaired cell-mediated immunity in humans.[49, 50] Iron deficiency has been shown to decrease lymphocyte counts and proliferation, IFNγ and IL-2 production, and to impair NK cell activity. Omara and Blakley found that mice fed an iron deficient diet had a lower delayed type hypersensitivity (DTH) response, in which CD4+ lymphocytes are key players, than mice fed a normal or supplemented iron diet.[50] Furthermore, mice fed an iron deficient diet had significantly lower concanavalin A (Con A)-induced lymphoproliferation than the normal or supplemented diet groups. These results suggest that iron deficiency leads to impaired T cell response. Another study showed that NK cell cytotoxicity was impaired in rats fed an iron deficient diet, and adding IFNγ *in vitro* rescued the loss of function.[51]

Certain aspects of adaptive immunity have been shown to be affected by iron overload. For example, Omara et al. showed that contact sensitivity, a measure of lymphocyte response and immunoglobulin E (IgE) production, was impaired in mice fed high-iron diets with respect to those fed an iron-replete diet.[50] In addition, high CD4/CD8 T cell ratios, low numbers of CD28+ cells and impaired CD8 T cell function have been reported in patients with hemochromatosis,[48] although direct causality with iron overload cannot be proven in this scenario. Mouse knockout models of β2-microbulin ($\beta 2m^{-/-}$), a protein associated with MHC class I molecules, have abnormal

increase of iron absorption and increased plasma iron. Furthermore, *β2m*$^{-/-}$ and *Rag2*$^{-/-}$ mouse knockout models, the latter being a factor essential for B and T lymphocyte development, have immature lymphocytes and severe iron overload.[48] *In summary*, this evidence suggests different effects of iron deficiency and iron overload on the adaptive immune response.

Hepcidin's role in adaptive immunity has been defined more recently, but more evidence is needed. Pinto et al. showed that lymphocyte hepcidin production increased upon stimulation with anti-T cell receptor (anti-CD3/CD28) antibody, and that partial knockdown of hepcidin impaired T cell proliferation.[23] This effect was reversed by adding synthetic hepcidin peptide to the hepcidin-deficient lymphocytes. In addition, different subtypes of PBMC were shown to express hepcidin, and seem to play a role in iron homeostasis because they express ferroportin and internalize iron in the presence of hepcidin. Furthermore, it has been suggested that regulation of hepcidin through the Smad and STAT3 pathways may play a role in Th17 responses.[33]

Taken together, the evidence presented above suggests that iron homeostasis and immune response are closely related. The interactions between iron status and immune response are complex, implying that optimal iron status for immune response may depend on the nature of the pathogen. Iron plays an important role in both innate and adaptive immunity, but many questions remain unanswered, especially pertaining to hepcidin's involvement in adaptive immunity. The relationship between hepcidin and adaptive immunity will be studied further in chapters II and III.

Chronic inflammation of obesity is associated with high hepcidin and low iron status

Obesity and iron homeostasis

In the past few decades, several reports have shown that obese adults are at a high risk of becoming iron deficient.[4-8, 52] The first evidence came from Wenzel et al. in 1962, showing lower serum iron in obese adolescents with respect to normal weight adolescents.[53] Other studies in children and adults around the world followed with consistent findings. **Table 1** compiles these studies.

It was later observed that the identified association between obesity and iron deficiency was independent of iron intake or other dietary factors.[5, 8-10] In fact, studies found that obese individuals become iron deficient despite having adequate iron consumption. Furthermore, it was observed that iron absorption was affected by obesity. A recent study related poor iron absorption to obesity and adiposity-related inflammation in women and children through the measurement of stable iron isotope incorporation.[10] These groups were in transition countries and were less responsive to iron fortification than their non-obese counterparts. The authors found iron status was negatively correlated with C-reactive protein (CRP) and BMI in women. In children, they found an inverse relationship between BMI *z*-score and body iron. They also showed that change in body iron after iron supplementation was inversely related to BMI, suggesting that absorption was reduced in obese children. Another study found that obesity was a strong predictor of iron deficiency in obese women and children from Mexico, regardless of iron intake.[9] Obese women were twice as likely as lean women, and obese children were four times as likely as lean children, to be iron deficient.[9] *In summary*, these studies identified

iron deficiency as a co-morbidity of obesity in different age groups, independent of iron consumption.

Obesity is characterized by low-grade chronic inflammation, i.e. an increase in circulating inflammatory molecules, such as IL-6 and CRP,[54] which in turn are associated with chronic disease risk. In recent years studies have shown an association between hepcidin, chronic inflammation and low iron status in obese populations,[6, 13, 55-59] identifying a possible mechanism by which iron deficiency occurs in the obese. These studies have been summarized in **Table 2** and they consistently show a significant association between serum hepcidin, inflammation, and low iron status. Of note, Tussing-Humphreys et al. found significantly higher serum hepcidin and CRP, together with significantly lower iron status in obese versus lean women.[6] Similar results were reported in other adult cohorts, as well as children,[55, 57, 59] and pregnant women.[56] As to the latter, we recently found significantly higher hepcidin and CRP levels in obese pregnant women with respect to lean pregnant women.[56] Iron status did not differ between the mothers, but it did for the newborns, with significantly lower Tsat and serum iron in cord blood from the obese group. In addition, we found strong negative correlations between maternal BMI and cord blood iron status, and between maternal serum hepcidin and cord blood iron status. *In summary*, these data suggest that hepcidin dysregulation causes iron deficiency in obese individuals. Further studies are needed to confirm these results and to elucidate the mechanism of hepcidin dysregulation in obesity.

Some publications in the last few years have shown that adipose tissue (AT) may be playing an important role in hepcidin dysregulation in obesity.[13, 58] Bekri et al.

showed that hepcidin expression is significantly higher in AT of obese individuals (BMI>42 kg/m^2) who had a high prevalence of iron deficiency, compared to a lean group.[13] The same group later showed that HJV may be a primary regulator of hepcidin in AT. They found significantly higher hepcidin, IL-6 and HJV expression in AT of obese subjects compared to lean.[58] BMI and AT HJV expression were significantly correlated. When cultured adipocytes were treated with BMP2, which is downstream of HJV signaling, hepcidin expression was upregulated. Adipose tissue may also be contributing to hepcidin dysregulation in other tissues through adipokine secretion. Chung et al. found hepcidin in HuH17 human hepatoma cells to be upregulated by leptin through the JAK/STAT3 pathway.[60] Leptin is significantly upregulated in obesity as it is secreted by AT,[61] and liver is the main producer of hepcidin[17] and, thus increased adiposity may be having an impact on hepatic hepcidin, but further research is needed.

The effect of obesity on AT hepcidin expression is not completely understood. Tussing-Humphreys et al. recently found no differences between arterial and venous hepcidin across subcutaneous AT of obese and lean subjects. There were no differences between groups either, suggesting that subcutaneous AT may not be a significant contributor of hepcidin secretion into plasma.[62] However, their study sample size was small, with only 9 subjects per group, and the subjects' iron status and serum hepcidin levels spanned a wide range, making it difficult to draw definite conclusions. Also, important differences may exist between subcutaneous and visceral AT, although Bekri et al. showed that hepcidin mRNA levels were not different between the two in morbidly obese patients.[13] Together, these findings suggest a mechanistic link between iron deficiency and obesity through inflammation, where AT hepcidin expression may play a

significant role. More research is needed to determine the role of AT on hepcidin levels and iron status. Further research on the impact of chronic hepcidin upregulation on iron absorption, a site of iron status regulation, as well as on the impact of obesity-associated hepcidin upregulation in other cell types, such as PBMC is needed. Some of these gaps in knowledge will be addressed in chapters II and IV.

Weight loss in obese people, inflammation and iron status

Weight loss studies, both through calorie restriction and restrictive bariatric surgery, are good approaches to studying the underlying mechanisms of iron deficiency and hepcidin dysregulation in obesity. To date, two intervention studies measuring the effect of weight loss on iron status, hepcidin and inflammation have been conducted (**Table 3**). The first one, by Tussing-Humphreys et al. showed that 6 months after bariatric surgery obese women had a significant decrease in systemic inflammation (measured as CRP and IL-6) and serum hepcidin, together with an improvement in iron status.[7] The second study, by Amato et al. found that obese children losing weight through calorie restriction experienced a significant decrease in serum IL-6 and hepcidin, accompanied by enhanced iron status.[14] They also observed a significant improvement in iron absorption after weight loss. No studies on the impact of calorie restriction on hepcidin and iron status in adults are available. Bariatric surgery may not be a good model to study changes in iron status and hepcidin. Malabsorptive bariatric interventions are the most effective for weight loss, but due to the excision of a considerable portion of the small intestine iron absorption is impaired. Iron deficiency and anemia are common complications among bariatric patients after surgery.[63] Therefore, calorie restriction studies in adults are needed in order to determine the effectiveness of weight loss on iron

homeostasis and to uncover the underlying mechanisms of iron deficiency and obesity. We have conducted such a study and the results are present in chapter II.

Determination of iron status in obese populations

Iron status can be determined through several parameters in serum or plasma, including serum iron, total iron binding capacity (TIBC), transferrin saturation (Tsat), ferritin, soluble TfR (sTfR) and the ratio sTfR/log-ferritin. For healthy individuals with no chronic or acute inflammation, serum ferritin is a good indicator of iron status. Another commonly used parameter is sTfR, which is directly related to cellular iron stores: the higher sTfR is in circulation the lower the iron status.[64] However, cutoff values and normal ranges for sTfR are variable between publications and assays,[64] thus, when sTfR is used as a measure of iron status it is necessary to indicate assay specifications. Serum iron, TIBC and Tsat are also effectively used to measure iron status. TIBC is a measure of total circulating transferrin levels, and Tsat is calculated by the formula: Tsat = (serum iron/TIBC) x 100%. Hemoglobin is used to determine the presence of anemia.

Determining iron status in the obese is more complicated and controversial because low-grade chronic inflammation affects several parameters of iron status. Also, definition of iron deficiency in the obese is not consistent, as publications have used different cutoff values to define iron deficiency in the obese. Ferritin levels are affected by chronic inflammation;[64] its expression in hepatocytes, macrophages and adipocytes is induced by IL-1β and TNFα,[65] which are known to be upregulated in obesity. In situations where there is iron sequestration in cells without real iron deficiency, as may

be the case with the obese, sTfR levels are normal. Tussing-Humphreys et al. suggested that in obesity chronic inflammation leads to real iron deficiency due to long term decreased iron absorption and unregulated iron loss.[30] The qualifiers for iron deficiency in the obese may therefore be similar to those seen in anemia of chronic disease coexisting with real iron deficiency. However, this has not always been the case in studies measuring iron status in obese populations and further research is needed.

The criteria used by Muñoz et al.[63] to diagnose iron deficiency in obese patients are chronic inflammation (CRP>1 mg/L), low hemoglobin (less than 12 g/dl for women and less than 13 g/dl for men), low Tsat (<20%), normal to high ferritin (30-100 µg/L), sTfR above the normal range (range specified by manufacturer), and a high ratio of sTfR/log-ferritin (>2). For anemia of chronic disease without real iron deficiency, sTfR would be normal and ferritin would be higher than 100 µg/L. Similarly, Tussing-Humphreys et al.[30] have defined iron status in the obese as unaffected or low hemoglobin, unaffected or increased ferritin, decreased serum iron and Tsat, unaffected or increased TIBC, and high sTfR.

Soluble TfR is not influenced by acute or chronic inflammation; instead, it is a reflection of cellular iron requirements and it is proportional to TfR on cell membranes.[7] Therefore, sTfR is a parameter that can be used to define true iron deficiency. The effect of weight loss through calorie restriction in the obese on sTfR has not been reported. *In conclusion,* for populations with chronic inflammation, such as those with obesity, it is necessary to take into account markers of chronic inflammation, specifically CRP, as well as a combination of iron status markers to define iron deficiency. In addition,

establishing a set of criteria used consistently for the diagnosis of iron deficiency in the obese will be essential for the advancement of the field.

Immune function is altered in obesity

Obesity and immune response

Several studies in high-fat fed rodent models and obese humans have shown immune response impairment, including decreased macrophage, dendritic and NKT cell function, and impaired lymphocyte response to mitogenic or antigenic stimulation.[1, 66] Specifically, there is T cell function impairment as shown by decreased thymic output of naïve T cells and lower lymphocyte proliferation, with evidence of altered circulating numbers of CD4+ and CD8+ T cells.[1, 66-68] There is also evidence for T cells to be skewed towards a Th1 phenotype with higher production of IFNγ in humans.[69, 70] These alterations in the immune system, which are associated with other obesity comorbidities such as type II diabetes, may explain the increased prevalence of susceptibility to infections in the obese.[1, 66]

A study comparing immune response in obese versus non-obese subjects found that the obese group had increased lymphocyte numbers, with a significant correlation between BMI and total leukocyte count.[67] Particularly, CD3+, CD3+CD4+ and CD19+ cells were elevated. The obese subjects had suppressed lymphocyte proliferation after stimulation with Con A and phytohemagglutinin (PHA). In addition, the obese group was found to have significantly higher monocyte and granulocyte phagocytosis and oxidative burst, but there was no difference in NKT or cytotoxic CD8+ T cell numbers when compared to lean subjects. Therefore, it seems that even though basal production

of inflammatory cytokines is higher, stimulated immune cell cytokine production is suppressed in obese compared to lean. While this might appear contradictory, it could indicate that the higher inflammation associated with obesity renders the immune cells less responsive to activation. Many of the studies conducted so far on the impact of obesity on immunity have small sample sizes and populations with mixed age groups and co-morbidities, and studies that account for these factors are needed.[1]

Iron homeostasis, although important for immune function as shown in previous sections of this review, has not been studied extensively together with immune response in the obese. Only one study so far has measured the effect of obesity and weight loss on iron status, hepcidin and immune response simultaneously.[71] Consistent with other studies, they found that despite higher serum levels of inflammatory cytokines, obese women had significantly lower production of IL-6, TNFα and IFNγ than lean women in PBMC stimulated with (LPS).[71] This study found that serum hepcidin and sTfR were significantly correlated with *ex vivo* production of IFNγ upon stimulation of PBMC. These results suggest that further research is needed to explore the role of hepcidin and iron status in immune dysregulation seen in obesity. *In conclusion*, immune function is impaired with obesity, but more information is needed to characterize this impairment in different age groups as well as linking it to clinical outcomes. Chronic inflammation seems to be desensitizing immune response upon encounter with stimuli, but more evidence is needed. Whether hepcidin and iron play a role in this desensitization remains to be elucidated. These questions will be explored in chapter III.

Obesity, adipose tissue remodeling and immune function

AT undergoes structural and functional changes with weight gain and obesity. There is tissue hypoxia, adipocyte death, increased circulation of free fatty acids, alterations to the adipokine secretory profile, formation of crown-like structures, and dysregulation of AT immune cell function.[61, 72] The latter is characterized by activation and increased recruitment of macrophages, granulocytes and lymphocytes.[72] Macrophages involved in AT can have a M1 or M2 phenotype. M1 macrophages produce pro-inflammatory signals and are associated with Th1 cell recruitment, while M2 macrophages have an anti-inflammatory profile associated with Th2 cell recruitment and extracellular matrix remodeling, angiogenesis and tissue repair.[72] AT macrophages in obesity are characterized by having M2 surface markers together with pro-inflammatory cytokine production.[73-76]

T regulatory cells (Tregs), eosinophils and invariant natural killer T cells (iNKT) are among the anti-inflammatory cells that prevent uncontrolled inflammation in AT under normal conditions, but their function becomes impaired in obesity. iNKT cells are reactive to lipids through CD1d, appear to be enriched in AT, and are important for maintenance of healthy adipocytes and insulin sensitivity.[77] This function appears to be particularly important in the context of low fat diets. The number of iNKT cells is decreased in obesity, and this may be related to onset of insulin resistance.[78] Changes in AT with obesity lead to the systemic elevation of pro-inflammatory cytokines, most notably TNFα, IL-1β and IL-6,[79] which in turn leads to chronic inflammation. Although inflammation of obesity has been correlated with comorbidities and complications seen in obesity, mechanistic evidence remains to be elucidated.

Weight loss in the obese and immune function

In general, weight loss is associated with improved health. As little as 1% weight loss has been associated with a significant decrease in systemic inflammation.[80] The most consistent systemic changes with weight loss are decreases in serum CRP and IL-6. Decreased inflammation after weight loss has been associated with improvement in insulin sensitivity and iron status, among other conditions.[7, 81-83] Most weight loss studies have also shown an improvement in cell-mediated immune response measured as lymphocyte proliferation.[1, 71, 84, 85] For example, Ahmed et al. showed a significant improvement in lymphoproliferation after a 10 or 30% calorie restriction over 6 months.[84] This was shown in whole blood stimulated with Con A and PHA. Antigenic stimulation was significantly improved in the 30% calorie restricted (CR) group, but not the 10% CR group. In addition, they found that DTH response, an *in vivo* measure of T cell function, improved with calorie restriction. In this study, percent change in BMI was correlated with percent increase in lymphoproliferation. These findings show that CR can improve T cell function. Given that not all studies have shown significant and positive effects on immune response, more research is needed.

AT inflammation[86, 87] and immune cell infiltration[88] have been shown to decrease with weight loss. However, AT remodeling during weight loss is not completely understood. A study by Capel et al. presented AT gene profiling at different stages of weight loss in obese women: a 4 week very low calorie diet, followed by a 2 month weight stabilization period on a low calorie diet, and a 3-4 month weight maintenance period. They showed that macrophage genes are initially upregulated during the very low calorie diet, together with a downregulation in genes involved in adipocyte metabolism.[89]

During the weight stabilization period they then showed a reversed gene expression profile, with attenuation of macrophage gene expression and increase in adipocyte metabolic genes. Therefore it is important to consider the stage of weight loss an individual is in when conducting CR studies. This study further showed a decrease in plasma CRP while AT macrophage markers were increasing in the energy restriction period, suggesting that adipose and systemic inflammation might, to a certain extent, be regulated separately.

In conclusion, evidence regarding the effect of weight loss on immune response and AT architecture seems to depend on the type and duration of the weight loss intervention and more research is needed to further define these relationships. Furthermore, the long term effects of weight loss on immune response have not been determined.[1] This gap in knowledge will be addressed in studies described in chapter IV.

Old age is characterized by increased adiposity and inflammation, impaired immune function, and low iron status

Old age is characterized by chronic inflammation and the decline of the immune response. Iron status is also impaired with aging, but the reason why is not completely understood. The prevalence of obesity in the elderly has increased at worrisome rates. With the expansion of the elderly population worldwide, expected to reach 20% of the world's population by 2050 from a current 8%,[2] and aging-associated morbidities placing a high burden on healthcare systems around the world, it is important to address issues such as iron deficiency in the obese elderly.

Obesity and increased adiposity are prevalent in the elderly

Older adults are becoming obese at a global scale, with an estimate of more than 20% obese elderly in the United States by 2030.[3] Elderly obesity can lead to frailty, and represents an increased challenge for mobility and strength, because it leads to added fat mass to the already increasing levels of body fat seen with aging. Being elderly and overweight (but not obese) is not associated with disease risk,[90] but a meta-analysis showed that BMI greater than 30 kg/m^2 in older adults (≥65 y) was associated with a modest increase in the risk of mortality.[91] Previously, intentional weight loss for older individuals was not recommended because it was considered to increase risk for disease and mortality.[90] However, more recently weight loss in elderly obese has become a recommendation,[92] as long as the approach includes caloric restriction together with increased physical activity. Where this approach proves challenging due to the physical constraints and changes in body composition in the elderly, other approaches like pharmacology and bariatric surgery can be used. Very few studies have been conducted to measure the effectiveness of weight loss on different health outcomes in obese elderly. In their review, Witham and Avenell[90] stress the need and importance for conducting such trials.

Aging, inflammation and immune response

The elderly experience low-grade chronic inflammation, characterized by an increase in circulating levels of IL-6 and CRP, among other inflammatory markers.[93, 94] Higher IL-6 is an independent predictor of mortality and morbidity in older adults.[93] In addition, old age is characterized by weakening of cell-mediated immunity, particularly that of T cells.[12, 95] Changes in the immune system observed with aging include thymic

involution, impaired response to evolving pathogens and newly encountered antigens, poor response to vaccinations, increased susceptibility to infection, as well as increased autoimmunity and inflammation,[94, 95] A consistent age-related defect observed across different models is the impaired ability of T cells to proliferate and produce IL-2 in response to antigenic or mitogenic stimulation.[12, 96]

Little is known about the effect of obesity on immune response in the elderly. A recent study showed that obesity negatively affects aging of the immune system, with acceleration of thymic involution, increase in perithymic AT, and reduction of T cell precursors,[97] narrowing the immune repertoire against antigens encountered later in life. Therefore, studies are needed to investigate the effect of obesity on the already declining elderly immune response.

Iron deficiency in the elderly

There is a high prevalence of anemia in the elderly. The World Health Organization reported that anemia increases after the age of 50, affecting 10% of elderly >65 years and 20% of elderly >85 years.[98, 99] Complications associated with anemia are cardiovascular disease, increased falls and fractures, and cognitive impairment. There is also decreased quality of life and higher risk of mortality.[99] The most common type of anemia in the elderly is anemia of chronic inflammation, caused by chronic conditions prevalent in older populations.[11] According to studies using data from NHANES III, one third of anemia cases in older people are caused by nutritional deficiencies of iron, folate or vitamin B-12, with iron deficiency accounting for over half of these deficiencies.[98, 99] Low dietary iron, impaired iron absorption due to reduced stomach acid production, and gastrointestinal bleeding also contribute to iron deficiency anemia in older adults.[11, 98-100].

As with obesity, diagnosis of iron deficiency in the elderly is challenging due to the presence of chronic inflammation.[100] There is very limited information on the impact of obesity on iron homeostasis in the elderly. Recently, we found that elderly women with high BMI and waist circumference from low-income areas of Quito, Ecuador had significantly higher serum hepcidin and CRP than their lean counterparts (Dao, et al., manuscript submitted, under review – see appendix). Even though iron status was inversely related to CRP, it did not differ between women with high and normal BMI. Dietary iron, as well as intake of other micronutrients, was low in this population. Further research is necessary to determine the factors regulating iron status in elderly populations with a high prevalence of obesity, accompanied by poor nutrition. Furthermore, evidence is needed on the effect of aging on hepcidin regulation, as well as the effect of weight loss on hepcidin, inflammation and iron status in older obese individuals. Studies described in chapters II and III address the impact of aging and obesity on iron homeostasis and immune response.

Conclusion

In this review the research conducted thus far regarding the impact of obesity on iron status, inflammation, hepcidin and immune response has been summarized. We have also described what is known about the role that iron and hepcidin play in immune response. Importantly, we have identified gaps in research regarding iron homeostasis, immune function, obesity and aging, where hepcidin may play an important role.

Taken together, the available evidence strongly suggests that chronic inflammation of obesity and aging may impair iron status through hepcidin, and that

hepcidin is not only crucial for iron homeostasis but also for immune response. Further research is needed with respect to: 1) the effect of inflammation of obesity in young and older individuals on iron homeostasis and immunity; 2) the role of hepcidin in the adaptive immune response; 3) the impact of weight loss on iron homeostasis and immune response in the obese; and 4) the effect of aging on these processes. The studies presented in chapters II-IV are designed to address the above objectives.

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Table 1. Cross-sectional or iron supplementation studies reporting iron status in relation to obesity.

Author	Yr	Population	Relevant primary measurements	Key findings
Wenzel[53]	1962	Adolescents 11-19y (N=355) (United States).	BMI, Iron status (sFe, Hb).	Obese adolescents had significantly lower sFe than lean. No difference in Hb was found.
Seltzer[101]	1963	Children and adolescents 11-21y (N=321) (United States).	Iron status (sFe, Tsat, Hb, HCT, mean corpuscular Hb concentration), obesity defined using Wetzel Grid.	sFe and Tsat were significantly lower in obese vs. non-obese subjects.
Micozzi[102]	1989	NHANES I: Adults 25-74y (N=13,834) (United States).	BMI, body composition (skinfold thickness, lean body mass, total fat mass), iron status (Hb, HCT, TIBC), serum albumin, total protein and cholesterol.	Body size was associated with lower sFe and Tsat, but higher Hb and HCT in men and women.
Pinhas-Hamiel[103]	2003	Children and adolescents (N=321) (Israel).	BMI (defined overweight using BMI percentiles), Iron deficiency (8µmol/l) and iron deficiency anemia (2 Std. Dev. below mean for age and gender).	Iron deficiency was as follows: 4.4% in normal weight children, 12.1% in overweight children, and 38.8% in obese children. Iron deficiency anemia was more prevalent with higher BMI.
Nead[104]	2004	NHANES III: children 2-16y (N=9,698) (United States).	BMI (defined overweight using BMI percentiles), iron status (Tsat, erythrocyte protoporphyrin, ferritin).	There were 14% children at risk for being overweight and 10% were overweight. The risk for iron deficiency was twice as high for children in these categories.
Moayeri[105]	2006	Overweight and obese children (N=540), and normal weight children (N=200) (Iran).	BMI, iron status (sFe, Tsat, ferritin, Hb).	The highest prevalence of iron deficiency (Tsat<16% and ferritin<12ng/ml) was in obese children (7% of total), and overweight children (5.3% of total), with 2.5% iron deficiency in the normal weight group. Iron deficiency was more prevalent in female subjects.
Lecube[4]	2006	Postmenopausal, non-diabetic, obese women (N=50), and non-obese healthy postmenopausal women (N=50) (Spain).	BMI, iron status (sFe, Tsat, sTfR, ferritin, Hb, reticulocytes, sTfR/log ferritin), insulin resistance (HOMA-IR).	Obese women had lower iron status (higher sTfR and sTfR/log-ferritin). BMI was positively correlated with sTfR and sTfR/log-ferritin. Using linear regression analysis, BMI was a positive predictor of sTfR. Insulin resistance was not correlated with sTfR
Yanoff[8]	2007	Obese (N=234) and non-obese adults (N=172) (United States).	BMI, iron status (sFe, TfR, ferritin), iron intake, CRP, body composition.	Iron status (sFe and sTfR) was lower, and CRP and ferritin were higher in the obese group. Approximately 25% of subjects in the obese group had iron deficiency.
Brotanek[106]	2007	NHANES IV: Representative sample of children 1-3y (N=1,641) (United States).	Weight-for-height percentiles, iron status (Tsat, erythrocyte protoporphyrin, ferritin).	There were 20% overweight, 8% at risk for overweight, and 7% normal weight subjects. Prevalence of iron deficiency was as follows: Hispanics (12%), whites (6%) and blacks (6%). Hispanic children were more likely to be overweight than other racial groups.

Table 1. continued

Ausk[107]	2008	NHANES III: BMI (kg/m^2) categories, BMI<25 (N=6,059), 25<BMI<30 (N=5,108), 30<BMI<35 (N=2,366), 35<BMI<40 (N=850), and BMI≥40 (N=465) (United States).	BMI, iron status (sFe, Tsat, H, ferritin), CRP, dietary iron (24hr dietary recall questionnaire).	Serum ferritin increased with BMI, while Tsat and sFe decreased. Hb did was not different across BMI categories. Anemia was not more prevalent in overweight or obese subjects.
Eckhardt[108]	2008	Lean (BMI<25) or overweight and obese (BMI≥25) women 18-49y from Mexico (N=11,965), Peru (N=5,078) and Egypt (N=6,841).	BMI, Hb, and anemia (Hb<12g/dl). They controlled for socio demographic factors.	Prevalence of women with BMI≥25 was greater than 50% in the three countries, with Egypt being the highest, at 77%. Odds ratio for anemia was greater in lean women in Egypt and Peru, and was not different across BMI categories in Mexico. Total prevalence of anemia was between 20% and 30% in all three countries.
Menzie[5]	2008	Obese (N=207) and non-obese adults (N=177) (United States).	BMI, dietary intake of iron and factors that affect iron absorption, iron status (sFe, Tsat, Hb, ferritin), body composition (DEXA).	Iron status (sFe, Tsat) was lower in obese compared to non-obese subjects. Obese group consumed more animal protein and heme iron, and less vitaminc C and calcium than non-obese. Total iron intake was not different between groups. Fat mass was a negative predictor of sFe.
Zimmermann[10]	2008	Premenopausal women in Thailand (N=92), and children in Morocco and India (N=1,688 for baseline studies and N=727 for intervention studies).	BMI and iron absorption using an isotope labeled reference meal (Thailand cohort). BMI *z*-scores, iron status (Hb, ferritin, sTfR, and body iron stores) (baseline and intervention studies in Morocco and India).	In Thai cohort, 20% of subjects were iron deficient and 22% were overweight. Inflammation and lower iron absorption were associated with higher BMI *z*-score. There was 42% iron deficiency and 6.3% overweight in Indian and Moroccan children. For these two cohorts BMI was inversely related to iron status at baseline and less improvement in iron status after an iron-fortified diet.
Tussing-Humphreys[109]	2009	NHANES III: female adolescents 12-17 y (N=210) (United States).	BMI, iron status (sFe, Tsat, sTfR, Hb, mean cell volume, erythrocyte protoporphyrin), CRP, physical activity level (metabolic equivalent score), dietary intake.	Overweight and obese subjects (BMI higher than 85th percentile) had more iron deficiency (31%) than lean subjects (14%). Groups did not differ in iron intake, age, physical activity or time of first menarche. Both high BMI and CRP were predictors of lower iron status. Iron status was significantly lower in the high-BMI group.
Cepeda-Lopez[9]	2011	Mexican Nutrition Survey (1999): Children (N=1,174) and women (N=621) (Mexico).	BMI (women) or BMI *z*-scores (children), iron status (Hb, sFe, Tsat), CRP, dietary intake of iron and factors that affect iron absorption.	Obesity was seen in 25% of women and 3.5% of children. There was higher risk for iron deficiency in obese women and children compared to lean subjects. CRP was higher in the overweight and obese groups and was a negative predictor of iron status. Iron intake did not differ between lean and obese subjects.

Abbreviations: BMI, body mass index; Tsat, transferrin saturation; sFe, serum iron; TIBC, total iron binding capacity; sTfR; soluble transferrin receptor; Hb, hemoglobin; HCT, hematocrit; CRP, C-reactive protein.

Note: Studies reporting only hemoglobin and/or ferritin as measured of iron status have been excluded.

Table 2. Cross-sectional or iron supplementation studies reporting iron status, inflammation, hepcidin in relation to obesity.

Author	Yr	Population	Relevant primary measurements	Key findings
Bekri[13]	2006	Three groups of obese participants: obese (N=8), obese with diabetes (N=7), obese with NASH (N=10). Lean controls (N=9) (France).	BMI, iron status (sFe, Tsat, Hb, ferritin), CRP and IL-6. Hepcidin expression in visceral and subcutaneous AT and liver biopsies. Hepcidin expression with stimulation in human AT cultured explants.	In the obese group, 68% had low Tsat (<25%) and 24% had anemia. Hepcidin mRNA and protein was expressed in liver and AT (no difference between visceral and subcutaneous), with significantly higher expression in AT of obese patients. There was no effect of diabetes or NASH. AT CRP and IL-6 strongly correlated with AT hepcidin levels. *In vitro* studies showed hepcidin stimulation in AT upon treatment with IL-6.
Aeberli[55]	2009	Lean (N=33) and overweight children (N=85) 6-14y (Switzerland).	Iron status (sTfR, ferritin, body iron), iron intake and bioavailability, hepcidin (N=30), CRP, IL-6 (N=68), and leptin.	Overweight group had lower iron status, seen by significantly higher sTfR. Hepcidin, CRP and IL-6 were significantly higher in the overweight group. No difference in iron intake or bioavailability was observed.
Giudice[57]	2009	Obese (N=60) and lean children (N=50) (Italy).	BMI and BMI *z*-score, iron status (sFe, Tsat, Hb, ferritin), iron absorption (iron load test), serum hepcidin, leptin and IL-6.	Iron status (sFe, Tsat) was lower in obese children. Hepcidin, IL-6 and leptin were higher in the obese group. Hepcidin was inversely correlated with iron absorption.
Tussing-Humphreys[6]	2009	Obese (N=20) and non-obese (N=20) premenopausal women. Groups were matched for Hb (United States).	BMI, waist circumference, iron status (sFe, Tsat, ferritin, sTfR), serum hepcidin, IL-6, CRP. Iron accumulation and hepcidin mRNA in AT and liver biopsies. Dietary iron and vitamin C.	Obese group had higher serum hepcidin, CRP, and sTfR. Hepcidin did not correlate with iron status in the obese group, but it did in the non-obese group. There was little iron accumulation in AT and liver of the obese subjects. Liver hepcidin mRNA strongly correlated with serum hepcidin.
Luciani[58]	2011	Two groups of morbidly obese women: group 1 (N=18), blood and AT samples; and group 2 (N=16), blood samples for serum HJV. Lean control group (N=9 women), blood and AT samples (France).	BMI, iron status (sFe, Tsat, ferritin, Hb), CRP. HJV expression in AT biopsies. Hepcidin expression after BMP stimulation in cultured adipocytes.	Serum CRP and HJV were higher in obese group compared to lean. Iron status (SFe, Tsat and Hb) was significantly lower in obese groups. HJV, Hamp and IL-6 mRNA was significantly higher in obese group compared to lean. HJV in AT correlated with BMI and AT hepcidin expression. Treatment of cultured adipocytes with BMP2 stimulated hepcidin expression.
Sanad[59]	2011	Three groups of children 7y old: obese with iron deficiency anemia (N=35), non-obese with iron deficiency anemia (N=35), and non-obese healthy (N=30). All groups were supplemented with iron for 3 months (Egypt).	BMI, iron status (sFe, Tsat, transferrin, ferritin, Hb), CRP, serum hepcidin.	Compared to the non-obese healthy children, serum hepcidin was lower in non-obese anemic children and higher in anemic obese children. CRP was highest in the obese group. Iron status was not different between the anemic groups. After 3 months of iron supplementation, serum hepcidin increased in non-obese anemic but not in the obese group. Iron status increased in both anemic groups but to a lesser extent in the obese group. In the obese group, hepcidin was inversely related to iron status.

Table 2. continued

Tussing-Humphreys[62]	2011	Obese (N=9) and lean (N=9) adults (UK).	Plasma hepcidin in arterialized vs. venous blood. Arterio-venous differences were measured across subcutaneous AT.	There was no difference between arterialized and venous blood. Authors conclude hepcidin in obesity is not secreted from AT to the plasma and does not contribute to hepcidin dysregulation.
Dao and Sen[56]	2012	Obese (N=15) and lean (N=15) pregnant women. Cord blood from obese (N=8) and lean (N=9) group (United States).	BMI, and maternal and cord blood iron status (sFe, Tsat, ferritin, Hb, HCT), CRP and IL-6.	Hepcidin, CRP and IL-6 were significantly higher in the obese group, but not in cord blood from this group. Iron status was not different between obese and lean women, but cord blood from the obese group had significantly lower iron status (sFe, Tsat) than the lean group. Serum hepcidin and BMI in women were negatively correlated with cord blood iron status.

Abbreviations: BMI, body mass index; AT, adipose tissue; NASH, non-alcoholic steatohepatitis, Tsat, transferrin saturation; sFe, serum iron; TIBC, total iron binding capacity; sTfR; soluble transferrin receptor; Hb, hemoglobin; HCT, hematocrit; CRP, C-reactive protein; IL-6, interleukin-6; HJV, hemojuvelin.

Table 3. Studies that have reported the impact of weight loss interventions on in iron status, inflammation and hepcidin.

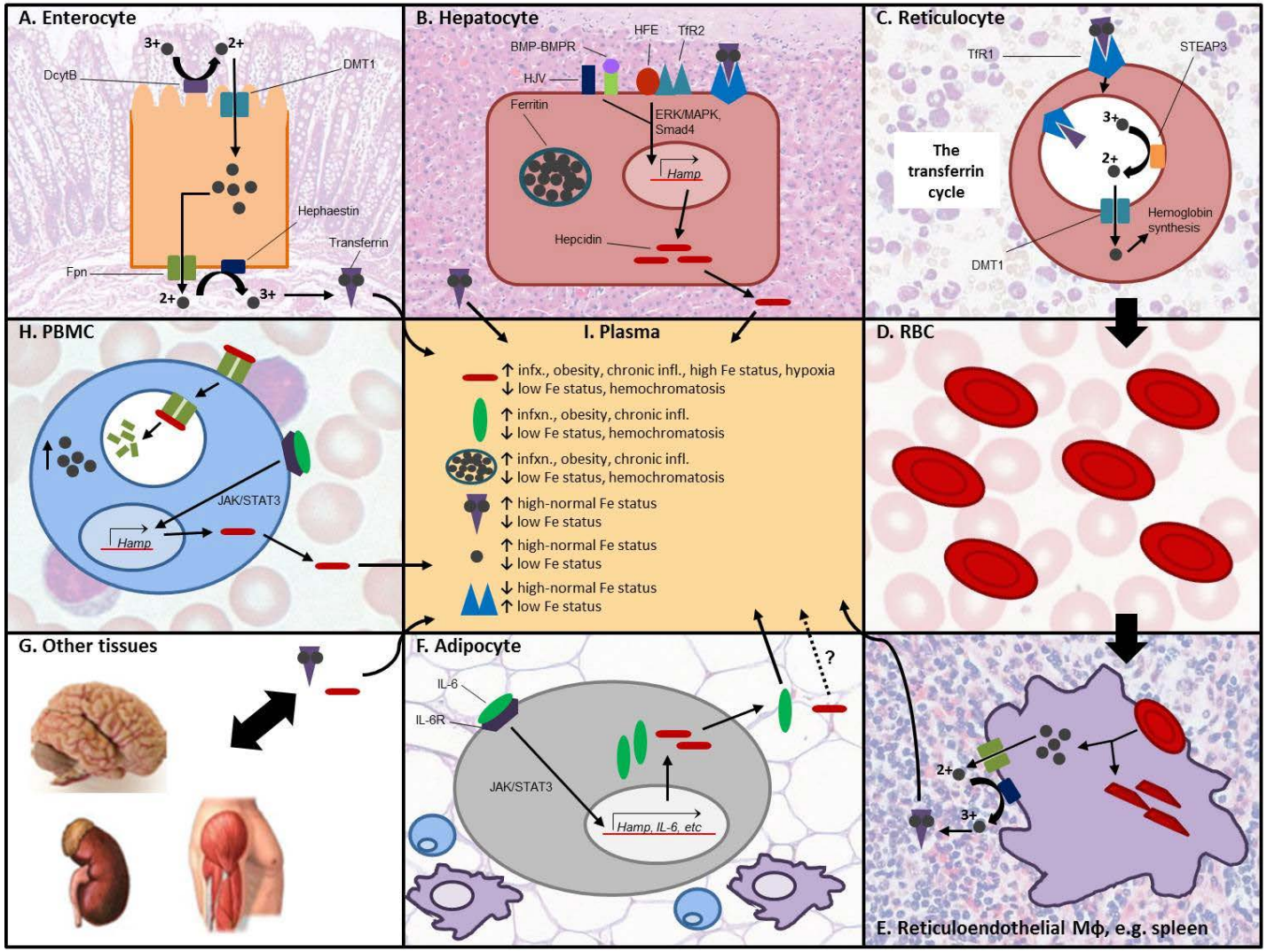
Author	Year	Population	Relevant primary measurements	Key findings
Ausk[107]	1997	Severely obese women (N=43) on three different very low energy diets (VLED): lowest calorie, middle calorie supplemented with iron (27mg/d), and highest calorie (United States).	BMI, body composition (fat mass and fat-free mass), iron status (Tsat, ferritin, Hb, HCT)	Initially Tsat decreased by 30% in all groups, but only the iron supplemented group had an increase in iron status back to normal levels.
Di Toro[110]	1997	Obese children and adolescents (N=55) on a 13-week hypocaloric diet (N=22) or a 10-week (N=33) protein sparing modified fast diet (Italy).	Ideal body weight (IBW), arm fat area, arm muscle area, iron status (sFe, Tsat, ferritin), dietary intake (24hr recall).	There was significant weight loss with both diets but no change in iron status.
Anty[111]	2008	Morbidly obese women (N=178) undergoing bariatric surgery with a 6-month follow-up (N=55) (France).	BMI, iron status (plasma iron, Tsat, ferritin, Hb), CRP.	At baseline there was a high prevalence of iron deficiency (53%, Tsat<20%), and 6% of the subjects were anemic (Hb<12g/dl). Six months after bariatric surgery there was a decrease in CRP, and an increase in Tsat, from 18% to 25%.
Amato[14]	2010	Obese children (N=15) undergoing a 6-month calorie restricted diet (Italy).	BMI, iron status (sFe, Tsat, ferritin), iron absorption (iron load test), serum hepcidin, IL-6 and leptin.	There was a decrease in BMI, serum hepcidin and leptin and a trend for decrease in IL-6. Also, iron status (sFe, Tsat) and iron absorption increased significantly after weight loss. Hepcidin was positively correlated with serum leptin.
Tussing-Humphreys[7]	2010	Premenopausal obese women undergoing bariatric surgery (N=20) with a 6-month follow-up (United States).	BMI, waist circumference, iron status (sFe, Tsat, sTfR, ferritin, Hb, HCT), serum hepcidin, CRP, IL-6, dietary iron.	At baseline, iron deficiency was seen in 45% of the subjects (sTfR>28.1nmol/L). There was a decrease in BMI, CRP, serum hepcidin, and sTfR. There were increases in Hb and HCT. Change in hepcidin was not associated with change in BMI.
Tussing-Humphreys[71]	2011	Obese (N=17) and lean (N=19) premenopausal women. Outcomes were also measured in obese group 6-months after bariatric surgery (United States).	BMI, waist circumference, iron status (sTfR, Hb), serum hepcidin, CRP, IL-6. Cytokine production (IL-6, IL-10, IL-22, IFNγ, TNFα) by WBC after stimulation with LPS or ZY.	At baseline, cytokine production (IL-6, IFNγ, and TNFα) after stimulation with LPS or ZY was significantly lower in the obese vs. lean women. After weight loss, IL-6 and TNFα production in the obese group increased and became equal to the non-obese. AT baseline, IFNγ *ex vivo* production in the obese group correlated negatively with serum hepcidin and positively with sTfR.

Abbreviations: BMI, body mass index; AT, adipose tissue; Tsat, transferrin saturation; sFe, serum iron; TIBC, total iron binding capacity; sTfR; soluble transferrin receptor; Hb, hemoglobin; HCT, hematocrit; CRP, C-reactive protein; IL-6 (-10, -22), interleukin-6 (-10, -22); IFNγ, interferon γ; TNFα, tumor necrosis factor α; WBC, white blood cells; LPS, lipopolysaccharide; ZY, zymosan.

Figure Legends

Figure 1. Post-translational regulation of iron homeostasis. (A) Iron is absorbed through the enterocyte. It is first reduced by DcytB and transported by DMT1 into the cell, and it is either stored or exported from the cell by the action of hephaestin and ferroportin. Two iron atoms bind transferrin which transports iron to target tissues. (B) Iron can be stored in hepatocytes in the storage protein ferritin. The liver is the main producer of hepcidin, and its expression can be induced by hemojuvelin (HJV)/BMP-BMP receptor, or by TfR2/HFE through the Smad4 or ERK/MAPK pathways, respectively. (C) In the bone marrow, reticulocytes take in transferrin-bound iron to make hemoglobin. The TfR/transferrin-iron complex is internalized by endocytosis. Acidification of the endosome induces the release of iron from transferrin. Iron is reduced and exported into the cytoplasm and used for hemoglobin synthesis in the mitochondria. TfR and transferrin are recycled back to the cell membrane. (D) RBCs transport the synthesized hemoglobin and are involved in oxygen and CO_2 exchange with different tissues. (E) Effete RBCs are recycled via the reticuloendothelial system. Macrophages phagocytose the RBCs and recycle the iron. (F) AT secretes adipokines, including pro-inflammatory cytokines such as IL-6, that induce hepcidin expression via the JAK/STAT3 pathway. It is not confirmed whether hepcidin made in AT goes into circulation. (G) Transferrin and hepcidin go to different tissues in the body that utilize iron. (H) PBMCs are one of the cell types that express ferroportin. Hepcidin binds ferroportin and induces its internalization and degradation, increasing the intracellular iron pool. (I) Different proteins are found in circulation, and their levels affect or are affected by iron status. Adapted from reviews by Hentze et al[16] and Andrews.[17]

Figure 1.


A. Enterocyte
3+
2+
DcytB
DMT1
Hephaestin
Transferrin
Fpn
2+
3+
B. Hepatocyte
HFE
TfR2
BMP-BMPR
HJV
Ferritin
ERK/MAPK, Smad4
Hamp
Hepcidin
C. Reticulocyte
TfR1
STEAP3
3+
2+
The transferrin cycle
Hemoglobin synthesis
DMT1
H. PBMC
JAK/STAT3
Hamp
I. Plasma
↑ infx., obesity, chronic infl., high Fe status, hypoxia
↓ low Fe status, hemochromatosis
↑ infxn., obesity, chronic infl.
↓ low Fe status, hemochromatosis
↑ infxn., obesity, chronic infl.
↓ low Fe status, hemochromatosis
↑ high-normal Fe status
↓ low Fe status
↑ high-normal Fe status
↓ low Fe status
↓ high-normal Fe status
↑ low Fe status
D. RBC
G. Other tissues
F. Adipocyte
IL-6
IL-6R
JAK/STAT3
Hamp, IL-6, etc
?
2+
3+
E. Reticuloendothelial Mϕ, e.g. spleen

Chapter II

Effect of calorie restriction and aging on iron status, hepcidin and inflammation in young and older obese women

Effect of calorie restriction and aging on iron status, hepcidin and inflammation in young and older obese women

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Key words: Obesity, inflammation, hepcidin, iron status, calorie restriction, PBMC.

Running title: Weight loss, iron status and hepcidin.

Abbreviations: WL, weight loss; CR, calorie restriction; PBMC, peripheral blood mononuclear cells; IL-1β, interleukin β; IL-6, interleukin 6; Tsat, transferrin saturation; sTfR, soluble transferrin receptor; MFI, mean fluorescence intensity; YG, young group; OG, older group.

Abstract

Background: Obesity leads to higher risk of iron deficiency suggested to be through dysregulation of hepcidin induced by chronic inflammation. Little is known about the impact of obesity on iron homeostasis of older adults. Furthermore, there is no evidence on the effect of weight loss (WL) through calorie restriction (CR) on iron homeostasis and hepcidin in young or older obese adults. Hepcidin and ferroportin are expressed in peripheral blood mononuclear cells (PBMC), and iron and hepcidin are essential for PBMC function, but no information is available on the impact of obesity and aging on PBMC iron homeostasis.

Objective: We investigated the effect of WL through CR on serum inflammatory markers, hepcidin, soluble transferrin receptor (sTfR) and transferrin saturation (Tsat) as well as PBMC iron homeostasis in young and older obese adults.

Design: This was a non-randomized, non-controlled CR intervention in 20 young (18-45 y) and 8 older (>60 y) obese women. Iron status markers (sTfR and Tsat), serum hepcidin, inflammation, PBMC hepcidin and ferroportin expression, and total PBMC iron content were measured at baseline and after 3 months of CR.

Results: At baseline, hepcidin was significantly higher in the older group (OG) than the young group (YG) ($p<0.05$). Also, sTfR was inversely related with hepcidin in the OG at baseline. After WL, there was no change in hepcidin in either group when all subjects were considered, but there was a significant enhancement of Tsat and a decrease in IL-1β in OG with >1% WL ($p<0.05$). Serum IL-1β was inversely associated with PBMC ferroportin ($p<0.05$, OG), and PBMC ferroportin and total PBMC iron were inversely

related ($p<0.05$, OG). Serum IL-1β was directly related with PBMC hepcidin ($p<0.01$, YG).

Conclusion: Moderate WL is sufficient to enhance Tsat and decrease inflammation in older obese women but not young obese women. The relationship between IL-1β and iron does not appear to be mediated through hepcidin.

Introduction

Hepcidin is a peptide hormone implicated in obesity-associated iron deficiency.[1] The function of hepcidin is to regulate circulating iron levels by inhibiting ferroportin, the only known iron exporter.[2] Hepcidin is induced through chronic or acute inflammation, specifically though interleukin (IL-) 6 and IL-1β signaling,[3-5] which may be the mechanism that leads to iron deficiency in obesity. There is evidence of IL-1β to be a stronger inducer of hepcidin than IL-6 in mice,[4] but this has not been studied in humans to a great extent. It has also been proposed that IL-6 and IL-1β have synergistic activity when inducing hepcidin, leading to a greater response than that induced by either cytokine separately.[3] The elevation of hepcidin in obesity has been observed in children[6-8] and adults,[9-11] but there is little evidence in obese elderly.

Weight loss (WL) interventions have been conducted to address the mechanism of iron deficiency in obesity. A bariatric surgery intervention showed significant decreases in hepcidin and inflammation and significant iron status enhancement in obese women.[12] A calorie restriction (CR) intervention in children showed similar results.[13] In addition, this study found iron absorption to increase after WL. No CR studies in obese young or older adults measuring the effect of WL on hepcidin, inflammation and iron status have been conducted.

Several tissues express hepcidin and ferroportin. Hepcidin expression is highest in hepatocytes,[14] but it also occurs in other cell types, such as peripheral blood mononuclear cells (PBMC),[15] and adipocytes, especially in the obese[9, 16] Ferroportin is expressed in enterocytes, monocytes, macrophages, and PBMC, among others, and has been shown to be inhibited by hepcidin in these cell types.[14, 15, 17-19] PBMC are a

convenient and interesting model to study iron homeostasis in humans due to their accessibility and the crucial role of iron in immune function. Importantly, PBMC may reflect changes in iron homeostasis that occur in other tissues. There is no information on the effect of obesity or WL on hepcidin and ferroportin expression, or iron content in PBMC.

In this study, we sought to identify the effect of WL through CR on hepcidin, inflammation, iron status and PBMC expression of hepcidin and ferroportin, as well as PBMC total iron content, in obese young and older women. We hypothesized that WL through CR in young and older obese women would lead to an enhancement in iron status, and a decrease in serum hepcidin and inflammation. This study will shed light on the underlying mechanism of iron homeostasis dysregulation in obesity, and it will serve to clarify the role of WL as a potential therapy for iron deficiency in different age groups.

Subjects and Methods

Study population

Study participants were recruited from the Weight and Wellness Center, a medical and surgical weight loss clinic at Tufts Medical Center. Subjects were excluded if they were pregnant or anticipated becoming pregnant while participating in the study, or if they had given birth or had been breastfeeding within 6 months prior to screening. They were also excluded if they had lost more than 3% body weight within three months before recruitment, had a history of eating disorders, renal or hepatic disease, gastrointestinal disorders, prior gastric restrictive surgery, hematological malignancies, iron overload disorders, cancer, chronic infections, autoimmune disease, or intake of iron

supplements. All subjects signed an informed consent form. This study was approved by the Tufts Medical Center/Tufts University Institutional Review Board.

The purpose of this study was to determine how collected measurements changed over a period of calorie restriction. All anthropometric and blood measurements were assessed at baseline and immediately after a calorie restriction period of approximately 13 weeks, which is roughly the average duration of the weight loss programs at the Weight and Wellness Center. Details of these measurements are delineated below.

Anthropometric measures

Height, weight and waist circumference (WC) were measured before and after CR. All measurements were taken to the nearest 0.1cm or 0.1kg. For each time point, WC was measured 3 times around the umbilical area and the average was taken.

Serum measurements

Full chemistry profile and CBC differentials were assessed by the Nutrition Evaluation Laboratory, a core unit at the Human Nutrition Research Center on Aging. Competitive ELISA was used to measure serum hepcidin (Peninsula Laboratories, Bachem, San Carlos, CA). CRP was measured with the high sensitivity IMMULITE-100 immunoassay (Siemens Healthcare Diagnostics, Los Angeles, CA), ferritin was also measured with the IMMULITE-100 immunoassay (Siemens Healthcare Diagnostics, Los Angeles, CA), and soluble transferrin receptor (sTfR) with ELISA (Ramco Laboratories, Stafford, TX). Leptin, adiponectin, IL-6 and IL-1β were measured using multi-spot electrochemiluminescence assays (Meso Scale Discovery, Gaithersbug, MD). Colorimetric endpoint assays were used to measure serum iron and total iron binding capacity (TIBC) (Diagnostic Chemicals).[20] Transferrin saturation (Tsat) was calculated

by the formula: Tsat = (serum iron/TIBC) x 100%. We used the definition of iron deficiency for obese populations described in Muñoz et al., which is similar to the definition of anemia of chronic disease with real iron deficiency (ACD+ID),[26] to define iron deficiency in this population. Subjects were iron deficient if they had a CRP>1mg/L, sTfR in the normal or above normal rage (normal range according to manufacturer: 2.9-8.3 μg/ml), normal or low hemoglobin (low is <12g/dl for women), Tsat<20%, and normal or high ferritin (normal range is 30-100 ng/ml).

Dietary intake assessment

Habitual dietary intake was estimated using 3-day food records collected for 3 days prior to each blood draw at baseline and at the end of study (before and after CR) as well as once per month between blood draws. Dietary intake data, including dietary supplement data, were collected and analyzed using Nutrition Data System for Research software version 2010 developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. Dietary components used in our analysis include mean calories per day, macronutrients, iron, inhibitors of iron absorption (fiber, caffeine, calcium and phytates), and vitamin C, an enhancer of iron absorption. Heme iron was calculated as in Monsen et al.,[21] where it was estimated that 40% of iron from meat, poultry and fish products is heme iron. Non-heme iron was calculated by subtracting estimated heme iron from total daily iron.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood was collected in EDTA tubes, and PBMC were isolated with gradient centrifugation using a Histopaque matrix (Sigma-Aldrich, St. Louis, MO). Cell culture media supplemented with 5% FBS was used for the separation step, but the rest of the

PBMC isolation was done under serum-free conditions. PBS (1x) was used for washing the isolated PBMC. After the last wash, PBMC were suspended in serum-free media at a concentration of 20 million/ml. Cells from this suspension were used for hepcidin and ferroportin expression measurement with flow cytometry and total PBMC iron content measurement with inductively coupled plasma mass spectrometry (ICP-MS).

Hepcidin and ferroportin in PBMC

Flow cytometry was used for determination of hepcidin and ferroportin expression in total PBMC or in PBMC subpopulations. PBMC surface staining of different white blood cell markers was done using BD Biosciences reagents and protocols (BD Biosciences, San Jose, CA). The antibodies used were specific to CD3, CD4, CD8, CD19, NK, NKT (CD3+NK+) and CD14. After cells were fixed, intracellular staining was done to determine hepcidin and ferroportin protein expression. Primary and FITC-labeled secondary antibodies were used for hepcidin (Abcam, Cambridge, MA), ferroportin (Santa Cruz Biotechnology, Inc., Dallas, TX), as well as their respective isotype controls. An Acuri C6 flow cytometer was used for data collection. The FACS analysis software FloJo version 10.0.6 was used to determine mean fluorescence intensity (MFI) as a measure of protein expression. For normalization, hepcidin and ferroportin MFI were divided by isotype control (background) MFI and the ratio was used for statistical analysis.

Total PBMC iron content

Total intracellular PBMC iron levels were measured using ICP-MS.[22] Upon isolation, 100k or 500k PBMC aliquots (depending on availability) were immediately frozen at -80^{o}C. Frozen samples were shipped to Dr. Abrams' laboratory at Baylor

College of Medicine for analysis. All samples were collected in duplicate. Prior to data collection, it was determined that the media in which these cells were suspended had no traceable iron content.

Statistical analysis

Student's t test and paired t test were used for comparisons between the young and older group or within groups, respectively. These comparisons included anthropometric measurements (BMI, WC), blood chemistry profile values (glucose, blood lipids, liver enzymes), iron status markers (hemoglobin, serum iron, Tsat, sTfR), hepcidin, markers of inflammation (CRP, IL-1β, IL-6), and dietary intake of iron or nutrients that may enhance (vitamin C) or inhibit (calcium, fiber, phytates) dietary iron absorption. Paired t test was used to determine significance of change in Tsat, IL-1β, serum hepcidin, or changes in PBMC hepcidin and ferroportin. Correlation analysis between markers of iron status or hepcidin and BMI, WC, CRP, IL-1β, and IL-6 were corrected for race and total dietary iron. Pearson correlation coefficient was used for correlations between inflammation and BMI or WC. Pearson correlation coefficienct was also used for correlation analysis between total PBMC iron or IL-1β and PBMC hepcidin and ferroportin. Correlations between %WL or change in WC and change in IL-1β were determined using Pearson correlation coefficient analysis. Partial correlation coefficient adjusting for race and change in iron intake was used for correlations between %WL or change in WC and change in Tsat and hepcidin. Mean ± standard deviation (SD) is shown for normally distributed variables, and median (Q1, Q3) for variables with a skewed distribution. Statistical significance was set at alpha = 0.05. SAS 9.2 for Windows (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Population characteristics and baseline comparisons

This was a non-randomized, non-controlled CR intervention in obese young (18-45y) and older (>60y) women. The CR period was on average 13±4 weeks and it did not differ between groups. Initially, 141 obese women pre-screened and determined eligible based on age (18-45y or >60y) and BMI (30-55 kg/m^2) were offered to participate in the study (**Figure 1**). Of these, 89 women were screened for eligibility based on exclusion criteria (see below), 66 were eligible to participate in the study, and 60 enrolled. The total number of dropouts was 29 and the main reason for study dropout was voluntary withdrawal. At baseline, there were 23 young and 15 older participants. The study was completed by 20 young and 8 older participants.

All baseline comparisons included all subjects, even those that did not finish the study. The young group (YG) was on average 36 ± 6 years old and the older group (OG) was 66 ± 7 years ($p<0.0001$) (**Table 1**). At baseline, BMI in the YG was significantly higher than the OG ($p<0.05$), but WC did not differ between groups. There were 23 young and 15 older obese women at baseline, but 20 young and 8 older participants at follow-up (**Figure 1**). There was no significant difference in glucose, cholesterol, triglycerides, liver enzymes, leptin, inflammatory markers, or iron status between groups. Adiponectin was significantly higher in the OG ($p<0.01$). Dietary intake of iron, or of enhancers and inhibitors of iron absorption did not differ between groups, except for heme iron intake which was higher in the YG ($p=0.05$). Finally, the OG had significantly higher serum hepcidin ($p<0.05$) and ferritin ($p<0.05$) than the YG ($p=0.02$).

Adjusting for baseline BMI did not change any of these comparisons between YG and OG (data not shown).

Baseline correlations between iron status, inflammation and hepcidin

All baseline correlation analysis included all subjects (YG N=23 and OG N=15). Partial correlation coefficient analysis adjusting for total iron intake and race was used for all comparisons involving markers of iron status or hepcidin. For the other comparisons, Pearson correlation coefficient analysis was used. In the YG, there was a significant inverse correlation between BMI and serum iron ($r=-0.48$, $p<0.05$), and Tsat ($r=-0.47$, $p<0.05$) (**Table 2**). Ferritin is upregulated by inflammation, and consequently it has not been used as a marker of iron status in this population, but rather as a quality control for hepcidin, as they are usually found to be strongly correlated.[12, 23, 24] Contrary to our expectations, serum hepcidin was positively associated with sTfR ($r=-0.51$, $p<0.05$) and Tsat ($r=0.52$, $p<0.05$) in the YG. BMI, inflammation and hepcidin were not correlated in the YG.

In contrast, baseline correlations in the OG showed a positive association between BMI and inflammation, as indicated by IL-6 ($r=0.52$, $p<0.05$), and between IL-1β and WC ($r=0.54$, $p<0.05$) (**Table 2**). There was a negative association between hepcidin and a marker of iron status, as indicated by a positive association with sTfR ($r=0.67$, $p<0.05$). Neither the inflammatory cytokines nor BMI correlated with hepcidin.

Changes in markers of iron status, hepcidin, and inflammation with weight loss in young and older groups

This analysis included only subjects that completed the study (YG N=20 and OG N=8). For both groups there was a significant decrease in BMI, but not WC (**Table 3**). YG had a significant decrease in total calories and intake of carbohydrate, fat, heme iron, fiber and caffeine, but a significant increase in total iron, vitamin C, and calcium intake. There was, however, no change in markers of iron status in the YG. After WL, fat intake in the YG was higher than in the OG ($p<0.05$). The lack of change in total calorie intake in the OG was perhaps due to underreporting in this group.

There was a trend towards a decrease in IL-1β ($p=0.06$) and adiponectin ($p=0.06$) after WL in the OG. There was no change in iron status or dietary intake of iron or enhancers/inhibitors of iron absorption in the OG (**Table 3**). After WL hepcidin ($p<0.01$) and adiponectin ($p<0.05$) were still significantly higher in the OG vs. the YG. Even though there was no significant increase in markers of iron status in the OG, serum iron was higher in the OG vs. YG after WL ($p<0.05$).

It has been shown that at least 1% WL is associated with positive health outcomes.[25] Due to the moderate WL in this cohort, we categorized subjects into those that lost less than 1% body weight or gained weight (no WL) and those that lost at least 1% body weight (>1% WL) (**Figure 2**). After this categorization, there was a significant increase in Tsat, from 23.2 ± 4.2 % to 28.4 ± 5.1 % ($p<0.05$) (Figure 2a), and a decrease in serum IL-1β from 0.96 ± 0.27 pg/ml to 0.66 ± 0.27 pg/ml ($p<0.01$) (Figure 2b) in the OG with >1% WL. Serum hepcidin did not change significantly in any of the

subcategories (Figure 2c). All other comparisons were the same between categorized and pooled data (data not shown).

When using the criteria for iron deficiency described by Muñoz et al.,[26] the prevalence of iron deficiency before WL was 18% in the total study population (7 out of 38). At baseline, the prevalence of ACD+ID was 22% in young subjects (5 out of 23) and 13% in the OG (2 out of 15). After WL, the prevalence decreased to 11% in the total study population (3 out of 28), with 15% in the YG (3 out of 20) and no subjects with iron deficiency in the OG (0 out of 8). From the 10 subjects lost to follow-up, one young and one older subject had ACD+ID at baseline.

Correlation of change between iron status, inflammation, hepcidin and weight loss

Correlation of change involving hepcidin and markers of iron status was adjusted for change in total iron intake and race. In the YG (N=20, trend, r=0.5, p=0.06) and OG (N=8, r=0.9, p=0.02), %WL was associated with an increase in Tsat (**Figure 3**). There was no association between the change in hepcidin and anthropometric changes. Unexpectedly, unadjusted correlation coefficient analysis showed a positive correlation between %WL and hepcidin increase (data not shown), perhaps due to the significant increase in iron consumption in the YG. Lower IL-1β was associated with a decrease in WC in the YG ($r=0.5$, $p<0.05$), and with %WL in the OG ($r=-0.8$, $p<0.05$). No associations were seen between other markers of iron status or inflammation and WL.

Hepcidin, ferroportin and total iron content in PBMC

Only subjects that finished the study for whom data was available both before and after CR were included in this analysis. Monocytes (CD14+) had the highest expression of both hepcidin and ferroportin in both groups, as expected.[2] All other cell types had comparable levels of these proteins. At baseline, there was no difference between hepcidin or ferroportin expression in PBMC between the YG and OG (data not shown). In the YG, there was a significant decrease after WL in total PBMC ferroportin ($p<0.05$) (**Figure 4C)**, as well as ferroportin expression in CD3+ lymphocytes ($p\leq0.01$), CD4+ and CD8+ T lymphocytes ($p<0.05$ for both), B cells (CD19+ cells; $p<0.001$), natural killer T cells ($p\leq0.01$), and monocytes (CD14+ cells; $p<0.05$). This was seen both in the total YG population (data not shown), and in those that lost >1% WL (Figure 4).

Hepcidin expression in total PBMC or PBMC subpopulations did not change in the pooled YG (data not shown). In YG subjects with >1% WL hepcidin expression decreased significantly in total PBMC ($p<0.05$), but not in PBMC subpopulations (**Figure 4A**). However, there was a significant increase in hepcidin expression in YG subjects with no WL, specifically in CD3+ lymphocytes ($p\leq0.01$), CD4+ ($p\leq0.01$) and CD8+ ($p<0.05$) T cells and B cells ($p<0.05$). Neither hepcidin nor ferroportin expression changed in the pooled OG population, or when divided into no WL and >1% WL (**Figure 4**).

There was no change in total PBMC iron after WL in either group. For the OG, but not for the YG, baseline total PBMC iron was negatively correlated with PBMC ferroportin before ($r=-0.57$, $p<0.05$) and after ($r=-0.74$, $p<0.05$) WL, suggesting that there is greater PBMC iron retention when there is less ferroportin in PBMC, as expected

(**Table 4**). Additionally, PBMC ferroportin was inversely correlated with serum IL-1β in the OG at baseline ($r=-0.59$, $p<0.05$). In the YG, serum IL-1β was positively correlated with PBMC hepcidin before WL (0.59, $p<0.01$), and there also was a significant correlation of change between IL-1β and PBMC hepcidin in this group ($r=0.62$, $p<0.01$). Markers of iron status were not correlated with PBMC hepcidin, ferroportin or total iron content (data not shown).

Discussion

In this study we have identified age differences with respect to iron homeostasis, inflammation and WL. The OG had the expected inverse association between iron status and hepcidin, while the opposite was true for the YG. In addition, the OG had significantly higher hepcidin than the YG before and after WL. Yet, the OG experienced an increase in Tsat and lower inflammation than the YG, which despite having higher iron intake upon completing the study had no improvement in iron status. Differences were also present in PBMC expression of hepcidin and ferroportin and total iron content, suggesting that PBMC iron homeostasis is impacted by obesity, aging and WL.

At baseline, even though BMI was significantly higher in the OG, WC was not different between groups, suggesting that the OG had higher or equivalent central adiposity and possibly higher or equivalent fat mass. We propose that these two populations may be considered equivalent with respect to anthropometric measurements. Older subjects had significantly higher hepcidin than the YG before and after WL, and yet the older subjects experienced an improvement in Tsat and their serum iron was greater than the young subjects' after WL. Furthermore, markers of iron status in the YG

did not increase even though they had increased iron intake, decreased intake of iron absorption inhibitors (fiber and caffeine), and increased vitamin C intake which is an iron absorption enhancer. The observed increase of change in hepcidin with %WL (Figure 3) in this group was perhaps associated with this increase in iron intake, given that higher iron consumption leads to higher hepcidin.[14] In addition, contrary to our expectations, hepcidin was positively correlated with iron status (measured as Tsat and sTfR) in the YG at baseline, and there was no association between inflammation and BMI or hepcidin. This perhaps is due to the uniformly high BMI in this group and a wider BMI range may be needed to detect significant correlations.

Conversely, BMI was positively correlated with inflammation, and hepcidin was inversely related with iron status in the OG. The observed association between IL-1β and sTfR was unexpected and warrants further investigation. Even though hepcidin was higher in the OG at baseline, it did not change significantly with WL in either group. There was a non-significant trend for it to be lower after WL in the OG, but the sample size is small and perhaps a larger sample size and greater WL may be needed to induce a significant change in hepcidin in young and older obese individuals. Alternatively, hepcidin upregulation in obesity may not be the only mechanism for low iron status. For example, a recent study in mice found that high fat diets led to low iron absorption independent of hepcidin.[27] The YG in this cohort had higher fat intake after WL than the OG, which may partly account for the lack of improvement in iron status. This should be explored further in future studies. The novelty of our findings partially stems from the suggestion that even though the elderly are known to have lower iron status than young adults,[28, 29] obese elderly subjects in this cohort experienced an improvement in iron

status after mild WL while young adults did not. They may have required greater WL to experience the same improvement as OG.

We have uncovered a potential role for IL-1β in obesity and iron homeostasis. There was not only a significant correlation between WL and decrease in IL-1β in both groups, but also IL-1β was positively correlated with PBMC hepcidin, and negatively correlated with PBMC ferroportin suggesting a direct impact of obesity-induced inflammatory signals on PBMC iron homeostasis. Hepcidin has been shown to be induced by IL-1β in murine hepatocytes and hepatoma cells lines.[4] Another study found that IL-1α does not induce hepcidin in human hepatocytes,[30] but they did not study other cell types or the effect of IL-1β on hepcidin expression. In their review, Bode et al. discuss how both IL-6 and IL-1β have the potential to induce hepcidin expression either separately, or synergistically.[3] IL-6 signaling is considered the most direct inflammatory pathway to induce hepcidin expression, but perhaps IL-1β has a more significant role than previously thought. This is the first implication of IL-1β in obesity-associated hepcidin dysregulation and iron deficiency.

Our results from PBMC analysis confirm that ferroportin and total intracellular iron are inversely related. This was seen in older but not young subjects. There was a significant decrease in PBMC ferroportin in the YG, but an increase in PBMC hepcidin in YG subjects that did not lose weight, while no changes were detected in the OG. The reason for these age differences needs to be investigated further. Aging is accompanied by immune impairment,[31, 32] and this may be related to the lack of changes in PBMC hepcidin or ferroportin in the OG. The decrease in PBMC ferroportin in the YG may be associated with their increase in iron intake and the trend towards hepcidin increase with

%WL. Others have found that hepcidin is essential in PBMC function,[15] and both iron and ferroportin are important for immune response,[17] but this is the first evidence of PBMC iron homeostasis in the context of obesity. More research is needed to determine whether PBMC are significant contributors of iron status dysregulation in obesity.

Several studies have investigated the effect of obesity on iron homeostasis in obese adults,[9-11] but this is the first study establishing comparisons between young and older obese adults. Furthermore, we have determined for the first time the effect of CR and WL on iron status, hepcidin and inflammation in adults. Finally, this study provides the first evidence of iron homeostasis in PBMC in the context of obesity and aging.

Limitations of this study include the high attrition rate and limited sample size, especially for the OG. In this study subjects were recruited from a weight loss clinic and there was no control over dietary intake, leading to dietary variations within and between groups. However, this has been remedied in part by assessing dietary intake once per month and correcting for iron intake in our analysis. Also, some of these subjects were weight-unstable at the time of the second sample collection, making the intervention uneven. There was also a lack of data on body composition or iron absorption, which should be included in further studies.

In conclusion, there are marked differences in iron homeostasis between young and older obese women. The moderate WL seen in this study is associated with an enhanced Tsat in the older and, to a lesser extent, young groups, but was not sufficient to induce a significant decrease in serum hepcidin. In addition, this study shows a potential new role for IL-1β in obesity-associated hepcidin dysregulation. Finally, PBMC iron

homeostasis seems to be impacted by obesity, aging and WL. The role of PBMC in iron deficiency remains to be elucidated.

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Table1. Age comparisons at baseline.

	Young (N=23)	**Older (N=15)**	**P**
Age (y)	36 ± 6	66 ± 7	<0.0001
Race (N)			
White	16	13	
Black	5	2	0.09
Hispanic	2	0	
BMI (kg/m^2)	43.6 ± 4.2	39.8 ± 5.3	0.03
Weight (kg)	118.6 ± 12.0	104.8 ± 13.9	0.003
WC (cm)	121.2 ± 10.5	115.0 ± 11.6	-
Glucose (mg/dl)	103.6 ± 37.6	94.9 ± 9.2	-
Cholesterol (mg/dl)	188.0 ± 33.7	206.9 ± 30.5	0.09
Triglycerides (mg/dl)	96 (71, 170)	100 (73, 134)	-
Albumin (g/dl)	4.3 ± 0.3	4.3 ± 0.2	-
SGPT (mu/ml)	18 (13, 25)	15 (13, 22)	-
SGOT (mu/ml)	17 (14, 22)	17 (16, 21)	-
Red blood cell (mil/µl)	4.7 ± 0.3	4.6 ± 0.4	-
Hemoglobin (g/dl)	13.2 ± 1.2	13.7 ± 1.1	-
Hematocrit (%)	39.9 ± 3.1	41.0 ± 3.1	-
Serum iron (µg/dl)	75.4 ± 34.0	82.9 ± 27.4	-
Tsat (%)	24.0 ± 11.7	26.5 ± 9.5	-
Ferritin (ng/ml)	72.2 ± 50.1	159 ± 105	0.02
sTfR (µg/ml)	4841 ± 2108	4730 ± 1622	-
Hepcidin (ng/ml)	22.7 ± 16.4	48.9 ± 35.9	0.02
CRP (mg/L)	11.2 ± 9.8	7.3 ± 8.5	-
IL-6 (pg/ml)	7.7 ± 3.8	8.4 ± 3.7	-
IL-1β (pg/ml)	0.66 ± 0.40	0.85 ± 0.40	-
Leptin (ng/ml)	417.9 ± 169.6	397.5 ± 178.8	-
Adiponectin (µg/ml)	9.4 ± 3.2	18.0 ± 6.3	0.002
Dietary iron (mg/d)	16.3 ± 7.1	13.1 ± 6.4	-
Heme (mg/d)	1.2 ± 0.6	1.0 ± 0.5	-
Non-heme (mg/d)	13.2 ± 5.4	9.9 ± 3.8	0.05
Dietary vitamin C (mg/d)	103.7 ± 120.7	131.8 ± 105.4	-
Dietary calcium (mg/d)	990.1 ± 521.8	1044.3 ± 556.0	-
Dietary fiber (g/d)	17.3 ± 6.3	20.3 ± 6.8	-
Dietary phytate (mg/d)	625 ± 397	476 ± 180	-
Dietary oxalic acid (mg/d)	216 ± 168	170 ± 133	-
Caffeine intake (mg/d)	143 ± 151	114 ± 79	-

Fisher's exact test was used for race comparisons and Student's t test was used for all other comparisons. Mean ± SD, median (Q1, Q3), and p values less than 0.1 are shown.

Table 2. Baseline correlations between iron status, hepcidin and inflammation.

Correlation coefficients - Young (18-45 y) (N=23)								
	Hepcidin	Serum iron	Tsat	Ferritin	sTfR	CRP	IL-6	IL-1β
BMI	-0.26	**-0.48***	**-0.47***	**-0.48***	0.10	0.09	0.21	0.11
WC	-0.41	-0.36	-0.38	0.14	0.07	0.12	-0.01	0.19
IL-1β	-0.11	-0.05	0.06	0.09	0.03	0.14	**0.60****	
IL-6	0.01	0.12	0.13	0.05	-0.04	0.34		
CRP	0.06	0.09	0.09	0.20	-0.03			
sTfR	**-0.51***	-0.29	-0.37	-0.36				
Ferritin	**0.46***	**0.58****	**0.69*****					
Tsat	**0.52***	**0.95******						
Serum iron	0.36							
Correlation coefficients - Older (>60 y) (N=15)								
	Hepcidin	Serum Iron	Tsat	Ferritin	sTfR	CRP	IL-6	IL-1β
BMI	0.10	-0.47	-0.33	0.43	-0.09	0.37	**0.52***	0.47
WC	0.01	-0.31	-0.23	0.29	-0.01	0.02	0.39	**0.54***
IL-1β	-0.54	0.39	0.48	-0.12	**-0.74***	-0.06	**0.54***	
IL-6	0.05	-0.43	-0.22	0.32	-0.36	**0.76****		
CRP	0.40	-0.50	-0.36	0.45	-0.10			
sTfR	**0.67***	-0.58	-0.63	0.36				
Ferritin	**0.86****	-0.50	-0.27					
Tsat	-0.50	**0.89****						
Serum iron	-0.58							

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Partial correlation coefficient analysis adjusting for race and iron intake was used for correlations involving hepcidin and iron status. Pearson correlation coefficients were used for the rest.

Table 3. Anthropometric, systemic and dietary intake changes with calorie restriction.

	(a) Young (18-45y) (N=20)			(c) Older (>60y) (N=8)			Young vs. Older		
	Before	**After**	**P (Young)**	**Before**	**After**	**P (Older)**	**P (Before)**	**P(After)**	**P(Change)**
BMI (kg/m2)	42.8 ± 4.2	42.0 ± 4.3	0.01	41.0 ± 4.9	40.1 ± 4.8	0.04	-	-	-
Weight (kg)	117.3 ± 12.1	115.0 ± 11.2	0.01	110.2 ± 12.1	107.8 ± 12.6	0.04	-	-	-
%WL		1.9 ± 3.1			2.3 ± 2.7		-		-
WC (cm)	121.3 ± 10.8	120.9 ± 10.2	-	118.1 ± 7.5	117.1 ± 8.7	-	-	-	-
Glucose (mg/dl)	103.8 ± 40.4	106.8 ± 53.5	-	94.1 ± 8.9	96.3 ± 6.4	-	-	-	-
Cholesterol (mg/dl)	191 ± 32	197.1 ± 37.7	-	217 ± 31	213 ± 21	-	0.06	-	-
Triglycerides (mg/dl)	103 (80, 172)	107 (82, 153)	-	107 (90, 143)	137 (81, 154)	-	-	-	-
Albumin (g/dl)	4.3 ± 0.3	4.4 ± 0.3	-	4.3 ± 0.3	4.3 ± 0.2	-	-	-	-
SGPT (mu/ml)	18 (13, 23)	18 (16, 24)	-	17 (14, 23)	18 (14, 21)	-	-	-	-
SGOT (mu/ml)	17 (14, 22)	18 (15, 21)	-	18 (15, 21)	19 (16, 20)	-	-	-	-
Red Blood Cell (mil/µl)	4.7 ± 0.3	4.7 ± 0.4	-	4.6 ± 0.4	4.5 ± 0.4	-	-	-	-
Hemoglobin (g/dl)	13.2 ± 1.3	13.2 ± 1.2	-	13.9 ± 1.3	13.7 ± 1.1	-	-	-	-
Hematocrit (%)	39.8 ± 3.2	39.8 ± 2.7	-	41.3 ± 3.8	40.7 ± 3.1	-	-	-	-
Serum iron (µg/dl)	75.2 ± 36.1	69.3 ± 28.2	-	82.5 ± 22.3	95.6 ± 24.9	-	-	0.03	-
Tsat (%)	23.6 ± 12.2	21.6 ± 10.5	-	25.5 ± 6.0	27.1 ± 5.0	-	-	0.07	-
Ferritin (ng/ml)	73.9 ± 52.4	73.3 ± 63.8	-	133.3 ± 91.2	120.1 ± 81.2	-	0.04	-	-
sTfR (µg/ml)	4879 ± 2171	5019 ± 2640	-	4122 ± 938	4056 ± 1141	-	-	-	-
Hepcidin (ng/ml)	22.4 ± 17.2	19.4 ± 10.9	-	46.8 ± 23.5	39.8 ± 20.1	-	0.005	0.02	-
CRP (mg/L)	10.5 ± 10.1	9.5 ± 7.0	-	7.5 ± 9.9	6.1 ± 6.3	-	-	-	-
IL-6 (pg/ml)	7.6 ± 3.9	7.0 ± 3.3	-	9.0 ± 4.3	7.9 ± 3.3	-	-	-	-
IL-1β (pg/ml)	0.65 ± 0.37	0.64 ± 0.33	-	0.86 ± 0.29	0.69 ± 0.24	0.06	-	-	0.08
Leptin (ng/ml)	411 ± 175	359 ± 133	-	446 ± 180	413 ± 186	-	-	-	-
Adiponectin (µg/ml)	8871 ± 3057	8749 ± 3018	-	15171 ± 5708	13761 ± 5490	0.06	0.03	0.04	0.04
Total energy intake (kcal/d)	1862 ± 486	1391 ± 434	0.002	1507 ± 590	1536 ± 351	-	-	-	-
Carbohydrate (g/d)	225 ± 80	166 ± 65	0.005	183 ± 62	153 ± 37	-	-	-	0.06
Fat (g/d)	70 ± 20	48 ± 24	0.01	58 ± 39	32 ± 9	-	-	0.05	-
Animal protein (g/d)	39 ± 19	38 ± 19	-	34 ± 7	32 ± 9	-	-	-	-
Vegetal protein (g/d)	1.8 ± 1.9	0.5 ± 1.2	0.03	0.8 ± 1.2	1.7 ± 2.3	-	-	-	0.07
Dietary iron (mg/d)	16.1 ± 7.5	26.7 ± 14.3	0.005	10.0 ± 4.4	19.1 ± 9.7	0.09	0.04	-	-

Table 3. continued

Heme (mg/d)	1.1 ± 0.6	0.9 ± 0.3	0.04	1.0 ± 0.4	0.9 ± 0.5	-	-	-	-
Non-heme (mg/d)	12.9 ± 5.5	12.1 ± 5.0	-	8.7 ± 4.4	9.7 ± 3.4	-	0.06	-	-
Dietary vitamin C (mg/d)	109 ± 125	179 ± 153	0.009	105 ± 94	131 ± 108	-	-	-	-
Dietary calcium (mg/d)	991 ± 549	1260 ± 568	0.007	1185 ± 654	1105 ± 430	-	-		0.06
Dietary fiber (g/d)	17.7 ± 6.3	13.8 ± 4.2	0.008	17.9 ± 6.6	17.3 ± 3.6	-	-	0.07	-
Dietary phytate (mg/d)	641 ± 412	429 ± 226	-	407 ± 174	454 ± 202	-	0.04	-	-
Dietary oxalic acid (mg/d)	225 ± 174	171 ± 128	-	130 ± 128	148 ± 106	-	-	-	-
Caffeine Intake (mg/d)	126 ± 137	81 ± 96	0.03	128 ± 89	61 ± 74	-	-	-	-

Student's t test was used for young vs. older comparisons. Paired t test was used for within group changes. P values less than 0.1, Mean ± SD and median (Q1, Q3) are shown.[12]

Table 4. Correlations between PBMC hepcidin and ferroportin expression, PBMC iron content, serum hepcidin, and inflammation.

	PBMC Hepcidin			PBMC Ferroportin		
Young (18-45 y)	**Before**	**After**	**Change**	**Before**	**After**	**Change**
PBMC Iron	0.17	0.08	0.02	0.09	-0.24	-0.17
IL-1β	**0.59****	0.36	**0.62****	-0.17	-0.07	0.38
Older (>60 y)						
PBMC Iron	0.35	0.20	-0.48	**-0.57***	**-0.74***	-0.31
IL-1β	0.40	0.32	-0.42	**-0.59***	-0.32	-0.59

No correlations with iron status were found. *$p \leq 0.05$; **$p < 0.01$.

Figure Legends.

Figure 1. Flow chart for subject recruitment and enrollment.

Figure 2. Changes in iron status, hepcidin and inflammation with and without WL. Changes in iron status and serum IL-1β in young and older obese women that lost no weight or at least 1% body weight. Sample sizes are as follows: YG no WL (N=8), YG >1% WL (N=12), OG no WL (N=3), OG >1% WL (N=5). Mean and standard error bars are shown; *$p<0.05$.

Figure 3. Correlations between WL and change in iron status, hepcidin and inflammation. For analysis involving iron status or hepcidin, partial correlation coefficient adjusting for race and change in iron intake was used. Pearson correlation analysis was used for all other comparisons. Change = after – before.

Figure 4. Changes in hepcidin and ferroportin expression in PBMC subpopulations with calorie restriction. The MFI of hepcidin and protein obtained through flow cytometry has been normalized to the MFI of their corresponding isotype control. The ratio of protein MFI to background MFI is shown. Groups have been categorized into no WL and >1% WL, with YG in panels A) and C) and OG in panels B) and D). Sample sizes are: Young no WL (N=7), young >1% WL (N=12), older no WL (N=3), and older >1% WL (N=5). Mean and standard error bars are shown; *$p<0.05$; **$p\leq0.01$; ***$p<0.001$.

Figure 1.

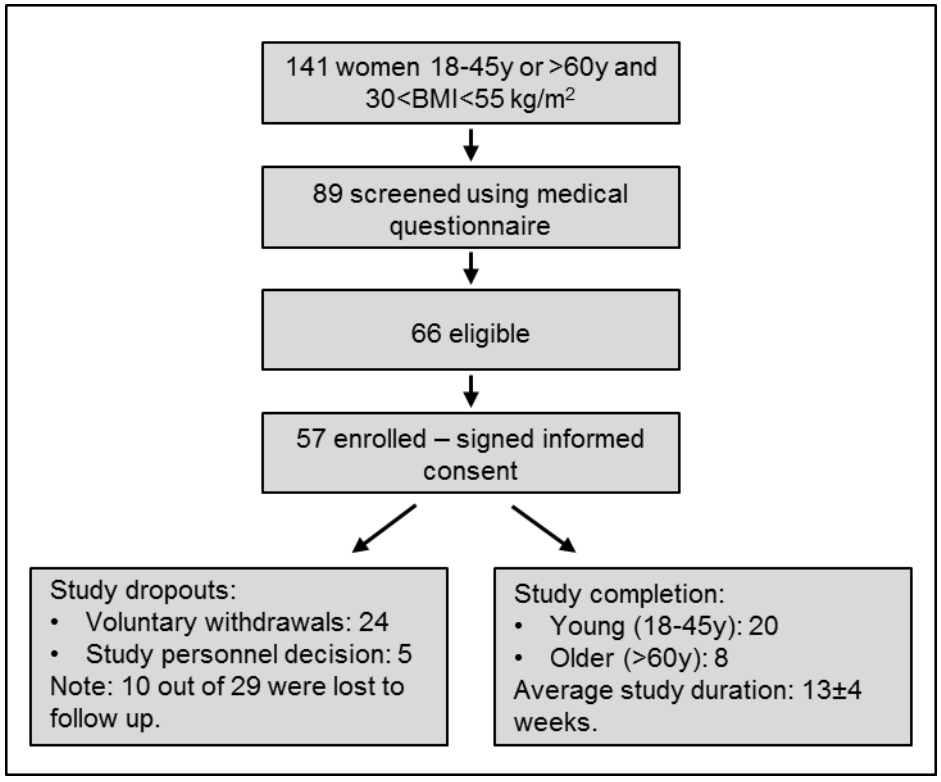

141 women 18-45y or >60y and 30<BMI<55 kg/m2
89 screened using medical questionnaire
66 eligible
57 enrolled – signed informed consent
Study dropouts:
• Voluntary withdrawals: 24
• Study personnel decision: 5
Note: 10 out of 29 were lost to follow up.
Study completion:
• Young (18-45y): 20
• Older (>60y): 8
Average study duration: 13±4 weeks.

Figure 2.

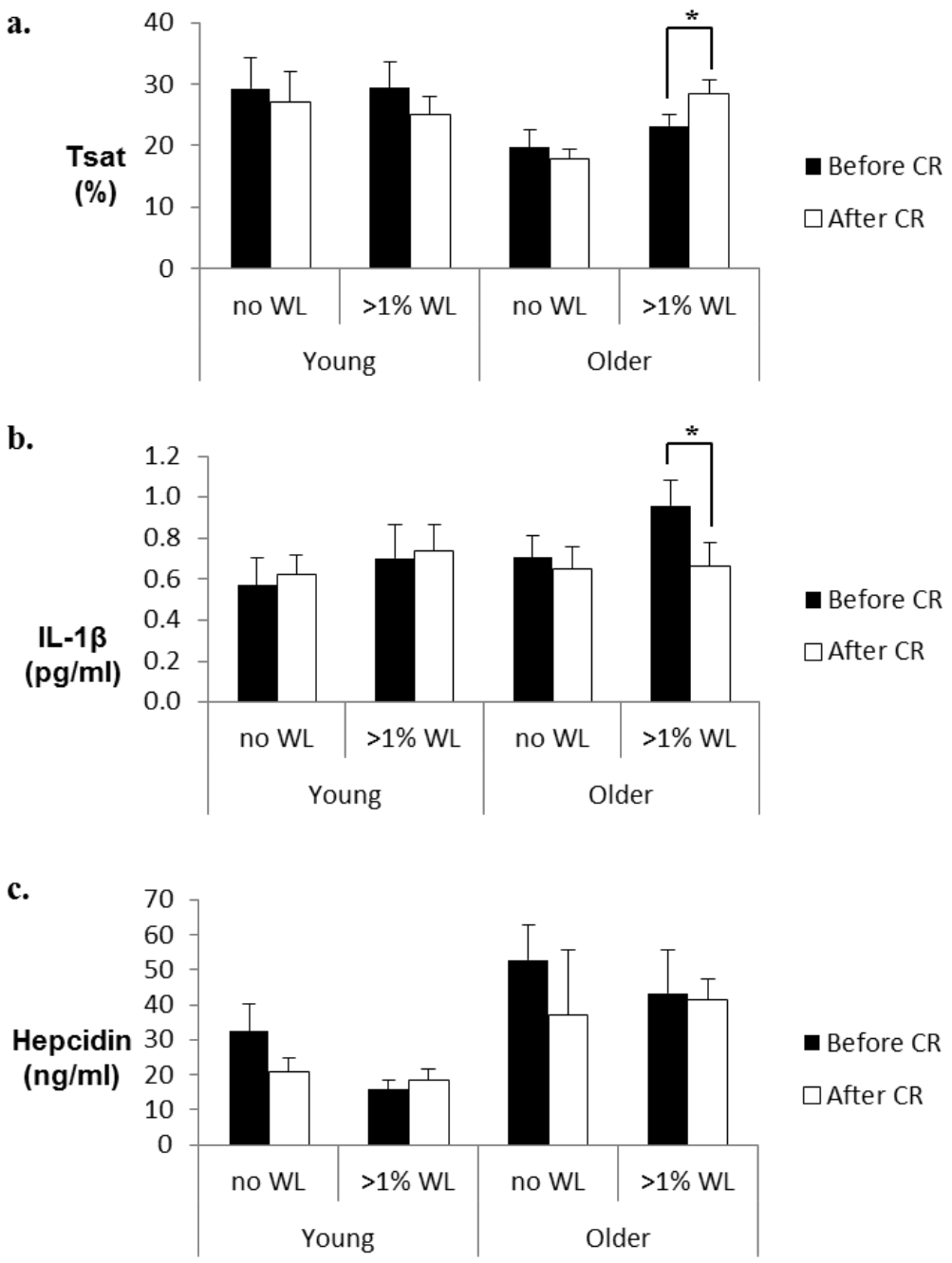

a.
Tsat (%)
40
30
20
10
0
*
no WL
>1% WL
no WL
>1% WL
Young
Older
Before CR
After CR
b.
IL-1β (pg/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
no WL
>1% WL
no WL
>1% WL
Young
Older
Before CR
After CR
c.
Hepcidin (ng/ml)
70
60
50
40
30
20
10
0
no WL
>1% WL
no WL
>1% WL
Young
Older
Before CR
After CR

Figure 3.

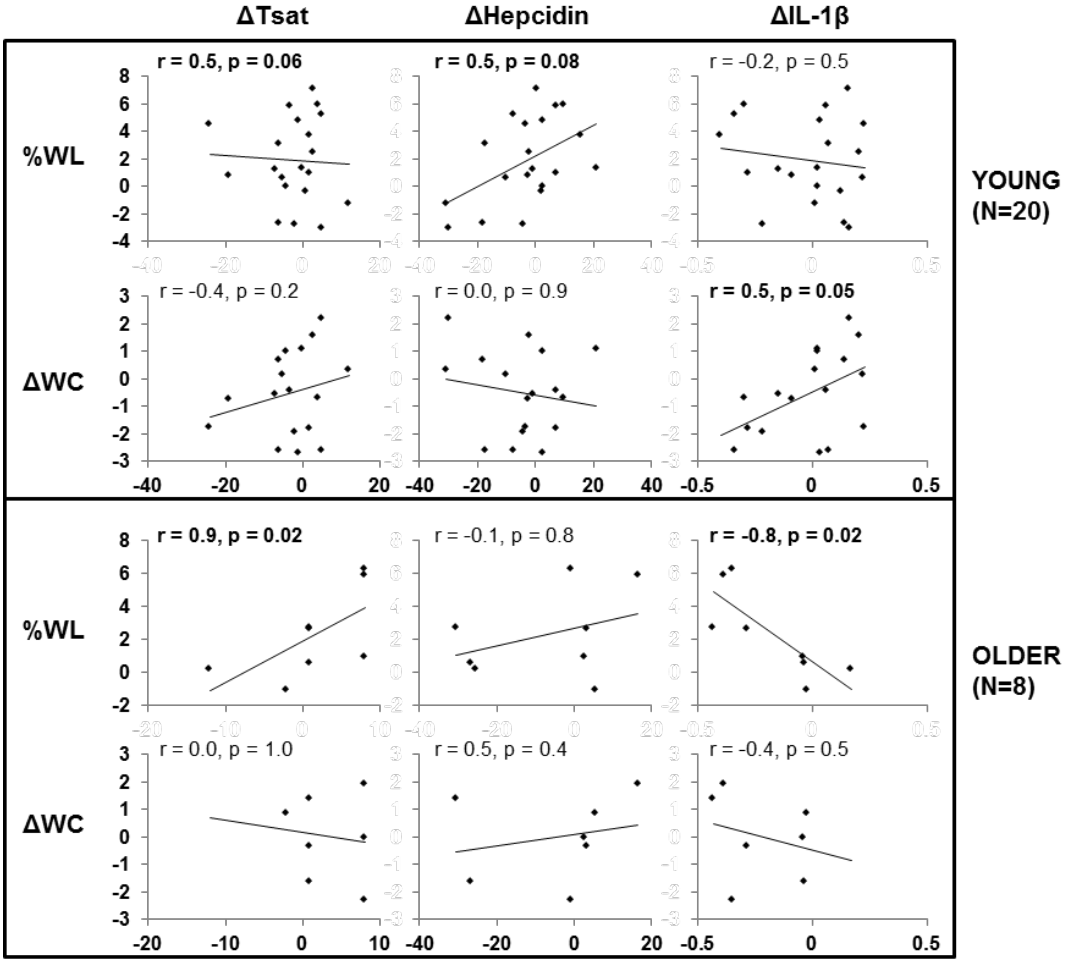
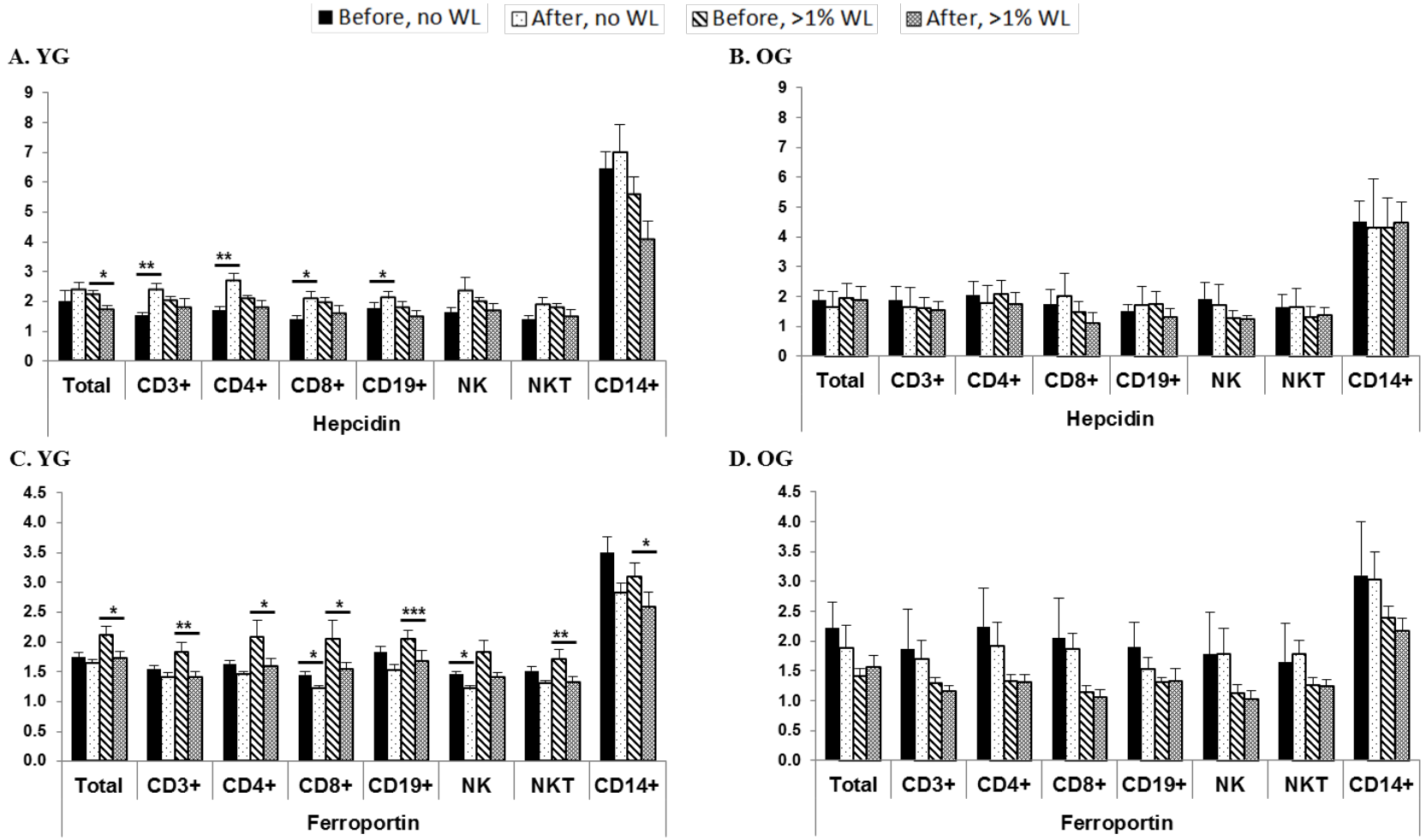

ΔTsat
ΔHepcidin
ΔIL-1β
%WL
ΔWC
YOUNG (N=20)
OLDER (N=8)
r = 0.5, p = 0.06
r = 0.5, p = 0.08
r = -0.2, p = 0.5
r = -0.4, p = 0.2
r = 0.0, p = 0.9
r = 0.5, p = 0.05
r = 0.9, p = 0.02
r = -0.1, p = 0.8
r = -0.8, p = 0.02
r = 0.0, p = 1.0
r = 0.5, p = 0.4
r = -0.4, p = 0.5

Figure 4.


Before, no WL
After, no WL
Before, >1% WL
After, >1% WL
A. YG
B. OG
C. YG
D. OG
Total
CD3+
CD4+
CD8+
CD19+
NK
NKT
CD14+
Hepcidin
Ferroportin

Chapter III

Hepcidin expression and immune response in stimulated PBMC of young and older obese women undergoing calorie restriction

Hepcidin expression and immune response in stimulated PBMC of young and older obese women undergoing calorie restriction

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Key words: Obesity, aging, immune response, hepcidin, PBMC, calorie restriction.

Running title: PBMC hepcidin and proliferation.

Abbreviations: WL, weight loss; CR, calorie restriction; PBMC, peripheral blood mononuclear cells; FBS, fetal bovine serum; HS, human serum; MFI, mean fluorescence intensity; YG, young group; OG, older group; PHA, phytohemagglutinin; IL-6, interleukin 6; IL-1β, interleukin 1β.

Abstract

Background: Immune response and iron homeostasis become impaired with aging and obesity. Hepcidin may be involved in this dysregulation. There is little evidence on the effect of obesity on immune response and iron homeostasis of the elderly. Hepcidin is essential for innate immunity, but its role in adaptive immunity remains unexplored. The effect of weight loss (WL) through calorie restriction (CR) on iron homeostasis and immune response in the elderly is not known.

Objective: Our aim was to investigate changes in immune response and its relation to hepcidin in young and older obese women undergoing WL through CR.

Design: This was a non-randomized, non-controlled CR intervention in young (18-45y) and older (>60y) obese women. We determined lymphocyte proliferation and hepcidin expression in unstimulated and stimulated lymphocytes before and after CR.

Results: There was a strong correlation between hepcidin expression in CD3+ cells and lymphoproliferation upon antigenic stimulation at baseline. We found no difference in lymphoproliferation upon antigenic stimulation between young and older obese subjects, and a higher number of CD4+ T cells in the older obese group. The young obese group had higher numbers of CD8+ and NKT cells. Neither immune response nor hepcidin expression changed significantly with CR, except for an increase in NKT cells in the older group.

Conclusion: Hepcidin expression in stimulated lymphocytes strongly correlated with lymphocyte proliferation supporting the evidence that hepcidin plays an important role in adaptive immunity. Further, our data suggest that obesity may be affecting immunity in young individuals in a similar way to that observed with aging. WL through CR did not

impact immune response or PBMC hepcidin expression, but WL was moderate (about 2% WL per group); more research is required to clarify whether greater WL is associated with significant changes in these parameters.

Introduction

The adaptive immune system becomes impaired with aging. There is decreased lymphocyte proliferation, impaired production of naïve lymphocytes, a decline in the number and function of CD4+ T cells, and impairment in CD8+ cytotoxic effector T cell function, among other changes.[1, 2] Obesity is also associated with impaired immunity, similar in certain aspects to immune senescence. Several studies have shown that obese individuals have decreased lymphocyte proliferation,[3, 4] lower naïve lymphocyte output and a skewed T cell phenotype towards Th1 cells.[5, 6] Nieman et al. found that obese subjects had significantly higher total lymphocytes, CD19+ and CD4+ cells than lean subjects,[7] and no difference in CD8+ or NKT cells. There is no evidence on the impact of obesity on immune response in the elderly.

Iron homeostasis is essential for both the innate and adaptive immune response.[8, 9] Hepcidin is a peptide hormone that was originally identified as an acute phase protein.[8, 10] Its main role in immune response, as far as has been studied, is to deplete iron pools from extracellular pathogens by binding to the iron exporter ferroportin, causing its degradation and leaving iron sequestered within cells, therefore preventing its access from pathogens.[11] Hepcidin is induced by pro-inflammatory cytokines, primarily interleukin (IL-) 6 and IL-1β.[12-14] Recently hepcidin was identified to be essential for lymphocyte proliferation *ex vivo* and *in vitro*. Pinto et al. showed that hepcidin knockdown in human lymphocytes caused proliferation impairment upon CD3/CD28 stimulation.[15] Lymphocyte proliferation was then rescued with the addition of synthetic hepcidin. This study also showed that hepcidin mRNA expression was found in different

lymphocyte subpopulations. Hepcidin peptide levels have not been measured in peripheral blood mononuclear cell (PBMC) subpopulations.

The elderly and obese are at higher risk of iron deficiency. It is believed that, due to the state of chronic inflammation present both with aging and obesity, upregulation of hepcidin may lead to iron deficiency through a mechanism similar to anemia of chronic disease.[16, 17] However, the mechanism of iron deficiency in obesity requires further investigation. No studies have measured hepcidin and iron status in obese elderly cohorts.

Calorie restriction (CR) may be an effective approach in reversing the effects of obesity on immune response and iron homeostasis. The limited available evidence has shown a decrease in systemic inflammation[5, 18, 19] and an improvement in immune response measured as lymphocyte proliferation[20, 21] with CR. Only two studies have determined the effect of weight loss (WL) on hepcidin and iron status in children and adults,[22, 23] and none have been conducted in the elderly. One study so far, a bariatric surgery intervention, has simultaneously measured the effect of WL on iron status, hepcidin and immune response. Through *ex vivo* assays in PBMC they found that there was impaired cytokine production upon stimulation with lipopolyssacharide (LPS) and that serum hepcidin was negatively correlated with interferon γ in stimulated PBMC. Cytokine production upon stimulation improved after WL. The findings from this study suggest a role for hepcidin in obesity-associated immune response dysregulation.

We have determined the effect of aging and calorie restriction on PBMC subpopulation percentages and lymphocyte proliferation, and hepcidin expression in stimulated and unstimulated PBMC in young and older obese women. We hypothesized

that lymphoproliferation would be associated with hepcidin and enhanced with WL through CR. The effect of WL on serum hepcidin, iron status and inflammation was presented in Chapter II.

Subjects and Methods

Study population and sample collection

This was a non-randomized, non-controlled CR intervention in young (18-45y) and older (>60y) obese women. At baseline there were 23 subjects in the young group (YG) and 15 in the older group (OG). After CR, there were 20 subjects in the YG and 8 subjects in the OG. This population has been described in detail in Chapter II. After an overnight fast, a 30-ml venous blood sample was collected into EDTA-tubes and serum separation tubes. Participants reported having no infections or illness, taking antibiotics or receiving vaccinations in the 2 weeks prior to blood collection. Further, participants reported not taking non-steroidal anti-inflammatory drugs (NSAIDs) or anti-histamine medications for 72 hours before each blood draw.

PBMC isolation and culture conditions

PBMC were isolated with gradient centrifugation using a Histopaque matrix, as described in Chapter II. Isolated PBMC were cultured under different conditions either for determination of hepcidin expression or lymphoproliferation. Cell culture schematics are presented in **Figure 1**. Serum from each subject was stored before and after CR. Before CR PBMC were cultured either in standard conditions (media enriched with 5% fetal bovine serum, FBS), or in media with 10% autologous human serum (HS). Serum collected at baseline was also frozen for later use. After CR, cells were cultured in media

enriched with serum collected at baseline (PreHS, 10%), serum collected after calorie restriction (PostHS, 10%), or 5% FBS. PBMC were cultured at a density of 10^6 cells/well in 24-well culture plates or at 100k cells/well in 96-well culture plates. Cells at each culture condition were either untreated or stimulated with the T cell mitogen phytohemagglutinin (PHA), or with antibodies against CD3 T cell receptor and CD28 T cell co-receptor (anti-CD3/CD28) as delineated below. All incubations were done at 37^oC, 5% CO_2 and 95% humidity.

PBMC subpopulations

To determine percent PBMC subpopulations, surface staining of PBMC was done using BD Biosciences reagents and protocols (BD Biosciences, Mountain View, CA). The antibodies used were specific to CD3, CD4, CD8, CD19, NK, and CD14 cell surface markers (eBioscience, San Diego, CA). Natural Killer T (NKT) cell frequency was determined as the percentage of cells expressing both NK and CD3 markers. Cells were fixed and subsequently analyzed with flow cytometry using an Acuri C6 instrument and Flojo version 10.0.6 for analysis. Isotype controls for each antibody class were used as negative controls.

Lymphocyte proliferation

Lymphocyte proliferation was assessed by [^{3}H]-thymidine incorporation in cells that were untreated or treated with anti-CD3/CD28 (eBioscience, San Diego, CA) or PHA (Difco Laboratories, Detroit, MI),[20] and cultured in 5% FBS or 10% HS. First, 96-well round bottom cell culture plates were coated with serially diluted anti-CD3 in 1X PBS (1 µg/ml, 5 µg/ml, or 10 µg/ml) and incubated for 2 hours. Coated plates were washed twice with sterile 1X PBS. Cells in 5% FBS or 10% HS, and anti-CD28 (2

μg/ml) were added to the wells. Stimulation was also done with PHA (2 μg/ml, 5 μg/ml, or 25 μg/ml). All wells were done in triplicate. After stimulation for 68 ± 1 hours, cells were pulsed with 0.5 μCi [^{3}H]-thymidine (Perkin Elmer, Shelton, CT) and incubated for exactly 4 hours. Plates were then frozen at -80^{o}C for later analysis. Thawed plates were trypsinized for 1 hour and harvested onto glass fiber mats using a Perkin Elmer cell harvester (Perkin Elmer, Boston, MA). Incorporation of [^{3}H]-thymidine was determined by liquid scintillation counting using a Micro Beta 2 MicroPlate counter (Perkin Elmer, Boston, MA). Results, expressed as counts per minute (CPM), were used as a measure of lymphocyte proliferation.

Hepcidin expression in PBMC

Isolated PBMC were cultured with and without PHA (10 μg/ml) or anti-CD3 (5 μg/ml) / CD28 (2 μg/ml) in 5% FBS or 10% HS. Cell culture 24-well plates were coated with anti-CD3 as described above. Cells were incubated for 72 hr, after which each well was counted using a hemocytometer. Dead cells were excluded with Trypan blue staining. A total of 25k cells were used per sample for flow cytometry. Surface staining was done to determine the percentage of CD3+ cells within PBMC, as described above. Fixed cells were permeabilized to measure intracellular hepcidin. To this end, a hepcidin primary antibody with a FITC-labeled secondary antibody was used (Abcam, Boston, MA). An isotype control was used as a negative control. Given the increase in background signal with PBMC stimulation, an isotype control was used for each stimulation condition. Mean fluorescent intensity (MFI) was determined for hepcidin and the isotype control. The relative expression of hepcidin was calculated as the ratio of the

hepcidin MFI to the isotype MFI. In this way, we measured hepcidin expression either in total PBMC or in CD3+ lymphocytes.

Statistical Analysis

Student's t test was used for all comparisons between the YG and the OG, including %PBMC subpopulations and lymphocyte proliferation. Student's t test was also used to compare %PBMC subpopulations and lymphocyte proliferation before and after CR. One-way ANOVA was used to assess the change in hepcidin expression between unstimulated and stimulated PBMC in different culture conditions (5% FBS, 5%HS, 10%HS) for YG and OG separately. Pearson correlation coefficient analysis was used to determine the association between hepcidin expression in CD3+ cells and lymphocyte proliferation upon stimulation with anti-CD3/CD28. Statistical significance was set at alpha = 0.05. SAS 9.2 for Windows (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Population characteristics

Population characteristics have been described in Chapter II. Briefly, there were 23 subjects in the young group (YG, 18-45y) and 15 in the older group (OG, >60y) at baseline. There were 20 subjects in the YG and 8 in the OG after CR. The CR period lasted 13 ± 4 weeks and there was approximately 2% WL in each group ($p<0.05$).

Baseline age comparisons in PBMC subpopulations and immune response

Analysis in PBMC subpopulations showed a significantly higher percentage of CD8+ and NKT cells in the YG than the OG ($p<0.01$ and $p<0.0001$, respectively)

(**Figure 2A**). In contrast, the OG had significantly more CD4+ T cells ($p<0.01$). Lymphoproliferation was higher in the YG only under certain conditions: in PBMC cultured in 5% HS and stimulated with PHA (2 μg/ml and 5 μg/ml), and PBMC cultured in 10% HS and stimulated with PHA (5 μg/ml) (**Figure 2B**). The highest dose of PHA stimulation led to no differences in proliferation between the two age groups. There was no difference in anti-CD3/CD28 stimulation between the YG and OG (**Figure 2C**).

Changes in lymphocyte proliferation and PBMC subpopulations with calorie restriction

We found no significant differences in lymphocyte proliferation after CR in the YG or the OG for any culture (5% FBS, and 5% or 10% HS) or stimulation (PHA or anti-CD3/CD28) condition (**Figure 3C and D**). In addition, the YG did not experience any changes in PBMC subpopulations (**Figure 3A**). In the OG, there was a significant decrease in percentage of B cells (CD19+, $p<0.05$) and an increase in NKT cells ($p<0.01$) (**Figure 3B**). Correlation of change between WL and lymphocyte proliferation was not significant (data not shown).

Hepcidin expression in stimulated PBMC

PBMC hepcidin expression was not different between the YG and OG in any culture or stimulation condition (**Figure 4**). Additionally, PBMC hepcidin expression was not significantly upregulated upon stimulation (either PHA or anti-CD3/CD28) when PBMC were cultured with 5% FBS (**Figure 4A and C**). On the other hand, PBMC hepcidin expression was significantly upregulated in PBMC that were cultured with 10% HS and stimulated with PHA (**Figure 4B**). ANOVA showed significant models for both YG and OG ($p\leq0.05$), but individual comparisons were not significant. No significant differences in hepcidin expression were found for CD3/CD28 stimulation in 10% HS

(**Figure 4D**). After CR, hepcidin expression of PBMC cultured with 10% HS obtained before CR (PreHS) was not different from culture with serum obtained after CR (PostHS). This section shows that hepcidin is upregulated in PBMC stimulated with PHA (10% HS), in both the YG and OG, but not in PBMC stimulated with anti-CD3/CD28.

Lymphocyte proliferation and hepcidin

We used Pearson correlation coefficient analysis to determine associations between lymphocyte proliferation and hepcidin expression in stimulated or unstimulated CD3+ cells. We found that hepcidin expression in lymphocytes stimulated with anti-CD3/CD28 (10% HS) was strongly correlated with antigenic lymphocyte proliferation (5 μg/ml and 10 μg/ml anti-CD3) in the OG, but not in the YG. Specifically, the correlation coefficient between stimulated lymphocyte hepcidin and lymphoproliferation was $r=0.75$ ($p\leq0.05$) for stimulation with 5 μg/ml anti-CD3, and $r=0.77$ ($p\leq0.05$) for stimulation with 10 μg/ml anti-CD3. No significant correlation was found for PBMC cultured in 5% FBS (data not shown).

Discussion

We have demonstrated that immune response, measured as lymphoproliferation, does not differ between obese young and older women, which is opposite to the differences reported between lean young and older individuals. Previous studies have found notable reductions in proliferation with PHA or anti-CD3/CD28 stimulation in elderly lean subjects with respect to young.[3, 4] This suggests that obesity may be impairing the immune system of young individuals in a manner similar to immune

senescence. In further support of this observation, we found %CD4+ cells to be significantly higher in the older subjects even though CD4+ T cell number is known to decrease with aging.[1, 2]

CD8+ T cells and NKT cells are involved in immune cytotoxicity. We have shown that older obese subjects have lower numbers of these cell populations than young subjects. Nieman et al. found no difference in CD8+ or NKT cell cytotoxic activity or numbers between lean and obese individuals,[7] and so the difference that we found is likely due to the impact of aging rather than obesity. Upon WL the older subjects had a significant increase in NKT cells which suggest WL in older individuals could potentially reverse certain aspects of immune impairment.

In this study there were no changes in immune response with CR, and the change in proliferation was not correlated with percent WL (data not shown). Previously, lymphocyte proliferation with both PHA and anti-CD3/CD28 stimulation was shown to increase significantly after a 6-month calorie restriction period in adults, and the change in proliferation correlated with percent WL in this cohort.[20] Therefore, perhaps greater WL or a longer CR period is needed to have a significant impact on immune response.

PHA, but not anti-CD3/CD28, stimulation significantly induced PBMC hepcidin expression in the YG and OG in cells cultured in human serum (**Figure 4B**). This is the first evidence that mitogenic stimulation induces hepcidin expression in lymphocytes. We also expected hepcidin to be significantly upregulated upon CD3/CD28 stimulation as it has been shown previously with hepcidin mRNA.[15] Looking at each individual's response, we found that, upon CD3/CD28 stimulation, hepcidin expression increased for some subjects, decreased for others and remained unchanged for the rest. Comparing

these results with a lean group would be necessary in order to determine whether the lack of hepcidin upregulation upon antigenic stimulation is due to obesity.

Even though hepcidin appeared not to increase with antigenic stimulation, we observed a strong correlation between lymphocyte hepcidin expression and proliferation upon CD3/CD28 stimulation. This corroborates previous evidence showing that hepcidin plays an important role in lymphocyte proliferation. More mechanistic evidence is needed to further characterize this function of hepcidin.

Previous evidence has shown that basal cytokine levels are increased in obesity, but cytokine production upon stimulation is somewhat inhibited.[24] We had anticipated a similar scenario for hepcidin expression in PBMC. To confirm this, we sought to explore the impact of the inflammatory environment of obesity on PBMC hepcidin expression by culturing the cells in autologous serum obtained from before and after CR. However, WL was moderate (only about 2% per group), and we saw no significant decreases in systemic inflammation, except for IL-1β (Chapter II), and found no difference in hepcidin expression. Studies with more significant WL are needed to confirm this hypothesis.

Limitations to this study are the small sample size after CR, especially for the OG. In addition, the limited amount of WL possibly prevented the determination of significant differences in hepcidin. Our results are mainly correlational, and a direct involvement of hepcidin in lymphocyte proliferation cannot be established with our study design. Yet our findings support the need for further mechanistic studies. This study is novel because it shows for the first time a direct comparison of immune function and hepcidin between young and older obese groups. Furthermore, it shows the first evidence of a direct

involvement of endogenous PBMC hepcidin peptide in lymphocyte proliferation, suggesting that hepcidin's role in immune function goes beyond innate immunity.

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Table 1. Pearson correlation coefficient analysis between lymphocyte proliferation and lymphocyte hepcidin expression.

				Lymphoproliferation with anti-CD3 (concentration below)/CD28 (2µg/ml) simulation		
	Culture conditions for hepcidin expression			**1µg/ml**	**5µg/ml**	**10µg/ml**
Hepcidin expression in CD3+ cells	YG	10% HS	Unstimulated	0.24	0.28	0.32
			anti-CD3/CD28	0.52**	0.41	0.38
	OG	10% HS	Unstimulated	-0.13	-0.09	-0.06
			anti-CD3/CD28	0.67	0.75*	0.77*

PBMC were stimulated with antiCD3/CD28 (1 µg/ml, 5 µg/ml, or 10 µg/ml) and cultured in 10% HS. *p≤0.05; **p=0.06. Sample sizes are: YG (N=14); OG (N=8).

Figure Legends

Figure 1. Diagram for cell culture assays. PBMC and serum were obtained before and after CR. At baseline, PBMC were cultured in 5% FBS or 10% HS. After CR, PBMC were cultured in 5% FBS, or 10% HS obtained at baseline (PreHS) or after CR (PostHS). All cultured cells were treated with PHA or anti-CD3/CD28. Unstimulated (Unstim.) cells were used as controls.

Figure 2. Baseline age comparisons of immune parameters. A) PBMC subpopulations expressed as % of total PBMC. **B)** Lymphocyte proliferation after 72hr stimulation with PHA (2 µg/ml, 5 µg/ml, or 25 µg/ml) or **C)** anti-CD3 (1 µg/ml, 5 µg/ml, or 10 µg/ml) / CD28 (2 µg/ml) with subsequent [^{3}H]-thymidine pulse. Student's t test was used for comparison between YG and OG, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.0001$. Sample sizes are: A) YG (N=22), OG (N=15), B) YG (FBS N=22, HS N=14); OG (FBS N=15, HS N=10).

Figure 3. Changes in immune parameters with CR. A and B: PBMC subpopulation changes with CR in YG (A) and OG (B). *$p < 0.05$, **$p < 0.01$, Student's t test. **C and D:** Proliferation of PBMC cultured in 5% FBS, and stimulated with PHA (2 µg/ml, 5 µg/ml, or 25 µg/ml) or anti-CD3 (1 µg/ml, 5 µg/ml, or 10 µg/ml) / CD28 (2 µg/ml) in YG (C) and OG (D). Sample sizes are: A) N=20; B) N=8; C) N=12; and D) N=7.

Figure 4. Change in PBMC hepcidin expression. Stimulation with PHA **(A and B)** or anti-CD3/CD28 **(C and D)** is shown. Cells were cultured in 5% FBS **(A and C)** or 10% HS **(B and D)**. In all plots hepcidin expression is calculated as the ratio of hepcidin MFI

over isotype (background) MFI. * $p \leq 0.05$ for one-way ANOVA model, multiple comparisons were not significant. Age comparisons were not significant. U = unstimulated, PreHS = serum isolated before CR, PostHS = serum isolated after CR. Sample sizes are: A and C) YG N=19 and OG N=7; B and D) YG N=12 and OG N=5.

Figure 1.

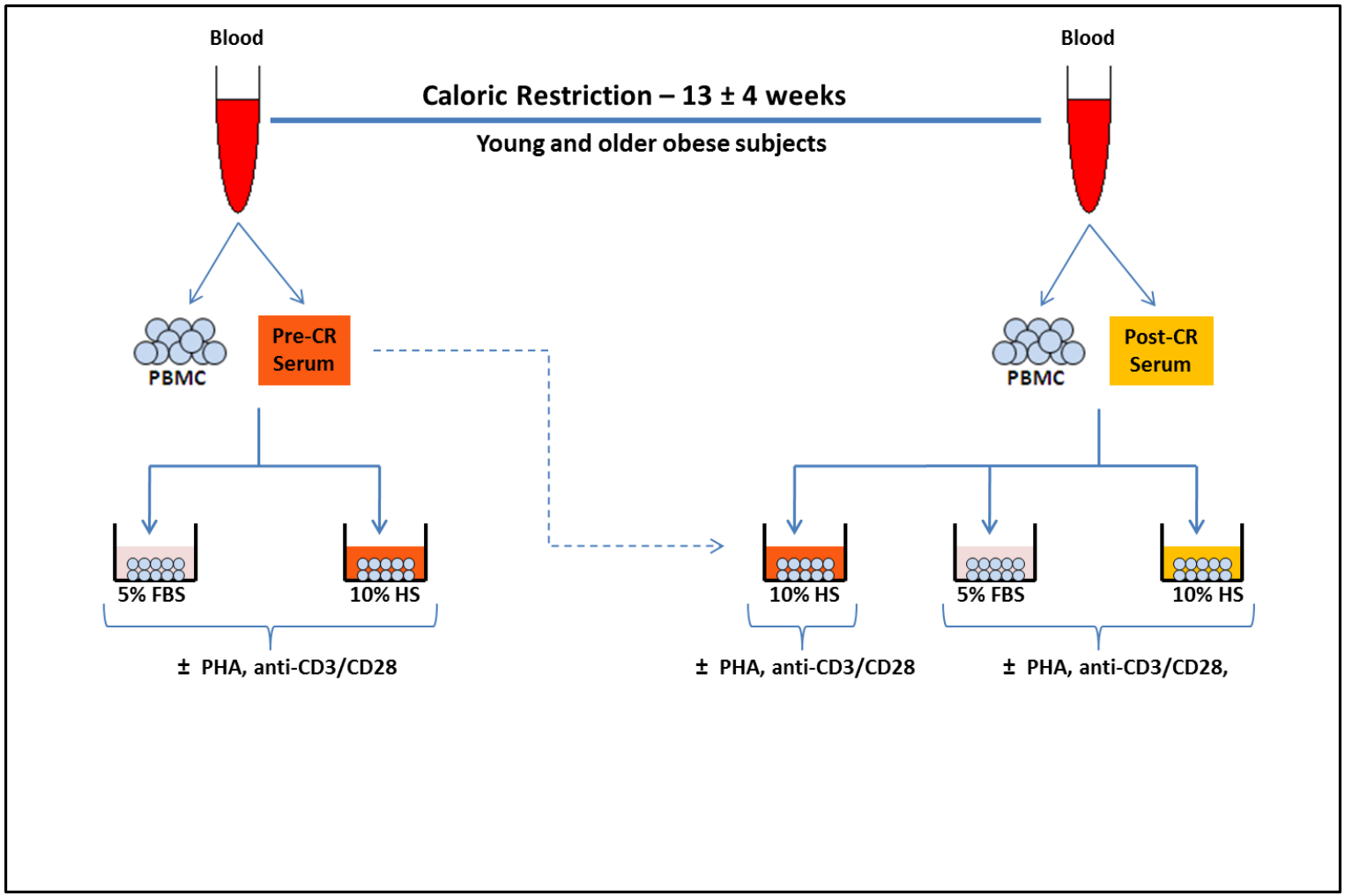

Blood
Blood
Caloric Restriction – 13 ± 4 weeks
Young and older obese subjects
PBMC
Pre-CR Serum
PBMC
Post-CR Serum
5% FBS
10% HS
10% HS
5% FBS
10% HS
± PHA, anti-CD3/CD28
± PHA, anti-CD3/CD28
± PHA, anti-CD3/CD28,

Figure 2.

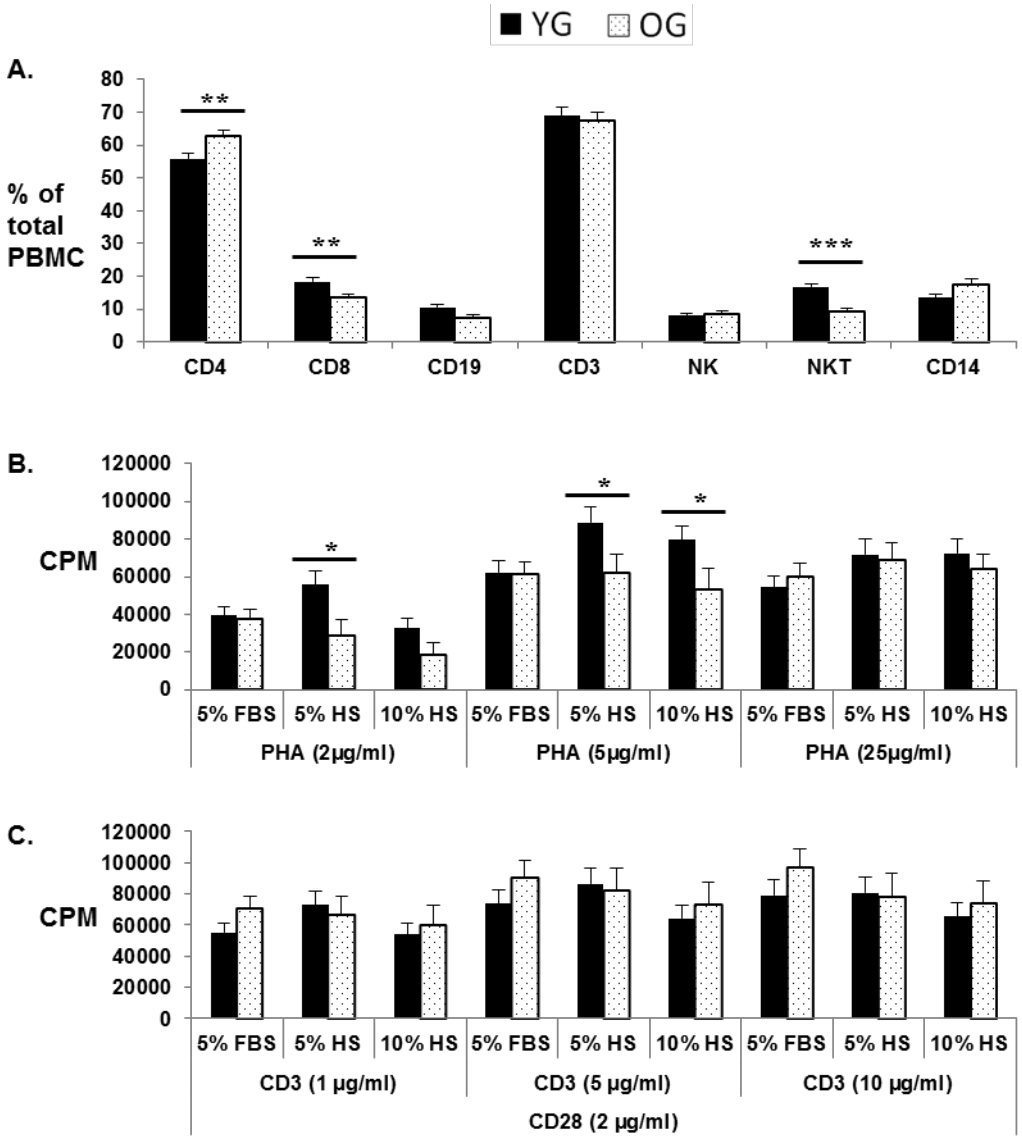

YG
OG
A.
% of total PBMC
80
70
60
50
40
30
20
10
0
**
**
***
CD4
CD8
CD19
CD3
NK
NKT
CD14
B.
CPM
120000
100000
80000
60000
40000
20000
0
*
*
*
5% FBS
5% HS
10% HS
PHA (2µg/ml)
PHA (5µg/ml)
PHA (25µg/ml)
C.
CPM
CD3 (1 µg/ml)
CD3 (5 µg/ml)
CD3 (10 µg/ml)
CD28 (2 µg/ml)

Figure 3.

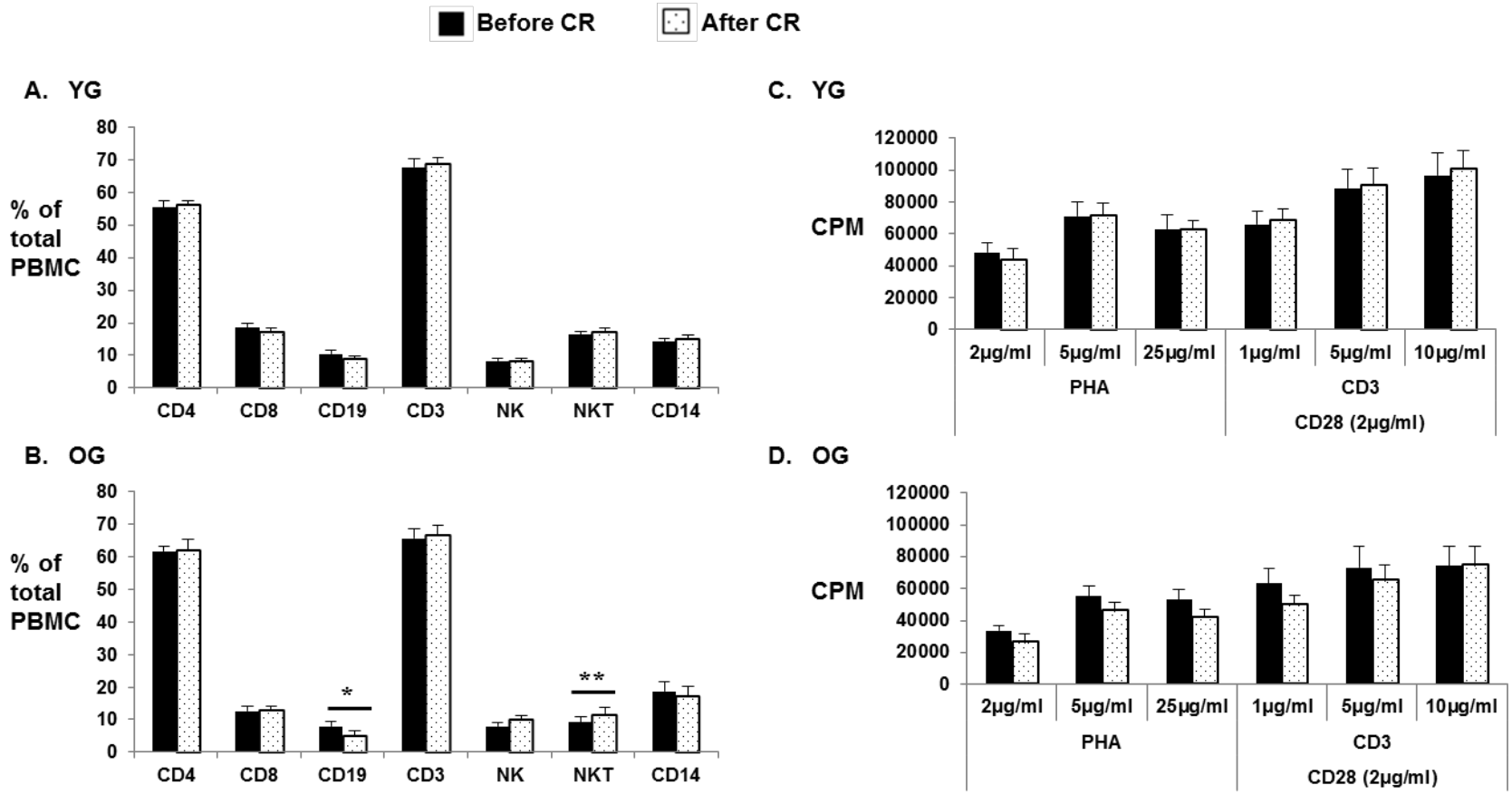
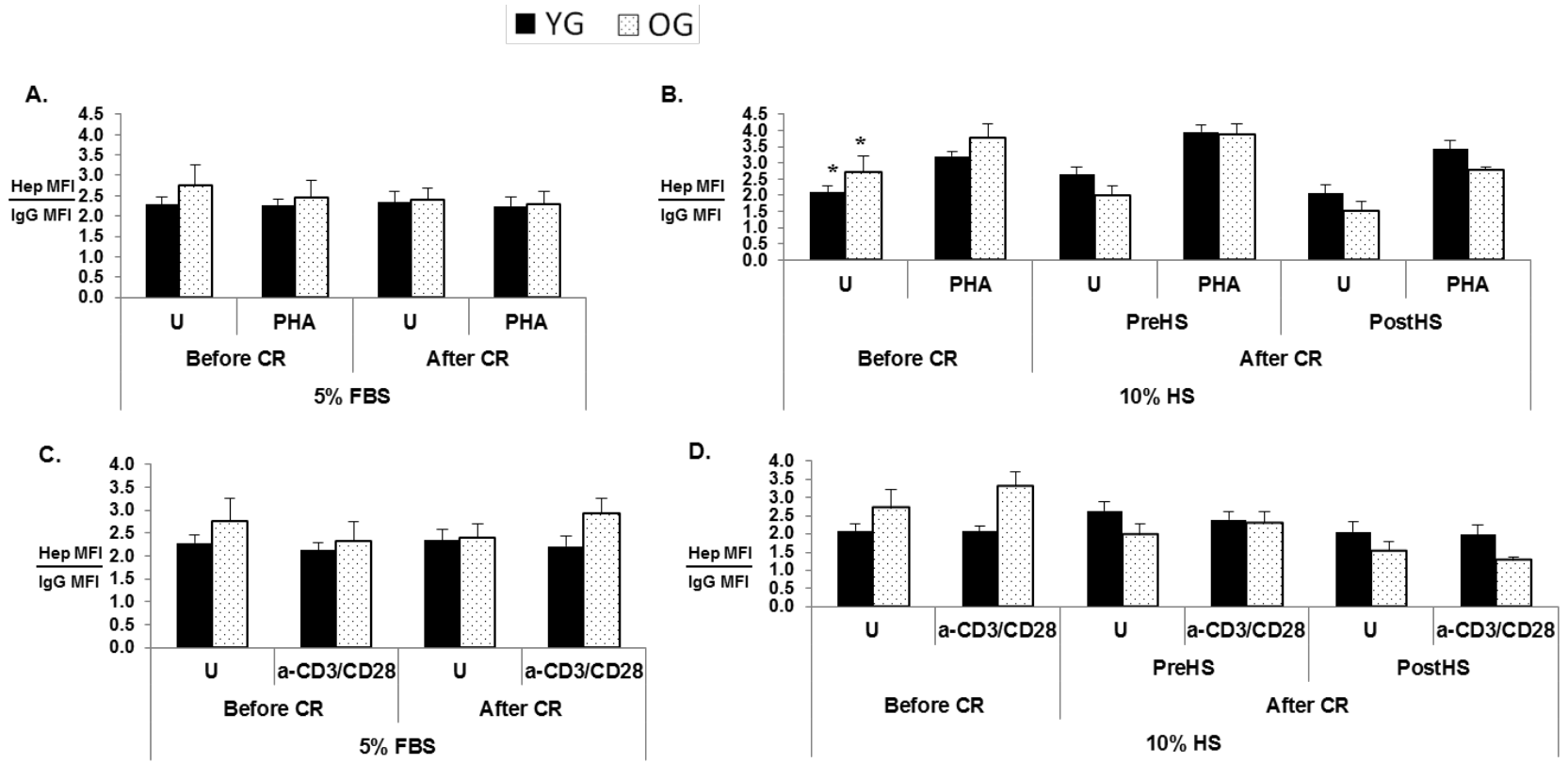

Before CR
After CR
A. YG
% of total PBMC
80
70
60
50
40
30
20
10
0
CD4
CD8
CD19
CD3
NK
NKT
CD14
B. OG
% of total PBMC
*
**
C. YG
CPM
120000
100000
80000
60000
40000
20000
0
2μg/ml
5μg/ml
25μg/ml
1μg/ml
5μg/ml
10μg/ml
PHA
CD3
CD28 (2μg/ml)
D. OG
CPM

Figure 4.


YG
OG
A.
Hep MFI / IgG MFI
U
PHA
U
PHA
Before CR
After CR
5% FBS
B.
Hep MFI / IgG MFI
U
PHA
U
PHA
U
PHA
PreHS
PostHS
Before CR
After CR
10% HS
C.
Hep MFI / IgG MFI
U
a-CD3/CD28
U
a-CD3/CD28
Before CR
After CR
5% FBS
D.
Hep MFI / IgG MFI
U
a-CD3/CD28
U
a-CD3/CD28
U
a-CD3/CD28
PreHS
PostHS
Before CR
After CR
10% HS

Chapter IV

Impact of weight loss through calorie restriction on iron status, inflammation and hepcidin expression in circulation and adipose tissue of obese individuals

Impact of weight loss through calorie restriction on iron status, inflammation and hepcidin expression in circulation and adipose tissue of obese individuals

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Key words: Obesity, adipose tissue, inflammation, hepcidin, iron status, calorie restriction.

Running title: Weight loss, adipose tissue and hepcidin.

Abbreviations: AT, adipose tissue; WL, weight loss; CR, calorie restriction; WC, waist circumference; BMI, body mass index; Tsat, transferrin saturation; sTfR, soluble transferrin receptor; CRP, C-reactive protein; IL-6, interleukin 6; HJV, hemojuvelin; TNFα, tumor necrosis factor alpha; MCP-1, monocyte chemotactic protein-1; IL-2, interleukin 2; IFNγ, interferon gamma; PPIA, peptidylprolyl isomerase A.

Abstract

Background: Hepcidin is a hormone that regulates cellular iron export. Obesity is characterized by a state of low-grade chronic inflammation and elevated hepcidin both in circulation and adipose tissue. Chronic overexpression of hepcidin in obesity may lead to iron status impairment. The effect of weight loss (WL) through calorie restriction (CR) on hepcidin and iron status in obese adults is not known. Hepcidin is expressed in adipose tissue (AT) in obesity, but the effect of WL on AT hepcidin expression has not been determined.

Objective: We investigated the effect of WL through CR on systemic inflammation, hepcidin and iron status, and WL-induced changes on AT hepcidin and macrophage marker expression.

Design: A CR intervention trial was conducted in 26 obese adults. Serum hepcidin, C-reactive protein (CRP), iron status, and AT expression of hepcidin, adipokines, and expression of macrophage markers were assessed before and after WL.

Results: AT hepcidin was inversely correlated with iron status at baseline ($p<0.05$). WL correlated with decreased serum hepcidin in subjects who had at least 5% WL ($p<0.05$, N=14). Change in waist circumference also correlated with decreased serum hepcidin and CRP ($p<0.05$). There was no significant change in iron status with WL. AT expression of CD68, a macrophage marker, was highly correlated with AT hepcidin expression before ($p<0.001$) and after ($p<0.0001$) WL. CD14 and TNFα, correlated with hepcidin at baseline only.

Conclusion: Our data show a significant association between WL through CR and decrease in serum hepcidin and inflammation, but not iron status. However, there was an

inverse association between AT hepcidin and iron status, as well as a tight association between AT macrophages, AT inflammation and hepcidin. Further research is needed to determine the role of inflammatory dysregulation of AT in obesity-related iron status impairment.

Introduction

Weight gain and obesity result in a wide array of structural and functional changes in adipose tissue (AT). These changes include tissue hypoxia, adipocyte enlargement and death, and outflow of free fatty acids from adipocytes into circulation.[1, 2] The secretory profile of AT, an important endocrine organ, also changes. There is a decrease in adiponectin expression, and increase in leptin and production of pro-inflammatory cytokines.[1] Further, there is local activation and increased recruitment of immune cells, primarily macrophages to AT.[2] Macrophages involved in AT can be classified as having a M1 or M2 phenotype. M1 macrophages produce pro-inflammatory signals, while M2 macrophages have an anti-inflammatory profile and are involved in extracellular matrix remodeling, angiogenesis and tissue repair.[2] AT macrophages in obesity are characterized as having M2 surface markers together with pro-inflammatory cytokine production.[3-6]

Iron status impairment has been identified as a comorbidity of obesity. Several studies in adults have shown associations between obesity and iron deficiency.[7-12] This association is likely mediated by hepcidin, a peptide hormone with dual action: it maintains iron homeostasis by controlling iron levels in circulation, and it is important for innate[13, 14] and adaptive[15] immune function.[16] Hepcidin acts by binding and inducing the degradation of ferroportin, leading to intracellular iron sequestration in enterocytes[17, 18] and macrophages,[19] among other cell types. Hepcidin is upregulated by iron overload and by pro-inflammatory signals, primarily through the interleukin 6 (IL-6)/JAK/STAT3 and BMP/Smad4 pathways.[20-22] Evidence suggests that obesity-induced upregulation of pro-inflammatory cytokines, such as IL-6, induce hepcidin overexpression, which leads

to lower iron status.[23] Several studies in obese adults have shown upregulation of hepcidin and pro-inflammatory signals in parallel with lower iron status.[24-26] Furthermore, two weight loss (WL) studies, a bariatric intervention in obese women with a 6 month follow-up[27] and a 6-month calorie restriction (CR) intervention in obese children,[28] have shown an improvement in iron status and a decrease in chronic inflammation and hepcidin. No CR studies measuring these outcomes have been conducted in obese adults.

For the most part, WL in obesity is accompanied by decrease in systemic inflammation[27, 29-31] and lower inflammation in AT in humans.[32, 33] It has also been shown that WL after bariatric surgery is associated with reduced macrophage infiltration and crown-like structures in AT of obese patients.[34] However, not all studies have been linked with beneficial changes in inflammation and some have argued that optimal design and duration of WL interventions need to be determined.[35]

AT, both adipocytes and stromal vascular fraction, produces hepcidin in obesity. Bekri et al. discovered that hepcidin is expressed in AT of obese individuals in significantly higher levels than their lean counterparts.[24] They hypothesized that AT could be an important contributor of hepcidin in circulation and therefore of dysregulaton of iron homeostasis in obesity. Luciani et al. also studied hepcidin expression in AT and found that gene expression of hemojuvelin (HJV), a protein upstream of hepcidin expression through the HJV/BMP pathway, hepcidin and IL-6 were higher in obese compared to lean subjects.[25] These studies show that hepcidin expression in AT in the context of obesity is affected by inflammation, but the effect of AT hepcidin upregulation on iron status needs to be elucidated.

Thus, in this study we investigated the effect of WL through CR in obese adults on systemic changes in iron status, inflammation and hepcidin, as well as AT expression of hepcidin, adipokines, and macrophage markers. We hypothesized that WL would be associated with a decrease in blood and AT levels of inflammation, hepcidin, and macrophage marker expression, and an enhancement in iron status.

Subjects and Methods

Study population

This was a 12-week CR intervention trial in obese men and women. Participants were either on a low calorie low fat or low calorie low carbohydrate diet. For the purposes of this ancillary study we pooled all participants together and accounted for the two diet types in our analysis. Eligible participants were adults between the ages of 18 and 64 with a body mass index (BMI) higher than 30kg/m^2, and if diabetic had hemoglobin A1c $\leq$ 8% within one month of enrolling in the study. Exclusion criteria were pregnancy, diabetes with a hemoglobin A1c > 8% or insulin treatment, known peripheral arterial or cardiovascular disease, use of angiotensin receptor blockers, WL greater than or equal to 3% three months prior to recruitment, use of WL medications within 4 weeks prior to enrollment, history of eating disorders, history of renal or hepatic disease, prior gastric restrictive surgery, or habitual tobacco use. This study was approved by the Boston Medical Center and Tufts Medical Center/Tufts University Institutional Review Boards, and all subjects provided signed informed consent.

Low calorie diets

The study was conducted at the Nutrition and Weight Management Center at Boston Medical Center and was originally designed to compare a low fat vs. a low calorie diet. For the purposes of this study we have combined the two diet groups. Subjects consumed hypocaloric self-prepared diets with a goal of 500 kcal/day less than maintenance needs. Subjects were assigned to either a low fat/low calorie diet (goals: 60% of energy from complex carbohydrates; 25% from fat, the majority being mono and polyunsaturated; and 15% from protein) or a low carbohydrate/low calorie diet (goal up to 60% from fat). Subjects received regular nutrition counseling from a registered dietitian either in group or individual settings. There was not a formal exercise component in this program, but subjects were encouraged to exercise for 30 minutes at least 3 times per week.

Anthropometric measures

Anthropometric measures included weight, height, BMI, waist circumference (WC) and waist-to-hip ratio. All measurements were taken to the nearest 0.1cm or 0.1kg. All measurements were taken before and after CR, except for weight which was also taken on a weekly basis.

Serum measurements

Serum for iron status, hepcidin and CRP was assessed from participants who lost more than 5% of their body weight (N=14). ELISA was used to measure serum hepcidin (Peninsula Laboratories, Bachem, San Carlos, CA), CRP (Quest Diagnostics, Boston, MA) and soluble transferrin receptor (sTfR) (Ramco Laboratories, Stafford, TX). Serum iron and total iron binding capacity (TIBC) were used for the calculation of transferrin

saturation (Tsat), and were measured with colorimetric endpoint assays (Diagnostic Chemicals).[36]

Adipose tissue measurements

Subcutaneous AT biopsies were taken at baseline and after the CR period. Briefly, AT samples of approximately 1.5g were obtained through incisions at the suprailiac crest skinfold. An area of approximately 10cm was anesthetized with 1% lidocaine and 2 to 6 passes were required per sample. Incisions were sutured, and the wounds were monitored and sutures removed one week after the procedure. AT samples were immediately frozen at -80^{o}C for later analysis.

RNA was isolated from AT samples and subsequently 1µg RNA was reverse transcribed (Roche, Indianapolis, IN). Gene expression was assessed with RT-PCR using the FastStart Universal Probe Master (Rox) (Roche, Indianapolis, IN), LightCycler 480 Multiwell 384-well plates (Roche, Indianapolis, IN), and the Taqman Gene Expression Assay system using FAM dye label (Applied Biosystems, Foster City, CA). Gene expression of *hamp* (the hepcidin gene), IL-6, tumor necrosis factor alpha (TNFα), monocyte chemotactic protein-1 (MCP-1), CD14, CD68, leptin, adiponectin, IL-2 and interferon gamma (IFNγ) was assessed. All values were normalized to peptidylprolyl isomerase A (PPIA), which was used as a housekeeping gene. Standard curves, blank samples, and a 25ng positive control were run in every plate, and all samples were loaded in duplicate.

Statistical analysis

All changes from baseline to after CR were analyzed using paired t test. Partial correlation coefficient analysis was used to determine associations at baseline, after CR

or correlation of change after WL. Weight loss was expressed as a percent of initial weight; greater loss is indicated by a greater % change. For all correlation analysis we adjusted for age, gender and diet group. For normally distributed variables mean ± standard deviation (SD) is reported. For variables that were not normally distributed median with first and third quartile (Q1, Q3) is reported. Statistical significance was set at alpha = 0.05. SAS 9.2 for Windows (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Population characteristics and anthropometric changes with calorie restriction (CR)

There were 22 women and 4 men in this study group with an average age of 42 years (range 21-62 y) (**Table 1**). The BMI range at baseline ranged from 29.1 to 44.6 kg/m^2. Mean WL was 6.1 ± 3.8% and BMI decreased from 36.1 ± 4.4 to 33.9 ± 4.3 kg/m^2 ($p<0.0001$) over the 12 weeks of CR. Significant decreases were also observed for WC, from 109.3 ± 9.9 to 101.5 ± 11.0cm ($p=0.004$), and hip circumference, from 122.6 ± 11.3 to 114.2 ± 12.6cm ($p<0.0001$).

Baseline correlations between iron status and adipose tissue hepcidin expression

At baseline, after adjusting for age and sex, AT hepcidin mRNA expression was strongly and positively correlated with sTfR, which is increased with lower iron status ($r=0.75$, $p=0.01$, $N=12$) (**Figure 1**). AT hepcidin expression did not correlate with serum iron, Tsat, ferritin, or CRP. Notably, AT hepcidin did not correlate significantly with serum hepcidin or CRP. After CR the correlation between AT hepcidin expression and sTfR was not significant.

Systemic changes in inflammation, iron status and hepcidin with calorie restriction

Serum was available from participants with at least 5% WL (N=14). There were no statistically significant changes in serum hepcidin or iron parameters (**Table 2**) with 5% WL. There was a trend towards decrease in CRP (p=0.07). Changes in serum hepcidin and iron status were not significant when stratifying the analysis by diet (data not shown).

Associations between change in anthropometric and biochemical variables

Because there was a wide range of weight change in this population, ranging from 1.6% gain to a 13.4% loss, we determined correlations between the change in anthropometric measurements and changes in iron status, serum hepcidin and CRP. All changes were calculated by subtracting values before CR from values after CR, and all partial correlations were adjusted for age, sex and diet. There was a significant inverse association between WC reduction and decrease in CRP (r=-0.62, p=0.04). Further, there was a significant negative association between change in serum hepcidin and both percent WL (%WL) (r=-0.73, p=0.01) and WC change (r=-0.64, r=0.03) (**Figure 2**). There was no association between changes in anthropometric measures and iron parameters.

Adipose tissue hepcidin and macrophage marker expression

AT samples were available from all study participants (N=26), who had a weight change range between a 1.6% weight gain and a 14.3% loss. Expression of AT CD68, a macrophage marker, was highly correlated with hepcidin expression before (r=0.68, p=0.001) and after (r=0.67, p=0.002) CR (**Figure 3**). Change with CR in AT hepcidin expression was also significantly correlated with change in AT CD68 (r=0.56, p=0.01).

In addition, baseline AT hepcidin expression was significantly correlated with AT CD14 ($r=0.53$, $p=0.02$) and TNFα ($r=0.46$, $p=0.05$), but not after CR. No significant associations were found between AT hepcidin expression and AT expression of IL-6, MCP-1, IL-2, IFNγ and leptin. There were significant correlations between CD14 and TNFα before ($r=0.55$, $p=0.01$) and after ($r=0.53$, $p=0.02$), as well as a correlation of change between these two markers ($r=0.62$, $p=0.004$). There was a trend for correlation between baseline TNFα and CD68 ($r=0.43$, $p=0.07$) and for correlation of change between these two markers ($r=0.42$, $p=0.07$). Using paired t test we saw no change in hepcidin expression or any of the immune markers after CR.

Discussion

In this study, reductions in weight and waist circumference correlated with decreases in serum hepcidin and CRP, but not with changes in iron parameters. At baseline, AT hepcidin expression was significantly correlated with sTfR, a marker of iron status not affected by inflammation, suggesting that AT hepcidin may contribute to obesity-associated dysregulation of iron homeostasis. Also, there was a strong association between AT macrophage marker CD68 and hepcidin gene expression before and after CR, suggesting that AT macrophages may be a primary source of hepcidin expression or participate in hepcidin regulation in AT.

Our results showed no significant change in circulating hepcidin, CRP or iron parameters with WL. This may be due to the wide range of WL, which is supported by the significant correlations between WL, hepcidin and CRP. In addition, some participants may have been at different stages of WL at the final assessment. For this

analysis serum samples were available only from subjects who lost at least 5% of their body weight. Including all participants may have allowed us to see more significant changes and perhaps even an effect on iron status as identified in other WL interventions.[27, 28] Also, given a lack of dietary data we could not account for the impact of dietary iron on iron status. Of note, when using a Tsat less than or equal to 16% as a definition of iron deficiency,[37] three subjects who were iron deficient before WL, became iron replete after the intervention.

We saw an inverse relationship between AT hepcidin expression and iron status as measured by sTfR (**Figure 1**), which is a marker of iron status not affected by the acute phase response and chronic inflammation.[11] This association, however, was subclinical because the sTfR values for this population remained within the normal range specified by the manufacturer (2.9-8.3 μg/ml). When iron deficiency anemia coexists with anemia of chronic inflammation, as one would expect in obesity, sTfR should be above the normal range.[38] Even though this is not the case, the observed correlation between AT hepcidin and sTfR is consistent with findings by Bekri et al, showing increased AT hepcidin and lower iron status in obese vs. lean patients.[24] Even though sTfR remained unchanged after WL, the correlation between sTfR and AT hepcidin that was observed at baseline was lost after WL; this suggests that AT hepcidin is influenced by WL differently than iron status. The contribution of AT hepcidin expression to hypoferremia in the setting of obesity and its relation to hepatic hepcidin expression require further investigation. Alternatively, liver hepcidin may be the main contributor of iron status impairment.

There were strong correlations between AT hepcidin and macrophage marker gene expression at baseline. After WL, hepcidin was still strongly correlated with CD68. The correlation between change in hepcidin and change in CD68 also was significant (**Figure 3**). This tight association with CD68 suggests not only that hepcidin is secreted to a greater extent by macrophages in AT of obese individuals, but they are affected in similar ways by CR. In addition, CD14 and, to a lesser extent, CD68 were correlated with TNFα suggesting a link between AT inflammation and macrophage markers.

Both CD14 and CD68 are markers of macrophages, and TNFα is a pro-inflammatory cytokine produced mainly by macrophages. CD14 is a receptor for lipopolysaccharide and is involved in innate immunity. CD68 is part of the scavenger receptor family and is involved in lipid metabolism and phagocytosis. CD68, CD14 and TNFα were associated with hepcidin at baseline. However, after WL only CD68 remained correlated with AT hepcidin expression. Due to the changes in AT architecture with WL, CD68 may be reflecting the changes in macrophage numbers and activity. CD14 and TNFα were associated with hepcidin at baseline, but not after WL. This suggests regulation of AT hepcidin expression with CR may differ from regulation of the CD14 and TNFα genes. Hepcidin expression was not associated with IL-6 expression. This was contrary to our expectations, as IL-6 has been shown to induce hepcidin through the JAK/STAT3 pathway,[22] and IL-6 AT expression has been correlated with AT hepcidin in obesity.[24] Perhaps IL-6 protein expression would correlate with the hepcidin peptide rather than mRNA, but this remains to be studied.

When analyzing all subjects, rather than dividing them by diet group, we found no significant changes with WL in AT hepcidin or immune marker expression. The reason

may be that the stage of WL at which AT biopsies were collected was not uniform. In fact, WL had not been stable for 9 of the participants included in this analysis at the time the final sample was collected (data not shown). Capel et al. have shown that the stage of WL - energy restriction, weight stabilization or weight maintenance, needs to be taken into consideration because adipose gene expression profiles differ between these stages.[39] Studies where a homogenous population of individuals loses weight at a similar rate for the same amount of time, and where there is strict control of their dietary intake, are challenging to perform but are needed. Other publications have shown that AT expresses hepcidin in obesity,[24, 25] but we have shown for the first time a direct association between AT hepcidin and macrophage markers before and after CR.

This study has several limitations, including heterogeneity of the population. However, this heterogeneity allows for generalizations that would otherwise not be possible in a more homogenous population. Other limitations include the difference in diet composition between the low fat and low carbohydrate group, although no significant differences were seen between diet group in our primary outcomes, and the lack of data pertaining to body composition, and dietary intake data. Strengths of this study include the significant amount of WL through CR in adults, and the complete assessment of iron status using several markers, including sTfR.

A role for AT hepcidin in iron homeostasis is suggested by our data but ultimately remains undefined. A recent study compared arterial and venous hepcidin expression from AT in obese and lean individuals. They showed no difference in hepcidin between arterial versus venous blood and concluded that AT does not significantly contribute to the iron status impairment seen in obesity.[40] However, their sample size was small and

their population had mixed iron status and hepcidin levels. In order to make a definite conclusion, obese individuals with high hepcidin and low iron status need to be compared with individuals with normal iron status, hepcidin and inflammation levels. A better understanding of the role that AT plays in iron homeostasis, along with further elucidation of the impact of obesity on AT architecture and gene expression profile, should eventually lead to therapeutic approaches to target hepcidin dysregulation in iron deficient obese individuals.

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Table 1. Population characteristics and anthropometric changes with weight loss.

Variable	N (Women=22, Men=4)	Before CR	After CR	Change (after-before)
BMI (kg/m^2)	26	36.1 ± 4.4	33.9 ± 4.3	-2.2 ± 1.3*
Weight (kg)	26	99.5 ± 13.9	93.2 ± 12.4	-6.2 ± 4.0*
Percent weight loss (%)	26	-	-	6.1 ± 3.8
Waist circumference (cm)	14	109.3 ± 9.9	101.5 ± 11.0	-7.8 ± 8.2**
Hip circumference (cm)	13	122.6 ± 11.3	114.2 ± 12.6	-8.4 ± 4.7*
Waist-to-hip ratio	13	0.89 ± 0.07	0.88 ± 0.08	-0.01 ± 0.05

Mean ± SD is shown. *$p<0.0001$, **$p<0.01$; paired t test.

Table 2. Systemic changes with weight loss in subjects that lost 5% or more body weight.

Variable	N	Before CR	After CR	Change (after-before)
Serum hepcidin (ng/ml)	14	33.3 (19.8, 59.4)	35.5 (27.4, 43.5)	0.8 (-13.8, 8.6)
Serum iron (µg/ml)	14	87.9 ± 36.8	84.6 ± 23.2	-3.3 ± 32.1
Tsat (%)	14	26.5 ± 11.6	25.8 ± 6.3	-0.7 ± 10.3
Ferritin	14	132.8 ± 104.1	129.6 ± 92.1	-3.1 ± 31.7
sTfR (µg/ml)	14	4.71 ± 0.89	4.69 ± 0.88	-0.02 ± 0.56
CRP (mg/L)	14	1.9 (1.4,7.0)	1.5 (1.2, 3.6)	-0.6 (-2.7, 0.1)*

Mean ± SD or median (Q1, Q3) is shown. *$p<0.1$; paired t test.

Figure Legends

Figure 1. AT hepcidin gene expression is positively correlated with sTfR at baseline. Partial correlation coefficient was used, adjusting for age and sex. Serum was available only from participants that lost at least 5% body weight (N=14).

Figure 2. WL and WC reduction are associated with a decrease in serum hepcidin and CRP. Partial correlation coefficient adjusting for age, sex and diet (low calorie and low fat, or low calorie and low carbohydrate) was used. Serum samples were available only from participants that lost at least 5% body weight (N=14**).** Change in WC (ΔWC) = WC after CR – WC before CR.

Figure 3. Expression of macrophage markers positively correlates with hepcidin expression in AT. Values before CR (triangles and dashed lines) and after CR (squares and solid lines) are shown. This analysis included all participants that finished the study for whom gene expression data was available (N=22). Partial correlation coefficient adjusting for age, sex and diet (low calorie and low fat, or low calorie and low carbohydrate) was used. Partial correlations before and after CR, as well as correlations of change (expression after CR – expression before CR), were calculated between AT hepcidin and AT CD68, CD14, or TNFα.

Figure 1.

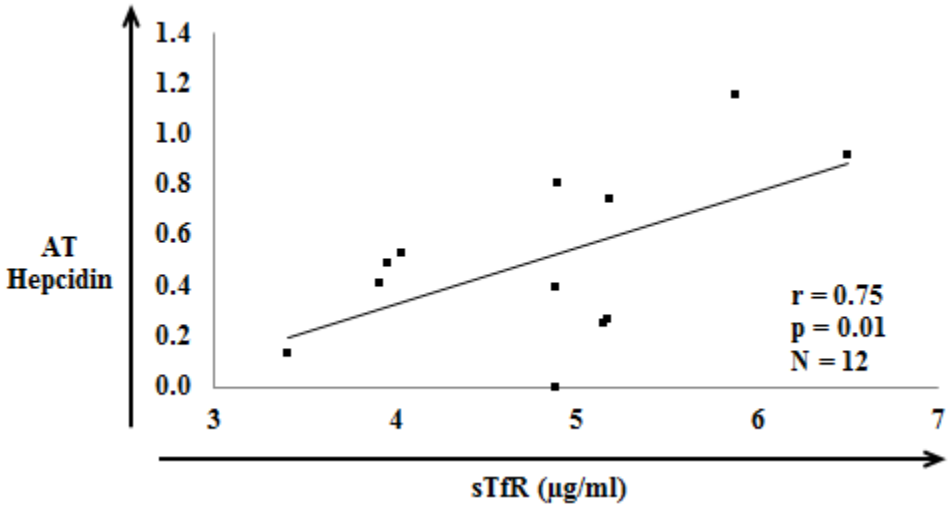

AT
Hepcidin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
4
5
6
7
sTfR (µg/ml)
r = 0.75
p = 0.01
N = 12

Figure 2.

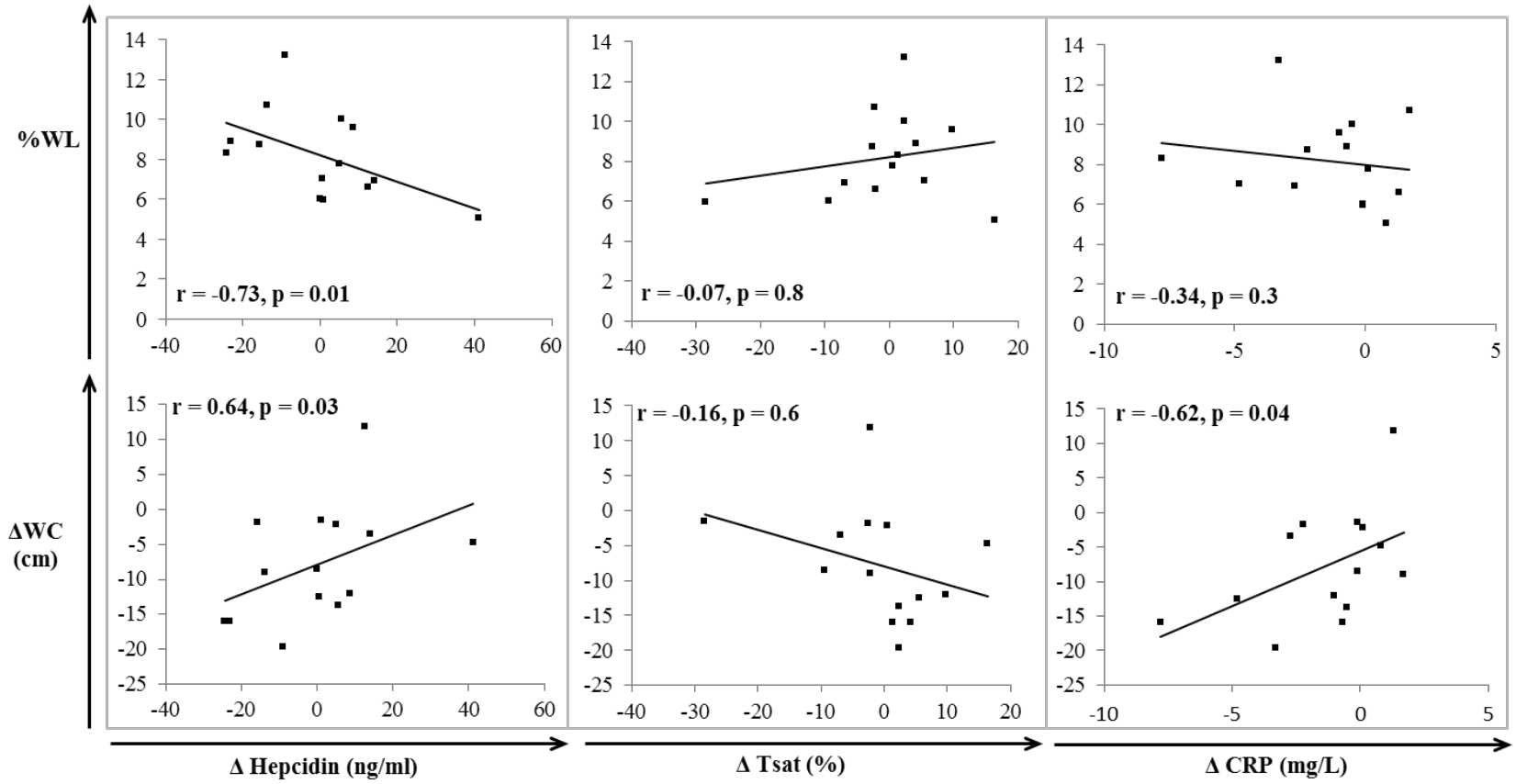
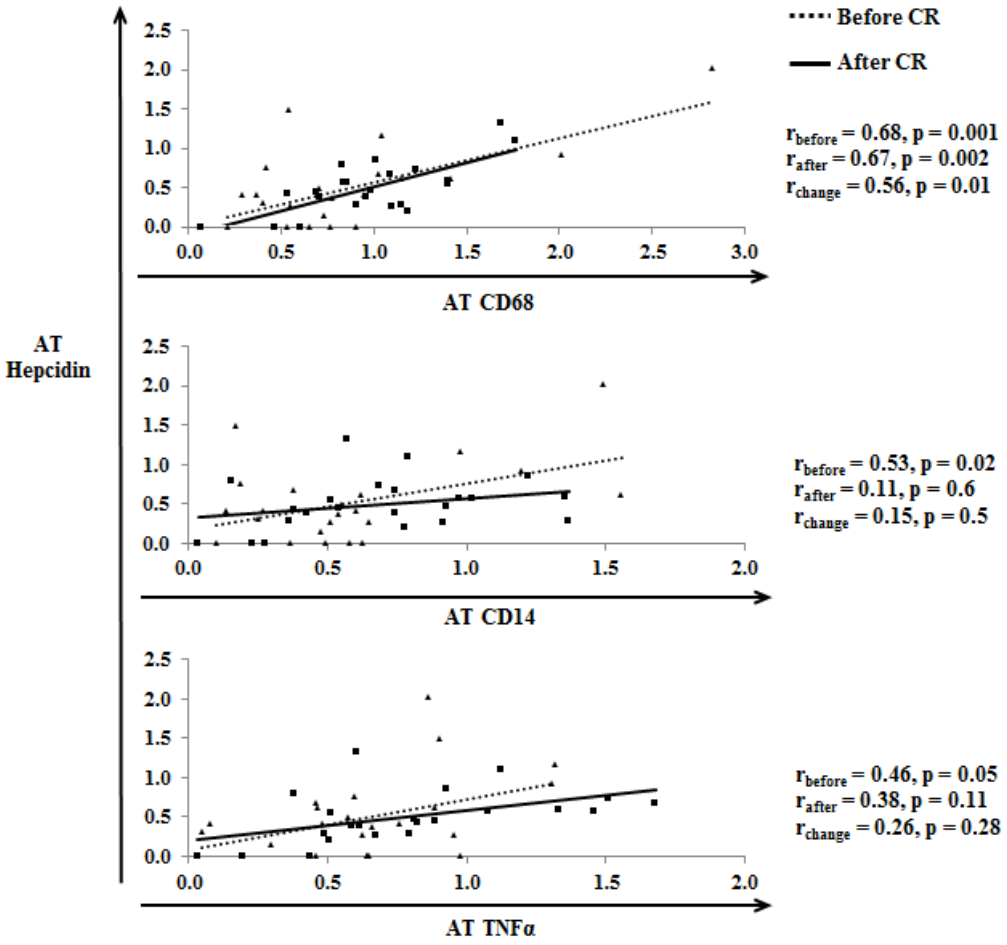

%WL
ΔWC (cm)
r = -0.73, p = 0.01
r = -0.07, p = 0.8
r = -0.34, p = 0.3
r = 0.64, p = 0.03
r = -0.16, p = 0.6
r = -0.62, p = 0.04
Δ Hepcidin (ng/ml)
Δ Tsat (%)
Δ CRP (mg/L)

Figure 3.


Before CR
After CR
AT Hepcidin
AT CD68
r_{before} = 0.68, p = 0.001
r_{after} = 0.67, p = 0.002
r_{change} = 0.56, p = 0.01
AT CD14
r_{before} = 0.53, p = 0.02
r_{after} = 0.11, p = 0.6
r_{change} = 0.15, p = 0.5
AT TNFα
r_{before} = 0.46, p = 0.05
r_{after} = 0.38, p = 0.11
r_{change} = 0.26, p = 0.28

Chapter V

Summary and Discussion

Conclusion and Future Directions

Summary and Discussion

Through this work we sought to determine the impact of weight loss (WL) through calorie restriction (CR) on iron homeostasis, inflammation and immune response of young and older adults. Our purpose has been to expand the current knowledge on the mechanism of obesity-associated dysregulation of iron homeostasis and immune response. To this end, we conducted two studies: a CR study in young and older obese women where we determined inflammation, hepcidin, iron status and immune response before and after WL (Chapters II and III), and a CR study in obese adults studying the effect of WL on serum and adipose tissue (AT) hepcidin, systemic inflammation, iron status, and AT expression of inflammatory and macrophage markers (Chapter IV). To complement this work, we have also conducted two cross-sectional studies included in the Appendix section: a study in elderly subjects from Ecuador, where we determined associations between BMI, iron status, inflammation and serum hepcidin; and a study comparing systemic inflammation, iron status and hepcidin between lean and obese pregnant women, and their cord bloods.

Chapter II findings

For the study in young and older women described in Chapter II, we measured iron status, serum hepcidin, and serum inflammatory cytokines before and after CR. We also assessed dietary intake of macronutrients, vitamins and minerals, and calculated heme and non-heme iron. To elucidate the effect of aging and WL on iron homeostasis and its association with immune response, we measured total hepcidin, ferroportin and

iron content in peripheral blood mononuclear cells (PBMC). We propose that PBMC also serve as a proxy for other tissues.

We found age-related differences in the relationship between iron status, BMI, inflammation and hepcidin at baseline. In the older group we found an inverse correlation between hepcidin and iron status, which would be expected of an obese population, while the opposite was true for the younger group. Our results suggest that the relationship between iron status and hepcidin in the elderly is dominated by chronic inflammation of obesity and aging to a greater extent than iron intake, while the opposite seems to hold true for the young group. In addition we found hepcidin to be significantly higher in the older group. These age differences suggest that obesity has a greater impact on iron homeostasis in the older group than the young group. The older group experienced an improvement in iron status and a reduction in IL-1β, but not hepcidin, with CR. Even though the young group had a significant increase in iron intake they underwent no significant improvement in iron status. After WL, the iron status in the older group was significantly higher than the young group.

Inflammation, measured as IL-1β, was correlated with PBMC hepcidin in the young group. In the older group, PBMC ferroportin was inversely related with IL-1β and total intracellular iron, suggesting that PBMC hepcidin and ferroportin are impacted by inflammation of obesity. In conclusion, this chapter shows that moderate WL is not sufficient to induce significant changes in hepcidin, but it may induce significant changes in iron status and inflammation in older individuals. PBMC iron homeostasis may be influenced by inflammation of obesity, but studies with lean controls are needed to corroborate this.

This is the first study showing the effect of aging and WL through CR on hepcidin, iron status and inflammation. We also show for the first time hepcidin and ferroportin expression in PBMC subpopulations of obese individuals and their relationship to systemic inflammation. We uncovered a role for IL-1β that is perhaps more relevant than that of IL-6 in obesity-associated dysregulation of iron homeostasis.

Chapter III findings

In Chapter III we 1) compared PBMC subpopulations between young and older subjects, before and after WL, 2) measured lymphocyte proliferation upon mitogenic or antigenic stimulation, 3) determined hepcidin protein levels in total or CD3+ lymphocytes under different stimulation conditions, and 4) correlated hepcidin expression with lymphoproliferation. All assays were done either in 5% FBS or 10% autologous serum (10% HS).

In this study we found age-related differences in immune phenotype and function. Older subjects had lower CD8+ T and NKT cells, which play a role in cytotoxicity. The number of NKT cells increased after WL for the older group, but the clinical significance of this needs to be studied further through the use of cytotoxicity functional assays. CD4+ T cells are known to decrease with aging, but in this study the young group had significantly lower T cells than the older group. In addition we found no difference in lymphocyte proliferation upon antigenic stimulation between the two groups. These results suggest that obesity in young individuals may negatively impact immune function to the extent of resembling immune senescence.

Even though hepcidin mRNA has been shown to become upregulated with CD3/CD28 stimulation,[1] we found no significant hepcidin upregulation with antigenic stimulation in PBMC cultured in FBS or human serum. We did observe a significant increase in hepcidin levels with mitogenic stimulation. This impaired response of hepcidin may be due to obesity and needs to be studied further by comparing hepcidin expression in stimulated lymphocytes between lean and obese groups. Finally, we found a strong correlation between hepcidin expression and proliferation of lymphocytes stimulated with anti-CD3/CD28 in older, but not young, subjects. This supports previous evidence suggesting that hepcidin is involved in lymphocyte proliferation. The impact of aging, however, needs to be studied further. With this section of the study we have found for the first time an association between hepcidin and immune response in the context of obesity. We have also established unexpected similarities between the immune system of obese young and older individuals that require further study. Additional studies are also needed to elucidate the specific role of hepcidin in lymphocyte function.

Chapter IV findings

For Chapter IV we conducted a CR study in obese adults. WL in this study was greater than in the previously explained study, and there was also a significant reduction in waist circumference. We measured BMI, waist circumference, iron status, inflammation, serum hepcidin, AT hepcidin and AT markers of inflammation and macrophages before and after WL. There was a significant inverse relationship between AT hepcidin and iron status, which suggests that AT hepcidin either may be involved in obesity-associated iron status dysregulation or may be impacted by obesity. In addition,

we found strong positive correlations between AT hepcidin and AT macrophages and inflammation markers before and after WL, suggesting not only that AT hepcidin and macrophages are impacted similarly by WL in obese people, but also that macrophages may be the primary source of hepcidin in AT. Bekri et al. had found that adipocytes were the main producers of hepcidin in AT,[2] but in AT fraction separation the adipocyte fraction is commonly contaminated with cells from the stromal vascular fraction. In this study the authors did not measure macrophage markers in the adipocyte fraction to ensure that there was no contamination. It seems likely that adipocytes produce hepcidin, but we argue that AT macrophages are the major source of hepcidin in AT. They confirmed successful tissue fraction separation by measuring enrichment of the GLUT4 gene in the adipocytes, but they did not measure macrophage genes in both fractions.

Through this study we found that 5% WL is sufficient to induce a decrease in hepcidin and inflammation, but not an increase in iron status. This is consistent with our observation in Chapter II, where there was no significant change in iron status in the young group, but waist circumference reduction was associated with decrease in IL-1β. In this section we have: 1) shown that WL can lead to decreases in hepcidin in obese adults, 2) provided the first evidence of the relationship between AT hepcidin and low iron status in obesity and, 3) contrary to previous evidence, we have identified macrophages as the likely main source of hepcidin in AT. Further research is needed to establish the extent of the contribution of AT hepcidin in iron status dysregulation in obesity.

Limitations

The main limitations for the two CR studies presented are the small sample size, the lack of data on body composition or iron absorption, and not having a lean control group. In the study presented in Chapters II and III there was moderate WL and no significant reduction in central adiposity, and there likely was underreporting of dietary intake. In fact, our results show that the older group did not have a significant decrease in total calorie intake even though they lost weight. We speculate that there was underreporting, especially at baseline. Obese groups tend to under report dietary intake,[3, 4] so the recorded data may not be an accurate portrayal of habitual dietary intake. Conversely, the subjects may have reported accurately but due to their motivation to lose weight, they may have begun dieting earlier than the study specified. Finally, we had little control over the participants' diet, as these subjects were recruited from a weight loss clinic and followed diets based on advice by the clinic's dietitians. Future studies are needed that ensure constant iron intake throughout the intervention. Meal replacement interventions would be ideal to increase diet uniformity. Additional limitations of the study in Chapter IV are that this study was part of a larger CR intervention designed to compare a low fat with a low carbohydrate diet. Given recent evidence that high fat diets may inhibit iron absorption independently of the hepcidin-ferroportin mechanism,[5] diet diversity may have confounded the study. We addressed this by adjusting for diet group in our analysis.

Appendix section

In the Appendix section of this dissertation we have included a review on micronutrient deficiency and immune senescence in elderly populations from less developed countries, which delineates 1) the lack of data on the prevalence of micronutrient deficiency in elderly populations from less developed countries, 2) how micronutrient deficiencies exacerbate the already impaired immune response of older individuals, 3) and how the nutrition transition, together with the demographic transition towards an older population worldwide, have introduced new burdens to the health of these individuals.

In the second section of the appendix we show a cross-sectional study conducted in an elderly population in low-income periurban areas of Quito, Ecuador. In this study we measured the association between BMI and central adiposity with inflammation (CRP), serum hepcidin, and iron status (serum iron). We found that BMI and central adiposity were positively correlated with inflammation and hepcidin in women. However, we found no correlation with iron status. This was a cohort with iron consumption that was lower than the iron DRI for this age group (>65y). If we were to apply the hypothesis suggesting that hepcidin levels are independently regulated by inflammation and iron status/requirements, and are determined by the strength of these signals,[6] then we would conclude that hepcidin levels in this population are more influenced by true iron deficiency caused by poor diet, than by chronic inflammation seen in the overweight elderly.

The final section of the appendix is from a study conducted at Tufts Medical Center comparing iron status, hepcidin and inflammation between obese and lean

pregnant women and their cord blood. It was previously known that hepcidin during pregnancy is kept at very low levels in order to maximize iron transfer to the fetus.[7] We showed that inflammation and hepcidin in obese pregnant mothers is significantly higher than lean mothers. Even though maternal serum iron status did not differ between groups, cord blood serum iron status was significantly lower in the obese group, suggesting that fetal iron transfer may be impaired in obesity. We further showed that maternal BMI and maternal serum hepcidin were inversely related with cord blood iron status. This study is cross-sectional and further research is needed on the mechanism of placental iron transfer in lean mothers compared to obese.

Conclusion and Future Directions

In conclusion, WL through CR has the potential of inducing changes in iron homeostasis and inflammation in young and older subjects. Aging has an effect on: 1) immune phenotype and response, 2) hepcidin expression and association with iron status in the context of obesity, and 3) the impact of WL on iron status and inflammation. Our results are consistent with the hypothesis that the strongest of the different signals that regulate hepcidin, namely inflammation, iron status and erythropoietic requirements, determines the level of hepcidin expression. Furthermore, we showed that obesity may have a similar impact on immune response as aging.

Future studies are needed to investigate the impact of adipose tissue hepcidin on obesity-associated iron status impairment, and the effect of obesity on hepcidin expression in stimulated PBMC. Comparisons between lean and obese groups are needed, especially pertaining to PBMC function and the role of hepcidin. Comparisons

between well-nourished populations and those suffering from the double-burden of malnutrition are also needed.

It is possible that alternative pathways to the one we have suggested are partly responsible for the dysregulation of iron homeostasis seen in obesity. Inflammation may be having a direct effect on the expression of proteins involved in iron homeostasis other than hepcidin. For example, ferroportin is directly downregulated at the transcriptional level by inflammatory cytokines in monocytes.[8, 9] We showed in Chapter II that IL-1β was inversely correlated with PBMC ferroportin, supporting this alternative hypothesis. Further research should determine the relative contribution of this pathway with respect to that of hepcidin upregulation. In addition, we have not evaluated the effect of insulin which can stimulate iron intake by adipocytes through a mechanism involving translocation of transferrin receptor to the plasma membrane.[10] Another potential explanation for iron deficiency in obesity is impaired iron absorption through a mechanism independent of ferroportin. Sonnweber et al. showed that high fat diets in mice impair iron absorption.[5] Evaluation of the effect of CR and diet composition on iron absorption is required.

Finally, another alternative hypothesis for iron deficiency in obesity is that iron requirements increase with obesity. Other studies should determine the validity of this hypothesis. It seems unlikely, however, because iron supplementation studies in obese populations have shown that obese subjects have lower iron absorption compared to lean subjects.[11] In addition, lower iron status has been found in obese populations that consume diets with the same iron bioavailability as lean groups.[12, 13]

This research has contributed to the elucidation of the mechanism of iron homeostasis and immune dysregulation in obesity and aging. We have for the first time measured changes in hepcidin, iron status, inflammation, immune response, and AT hepcidin with CR within the same study. We offer the first evidence of ferroportin and hepcidin expression in different PBMC subpopulations in the context of obesity and aging. Furthermore, our results are novel in that they suggest for the first time that the immune response of young obese individuals may resemble immune senescence.

With this project we have addressed important public health problems that are rapidly developing worldwide. Our results suggest that the extent of immune response impairment in young obese individuals warrants preventive care against chronic and infectious diseases. We have also identified WL through CR in older individuals as a possible therapy to enhance iron status and decrease inflammation. Given the small sample size in our cohort, however, larger controlled interventions are needed. Iron deficiency is the most prevalent micronutrient deficiency worldwide, and it will only increase with the obesity epidemic if not addressed. This issue is of particular importance for vulnerable groups, such as the elderly and children, but also women of reproductive age.

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Chapter VI

Appendix

Appendix

Part I

Micronutrient status, immune response and infectious disease in elderly of less developed countries

Micronutrient status, immune response and infectious disease in elderly of less developed countries

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Introduction

The world's population, especially in less developed countries, is expected to continue expanding. This growth is occurring together with a demographic transition due to increase in lifespan, and decrease in mortality and fertility. As a result, the number of people aged above 60 years in less developed countries is expected to increase from a current 8% to 20% in 2050, with the group above 80 years growing almost five fold [1]. When using country median age as an indicator of ageing, the 2006 Revision of the U.N. World Population Prospects shows that the overall world population will age (**Figure 1**) and that this shift will occur mainly in developing countries. But, even though lifespan has increased, quality of life has not improved for this age group [2], leading to unhealthy ageing and increased morbidity. As it has been eloquently expressed on the 1995 State of World Health, "For most of the people in the world today every step in life, from infancy to old age, is taken under the twin shadows of poverty and inequity, and under the double burden of suffering and disease. For many, the prospect of a longer life may seem more like a punishment than a prize." A primordial objective is not only to increase lifespan but to achieve successful ageing, which is defined as minimizing the time between the onset of illness and death [3].

Elderly in the less developed world play an important role in society and in their country's economy [4]. However, they are vulnerable to malnutrition and suffer from infectious diseases. Additionally, in the past years many elderly from less developed countries have experienced an increase in chronic diseases as a consequence of the double burden of malnutrition [5, 6]. The increase of infectious and non-communicable

disease within this expanding population has translated into poor quality of life and an increased burden on the healthcare systems of their countries.

Subclinical levels of micronutrients have been associated with impaired immune function in people above the age of 60 [7], and it has been found that micronutrient supplementation and improved nutrition can enhance immune function [8, 9]. It is important, however, that more information is obtained on regional micronutrient status and the benefits a nutrition intervention might have. Such studies on elderly populations in the developing world are limited. Often data obtained from developed countries is applied to less developed countries, or has been extrapolated from younger age groups within the same country. Many aspects of populations in developed countries are not applicable to groups in less developed countries. Also, changes in health, physiology, and immune function during ageing make younger groups an inappropriate model for an older group.

In this review we will describe age-associated changes in immune response, summarize the impact of micronutrients on immune status in the elderly, assess the current micronutrient status of the elderly in less developed countries and its relation to their immune response, and review current interventions to determine what they teach us for improving the health outcomes of this growing population, as well as the obstacles we need to overcome.

Ageing and immunity

Both innate and acquired immunity weaken with age even in “healthy” elderly [10]. Age-related changes detrimental to the immune system include thymic involution, poor

response to vaccinations, impaired response to evolving pathogens and newly encountered antigens, increased vulnerability to infection, increased autoimmunity, and inflammation [11-13].

The most widely studied cells of innate immunity with respect to ageing are macrophages. Some, but not all studies have shown that macrophage chemotaxis, phagocytosis, cytokine production and bone marrow population are compromised with age [14]. Macrophages are part of the defense barrier in the skin; they detect pathogens and defend the body against bacteria [14]. Because skin is affected with ageing, its efficiency as a protective barrier declines, and along with it macrophage function is altered. This change leads to increased colonization of bacteria and yeast on the skin and on mucosal surfaces [14]. Wound repair is also affected with ageing partly due to delayed macrophage infiltration and function. This causes delayed symptom manifestation and diagnosis of infection, which exacerbates disease [14]. Therefore, in general, macrophage ability to fight infection is impaired and there is deregulation of the molecules they produce. For example, production of prostaglandin E2, an inflammatory molecule which has been shown to suppress T cell function in aged [15], as well as contribute to several chronic diseases associated with aging such as cardiovascular and inflammatory diseases, is increased with age [16, 17].

Many aspects related to changes in other innate immune system cells such as neutrophils, eosinophils, mast cells and NK cells remain undiscovered or controversial. However, it is known that neutrophil phagocytosis and superoxide (O2-) and hydrogen peroxide (H2O2) production is impaired, and mast cell number seems to decrease in the skin [18]. Also, eosinophil function becomes impaired, leading to increased responses to

allergens, explaining in part the exacerbation of asthma with older age [18]. Dendritic cell (DC) function decreases with age [19] and chemotaxis may be compromised [18] but it is not clear whether the number of DCs decreases [11].

The adaptive immune system, whose main players are T and B cells, is widely modified with ageing. Even though peripheral B cell number and secreted immunoglobulin levels stay constant with age there is impaired naïve B cell production [11], less affinity from antibodies to antigen, and more autoantibodies produced [20]. These changes may reduce the response to newly encountered antigens [19].

It has been established that that T cell decline is the main cause of immune senescence [3, 19]. There is reduced number of naïve T cells, and an expansion of memory T cells [11]. This imbalance results in part from thymic involution and the expansion of memory T cells as a result of persistent or latent pathogens [13]. The imbalance leads to a decreased response to new antigens, such as new strains of influenza [11]. Additionally, CD4 T helper cell number and function declines, and CD8 cytotoxic effector T lymphocytes (CTLs) have reduced intensity in their response against influenza vaccine and less interferon-gamma (IFNγ) production during viral infections [19]. Influenza is the fifth cause of death in people older than 50 years [19]. This age group is a target for vaccination campaigns, but influenza virus vaccines have only 30–40% efficacy in the elderly [11, 13].

In addition to age-associated immunological changes which predispose the elderly to higher incidence of infectious diseases, a recent report by Gay et al. [21] showed that passage of an avirulent coxsackie B3 virus (COXB3-0), which normally does not cause morbidity and mortality in young mice, through old host resulted in several mutations in

the virus which increased its virulence, transforming into a morbidity and mortality causing virus for the young mice. These results indicate that in addition to immunological changes, increased viral virulence in the aged host could contribute to their higher susceptibility to infection. Given that Beck et al. [22] have shown that micronutrient deficiencies such as those of selenium and vitamin E also increase viral virulence, and there is a high prevalence of nutritional deficiencies in less developed countries and an increasing number of older people in these countries, these findings could have significant public health implications worldwide and emphasize the need to address nutritional deficiencies in the elderly of less developed countries.

Micronutrient status, ageing and immunity

Adequate nutritional status is essential for efficient immune function. Investigating this relationship and its relevance to ageing is of great importance for the health of elderly and for disease prevention [8]. Many postulate that improvements of nutritional status in elderly populations will enhance their immune system [8, 20]. In turn, this would lead to enhanced nutritional status by preventing consequences of infectious disease, such as nutrient malabsorption, nutrient and energy store loss, and reduced appetite [3]. There is a large body of evidence on the potential benefits micronutrient enhancement can have for the ageing immune system. However, much of this evidence comes from the developed world and has yet to be extended to less developed countries.

There are several comprehensive reviews of micronutrient supplementation studies in the elderly [3, 23]. Single nutrient supplementation studies have shown improvement in the immune response of the elderly. These nutrients include vitamin B6

[24, 25], vitamin C [26, 27], vitamin E [9, 28, 29] and zinc [30, 31]. Also, there may be a role of vitamin D in age-related deregulation of the immune response in elderly [32, 33]. However, more studies relate to this topic are needed. Additionally, there are studies supporting that certain micronutrients, such as antioxidants, be given as a mixture so that they work synergistically and to prevent an imbalance that may lead to pro-oxidant production [34]. However, such evidence of supplementation in the elderly remains controversial. A systematic review by Stephen and Avenell [35] showed that there was no significant effect of micronutrient mixture supplements in the elderly. But, subgroup analysis within that study showed that elderly undernourished at baseline and consuming supplements for 6 months experienced the greatest benefit. A high proportion of elderly in developed countries take multi-vitamins, which may bias results of an intervention trial. Therefore the lack of the effect observed following micronutrient supplementation in developed countries might not be applicable to less developed countries.

A randomized controlled trial in which Girodon et al. [36] supplemented institutionalized elderly with zinc, selenium, and vitamins A, C, and E for two years showed significant improvement in antibody production in response to influenza vaccine in groups receiving single supplements or combinations thereof. Also in this study a correlation was observed between zinc and selenium supplementation and reduction of respiratory infections. This study suggests that elderly vaccine response can be enhanced through micronutrient supplementation, which would not only prevent disease and malnutrition but also reduce the economic strain on the health care system by decreasing costs associated with hospitalization.

The nutritional status and presence of other diseases could influence the impact of nutritional intervention on immune response and resistance to infection in elderly. For example, Graat [34] showed that supplementing a group of elderly individuals from the Netherlands replete with vitamin C and E with a 200 mg daily of vitamin E had no effect on infectious disease outcomes. On the other hand, a vitamin E trial by Meydani et al. [37] showed that supplementing nursing home residents with 200 IU per day of vitamin E reduced upper respiratory tract infections, such as the common cold. Differences in disease status, genetic background, as well as study design and documentation of infection could have attributed to the observed [38]. Further analysis from the study by Meydani et al. [39] also found that low serum zinc levels in this population correlated with higher incidence and duration of pneumonia. A recent study also showed that genetic variation in cytokine genes can influence the impact of vitamin E supplementation on cytokine production in elderly [40].

Finally, research on probiotic supplementation to prevent or reduce infection is being developed and offers potential for immune system enhancement. Probiotics help maintain immunologic balance in the mucosal sites of the body, protect against pathogens, and it has been suggested that they help restore impaired innate immunity in the mucosal epithelia [41]. Long-term daily supplementation of 479 healthy adults (18–67 years old) with vitamins and minerals, with or without probiotics, did not show a difference in the incidence of common colds; but, shorter duration of colds, decreased severity of symptoms, and enhanced T cell responses were observed [42]. Another study showed that elderly supplemented with probiotic supplements had higher antibody titers after influenza vaccination than those given a placebo, indicating the potential of

probiotics in enhancing response to vaccine in the elderly [43]. Since the "indigenous microbiota" population in the intestinal mucosa changes with age [41], probiotic supplementation, together with micronutrient supplementation may have a positive impact on elderly immunity. In summary, the studies described above conducted in developed countries indicate that micronutrient and other dietary interventions could be of benefit to the elderly of less developed countries in protecting the elderly against infection and other immune/inflammation related diseases [11], minimizing the number of years in a person's life during which he or she will suffer from recurring disease (**Figure 2**) and allowing healthy ageing.

Micronutrient status, immune response and infectious diseases in elderly from less developed countries

The most prevalent and targeted causes of malnutrition worldwide are protein energy malnutrition and vitamin A, iodine, iron, and zinc deficiency. Data on micronutrient status in less developed countries is abundant for vulnerable groups, particularly children and pregnant women, but scarce in the elderly. The reasons for less developed country elderly vulnerability to malnutrition include poor diet, food insecurity, lack of public health measures and low allocation of government funds to the health care system [44], resulting in higher incidence of diseases. In many less developed nations consumption of foods from animal origin is very low due to inaccessibility and/or religious practices, limiting micronutrient consumption. This scenario also translates into low protein consumption, which has been shown to impair the immune system in the elderly [45]. In addition, many regions have high consumption of phytates, lowering further

the absorption of minerals in a group that already has limited access to nutrients. All these factors lead to higher incidence of communicable diseases and, due to the nutrition transition and consumption of low quality (empty calories) food, to obesity and chronic disease as well [6].

In order to target appropriate micronutrient interventions and develop effective public health measures more detailed nutritional data is needed from less developed nations. Nutritional status, even though generally impaired in poor elderly populations, differs greatly between regions. The recommended micronutrient dosages to be used in supplementation in less developed countries, listed elsewhere [46], divide individuals into three age groups: 1 to 3 years, 4 to 13 years, and >14 years. Elderly are grouped together with adults, but due to their impaired nutrient absorption and intake, not to mention disease status, they may require different doses for certain nutrients.

Table 1 summarizes studies that have reported micronutrient status in less developed country elderly and the relationships between micronutrients and immune response and infection. From this limited data it is clear that micronutrient deficiencies vary greatly from region to region, even within the same country. Even though vitamins C and E play an important role in immune function and in the prevention of chronic diseases due to their antioxidant properties very little data has been acquired from elderly in less developed countries on these micronutrients (Table 1). Hamer et al. reported that 92.4% of elderly Ecuadorians were deficient in vitamin C and that plasma vitamin C and zinc levels correlated with impaired immune cell's ability to produce IFNγ. A little more is known about B vitamins, which are important in the development of chronic disease, anemia, and cognition impairment, and are involved in a wide array of cellular functions,

including immune response. Vitamin B12 deficiency is common in the elderly both in developed and less developed countries. In the less developed world, however, there is higher prevalence and it starts earlier in life [5, 47] because of low dietary intake and other environment factors. *Helicobacter pylori* infection has been identified as one of the causes for poor vitamin B12 absorption. There is evidence that probiotic supplementation can help displace harmful bacteria and repopulate harmless or beneficial intestinal flora. Parasitic infections are common in less developed countries both among children and the elderly. Hamer et al. found that most elderly Ecuadorians in their study had parasites [5]. In addition, as shown in table 1 other B vitamin deficiencies are also prevalent. For example, riboflavin deficiency has been found in several countries in the past few decades.

Iron Deficiency Anemia (IDA) affects about one quarter of the world's population [48]. The causes for this deficiency in elderly include low iron intake, high levels of dietary phytates and low animal food consumption, as well as atrophic gastritis, intestinal atrophies and in some instances *Helicobacter pylori* infection [49]. National data on elderly anemia prevalence is missing from many countries. According to Deitchler et al. [50] only three countries out of 12 countries studied, Indonesia, Laos, and the Philippines, have recorded anemia prevalence in the elderly and found it to be greater than 30%. In a study by Hamer et al. [5] it was found that 39% of the elderly Ecuadorians in the study had serum iron below the reference range. Furthermore, they found that iron status correlated with immune cell ability to produce interleukin-2 (IL-2).

Several organizations and governments estimate micronutrient status in specific regions, but direct measurements are scarce. For example, McLean et al. [48] gathered

global and regional data from the WHO Vitamin and Mineral Nutrition Information System for 1993-2005 and determined anemia prevalence in different vulnerable groups based either on actual data or estimations. Data on the elderly were unavailable in almost every country, so only global estimations were made. It was determined that 24%, or 163 million, of elderly were suffering from iron deficiency anemia, with the highest proportion being in low-income countries.

Zinc deficiency is prevalent in elderly of both developed and less developed countries. Hamer et al. [5] reported that close to 50% of elderly Ecuadorians had low serum zinc level and low serum zinc levels correlated with low IL-2 and INFγ levels. Interestingly, Meydani et al. [39] reported that 30% of nursing home residents in US also had low serum zinc levels which were associated with higher incidence of pneumonia. Results from study by Hamer et al. indicated that elderly Ecuadorians had much higher prevalence of micronutrient deficiencies compared to those living in US and that corresponded with their lower immune response compared to those living in US. For example, zinc deficiency was found to be two to three times (depending on whether independently living or NH residents) higher in elderly Ecuadorians compared to those in US, and their delayed type hypersensitivity response (a measure of cell-mediated immunity) was half that of their counterparts in US. Hamer et al. also showed that elderly Ecuadorians have higher incidence of infectious disease than those living in US and that a significant correlation exist between micronutrient deficiency and infection in these elderly. While several factors including sanitation could contribute to higher incidence of infection in elderly Ecuadorians compared to US elderly, these data point to micronutrient deficiencies as an important contributor.

Summary and Conclusions

In summary, the elderly population is increasing worldwide and is suffering from the double burden of disease, i.e. both chronic and infectious diseases, and as such face significantly more health problems compared to other age groups. Many factors contribute to higher susceptibility of infection in elderly (**Figure 3**), chief among them are age-related immunological changes. In addition recent data suggest that the environment of old host might increase viral virulence and the morbidity and mortality caused by them. The limited data available suggest that the elderly in less developed countries suffer from high prevalence of several micronutrient deficiencies. Furthermore, these reports indicate that the prevalence and type of micronutrient deficiencies differ by region, and are correlated with low immune response and high incidence of infection. Micronutrients are needed for immune response and their deficiency not only impairs the immune response, but could also increase viral virulence by causing mutations in the virus. Thus, the combined impact of immunological defects, increase viral virulence in the aged, and presence of micronutrient deficiencies in the aged not only puts the elderly of less developed countries at high risk of infection and morbidity and mortality from them, but could pose a public health problem for all age groups by helping spread of more virulent viral species. Therefore, there is an urgent need to address the nutritional problems of elderly of less developed countries so that effective intervention strategies can be devised. Region-specific studies to determine micronutrient status are needed so that cost-effective supplementation strategies could be proposed, and for those countries in which specific deficiencies have already been described, studies are needed to

demonstrate the efficacy of specific micronutrient supplementation to improve immune response and decrease infectious diseases.

Even though much data remains to be gathered with respect to micronutrient status in elderly from less developed countries, especially as it pertains to immune response, some initiatives have been taken to improve elderly health and quality of life. In response to the demographic transition and the economic strain of elderly disease, Chile's government developed a program to distribute a micronutrient mix fortified with vitamins and minerals to low-income elderly [51]. It would be interesting to determine whether this program has led to disease prevention and improvement of Chilean low-income elderly nutritional status and quality of life.

In conclusion, there is an urgent need for acquiring more data on nutritional status of elderly in less developed countries and implementing specific interventions. Generation of this information will improve nutritional status of elderly in a cost-effective manner, which in turn could result in reduction of both infectious and chronic diseases, increase health span, improved quality of life in this age group and significant saving of health care resources in these countries. Furthermore, improving the nutritional status of elderly in less developed countries could reduce the global burden of infectious disease.

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Table 1. Studies on micronutrient status in less developed countries.

Location	Reference	Age (yr)	N	Study Description	Findings - Micronutrient Status	Immune Response Findings
Central Guatemala	Boisvert et al, 1983	50+	433	Cross-sectional. Dietary assessment and riboflavin status.	Riboflavin deficiency prevalence was 50-76%. Levels correlated with milk intake. Small intervention trial revealed strong correlation between riboflavin status and dietary intake.	Study did not look at immune response
Bangkok, Thailand	Prayurahong et al, 1993	NA (elderly)	147	Cross-sectional Study. Hematological data.	15% of subjects had anemia, 21% were folic acid deficient, and 7% were B12 insufficient.	Study did not look at immune response
Zimbabwe	Allain et al, 1997	65+	278	Cross-sectional Study. Hemoglobin, folate and B12 levels in rural and urban elderly.	Anemia seen in 23% of subjects, 30% had low folate level, and 13% had low serum B12 level. Folate was lower in urban subjects and B12 was lower in rural subjects.	Study did not look at immune response
Chile	Olivares et al, 2000	60+	274	Cross-sectional Study. Anthropometric measurements and biochemical measures of iron, copper, folate, vitamins B12 and A and C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR).	5% men and 4% women were anemic. Abnormal serum retinol was seen in 14% of men and 16% of women. Folate deficiency was 50% in men and 33% in women. B12 deficiency seen in 51% of men and 31% of women. Almost no iron and copper deficiencies.	10% subjects had inflammation (high ESR and CRP values, and high white blood cell count). They had higher prevalence of anemia (22% men and 32% women).
Chile	Bunout et al, 2001	70+	98	Randomized controlled trial; lasted 18 months. Micronutrient supplement with or without exercise.	Compliance with supplement was 48%. Supplemented, and supplemented + exercise maintained weight, lean mass, bone mineral density, serum cholesterol, and had greater muscle strength.	Study did not look at immune response
Chile	Hirsch et al, 2002	70+	108	Prospective study. Six months after folic acid fortification started determine effect on folic acid and B12 status and plasma homocysteine	Folic acid increased, plasma homocysteine decreased, and B12 stayed the same. Authors recommend elderly B12 supplementation.	Study did not look at immune response
Chile	Bunout et al, 2004	70+	60	Randomized controlled trial. Micronutrient (vitamin E, B12, folate), probiotic and protein supplementation, and placebo.	Micronutrient status was not reported in this study.	Response to influenza and pneumococcal vaccination. They observe enhanced NK cell activity. There was no change in IL-2 production. Subjects on supplement reported less infections, measured by scheduled hospital visits.
Cape Town, South Africa	Charlton et al, 2001	68.9 (SD=5.7)	148	Cross-sectional Study. 24-hr recall and anthropological measurements.	About one third had intake <67% RDA. Low intakes of calcium, vitamin D, zinc, and B6. Low fruit and vegetable consumption. Over half of women and 18% men were obese.	Study did not look at immune response

Beirut, Lebanon	Sibai et al, 2003	65+	200	Cross-sectional. Questionnaires, anthropometric measurements, hematological and biochemical analyses	Deficiencies in zinc, magnesium, alpha-tocopherol, and vit A, D and B6 were observed in both institutionalized and free-living elderly. Also, they were anemic and had low albumin levels.	Study did not look at immune response
Cape Town, South Africa	Charlton et al, 2005	72.7 (SD=8.3)	285	Cross-sectional. 24-hr recall, plasma micronutrient levels, anthropometric measurements.	Micronutrient levels (Thiamin, riboflavin, niacin, vit B6, folate, panthotenate, biotin, vit C, calcium, iron, magnesium, phosphorus, zinc, copper, and selenium) were inversely related to added sugar intake. Sugar has a nutrient-diluting effect.	Study did not look at immune response
Taiwan	Cheng et al, 2005	65+	2373	Crossectional Study (Elderly NAHSIT). Plasma retinol and tocopherol measurements.	Low prevalence of plasma retinol or a-tocopherol deficiency.	Study did not look at immune response
Taiwan	Wang and Shaw, 2005	65+	2354	Crossectional Study (Elderly NAHSIT). Plasma iron measurements.	Low prevalence of iron deficiency or iron deficiency anemia in men and women. Some subjects had elevated iron stores.	Study did not look at immune response
Taiwan	Wang et al, 2005	65+	1911 (diet); 2225 (plasma Mg)	Crossectional Study (Elderly NAHSIT). 24-hr recall and biochemical measurements of magnesium.	Dietary magnesium intake was about 70% of DRI, and 8-9% had low plasma magnesium levels. Magnesium levels and diabetes inversely related.	Study did not look at immune response
Taiwan	Yang et al, 2005	65+	2379	Crossectional Study (Elderly NAHSIT). Biochemical measurements of thiamin and riboflavin.	17% men and 14% women were thiamin deficient. 6.6% men and 4% women were riboflavin deficient. A large proportion (>11% for thiamin and >20% for riboflavin) were marginally deficient for both vitamins.	Study did not look at immune response
Quito, Ecuador	Semperteg ui et al, 2006	74.3 (SD=6.9)	145	Cross-sectional. Nutritional assessment through 24-hr recall, DTH, biochemical and anthropometric measurements.	50% of subjects had low plasma B12, Zn, and Fe. About 30% had low B6, and 19% were low in folate and vitamin D	Low DTH response. In previous 6 months, 54% and 21% had at least one episode of RI or diarrhoea, respectively
Quito, Ecuador	Hamer et al, 2008	74.4 (SD=6.4)	352	Cross-sectional.CRONOS Questionnaires, anthropometric, blood micronutrient and immuno assays.	Deficiencies for vitamins C, D, B6; zinc and folate.	Plasma Vit C associated with IFNg production. Zinc associated with IFNg and IL2 production. Micronutrient deficiency and poor immune response and burden of RI like pneumonia and common cold. Micronutrient deficiency associated with history of recent infection

Figure Legends

Figure 1. Demographic transition towards and older population (United Nations, Population Division, 1999).

Figure 2. Hypothetical effect of micronutrient status assessment and nutritional interventions in elderly from developing countries *(adapted from Dr. Jose Ordovas, JM-USDA HNRC at Tufts University).*

Figure 3. Holistic view of factors that influence the immune system.

Figure 1.


2030
AGE
1830
Male
Female
Male
Female
100-108
90-99
80-89
70-79
60-69
50-59
40-49
30-39
20-29
10-19
0-9
10 5 0 5 10
15 10 5 0 5 10 15

Figure 2.

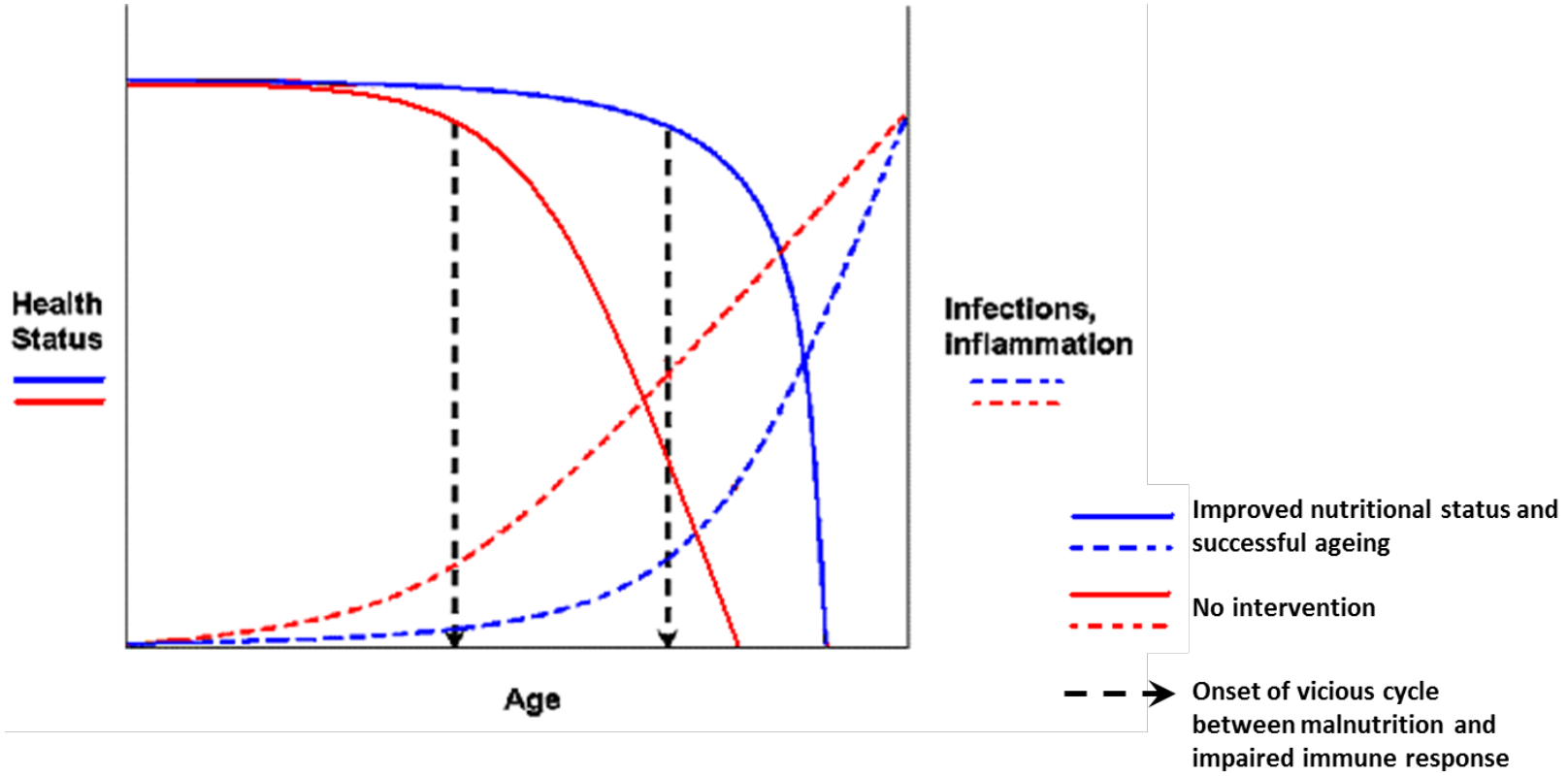
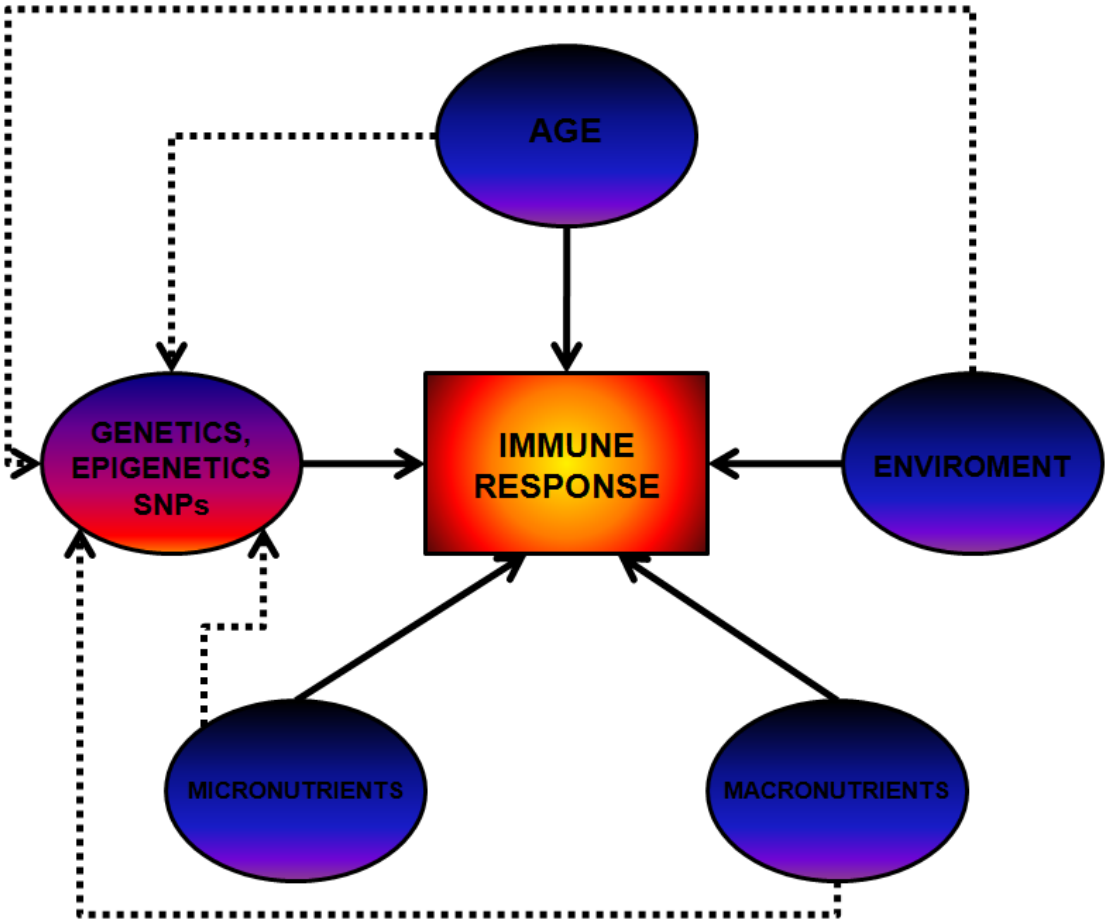

Health Status
Infections, Inflammation
Age
Improved nutritional status and successful ageing
No intervention
Onset of vicious cycle between malnutrition and impaired immune response

Figure 3.


AGE
GENETICS,
EPIGENETICS
SNPs
IMMUNE
RESPONSE
ENVIROMENT
MICRONUTRIENTS
MACRONUTRIENTS

Appendix

Part II

Inflammation of obesity and iron status in an elderly Ecuadorian population

Inflammation of obesity and iron status in an elderly Ecuadorian population

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Manuscript under review.

Abstract

Background: Obesity among elderly populations in low-income countries is increasing rapidly. Obese adults are at higher risk of iron deficiency, likely due to chronic inflammation of obesity and upregulation of hepcidin. Little is known about this process in elders.

Objective: We determined the association between inflammation, iron status, body mass index (BMI), waist circumference (WC), and hepcidin in a population of elderly Ecuadorians with a high BMI prevalence. We hypothesized that obesity and central adiposity would correlate with inflammation and hepcidin, and that these would be inversely related to iron status.

Design: This cross-sectional analysis was conducted with 258 elderly Ecuadorians. We studied correlations between iron status, serum hepcidin and C-reactive protein (CRP) in elderly men and women with normal or high BMI and WC.

Results: We found no difference in iron status between lean and obese participants. Women, but not men, with high BMI or WC had significantly higher CRP and hepcidin than lean women. Systemic inflammation (CRP), but not hepcidin, was inversely related to iron status.

Conclusions: In this population obesity was not correlated with lower iron status. However, there was a significant inverse relationship between inflammation and iron status. Further research is required to explore other mechanisms of low iron status mediated by inflammation in older adults, as well as the impact of the double burden of malnutrition on elderly populations from developing countries.

Introduction

Iron deficiency and obesity are prevalent public health concerns worldwide, affecting developing countries at a growing rate. The elderly population in developing countries is rapidly increasing, and it is estimated to grow from the current 8% to 20% of the world's population by 2050 [1]. Low-income elders in developing countries have been affected by the double burden of malnutrition, where obesity-related chronic disease and undernutrition occur simultaneously [1-3].

Obese adult populations are at risk of becoming iron deficient [4-8], as increased adiposity is associated with decreased iron absorption [9]. This may be due to upregulation of hepcidin, a peptide hormone synthesized primarily in the liver, but also in other tissues, including adipose. As such, hepcidin may play a crucial role in the association between obesity and iron deficiency [10, 11]. The main function of hepcidin is to bind the iron exporter ferroportin, and to induce its internalization and degradation, leading to intracellular iron retention. Therefore, upregulation of hepcidin results in iron sequestration in cells expressing ferroportin, such as reticuloendothelial macrophages and enterocytes, leading to decreased iron absorption and availability in circulation. Inflammatory signals directly upregulate hepcidin expression [12, 13], and due to chronic inflammation in obesity [14], hepcidin may contribute to iron deficiency in obese individuals. Previous studies have shown that hepcidin is associated with iron deficiency in the obese, and two recent reports showed that obese women undergoing bariatric surgery [15] and obese children on caloric restriction [16] experienced improvement in iron status and reduction in hepcidin and inflammatory markers, associated with weight loss.

Chronic inflammation increases with aging [17, 18], but little is known about hepcidin and inflammation in obese elders. A previous study showed no association between urinary hepcidin and inflammation in the elderly [19]. However, this may differ from the association between bioactive serum hepcidin and inflammation. Another study found elevated serum hepcidin in elders with anemia of chronic inflammation, but not in unexplained anemia [20]. Few studies have reported serum hepcidin concentrations in low-income country settings and, to our knowledge, none have measured hepcidin in elderly individuals in low-income countries. Therefore, we investigated serum hepcidin and its association with obesity, inflammation and iron status in elderly individuals from low-income peri-urban areas of Quito, Ecuador.

Subjects and Methods

Study population

The population for this cross-sectional study has been previously described in Hamer et al. [21] and Sempértegui et al. [22]. Participants were elderly (≥65 yrs) Ecuadorians from low-income peri-urban areas of Quito, Ecuador. Stored serum was available from 258 of the original 352 participants. Anthropometric measures, dietary assessment and blood collection for analysis of micronutrient status were done by trained nurses and nutritionists. Dietary intake was collected with a modified 24 hr recall-weighing method, described previously [22]. Blood samples were collected in 2003-2004, and serum was separated and stored until present. The study was approved by Tufts Institutional Review Board (IRB) and the Ethical Committee of the Corporación Ecuatoriana de Biotecnología.

Anthropometric measures

Anthropometric measures for this cohort are described in Hamer et al. [21]. Briefly, subject weight, height, and waist circumference were measured. For subjects whose standing height was not possible to collect, knee height was measured and estimated height from that measure was used in the calculation of BMI [23]. All measures were taken to the nearest 0.1cm.

Laboratory measures

Fasting blood was centrifuged shortly after collection. Serum was collected, stored at -20°C and transported to the Human Nutrition Research Center on Aging (HNRCA) in Boston, MA for micronutrient analysis. All samples were frozen at -80°C upon arrival at the HNRCA. Among others, vitamin C, calcium and iron were measured. The methods and reference ranges used to establish deficiency have been described elsewhere [24]. Serum C-reactive protein (CRP) and ferritin were measured, and the methods and reference ranges are also described elsewhere [24]. Serum iron was measured using atomic absorption spectroscopy.

Hepcidin was measured using the human serum hepcidin-25 competitive ELISA (c-ELISA) assay (Peninsula Laboratories, Bachem, San Carlos, CA). This assay has a detection range of 0-25 ng/ml. Serum samples were diluted 1:15 and added to the pre-coated 96-well plates. Hepcidin in 252 of the 258 serum samples was assayed during a one week period. There was insufficient sample from six specimens to measure hepcidin. All samples were assayed in duplicate. The assay was repeated for a subgroup of samples to determine inter-assay variability. For a sample with a hepcidin concentration

of 1.56 ng/ml, there was an inter-assay variability of 2.54% and an intra-assay variability of 5.24%, based on seven assay replications.

Statistical analysis

For variables that were not normally distributed, median values (minimum-maximum) are reported, and $\log_{10}$-transformed data were used in the statistical analysis. For normally distributed variables, the mean ± standard deviation (SD) is reported. Statistical significance was set at alpha=0.05. Differences in linear variables between men and women were tested using Student's t test. Fisher's exact test was used to determine differences in BMI categories between men and women. Pearson's correlation coefficients and partial correlation coefficients, adjusting for age and gender, were used to determine associations between hepcidin, iron status, inflammation and BMI. Hepcidin, serum iron, ferritin, and CRP concentrations were stratified by gender and BMI: obese, BMI≥30 kg/m^2; overweight, 25≤BMI<30 kg/m^2; and lean, 20≤BMI<25 kg/m^2. Hepcidin, ferritin, and CRP data were not normally distributed, and thus were log transformed for two-way analysis of variance (ANOVA), adjusting for age, and for partial correlation coefficient analysis. When an ANOVA model was significant, Tukey's Honestly Significant Difference (HSD) test was used to adjust for multiple comparisons. Log-transformed variables were also used in linear regression analysis. SAS 9.2 for Windows (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Population characteristics

Descriptive participant characteristics, including socio-economic status and education level, have been described previously [21]. On average, men had higher income and education level than women. Men were significantly older than women, 76 ± 6 years and 74 ± 6 years, respectively ($p<0.05$) (**Table 1**). Women's mean BMI, 25.7 ± 4.1 kg/m^2, was significantly greater than the mean BMI for men, 24.0 ± 3.1 kg/m^2 ($p<0.001$). There were significantly more overweight and obese women than men ($p<0.01$; Fisher's exact two-sided test). The mean waist circumference (WC) of women was 88.6 ± 10.6 cm, which is considered high both by the International Diabetes Federation (IDF) and Adult Treatment Panel III metabolic syndrome definitions [25, 26]. In contrast, men had a mean WC of 87.6 ± 9.4 cm which, by these definitions, is considered normal.

Correlations between iron status, CRP and hepcidin

After logarithmic transformation ($\log_{10}$), hepcidin, CRP and ferritin values became normally distributed. Partial correlation analysis, adjusting for sex and age, showed positive correlations between hepcidin and both serum ferritin ($r=0.69$, $p<0.0001$) and CRP ($r=0.27$, $p<0.0001$) (**Table 2**). There was a significant negative correlation between serum iron and CRP ($r=-0.21$, $p=0.001$). However, serum iron was positively correlated with hepcidin ($r=0.13$, $p<0.05$). A positive correlation between serum iron and ferritin ($r=0.19$, $p=0.002$) was also observed.

Iron status, inflammation, and hepcidin by BMI and gender

Serum iron was significantly higher in men than in women (131 ± 41 vs. 110± 37 µg/dl; p<0.0001) (**Table 3**). There were no differences in the intake of iron, vitamin C or calcium, or in serum ferritin, hepcidin or CRP between men and women (Table 3).

When dividing women into obese, overweight and lean BMI categories, there was a trend toward higher hepcidin across categories (lean to obese) (p=0.08; ANCOVA, adjusting for age) (**Figure 1a**). Median (min-max) values were: 38 (2.5-113) ng/ml for the obese, 37 (1.7-230) ng/ml for overweight participants, and 24 (0.5-310) ng/ml the lean (Figure 1). The trend was not significant for men (data not shown), perhaps due to the small sample size in the obese group (n=4). CRP concentration showed a pattern similar to that seen for hepcidin (p=0.01, ANCOVA, adjusting for age); CRP was more than twice as high in obese than in lean women (p=0.005, Tukey's HSD) (Figure 1b). Serum iron was not different among obese, overweight and lean women (Figure 1c). We found no significant differences in income level, education, or dietary intake of iron, calcium or vitamin C between lean, overweight and obese women (data not shown).

There was a significant interaction between age and BMI in relation to hepcidin in women with BMI>20kg/m^2 (Figure 1d). BMI was associated positively with CRP. Finally, BMI was positively associated with serum iron, suggesting that the originally anticipated inverse association between serum iron and BMI may not hold for this population of elderly women.

Because central obesity is a strong predictor of systemic inflammation, we compared CRP and hepcidin between participants with high and low WC, using the IDF definition (80 cm for women and 90 cm for men). We found that women with high WC had

significantly higher hepcidin and CRP than women with WC <80 cm (**Table 4**). The values (median [min-max] were as follows for women with WC >80 cm vs. women with WC <80 cm: 34 [1-310] ng/ml vs. 25 [0.5-204] ng/ml for hepcidin (p=0.03); and 3.6 [0.3-145] mg/L vs. 2.0 [0.2-18] mg/L for CRP (p=0.02). No differences for hepcidin or CRP were found across WC in men, or for iron status in men or women (data not shown). CRP was negatively associated (p=0.0001), and hepcidin positively associated (p=0.003) with serum iron, after adjusting for WC (**Table 5**).

Discussion

In contrast to our hypothesis, we did not find an inverse association between BMI and iron status, even though CRP was higher in obese and overweight subjects and there was a significant inverse association between systemic inflammation and iron status. Furthermore, CRP and hepcidin, but not serum iron, were significantly higher in women with high central adiposity. Other studies are needed to explore the underlying mechanisms of low iron status mediated by inflammation in overweight and obese elderly populations from developing countries.

We found higher systemic inflammation, measured as CRP, in obese and overweight women when compared to lean women, confirming a hypothesized association between BMI and inflammation in this low-income elderly population from a developing country. Hepcidin tended to be highest in women with higher BMI, but this trend did not reach significance (p=0.08) (Figure 1). In addition, women with high WC had significantly higher CRP and hepcidin than women with WC <80 cm, showing that inflammation and

higher hepcidin occur in women with high central adiposity in this population. CRP was negatively correlated with serum iron as hypothesized.

Contrary to our expectations, however, we saw a positive association between hepcidin and serum iron. This could be due to the predominance of overweight rather than obese subjects in this population, suggesting that the negative relationship between hepcidin and iron occurs in populations with higher prevalence of obesity. There is also the possibility that negative associations between iron status, hepcidin and BMI do not occur in an elderly overweight and obese population in a low-income country setting, where both undernutrition and the complications of high adiposity are playing a role. In other words, there may be two factors with opposing effects on hepcidin in this population. On the one hand, there is low dietary iron (Table 3), which would cause hepcidin to decrease to enhance iron absorption. On the other hand, there is chronic inflammation of aging and obesity, which would increase hepcidin in circulation, thereby lowering iron status. These two opposing signals appear to be influencing iron homeostasis and the expression of hepcidin at the same time. In this population, low iron status due to undernutrition may be overwriting the influence of chronic inflammation of obesity on hepcidin expression. In this way, the nutritional deficiencies in elders from low-income countries may contribute to differences in the metabolic complications of obesity than those seen in elderly populations of high-income countries. Further studies are needed to investigate these possibilities.

Hepcidin was positively correlated with CRP and ferritin, as anticipated and previously reported [15, 27, 28], although one study showed no association between CRP and urinary

hepcidin in elders [19]. However, serum hepcidin is a more direct measure of the bioactive form of hepcidin than urinary hepcidin [27].

Given that we found a positive correlation between CRP and BMI, and a negative correlation between CRP and serum iron our data also suggest that, at least in this population, high CRP is more predictive of lower iron status than BMI or hepcidin. Aging is associated with low-grade inflammation and, thus, other factors than obesity may contribute to the CRP concentrations reported here. Obesity alone as a categorical variable was not a predictor of iron status in this study. When assessing iron status, specific population characteristics need to be taken into consideration. We measured serum iron and ferritin concentration. Ferritin is known to be affected by inflammation and may therefore not accurately portray iron status in this population [29]. Conversely, more sensitive measures of iron status such as soluble transferrin receptor might have better reflected the impact of obesity alone on iron status.

We also identified possibly important differences between men and women in this population that may need to be taken into consideration for future studies. Women who participated in this study had significantly lower serum iron and higher BMI than men. No significant differences, however, were found for CRP and hepcidin (Table 3). Dietary intake of iron, or some of the dietary factors that impact iron absorption, including vitamin C and calcium, did not differ by gender. Further, there was no association between iron intake and BMI (data not shown). Whether the difference seen in iron status is due to differences in BMI, or merely to gender differences requires further investigation.

To our knowledge, this is the first report of an association between inflammation, obesity and serum iron in an elderly population from a developing country. Hepcidin did not have the expected association with serum iron reported from high-income country adult populations, suggesting that differences in this relationship between adult and elderly populations, as well as between high- and low-income country populations, may exist. Strengths of this study include the large sample size, origin and characteristics of this population, and the extensive data available from these subjects, including dietary intake. Limitations include lack of body composition measures (e.g., fat mass and fat free mass), which would have provided useful information, especially when dealing with the elderly, who tend to develop more central visceral fat mass and lose fat free mass in the periphery [30]. Also, information on phytic acid consumption and other inhibitors of non-heme iron bioavailability was unavailable.

Iron deficiency in developing countries is usually attributed to low dietary iron consumption, concurrent to deficiencies of zinc or other micronutrients, or to infection. The effect of obesity, which has become prevalent in these countries, on iron status is only now beginning to be explored. Two reports have shown that adiposity and inflammation of obesity in women and children in transition countries are predictive of low iron status and are associated with lower iron absorption [9, 31], implying that hepcidin may play a role. We show that inflammation of obesity was associated with low serum iron, but this was not correlated with hepcidin, at least is this predominantly overweight population. Evaluating other mechanisms of low serum iron mediated by inflammation will need further research.

In summary, we have shown relationships between obesity, inflammation, and iron status in a low-income elderly population from a developing country which differ from those that have been previously identified in higher income and younger populations. Our findings suggest that in low-income country populations, where both obesity and undernutrition are prevalent, additional research will be needed to understand how different regulatory mechanisms affect the elderly.

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Authors report no conflict of interest for the research presented herein.

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Table 1. Population characteristics.

Variable	**Women (n=164)**	**Men (n=94)**	**P**
Age (y)	73.7 ± 6.0	75.7 ± 6.3	<0.05*
BMI (kg/m2)	25.7 ± 4.1	24.0 ± 3.1	<0.001*
Obese: BMI>30 (n)	21	4	
Overweight 25<BMI<29.9 (n)	65	24	<0.01**
Normal: 20<BMI<24.9 (n)	68	57	
Underweight: BMI<19.9 (n)	9	8	
Waist circumference (cm)	88.6 ± 10.6	87.6 ± 9.4	NS*

Mean ±SD is shown. *Student's t test; **Fisher's exact test.

Table 2. Correlation between iron status, inflammation and hepcidin.

	Log(Ferritin) (ng/ml)	**Iron (µg/dl)**	**Log(CRP) (mg/L)**
	r	**r**	**r**
Log(Hepcidin) (ng/ml)	0.69***	0.13*	0.27***
Log(CRP) (mg/L)	0.12#	-0.21**	-
Iron (µg/dl)	0.19*	-	-

Partial correlation coefficients adjusting for sex and age are shown. *p<0.05, **p=0.001, ***p<0.0001, #p<0.1

Table 3. Iron status and hepcidin in elderly men and women.

Variable	Women	N	Men	N	p*
Serum iron (µg/dl)	110 ± 37	160	131 ± 41	93	<0.0001
Ferritin (ng/ml)	137 (5-1219)	164	143 (14-627)	94	NS
Dietary iron (mg)	6.4 (0.5-54.5)	161	6.6 (1.0-29.9)	91	NS
Dietary vitamin C (mg)	24 (0.3-142)	161	24.4 (0.7-173)	91	NS
Dietary calcium (mg)	221 (10-865)	161	223 (40-989)	91	NS
CRP (mg/L)	2.8 (0.2-145)	164	3.7 (0.0-119)	94	NS
Hepcidin (ng/ml)	32.1 (0.5-310)	158	23.9 (0.5-313)	94	0.088

Values shown are mean ± SD, or median (minimum-maximum) for non-normally distributed variable. *Student's t test. For non-normally distributed variables, t test was done in log scale.

Table 4. Hepcidin and CRP in men and women with normal and high waist circumference.

	Hepcidin (ng/ml)			CRP (mg/L)		
	N	**Median (Min-Max)**	**p-value**	**N**	**Median (Min-Max)**	**p-value**
Women						
High WC (>80 cm)	127	33.6 (0.96-310)	0.03	129	3.60 (0.30-145)	0.02
Normal WC (<80 cm)	31	24.8 (0.48-204)		35	2.20 (0.20-17.9)	
Men						
High WC (>90 cm)	37	21.4 (0.45-142)	0.58	37	2.90 (0.20-24.8)	0.19
Normal WC (<90 cm)	57	25.4 (0.80-313)		57	4.00 (0.00-119)	

Student’s t test using log-transformed data.

Table 5. Linear regression analysis using serum iron as dependent variable.

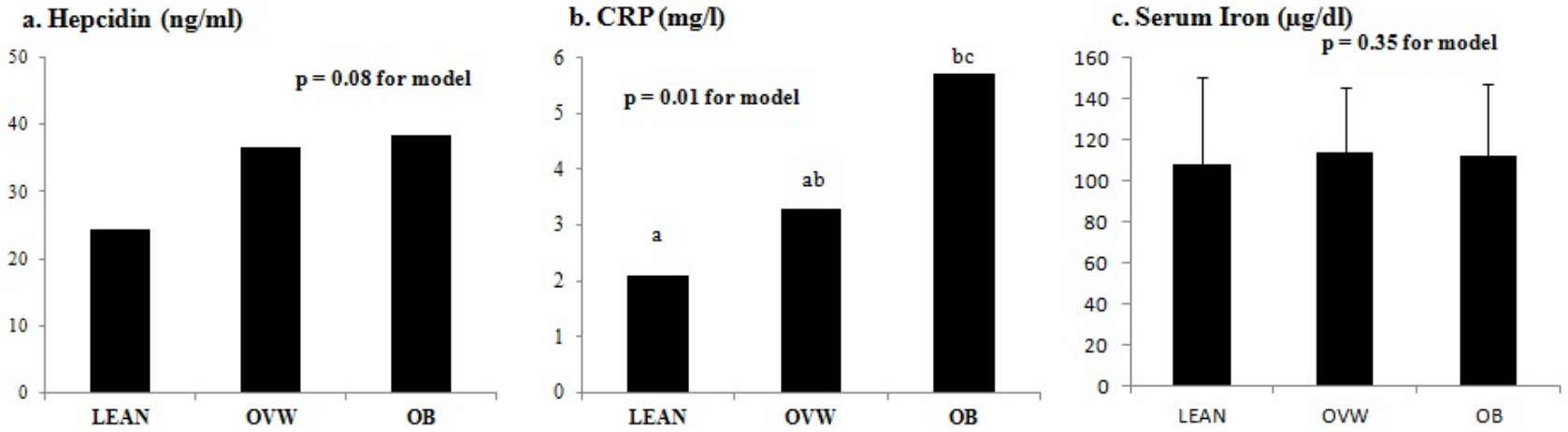
	Serum Iron (µg/dl)		
	ß	**SE**	**P**
Log(Hepcidin) (ng/ml)	16.3	5.49	0.0033
Log(CRP) (mg/L)	-18.3	4.69	0.0001
WC (high) (cm)	7.76	5.48	0.16
Sex (male)	25.3	5.40	<.0001

P value for model <.0001. Waist circumference cutoff was set according to IDF specifications (ß = estimated variation of serum iron by the independent variables; SE = standard error).

Figures Legends.

Figure 1. Hepcidin, CRP and iron in obese, overweight and lean women. Median values are shown in histogram plots for hepcidin (a) and CRP (b), and mean (SD) values are shown for iron (c). ANCOVA was used and p-value for model is shown. Log transformations were used for hepcidin and CRP. Tukey's HSD was used for significant models. (d) Linear regression analysis was done for three different models: the first one with log-hepcidin as the dependent variable, with centered values for age and BMI and including an interaction term, and two other models with log-CRP and iron as the dependent variables, with age and BMI as independent variables. Values shown are ß ± SE (estimated variation of dependent variable by the independent variables ±standard error). [a] values have been centered around the mean. [#]p<0.1; *p<0.05; **p<0.01; ***p<0.0001.

Figure 1.



d.

Dependent Variable	Age	BMI	Age*BMI
log(Hepcidin) (ng/ml)	$(-0.01 \pm 0.01)^{a\#}$	$(0.01 \pm 0.01)^{a}$	$(0.004 \pm 0.002)^{*}$
log(CRP) (mg/L)	(0.002 ± 0.007)	$(0.03 \pm 0.01)^{**}$	-
Serum Iron (µg/dl)	$(0.94 \pm 0.53)^{\#}$	$(1.77 \pm 0.82)^{*}$	-

Appendix

Part III

Obesity during Pregnancy and Fetal Iron Status: is Hepcidin the link?

Obesity during Pregnancy and Fetal Iron Status: is Hepcidin the link?

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Abstract

Objective: To ascertain the effect of obesity-related inflammation on maternal and fetal iron status. We hypothesized that obese pregnant women would have increased inflammation, hepcidin levels, and that their infants would have impaired iron status compared to lean controls.

Study Design: Fifteen obese (Ob) and fifteen lean (Lc) women were recruited in their second trimester of pregnancy. Markers of iron status, inflammation and hepcidin were measured in maternal and cord blood. Student's t test was used to compare obese and lean groups, and Pearson correlation coefficients were determined between maternal and cord blood values.

Results: Maternal C-reactive protein (CRP) ($p<0.01$) and hepcidin ($p<0.01$) were higher, and cord blood iron ($p<0.01$) was lower in the obese group. Maternal BMI ($p<0.01$) and hepcidin ($p<0.05$) were negatively correlated with cord blood iron status.

Conclusions: Maternal obesity is associated with impaired maternal-fetal iron transfer, potentially through hepcidin upregulation.

Keywords: Maternal obesity, iron deficiency, inflammation.

Introduction

Over half of all reproductive age women in industrialized nations are overweight or obese and this burden is growing rapidly in developing nations as well [1-3]. Epidemiologic data has shown that infants and children born to obese women are more likely to develop chronic health conditions such as asthma and diabetes, but there have been no studies describing the effect of maternal obesity on infant iron status. Hepcidin, a regulator of iron homeostasis, has been shown to be overexpressed in obesity and to correlate with low iron status in the obese [4-8]. Iron reaches the fetus through active transport in the placenta and hepcidin is known to be one regulator of this process [9].

Obesity leads to chronic overexpression of hepcidin as a downstream effect of low-grade chronic inflammation. Specifically obesity leads to increased interleukin (IL)-6 and IL-1 levels which upregulate hepcidin [10-12]. Recently, it was reported that hemojuvelin is overexpressed in adipose tissue of obese individuals, and directly upregulates hepcidin through the bone morphogenic protein-hemojuvelin (BMP-HJV) pathway [13]. Conversely, hepcidin is kept at a minimum during pregnancy in order to maximize iron transfer to the fetus [14]. During the late fetal and early neonatal period, the infant experiences rapid growth, and the nervous system is particularly vulnerable to alterations in the regulation of iron during this time. Impaired fetal iron transport is thought to have lifelong and irreversible effects on neurodevelopment [15-17]. In addition, maternal iron deficiency is associated with poor fetal growth and poor weight and height gain during childhood. Thus, identifying factors that affect fetal iron transport is of critical importance.

Subjects and Methods

We conducted a prospective case control study to determine the impact of obesity during pregnancy on maternal and fetal iron status. The study protocol and procedures were approved by Tufts University/Tufts Medical Center IRB and was conducted in accordance with HIPAA regulations. All participants gave written informed consent to participate in this study. All authors had access to collected clinical data.

Study Participants

Thirty women, 15 obese (Ob) and 15 lean controls (Lc), were recruited for this study from the Tufts Medical Center Obstetrics clinic between 24-28 weeks of pregnancy between May, 2010 and December, 2010. Potential subjects were identified from the pre-pregnancy body mass index (BMI) noted on their prenatal records. Subjects were assigned to the control (BMI 20-25 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2) group based on their pre-pregnancy BMI. Subjects with pre-gestational diabetes, preeclampsia, autoimmune disease, acute infectious process or the pregnancy complications PPROM (preterm premature rupture of membranes) and chorioamnionitis were excluded from the analysis. All subjects reported taking a standard prenatal vitamin with iron during the current pregnancy. Cord blood was harvested from the neonates of 10 obese women and 11 control women.

Measurements in maternal and cord blood

Maternal blood was collected at 24-28 weeks of gestation, after an 8-14 hour fast, one hour after ingestion of a 50g glucose drink. Blood was collected at this time to minimize venipuncture in subjects. Cord blood was collected after delivery via syringe aspiration from the umbilical vein. Cord blood could not be collected from all subjects. Iron status,

specifically serum iron and transferrin saturation (Tsat), were measured with colorimetric endpoint assays (Diagnostic Chemicals Ltd., Oxford, CT, USA). Hematocrit (HCT) was measured using a hematology analyzer (Horiba, Irvine CA). Serum C-reactive protein (CRP) (Abnova, Walnut, CA, USA) and IL-6 (eBioscience, San Diego, CA, USA) were measured with ELISA and hepcidin (Bachem Group, Torrance, CA, USA) was measured with competitive ELISA (c-ELISA). Reduced, oxidized and total glutathione were measured from serum per manufacturer's instruction using the Glutathione Kit (Biovision, Mountainview, CA, USA).

Statistical Analysis

We used Student's t test for Ob vs. Lc group comparisons, and Pearson correlation coefficient analysis to determine correlations between maternal and cord blood parameters. All tests were two sided and judged statistically significant at $p< 0.05$. SAS 9.2 for Windows (SAS Institute, Cary, NC, USA) was used for all analyses. For normally distributed variables, mean ± standard deviations (SD) are reported. A logarithmic transformation was applied to non-normally distributed variables and t tests were done on transformed data. In results, median and interquartile range (IQR) values are reported.

Results

Study population characteristics

The mean pre-pregnancy BMI was 38.6 ± 7.0 kg/m^2 for the Ob group, and 22.8 ± 1.5 kg/m^2 for the Lc group ($p<0.0001$) (Table 1). There were 6 African Americans in the Ob group and none in the Lc group ($p<0.05$). We found no significant differences in iron status, inflammation or hepcidin between obese African American and obese Caucasian

women. Average age was not different between the Ob and Lc groups. Importantly, rates of gestational diabetes did not differ between the two groups: one subject in each group developed gestational diabetes and there was no difference in the mean serum glucose level between the two groups based on the glucose tolerance test: 117 ± 33 mg/dl for Ob, and 109 ± 27 mg/dl for Lc. There was no association between maternal BMI and birth weight (data not shown). We found no significant association between maternal BMI and birth weight or Apgar scores (data not shown).

Obese pregnant women have increased oxidative stress, inflammation and higher hepcidin levels

The ratio of serum oxidized to reduced glutathione (oxidized/reduced x 100) was higher in the Ob compared to Lc [Ob: 9.3 ± 1.1 vs. Lc: 7.9 ± 1.2, $p<0.01$], indicating increased oxidative stress in the Ob group. Inflammation, measured as serum CRP, was significantly higher in the Ob compared to Lc [Ob: 14.3 (11.5) mg/L vs. Lc: 5.0 (4.4) mg/L, $p<0.01$] (Figure 1a). There was no statistically significant difference serum IL-6 between the two groups (Figure 1b). Hepcidin was significantly higher in the Ob vs. Lc group [Ob: 13.5 ± 9.0 ng/ml vs. Lc: 5.1 ± 2.7 ng/ml, $p<0.01$] (Figure 1c). However, serum iron and Tsat were not significantly lower in Ob compared to Lc (Figure 1d-e). HCT was not different between the Lc and Ob group (data not shown).

Infants of obese women have impaired iron stores

We found no statistically significant differences in CRP, IL-6 or hepcidin levels in cord bloods between the Ob and Lc groups (Figure 1f-h). Consistent with Rehu's previous report, cord blood hepcidin was approximately ten times higher than hepcidin levels in the mothers [14]. Adjusting for mode of delivery did not affect hepcidin or inflammation

differences in cord blood. Serum iron and Tsat were found to be significantly lower in cord blood from Ob compared to Lc [Iron: Ob, 97.3 ± 29.9 µg/dl vs. Lc, 147.7 ± 21.7 µg/dl, $p<0.01$; and Tsat: Ob, 39.6% vs. Lc, 63.5%, $p=0.01$] (Figure 1i-j). On average, HCT was not significantly different between the two groups (data not shown). These results suggest that iron transfer to the fetus is hindered in obese pregnant women.

Maternal BMI correlates with cord blood outcomes

There was a moderate and statistically significant correlation between maternal BMI and maternal CRP ($r=0.5$, $p=0.006$) and maternal BMI and maternal hepcidin ($r=0.4$, $p=0.04$). Correlation coefficient analysis also showed that maternal BMI was strongly negatively correlated with iron status in cord blood, both for serum iron ($r= -0.8$, $p=0.002$) and Tsat ($r= -0.7$, $p=0.009$) (Figure 2a and c). In addition, there was a significant moderate negative correlation between maternal hepcidin and cord blood serum iron ($r=-0.6$, $p=0.02$) and cord blood Tsat ($r=-0.6$, $p=0.02$) (Figure 2b and d). This suggests that BMI contributes to higher hepcidin levels and inflammation in mothers, and is a strong contributor to impaired maternal-fetal iron transport and fetal iron stores.

Discussion

This is the first study to report the effect of obesity in pregnancy on hepcidin levels and maternal-fetal iron transfer. In our population, obesity was associated with lower income and African American race. These factors have been shown to be associated with obesity in the US overall. Surprisingly, we did not have more subjects with gestational diabetes or asthma in our Ob group. The number of neonates that were delivered by Cesarean section did not differ between the Ob and Lc groups. It is interesting that there was no

significant association between maternal weight and birth weight in the Ob group which has been reported in large epidemiologic studies. This could be due to the size of our cohort or due to the slightly higher number of preterm infants born to obese subjects, which decreased the mean birth weight in that group.

There is extensive evidence showing obese individuals to have significantly higher hepcidin and greater risk of iron deficiency than lean individuals, but this is the first study showing this phenomenon in obese pregnant women. We have shown that inflammation and hepcidin are higher in obese than in lean pregnant women. More importantly, we demonstrate that this is associated with lower iron status in their neonates.

Neonatal iron deficiency has been best studied in the context of undernutrition. In our study, neonates born to obese women have iron profiles which closely resemble those of infants born to iron deficient women [18], although their mothers were not iron deficient. This further supports the idea that obesity-related inflammation may be contributing to impaired maternal-fetal iron transport. Under non-inflammatory conditions a pregnant population with low iron status would have low levels of hepcidin in order to maximize iron absorption and availability [14], thereby enhancing iron transfer to the fetus. However, obese pregnancy is characterized by inflammation, which upregulates hepcidin and decreases circulating iron transfer to the fetus. Although the mechanism leading to low iron status in neonates may be different in obese pregnancy compared to maternal undernutrition, the impact of a relative deficiency in this critical nutrient on fetal and infant neurodevelopment bears close monitoring.

IL-6 upregulates hepcidin expression through the Jak/STAT pathway. It has been recently shown that hepcidin is also upregulated through the BMP pathway by hemojuvelin

production in adipose tissue of obese individuals. Although we did not observe a statistically significant difference in serum IL-6 between the Lc and Ob group, further studies should evaluate the contribution of these pathways to low fetal iron status and diminished iron transfer in placenta.

Obese women and their infants are at risk for chronic inflammation and oxidative stress [19]. Our Ob cohort had increased inflammation and oxidative stress compared to the Lc cohort. Our data suggest that this chronic inflammation and oxidative stress may upregulate hepcidin, thereby impairing iron transport to the fetus. However, iron supplementation in obese pregnant women and their infants is a complex question since providing free iron, a potent oxidant, may further exacerbate the already present oxidative stress.

Strengths of this study are that we have been able to follow women through pregnancy and delivery, allowing accurate pairing of maternal and fetal data. Given the prospective data collection we were able to exclude confounding conditions, such as acute infections. We used BMI as an indicator of obesity, but body composition, percentage body fat and information on dietary intake of iron absorption enhancers and inhibitors would have provided additional valuable information. The sample size is small, but despite this we have shown a strong relationship between maternal obesity and offspring iron status. A Tsat< 20% has been used to define iron deficiency (18). In our control subjects, 57% of the women had Tsat<20% and in our obese subjects, 75% of the women had Tsat<20%; however, these differences were not statistically significant. Subgroup analysis comparing iron deficient and iron replete obese and lean mothers and infants was not

possible due to the small sample size of the subgroups, and therefore such analysis is needed in future studies.

In conclusion, we have shown, for the first time, that maternal obesity is associated with impaired iron transfer to the fetus. We speculate that this is due to the effects of a chronic pro-inflammatory environment and increased levels of hepcidin.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Maternal population characteristics. Participants were recruited as described in the methods section, and the following self reported data was obtained at the recruitment visit. Mean ± SD are shown.

	Obese (n=15)	**Lean (n=15)**
BMI (kg/m^2)*	38.6 ± 7.0	22.8 ± 1.5
Age (years)	30.0 ± 3.9	32.1 ± 5.8
Education (N with college degree)	7	11
Race (N)**		
Caucasian	6	9
African American	6	0
Hispanic	2	5
Asian	1	1
Mode of delivery (N)		
Vaginal Delivery	8	11
Cesarean	7	4

*Significantly different by Student's t test; $p<0.0001$
**Significantly different by Student's t test; $p<0.05$

Figure Legends

Figure 1. Hepcidin, inflammation and iron status in maternal and cord blood. Maternal and cord blood CRP, IL-6, hepcidin, serum iron and Tsat were measured as described in methods and Student's t test was used to determine differences between Ob and Lc groups (*p=0.01, **p<0.01). Mean ± SE are shown.

Figure 2. Correlations between maternal BMI and maternal hepcidin with cord blood iron status. Pearson correlation coefficient analysis was used to determine correlations between maternal BMI and cord blood iron status, and between maternal hepcidin and cord blood iron status. Correlation coefficients (r) and p values are shown. Cord blood data was not available from all subjects due to technical limitations.

Figure 1.

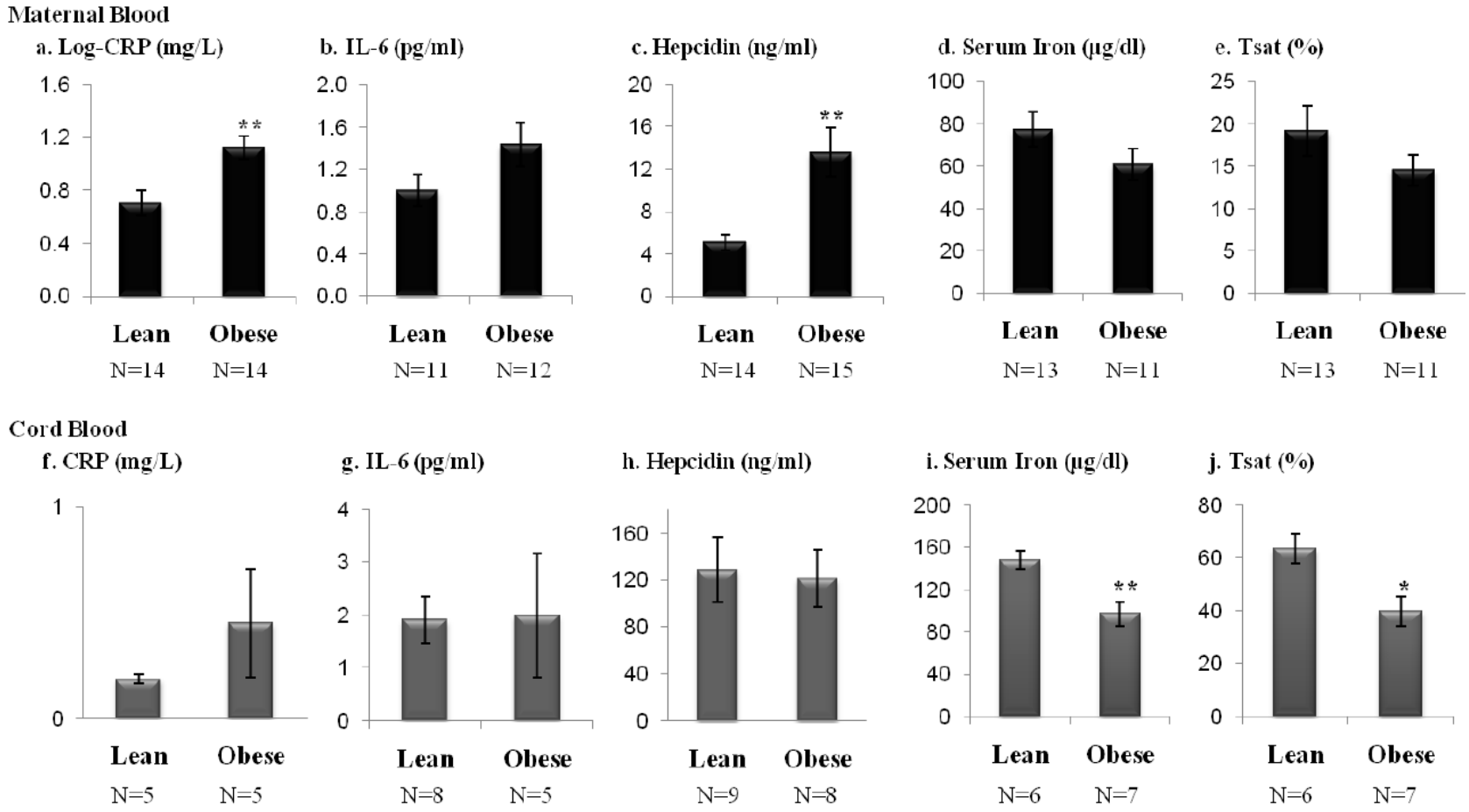
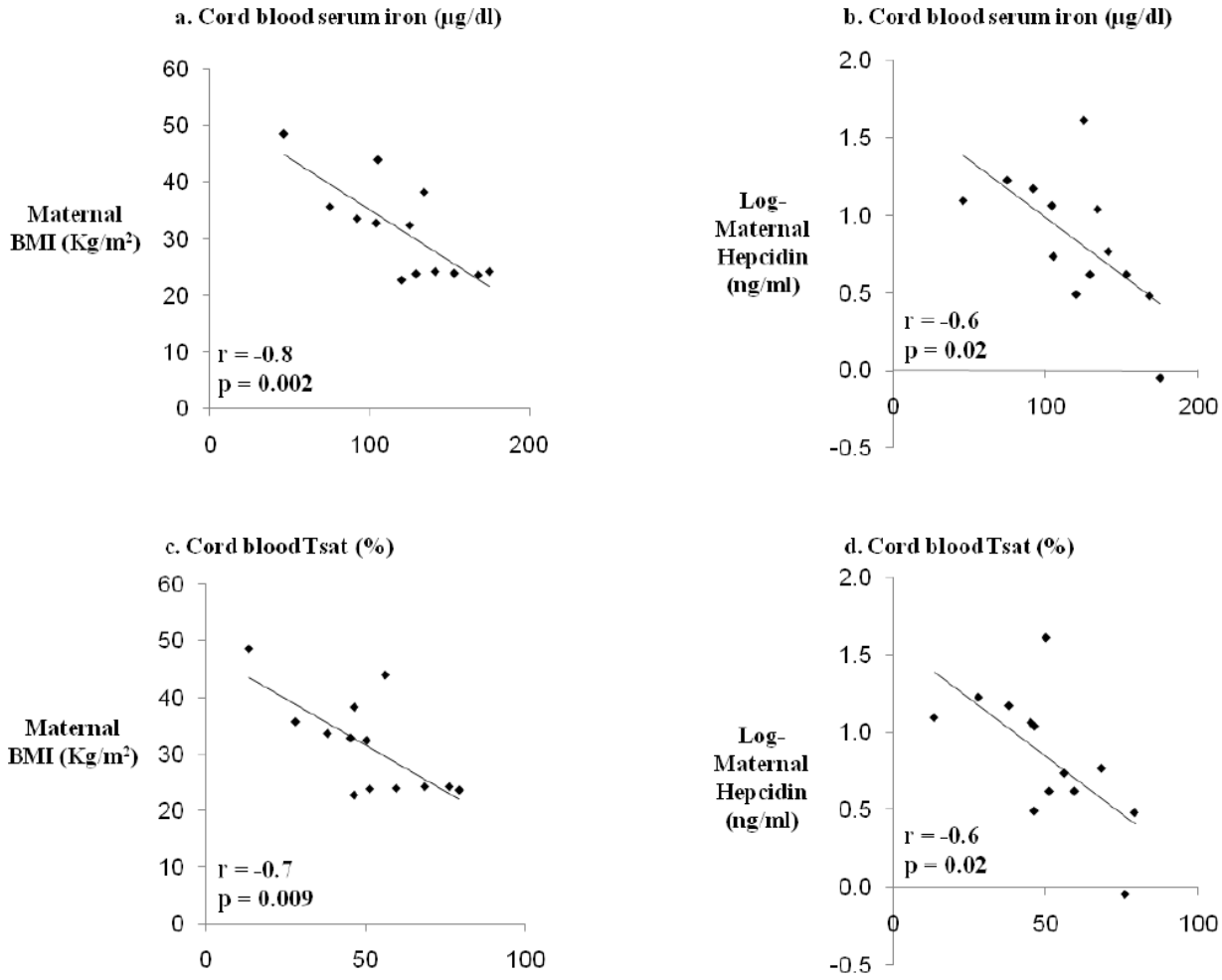

Maternal Blood
a. Log-CRP (mg/L)
b. IL-6 (pg/ml)
c. Hepcidin (ng/ml)
d. Serum Iron (µg/dl)
e. Tsat (%)
Lean
Obese
N=14
N=14
N=11
N=12
N=14
N=15
N=13
N=11
N=13
N=11
Cord Blood
f. CRP (mg/L)
g. IL-6 (pg/ml)
h. Hepcidin (ng/ml)
i. Serum Iron (µg/dl)
j. Tsat (%)
N=5
N=5
N=8
N=5
N=9
N=8
N=6
N=7
N=6
N=7

Figure 2.


a. Cord blood serum iron (µg/dl)
Maternal BMI (Kg/m²)
r = -0.8
p = 0.002
b. Cord blood serum iron (µg/dl)
Log-Maternal Hepcidin (ng/ml)
r = -0.6
p = 0.02
c. Cord blood Tsat (%)
Maternal BMI (Kg/m²)
r = -0.7
p = 0.009
d. Cord blood Tsat (%)
Log-Maternal Hepcidin (ng/ml)
r = -0.6
p = 0.02