SYSTEMIC IRON REGULATION AND ADIPOSE TISSUE INFLAMMATION IN HEALTH AND DISEASE

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Kinesiology)
in the University of Michigan
2015

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Acknowledgements

I would like to thank my thesis committee, namely Drs. Pete Bodary, Jeff Horowitz, Gregory Cartee, Yatrik Shah, and Rami Khoriaty, for their contribution to the development and critical analysis of this project. I could not have reached this point without the help of my committee members as well as fellow students, Kinesiology staff, willing participants, friends and family. But most importantly this opportunity was made possible by my advisor Dr. Pete Bodary.

I would like to express my deepest gratitude to my advisor Dr. Pete Bodary, whose excellent guidance, caring, patience, and providing me with support, encouragement and all the resources with which this dissertation was brought to fruition. Dr. Bodary you have been an invaluable mentor. Your passion for science, your attitude towards teaching and learning, your enduring encouragement, and your practical advice has been an inestimable source of support for me during this process. Thank you very much for encourage my research and for allowing me to grow as a graduate student. Your advice on both research and life is priceless.

I would also like to thank my committee for their continued support and encouragement. Dr. Horowitz, I can't thank you enough for your encouragement and guidance. From the very beginning of my study, you were always patient with my questions and prompt with feedback on my scientific writing. You also provided me with the opportunity to join your lab's journal club, which improved my presentation skills and helped me develop critical thinking. Dr. Cartee, you are my role model for my research life, your dedication to research and diligence towards muscle

biology always encouraged me to do better with my own research. Dr. Shah, thank you for being so helpful and generous. I couldn't have finished my data collection without the assays you provided. Thanks for taking your time and helping me with my dissertation projects. Dr. Khoriaty, thank you for giving me feedback from a clinical standpoint and helping me with other projects in addition to my dissertation.

My completion of this dissertation could not have been accomplished without the support of VBL (Vascular Biology Laboratory) members present and past. I have learned so many laboratory skills from working with Chester Kao, Danielle Trakimas, Monica Humby and Justin Kang. I appreciate the patience you showed me and the knowledge you shared. The work presented in this dissertation would not have been possible without the helping hands of our recent lab members including: Katlyn Patterson, Kayla Gieschen, Vinh Pham, Maria Ward, Lydia Proctor and Kayla Hanses. Thank you for your help with serum preparation, monocyte isolation and assistant with data collection with the participants. It was all of you that made my transition from my undergraduate in China to graduate life in U.S. much easier. Last, but by no means least, I want to thank my lab mate Justin, who has been a great colleague. Your help with a lot of tissue collection and preparation of our qualifying exam means a lot to me.

To the faculty and staff members in the School of Kinesiology, thanks for making my graduate life more productive and smooth. Thank you to Charlene Ruloff, you are always supportive and helpful for each step of my doctoral process. Thank you and Dr. Ketra Armstrong for organizing the get-togethers for all of the graduate students in Kinesiology. Thank you to Leona Cranford for helping with all the assays ordering and dealing with packages.

I would like to thank Dr. Carey Lumeng and his lab, where they generously taught me flow cytometery and helped me with data analysis. It was a productive term when I was in their lab. I also want to express my gratitude to Dr. Peter Mancuso, who was on my prelim committee and taught me so much in his class.

I would not be at this point without the help and mentorship of my Bachelor's Degree advisor, Dr. Yang Hu. The skills and the knowledge I gained from Dr. Hu's lab at Beijing Sport University built a strong base for my development as a doctoral student here in Michigan.

To my best friend Ramses, thank you for taking care of me, supporting me to get through bad times and celebrating good times with me. To my caring, loving, and supportive parents, they are the best for both my brother and I. I am so fortunate to be raised by them as they always instilled a strong belief in education and supported me through my entire professional career. To my dearest brother, I couldn't say thank you enough. Your encouragement gave me the motivation and confidence to seek for greater opportunities. Whenever I go through difficult times, you are always there to listen to me and provide me with your wisdom.

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Abstract

Iron dysregulation can lead to serious health concerns resulting from either too much or too little iron storage and availability. For example, iron deficiency anemia results in a reduced exercise tolerance, while chronic conditions such as obesity and type 2 diabetes may predispose individuals to tissue iron overload. The relatively recent discovery of hepcidin, the major iron-regulating hormone, has led to new hypotheses regarding conditions of iron dysregulation, including exercise-induced iron deficiency. A series of recent studies have suggested that exercise-induced iron deficiency might result from a transient increase in circulating hepcidin following acute exercise. However, it is unclear whether there is a cumulative effect of multiple acute excursions of hepcidin in response to everyday training. On the opposite end of the iron dysregulation spectrum, excess iron deposition is a potential contributor to the pathology of obesity-related metabolic complications. However, the underlying mechanisms are still unclear. The major findings from my dissertation studies include: in STUDY#1, the iron-regulating hormone, hepcidin, is not chronically elevated with sustained training in competitive collegiate runners, who have a high risk of iron deficiency ($p>0.05$); In STUDY#2, a high level of iron in the epididymal adipose tissue was accompanied by a robust adipose tissue remodeling, characterized by increased macrophages, fibrosis, cell death and elevated inflammation; In Study#3, five weeks voluntary exercise reduced weight, improved glucose intolerance and altered adipose tissue inflammatory gene expression in female polygenic obese KK mice. Contrary to our hypothesis, in STUDY#3, exercise did not improve the serum iron levels in KK mice and in STUDY#2 we observed no relationship between adipose tissue iron deposition and

glucose homeostasis. Together the three projects enhanced our understanding of the underlying cause of exercise-induced iron deficiency anemia in female athletes as well as the relationship among the risks of diabetes, iron overload and exercise.

Chapter 1

Statement of Problems

Iron is vital for oxygen transport, enzymatic activity and other metabolic reactions within the body. It plays an essential role as a cofactor for fuel oxidation and electron transport, but it also has the potential to cause oxidative damage if not carefully regulated [1]. Iron dysregulation can lead to serious health concerns, for example, insufficiency leads to anemia and excess iron leads to organ damage (e.g. heart, liver and pancreas). In order to further understand iron regulation in health and disease, my dissertation focused on: 1) investigating the abundance of iron-regulating hormone, hepcidin, in highly trained female distance runners; 2) analyzing polygenic obese mouse model with iron overload in adipose tissue; and 3) examining the influence of exercise on iron homeostasis and adipose tissue inflammation in a polygenic obese female mouse model.

Iron deficiency can directly affect health and well-being, and even modest iron deficiency (ID) can decrease athletic performance. Nutritional iron deficiency is the most frequent cause of anemia. The diagnosis of anemia includes iron-related indicators such as low hemoglobin, decreased serum ferritin (an iron storage protein), reduced mean corpuscular volume (mean red blood cell size) [2], and low transferrin saturation (an indicator of iron binding capacity) [3, 4]. Female endurance athletes are highly susceptible to the development of ID [5, 6] and iron deficiency anemia (IDA) [7, 8]. This has typically been attributed to one or more of the following mechanism: excessive

menstrual bleeding[6], hemolysis[9, 10], hematuria[11-13], gastrointestinal bleeding [14-16], sweating [13, 14, 17, 18], increased red blood cell turnover [19, 20], and exercise-related iron absorption disorders [19]. A recently discovered protein, hepcidin, is critical to iron regulation and may be important in exercise-related iron deficiency [21-23]. Hepcidin is a hormone expressed primarily in the liver and released into circulation [24, 25]. It degrades and internalizes the iron exporter, ferroportin, from the cell surface of important iron-regulating cells including the macrophage of the reticuloendothelial system and epithelial cells of the intestine. An elevation in hepcidin thereby results in increased iron retention in cells and decreased iron absorption from the intestine [26, 27]. This combination ultimately reduces the circulating iron level and thereby suppresses iron availability for erythropoiesis [28, 29]. In light of the increased prevalence of iron deficiency in athletes, the discovery of this important iron regulatory hormone led to the hypothesis that exercise might directly promote iron deficiency through increasing hepcidin. In support of this, several studies have demonstrated a transient increase in urinary/serum hepcidin after acute exercise and led to the hypothesis that this change contributes to the high prevalence of IDA in athletes [9, 30-33]. Although a transient hepcidin increase was observed after acute exercise, it is still unclear whether there is an additive or cumulative effect of multiple acute excursions of hepcidin in response to everyday training. As such, in this dissertation, the main objective of **my first project was to investigate whether hepcidin is chronically up-regulated at rest in highly trained female distance runners**.

In addition to the issues resulting from iron deficiency, iron accumulation can also lead to serious consequences. **My second project focused on the investigation of excessive iron accumulation and the related metabolic outcomes.** In particular, iron has been suggested to be a possible

contributor to the pathology of obesity and insulin resistance in Type 2 Diabetes Mellitus (T2DM) [34-37]. Excessive iron has been shown to increase T2DM risks, while lowering iron level can improve insulin sensitivity in obese individuals [37-39]. To better understand the mechanism of how iron increases T2DM risks, my second project was to study iron in a polygenic obese mouse model, the inbred KK/HIJ (KK) mouse. This mouse strain has often been used for studying metabolic syndrome because of the presence of impaired glucose homeostasis [40]. Their elevated serum iron concentration also provides an intriguing model for evaluating the influence of iron in the setting of obesity and insulin resistance. We have recently observed a >100-fold increase of iron levels in the epididymal fat pad of KK males mice. In my dissertation, the second project investigated the localization of iron deposition, examined the association between adipose tissue iron overload and adipose tissue inflammation as well as the metabolic dysfunction in this polygenic obese male mouse model.

My third project focused on the influence of exercise on iron homeostasis and adipose tissue inflammation in a polygenic obese female mouse model. Recently, adipose tissue iron metabolism has received attention because iron dysregulation has been recognized as a potential contributor to the pathology of obesity-related metabolic complications, such as type 2 diabetes mellitus (T2DM). Some studies have demonstrated elevated iron stores to precede insulin resistance [41, 42], while lowering serum iron was demonstrated to increase insulin sensitivity [43, 44]. Although exercise has been observed to contribute to weight loss and improvements in insulin resistance [45], the influence of exercise on adipose tissue inflammation is still being explored. Several recently published studies suggested a decrease of adipose tissue inflammation after short-term exercise intervention in high fat diet-induced obese male mice or rats [46-48]. However, there

is a paucity of data regarding the role of exercise on adipose tissue inflammation in obese females, primarily because effective models for such studies have not been developed; the importance of studying female models is becoming recognized as a vital effort due to important clinical differences between female and male physiology and pathophysiology [49]. Therefore, **the aim of my third project was to evaluate the influence of exercise on metabolic function, iron homeostasis and adipose tissue inflammation in a mouse strain with inherently obese females.**

The outline of my dissertation is as follows:

A. Iron regulation project with human subjects

- Determination of resting serum hepcidin in highly trained female distance runners [**PROJECT 1]**.

B. Iron regulation projects with animal model

- The association between adipose tissue iron overload and adipose tissue inflammation and metabolic dysfunction in a polygenic obese male mouse model **[PROJECT 2]**.
- The influence of exercise on adipose tissue inflammation and iron homeostasis in a polygenic obese female mouse model [**PROJECT 3]**.

Overall, the significance of my first project was to broaden our knowledge of iron deficiency in female athletes, which may ultimately help to reduce the prevalence and improve the treatment of this condition. The second and third projects, related to iron regulation in a polygenic obese mouse model, which facilitate our understanding of the role of iron homeostasis and adipose tissue inflammation in the setting of metabolic dysfunction. Together, **these studies advance our understanding about iron regulation in health and disease by expanding our knowledge in**

the areas of 1) hepcidin in iron homeostasis in female athletes; 2) adipose tissue iron deposition; and 3) the effect of exercise on iron homeostasis and adipose tissue inflammation.

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Chapter 2

Review of Literature

Epidemiology of iron deficiency and iron overload

Iron deficiency (ID) affects almost 50% of the population in developing and ~10% in developed countries, making it the most common nutritional deficiency [1, 2]. Iron deficiency anemia (IDA) is the end state of iron deficiency and IDA is the most common form of anemia. To date, this fundamental health issue continues to affect the health and quality of life in billions of people all over the world. The World Health Organization (WHO) estimates that anemia affects nearly two billion people worldwide, which is nearly one third of the rapidly growing world population of approximately seven billion people [3]. These figures indicate that anemia constitutes a big health problem in many countries. In addition, there is a clear gender difference, with iron deficiency being more prevalent among women [4, 5].

The female athletic population is at a heightened risk for IDA [6]. Iron is essential to produce adequate hemoglobin for red blood cell production (erythropoiesis) and IDA results in profound decrements in endurance performance [7-9]. The classic criteria used to diagnose IDA in females is ferritin levels below 12 μg/L accompanied by hemoglobin concentrations below 12 g/dL. Additional indicators include small red blood cell size (microcytic) and pale red blood cell color

(hypochromic), low hematocrit (the red blood cell volume to the total blood volume), low mean corpuscular volume [10], and high soluble transferrin receptor concentration [11]. Maintaining adequate iron stores is a high priority to athletes and coaches; however, iron deficiency continues to be prevalent in this population.

In addition to the issues resulting from iron deficiency, iron accumulation can also lead to serious consequences. In particular, iron has been suggested to be a possible contributor to the pathology of obesity and insulin resistance in T2DM. This is of great importance as obesity is a common and increasing worldwide health problem with two-thirds of the U.S. population classified as overweight or obese [12]. Along with this alarming prevalence of obesity, there is also a very high incidence of obesity-related diseases, such as Type 2 Diabetes Mellitus (T2DM) [13, 14]. Over twenty-five million people in the United States have T2DM, and there are nearly two million new cases reported each year [15]. Approximately 35% of U.S. adults have impaired fasting glucose and are at heightened risk of developing T2DM (Centers for Disease Control and Prevention). These health consequences also contribute to the growing financial burden of society and individuals. For example, according to American Diabetes Association, the estimated costs of diagnosed diabetes care in the United States was $245 billion in 2012 and average medical expenditures exceeded $13,000 per year for people with diabetes (American Diabetes Association) [16]. So far, the studied mechanisms include an abnormality in insulin signaling (reviewed in [17]), beta cell failure[18], activation of stress pathways [19, 20], mitochondrial dysfunction [21], alteration of hepatic fuel homeostasis [22], central nervous system dysregulation [23] and adipose tissue inflammation [24, 25]. However, the direct cause(s) of type 2 diabetes and underlying mechanisms remain largely unknown. Since obesity is a well-known contributor to this disorder,

close attention has also been paid to the contribution of nutrients and nutrient-sensing pathways in situations of chronic caloric excess. Most of the interest in the role of nutrients in diabetes is centered on macronutrients, but a micronutrient, iron, is also associated with diabetes risk. Understanding the mechanisms by which increased iron deposition might increase diabetes risk is critical to optimize the prevention and treatment of the disease.

Iron recycling is a highly regulated process

Iron plays an important role in energy production, oxygen utilization, and cellular proliferation. It can act as an electron donor as well as an electron acceptor because of the flexibility of interconverting between ferric (Fe^{3+}) and ferrous (Fe^{2+}) oxidation states. This makes iron an irreplaceable component of oxygen transport (hemoglobin) and storage (myoglobin), cytochromes, and enzymes (containing heme and/or nonheme, reviewed in [26]). However, the ease with which iron changes its oxidation state also allows iron to form the reactive oxygen species, including oxygen ions and peroxides, which can damage DNA, lipids, and protein enzymes. Subsequently these alterations can result in cellular dysfunction, apoptosis, and necrosis [27] (also reviewed in book [28]). In other words, if too little iron is available (iron deficiency), limitations on the synthesis of physiologically active iron-containing compounds can have harmful consequences. Alternatively, if too much iron accumulates (iron overload) and exceeds the body's capacity for safe transport and storage, iron toxicity may produce widespread organ damage and death. Collectively, both total body iron and cellular iron concentrations need to be carefully regulated to ensure adequate iron availability without leading to excess iron toxicity.

The iron cycle is a highly regulated process. Iron is absorbed into the duodenal enterocytes and can be stored or transported out of the basolateral surface of the enterocyte. If it enters the circulation, it is bound to transferrin, a protein that subsequently transports iron to target cells. Most iron is stored in bone marrow and is used for the production of red blood cells. After about 120 days, this iron is typically recycled as senescent red blood cells are engulfed by macrophages. The iron is then stored as ferritin in hepatocytes and macrophages as part of the reticuloendothelial system (**Figure 2-1**).

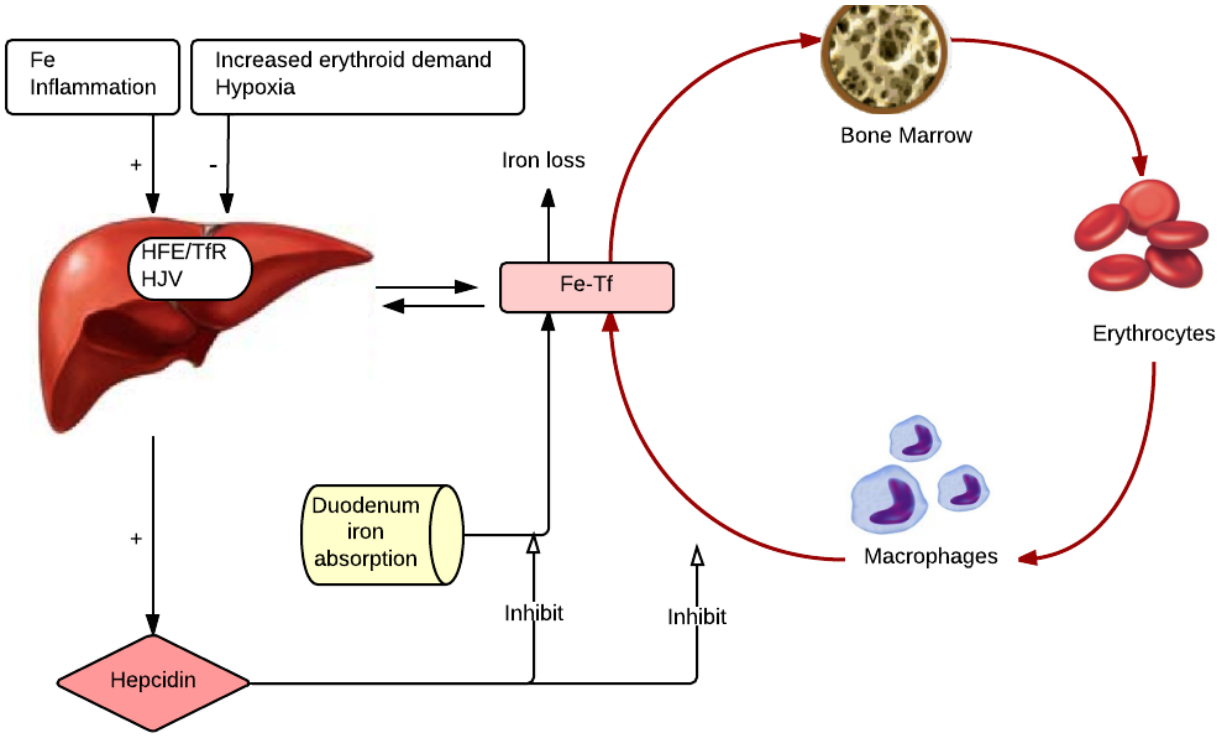


Figure 2-1. Iron recyclng in human body.

This figure depicts the flow of iron in mammals: from iron absorption (in the duodenum), to iron-transferrin (Fe-Tf) formation, to major iron utilization (the erythroid bone marrow), to circulating erythrocytes (red blood cells), to tissue macrophages that phagocytose senescent erythrocytes and recycle iron in the spleen (step not shown), to storage in liver hepatocytes, and back to Fe-Tf. Hepcidin is expressed and secreted mainly from liver via factors such as HFE, TfR and HJV. Elevated iron concentration in hepatocytes and inflammation both promote hepcidin production, whereas erythroid demands and hypoxia decrease its production. Hepcidin controls plasma iron concentration by both inhibiting iron absorption from the diet and sequestering iron in cells. *Abbreviations*: HFE: gene encoding human hemochromatosis protein; TfR: transferrin receptor; HJV, gene encoding hemojuvelin protein. Adapted from [29] and [28].

As shown in Figure 2-1, iron homeostasis is mainly regulated by hepcidin, a small peptide hormone [30]. At times of iron overload, hepcidin, produced mainly from liver due to various stimuli, can prevent cellular iron export [31]. Hepcidin regulates the exclusive cellular iron exporter, ferroportin [32, 33]. This iron regulator inhibits iron export from macrophages and enteric cells by binding to and inducing degradation of ferroportin, causing sequestration of iron in these cells (**Figure 2-2**). This reduces both intestinal iron absorption and iron release from macrophages into the circulation [34]. Hepcidin is encoded by the *HAMP* gene which results in the production of the 80 amino acid pre-pro-hormone [35]. Numerous pro-protein convertases can then cleave pre-pro-hepcidin to the 25 amino acid active peptide [36]. *HAMP* expression is induced by iron overload and inflammation as the body's main pathway avoiding excessive iron. Hepcidin levels can also increase during states of inflammation as a protective mechanism to reduce free iron in the presence of possible bacterial infection [32, 37, 38]. This response is mediated by the action of various cytokines including the pro-inflammatory cytokine interleukin-6 (IL-6), which induces HAMP expression [39]. In addition to liver-derived hepcidin, the monocyte and macrophage have been demonstrated to produce and secrete hepcidin and attenuate iron release through autocrine and paracrine mechanisms [40, 41]. Thus, monocyte hepcidin has the potential to sequester iron and thereby reduce the amount of iron available for erythropoiesis. In addition, iron sequestration in macrophage may lead to an increased pro-inflammatory state in the cell [42].

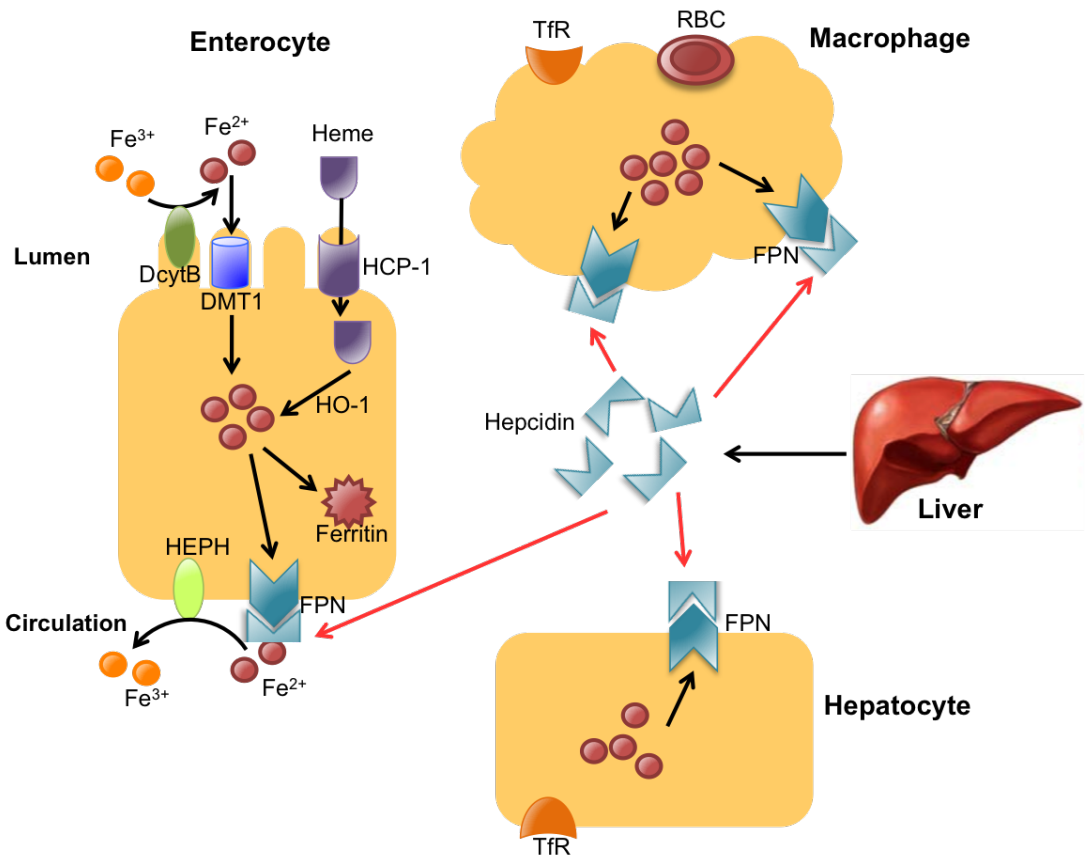


Figure 2-2. The role of hepcidin in systemic iron homeostasis.

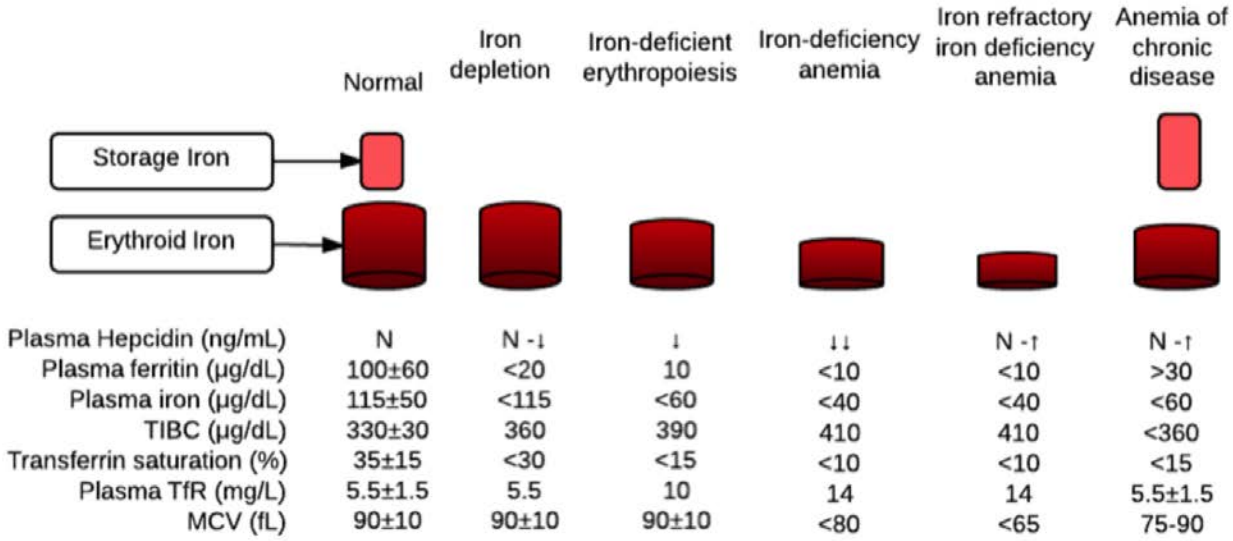
In the duodenal lumen, dietary iron is presented to the enterocyte as heme or nonheme iron. Heme iron is taken up by heme carrier protein (HCP-1) and heme-oxygenase 1(HO-1) is required for releasing iron from heme. Nonheme iron is predominantly ferric (Fe^{3+}), which must first be converted to ferrous (Fe^{2+}) by ferrireductase, such as duodenal cytochrome B (DcytB), before absorption. Ferrous iron is then transported across the apical membrane by the divalent metal transporter 1 (DMT1) in to the cell. Once inside the enterocyte, the absorbed iron enters into a common cytosolic iron pool. If the iron is not required for hemoglobin production, it is stored in ferritin protein in the cell. Iron required by the body is transported across the basolateral membrane by ferroportin (FPN), and the exported iron requires ferroxidase hephaesin (HEPH). In addition, hepcidin, expressed primarily in liver, inhibits iron absorption from the diet and the release from macrophages and hepatocytes. *Abbreviations*: HCP-1, heme carrier protein; HO-1, heme oxygenase 1; DcytB, duodenum cytochrome B; DMT1, divalent metal transporter 1; FPN, ferroportin; HEPH, hephaesin; TfR, transferrin receptor. Adapted from [26] and [43].

Different types and causes of iron deficiency

Iron deficiency occurs when the amount of body iron decreases due to insufficient iron supply. Three consecutive stages of decreased iron have been described [29] (**Figure 2-3**). The first stage is iron depletion, which begins when iron losses continually exceed iron absorption and recycling. This results in an isolated decrease in plasma/serum ferritin levels and a decrease in iron storage without a decline in functional iron level. The second stage is iron-deficiency erythropoiesis. After the exhaustion of iron stores, the production of hemoglobin and other iron-dependent metabolically active compounds become limited. In response, plasma/serum ferritin levels are low, transferrin saturation is decreased (<15%) and the total iron binding capacity is increased (>390 μg/dL). At the third stage, a further decrease in body iron leads to frank iron-deficiency anemia, where the levels of hemoglobin (<12g/dL), iron (<40 μg/dL), and transferrin saturation (<10%) are decreased and total iron binding capacity (>410 μg/dL) is increased (**Figure 2-3**).

There are various types and causes of iron deficiency. The ability to differentially diagnose the type and/or cause of the iron deficiency is critical for identifying optimal treatment strategies. Parameters including hemoglobin, mean corpuscular volume (MCV), plasma/serum iron, plasma/serum ferritin and total iron binding capacity (TIBC) are commonly used in the determination of iron deficiency. However, in order to differentiate between IDA, iron refractory iron deficiency anemia (IRIDA), anemia of chronic disease (ACD; also referred to as anemia of inflammatory response, **Figure 2-3**) and exercise-induced anemia, more tests are necessary. An elevation of circulating (plasma or serum) hepcidin concentration has very recently been used to characterize cases of IRIDA [44-46]. This measure has been used to separate IRIDA from IDA because IDA patients have low plasma/serum hepcidin levels. This discrepancy between the two

conditions relates to the important role of TMPRSS6 (which is mutated in IRIDA) to suppress HAMP expression when iron stores are low [47, 48]. In addition, the circulating concentration of soluble transferrin receptor (TfR) is also a useful indicator for iron deficiency [49-53]. TfR promotes iron uptake by binding diferric transferrin and transporting iron into the cytosol of iron requiring cells (e.g. erythroblasts). Cells deficient in iron upregulate TfR expression to compete for the circulating transferrin-bound iron [54, 55]. The soluble form of TfR (sTfR) is typically measured in plasma or serum and is used diagnostically because it is proportional to cellular TfR [56]. Consistent with this, concentrations of sTfR are increased in IDA and IRIDA patients but are within the normal range in ACD patients [57], which provide additional diagnostic measures to differentiate between the different types of anemia. Unfortunately, testing to conclusively differentiate exercise-related iron deficiency anemia from other forms of anemia will still require a more complete understanding of the specific mechanisms that cause exercise-related anemia(s).



	Normal	Iron depletion	Iron-deficient erythropoiesis	Iron-deficiency anemia	Iron refractory iron deficiency anemia	Anemia of chronic disease
Plasma Hepcidin (ng/mL)	N	N -↓	↓	↓↓	N -↑	N -↑
Plasma ferritin (μg/dL)	100±60	<20	10	<10	<10	>30
Plasma iron (μg/dL)	115±50	<115	<60	<40	<40	<60
TIBC (μg/dL)	330±30	360	390	410	410	<360
Transferrin saturation (%)	35±15	<30	<15	<10	<10	<15
Plasma TfR (mg/L)	5.5±1.5	5.5	10	14	14	5.5±1.5
MCV (fL)	90±10	90±10	90±10	<80	<65	75-90

Figure 2-3. The changes of erythroid iron and storage iron (hepatocyte and reticuloendothelial macrophage) in the presence of decreased body iron content.

Different indicators of iron status are shown in the development of anemia from normal to iron deficiency anemia (including the 3 stages of iron deficiency anemia development). Plasma hepcidin, ferritin, iron, transferrin saturation and MCV decrease, whereas TIBC and plasma TfR increase with increased iron deficiency. Iron refractory iron deficiency anemia (IRIDA) is similar to iron deficiency anemia with the exception of hepcidin concentration, which is significantly higher in IRIDA. In anemia of chronic disease, plasma TfR is normal. *Abbreviations*: TIBC, total iron binding capacity; MCV, mean corpuscular volume; TfR, transferrin receptor; N, normal. ↑, high; ↓, low. Adapted from [29] and [28].

Mechanisms of exercise-related iron deficiency anemia

It is important to note that the treatment for iron deficiency in athletes should be carefully governed. Endurance trained athletes, especially female athletes, often have low blood hemoglobin levels. It is so common for female endurance athletes to have reduced hemoglobin that it has given rise to the concept of "sports anemia" [58, 59]. However, this concept is controversial because: 1) not all highly trained athletes acquire an iron deficient state; and 2) it does not consider hemodilution and other parameters, such as transferrin saturation, total iron binding capacity, serum ferritin or mean corpuscular volume to determine if iron levels are sufficient. Therefore, some scientists in the field have considered "sports anemia" as nothing more than "dilutional pseudo-anemia" suggesting that it is not truly an anemic state but simply the dilution of the hemoglobin due to an enhancement in plasma volume. Studies have demonstrated that many highly trained athletes, who have relatively low hemoglobin concentration, maintain a high red blood cell mass and normal body iron stores [60-64]. In addition to the increase in plasma volume, training also can induce an increase in erythropoiesis [60]. However, these two responses are independent and the result of different stimuli. The increase in plasma volume is regulated by changes in osmotic pressure, which depends on hormonal and protein responses to short and long-term exercise [60]. On the other hand, erythropoiesis depends on the production and release of erythropoietin, which is regulated by oxygen content of the blood perfusing the kidneys [60]. Therefore, "sports anemia" is not a true iron deficiency anemia.

Because of the possibility for athletes to have pseudoanemia and therefore to maintain adequate iron stores, the practice of continuous iron supplementation is not prudent for athletes without demonstrated evidence of iron deficiency. Henegauer et. al and Tsalis et. al. showed that iron

intake did not affect serum ferritin, serum iron, transferrin saturation, final hemoglobin, or hematocrit with high intensity exercise training. Thus, it seems that neither dietary iron availability nor iron supplementation appears to have an influence on the phenomenon of "sports anemia"[65, 66]. As such, the treatment for “sports anemia” (or “pseudo-anemia”) should be carefully examined. Self-medication should not be encouraged because of potential iron intolerance, the risk of overdose, and potential interaction with other medications.

Despite the possibility that some athletes will maintain adequate iron stores, the evidence for female endurance athletes is that many experience a continuous struggle to avoid iron deficiency. This has been the focus of many research studies and several mechanisms of iron loss have been attributed to exercise training. The following list outlines the most commonly touted potential mechanisms:

1) Gastrointestinal blood loss [67]: during exercise, blood flow to the gastrointestinal tract is compromised due to the increased blood requirement to muscles and skin [68, 69]. A 56% blood flow reduction can occur during exercise due to increased sympathetic nervous system activity driven by a high exercise intensity [70, 71]. As a result, the gastrointestinal tract may be deprived of oxygen and metabolic substrates, leading to necrosis and mucosal bleeding [69].

2) Hematuria [72], which is the presence of blood in the urine due to intense exercise. Increased bladder movement during exercise, especially running, may cause bleeding of the interior wall [73].

3) Hemolysis, the destruction of red blood cells during exercise. This implicates a mechanism of iron loss, since the destruction of the red blood cell membrane allows the hemoglobin and associated iron within the cell to be released to the plasma [74].

4) Iron-loss from sweating during exercise. This is another mechanism by which the body may lose iron. One study outlined that sweat can lead to about 0.14mg/L of iron loss, and the iron loss from prolonged time and multiple training sessions may impact on body iron status [75].

5) Excessive menstruation in female athletes [8, 76]. Rowland et al. showed an inverse correlation between the amount of menstrual flow and serum ferritin level [76].

6) Elevation of the iron-regulatory hormone, hepcidin. Several studies have suggested that increased hepcidin, associated with exercise-induced inflammation, may represent an important mechanism for exercise-induced iron deficiency anemia [77-80].

The following section will introduce detailed information about exercise and hepcidin.

Does exercise induce iron deficiency via increasing hepcidin?

Studies have shown that exercise can transiently elevate hepcidin. The scientists in this field have speculated that these transient elevations result from acute exercise-induced inflammation and that long-term training could promote an iron-depleted state over time [77, 78, 80-82]. Iron is absorbed by enterocytes in the duodenum and proximal jejunum; however, only about 5% (non-heme sources) to 25% (heme sources) of the iron in the diet is typically absorbed [83, 84]. The majority of the body's iron stores are recycled from senescent red blood cells by splenic and hepatic tissue macrophages [85]. Circulating monocytes also play an important role in iron recycling by clearing hemoglobin-haptoglobin complexes that result from intravascular hemolysis. As described in the previous section, the iron recycled by monocytes/macrophages after phagocytosis of senescent red blood cells represents the main iron supply for erythropoiesis in the bone marrow [10]. Hormonal control, exerted primarily through hepcidin, contributes to iron homeostasis through influencing

iron export from the macrophage and enterocyte (**Figure 2-2**) [86]. Considering the high prevalence of iron deficiency anemia in the highly-trained population, hepcidin might be a mechanistic connection between exercise and iron deficiency anemia in athletes.

A significant elevation in hepcidin has been observed in specific groups with anemia associated with inflammation and/or chronic diseases (for example, anemia of chronic disease (ACD)) [35, 39, 42, 87-90]. In these conditions, anemia is secondary to an up-regulation of hepcidin induced by chronic inflammation [90]. Exercise training-induced inflammation, possibly through activation of the acute phase response and/or elevation in circulating IL-6, may promote a similar set of symptoms including elevated hepcidin [91-93]. Recent studies have described a transient elevation in serum hepcidin levels approximately three hours after exercise [93-95]. However, other studies demonstrated that hepcidin is decreased in IDA and low iron stores directly suppress hepcidin concentration [48, 96]. In addition, many of the published studies have focused on urinary hepcidin and/or the response to a single exercise stimulus [93, 97-101]. Despite the growing body of evidence on the topic, the clinical significance of the hepcidin response to exercise and its relevance to IDA has yet to be established.

Cellular hepcidin regulation and iron homeostasis

As discussed above, iron homeostasis is maintained by the hormone, hepcidin, through effects mediated by the iron export protein, ferroportin. Ferroportin, which is expressed on the surface of iron-releasing cells, is triggered for degradation by hepcidin binding [35, 96]. Hence, hepcidin binding to cellular ferroportin leads to iron sequestration and reduces plasma iron levels. As described above, it regulates intestinal iron absorption, macrophage-mediated iron recycling from

senescent erythrocytes, and iron mobilization from hepatic stores [102]. On the other hand, hepcidin expression is influenced by systemic stimuli such as iron stores, systemic iron availability, the rate of erythropoiesis, inflammation, hypoxia and oxidative stress. These stimuli control hepcidin levels by acting through hepatocyte cell surface proteins including human hemochromatosis protein (HFE), transferrin receptor 2 (TfR2), hemojuvelin (HJV), TMPRSS6 and the interleukin 6 receptor (IL-6R) [103].

Iron availability for erythropoiesis and cellular functions is determined by the amount of iron bound transferrin that circulates in the plasma. In the duodenum, in order to be absorbed and pass iron transporters, ferric iron (Fe3+) is first reduced to ferrous iron (Fe2+) by ferrireductase duodenal cytochrome b (DCTB). Ferrous ions then enter the enterocyte through the divalent metal ion transporter1 (DMT1 or *SLC11A2*). Iron is next transported to the basolateral side of the cell and exits the enterocyte through ferroportin (FPN or *SLC40A1*), which is the only known iron export channel [104]. The iron is oxidized to Fe3+ by ferroxidase hephaestin (HEPH), whereupon it is bound to transferrin Fe(III)-Tf and can be readily taken up by all cell types via the ubiquitously expressed transferrin receptors (TfRs), TfR1 and TfR2 [50, 105]. In addition, transferrin saturation is the ratio of serum iron to the total iron-binding capacity. Its measure in serum is a sensitive indicator of functional iron deficiency [50]. Transferrin-bound iron also interacts with the hepatocyte TfR2 and the protein HFE on the surface of hepatocytes [106]. A signaling pathway including hemojuvelin (HJV), bone morphogenic protein 6 (BMP6) [107, 108], and SMAD (Human homolog of Drosophila mad) [109] stimulates the production of hepcidin (**Figure 2-4**). Therefore, the membrane proteins HFE, TfR2 and HJV contribute to hepcidin regulation. Furthermore, HJV is a glycophosphatidylinositol (GPI)-anchored protein that acts as a bone-

morphogenetic protein (BMP) co-receptor, driving hepcidin transcription via the BMP/SMAD signaling cascade [110]. Disease-associated dysregulation in HJV causes a severe phenotype of iron overload, indicating that the HJV/BMP pathway plays a critical role in maintaining basal hepcidin levels [108, 111].

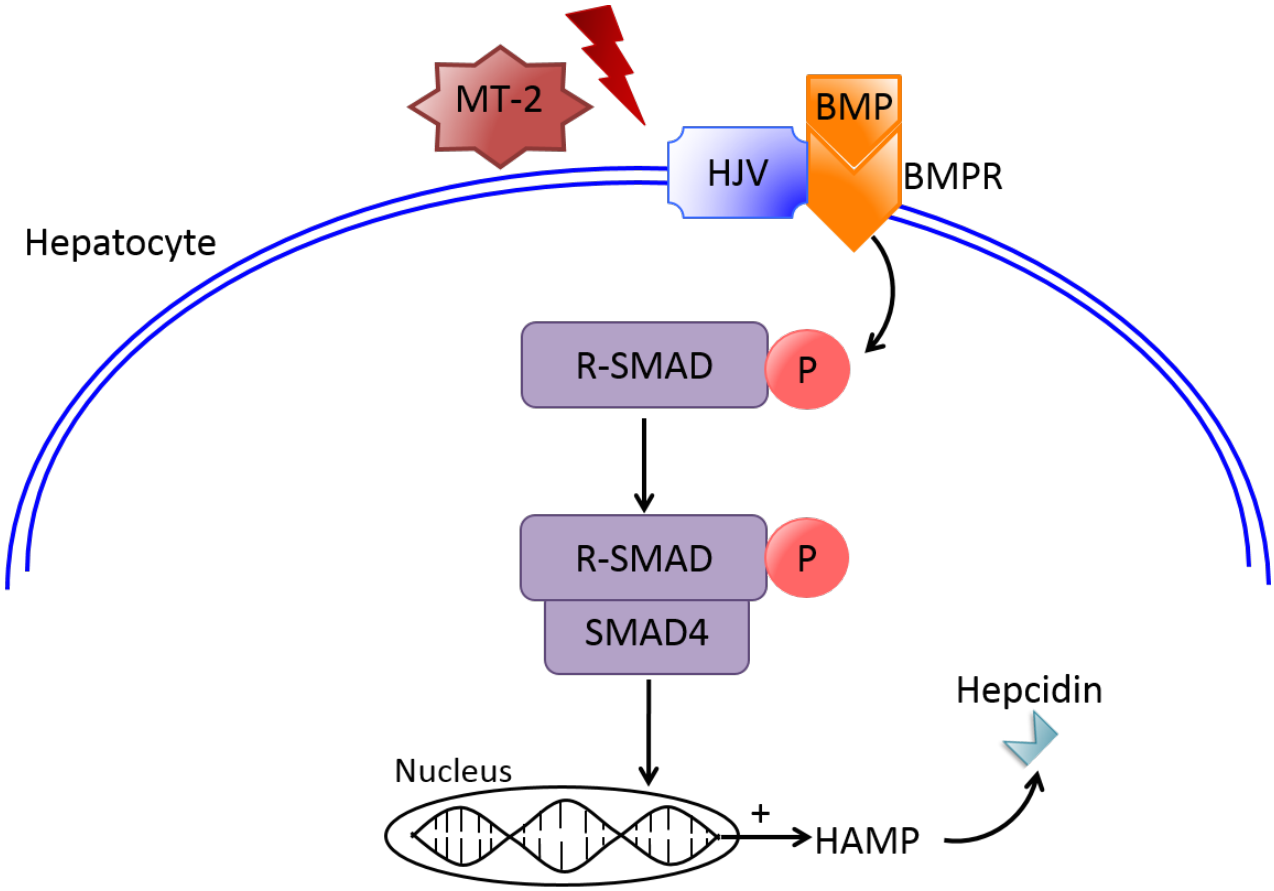


Figure 2-4. Schematic representation of the regulation of hepcidin expression.

Matriptase-2 is encoded by TMPRSS6 gene. TMPRSS6 gene mutation suppresses the interaction of MT-2 and hemojuvelin (HJV) (indicated by the red lighting bolt), which prevents the fragmentation of HJV. The reaction of BMP-BMPR and BMP co-receptor HJV activates the signaling cascade. BMP receptors (BMPR) at the plasma membrane induce phosphorylation of receptor-activated SMAD (R-SMAD) proteins. The subsequent formation of active transcriptional complexes involves the co-SMAD factor SMAD4. The complexes activate hepcidin transcription via bone morphogenetic protein (BMP)/SMAD signaling. *Abbreviations*: MT-2, Matriptase-2; HJV, hemojuvelin; BMP, bone morphogenetic protein; BMPR, BMP receptor; SMAD, Smad protein; HMAP, gene encodes hepcidin. Adapted from [44].

The association between iron regulation and T2DM risks

Interestingly, iron stores are also associated with T2DM. The Third National Health and Nutrition Examination Survey (NHANES III) studied ~ 9,500 participants and demonstrated that increased serum ferritin, a marker of body iron stores, was strongly associated with newly diagnosed diabetes [112]. Furthermore, elevated serum hepcidin levels have been observed in prediabetic patients with impaired fasting glucose [113]. Finally, insulin resistance has also been observed in beta thalassemia major patients with iron overload [114, 115]. However, all of these associations do not provide evidence for iron playing a causative role in glucose dysregulation. Nevertheless, many studies have demonstrated elevated iron stores to precede insulin resistance and/or T2DM. For example, Jiang et al. used a prospective cohort study design to demonstrate that an elevation of baseline serum ferritin was associated with an increased risk of T2DM during a 10 year follow-up of ~33,000 participants in the Nurses Health Study [116]. Additionally, without age, BMI, gender, family history, physical inactivity, smoking and diet factors, Forrouhi et al. showed that serum ferritin can predict the development of diabetes in the EPIC-Norfolk study [117]. Furthermore, recent studies have demonstrated that lowering serum iron decreased T2D risks. For example, in subjects with diabetes and high ferritin levels [118], as well as in healthy subjects with normal serum ferritin levels [119], a phlebotomy intervention reduced body iron stores and increased their insulin sensitivity This growing body of evidence further suggests that iron may directly contribute to T2DM risks.

Dysregulation of iron homeostatic pathways may be especially prominent in the setting of chronic inflammation. It has been well accepted that obesity, an important risk factor to T2DM, is

associated with chronic low-grade inflammation. White adipose tissue (WAT) is not only a major site for fat storage, it is also a major endocrine and secretory organ, which releases a wide range of protein signals and factors such as adipokines. A number of adipokines, including leptin, adiponectin, tumor necrosis factor α (TNFα), IL-1β (interleukin 1β), interleukin-6 (IL-6), monocyte chemotactic protein-1, macrophage migration inhibitory factor, vascular endothelial growth factor, and plasminogen activator inhibitor 1, are linked to inflammation and the inflammatory response [120]. Obesity alters adipose tissue metabolic and endocrine function and leads to an increased release of fatty acids and pro-inflammatory molecules that contribute to obesity-associated complications [120]. In 1995, Spiegelman and colleagues first characterized the link between inflammation, obesity and insulin resistance via tumor necrosis factor-alpha (TNFa), which is a pro-inflammatory cytokine that was found to impair insulin signaling via serine site phosphorylation on insulin receptor substrate 1 (IRS1) [121].

Macrophages in WAT can also play an active role in obesity, and macrophage-related inflammatory activities may contribute to the pathogenesis of obesity-induced insulin resistance [122]. The metabolic disorders in the setting of obesity may partially result from increased macrophage infiltration in the adipose tissue and elevated levels of circulating inflammation markers such as interleukin-6 (IL-6), tumor necrosis factor (TNFα), C-reactive protein (CRP), and macrophage chemoattractant protein (MCP-1) [123]. In 2003, Ferrante et. al characterized the macrophage changes that occur in the adipose tissue with increasing adiposity. Adipose tissue macrophages are responsible for the secretion of many cytokines, including TNF-α, iNOS and IL6 [124]. This indicates that adipose tissue macrophage numbers increase in obesity and participate in inflammatory pathways that are activated in adipose tissues of obese individuals [124]. It is

important to recognize that subsets of macrophages play differing roles in inflammation. Two subsets are most commonly described: M1 macrophages are thought to promote a chronic low-grade inflammation in adipose tissue, while M2 macrophages are thought to provide an anti-inflammatory influence [125]. In addition to the difference in their numbers, adipose tissue macrophages in lean and obese animals exhibit distinct cellular localizations and inflammatory potentials [126]. Using a special dye (PKH26) technique, Oh et al. demonstrated that high fat diet induces an increase of macrophage infiltration to the adipose tissue [127]. In addition, this study also demonstrated that monocytes are not pre-programmed to become inflammatory ATMs, but rather become pro-inflammatory in response to the tissue signals present in the obese condition [127]. One possible iron signal that promotes inflammation in the setting of obesity is iron. For example, the increased oxidative stress via elevated iron has been demonstrated to promote inflammation [128, 129]. Tajima et al. studied an obese and diabetic mouse model (KK/Ay) and demonstrated that a reduced iron diet resulted in an amelioration of adipocyte hypertrophy by suppressing oxidative stress, inflammatory cytokines (such as TNF-α, IL-6, IL-1β, and MCP-1), and macrophage infiltration, thereby breaking a vicious cycle in obesity [130]. Adiponectin, an adipokine secreted from adipose tissue, is inversely associated with adipose tissue mass and causally linked to insulin sensitivity. Studies in mice, human and cell culture have demonstrated that iron lowers adiponectin production and increases diabetes risk [131]. To this end, iron has been demonstrated to play an important role in metabolic syndrome including detrimental effects on adipocyte function and modulation of metabolism through inflammation and oxidative stress. However, it should be noted that the pathology of diabetes and iron overload is controversial. Thus, a better understanding of the influence of iron on obesity and insulin sensitivity is needed.

Adipose tissue iron metabolism has recently received considerable attention. Adipocytes express not only common iron homeostasis regulators such as ferritin and iron-regulatory proteins [132], but also iron-related proteins with restricted tissue expression (TfR2, HFE, and hepcidin) [133, 134]. Iron overload has been demonstrated to promote adipocyte insulin resistance [131, 135], whereas strategies to reduce iron concentration (e.g., low iron diet, chelation therapy, and phlebotomy) have led to improvements in insulin sensitivity in obese animal models [130, 136, 137] and humans [118, 131, 138, 139]. Regarding the effect of insulin metabolism on iron regulation, insulin treatment promotes iron uptake by increasing cell-surface expression of TfR1 in adipocytes [140, 141]. These studies, combined with the fact that macrophages play the predominant role in controlling systemic iron recycling [142], raise the possibility that ATM iron handling contributes to AT homeostasis. Furthermore, animal studies have demonstrated that obesity induces an increase in M1 polarization [126, 143], which, based on in vitro studies, may further promote iron deposition [144, 145]. Interestingly, Orr et. al. recently indicated that high fat diet promotes iron partitioning to adipocytes and also reduces the iron handling capacity in adipose tissue macrophage [146]. Clearly, more studies are needed to understand adipose tissue iron deposition and its influence on obesity-related metabolic consequences.

The influence of exercise on T2DM and adipose tissue inflammation

It is importatnt to note that both exercise and weight loss have been shown to improve glucose tolerance. For example, acute exercise or exercise training without weight loss can increase skeletal muscle glucose uptake and improve insulin sensitivity [147-149]. In addition, calorie restriction or bariatric surgery induced weight loss can also improve insulin sensitivity by decreasing fat mass, changing the adipokines release (reviewed by [150]) and reducing fatty acid mobilization [151]. Therefore both exercise and weight loss are important and independent factors

resulting in improved insulin sensitivity. In this literature review, I will focus on the inflence of exercise.

Physical inactivity and sedentary behavior also increase the risk of metabolic disorder. An inactive lifestyle leads to the accumulation of visceral fat, and this is accompanied by adipose tissue infiltration by pro-inflammatory immune cells, increased release of adipokines and the development of a low-grade systemic inflammatory state [152]. Again, this low-grade systemic inflammation has been associated with the development of insulin resistance in T2DM[123]. Exercise, on the other hand, has a beneficial effect on metabolic control. It may reduce the risk of developing obesity and excessive adiposity by increasing energy expenditure. In addition, regular exercise can improve the blood lipid profile and therefore promote cardiovascular health, for example, exercise increases the concentration of protective high-density lipoprotein (HDL) cholesterol and decrease the concentration of low-density lipoprotein (LDL) and plasma triglycerides. [153]. These beneficial adjustments in plasma lipids are considered to limit the development of obesity and T2DM [153]. However, it has been suggested that exercise exerts an anti-inflammatory influence on adipose tissue [154], which might be another important benefit of exercise against obesity- related complications. The proposed mechanisms of this anti-inflammatory effect include: increased levels of cortisol and adrenaline [155, 156]; elevated IL-6 and other myokines from working skeletal muscle [157]; a rising number of circulating T-regulatory cells [158]; a decrease in the monocyte / macrophage expression of Toll Like Receptors (TLRs) [154, 157, 159]; and/or a reduced number of circulating pro-inflammatory monocytes [154, 157, 158, 160, 161]. In addition, gene expression studies of adipose tissue RNA have led to speculation that exercise accelerates the polarization of macrophage toward an anti-inflammatory (M2) phenotype from a pro-inflammatory (M1) phenotype [162]. However, despite the evidence

to data, future studies are needed to delineate the direct role of exercise on changes in adipose tissue inflammation.

Although exercise has been observed to contribute to weight loss and improvements in insulin resistance [154], the influence of exercise on adipose tissue inflammation is still being explored. Several recently published studies suggested a decrease of adipose tissue inflammation after short-term exercise intervention in high fat diet-induced obese male mice or rats without weight loss [160, 161, 163]. However, there is a paucity of data regarding the role of exercise on adipose tissue inflammation in obese females, primarily because effective models for such studies have not been developed; the importance of studying female models as well as males is becoming recognized as a vital effort due to important clinical differences between female and male physiology and pathophysiology [164]. In addition, iron dysregulation is a potential contributor to the pathology of obesity-related metabolic complications, such as type 2 diabetes mellitus (T2DM). Studies have demonstrated elevated iron stores to precede insulin resistance [116, 117], while lowering serum iron can increase insulin sensitivity [118, 134]. Therefore, the aims of the third study were to evaluate the influence of exercise on metabolic function, adipose tissue inflammation and iron homeostasis in a mouse strain with inherently obese females.

Mouse model, KK/HIJ polygenic obese mice

The KK/HIJ strain is a model of metabolic dysfunction, displaying severe insulin resistance, hyperglycemia, and obesity without dietary intervention [165, 166]. This inbred mouse strain was first published in 1944, from studies resulting from K. Kondo's breeding of Nishiki-nezumi Japanese fancy mice in Kasukabe and subsequent inbreeding of the so-called "KK substrains" [167-169]. These KK substrains have often been used for studying metabolic dysfunction because of their glucose intolerance and insulin resistance, which result in hyperglycemia [170-172]. This

mouse strain also has elevated serum iron level, which makes it a suitable model for studying iron and metabolic dysfunction in my third project.

Summary of review of literature

The overall objective of my dissertation projects is to examine iron regulation in both health and disease. Although recent studies suggest that the transient increase in circulating hepcidin after acute exercise might promote iron deficiency anemia in athletes [77-79, 82, 173], it is unclear whether there is an additive or cumulative effect of multiple acute excursions of hepcidin in response to everyday training. Therefore, the first project was to broaden our knowledge of iron deficiency anemia with the aim to improve the treatment and reduce the prevalence of anemia. On the opposite end of the iron dysregulation spectrum, excess iron storage is a potential contributor to the pathology of obesity-related metabolic complications. Iron overload has been demonstrated to promote adipocyte insulin resistance [131, 135], whereas strategies to reduce iron concentration (e.g., low iron diet, chelation therapy, and phlebotomy) have led to improvements in insulin sensitivity in obese animal models [130, 136, 137] and humans [118, 131, 138, 139]. However, the mechanisms underlying adipose tissue iron deposition and its influence on obesity-related metabolic consequences are still unclear. Therefore, the second project used a unique mouse model to facilitate our understanding of the role of adipose tissue iron in metabolic dysfunction (e.g. glucose homeostasis). Although exercise has been observed to promote improvements in insulin resistance [154], the influence of exercise on adipose tissue inflammation and iron regulation in the setting of obesity is still being explored. As a close association has been observed between iron dysregulation, inflammation and diabetes [130, 174, 175], a better understanding of iron regulation could greatly improve the prevention and treatment of iron disorders research and treatment of iron disorders including iron deficiency and iron overload and their subsequent complications. Together, these studies will call attention to the importance of iron regulation in health and disease and specifically expand our knowledge in the areas of 1) hepcidin and exercise; 2) adipose tissue iron deposition and metabolic dysfunction; and 3) the influence of

exercise on adipose tissue inflammation and iron homeostasis in a polygenic obese female mouse model.

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Chapter 3

Serum hepcidin levels are not chronically elevated in collegiate female distance runners

Abstract

The prevalence of iron deficiency tends to be higher in athletic populations, especially among endurance-trained females. Recent studies have provided evidence that the iron-regulating hormone, hepcidin, is transiently increased with acute exercise and suggest that this may contribute to iron deficiency anemia in athletes. This is mainly through the suppression of iron exporting protein, ferroportin, by hepcidin. The purpose of this study was to determine whether resting serum hepcidin is significantly elevated in highly trained female distance runners compared to a low exercise control group. Due to the importance of the monocyte in the process of iron recycling, monocyte expression of hepcidin was also measured. A single fasted blood sample was collected mid-season from twenty female distance runners (RUN) averaging 81.9 ± 14.2 km of running per week. Ten age-, gender-, and BMI-matched low exercise control subjects (CON) provided samples during the same period using identical collection procedures. There was no difference between RUN and CON for serum hepcidin levels ($p=0.159$). In addition, monocyte hepcidin gene expression was not different between the two groups ($p=0.635$). Furthermore, no relationship between weekly training volume and serum hepcidin concentration was evident among the trained runners. The results suggest that hepcidin is not chronically elevated with sustained training in competitive collegiate runners. This is an important finding because the current clinical conditions that link hepcidin to anemia include a sustained elevation in serum hepcidin, Nevertheless,

additional studies are needed to determine the clinical relevance of the well-documented, transient rise in hepcidin that follows acute sessions of exercise.

Keywords: Exercise, Iron deficiency, Monocytes

Introduction

The female athletic population is at a heightened risk for iron deficiency anemia [1]. Because iron is essential to produce adequate hemoglobin for erythropoiesis, iron deficiency anemia results in profound decrements in endurance performance [2-4]. Iron deficiency anemia is often diagnosed in females with ferritin levels below 12 ug/L accompanied by hemoglobin concentration below 12 g/dL. Low hematocrit, low mean corpuscular volume (MCV), and high transferrin receptor values are additional indicators of iron deficiency anemia [5]. Maintaining adequate iron stores is a high priority for endurance performance; however, iron deficiency continues to be prevalent in female endurance athletes.

Iron recycling is a highly regulated process. Iron depletion occurs when iron losses continually exceed iron absorption and recycling. Exercise can contribute to iron loss in several ways, including gastrointestinal blood loss [6], hematuria [7], and increased red cell turnover [8]. Menstruation also contributes to iron loss in females. Iron is absorbed by enterocytes in the duodenum and proximal jejunum; however, only about 5% (non-heme sources) to 25% (heme sources) of the iron in the diet is typically absorbed [9,10]. The majority of the body's iron stores are recycled from senescent red blood cells by splenic and hepatic tissue macrophages [11]. In addition, the iron recycled by macrophages after phagocytosis of senescent red blood cells represents the main iron supply for erythropoiesis in the bone marrow [12]. Hepcidin contributes to iron homeostasis primarily through the macrophage and enterocyte [13].

Iron homeostasis is mainly regulated by hepcidin, a small peptide hormone produced primarily by the liver [14]. It is encoded by the HAMP gene, which results in the production of the 80 amino

acid pre-pro-hormone [15]. Numerous pro-protein convertases can cleave pre-pro-hepcidin to the 25 amino acid active peptide [16]. HAMP is known to be induced by iron overload and inflammation, and hepcidin regulates the exclusive cellular iron exporter, ferroportin [17, 18]. Hepcidin inhibits iron export from macrophages and enteric cells by binding to and inducing degradation of ferroportin causing sequestration of iron in these cells. This reduces both intestinal iron absorption and iron release from macrophages into the circulation [19]. Moreover, hepcidin is proposed to be increased in states of inflammation as a protective mechanism to reduce free iron in the presence of possible bacterial infection [17, 20, 21]. This response is mediated by the action of various cytokines including the pro-inflammatory cytokine interleukin-6 (IL-6), which has been shown to induce HAMP expression [22]. In addition to liver-derived hepcidin, the monocyte and macrophage have been demonstrated to secrete hepcidin and attenuate iron release through an autocrine mechanism [23] [24]. Thus, monocyte hepcidin has the potential to be detrimental to health by reducing the amount of iron available for erythropoiesis, increasing the intracellular macrophage iron content and increasing the pro-inflammatory state of the cell [25].

A significant elevation in hepcidin has been observed in specific groups with anemia associated with inflammation and/or chronic diseases [15, 22, 25-29]. In these conditions, anemia is secondary to an upregulation of hepcidin induced by chronic inflammation [29]. Exercise training-induced inflammation, possibly through activation of the acute phase response and/or elevation in circulating IL-6, has been speculated to promote a similar set of symptoms including elevated hepcidin and iron deficiency anemia [30-32]. Several studies have described a transient elevation in serum hepcidin levels approximately three hours after exercise [32-34]. However, many of the published studies have focused on urinary hepcidin and/or the response to a single exercise

stimulus [32, 35-39]. Despite the growing literature on the topic, the clinical significance of the hepcidin response to exercise and its relevance to iron deficiency anemia have yet to be established.

The purpose of this study was to determine whether a chronic hepcidin elevation is evident during periods of high training in female runners. This was investigated through measurement of serum hepcidin levels and other hematologic indicators in a cohort of collegiate female distance runners and low exercise control subjects. In addition, isolated monocytes were examined to investigate a potential influence of endurance exercise training on inflammatory and iron regulatory gene expression.

Methods

Subjects. A cohort of twenty female runners (RUN) were recruited from a NCAA Division I Varsity Cross Country team with cross-country 5 kilometer personal best times between 17 and 20 minutes. A control group (CON) was comprised of ten age- and BMI-matched female subjects with low levels of exercise (**Table** 3-1). The study was approved by the Institutional Review Board of the University of Michigan. Subjects were informed of the requirements of the study before consenting to participation. All subjects were female between 18 and 23 years of age. None of the subjects were pregnant or lactating, and there was no evidence or history of cardiovascular or metabolic disease.

A preliminary questionnaire was conducted before the blood collection to gather information regarding vitamin and iron supplementation, training level, lifestyle trends, and menstrual history. RUN reported average training distance per week, which was converted to minutes per week.

Minutes of weekly training in RUN also included bi-weekly weight lifting sessions (~60 minutes per week). Each subject's blood collection date was standardized for menstrual cycle in regularly cycling subjects, occurring between the 15th and 19th day of the cycle. On the day of blood sample collection, subjects reported the most recent exercise session, iron supplementation, and recent use of anti-inflammatory medications. Subject height and weight were measured to calculate BMI. A 20 mL blood sample was collected from the antecubital vein following a 12 hour overnight fast.

Hematologic measures. A 2 mL blood sample was collected into a commercially available *Acid Citrate Dextrose tube* (BD vacutainer, Franklin Lakes, NJ); complete blood count was performed by an Advia 120 Hematology System analyzer according to manufacturer instructions (Bayer Diagnostics, Tarrytown, NY). Whole blood was collected into a *Plus Plastic Serum tube* (BD vacutainer, Franklin Lakes, NJ). The sample was kept at room temperature for 30 minutes and was then centrifuged at 3000 x g for 10 minutes. Serum was stored in a -80℃ freezer until cytokine assays were performed. Serum iron, transferrin and transferrin saturation were determined using the Total Iron Binding Capacity (TIBC) assay (Pointe Scientific Inc., Canton, MI). Serum ferritin concentration was determined using the DRG ferritin immunoassay (DRG International, Inc., Mountainside, NJ). Hepcidin was measured by a commercially available enzyme immunoassay (EIA) kit (Peninsula Laboratories, San Carlos, CA). Serum soluble transferrin receptor concentration was determined using a commercially available ELISA assay kit (Human sTfR Quantikine IVD ELISA Kit, R&D Systems, MN).

Isolation of monocytes. Peripheral blood mononuclear cells were freshly isolated from whole blood by *Histopaque-1077* (Sigma-Aldrich; St. Louis, MO) density gradient centrifugation as

previously described [40]. Monocytes from 6 ml whole blood were prepared for RNA isolation and reverse transcription Polymerase Chain Reaction (rtPCR). Monocytes isolated from an additional 3 ml whole blood were prepared for cell cytokine examination.

Monocyte CD163 determination. Following monocyte isolation, the monocyte cell pellet was homogenized with 400ul RIPA buffer (Boston Bioproducts, Ashland, MA) to produce a cell lysate. The total protein of the cell lysate was determined by Bradford assay. CD163 concentration of the cell lysate was then measured by an immunoassay kit obtained from R&D (Quantikine HS ELISA kit; Minneapolis, MN) and normalized to total protein.

Determination of human monocyte gene expression. Total RNA was extracted from monocytes using the RNAqueous kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Total RNA was quantified by Nanodrop (NanoDrop Technologies, Wilmington, DE), and equal amounts of RNA (2ug) were reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Bio-systems, CA). Quantitative PCR (qPCR) was used to amplify the cDNA with gene specific primers using Fast SYBR green method (Applied Biosystems, Grand Island, NY). Different monocyte gene primers were constructed by the Perl Primer program [41]. GAPDH was used as a housekeeping gene. Table 3-2 shows the genes and sequences of primers used in this study. qPCR was carried out by StepOne plus software (Applied Biosystems, Foster City, CA). Results were analyzed by $2^{-\Delta\Delta CT}$ method described previously [42, 43] and were expressed relative to CON.

Statistical analysis. Each dependent variable was tested for evidence of a normal distribution by

use of a Q-Q plot. For measures that were normally distributed, we used an independent student's t-test to compare group means. For serum hepcidin, which had a non-normal distribution, we performed a log-transformation of the data, and then an independent student's t-test was applied to the normally distributed log-hepcidin data. The non-parametric Mann-Whitney U test was used to test for statistical significance among other variables with a non-normal distribution. The statistical analysis was carried out using the SPSS statistics package (IBM SPSS statistics 19) and results are expressed as Mean (±SD). P-values less than 0.05 were considered statistically significant.

Results

Questionnaire. Forty percent of RUN reported that they had been previously diagnosed with anemia, and 85% of RUN were regularly taking an iron supplement in the form of ferrous sulfate. Regular menstrual cycles were reported in 80% of CON and 70% of RUN. Approximately 75% of subjects across both groups reported two or less servings of red meat per week. Consistent with our recruitment strategy for CON and RUN, there was a distinct difference in the amount of weekly exercise between groups (**Table** 3-1).

Serum Hepcidin and Training Level. Hepcidin levels were within a range of 1.3-41.2 ng/ml in RUN and 0.9-41.1 ng/ml in CON. These values are reasonably consistent with a recently published normal reference range for 18-24 year old females (1.95 to 29.28 ng/ml) where a median value of 7.25 ng/mL was observed [44]. As the measurement was not normally distributed, a log-transformation was performed and independent t-test was used to compare the groups (**Figure 3-1**). There was no significant difference for serum hepcidin concentration between CON and RUN ($p=0.159$). The average weekly running distance in RUN ranged from 56.3 to 104.6 km and the

average distance ran in the 24 hours prior to the blood collection was 10.5 ± 6.4 km and ranged from 0 to 19.3 km. The training session preceding the blood sampling was a continuous run at a moderate aerobic effort (corresponding to a running pace of 12.9 to 13.7 km/hour). To examine whether these factors related to the variation in serum hepcidin concentration, we performed correlational analyses using the Spearman's rank-order correlation. However, there was no relationship evident between serum hepcidin and the weekly training distance or the running distance in the 24 hours preceding the blood sample (**Figure 3-2**a and **3-2**b). To investigate any impact of previous anemia, RUN was divided into two groups: 8 with and 12 without a self-reported history of anemia. However, there was no difference in serum hepcidin based on the history of anemia (p=0.173) (**Figure 3-2**c).

Monocyte Gene Expression. Gene expression studies were successfully performed on 27 of the 30 subjects (CON: n=10; RUN: n=17). No differences were observed between RUN and CON in the primary iron export protein, ferroportin (SLC40A1). Similarly, no differences were evident for the heme oxygenase gene, HMOX1, the inflammatory related genes, CCL2 and IL-6, or the hepcidin gene (HAMP) between RUN and CON. However, gene expression of the cell surface iron scavenger, CD-163, was significantly higher in RUN than CON ($P \leq 0.05$) (**Table 3-3**).

Monocyte CD163 protein. To further investigate the observed increase in monocyte CD163 gene expression, we used monocyte lysates to determine the monocytic CD163 protein level in RUN and CON (n=10 per group). However, there was no significant difference between RUN and CON for monocytic CD163 protein (CON: 20.6±10.4 ng/mg, RUN: 23.8±5.33 ng/mg; p=0.399).

Hematology. Complete blood count and iron status measures are shown in Table 3-4. White blood cell (WBC) count and several WBC sub-populations, such as leukocytes and eosinophils, were significantly lower in RUN ($p \leq 0.05$). This is consistent with previous studies regarding exercise and immune function [45]. Monocyte count tended to be lower in RUN ($p=0.064$), but there was no significant difference between RUN and CON (**Table** 3-4). However, mean corpuscular volume (MCV) was significantly higher in RUN versus CON ($p=0.015$). This may be indicative of increased hemolysis in the runners [46] and is not consistent with the microcytosis present in iron deficiency anemia. However, all subjects fell within the normal MCV clinical range of 80-99 fL. In both groups, the hemoglobin levels were relatively low across all subjects (range: 10.5-13.3 g/dL) compared to a typical normal range of 12.1-15.1g/dL, with no significant difference between RUN and CON. Mean serum ferritin and soluble transferrin receptor concentration were not significantly different between groups (**Table** 3-4).

Discussion

This is the first study using highly trained female collegiate distance runners with sustained high level training that investigates resting hepcidin levels. Evidence of elevated hepcidin at around 3 hours following moderate to high intensity exercise is well documented. However, the question of an additive or cumulative effect of multiple acute excursions of hepcidin in response to everyday training on elevating resting levels is still unclear. The main finding of this study was that highly trained female athletes did not have significantly higher resting hepcidin levels compared to control subjects, suggesting that there is no cumulative effect of chronic, daily endurance training on plasma hepcidin accumulation. A chronic elevation of hepcidin in response to training would

represent an important finding due to hepcidin's critical role in iron homeostasis and the relatively high prevalence of iron deficiency anemia in female athletes. However, we observed that serum hepcidin levels in highly trained female runners did not differ significantly from an age- and BMI-matched low exercise control group in a single resting measure. This result is in agreement with recently published training studies investigating serum hepcidin concentration where there was no significant increase in resting hepcidin following weeks of training [47, 48].

Studies have demonstrated that low iron stores directly suppress hepcidin concentration [49, 50]. In the current study, the relatively low serum ferritin levels were evident in both control and experimental groups compared to population reference standards (female normal range: 12-150 ng/mL). Therefore, the similar levels of serum hepcidin may be attributed to the low iron stores evident in both RUN and CON. Even an inflammatory stimulus that would normally increase hepcidin (e.g. exercise-induced IL-6) may be inhibited in the presence of low iron stores [26]. This concept is not without precedent as previous exercise studies have speculated that the transient rise in hepcidin in response to a single exercise session is suppressed in athletes with low serum ferritin [36] or low serum iron [32]. The cause of the low iron stores in the present study is also of interest. Although extensive dietary records were not collected, both CON and RUN subjects self-reported low intake of red meat. Red meat represents a source of heme iron which is most readily absorbed from the gut. A limited consumption of this type of dietary iron may have contributed to the observed low serum ferritin in both groups.

The monocyte, as a precursor to the macrophage, plays important roles in both inflammation and iron homeostasis. There is evidence of a two-fold elevation of hepcidin (*HAMP*) gene expression

from the circulating monocytes of anemia of chronic disease (ACD) patients versus non-ACD controls in the literature [24]. As high intensity endurance exercise has been hypothesized to promote hepcidin production and induce an inflammatory response similar to ACD, we isolated monocytes from RUN and CON and determined *HAMP* gene expression. However, despite the high training level of RUN (on average more than 80 km per week), we did not observe a significant increase in *HAMP* gene expression compared with CON. This result is consistent with other ACD-related measures in our study where we observed no difference between RUN and CON including serum levels of iron, ferritin, and hepcidin. Overall, the results from our study do not suggest an ACD-like phenotype resulting from the rigors of collegiate distance running in female athletes.

In addition to the measure of *HAMP* expression from our isolated monocytes, we also determined several other iron- and inflammation-related genes expressed in monocytes. Consistent with our lack of difference in *HAMP*, we did not observe differences in the gene expression of ferroportin (*SLC40A1*), *IL-6* or *CCL2* between RUN and CON. However, we did observe a modest increase in monocyte gene expression of *CD163* in RUN. *CD163* is expressed in monocytes and macrophages and is responsible for scavenging the hemoglobin-haptoglobin complex [27] as well as free hemoglobin [51]. This is especially interesting since others have established that increased intravascular hemolysis in runners generates hemoglobin-haptoglobin complexes and free hemoglobin [39, 52, 53]. In addition, *CD163* promotes an anti-inflammatory phenotype in monocytes and macrophages that promotes iron recycling [54, 55]. Although intriguing, this potential of an upregulation of *CD163* is tenuous in the present study, as the effect was small and was not confirmed in our measure of monocytic CD163 protein. Nevertheless, we speculate that

this may be an interesting iron-regulating response in the monocyte resulting from endurance exercise training.

Our study design had several limitations. One limitation is that we solicited blood samples from just one collegiate cross country team at one time-point in the middle of their competitive season. Therefore, our study had a small sample size and a limited power for detecting differences between the RUN and CON group. Another limitation was the partial confounding resulting from a high prevalence of iron supplementation in the athletes. Although the intent was for exercise volume to be the only variable that differed between RUN and CON, most of RUN (85%) were taking an oral iron supplement (ferrous sulfate) while none of CON were supplemented. However, the evidence to date suggests that iron supplementation results in an increase rather than a decrease in hepcidin concentration [22, 56]. In addition, comparing the hepcidin level between the athletes that had or did not have iron supplementation did not provide evidence of differences in hepcidin level due to supplementation. Therefore, the presence of the iron supplementation should increase the likelihood of detecting higher serum hepcidin in the athletes rather than conceal a difference between the groups.

Our study was also limited by variable sample collection schedule. Blood sampling was carefully scheduled to control for menstrual cycle phase and the time of day of collection. However, athletes were not asked to alter their training plan. As a result, the time between the last training session and the blood collection was either 16 or 23 hours in subjects training the previous day. There was variable distance of their most recent run as well as three subjects who did not train on the day preceding the blood collection. We expect that this was not problematic, as our goal was to

determine the cumulative effect of training at a single time point rather than the transient effect of training, which has been previously established [32, 35, 36, 38]. Additionally, we found no correlation between hepcidin level and the distance of the most recent training bout or the total average weekly training volume. Nevertheless, our study design would have benefitted from additional time points and/or additional control over the training sessions of athletes.

Conclusion and Clinical Relevance:

We have evaluated the resting serum hepcidin concentrations of collegiate female distance runners and observed no difference compared with a well-matched low exercise control group. Although hepcidin has been demonstrated to be transiently increased with strenuous exercise, we did not observe evidence of an elevation of resting hepcidin in highly-trained female athletes. However, the relatively low iron stores in both CON and RUN may confound the interpretation. In addition, studies of monocyte gene expression resulted in higher CD163 gene expression in the athletes compared with controls, suggesting a potential anti-inflammatory response in the monocyte resulting from endurance training. Further research should be conducted to determine the importance of the exercise-induced, transient elevation in hepcidin to the incidence of iron deficiency anemia. Moreover, iron deficiency in female is a widespread and multi-factorial problem, which will likely benefit from studies beyond the realm of the exercise-induced hepcidin response.

Acknowledgements

The authors would like to thank all of the subjects for their participation in this study. I wish to acknowledge Kaitlyn Patterson as co-first author, Kayla Gieschen for project management, and Monica Humby and Justin Kang for technical support.

Funding

The authors acknowledge funding received from the School of Kinesiology at the University of Michigan.

Table 3-1. Subject information

	Age (years)	BMI (kg/m^2)	Training level (min/week)	Percent Iron supplemented	Percent anti-inflammatory medications used
CON (n=10)	19.5 ± 1.35	20.5 ± 2.27	51.5 ± 39.7	0%	45%
RUN (n=20)	19.5 ± 1.07	20.5 ± 1.32	441.8 ± 64.2	85%	20%

Table 3-2. Primer sequences for monocyte gene expression. *Abbreviations*: GAPDH, housekeeping gene; HAMP, hepcidin; Hmox1, Heme-oxygenase 1; SLC40A1, Ferroportin; CCL2, chemokine (C-C motif) ligand 2; IL6, Interleukin 6; CD163, Cluster of Differentiation 163.

	Forward (5'→3')	**Reverse (5'→3')**
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HAMP	CTGCAACCCCAGGACAGAG	GGAATAAATAAGGAAGGGAGGGG
HMOX1	TCAACATCCAGCTCTTTGAGG	TAAGGACCCATCGGAGAAGC
SLC40A1	AATGCTAGACTTAAAGTGGCCC	GATATAGCAGGAAGTGAGAACCC
CCL2	GAAGCTGTGATCTTCAAGACC	GGAGTTTGGGTTTGCTTGTC
IL6	TGGCTGAAAAAGATTGGATGCT	AACTCCAAAAGACCAGTGATGATTT
CD163	ACATAGATCATGCATCTGTCATTTG	CATTCTCCTTGGAATCTCACTTCTA

Table 3-3. Iron- and inflammation- related monocyte gene expression *Abbreviations*: HAMP, hepcidin; Hmox1, Heme-oxygenase 1; SLC40A1, Ferroportin; CCL2, chemokine (C-C motif) ligand 2; IL6, Interleukin 6; CD163, Cluster of Differentiation 163.

	CON (n=10)	**RUN (n=17)**	**p-value**
HAMP (AU)	1.00 ± 0.39	1.11±0.61	0.635
IL6 (AU) §	1.00 ± 0.68	0.62 ± 0.34	0.083
CD163 (AU)	1.00 ± 0.17	1.18 ± 0.21 *	**0.039**
CCL2 (AU) §	1.00 ± 0.67	1.01 ± 0.61	0.711
SLC40A1 (AU)	1.00 ± 0.19	0.98 ± 0.25	0.834
Hmox1 (AU)	1.00 ± 0.24	1.02 ± 0.23	0.843

§ Nonparametric Mann-Whitney U test; *P≤0.05

Table 3-4. Complete blood cell count and hematologic results for CON and RUN.
Abbreviations: WBC, white blood cell; Lymph, lymphocytes; Mono, monocyte, Eos, Eosinophil; RBC, red blood cell; Hgb, hemoglobin; MCV, mean corpuscular volume; UIBC, unsaturated iron-binding capacity; TIBC, total iron-binding capacity; sTfR, soluble transferrin receptor.

	CON (n=10)	**RUN (n=20)**	**P-value**
WBC $\times 10^3$ (cells/ul)	6.3 ± 1.64	5.1 ± 0.82 *	**0.009**
Lymph $\times 10^3$(cells/ul)	2.3 ± 0.47	1.8 ± 0.39 *	**0.005**
Mono $\times 10^3$(cells/ul)	0.4 ± 0.15	0.3 ± 0.11	0.064
Eos $\times 10^3$(cells/ul)	0.2 ± 0.10	0.1 ± 0.06 *	**0.006**
RBC $\times 10^6$ (cells/ul)	3.9 ± 0.31	3.8 ± 0.24	0.283
Hgb (g/dl)	11.9 ± 0.76	11.8 ± 0.71	0.737
MCV (fL)	90.8 ± 4.43	94.52 ± 3.45*	**0.015**
Serum Iron (ug/dl)	113.3 ± 30.62	101.8 ± 34.65	0.384
UIBC (ug/dl)	200.2 ± 70.36	213.8 ± 82.70	0.659
TIBC (ug/dl)	313.5 ± 55.66	315.7 ± 72.46	0.933
Transferrin saturation (%)	37.3 ± 12.25	33.4 ± 11.36	0.384
Serum ferritin (ng/ml) §	25.0 ± 29.27	32.5 ± 24.23	0.120
Soluble transferrin receptor	13.8 ± 3.30	15.5 ± 2.94	0.154

§ Non-parametric Mann-Whitney U test; *P≤0.05

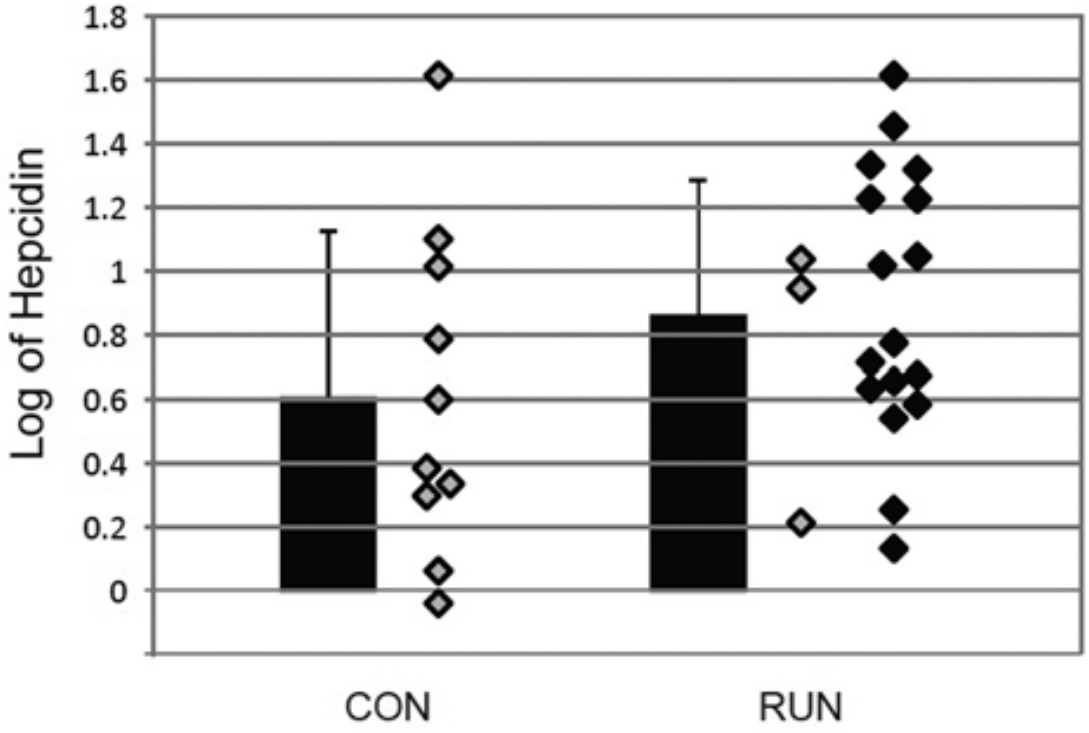

Figure 3-1. Serum hepcidin

Log-transformed serum hepcidin levels in both CON (n=10) and RUN (n=20). The individual subject values are provided for each group adjacent to the mean value. Subjects without iron supplementation are depicted by gray diamonds while iron supplemented subjects are depicted with black diamonds.

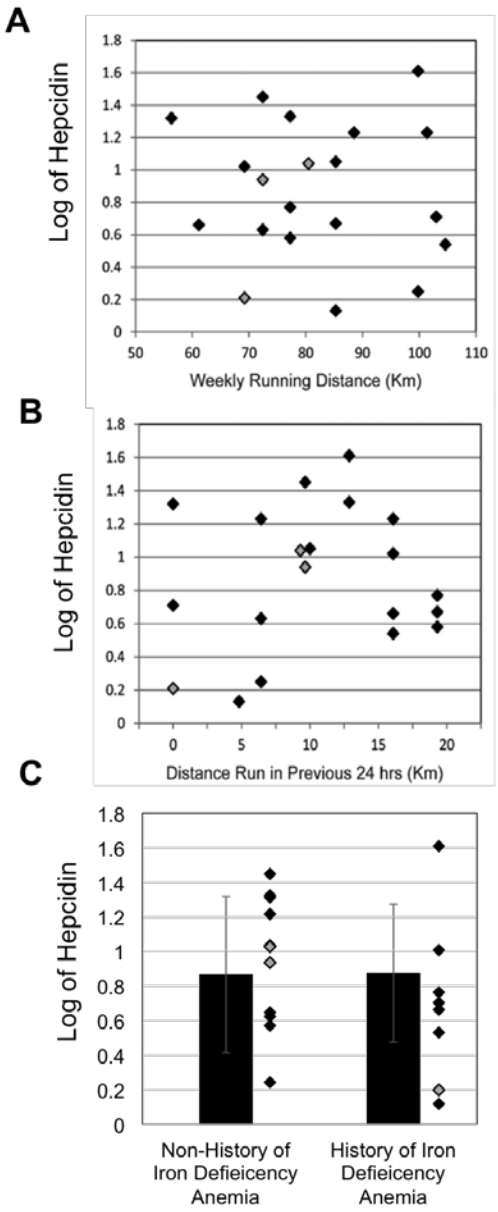

Figure 3-2. Correlation between serum hepcidin and training levels, recent training, and history of iron deficiency anemia in RUN (n=20)

A) The relationship between weekly running distance and log-transformed serum hepcidin; B) The relationship between distance run in 24 hours prior to the blood sample collection and log-transformed serum hepcidin; C) Bar graph of log-transformed serum hepcidin in runners with (n=8) and without (n=12) history of iron deficiency anemia. Black diamonds represent iron-supplemented runners (n=17), gray diamonds represent runners without iron supplementation (n=3).

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Chapter 4

Tissue-specific Iron Elevation and Adipose Tissue Remodeling in a Polygenic Obese Mouse Model

Abstract

Iron dysregulation is a potential contributor to the pathology of obesity-related metabolic complications. KK/HIJ (KK) mice, a polygenic mouse model of obesity and insulin resistance, have elevated serum iron levels and a propensity for tissue iron deposition. In previous assessments of older KK males, we observed a subset of mice with a discoloration of epididymal adipose tissue (eAT) associated with >100-fold ($p<0.001$) higher iron concentration. To further phenotype and characterize the adipose tissue iron overload and associated systemic effects, in this study, 27 male KK mice aged 47-79 wks were evaluated. Fourteen mice had discolored eAT (high iron, HI) and 13 had normal colored eAT (normal iron, NI). Fasting serum was collected and tissues were harvested for iron content, gene expression, and histology. Robust elevations of iron were confirmed in the eAT of HI versus NI mice. Surprisingly, iron levels in subcutaneous and brown adipose depots were not statistically different between groups ($p>0.05$). However, tissue iron levels were significantly higher in the liver (27%, $p<0.01$), pancreas (44%, $p<0.01$) and heart (30%, $p<0.01$) of the HI group. The eAT histology using H&E and F4/80 staining revealed a robust macrophage clustering, while trichrome and caspase3 staining revealed more fibrosis and cell death in the HI eAT compared with NI. rtPCR of eAT showed significantly decreased *Lep* (leptin)

and *Adipoq* (adiponectin), whereas *Tnfα* (tumor necrosis factor*α*) and the iron exporter, *Slc40a1* (ferroportin), were up regulated in HI compared with NI ($p<0.05$). No

significant difference of fasting serum glucose, serum insulin and insulin sensitivity index was observed between groups ($p>0.05$). Our data suggest that the deposition of iron in adipose tissue is limited to the epididymal depot in male KK/HIJ mice. The increased macrophages, collagen and cell death indicate a robust adipose tissue remodeling that is concomitant with the high iron concentration of the eAT. However, no association was evident between eAT iron deposition and glucose homeostasis.

Keywords: Tissue Iron, Glucose homeostasis, Adipose tissue macrophages

Introduction

Iron dysregulation is a potential contributor to the pathology of obesity-related metabolic complications, such as type 2 diabetes mellitus (T2DM). Studies have demonstrated elevated iron stores to precede insulin resistance [1,2], while lowering serum iron can increase insulin sensitivity [3,4]. This association between iron and diabetes is hypothesized to be linked by inflammation, and promoted by increased oxidative stress via elevated iron [5, 6]. Tajima et al. studied an obese and diabetic mouse model (KKay mice) and demonstrated that a reduced iron diet resulted in an amelioration of adipocyte hypertrophy by suppressing oxidative stress, inflammatory cytokines (such as TNF-α, interlukin-6 (IL-6), and monocyte chemotactic protein 1 (MCP-1)), and macrophage infiltration, thereby breaking a vicious cycle in obesity [7]. Adiponectin, an insulin-sensitizing adipokine secreted from adipocytes, is inversely associated with adipose tissue mass [8, 9]. Studies in humans, mice and cultured cells have demonstrated that excessive iron lowers adiponectin production and increases diabetes risk [10]. Both an increase in inflammatory cytokines and a decrease in adiponectin, can lead to the interruption of insulin signaling pathways and consequently to a decrease in insulin sensitivity [11-14]. In addition, strategies to reduce iron concentration (e.g. low iron diet, chelation therapy, and phlebotomy) have led to improvements in insulin sensitivity in obese animal models [7, 30, 31] and humans [3, 10, 32, 33]. To this end, iron has been demonstrated to play an important role in metabolic syndrome, including detrimental effects on the modulation of metabolism through inflammation and oxidative stress. However, it should be noted that the pathology of diabetes and iron overload is controversial. Thus, a better understanding of the influence of iron deposition in the setting of obesity is needed.

Adipose tissue iron metabolism has recently received considerable attention. The dual role of

adipose tissue macrophages in both inflammation and iron metabolism may link these recent observations. Adipose tissue inflammation has been considered an important factor contributing to the increased diabetes risk associated with obesity [12-18]. Obesity is often accompanied by a phenotypic switch of macrophages from anti-inflammatory "alternatively activated" M2 macrophages that primarily accumulate during healthy metabolic function [19], to the pro-inflammatory "classically activated" M1 macrophages [20]. The M1 population has been demonstrated to contribute to insulin resistance because of the production of pro-inflammatory cytokines such as TNF-α, IL-6, and MCP-1 [20-24]. Since the macrophage is one of the most important iron storage cells, the role of iron regulation in adipose tissue macrophages may provide important insights to the connection between iron and metabolic disease.

In addition to tissue macrophages, adipocytes also express common regulators of iron homeostasis including ferritin and iron-regulatory proteins hepcidin [26], as well as iron-related proteins with restricted tissue expression (e.g. TfR2-Transferrin Receptor 2, HFE-encoding Human hemochromatosis protein, and HAMP-hepcidin) [27, 28]. As mentioned, iron has been observed to be associated with inflammation in the setting of T2DM. More directly, iron overload has been demonstrated to promote adipocyte insulin resistance [10, 29]. Regarding the effect of insulin metabolism on iron regulation, insulin treatment promotes iron uptake by increasing cell-surface expression of TfR1 (Transferrin Receptor 1) in adipocytes [34, 35]. These studies, combined with the fact that macrophages play the predominant role in controlling systemic iron recycling [36], raise the possibility that iron handling by adipose tissue macrophage (ATM) contributes to normal adipose tissue function. Furthermore, animal studies have demonstrated that obesity induces an increase in M1 polarization [16, 25], which, based on in vitro studies, may further promote iron

deposition [37, 38]. Interestingly, Orr et. al. recently identified a population of adipose tissue macrophage with an iron handling phenotype and indicated that high fat diet promotes iron partitioning to adipocytes and also reduces the iron handling capacity of adipose tissue macrophage [39]. However, the direct metabolic consequences of iron deposition are still unclear. In summary, more studies are needed to understand adipose tissue iron deposition and its influence on obesity-related metabolic consequences.

KK/HIJ (KK) mice have been used as a polygenic mouse model of obesity and insulin resistance. Interestingly, the serum iron level is elevated (>2-fold) in this strain compared with other more commonly used mouse strains such as C57BL/6J or C57BL/10J [40]. The combination of these factors suggests the KK/HIJ mouse to be a useful model for iron and obesity-related research studies. In subsets of KK males, we have observed a grossly evident adipose tissue remodeling characterized by iron deposition within the epididymal adipose depot. In our pilot study, we observed that approximately 50% of KK males have distinctly discolored epididymal adipose tissue depots. We determined that this was directly associated with a striking increase in iron concentration in this adipose depot. Furthermore, we noted that the remainder of the KK males had normal adipose tissue iron levels (in line with KK females and both genders of the C57BL/6J strain). This observation motivated us to further characterize this phenomenon in male KK/HIJ mice. To our knowledge, there has not been any study addressing this increased tissue iron phenotype.

In this study, we identified two distinct groups of KK males: a normal iron adipose tissue group (NI) and a high iron adipose tissue group (HI). Our aim was to characterize the iron dysregulation

phenotype in the epididymal adipose tissue and evaluate the adipose tissue inflammation and remodeling in this polygenic obese male mouse model.

Methods

Animals.

Male KK/HIJ mice were obtained through in-house breeding at the University of Michigan from mice originally purchased from Jackson Laboratories (Strain #002106). Twenty-seven male KK mice aged 47-79 weeks were euthanized and checked for the presence of epididymal adipose tissue (eAT) discoloration (and subsequently eAT iron concentration). The mice with discolored eAT were assigned to "High Iron adipose tissue" group (HI, n=14), mice with normal colored eAT were assigned to "Normal Iron adipose tissue" group (NI, n=13). Five female KK mice (~ 34 weeks of age) from the same colony living under the same conditions were also euthanized for gonadal adipose tissue iron concentration. The animal care and experimentation were overseen and approved by the University of Michigan Committee on Use and Care of Animals.

Tissue iron assay.

The liver, spleen, heart, and pancreas as well as epididymal, subcutaneous (inguinal fat depot), and brown adipose tissue were weighed and harvested from each mouse. Gonadal adipose tissue samples were also harvested from the five KK female mice. Tissue iron concentration was determined using a commonly used, non-commercial iron chromogen colorimetric assay (main compositions including hydrochloric acid and trichloroacetic acid) described previously [41].

Histology.

eAT samples were fixed and stored in formalin after being harvested from the animals. Tissues then were transferred to histology cassettes individually and stored in 70% ethanol. The UM Histology Core performed paraffin processing, embedding, sectioning, and staining. The staining included hemotoxylin and eosin (H&E), F4/80, trichrome, and Caspase3 staining.

Serum measurements

Fasting serum insulin, adiponectin and leptin were measured using commercially available ELISA kits (Crystal Chem, Downers Grove, IL) according to manufacturer's instructions. Serum iron was analyzed using the QuantiChrom iron assay kit (Bioassay Systems, Hayward, CA) following the manufacturer's protocol. Insulin resistance was indicated by the homeostasis model assessment – estimated insulin resistance (HOMA-IR) index, which was calculated according to the following equation:

$$HOMA - IR = \frac{\text{Fasting Glucose(mg/dl)} \times \text{Fasting Insulin (μU/mL)}}{405}$$

Gene expression qRT-PCR

Total RNA was extracted from eAT and liver using the RNAqueous kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Bio systems, CA). Real-time PCR was used to amplify the cDNA with gene specific primers using Taqman gene expression assay (Applied Biosystems, FosterCity, CA) for TNFα and Leptin gene expression. Fast SYBR green Master Mix (Applied Biosystems, Grand Island, NY) was used for additional gene expression studies (**Table 4-1**). Real-time PCR was carried out by using StepOne plus software (Applied Biosystems, Foster

City, CA). Results then were analyzed using the $2^{-\Delta\Delta CT}$ method described previously [42].

Statistics

The primary comparison in this study was between the NI and HI groups, so an independent student's t-test was applied to determine significant differences between the groups. To evaluate the association between eAT iron and adipose tissue inflammation markers, Pearson's correlation tests were performed. Results were expressed as Mean (±SEM). The statistical analysis was carried out using the SPSS statistics package (IBM SPSS statistics 21). P-values less than 0.05 were considered statistically significant.

Results

Iron deposition is tissue and adipose depot-specific.

Iron concentrations in eAT were 115- fold higher in HI compared NI (NI: 0.0062 ± 0.002; HI: 0.71 ± 0.12 µg/mg tissue; $p<0.01$, **Figure 4-1a**). The gonadal adipose tissue (gAT) iron concentration in female KK mice was similar to eAT iron from the male NI group (gAT: 0.0082± 0.001 µg/mg tissue, $p>0.05$). In addition, the liver (27%, $p<0.01$), pancreas (44%, $p<0.01$) and heart (30%, $p<0.01$) had significantly higher iron concentration in HI compared with NI (**Figure 4-1b**). However, other adipose tissue depots, such as subcutaneous and brown adipose tissue, did not have significant differences in iron concentration between the two groups ($p>0.05$, **Figure 4-1b**). Duodenum, as the main dietary iron absorption site, was also not different between NI and HI ($p>0.05$, **Figure 4-1b**). Therefore, in addition to the major iron deposition organs including liver, pancreas and heart, iron overload in adipose tissue was specific to eAT in the HI group.

Iron deposition is associated with adipose tissue remodeling.

Tissue weight indicated that epididymal adipose tissue was smaller in HI compared with NI group ($p<0.05$, **Table 4-2**), while the heart, liver and spleen weights were not different between the two groups ($p>0.05$, **Table 4-2**). Histology data with H&E staining revealed robust adipose tissue cellularity changes in the HI group. The individual adipocyte size was in a much wider range and was irregular in HI compared with NI (**Figure 4-2**). In addition, a high number of non-adipocyte cells were observed residing between the adipocytes. F4/80 antibody staining revealed macrophage clustering among the adipocytes (**Figure 4-2**). By using Masson's trichrome stain for collagen fibers, we observed a significant amount of collagen fiber staining present among adipocytes in HI compared with NI (**Figure 4-2**). In addition, Caspase 3 staining suggested greater apoptosis in eAT of the HI group (**Figure 4-2**). Consistent with the robust remodeling of eAT in the HI group, the gene expression of leptin and adiponectin were markedly reduced compared with eAT of NI group ($p<0.01$, **Figure 4-3b**).

Iron deposition is associated with increased adipose tissue inflammation.

Gene expression studies of eAT were used to examine differences in genes relevant to adipose tissue inflammation and iron regulation between NI and HI. The pro-inflammatory gene marker TNFα was nearly 4-fold higher in eAT of HI group compared with NI group ($p<0.01$, **Figure 4-3a**), while the anti-inflammatory gene marker IL10 was not different in HI and NC. Gene expression of Vegfa (Vascular endothelial growth factor A) was on average 82% lower in the HI compared to NI, but this difference did not quite reach statistical significance ($p=0.058$). In addition, no statistical difference was evident between the groups for the gene expression of *Slc11a2* (encoding DMT1 the cellular iron intake marker) or *Hamp* (encoding hepcidin, which

degrades ferroportin) ($p>0.05$, **Figure 4-3a**). However, the gene expression of Slc40a1 (encoding iron exporter protein, ferroportin) was significantly higher (~3-fold) in the eAT of HI compared with NI ($p<0.05$, **Figure 4-3a).**

The glucose homeostasis was not changed with increased adipose tissue iron accumulation.

The analysis of metabolic markers including serum glucose (NI: 103.48 ±13.69 μg/dL; HI: 90.37 ± 24.83 μg/dL, $p>0.65$), serum insulin (NI: 1.83± 1.16; HI: 2.49 ± 1.13, $p>0.05$) and HOMA-IR ($p>0.05$, **Figure 4-4a**) were not different between HI and NI groups. Similarly, serum iron was not different (NI: 43.71 ± 16.32 μg/dL; HI: 47.78 ± 26.44 μg/dL, $p>0.05$, **Figure 4-4b**), despite differences in tissue iron deposition between the groups. Serum adipokines that are associated with adiposity and insulin sensitivity (e.g. serum adiponectin and serum leptin) were also investigated. Neither serum adiponectin (HI: 7.21 ± 0.58 μg /mL; NI: 7.42 ± 1.25 μg /mL, $p>0.05$) nor serum leptin (NI: 7.91 ± 1.62 ng/ml; HI: 6.48 ± 1.34 ng/ml, $p>0.05$) were significantly different between the HI and NI groups ($p>0.05$, **Figure 4-4c-4d**). Overall, iron deposition was localized to specific tissues and was not observed to produce overt differences in glucose homeostasis between NI and HI mice.

Discussion

In our study, we have characterized a polygenic obese mouse model with a propensity for tissue iron deposition in the epididymal adipose depot. We found that the adipose tissue deposition of iron in a subset of male KK/HIJ mice was specific to the eAT depot and not evident in the subcutaneous or brown adipose tissue depots. Moreover, these same animals had evidence of greater iron deposition in traditional tissues of iron overload including the liver, heart, and

pancreas. The increase in eAT macrophages, collagen and cell death suggests a robust tissue remodeling in the high iron subgroup. Furthermore, the marked increase in eAT *Tnfα* expression intimates that this depot has a marked increase in inflammation. The remarkably decreased levels of *Lep* and *Adipoq* expression suggest a localized adipocyte dysfunction. However, no systemic alterations were evident in mice with this localized eAT iron deposition including markers of glucose homeostasis and the serum concentration of the adipocytokines, leptin and adiponectin.

Analysis of the adipose tissue histology revealed a robust remodeling in the eAT of the HI group. In addition to the significantly increased eAT iron, our histology revealed an increase of macrophages (F4/80 staining) among the adipocytes as well as an increase of cell death (caspase 3 staining) and fibrosis (trichrome staining). It is not known whether either the observed elevation in ATMs or the high AT iron concentration are the cause or consequence of the robust adipose tissue remodeling. However, several published studies have suggested that adipocyte death and increased fibrosis can promote adipose tissue macrophage infiltration. For example, Cinti et al. showed that adipocyte necrosis is a significant phagocytic stimulus that regulates ATM infiltration [43]. Other studies have shown that macrophages can aggregate and form crown-like structures surrounding necrotic adipocytes [43-46]. In addition, the extracellular matrix (ECM) in adipose tissue plays an important role in supporting its function. For example, it can provide mechanical support for a fat pad as well as regulate physiological and pathological events through a variety of signaling pathways that are active in adipose tissue remodeling [47]. Adipose tissue fibrosis, however, is associated with reduced plasticity and therefore is a key indication of metabolic dysfunction [47]. We also observed a significantly lower tissue weight in the eAT of the HI group, which might be secondary to the adipose tissue remodeling. This finding is consistent with the

study from Dongiovanni et al. where high iron diet resulted in reduced epididymal adipose tissue mass [48]. The robust adipose tissue remodeling in HI mice was also associated with a greater than 80% reduction in adipose tissue *Vegfa* (vascular endothelial cell growth factor A) gene expression. This gene codes for VEGF-alpha and promotes pro-angiogenic activity as well as adipogenesis in adipose tissue through promoting preadipocyte proliferation [49]. Therefore, our results suggest that the HI group has evidence of localized disruption of normal adipose function and regulation in the eAT. However, interventional studies are needed to further our understanding of the direct cause of iron accumulation and adipose tissue remodeling and iron accumulation in the epididymal adipose tissue.

To further characterize the phenotype of the eAT with increased iron deposition; gene expression was performed to assess adipose tissue inflammation and iron signaling between the HI and NI groups. We observed higher expression of the inflammatory marker, *Tnfα,* in the eAT of the HI group. This is consistent with our histologic findings, where macrophage accumulation was strikingly increased in the HI eAT. As macrophages are one of the most important cells for iron storage, it is tempting to speculate that the macrophage infiltration may have preceded the elevation of iron. However, the current studies did not provide us the ability to temporally sequence the events leading to the observed increases in macrophage, inflammation and eAT iron. In addition, we are not aware of published evidence of macrophage infiltration promoting iron deposition in adipose tissue. Nevertheless, the remodeling in this depot was profound, as levels of *Lep* (leptin) and *Adipoq* (adiponectin) gene expression in the eAT were greatly reduced consistent with the observed loss of adipocytes. Of note to the overall phenotype, the gene expression studies also revealed significantly elevated *Slc40a1* (iron exporting protein ferroportin) in the HI eAT.

Previous studies have demonstrated that an increase in cytosolic iron promotes ferroportin transcription and ultimately promotes iron export through ferroportin protein on the cell surface [50] However, the gene expression of *Hamp* in our study also tended to be increased in the HI group (~7-fold), which could negate ferroportin function through autocrine or paracrine effects in the HI adipose tissues. Similar to our observations, Dongiovanni et al. demonstrated that mice provided an iron enriched diet had a ~10-fold elevation of iron concentration and a ~175-fold increase of HAMP gene express in the epididymal adipose tissue compared with normal diet control mice [48]. Therefore, our increase in both *Slc40a1* (~3-fold) and *Hamp* (~7-fold) expression is likely secondary to the deposition of iron in the eAT. However, despite iron deposition in many tissues, there was no evidence of iron sequestration from circulating iron, as serum iron levels were not different between HI and NI. Overall, we observed several changes in the HI mice that were consistent with C57BL/6 mice provided an oral iron overload, including iron deposition in and reduced size of the epididymal adipose tissue, reduced leptin expression and robust remodeling of the eAT [48]. Nevertheless, we had several findings inconsistent with Dongiovanni et al. including a decrease in adiponectin gene expression in the HI epididymal adipose tissue and no difference in serum iron levels associated with the iron deposition of the NI and HI groups.

Despite the robust local changes in eAT of the HI group, we did not observe an exacerbation of the metabolic function in KK male mice. Studies have shown that iron is negatively associated with adiponectin transcription and that loss of the adipocyte iron export channel, ferroportin, can result in adipocyte iron accumulation, decreased adiponectin, and insulin resistance in mice [10, 51]. Consistent with previous studies [10, 52], our results showed that the eAT iron has a strong

negative correlation with adiponectin gene expression in the epididymal adipose tissue (r^2=0.78). However, we did not observe a significant difference in circulating adiponectin between the HI and NI groups or a strong correlation between eAT iron and circulating (serum) adiponectin. Furthermore, unlike other studies showing that iron overload impairs glucose homeostasis and iron depletion improves insulin sensitivity [10, 33, 48], in our study, the analysis of fasting glucose and the insulin resistance index, HOMA-IR, revealed no difference between the HI and NI groups. We propose that the iron deposition in the KK adipose tissue is associated with the adipose tissue macrophages rather than the AT adipocytes, which would explain the considerable difference in the overall phenotype. Our histology supports this notion as adipocyte density appears considerably reduced and inflammatory cells are highly prevalent in the remodeled HI eAT.

To our knowledge, this is the first study demonstrating that about 50% of KK male mice are predisposed to iron accumulation and remodeling specifically in eAT depot. We observed a 115-fold higher iron in eAT of the HI group compared with NI group which was based initially on the discoloration of the eAT. Notably, these animals also had a significant elevation of tissue iron in liver, pancreas and heart (27-44%, $p<0.01$) compared with the NI group, suggesting that there was a systemic iron overload in tissues sensitive to iron deposition. However, no elevation of iron levels was evident in subcutaneous or brown adipose tissue depots indicating that the eAT may have a unique milieu regarding iron deposition. In addition, female KK mice were also evaluated for evidence of iron deposition in adipose tissue, but levels were similar to NI eAT iron and consistently low in gonadal adipose depot. This gender- and tissue-specific iron deposition represents an interesting phenomenon that may have important clues to our understanding of the prevalence of iron toxicity and iron deposition in adipose tissue, in particular. This eAT specificity

is consistent with other studies, where high iron diet or high fat diet-induced obese C57BL/6J showed a tissue iron increase in only the epididymal AT depot and not the subcutaneous adipose tissue depot [48, 53, 54]. In addition, a recent study evaluating only the epididymal adipose tissue in male C57BL/6 mice also observed an increase of eAT iron with high fat diet [52]. Consistent with this idea of a highly localized effect, we did not observe a significant difference of circulating (serum) iron levels between the NI and HI groups. However, we did observe robust differences in the adipose tissue histology and gene expression of the HI compared to the NI groups. The unique alterations of this specific adipose tissue depot may relate to variances in sympathetic innervation or other localized environmental differences. One considerable difference previously documented between the milieu of the eAT and other adipose depots is the presence of high levels of estrogen sulfotransferase (EST) in the eAT [55]. This enzyme promotes the sulfoconjugation of estrogen. High levels of EST in eAT can lead to the rapid inactivation of estrogen locally and may relate to this gender- and tissue-specific iron deposition in male KK mice. Consistent with this concept, increased adipose tissue iron has been observed in female mice following ovariectomy [56]. In addition, several publications have also demonstrated that EST deficiency can increase epididymal adipose tissue mass and adipocyte size; whereas EST overexpression decreased primary adipocyte differentiation and induced a similar adipose tissue remodeling (e.g. significantly decreased adipose tissue mass) as in our current study [55, 57]. Given the remarkable parallels between our observations and these findings, we speculate that the tissue- and gender-specific iron accumulation in the KK male mice may result from an elevation of epididymal adipose tissue estrogen inactivation by estrogen sulfotransferase.

Our study has several limitations that restrict the conclusions that we can make from our data. An important limitation of our study design was that the determination of the groups was based on post-mortem observation of the epididymal adipose tissue color. This is not ideal as it makes the phenotype appear qualitative and/or speculative. However, we have observed that the blinded measurement of eAT iron concentration has revealed a clearly defined quantitative endpoint that allowed the separation of mice into high iron eAT (HI) and normal iron eAT (NI) groups. In addition, to our knowledge, there is no published description of an inbred mouse strain with enhanced iron deposition in adipose tissue, which provided a unique opportunity for greater understanding of this phenomenon. Another important limitation was that we have a small number of animals for tissue weight, gene expression and hematologic measurements, and hence a low statistical power to detect differences between groups. This was the result of the study animals coming in two separate cohorts and fresh tissue samples for gene expression and hematologic measures were not available for one cohort. Although this did not prevent us from detecting several robust differences between the groups, it is a considerable limitation regarding our inability to detect differences between the groups regarding measures of glucose homeostasis and circulating adipokines (e.g. adiponectin and leptin). A third limitation of our study is that we were unable to determine the temporal sequence of the events surrounding the iron deposition and macrophage infiltration of the epididymal adipose tissue. Further study with iron manipulation (e.g. very low iron diet intervention) in KK male mice will be necessary to improve our understanding of the cause and consequences of this condition. However, the main focus of the current study was to better characterize the impact of the iron deposition on adipose tissue structure and function, as well as the overall metabolic consequences.

In conclusion, our study characterized the adipose tissue change and glucose homeostasis in KK mice with a grossly evident epididymal adipose tissue iron deposition. As the study of the male epididymal adipose depot is a commonly evaluated tissue, it is important to note that this depot may be unique regarding inflammation and iron deposition. We have observed in the KK strain a propensity for a site-specific iron accumulation in eAT that is associated with robust adipose tissue inflammation and remodeling. We speculate that the epididymal adipose tissue depot has a special propensity for iron deposition in the setting of iron overload. However, further studies are needed to characterize the systemic and local metabolic dysfunction as well as to identify the mechanism of the site-specific iron overload in the eAT. Overall, we propose that the KK mouse provides a robust model for examining the role of elevated iron in the setting of polygenic obesity.

Acknowledgements:

This study was supported by Rackham Graduate Research Grant from University of Michigan. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver NICHD/NIH (SCCPIR) grant U54-HD28934.

Table 4-1. Primers sequences for gene expression.

Abbreviations: Hamp, hepcidin; Slc11a2, DMT1; Slc40a1, ferroportin; Vegf, Vascular endothelial growth factor; Il10, interlukin 10; Adipoq, adiponection.

Gene	Forward (5'-->3')	Reverse (5'-->3')
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Hamp	CCTGAGCAGCACCACCTATCT	GCTTTCTTCCCCGTGCAAAGG
Slc11a2	TTGGCAATCATTGGTTCTGA	CTTCCGCAAGCCATATTTGT
Slc40a1	ATGGGAACTGTGGCCTTCAC	TCCAGGCATGAATACGGAGA
Vegf	CCACGTCAGAGAGCAACATCA	TCATTCTCTCTATGTGCTGGCTTT
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Adipoq	GGAGATGCAGGTCTTCTTGG	ATGTTGCAGTAGAACTTGCC

Table 4-2. Mouse tissue weights

Tissue weight	NI (n=6)	HI (n=4)	*P values*
Body Weight (g)	31.03 ± 1.19	28.98 ± 6.14	0.267
Epididymal Fat(g)	0.62 ± 0.12	0.14 ± 0.02	**0.026***
§%Epididymal fat	1.95 ± 0.33	0.50 ± 0.05	**0.019***
Perirenal Fat (g)	0.45 ± 0.10	0.25 ± 0.04	0.344
§%Perirenal fat	1.4 ± 0.30	0.90 ± 0.09	0.389
Heart (g)	0.15 ± 0.01	0.14 ± 0.00	0.586
§% Heart	0.47 ± 0.02	0.50 ± 0.02	0.606
Liver (g)	1.45 ± 0.08	1.17 ± 0.02	**0.035 ***
§% Liver	4.73 ±0.38	4.05 ± 0.11	0.201
Spleen (g)	0.12 ± 0.01	0.08 ± 0.00	**0.034 ***
§% Spleen	0.39 ± 0.03	0.28 ±0.03	0.064
Pancreas (g)	0.21 ± 0.03	0.24 ± 0.03	0.588

§ the percentage of the tissue weight relative to body weight

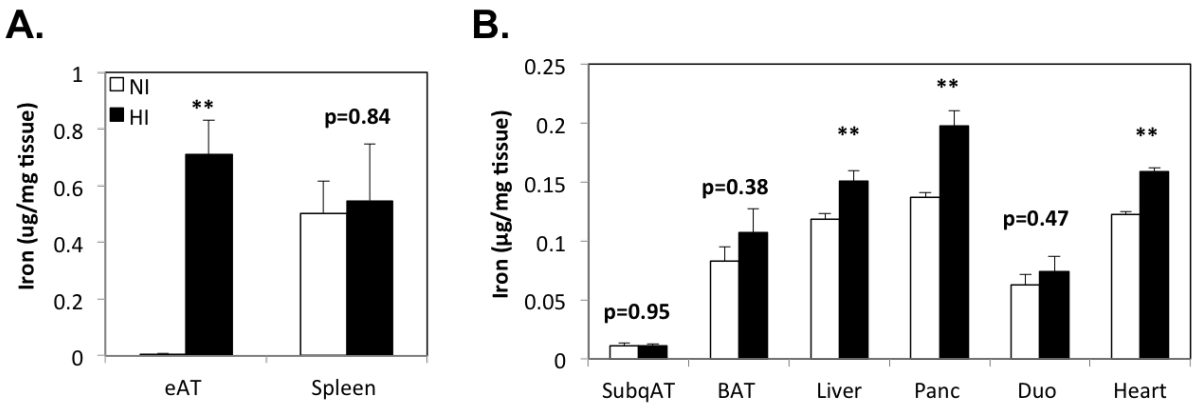

Figure 4-1 Iron deposition is tissue specific.

A), eAT iron was significantly elevated in HI group. B), common iron deposition tissues such as liver, pancreas and heart had elevated iron levels in HI. However, iron deposition in duodenum or other adipose tissue depots (i.e. subcutaneous and brown adipose tissue) was not elevated in HI group. *Abbreviations*: eAT, epididymal adipose tissue; SubqAT, subcutaneous adipose tissue; BAT, brown adipose tissue; Pan, pancreas; Duo, duodenum. **p<0.01 NI (open bar) *vs* HI (filled bar)

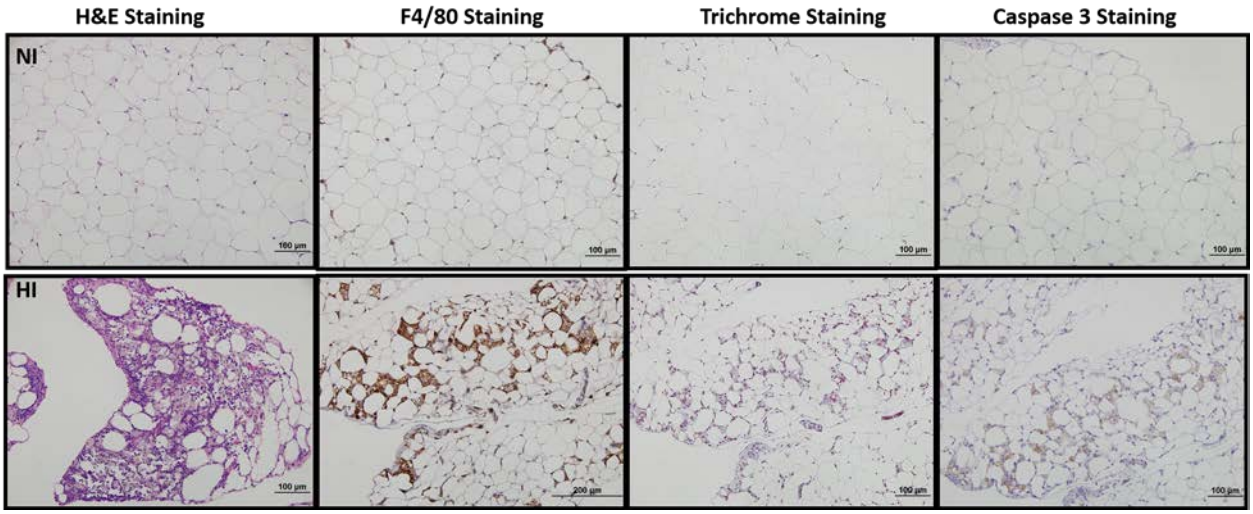

Figure 4-2. A robust tissue remodeling in the HI mice epididymal fat pads

Representitive adipose tissue histology from both NI (Top) and HI (Bottom) groups. *Colors*: F4/80, brown; Trichrome, blue; Caspase 3, brown.

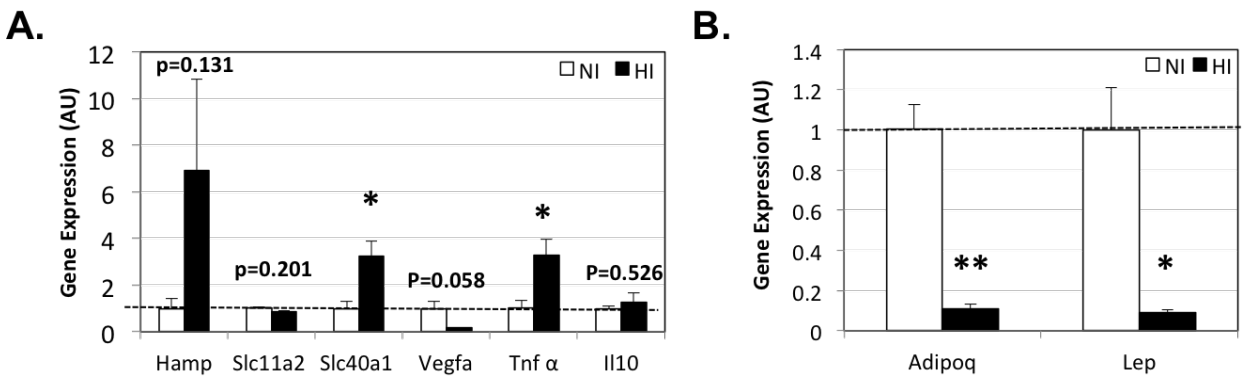

Figure 4-3. Epididymal adipose tissue gene expression

A) eAT gene expression with inflammatory and iron-regulating gene markers. B) eAT gene expression with adipokine gene markers. *Abbreviations:* *Hamp*, hepcidin; *Slc11a2*, DMT1; *Slc40a1*, ferroportin; *Vegfa*, Vascular endothelial growth factor A; *Tnf* α, tumor necrosis factor; *Il10*, interlukin 10; *Adipoq*, adiponection; *Lep*, leptin. NI (open bar, n=6) *v.s.* HI (filled bar, n=4). *p< 0.05, **p<0.01

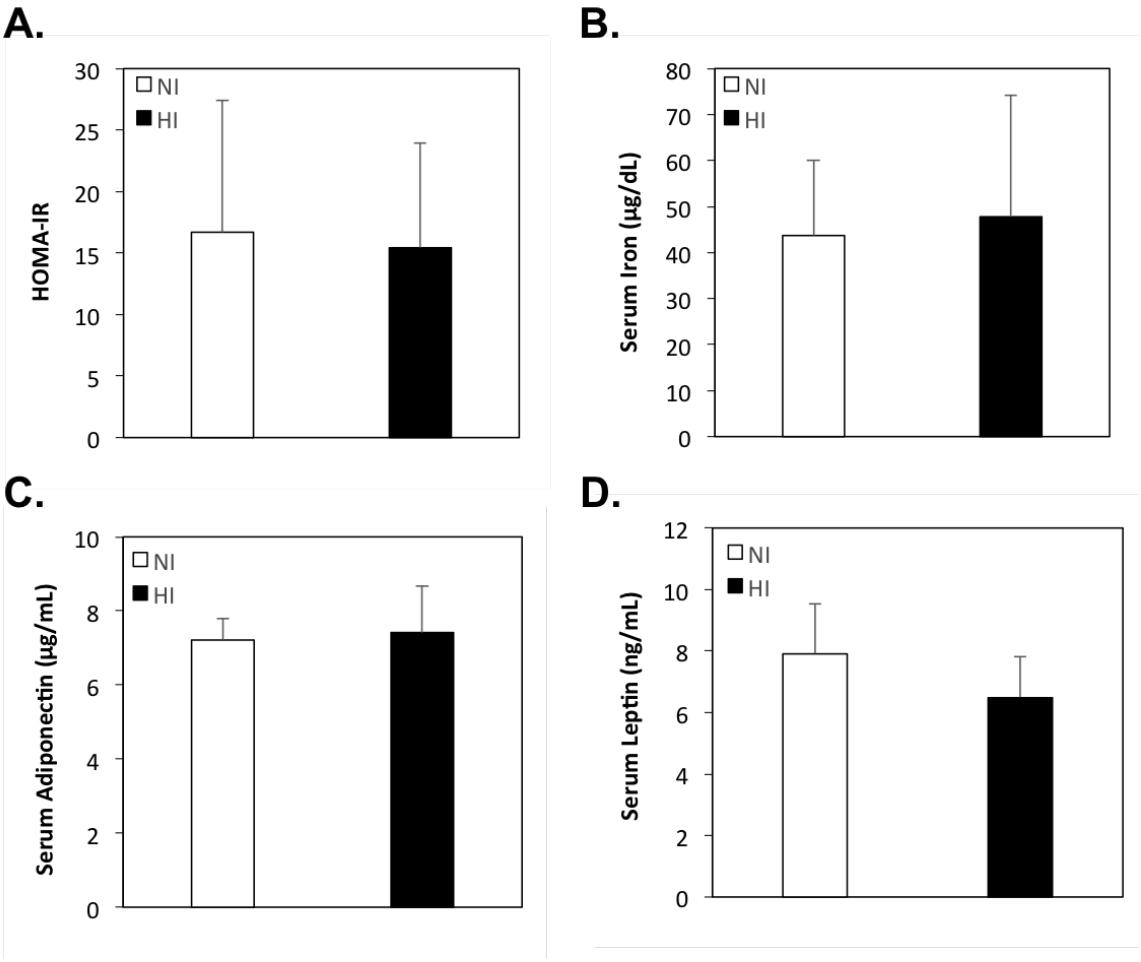

Figure 4-4. Comparison of hematologic measures between NI and HI.

A) Insulin resistance index HOMA-IR. B) = Serum iron levels between the two group. C&D) Serum adiponectin and leptin in NI (n=6) and HI (n=4) mice.

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Chapter 5

Effects of 5-week voluntary wheel running on adipose tissue inflammation in a female mouse model of polygenic obesity

Abstract

Background: Obesity-induced insulin resistance is associated with chronic low-grade inflammation in adipose tissue. Most studies of exercise and adipose tissue inflammation to date have examined male mice following a high fat diet. **Purpose**: In this study, we used female mice from a polygenic obese strain, the KK/HIJ mouse, to determine the effect of voluntary wheel running on adipose tissue inflammation, gene expression and whole-body glucose homeostasis. **Methods**: Female KK/HIJ mice (n=15) fed normal chow were randomly assigned to a control (CON) or voluntary exercise (EX) group for 5 weeks. Following the intervention, a complete blood count (CBC) was performed for blood hematological assessment; fasting serum iron, glucose, insulin, leptin, and adiponectin were measured and glucose tolerance was evaluated. In addition, adipose tissue was harvested and RT-PCR was used to evaluate adipose tissue gene expression. **Results**: Compared to CON, EX had decreased adiposity and significantly improved glucose homeostasis as evidenced by lower fasting insulin and increased glucose tolerance after the 5 weeks intervention ($p \leq 0.05$). Both serum leptin levels and adipose tissue *Lep* gene expression were significantly reduced ($p \leq 0.05$) following 5 weeks exercise intervention. Furthermore, adipose tissue gene expression of the pro-inflammatory marker, *Tnfα*, was down-regulated (49%, $p \leq 0.05$)

following the exercise intervention. Surprisingly, the adipose tissue anti-inflammatory gene markers including *Chi3l3* (chitinase 3-like 3), *Mgl1* (macrophage galactose *N*-acetyl-galactosamine–specific lectins 1) and *Mrc2* (mannose receptor, C type 2) were also down-regulated in EX ($p \leq 0.05$). **Conclusion**: The five week voluntary exercise intervention significantly lowered adiposity and improved glucose tolerance despite an increase in food intake. This short-term intervention also changed adipose tissue inflammatory gene expression in this female polygenic obese mouse model.

Keywords: Voluntary Exercise, Adipose tissue macrophages, Gene expression, KK/HIJ mice

Introduction

Obesity is a multi-faceted public health crisis that continues to grow and compromise the health of millions. It is evidently driven by changes in exposure to environmental factors, for example, reduced physical activity during work and leisure time, as well as the increased accessibility and reduced cost of calorie dense food [1]. In addition, an increasing number of studies have shown that hereditary factors play a significant role in determining human obesity [2]. A common metabolic perturbation in obese individuals is insulin resistance, which is known as a risk factor for chronic diseases including type 2 diabetes mellitus (T2DM) and cardiovascular diseases [3, 4]. Therefore, interventions that improve glucose tolerance are important therapeutic strategies for obese individuals. Exercise has been shown to improve insulin sensitivity [5, 6], assist in weight loss (or weight maintenance) [7], and exert anti-inflammatory effects [8-10]. However, the underlying mechanisms of the benefits of exercise are not completely understood, and few polygenic obese mouse models have been identified to address this issue.

Obesity is often characterized by chronic low-grade inflammation. Research studies suggest an association between metabolic disorders and a chronic low-grade inflammatory state in both obese humans [11-13] and mouse models of obesity [14, 15]. For example, the circulating levels of several inflammatory cytokines including tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), and C-reactive protein (CRP) are elevated in obesity and appear to contribute to metabolic dysfunction [16]. Studies evaluating TNFα and insulin signaling have demonstrated that TNFα impairs insulin-stimulated glucose storage [17] and insulin-stimulated glucose uptake [18] via serine site phosphorylation on insulin receptor substrate 1 [19]. Conversely, obese mice with TNFα deficiency were protected from insulin resistance [20] and inhibition of TNFα improved insulin

sensitivity in an insulin resistant mouse model [21]. In addition, anti-inflammatory cytokines such as interleukin-10 (IL-10) and the anti-inflammatory adipocytokine, adiponectin, are decreased in obese compared to normal weight individuals [22]. Furthermore, there is evidence from mouse models that the infiltration of macrophages into adipose tissue can contribute to the chronic low grade inflammatory state present in the obese condition [23]. Two macrophage subsets are commonly described: M1 or "classically activated" macrophages are thought to promote a chronic low-grade inflammatory state in adipose tissue, while M2 or "alternatively activated" macrophages are thought to provide an anti-inflammatory influence [24]. Adipose tissue macrophages (ATM) from lean mice have been observed to express many genes characteristic of M2 macrophages, including *Mgl1* (macrophage galactose *N*-acetyl-galactosamine–specific lectins 1) and *Mrc2* (mannose receptor, C type 2), *Chi3l3* (chitinase 3-like 3) and Il10. However, in the setting of diet-induced obesity, there appears to be a decrease in expression of these genes in ATMs and an increasing expression of genes such as *Tnfα* and *Il-6*, which are characteristic of M1 macrophages [25, 26]. In addition, overexpression of MCP-1 (monocyte chemoattractant protein-1) in adipose tissue increases ATM recruitment and mildly exacerbates obesity-induced insulin resistance [27, 28]. Together, these studies support the notion that ATMs contribute to the development of adipose tissue inflammation and systemic insulin resistance in obesity.

Although exercise has been observed to be capable of contributing to weight loss and improvements in insulin resistance [6], the influence of exercise on adipose tissue inflammation is still being explored. Several recently published studies suggested a decrease of adipose tissue inflammation after short-term exercise intervention in high fat diet-induced obese male mice or rats [29-31]. For example, in high fat diet-induced obese male C57BL/6 mice, four to 16 weeks of

treadmill training at 12-20 m/min for 2.5-6 km/week has been proposed to change the polarization of macrophage by up-regulating the anti-inflammatory (M2) and down-regulating the pro-inflammatory (M1) phenotype [32, 33]. However, there is a paucity of data regarding the role of exercise on adipose tissue inflammation in obese females, primarily because effective models for such studies have not been developed. The importance of studying female models as well as males is becoming recognized as a vital effort due to important clinical differences between female and male physiology and pathophysiology [34]. Therefore, the aim of this study was to evaluate the influence of exercise on metabolic function and adipose tissue inflammation in a mouse strain with inherently obese females.

Methods

Animals

KK/HIJ mice were obtained through in-house breeding at the University of Michigan from mice originally purchased from Jackson Laboratories (Strain #002106). The KK/HIJ strain is a model of metabolic dysfunction, displaying insulin resistance, hyperglycemia, and obesity without dietary intervention [35]. All mice were fed a commercially available chow diet (Lab Diet 5053, Lab Diet, Brentwood, MO), which contains 5.4% total calories from fat. The animal care and experimentation were overseen and approved by the University of Michigan Committee on Use and Care of Animals.

The KK/HIJ strain is relatively unique in the propensity of the female mice to accumulate fat on a standard low-fat diet. To further characterize this difference in adipose depots between genders of this strain, a total of 31 male and 27 female KK/HIJ mice were euthanized between 5 and 50 weeks

of age. Body weight, perirenal and gonadal/epididymal fat pad weights were recorded and the fat percentage was calculated by using the following equation:

Total Fat % = 100*(Perirenal fat + Gonadal fat)/Body weight

For the voluntary exercise intervention study, female mice at ~28 weeks of age were housed individually for two days before the start of data collection. The mice were maintained in a temperature controlled environment under a standard 12 hour light-dark cycle and provided *ad libitum* access to food and water throughout the study. Mice were randomly divided into two groups: control group (CON) (n=8) and exercise group (EX) (n=7).

Exercise Training

Mice in EX were provided a 12.7 cm running wheel (Petco, San Diego, CA). A wired odometer-Bell F12 Cyclocomputer (Easton-Bell Sports, Van Nuys, CA) was used to record wheel revolutions and running distance was recorded weekly.

Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (GTT) were performed 2 weeks before the exercise intervention began (PRE) and again after mice had undergone 5 wk of voluntary exercise intervention (POST). In each case, mice were fasted for 5 hrs (0800–1300) (POST GTT with access to the wheel) and were subsequently injected with glucose (1.5g/kg body wt *ip*). Tail blood was collected at 0, 30, 60, 90 and 120 min. Blood glucose concentrations were measured using a commercially available glucometer (Abbott Laboratories, Abbott Park, IL).

Hematologic Measures

Fasting blood samples were obtained PRE and POST exercise intervention after a 5hr food restriction (9am to 2pm) and were kept at room temperature for 30 min before being centrifuged at 3000 × g for 10 min. Serum was stored in a -80 °C freezer until cytokine assays were performed. Serum iron was analyzed using the QuantiChrom iron assay kit (Bioassay Systems, Hayward, CA) following the manufacturer's protocol. Fasting serum leptin and adiponectin levels were measured using commercially available ELISA kits (Crystal Chem, Downers Grove, IL) according to manufacturer's instructions. Insulin resistance was indicated by the homeostasis model assessment – estimated insulin resistance (HOMA-IR) index, which was calculated using the following equation: Fasting Glucose (mg/dL) × Fasting Insulin (μU/mL) / 405. Complete blood count (CBC) was performed with an Advia 120 Hematology System analyzer according to manufacturer instructions (Bayer Diagnostics, Tarrytown, NY).

Quantitative Real-Time PCR

Total RNA was extracted from gonadal adipose tissues using the RNAqueous kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Bio-systems, CA). Quantitative PCR (qPCR) was used to amplify the cDNA with gene specific primers using Taqman gene expression assay (Applied Biosystems, Foster City, CA) for TNFα, IL-6 and Leptin gene expression. Fast SYBR green Master Mix (Applied Biosystems, Grand Island, NY) was used for additional gene expression studies, and primer sequences are shown in **Table 5-1**. qPCR was carried out by StepOne plus software (Applied Biosystems, Foster City, CA). Results were then analyzed by $2^{-\Delta\Delta CT}$ method described previously [36, 37].

Statistical Analysis

Two-way ANOVA (Factor 1- Intervention: with or without voluntary exercise; Factor 2- Time: pre and post exercise intervention) was performed to determine whether there was a significant intervention by time interaction for the fasting serum glucose, insulin, HOMA-IR, GTT and iron parameters. In order to compare adipose tissue inflammation measures (i.e. gene expression) between EX and CON after 5 weeks exercise intervention, an independent student's t-test was applied. For correlational analyses, the Pearson's correlation coefficient test was performed. The statistical analysis was carried out using the SPSS statistics package (IBM SPSS statistics 19). Values are reported as group mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

Results

Female KK/HIJ mice have greater adiposity than males

Body weight of KK/HIJ male and female mice provided standard rodent chow increased with age between 5wks and 50wks and there was no significant difference between genders. However, the total fat percentage was markedly increased with age in females, while no change was observed in males (**Figure 5-1**). This is consistent with DXA body composition data from The Jackson Laboratory [38], where the body fat % is significantly higher in adult KK females than males with normal diet (Age: 14-18wks; Female: 43.5 ± 0.764%; Male: 32.7 ± 1.72%, $p<0.01$) [38].

5 Weeks of Voluntary Exercise Promotes Weight Loss in KK/HIJ Female Mice

Mice provided access to running wheels ran an average of 6.6 ± 0.7 km per day (**Figure 5-**2d). This was considerable but less than that previously observed in male [39] and female [40] C57BL/6 mice, documented to average ~10 and ~15 km per day, respectively. Following the 5 week intervention, the body weight of the mice in EX decreased by over 15% (**Figure 5-2a**; $p<0.01$), whereas mice in CON had no significant change in body weight during the same time period (**Figure 5-2a**). The gonadal and perirenal adipose depots were combined to determine the total fat pad weight from the tissue harvest. This measure of body fatness revealed a 65% lower fat mass in EX than CON (**Figure 5-2b**, $P\leq0.05$) despite an increased food intake in EX compared with CON during the 4th and 5th week of the intervention (**Figure 5-2c**). Serum leptin concentration was measured in the animals and, as expected, was tightly associated with the measured total fat weight ($r^2 = 0.89$,). A subset of both CON and EX groups had similar fasting serum levels of leptin before the intervention (PRE CON: 9.6 ± 1.3 ng/ml; PRE EX: 10.0 ± 1.6 ng/ml; n=4 per group), but consistent with the fat loss, the POST EX group had a strikingly lower leptin concentration following the five week intervention (POST CON: 10.17 ± 1.15 ng/ml, n=8; POST EX: 1.52 ± 0.69 ng/ml, n=7, $p\leq0.05$,). Overall, mice provided the voluntary running wheel were observed to have a 15% decrease in body weight due primarily to a reduction in adipose tissue and these changes were evident despite a modest but statistically significant increase in food intake during the 5-week intervention.

Voluntary Exercise Improves Glucose Tolerance in KK/HIJ Female Mice

Female KK/HIJ mice, in addition to having excess adipose tissue, have been documented to have considerable elevations in fasting serum insulin [41]. To evaluate the effect of exercise on glucose homeostasis in KK/HIJ female mice, we measured fasting glucose, fasting insulin, HOMA-IR, and

glucose tolerance both before and after intervention in both CON and EX groups. An index of the overall glucose tolerance was determined by calculating the incremental area under the curve (AUC) during the 2 hour GTT test. Two-way ANOVA analysis demonstrated that there was not a significant intervention (CON and EX) by time (PRE and POST) interaction for the measures taken in the fasted state: fasting glucose, fasting insulin and HOMA-IR ($p>0.05$). Indeed, fasting glucose was not different between CON and EX before or after the intervention ($p>0.05$; **Figure 5-3a**). Nevertheless, despite the lack of the intervention by time interaction, the fasting serum insulin was 30% lower in EX compared with CON following the intervention ($p<0.01$; **Figure 5-3b**) and HOMA-IR, was on average 35% lower in EX compared with CON after the intervention ($p\leq0.05$; **Figure 5-3c**). Importantly**,** the glucose tolerance test revealed robust effects of the wheel intervention compared to the control treatment as evidenced by the significant time by intervention interaction for the AUC ($p<0.001$; **Figure 5-3d, e**).

Effects of Voluntary Exercise on Hematologic Measures in KK/HIJ Female Mice

Complete blood count and iron status measures are shown in **Table 5-3**. Red blood cell (RBC) and white blood cell (WBC) count as well as several WBC subpopulations, such as lymphocytes, monocytes and eosinophils, were not different between CON and EX after 5-week voluntary exercise intervention ($p>0$ .05, **Table 5-3**). Hemoglobin levels, mean corpuscular hemoglobin (MCH), and hematocrit did not change after exercise intervention ($p>0.05$, **Table 5-3**). However, mean corpuscular hemoglobin concentration (MCHC) was lower while the mean corpuscular volume (MCV), proportion of marginally sized erythrocytes (%macro) and red blood cell distribution width (RDW) were significantly higher in EX compared to CON mice ($p\leq0.05$, **Table**

5-3). Regarding circulating iron, the two-way ANOVA revealed that there was no significant time (PRE and POST) by intervention (CON and EX) interaction for serum iron ($p>0.05$, **Figure 5-3f**).

Effects of Voluntary Exercise on Expression of Adipose Tissue Inflammatory Markers

To further characterize the effect of voluntary exercise on adipose tissue inflammation, gene expression was performed. *Tnfα* and *Il-6* were used as inflammatory cytokine markers, *F4/80* as an adipose tissue macrophage marker and *Itgax* as an M1 macrophage marker. We observed a decrease in gene expression of *Tnfα* in EX compared with CON ($P \leq 0.05$). However, gene expression analyses of *F4/80* ($p=0.07$) and *Itgax* ($p=0.09$) were not statistically different between the groups but tended to be decreased in EX compared with CON. There was no significant difference in *IL-6* expression between groups ($p=0.3$). Furthermore, voluntary exercise did not elevate, but rather lowered the M2 specific genes *Chi3l3*, *Mgl1* (macrophage galactose *N*-acetyl-galactosamine–specific lectins 1) and *Mrc2* (mannose receptor, C type 2) ($p<0.05$; **Table 5-2**). *IL-10* also had 63% reduction, but this difference did not reach statistical significance ($p=0.06$) (**Table 5-2**). Adipose tissue gene expression of the anti-inflammatory marker, adiponectin, was not different between CON and EX post intervention. This was found to be consistent with circulating levels of (serum) adiponectin, which were not different between the groups (POST-CON: 18.45 ± 1.39 µg/mL; POST-EX: 17.52 ± 1.33 µg/mL, $p>0.05$).

Discussion

To our knowledge, this is the first study focusing on the effects of voluntary exercise on adipose tissue inflammation in the polygenic obese female KK/HIJ mouse model. In these studies we observed the following: 1) Female KK mice have a robust age-associated accumulation of adipose

tissue; 2) A short term voluntary wheel running intervention decreased body weight and improved glucose tolerance despite an increase in food consumption; 3) The wheel intervention resulted in a down-regulation of both inflammatory and anti-inflammatory gene expression from the gonadal adipose tissue compared to the control group; and 4) though subtle alterations in hematologic measures were evident (e.g. higher RDW, MCV, %macro and lower MCHC) following the exercise intervention, no influence on circulating serum iron was observed. In summary, our study has demonstrated that 5 weeks voluntary exercise is adequate to induce a reduction in adiposity, decrease in inflammatory markers of gene expression in adipose tissue and an improvement in glucose tolerance.

In this study we used a rarely studied mouse model of polygenic obesity, the females of the KK/HlJ strain. Studies examining the importance of exercise on adipose tissue in the obese state have focused on male mice and rats, and most of the studies have used diet-induced obese animal models [6, 32, 42, 43]. Although dietary fat plays an important role in obesity, it is not the only factor [44-47]. This KK mouse strain was originally bred to produce a mouse with insulin resistance [35] and the males have been used in many studies as a model of hyperglycemia. However, the female KK/HIJ are remarkable in their predilection for greater fat storage than males of the same strain, which has been observed in our study as well as previous publication [48], making them notable for studies of adipose tissue inflammation in the setting of obesity. These mice were very responsive to the voluntary wheel running intervention and a robust weight loss effect was evident after 5 weeks of exercise intervention. The amount of weight loss in the mice provided the wheel was nearly equal to the difference in adipose tissue mass between the CON and EX groups, indicating that a high proportion of the weight loss was from the adipose depots harvested in this

study (i.e. perirenal and gonadal depots). Additionally, the remarkably reduced serum leptin level in EX was highly associated with the weight loss. This is consistent with human studies, where massive fat loss was paralleled by a robust decrease in circulating leptin [49-51]. The decreased leptin might also relate to the greater food intake in EX compared with CON. Furthermore, we observed approximately 5 grams of weight loss during 5 weeks of voluntary exercise in KK female mice whereas previous studies (of 6 weeks duration) have observed weight loss of only ~1 gram and ~3 grams in C57BL/6 mice provided normal and high fat diets, respectively [6, 52]. Therefore, the voluntary running wheel serves as an especially effective strategy for fat reduction in this KK/HIJ mouse model and provides a robust model of exercise-induced weight loss and adipose tissue remodeling.

Iron dysregulation is considered as a potential contributor to the pathology of obesity-related metabolic complications including T2DM. Studies have demonstrated elevated iron stores to precede insulin resistance [53, 54], while lowering serum iron has been demonstrated to increase insulin sensitivity [55, 56]. As the female KK mice have been observed to have greatly elevated serum iron concentration [57], we sought to examine the influence of exercise on iron metabolism in the context of their glucose intolerance. In the present study, we did not observe any change of the serum iron concentration in EX compared with CON. Furthermore, no alterations in hemoglobin or hematocrit were evident between groups. We did observe several differences in the complete blood count that suggest subtle alterations in hematopoiesis resulting from the wheel intervention. First, we observed a higher red blood cell distribution width (RDW) in EX which describes that there is an increase of the variation of red blood cell volume compared with CON. This has been used clinically as a marker of several types of anemia and has been described as an

early marker of iron deficiency anemia [58]. Secondly, we observed a significantly higher MCV and lower MCHC in EX compared to CON. Along with the higher RDW, the change of MCV and MCHC in EX mice is a phenomenon of increased red blood cell size and likely a compensatory response to increased red blood cell turnover, which has been observed in humans with increased hemolysis due to exercise [59]. Consistent with this, we observed significantly more macrocytic red blood cells in EX compared with CON. Nevertheless, despite subtle alterations in these RBC indices, no major alterations in serum iron or hemoglobin concentration were noted despite the robust changes in adiposity and glucose tolerance.

Short-term voluntary exercise resulted in a significant improvement in glucose homeostasis. This was manifest as a highly robust improvement in the AUC of the glucose tolerance test as well as lower fasting serum insulin between EX and CON following the voluntary wheel running intervention. However, given the robust difference in fat mass following the study, it is not possible to separate the direct effects of exercise on the improved glucose homeostasis from the indirect improvements provided by the reduced fat mass. Both exercise and weight loss have been shown to improve glucose tolerance. For example, acute exercise or exercise training without weight loss can increase skeletal muscle glucose uptake and improve insulin sensitivity [60-62] although this is often not observed when measured a few days after exercise[63]. In addition, calorie restriction or bariatric surgery induced weight loss can also improve insulin sensitivity by decreasing fat mass, changing the adipokines release (reviewed by [64]) and reducing fatty acid mobilization [65]. Therefore, our study was limited by the combined factors of the exercise and weight loss with the wheel intervention. However, given the robust effects demonstrated by this intervention, we propose that in future studies the voluntary running wheel access could be titrated, for example,

by locking the running wheel for a portion of the active period. This would allow a more consistent exercise dose and/or control of the energy expenditure and weight loss. Alternatively, a non-exercise control group could be evaluated to separate the effect of weight loss alone from the effects resulting from weight loss and exercise in the present study. Nevertheless, in these initial studies of voluntary exercise in the KK/HIJ females, we have demonstrated a robust improvement in glucose tolerance and adiposity that has not been observed previously in a mouse model of polygenic obesity.

Adipose tissue inflammation was lower in EX compared with CON mice following the 5-week voluntary exercise intervention. Low-grade inflammation has been noted in obese individuals with both insulin resistance and type 2 diabetes [15, 66, 67]. Previous studies have also shown that obesity-related adipose tissue inflammation is associated with increased adipose tissue macrophage infiltration [23]. However, there is evidence that insulin sensitivity can be recovered by inhibition of macrophage infiltration and the suppression of pro-inflammatory cytokines in the setting of diet-induced obesity [28, 67, 68]. Similarly, exercise has been demonstrated to decrease adipose tissue inflammation and improve glucose tolerance [6, 9, 52, 69]. However, most of these studies have focused on males and diet-induced obesity. In the present study, female KK mice from EX had a 50% lower adipose tissue *Tnfα* expression compared with CON following the 5 week intervention, suggesting that adipose tissue inflammation was suppressed by the voluntary wheel running or weight loss. In addition to *Tnfα,* which has been associated with both adipose tissue inflammation and insulin resistance [17, 18], *F4/80*, *Itgax* (Integrin alpha X, also known as CD11c) as well as IL-6 are also inflammatory markers or cytokines from ATMs or adipocytes [16]. However, our gene expression results for the pro-inflammatory genes *F4/80* and *Itgax*

(Integrin alpha X, also known as CD11c) were not statistically different between groups, though they tended to be decreased after exercise intervention in EX compared with CON. This may have been due to the small sample size and the variability in our samples. In the case of *IL-6*, its role in insulin resistance is highly controversial. Human studies have been equivocal, providing evidence that IL-6 may [70, 71] or may not [72] be associated with insulin resistance. Nevertheless, in our study, no difference in *IL-6* expression in adipose tissue was observed between CON and EX and therefore the improvement in glucose tolerance appears independent of changes in adipose tissue *IL-6* expression in the female KK/HIJ mouse model. However, due to the evident changes in both adiposity and adipose tissue inflammatory gene expression, it is not possible for us to delineate the specific mechanisms for the improved glucose homeostasis.

Recent studies evaluating exercise and adipose tissue inflammation have observed an increase in anti-inflammatory markers [6, 9, 29-32, 73]. For example, anti-inflammatory markers Interleukin 10 (IL-10) and Cluster of Differentiation 163 (CD163, a marker for M2 macrophages) were elevated following both acute and chronic exercise in C57/BL6 male mice [31, 32]. However, in our female KK mice, we observed lower expression of adipose tissue anti-inflammatory genes including *Mgl1* (macrophage galactose N-acetyl-galactosamine specific Lectin 1, which is encoded by CD301), and *Mrc2* (Mannose Receptor C Type2) in the exercise compared with the control group. We speculate that this might be partially due to the substantial decrease of the adipose tissue mass in those provided the voluntary wheel. However, as this is the first study of exercise-induced changes in adipose tissue inflammation in obese females, these differences may also be linked to sex-specific changes following exercise. Studies with obese women have shown that exercise training and long-term lifestyle intervention (hypocaloric diet and daily moderate

physical activity) with weight loss increased anti-inflammatory adipokine (adiponectin) and decreased inflammatory gene expression in adipose tissue [74-77]. To date, human studies have not directly compared men and women regarding the effect of exercise on adipose tissue macrophage markers. Although Brunn et al. examined both men and women in a small study (n=23 total) evaluating the effects of a 15-week lifestyle intervention, they did not directly compare the response of the men and women in the study. They did, however, demonstrate a significant weight loss (average of 13% of body weight) as well as a decrease in adipose tissue macrophage-specific markers (e.g. CD68 and CD14) and inflammatory adipokines (e.g. TNFα, IL-6 and MCP-1) [74]. On the other hand, in our study, since *Mgl1* and *Mrc2* are both macrophage markers, it is be important for future studies to isolate macrophages and directly evaluate the adipose tissue macrophage changes.

It is important to note that the concept of adipose tissue inflammation in the setting of obesity has implications beyond effects on glucose control and diabetes. For example, long-term up-regulation of inflammatory pathways in adipose tissue may contribute to increased fibrosis [78], atherosclerosis [79, 80] and/or CVD complications [81, 82]. We were specifically interested in the impact of exercise on adipose tissue macrophages and their gene expression within our obese female mouse model. However, in order to provide definitive answers regarding the role of exercise on macrophage polarization in the future, it will be necessary to directly determine the macrophage cell surface markers via flow cytometry or other direct measurement.

Conclusion

Five weeks of voluntary exercise intervention decreased body weight and improved glucose homeostasis despite increased food consumption in polygenic obese female KK/HIJ mice. These exercise-induced changes are coincident with an overall decrease in adipose tissue mass and the gene expression of both anti-inflammatory and pro-inflammatory macrophage markers.

Acknowledgement:

The authors acknowledge funding received from the School of Kinesiology, Rackham Graduate School and the Undergraduate Research Opportunity Program (UROP) at the University of Michigan.

Table 5-1 Primer sequences for adipose tissue gene expression.

Gene	Forward(5'-->3')	Reverse(5'-->3')
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
AdipoQ	GGAGATGCAGGTCTTCTTGG	ATGTTGCAGTAGAACTTGCC
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Itgax	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
Mgl1	TGAGAAAGGCTTTAAGAACTGGG	GACCACCTGTAGTGATGTGGG
Mrc2	TACAGCTCCACGCTATGGATT	CACTCTCCCAGTTGAGGTACT
Chi3l3	AGAAGGGAGTTTCAAACCTGGT	GTCTTGCTCATGTGTGTAAGTGA

Table 5-2 Adipose tissue gene expression

<u>*Abbreviations*</u>: Pro-inflammatory genes *F4/80*, *Itgax*, Integrin, Alpha X**;** *IL6*, Interleukin 6; and *Tnfα,* tumor necrosis factor α; Anti-inflammatory genes *Mgl1*, macrophage galactose *N*-acetyl-galactosamine–specific lectins 1; *Mrc2*, mannose receptor, C type 2 *Mrc2*, *IL10*, Interleukin10; *Adipoq*, Adiponectin; and *Lep*, Leptin gene expression (n=4 per group). * P≤0.05

		CON (Mean ± SEM)	**EX (Mean ± SEM)**	**P values**
Inflammatory Genes	*F4/80*	1.00 ± 0.33	0.28 ± 0.04	0.067
	Itgax	1.00 ± 0.38	0.25 ± 0.06	0.094
	Il6	1.00 ± 0.52	0.41 ± 0.19	0.329
	Tnfα	1.00 ± 0.20	0.54 ± 0.06	≤0.05
Anti-inflammatory Genes	*Chi3l3*	1.00 ± 0.10	0.59 ± 0.07	**<0.01**
	Mgl1	1.00± 0.06	0.70 ± 0.06 *	**0.004**
	Mrc2	1.00 ± 0.13	0.28 ± 0.03 *	**<0.01**
	Il10	1.00 ± 0.28	0.37 ± 0.07	0.064
Adipokine Gene	*Lep*	1.00 ± 0.14	0.28 ± 0.09 *	**<0.01**
	AdipoQ	1.00 ± 0.12	0.89 ± 0.14	0.577

Table 5-3 Hematologic Measures in CON and EX group after 5weeks voluntary exercise intervention. * CON vs EX $p \leq 0.05$.

	CON (Mean ±	**EX (Mean ± SEM)**	**P values**
WBC × 10^3 (cells/μl)	7.5 ± 0.74	7.1 ± 0.92	0.781
Lymph × 10^3(cells/μl)	5.0 ± 0.61	4.6 ± 0.66	0.694
Mono × 10^3(cells/μl)	0.3 ± 0.03	0.2 ± 0.06	0.386
Eos × 10^3(cells/μl)	0.4 ± 0.03	0.4 ± 0.03	0.689
RBC × 10^6 (cells/μl)	9.3 ± 0.07	9.0 ± 0.26	0.259
Hgb (g/dl)	12.5 ± 0.19	12.3 ± 0.47	0.666
MCV (fL)	50.0 ± 0.06	51.8 ± 0.14*	**<0.01**
% Macro	0.1 ± 0.04	0.4 ± 0.08	**0.006**
% Micro	0.4 ± 0.03	0.5 ± 0.04	0.666
RDW%	15.0 ± 0.16	16.0 ± 0.22*	**0.003**
MCH (pg)	13.5 ± 0.11	13.7 ± 0.12	0.326
MCHC (g/dL)	27.1 ± 0.15	26.4 ± 0.25*	**0.046**
HCT (%)	46.5 ± 0.33	46.7 ± 1.23	0.861

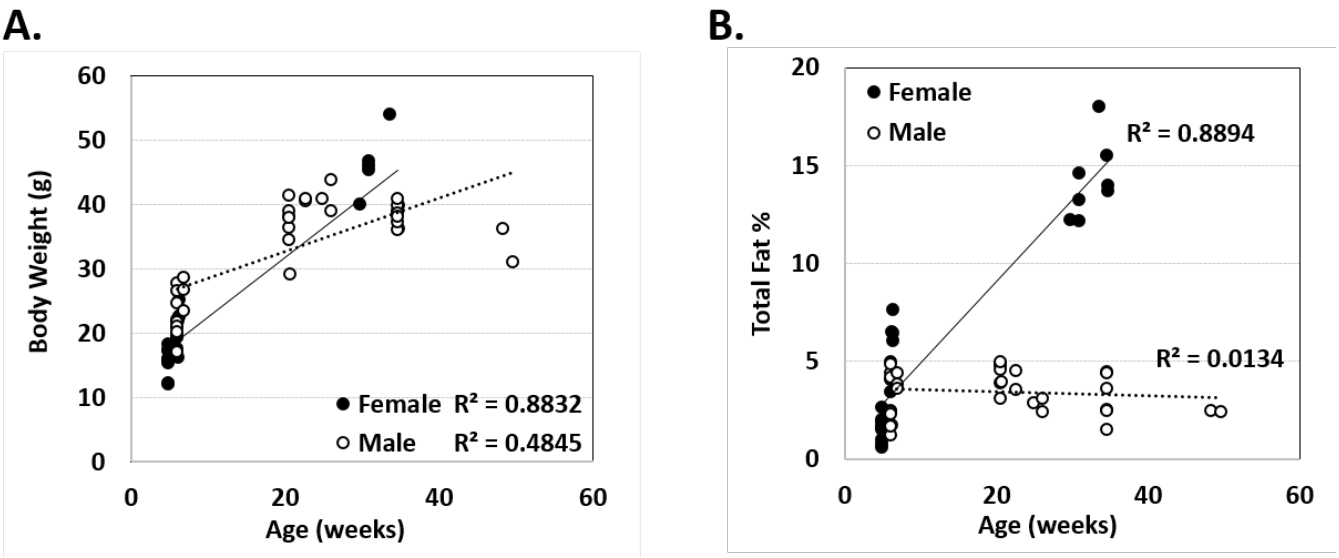

Figure 5-1 Female KK/HIJ mice are more prone to fat accumulation than males.

A) Body weight comparison between females and males of different ages. B) Age-related changes in total fat (gonadal and perirenal fat) percentage of male and female KK/HIJ mice. (Female: n=27; Male: n=31)

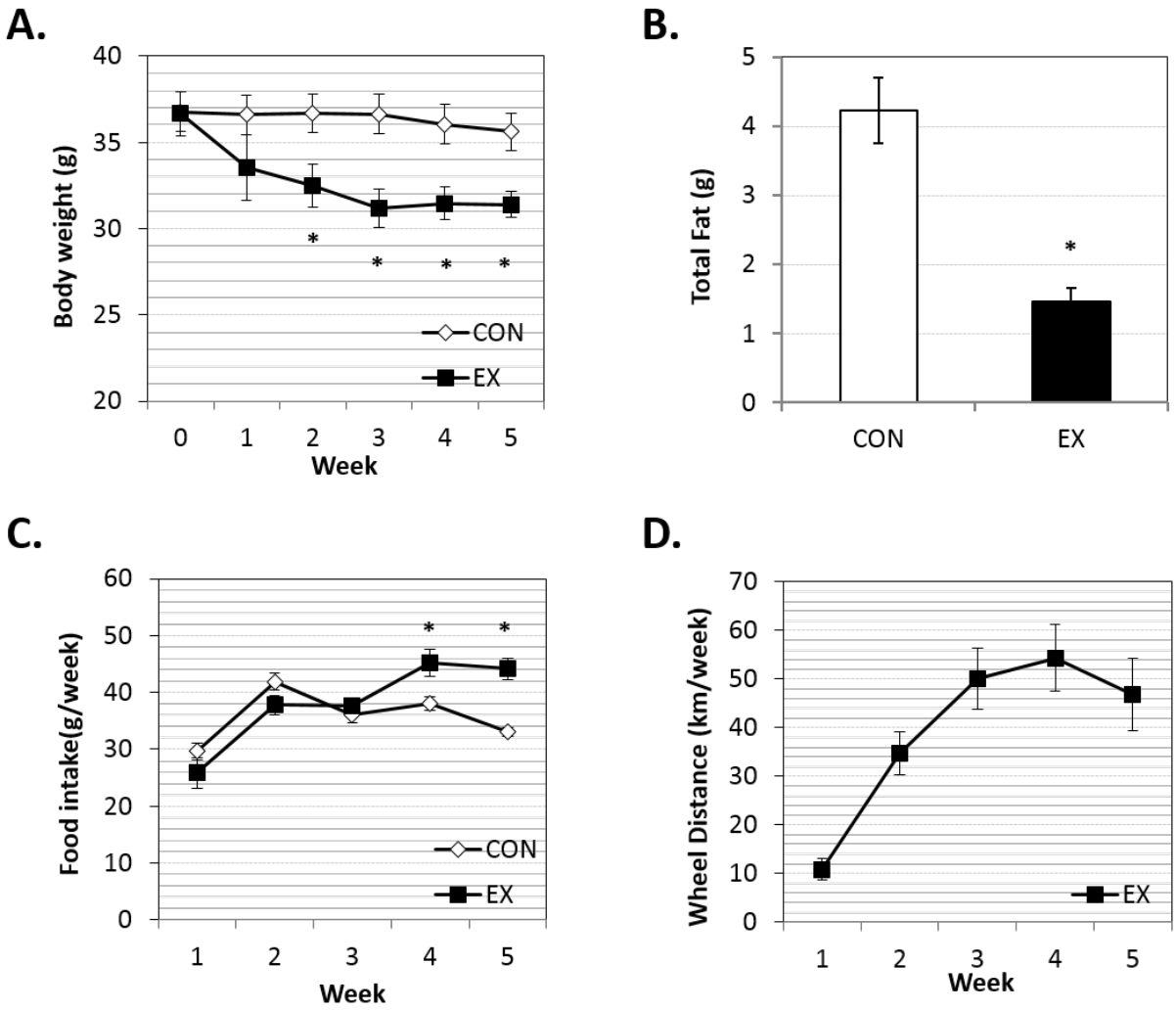

Figure 5-2 Voluntary exercise induced weight loss in KK/HIJ mice.

A), Body weight change during 5 weeks exercise intervention, B) Total fat weight including gonadal and perirernal fat pads in CON and EX after intervention, C) Food intake during the intervention in CON and EX groups, D) Weekly running distance in exercise group. *CON *vs* EX $P \leq 0.05$

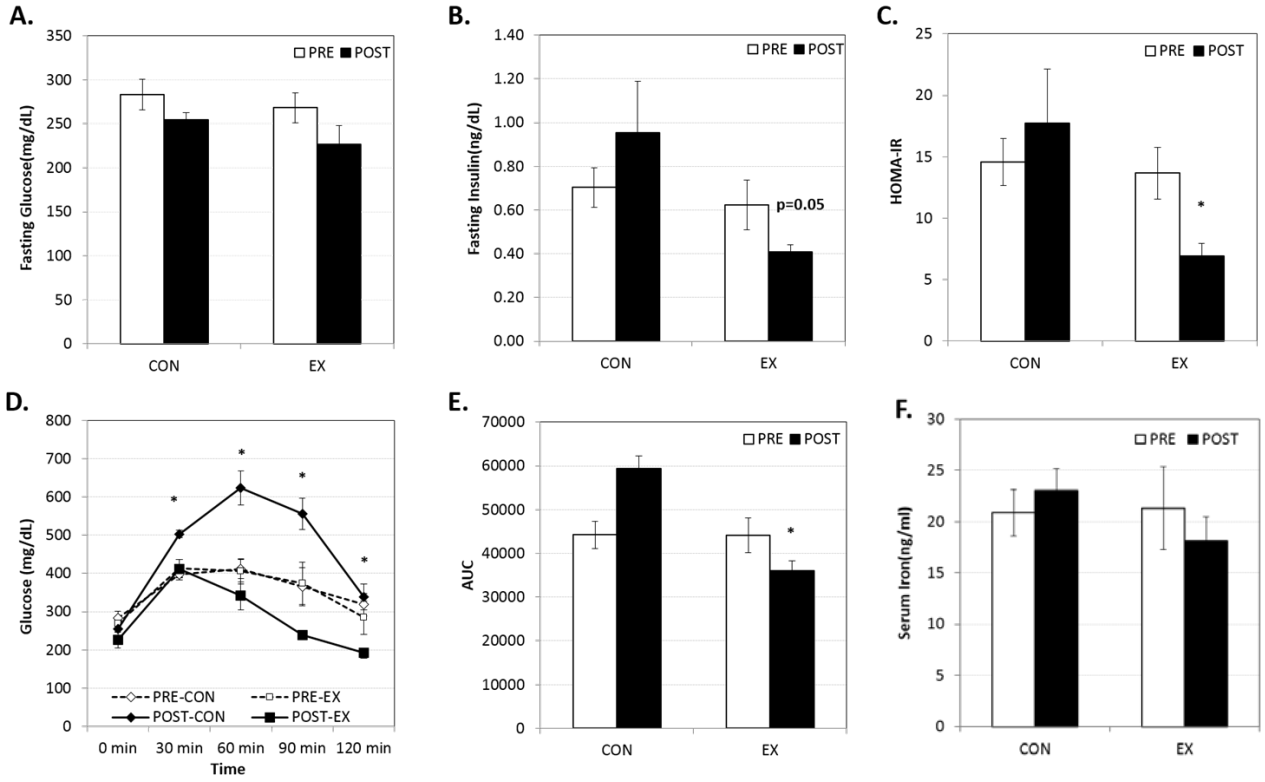

Figure 5-3 Voluntary exercise improves glucose tolerance and insulin sensitivity in KK/HIJ female mice.

A) Fasting serum glucose was not different in PRE and POST CON and EX groups; B) Fasting serum insulin did not change in PRE and POST CON and EX groups; C) HOMA-IR showed significant change in PRE and POST CON and EX groups; D&E) GTT and AUC had significant change in PRE and POST CON and EX groups; F) Serum iron in PRE and POST CON and EX groups was not different; * CON *vs* EX $p \leq 0.05$. (Glucose, time $p \leq 0.05$; HOMA-IR, intervention $p \leq 0.05$; Insulin, intervention $p \leq 0.05$)

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Chapter 6

Overall Discussion

Iron dysregulation can lead to serious health concerns resulting from either too much or too little iron storage or availability. The iron-regulating hormone, hepcidin, plays a primary role in iron regulation and alterations in the hepcidin signaling pathway are typically responsible for cases of iron dysregulation. For example, an absence of hepcidin promotes iron toxicity while elevated hepcidin promotes iron deficiency anemia. Iron deficiency is prevalent in athletic populations, especially among endurance-trained female athletes. Although recent studies suggest that a transient increase in circulating hepcidin following acute exercise might promote iron deficiency anemia in athletes, it is unclear whether there is an additive or cumulative effect of multiple acute excursions of hepcidin in response to everyday training. On the opposite end of the iron dysregulation spectrum, excess iron deposition (e.g. in adipose tissue) is a potential contributor to the pathology of obesity-related metabolic complications. However, the mechanisms underlying adipose tissue iron deposition and its influence on obesity-related metabolic consequences are still unclear. Although exercise has been observed to promote improvements in insulin sensitivity, the influence of exercise on iron regulation and adipose tissue inflammation in the setting of obesity is still being explored. As a close association has been observed between iron dysregulation, inflammation and diabetes, a better understanding of iron regulation could greatly improve the prevention and treatment of iron disorders including iron deficiency and iron overload as well as their subsequent complications (e.g. diabetes). Collectively, my dissertation was designed to emphasize the importance of iron in both athletic performance and the increased diabetes risks, by

examining the possible cause of iron deficiency anemia in female athletes, observations of adipose tissue iron overload in polygenic obese mouse model, and the incorporation of exercise in obese and diabetic mouse model with high serum iron.

Inflammation, obesity and diabetes may all be influenced by the small peptide hormone hepcidin, which is a critical factor in iron homeostasis. However, the relationships between these conditions and hepcidin are still poorly understood. Iron is necessary for proper function of both the innate and adaptive immune systems. Hence, iron deficiency will lead to a compromised inflammatory response such as abnormal cell-mediated immunity and an impaired ability of neutrophils to kill bacteria [1]. Therefore, since immune cells require iron for proper function, hepcidin may also play an important role in exercise-induced inflammatory responses. PROJECT#1 measured both circulating and monocyte gene expression of hepcidin in collegiate cross-country runners undergoing high-intensity training to examine the iron deficiency and possible anemia of chronic disease symptoms in this population. The roles of macrophage in iron recycling (iron uptake, metabolism, storage and transport) and inflammation (both pro-inflammatory and anti-inflammatory function) allowed us to extend my dissertation projects that focus on iron regulation to obesity-associated adipose tissue inflammation. It is well acknowledged that obesity-associated inflammation is characterized by increased levels of inflammatory mediators in plasma and in adipose tissue (e.g. TNFα). Macrophage infiltration and activation in the adipose tissue has provided a link between adipose tissue and inflammation. PROJECT**#2,** therefore, was to investigate the relationship between adipose tissue inflammation, iron deposition and glucose homeostasis. To further explore the relationship between adipose tissue inflammation and glucose homeostasis in a high-iron milieu, we evaluated the impact of voluntary running in polygenic obese

animals with high serum iron levels. To this end, PROJECT#3 was designed to evaluate the influence of exercise on iron homeostasis, adipose tissue inflammation, and glucose homeostasis.

Together the three projects of my dissertation enhanced our understanding of iron homeostasis in the setting of health (ie. exercise training in healthy female athletes) and disease (using animal models with elevated iron and obesity). Important findings from my dissertation studies include 1) the iron-regulating hormone, hepcidin, is not chronically elevated with sustained moderate to high intensity endurance exercise training in competitive collegiate runners (PROJECT#1), which is an important finding because this counters existing evidence which suggests hepcidin may induce anemia in endurance trained athletes due to a sustained elevation in serum hepcidin after each exercise session (PROJECT#1). 2) High level of iron in the epididymal adipose tissue is associated with a robust adipose tissue remodeling, which was evidenced by elevated inflammatory markers gene expression (e.g. *Tnfα*) and increased macrophages, collagen and cell death (PROJECT#2). 3) Five weeks voluntary exercise in obese female mice with high serum iron levels resulted in weight loss, improved glucose intolerance and significantly altered adipose tissue inflammatory gene expression (PROJECT#3). 4) However, exercise did not alter the serum iron level in polygenic obese mice and there was no association between adipose tissue iron deposition and glucose homeostasis (PROJECT#2&3). In the discussion that follows, I will focus primarily on the collective and integrative implications of these findings.

The causes of exercise-induced iron-deficiency anemia in athletes are still under debate. Physicians currently assist athletes with recovery from exercise-induced anemia by providing iron supplementation and educating athletes about optimizing iron absorption. However, it is essential

that we improve our understanding of the mechanisms of exercise-induced anemia in order to improve our treatment of, and ultimately prevent, this condition. Evidence of elevated hepcidin at around 3 hours following moderate to high intensity exercise is well documented. The possible mechanism that has been typically provided is an increase in the inflammatory cytokine IL-6 following the acute exercise. Non-exercise studies have demonstrated that IL-6 (both 1ng/ml and 20ng/ml) stimulation can up-regulate hepcidin production from the hepatic cells via the signal transducer and activator of transcription 3 (STAT3) signaling pathway (**Figure** 6-1) [2, 3]. Furthermore, acute exercise studies have demonstrated an increase in circulating IL-6 (from ~0.5pg/ml at resting to ~2pg/ml after exercise), which might be responsible for the transient hepcidin elevation at around 3-hour post-exercise [4]. However, it is not yet established whether the increased concentration of IL6 is necessary to induce the hepcidin elevation following exercise. Most importantly, the question of an additive or cumulative effect of multiple acute excursions of hepcidin in response to everyday training on elevating resting levels of hepcidin is still unclear. The main finding of PROJECT#1 was that highly trained female athletes did not have significantly higher resting hepcidin levels compared with control subjects, suggesting that there is no cumulative effect of chronic, daily endurance training on plasma hepcidin concentration. Overall, the results from our study did not suggest an anemia-like phenotype resulting from the rigors of collegiate distance running in female athletes. Our finding in PROJECT#1 is significant because we want to provide physicians with the most accurate information regarding exercise-induced anemia. The main message from our current result is that hepcidin does not appear to be the anemia cure-all for athletes that the scientific community had hoped.

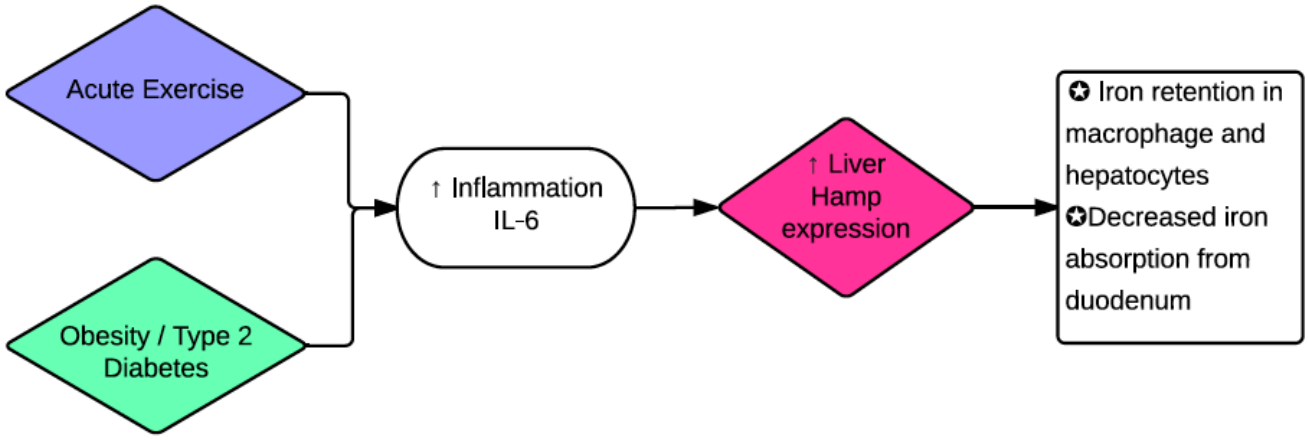

Figure 6-1 The role of hepcidin in exercise and obesity/type 2 diabetes.

On the opposite end of the iron dysregulation spectrum, excess iron storage is a potential contributor to the pathology of obesity-related metabolic complications. The role of major iron regulating hormone, hepcidin, has been explored beyond the realm of iron homeostasis and has been expanded to both immunology and diabetes. During chronic inflammation, which is hypothesized to exist at low levels in obese subjects, hepcidin expression may result in hypoferremia due to decreased duodenal iron absorption and increased iron sequestration by macrophages (**Figure** 6-1). This process with adequate time and intensity can result in *anemia of inflammation* [8]. As mentioned, hepcidin can be up-regulated by the inflammatory cytokine, IL-6, which some studies have suggested is involved in the development of obesity and insulin resistance [9-12]. IL-6 expression correlates with hepcidin expression in human adipose tissue, and direct IL-6 incubation of adipose tissue explants induces hepcidin expression [13]. In PROJECT#2, we found that the deposition of iron in a subset of the male KK/HIJ mice was specific to the eAT adipose tissue depot and not evident in subcutaneous or brown adipose tissue depots. This increased iron was associated with the increased HAMP trend in the HI group. A robust adipose tissue remodeling was also accompanied with the high iron deposition, which was evidenced by the increased macrophages, collagen, cell death and markedly up-regulated inflammatory gene expression (e.g. *Tnfα*). Despite the robust local remodeling in the eAT of the HI mice, there was no evidence of changes in glucose homeostasis or circulating adipokines. Our findings provided a new depth of insight into the specifically high iron eipididymal adipose tissue. KK male mouse model has a great potential in future studies to evaluate the mechanism of iron accumulation in the epididymal adipose tissue. We hope that our findings provide a starting point for many researchers to explore and identify novel roles of iron for adipose tissue function that can expand our knowledge and provide solution to obesity and diabetes. The important role of exercise

in health also drives us to examine how exercise influences obesity-related adipose tissue inflammation. Although exercise has been observed to promote improvements in many obesity-associated conditions (e.g. dyslipidemia, glucose tolerance, etc.), the influence of exercise on adipose tissue inflammation and iron regulation in the setting of obesity is still being explored. To further our knowledge on iron dysregulation, inflammation and diabetes from PROJECT#2, PROJECT#3 was designed to investigate the effects of voluntary exercise on adipose tissue inflammation in a polygenic obese female KK/HIJ mouse model with high serum iron concentration. We observed that a short-term voluntary exercise intervention improved glucose tolerance and decreased body weight despite increased food consumption in this mouse model. This result was consistent, though considerably more robust, with the exercise response observed in several published studies of high fat diet-induced obese male mice [14-16]. Gene expression data also indicated a decrease of the inflammatory signal in the adipose tissue. Consistent with the gene expression data, our limited adipose tissue macrophage flow cytometry data showed smaller ATMs and M1 population in the EX compared with CON (Appendix 1). However, there was no significant alteration of serum iron or evidence of ineffective erythropoiesis. Overall, the observed improvement in glucose tolerance was coincident with a decreased expression of adipose tissue derived inflammatory factors but occurred in the absence of systemic alterations in iron homeostasis. Although exercise did not alter the serum iron level in polygenic obese mice and there was no association between adipose tissue iron deposition and glucose homeostasis (PROJECT#2&3), both projects provided novel insight of iron in adipose tissue inflammation and glucose homeostasis. We hope this will inspire more researchers to explore this field and further study the dynamic roles of iron and adipose tissue. In addition, KK mouse model represents an

interesting anomaly, which may benefit translational research to understand current human clinical conditions.

The relationships among exercise, hepcidin and obesity are intriguing. Contrary to what recent studies have proposed [4, 17, 18], an accumulative effect of hepcidin with long-term, high-intensity training in female distance runners was not observed. However, the role of hepcidin in exercise-induced anemia should be further evaluated. What remains to be documented is whether the observed transient elevations in hepcidin are adequate to induce iron deficiency in athletes. This is especially controversial because as iron stores fall, even the transient rise in hepcidin following exercise is suppressed. However, as obesity and diabetes are associated with elevations in iron stores, the documented exercise-induced rise in hepcidin might provide a therapeutic reduction in iron bioavailability in this clinical population if it reduces duodenal iron absorption. Although we did not observe this in our voluntary wheel studies in mice, this is still an important and testable hypothesis in adults with obesity and iron excess. In addition, as insulin likely plays an important and direct role in iron regulation, exercise might also influence iron homeostasis through hepcidin-independent effects mediated by transient improvements in insulin sensitivity [19]. Moreover, the interactions of insulin sensitivity and iron homeostasis may reveal an important therapeutic role for exercise in patients where iron excess is promoted by insulin resistance. Therefore, our results provide a reference for future studies investigating the acute and chronic effects of exercise on iron regulation and insulin sensitivity.

In summary, my dissertation projects provided steps forward in our understanding of the role of iron regulation in the setting of a mouse model of disease as well as highly trained elite athletes at

high risk of iron deficiency anemia. My dissertation answered, “Whether there is a cumulative or additive effect of long-term high intensity training on serum hepcidin levels”, which is important for cooling down the controversy regarding “if hepcidin is the reason why athletes have iron deficiency anemia”. The study of elevated epididymal adipose tissue iron deposition in KK male mice, should further our understanding of iron deposition and metabolic alterations. My dissertation also utilized a unique mouse model to answer “how does short-term exercise intervention influence glucose tolerance, adipose tissue inflammation and iron regulation.” Together, these studies were designed to call attention to the importance of iron regulation in health and disease and expand our knowledge in the areas of 1) hepcidin in athletes at risk for iron deficiency; 2) adipose tissue iron deposition; and 3) exercise and adipose tissue inflammation in the setting of high iron availability and obesity.

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APPENDICES

APPENDIX 1

Additional Analysis for PROJECT #3 (Chapter 5)

Flow cytometry analysis examining adipose tissue macrophage change in KK female mice following short-term exercise intervention

In my dissertation proposal, I indicated that I would analyze the adipose tissue inflammation using flow cytometry in addition to adipose tissue gene expression data. In Chapter 5 of my dissertation, I have provided the manuscript from this study which we submitted for publication. Since the flow cytometry data were obtained from only four adipose tissue samples (2 for EX and 2 CON), the reviewers asked us to take out this part of the data because of the small sample size. Therefore, the flow cytometry method and data I proposed were not included in Chapter 5 of my dissertation. I have included this part of the data here.

Methods

Flow Cytometry

Mice were euthanized via exsanguination under sodium pentobarbital anesthesia. Adipose tissue was promptly perfused with ice cold PBS and harvested. Tissue was then minced and digested by collagenase (Sigma, St. Louis, MO), stromal vascular cells (SVC) were isolated by using the method previously described [1, 2]. The SVC was counted and $1x10^6$ cells were used for antibody staining. The SVCs were labeled with primary fluorophore conjugated antibodies: F4/80-phycoerythrin (F4/80-PE), CD11b-Allophycocyanin Cy7 (APC/Cy7), CD11c-PE Cy7, and

CD301-APC (eBiosciences) following the manufacturer's instruction. Cells were analyzed on a FACSCanto II Flow Cytometer (BD Biosciences), data were analyzed using FlowJo 10.0.6 software (Treestar, Ashland, OR).

Results

Effect of Voluntary Exercise on Adipose Tissue Inflammation and Adipose Tissue Macrophage Subsets

In order to characterize the effect of voluntary exercise on adipose tissue inflammation, flow cytometry was used to analyze the macrophage subset numbers post exercise intervention in a subset of the mice (n=2 per group). The adipose tissue macrophages (ATMs) were determined as $F4/80^{+}CD11b^{+}$ cells, M1 population as $F4/80^{+}CD11b^{+}CD11c^{+}CD301^{-}$ cells and M2 population as $F4/80^{+}CD11b^{+}CD11c^{-}CD301^{+}$ cells (**Figure A1-1**). The quantitation of ATM (among the total cell population), M1 (among the total ATMs) and M2 (among the total ATMs) is expressed as the frequency of each cell population as well as the actual concentrations in gonadal fat (cell numbers per gram of fat). Although no significant difference was observed between CON and EX in these parameters, M2% tended to be increased in EX group (**Table A1-1**). The individual mouse data are shown in Table A1-1.

Discussion:

An initial goal of this study was to evaluate the change in adipose tissue macrophages (ATM) between EX and CON at the end of intervention using flow cytometric studies. Although we digested the adipose and prepared and analyzed samples for all the mice in this cohort, we were unable to attain successful staining and sorting from the large cohort that we examined. We did

manage usable data from our first (small) cohort which included 2 mice each from the EX and CON groups and we provide that evidence here.

There are a considerable number of studies that have evaluated whole adipose tissue gene expression changes in response to exercise [3-7], however specific studies of adipose tissue macrophages have not been performed. For example, Kawanishi et al. proposed that exercise decreases adipose tissue inflammation by modulating the macrophage subsets. These conclusions were drawn from adipose tissue gene expression, however data from isolated macrophage were not included. Indeed, they determined that 16 weeks of exercise training resulted in suppressed adipose tissue gene expression of traditional pro-inflammatory M1-type markers, such as TNFα, F4/80 and CD11c, independent of weight loss. In addition, based on their adipose tissue gene expression studies, the authors speculated that exercise training in the setting of obesity might induce phenotypic switching from the classically activated M1 macrophage to the alternatively activated M2 macrophage [3, 5]. However, these studies were performed without analyzing a single macrophage and therefore cannot provide definitive answers regarding the role of exercise on macrophage polarization without directly determining the macrophage cell surface markers via flow cytometry or other direct techniques.

The gene expression data presented in Chapter 5 provided evidence that the pro-inflammatory markers of ATMs were down-regulated. Consistent with our gene expression data and others' studies (discussed in chapter 5), the flow cytometry data suggested a smaller total ATMs population and less M1%. However, the down-regulated anti-inflammatory ATM gene expression was not consistent with the flow cytometry data, where the M2% was slightly higher in EX compared with CON after the 5 weeks intervention. A possible explanation for this result

is that the lowered total ATMs population might lead to a decrease in the total population of M2 macrophage in the EX adipose tissue, which in turn contributes to the lowered adipose tissue gene expression. Therefore, the result from the flow cytometry, which is examining the number of M2 macrophage relative to the total number of macrophage is likely to reveal a different result than the gene expression data gathered from total RNA of the adipose tissue. Based on limited histological analyses, it was noted that there were very few crown like structures evident in the KK gonadal adipose, which further supports that macrophage infiltration may be limited in this animal model.

We speculate based on our limited flow cytometry data that the number of macrophages in the KK gonadal fat is limited and that the robust changes in adipose tissue mass and lower adipose tissue inflammation in the EX mice likely associated with less pro-inflammatory macrophages. As a result, flow cytometric analyses of isolated macrophage would be predicted to suggest a higher % of M2 macrophage (if the M1 population drops considerably). This observation has led to suggestions of macrophage phenotype "switching", but instead may simply relate to the cells that remain following adipose tissue remodeling following significant fat loss. Exercise studies designed to track the fate of adipose tissue macrophages will allow a better understanding of the temporal sequence of changes in macrophage polarization associated with exercise, adipose tissue remodeling and adipose tissue inflammation.

Table A1-1 Quantification of FACS analysis

The data include percentage of ATMs in alive cell, percentage of M1 and M2 in ATMs, and the cell numbers per gram of fat.

	ATM (% of alive cells)	M1 (%F4/80+ Cd11b+)	M2 (%F4/80+ Cd11b+)	ATM (x10^5 per gram of fat)	M1 (x10^4 per gram of fat)	M2 (x10^4 per gram of fat)	Total Gonadal Fat Weight
C1	29.7	21.9	28.6	1.92	4.21	5.49	4.13
C2	37.4	31.1	25.9	4.97	15.53	12.89	4.59
E1	22.1	15.7	45.1	2.69	4.23	12.13	1.88
E2	29.2	29.8	34.3	6.32	18.82	21.63	1.68

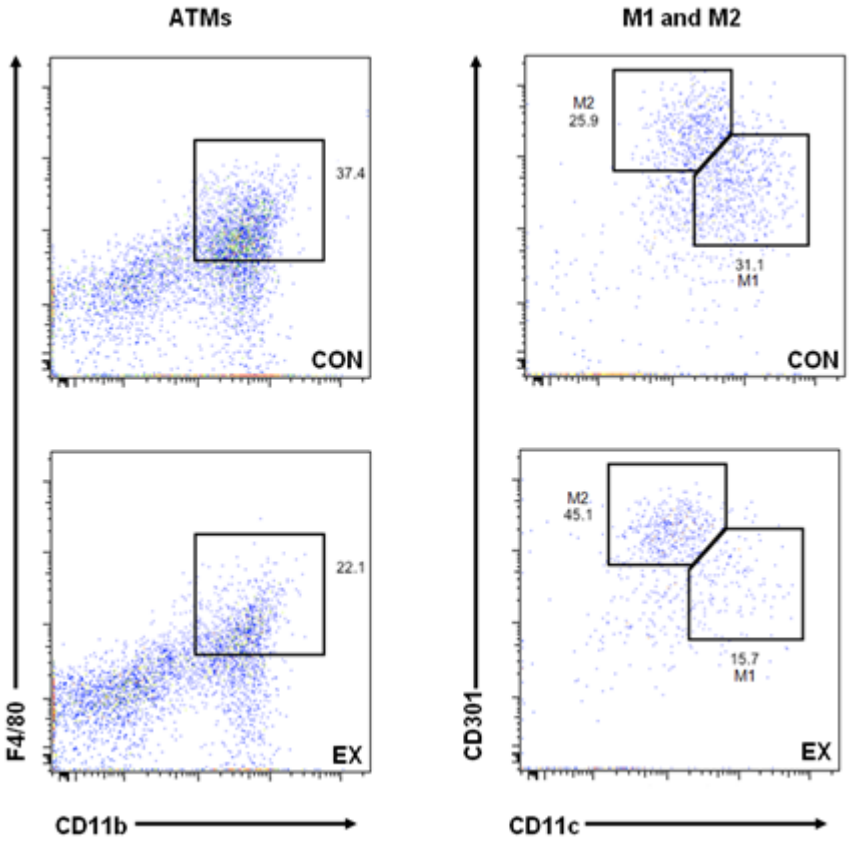

Figure A1-1 Gating strategy for adipose tissue macrophages and macrophage subsets Adipose tissue macrophages (ATMs) with positive F4/80 and CD11b staining were gated in the left panel for both CON and EX group. Among the ATMs, CD11c+CD301- cells were gated as M1 and CD301+CD11c- cells were gated as M2 macrophages in the right panel.

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APPENDIX 2

Iron Regulation in Exercise Questionnaire-For Control Subjects

The following questionnaire contains questions regarding lifestyle, supplementation, and exercise. Please answer each to the best of your ability.

Age:

Gender:

Subject ID:

1. Please describe your exercise trends:

Classify intensity as:

-**light-** heart rate is slightly elevated but you can talk normally

-**moderate-** you're working hard enough to raise your heart rate and break a sweat, but you are able to talk in short sentences

-**vigorous**- your breathing is hard and fast, your heart rate has increased and you can't say more than a few words without pausing for a breath

Mode of exercise	Minutes per week	Intensity

2. Do you take a multivitamin? yes no

If yes, how frequently?

What is the name of the vitamin? _____

Does it contain iron? yes no

3. Have you ever been diagnosed with iron deficiency anemia? yes no

   If you answered "No" to the question above, do you believe that you have ever been iron-deficient?" yes no

4. Have you ever taken iron supplements? yes no

5. Do you currently take iron supplements? yes no

   If yes, what form of iron supplement do you take? (circle one below)

   regular tablets liquid or drops

   coated or extended release tablets and capsules

   If yes, what type of iron supplement do you take? (circle one)

   ferrous sulfate ferrous fumarate ferrous gluconate

If yes, how often do you take the supplements per week?

At what time (or times) of day do you usually take your iron supplement? (morning, before bed, etc) _____

6. Do you take calcium supplements? yes no

7. Do you take calcium supplements at the same time as iron supplements? yes no

8. Do you take oral contraceptives? yes no

How long have you taken oral contraceptives? _____

What is the brand of the oral contraceptive? _____

9. Do you have a regular monthly menstrual cycle? yes no

10. What was the date of your last menstrual period? _____

11. How long is your cycle on average? _____

12. How frequently do you take anti-oxidative supplements or anti-inflammatory medication? (e.g. Aleve, tylenol, NSAIDs, etc.) -

_____

13. Please list any other medications you are taking.

14. Approximately how many times per week do you eat red meat? _____

15. Do you consciously think about including iron rich foods in your diet? yes no

16. Have you ever lived at high altitude for an extended period of time? yes no

If yes, at what elevation and for how long? _____

APPENDIX 3

Iron Regulation in Exercise Questionnaire-For Exercise subjects

The following questionnaire contains questions regarding lifestyle, supplementation, and exercise. Please answer each to the best of your ability.

Age:

Gender:

Subject ID:

1. How many miles per week do you run?

2. How many days per week do you run?

3. Do you take a multivitamin? yes no

   How frequently?

   What is the name of the vitamin? _____

   Does it contain iron? yes no

4. Have you ever been diagnosed with iron deficiency anemia? yes no

   If you answered "No" to the question above, do you believe that you have ever been iron-deficient? yes no

5. Have you ever taken iron supplements? yes no

6. Do you currently take iron supplements? yes no

   If yes, what form of iron supplement do you take? (circle one below)

   Regular tablets liquid or drops

   coated or extended release tablets and capsules

   If yes, what type of iron supplement do you take? (circle one)

   Ferrous sulfate ferrous fumarate ferrous gluconate

   How often do you take the supplements per week?

   At what time (or times) of day do you usually take your iron supplement? (morning, before bed, etc) _____

7. Do you take calcium supplements? yes no

8. Do you take calcium supplements at the same time as iron supplements? yes no

9. Do you take oral contraceptives? yes no

If yes, how long have you taken oral contraceptives? _____

What is the brand of the oral contraceptive? _____

10. Do you have a regular monthly menstrual cycle? yes no

11. What was the date of your last menstrual period? _____

12. How long is your cycle on average? _____

13. How frequently do you take anti-oxidative supplements or anti-inflammatory medication? (e.g. Aleve, tylenol, NSAIDs, etc.) _____

14. Please list any other medications you are taking.

15. Approximately how many times per week do you eat red meat? _____

16. Do you consciously think about including iron rich foods in your diet? yes no

17. Have you ever lived at high altitude for an extended period of time? yes no

If yes, at what elevation and for how long? _____

APPENDIX 4

Pre-Screening Survey - Exercise and Iron Regulation

Subject ID: _____ Date: _____ Time:_____

1. Do you currently have any type of infection?

   If so, <u>when</u> was the onset of this infection?

2. What day was your most recent iron supplementation?

3. <u>When</u> was your most recent use of anti-inflammatory medications?

4. <u>When</u> was your most recent use of cold remedies (if applicable)?

5. <u>When</u> was your last bout of exercise?

   What was the **<u>duration</u>** and **<u>intensity</u>** of this exercise?

6. How would you like your results returned to you?
   mail email

Email address_____

Campus mailing address_____

For research team only:

Height	
Weight	
BMI	

APPENDIX 5

Monocytes isolation protocol

Preparation: 6ml human blood; 6ml histopaque; 15-ml conical centrifuge tubes (three for each sample), isotonic phosphate buffered saline; centrifuge. For each human blood sample, we need two 15-ml conical centrifuge tubes of 3ml histopaque plus 3ml whole blood.

- Check the centrifuge in CCRB1208 at first, make sure it is available and set the temperature at 25ºC.
- Set up three 15 ml conical centrifuge tubes and one 2ml microcentrifuge tube
- Add 3.0ml Histopaque-1077(Sigma-Aldrich, St. Louis, MO) in two 15 ml conical centrifuge tubes. It is important to warm it up to room temperature.
- Then, carefully layer 3.0ml whole blood onto the histopaque.
    - Incline the conical centrifuge tube slightly, pipette 1000ml human blood slowly and allow the blood stay on top of the histopaque, repeat three times. Totally, 3ml histopaque and 3ml human blood.
- After laying blood, centrifuge at 400×g (set centrifuge in CCRB1208 at1500rpm) for exactly 30 minutes at room temperature. The reason is that centrifugation at a lower temperature, such as 4ºC, may result in cell clumping and poor recovery.
- After centrifugation, there would be four layers from top to the bottom of the tube: plasma, monocytes, histopaque and red blood cells.

- Carefully aspirate and discard the upper layer-plasma. Then, transfer the 0.5 cm of the opaque interface containing mononuclear cells into a clean conical centrifuge tube.
    - As we have two tubes of blood sample, transfer both of them to the new conical centrifuge tube.
- Add 10ml Isotonic Phosphate Buffer Saline Solution in this tube and mix the solution by gentle aspiration. Then centrifuge at 250×g (1150rpm) for 10min.
- Aspirate the supernatant and discard.
- Resuspend cell pellet with 1ml PBS and transfer that to a 2ml microcentrifuge tube, and then use 0.5ml PBS to make sure all the cells in 15ml tube transferred to 2ml tube.
- Centrifuge at 500×g for 10 min(using the centrifuge in our lab)
- Discard the supernatant and resuspend cell pellet in 400ul RNA lysis/binding solution.
- Continue the RNA isolation or put the sample in -80 ºC freezer for later RNA isolation.

APPENDIX 6

Tissue iron analysis

Preparing the Acid Solution:

30% HCl, 10% Trichloroacetic Acid

- Final Volume = 50 mL
- Add 31.145 mL DI water to a 100 mL glass bottle. DI water is located in the special DI spout at the south sink.
- Under the hood, pipette 6.25 mL of 80% trichloroacetic acis (TCA) into the glass bottle. TCA is stored in the 4 degrees C fridge.
- Pipette 12.605 mL of 12N HCL into the bottle. 12N HCl is stored in the cabinet below the hood.
- Store the acid solution at 4 C.

Preparing the Chromogen Reagent:

1.86 mM Bathophenathrolinedisulfonic Acid, 143 mM Mercaptoacetic Acid

- Final Volume = 100 mL
- Add 98.656 mL DI water to a 200 mL glass bottle.
- Mass out 100 mg of bathophenathrolinedisulfonic acid, found in the 4 C fridge. Under the hood, add this to the glass bottle and gently swirl to dissolve.

- Pipette 1.343 mL of 98% mercaptoacetic acid into the bottle. This reagent is stored in the fridge.
- Store chromogen reagent at 4 C.

Preparing the Working Chromogen Reagent:

1:5:5 Chromogen Reagent (1): Saturated Sodium Acetate (5): DI Water (5)

- Final Volume = 22 mL (Enough for a 96 well plate)
- Add 10 mL DI water to a 50 mL Falcon Tube.
- Add 10 mL saturated sodium acetate to this tube.
    - To prepare 100 mL saturated sodium acetate, add 46.4g sodium acetate to 100 mL DI water. Sodium Acetate is stored in the glad cabinet to the right of the hood. Store the sat'd SA solution at 4 °C.
- Pipette 2 mL chromogen reagent into falcon tube.
- Store at 4 °C.
- **NOTE:** Prepare fresh working chromogen reagent for each assay. Don't make excess because it will go to waste

Procedure:

1. Cut a sample of tissue weighing between 25 to 30 mg and transfer to a 1.5 mL microcentrifuge tube. Make sure to place sample at the very bottom of the tube.

2. Add 10x the sample weight in µL of the acid solution to each tube. For example: add 250 µL to a 25 mg sample and 275 µL to a 27.5 mg sample. Make sure the tissue is completely submerged in liquid.
3. Incubate tubes for ~20 hours, or overnight, at 65 degrees Celsius (this corresponds to setting "6" on the incubator).
4. Prepare 400 µL of a 1000 µg/dL iron standard by adding 4 µL of iron standard stock (1000 ppm) to 396 µL DI water. Prepare remaining iron standards by performing 5 serial dilutions from the 1000 µg/dL iron standard.
5. Pipette 20 µL of each standard and 20 µL DI water into the first column of a 96-well plate.
6. Add 20 µL of your acid solution to another well in the plate.
7. Pipette 20 µL of the acidic solution from each of your now digested samples into the 96-well plate. Try to pipette liquid from the middle of the tube and be sure not to disturb the solid at the bottom of the tube.
8. Add 200 µl of fresh working chromogen reagent to each well. Let sit for 5-10 minutes.
9. Measure the absorbance of the wells prepared in steps 3-6, containing samples and working chromogen reagent, in a spectrophotometer at a wavelength of 535, or at the closest wavelength to 535 available. (562 also works well.)

Notes:

- When preparing the standard solutions, be sure to vortex each tube in between dilutions to be sure they are evenly mixed.

- Prepare fresh standards often. All of them, but especially the ones containing the lowest iron molarities, begin to exhibit lower absorbencies as time goes on, throwing off your standard curve.
- Be sure to always add the sample into the well first, and THEN add chromogen reagent. Otherwise they don't mix right and the color is uneven, throwing off your results.
- You must prepare the HCl standard to be read. It has very visible iron content, and majorly skews results if not taken into account. To get accurate absorbencies of the samples, you simply subtract the HCl's absorbance from the sample's absorbance.
- Distilled water does not contain iron and therefore makes a suitable 0 standard.
- High standard values, such as 50 or 100, only fit into the standard curve when you use less than 20 µL in the wells. (To make 50 fit, used 10 µL. For 100, use 5 µL.) This is only necessary when some of your samples have extraordinarily high iron levels.
- Put the assay kit in the fridge at the end of every day.
- Be sure to record the weights of the samples before you place them into the test tube.
- Mincing the tissue doesn't do anything or yield better results.