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Background: Iron, copper, manganese, and zinc are essential trace elements critical for cellular and physiological process, such as catalytic enzymatic reactions, gene regulation, and signaling cascades. These elements must be maintained through homeostatic regulation for one's health. The importance of trace element homeostasis is seen in system organs, liver, spleen, and adipose tissues where dysregulation has been thought to be associated with liver disease, such as non-alcoholic fatty liver disease. Obesity is associated with increased risks for altered systemic trace element status, such as with iron, but the effects of diet-induced obesity (DIO) between strains and sexes remain unclear. Objective: The purpose of this study is to examine strain and sex differences in the distribution of systemic trace elements due to diet-induced obesity (DIO). Methods: Male and female C57BL/6J (n=36) and DBA/2J (n=36) mice were fed either a high-fat diet (60% Kcal from fat) or a control diet (10% Kcal from fat) for 16 weeks. Food intake and body mass were measured weekly. At the end of the 16 weeks, blood, liver, and spleen tissues were collected. Iron, Copper, Manganese, and Zinc concentrations were measured using graphite furnace atomic absorption spectroscopy. Trace element and inflammatory related genes were analyzed in the liver, spleen, and adipose tissue. Results: Key findings from this study included mouse models developing obesity-induced iron deficiency without anemia and decreased hepatic hepcidin expression in high fat diet group. DIO is sufficient in altering systemic trace elements and gene regulation. There were marked decreased levels of systemic copper, altered iron

homeostasis and the potential of hepatic manganese and zinc toxicity in high fat diet mice. There were mixed significant ceruloplasmin expression and increased gene expression of IRP-1 between strains and sexes, decreased HAMP expression, increased DMT-1in both sexes, only in C57 strain, and lastly HIF-1α gene expression remained near normal levels in male mice. Conclusions: These observations of altered trace element status and gene regulation indicates that these changes brought on by obesity may be amplified or attenuated depending on strain and sex.

THE EFFECTS OF DIET-INDUCED OBESITY ON THE DISTRIBUTION OF SYSTEMIC TRACE ELEMENTS. (2020)

by

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CHAPTER I

INTRODUCTION

Iron (Fe), Copper (Cu), and Manganese (Mn) are essential trace elements that are critical for health and must be maintained in an optimal physiological range. (1) When there is a disturbance of this regulation, such as obesity, pathologies ranging from anemia to neurotoxicity can occur. Inflammation from obesity is thought to induce hepcidin expression impairing iron absorption and retention of iron in liver and spleen macrophages leading to iron deficiency. (2) Due to the obesity epidemic much research has been conducted into the relationship between obesity and neurological disease. (3) There is a growing body of evidence has demonstrated that people suffering from child to midlife obesity, via dietary fat, have augmented risk for both Alzheimer's and Parkinson's Disease.(3,4) Erikson et al. has previously reported the effects of iron deficiency and brain development which can lead to other metal toxicity. (5,6) The objective of this current study is to examine strain and sex differences in the distribution of systemic trace elements due to diet-induced obesity (DIO). We hypothesize that high fat DIO causes significant systemic alterations of Fe, Cu, Mn distributions compared to low fat diet with sex and strain as contributing factors.

Aims

This current study aimed to expand on our previous work by using 2 mouse models of DIO, C57BL/6J and DBA/2J mice of both sexes according to NIH biological variable guidelines (7). Thus, the Aims of the study are:

1. To collect critical data on the effects of diet-induced obesity on system trace element biology in male/female C57BL/6J and DBA/2J mice.
    a. Based on previous data from the Erikson lab, we hypothesize that high fat DIO causes significant systemic alterations of Fe, Cu, Mn distributions compared to low fat diet with sex and strain main effects.

2. Identify alterations in the expression of both trace element and inflammatory related genes in liver, spleen, and adipose tissue due to diet-induced obesity.
    a. We hypothesize that high fat DIO causes significant changes in Fe, Cu, Mn, and inflammatory related genes in a sex and strain dependent manner.

Study Design

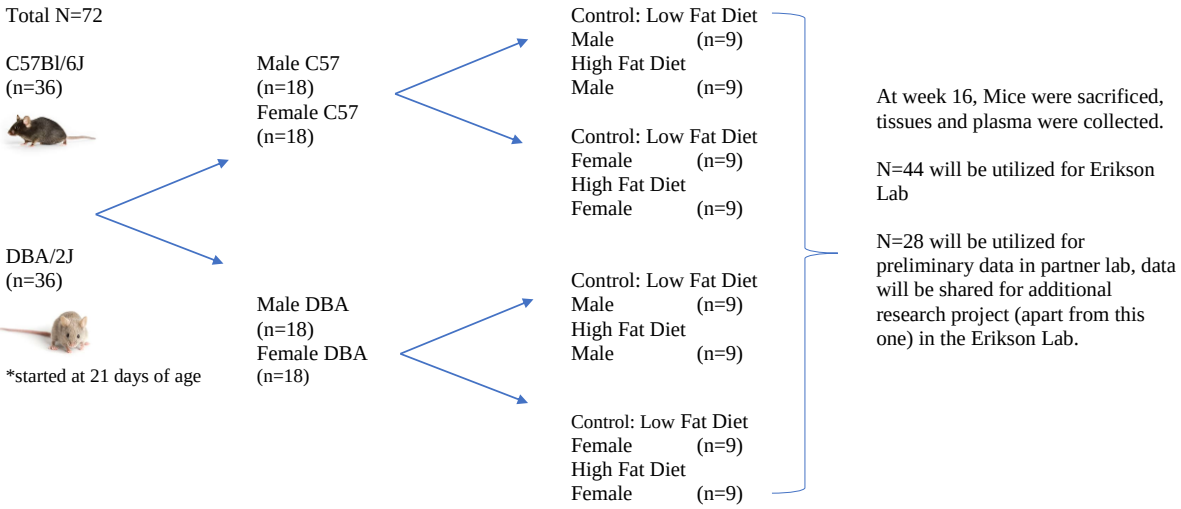

Figure 1: Study Design Twenty-one-day old male and female C57/6J and DBA/2J mice were randomly divided into 2 dietary groups, the control: low fat diet (10% Kcal from fat) and a high fat diet (60% of Kcal from fat). After 16 weeks of feeding, brain, liver, spleen, adipose and plasma were collected from each mouse. The systemic tissues, liver, spleen, and adipose, were assessed for trace element concentrations (μg/mg protein) and trace element related gene expression.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

Essential trace elements such as Iron (Fe), Copper (Cu), Zinc (Zn) and Manganese (Mn) are critical for many cellular and physiological process and must be maintained in optimal physiological range for one's health. (1,8) Some of these processes are, but not limited to, catalytic enzymatic reactions, regulatory functions for genes, or signaling cascades.

Iron is known for its role in oxygen regulation and homeostasis via hemoglobin and red blood cells.(9) Iron also functions in respiration, and genetic synthesis, energy metabolism, DNA synthesis and repair, and various signaling pathways. Other critical roles include the myelination of spinal cord in the central nervous system (CNS), synthesis, packaging, uptake and degradation of neurotransmitters. (1,9) This functionality of iron is largely due to the oxidation-reduction reactions of iron as a transition metal. Iron redox states are capable of -2 to +6, but primarily limited to ferrous (Fe^{+2}) and ferric (Fe^{+3}) and ferryl (Fe^{+4}) states. (10) This ability also can cause harm through the creation of free radicals or reactive oxygen species such as, converting hydrogen peroxide into hydroxyl free radical, which can in turn cause brain damage and carcinogenesis.(9,10) Under normal physiological conditions iron transport is a highly

conserved process which is controlled via transports, of which a major transporter being transferrin, and transporter receptors through negative feedback hormonal control of hepcidin. (11)

Copper facilitates many reactions through copper-dependent enzymes that participate in respiration pathways, such as the electron transport chain, antioxidant defense, and more. (1) Like iron, it is able to undergo oxidation-reduction reactions, as copper is also a transition metal, and biologically cycles between cupric (Cu^{+2}) and cuprous (Cu^{+}) states. It is because of coppers potential for redox reactions, thus oxidative stress and damage, that homeostasis is tightly regulated. (9,12) The efforts to examine copper's role in biological systems is limited to that of relative distribution between organs, tissues, and cell type. (13) Copper uptake is mainly controlled via a cell surface metalloreductase and transporter Ctr1 whereas ATP7 and ATP8 will export copper, preventing accumulation in the cell and is transported in plasma via ceruloplasmin.

Manganese is significant during enzymatic reactions such as destruction of reactive oxygen species as antioxidant defense, essential cofactor for many enzymes, and vital for development and brain function, and immune response. (1,9,14) Manganese also participates in redox reactions found biologically as Mn^{+2}, its most stable form and Mn^{+3}, a potent oxidant. (14)

Another highly related trace element is Zinc (Zn), which is also vital in catalytic function and provide structural roles in enzymes and proteins throughout metabolism and gene regulation and homeostasis is tightly regulated. (1,9) Unlike the other trace elements

mention, zinc lacks the ability of redox activity but is linked to excitation and inhibition signaling, brain function, memory and behavior and antioxidant health. (9)

Obesity

According to the World Health Organization (WHO) there are roughly 1.6 billion overweight and 400 million obese adults globally, 2005, and is expected to rise to 2.3 billion and 700 million by 2015. (15,16) Obesity increases the risk factors for chronic disease such as type II diabetes, cardiovascular disease, and stroke. (15) The prevalence of obesity is also on the rise in developing countries with the spread of calorie dense foods and sedentary behaviors. While an increase in adipose mass is hallmark for obesity, it's the functional changes of this tissue is most important, such as pro-inflammatory cytokines and low-grade systemic inflammation. (2) There have been links to obesity and iron status and the dysregulation of this trace element; obesity promoting iron deficiency. The liver is one organ greatly impacted in various ways by both obesity and trace element regulation. The liver is a major site of both iron and copper regulation. (2,17) In obesity as well as insulin resistance, the liver is characterized by accumulation of lipids, resulting in nonalcoholic fatty liver disease and potentially increasing via inflammation and severity to nonalcoholic steatohepatitis through. (2) There is mounting evidence that obesity plays an important role in altering iron, copper and manganese homeostasis and chronic disease, inflammation and neurodegeneration processes. (18–20) Few studies have evaluated manganese levels serum or various body materials in obese individuals, however, it has been seen that increased blood manganese levels are associated with increased adiposity and obesity. (20)

Liver Trace Element Transport

Iron

All tissues require Fe for its role as a cofactor in enzymes. (21) The majority of absorbed iron is used in hemoglobin and circulates between precursors, red blood cells, and macrophages in the spleen. Maintenance of proper iron levels, both storage and circulating, is critical to avoid physiological consequences of too low (Iron-deficiency anemia) or too high (hereditary hemochromatosis). (22) Systemic regulation of Fe is done by the protein Ferroportin to export Fe from cells which is regulated by the peptide hormone hepcidin. (21) Ferroportin is an iron exporter, releasing iron into the plasma, found on the surface of intestinal cells, macrophages, and hepatocytes. (18) Hepcidin, by inhibiting ferroportin, will mobilize internal iron and inhibit Fe acquisition from the diet by impeding enterocytes' ferroportin delivering iron into the plasma. (21) Hepcidin is transcriptionally regulated by high plasma transferrin-bound iron levels, the storage form of Fe, and is also stimulated by inflammation, specifically interleukin-6 (IL-6), IL-1, or IL-22. (21,23) With inflammation present, total iron is normalized, but availability for erythropoiesis is impaired causing anemia of inflammation, leaving iron trapped in the liver and spleen. (21) Additionally, it seems that male mice are sensitive to iron deficiency compared to female mice, similarly to that of rats.

Copper

The liver is the main regulatory organ for copper homeostasis and modulates carbohydrate and lipid metabolism. (17) Copper is first mobilized from epithelial cells via active transport into the hepatic portal circulation bound by albumin and transcuperin.

While iron-regulatory hormones have been identified, copper regulatory hormones have to-date, not been identified. (24) Once in hepatic cells it is either utilized for cellular processes, such as mitochondrial cellular respiration, or it is transported into the trans-Golgi network. (17) The known Cu- active transporters ATP7A and ATP7B are abundant in the liver, and disruptions in these transporters' function affects the central nervous system, suggesting that liver homeostasis of Cu is essential for normal brain function. (13) Mutations in ATP7A in males result in copper deficiency, while in contrast, mutations in ATP7B leads to copper toxicity found in the autosomal recessive Wilson's Disease. In hepatocytes, these transporters are located in the trans-Golgi network. (13,17) The function here is to supply Cu for the incorporation into Cu-dependent enzymes. When there is excess Cu, ATP7A and ATP7B are located on the basolateral and apical surfaces, respectively, to efflux Cu out of the cell. This copper is then removed from the hepatocyte by being secreted into bile, where it then is eliminated from the body via feces. (17) It should also be noted that Cu may also alter hepcidin activity as well as hypoxia-inducible factor (HIF) in the intestine modulating iron homeostasis. (24) Disruption in Cu homeostasis impacts both local and systemic processes. (17) Marginal Cu-deficiency has been linked to several disease states such as Alzheimer's, ischemic heart disease, non-alcoholic fatty-liver disease (NAFLD), and obesity. Many of which have been linked to dysregulation of lipid metabolism and finally dyslipidemia due to Cu-deficiency. NAFLD patients with mild inflammation present are at higher risk of disease progression to non-alcoholic steatohepatitis. Liver cirrhosis is caused by the development of regenerative nodules which are encompassed by fibrous bands in

response to chronic liver injury, which can lead to end-stage liver disease. (25) The liver is highly involved with homeostasis of both copper and zinc, which are widely studied for their implications in liver cirrhosis. Some research has suggested that hepatic fibrosis is the result of Zn depletion, among other negative health outcomes, such as impaired immune function. With liver damage there is an increase in impaired liver function which decreased the liver's ability to detoxify, which also impairs copper regulation. This impairment can lead to copper toxicity which contributes to liver cirrhosis. Clinical biomarkers are increased serum Cu/Zn ratio in patients receiving hemodialysis. Malavolta et al. described this ratio as an important tool as an inflammatory/nutritional biomarker of all-cause mortality. (26) This ratio has also been regarded as clinically more important together than the singular concentration of either trace element. (27)

Manganese

ZIP8 is best known for its role as a divalent metal ion transporter for zinc. As for Mn, hepatic ZIP8 regulates Mn homeostasis for the whole body, in conjunction with DMT1. (28) ZIP8, localized to the hepatocyte canalicular membrane, functions by reclaiming Mn from bile. DMT1 was believed to be the primary Mn transporter, but is dispensable in both the small intestine and liver. Current research indicates that hepatocytes will uptake Mn from the basolateral side, potentially with ZIP14, and excrete on the apical side via ZIP8, into bile.

Zinc

The liver plays a major role in the metabolism and homeostasis of zinc. (29) In dietary zinc deficient states, some tissues such as hair, zinc concentrations remain

constant. In other organs such as the liver, zinc concentrations will decrease. The liver characterizes a fast, exchangeable pool of zinc which is an important role in zinc metabolism as well as other trace elements. Other systemic signals such as glucocorticoids, endotoxins from bacteria, and cytokines decrease plasma concentrations of zinc, while zinc is redistributed in the liver. Intracellular zinc metabolism is regulated through ZIP proteins, which are involved in zinc uptake. Intracellular trafficking and compartmentalization of zinc is regulated by ZnT or zinc transporters and metalloproteins such as MTs. Cytokines like IL-1 and IL-6 and other metals regulate MT synthesis. In the presence of zinc MT synthesis increases, while deceases in zinc has the opposite effect. Reduced hepatic zinc levels have been correlated with impaired function of the liver as well as liver regeneration. Acute viral hepatitis has been associated with reduced serum zinc levels. Low levels of hepatic MT proteins sensitized the liver to alcohol induced fatty liver disease and liver injury. While increased MT levels enhanced zinc reserves, which makes zinc available with oxidative stress is present.

Spleen Trace Element Transport

Another important organ to maintain iron homeostasis is the spleen which possesses iron-recycling macrophages. (23) These specialized macrophages are red pulp macrophages (RPMs) and have the ability to recycle between 90-95% of body iron to maintain erythropoiesis. Splenic macrophages can uptake iron in various forms such as, transferrin-bound, heme iron, hemoglobin-bound, and free iron. Uptake mechanism also vary, such as transferrin receptor, DMT1, ZIP14, heme-hemopexin complexes. Once taken in it joins the pool of free iron, which is either stored as ferritin or utilized.

Exportation out of the cell occurs through ferroportin in conjunction with ceruloplasmin, which is then loaded onto transferrin to join systemic circulation and utilization. It has been shown in obesity that macrophages have an impaired ability to handle iron.

Similarly to iron recycling macrophages will also acquire some copper during recycling of red blood cells via copper transporter-1 or CTR-1-mediated uptake.(30) Macrophages will also acquire some zinc through recycling processes through the use of ZIP (uptake), ZnT (trafficking), and MT (Storage) proteins. (31) It was also shown that during hypoxic conditions, both CTR-1 gene expression and copper uptake was increased in a macrophage-like cell line. Ceruloplasmin also increased. (30) In addition, oxidative stress caused by altered levels of zinc can lead to dysfunction of the innate immune system during acute inflammation.

Obesity and Trace Elements

Obesity

The prevalence of overweight and obesity is a major health concern. (32) According to National Health and Nutrition Examination Survey (NHANES) date 2011-2012, almost 6% of 2-19 year-olds were severely obese. (33) Adults are defined overweight as a body mass index (BMI) of >25kg/m^2 and obese as 30kg/m^2 or more, while WHO defines it as abnormal or excessive fat accumulation. (32) Traditionally obesity has been described as an imbalance between energy intake and energy expenditure. (34) This imbalance was considered to be driven by the increased consumption of high calorie foods (usually nutritionally poor) and a sedentary lifestyle. In recent years obesity and comorbidities have been associated with a variety of other

factors such as sleep or gut flora which could cause weight gain. Obesity is associated with a chronic low-state of inflammation systemic organs such as the liver, brain, and adipose tissue. (32) Adipose tissue inflammation is considered to be a hallmark of obesity which is often in varying degrees in individuals. (34) In addition to the inflammatory response, adipose tissue will also secret inflammatory molecules, referred to as adipokines, largely secreting fatty acids and well over 50 proteins. (35) Several are directly linked to inflammation, specifically, adiponectin, necrosis factor-alpha (TNF-α), IL-1β, IL-6, IL-8, and IL-10, and many more. This inflammatory response leads to dysregulation. (32) Obesity also has links to diabetes, specifically type II, cardiovascular disease, neurological disease, and cancer to name a few.

There is growing amount of evidence proposing a potential link between obesity and dysregulation of iron in mice and humans. (28,36–39) Additionally, copper and manganese dysregulation have been seen in humans, especially children. (20) Significantly low serum iron concentrations were found in obese adolescents compared to non-adolescents. (40) Recent findings support previous findings that there is an association between obesity and iron-deficiency in adults, children, and adolescents. (35,40–42) A meta-analysis by Cheng et. al, showed significant higher ferritin concentrations, hemoglobin, and a trend for lower transferrin saturation compared to non-obese controls. (36) In the analysis, only one study measured hepcidin, and confirmed it to be higher in obese adults. Fan et. al observed an increase in blood copper and manganese concentrations were associated with obese children between the ages of 6-19years, as well as dysregulation of other metals such as zinc.(20) Lima et al. also seen

similar results in overweight and obese males, plasma copper concentrations were higher compared to control group. (43) Additional studies have also shown there were significantly higher serum copper levels in obese children and adolescents. (44,45) When there is excess in these, among other, trace metal ions, they can cause and contribute to mitochondrial dysfunction, autophagy dysregulation, stress on the endoplasmic reticulum, oxidative stress and damage, and apoptotic activation. (46)

Inflammation

With obesity rates on the rise over the last several decades, there have also been increases in insulin resistance, type II diabetes, and cardiovascular diseases, creating global concern. (47) Accompanied with obesity and other comorbidities, is increased white adipose tissue and chronic low-grade inflammation and the recruitment and activation of immune cells, macrophages. (47–49) In a healthy state, inflammation is a normal process which is necessary for the physiological healing process of wounded tissues. (50) The notion of obesity and inflammation is highly supported, also seeing an increase in plasma levels of many of the acute-phase proteins and cytokines. (35) Other effects have been proposed such as endoplasmic reticulum stress, oxidative stress, and adipose tissue hypoxia. (48) Inflammation markers mainly result from the increase in adipocyte size and infiltrated macrophages in the white adipose tissue. (47) Additionally, the changes in local macrophages are seen. (50) Adipose-residing macrophages will undergo morphology into M1-like polarization, from the M2 status, resulting in higher expression of proinflammatory mediators, which recruit additional macrophage infiltration, exacerbating the inflammation process. Activation of inflammatory cascades

and secretion of mediators, such as IL-6 cytokines, attract other M1 polarized macrophages, which become an adaptive immune response. (34) Several studies have described the cytokine messaging from adipocytes and increased macrophage infiltration. (51,52) There have been links associated with the inflammatory process and dysfunctional adipose tissue, as the expansion of adipose tissue are not met by compensatory capillary density and angiogenesis, resulting in hypoxia. (47,53) Under normal angiogenesis conditions, the vascular endothelial growth factor (VEGF) family of proteins are responsible for vasculature regulation. (54) During inflammation, macrophages will generate VEGF production to support the creation of new supply of blood in response to the increased adipose tissue. Research from Wu et al. also shows that the anti-inflammatory cytokine IL-10 is an important regulator in pro-angiogenic pathways, especially during hypoxic conditions. Recent studies have also corroborated decreased oxygenation in adipose tissue compared to that of lean controls resulting in hypoxia. (53) This hypoxic state drives inflammation and stimulation of angiogenesis via the above pathways, which is transcriptionally regulated by HIF1α. Lawler et al. expanded previous research by suggesting that this tissue hypoxia is a result of adipose growth and not causal in insulin resistance, however more research is needed in type and location of adipose, hypoxia, and insulin resistance/inflammation.

In the liver over time steatosis is often observed due to the increase in adipose, triglycerides accumulate in the hepatocytes. (50) This fat accumulation will alter metabolic responses, such as glucose and lipid metabolism, which can lead to additional obesity-related disorders. The Kupffer cells are morphed into a proinflammatory

phenotype which exacerbates inflammation which can further lead to non-alcoholic steatohepatitis or liver cirrhosis. (50,55) Martínez-Peinado et al. describes in cirrhotic patients both Cu concentrations and Cu/Zn ratios were higher while Zn concentrations were decreased. (25) The livers impairment to excrete excess copper leads to increased liver injury from the rise in oxidative stress. The Cu/Zn ratio has been suggested as a suitable biomarker of cirrhosis and liver disorders, as well as oxidative stress and inflammation.

In regards to immune function and blood filtration, the spleen plays an important role, as it is the largest lymphoid organ in the body. (56) IL-10 an anti-inflammatory cytokine is produced in the spleen as well as in other organs and work to inhibit (pro) inflammatory cytokines, such as IL-6. Specialized immune cells, B-cells, in the spleen produce large amounts of this anti-inflammatory cytokine, and it has been recently reported that obesity is associated with lower IL-10 production. These anti-inflammatory cytokines reduced capacity is largely due to the down-regulation of a cell surface molecule on B-cells due to high fat diets and increased apoptosis and oxidative stress in the marginal zone of the spleen. Spleen derived IL-10 also has effects in the brain, specifically in the hypothalamus. It has been seen that in mild reductions of splenic IL-10 leads to induction of hypothalamic inflammation giving way to hyperphagia, while severe reductions lead to hypophagia.

Lastly, as a whole, obesity is a low-grade systemic inflammation, which also includes the brain and central nervous system. (50) The blood brain barrier is a tightly controlled and regulated entry way into the brain, but there are multiple points of

communication between the CNS and the system which allow or physiological adaptation, such as insulin and leptin ques. When the blood brain barrier is chronically challenged, its permeability and homeostasis can be altered, which leads to neuroinflammation and neurodegeneration such as Alzheimer's and Parkinson's disease.

Trace Element and Obesity Associated Gene Expression

It goes without saying that oxygen is essential for normal metabolism, especially in mitochondrial function and erythropoiesis. (57–59) Hypoxia is a low oxygen state in the cell and depending on the cell type, metabolic demands, and adaptability can lead to cell death. (58) Recent studies show that obesity is associated with tissue hypoxia, especially in adipose, both in human and rodents and HIF-1α is increased with obesity. (47,60,61) These hypoxic conditions induce adipose inflammation, insulin resistance, hepatic steatosis and dyslipidemia and activation of hypoxia inducible factor-1 alpha (HIF-1α) as well as enterocyte DMT-1. (24,60,62) When there are hypoxic conditions, HIF-1α is upregulated. Additionally, HIF has been shown to be important in the function of the hypothalamus, which regulates energy balance and metabolism. (62) Hypoxia-inducible factors are master regulators in hypoxia and allow for the adaptation to hypoxia via angiogenesis and VEGF pathways. Chronic hypoxia, such as sleep apnea, is a comorbidity in both obesity and neurodegeneration such as Alzheimer's and Parkinson's disease and occurring more frequently in men than women. (63) It has also been seen that HIF-1α also plays a role in macrophage recruitment which mediate and release obesity related inflammation markers such as IL-6 and IL-1β, among other cardiac related inflammation markers accelerating and impairing cardiac function. (61)

Hepcidin is a negative regulator of ferroportin the only known cellular iron exporter. (57) It will inhibit Fe absorption from the diet and release Fe from storage both from the liver and spleen macrophages. Hepcidin helps to maintain safe levels of functional iron and storage by facilitating circulating iron into storage when iron levels are high or when there is an immune response or inflammation. (57,64) During hypoxic conditions, hepcidin is secreted from hepatocytes. (2) Although the liver is traditionally the main source of hepcidin, morbidly obese subjects have recently demonstrated the expression of hepcidin in adipose tissue.(2,35) It is suspected that inflammatory cytokines from adipose tissue induce hepcidin expression impairing Fe absorption and retention of Fe in liver and spleen macrophages leading to Fe deficiency. (2) A meta-analysis by Tussing-humphreys et al. examined the relationship between obesity and hepcidin and found in multiple studies serum hepcidin was significantly elevated obese people compared to leaner women and children. (18) They also noted overweight children having higher hepcidin and poor iron status, despite Fe intake. In a paradoxical aspect of hepcidin, inflammation in the brain increases iron import, while inflammation blocks export via hepcidin leading to neuronal oxidative damage causing neurodegeneration. (65) It has also been established that there are sex and strain differences in inbred strains with genetics playing some role. (66) Hepcidin expression differs between strains and models, especially with Hamp1, not Hamp2. Both sex and strain have effects on hepcidin, however effects on hepcidin on various diets are driven by sex.

Other genes of interest are IRP-1, DMT-1, CTR-1, and CP. IRP was chosen because it is a key regulatory protein for erythropoiesis and iron absorption by controlling other HIF factors (HIF2α) working as both an iron and oxygen sensor. (67) A characteristic feature of IRP is its indispensable function as mitochondrional aconitase, with causes the catalytic isomerization of citrate into iso-citrate. Both DMT-1 and CTR-1 are iron and copper transports, respectively. Cp is the oxidase protein known for loading onto plasma transferrin and transport to tissue. (68) It has been shown to oxidize both iron and copper as well as playing a role in iron mobilization, cellular iron uptake and efflux. Additionally, cp has been considered to have some antioxidant properties.

Conclusion

As described above the interplay between obesity and inflammation lead to dysregulation of trace element status, which can lead to a whole host of other physiological diseases, such as metabolic syndrome, type II diabetes, and even neurodegeneration and warrants further study. Obesity is known to alter metal status and cause inflammation that further affect metabolic processes and other regulatory proteins. Finally, the hypoxic conditions associated with obesity have been shown in some research to affect key regulators such as hypoxia-inducible factors and hepcidin. These effects are synergistic when coupled together of obesity, inflammation, metal dyshomeostasis, and hypoxia. These are largely intertwined with Fe status, and to lessor extents other trace elements, especially copper, due to how they are regulated. Additional assessment is essential to understand how obesity effects systemic trace element status.

CHAPTER III

THE EFFECTS OF DIET-INDUCED OBESITY ON THE DISTRIBUTION OF SYSTEMIC TRACE ELEMENTS

Abstract

Iron, Copper, Manganese and Zinc are essential trace elements that are critical for health and physiological processes, such as catalytic enzymatic reactions, gene regulation, signaling cascades and antioxidant/immune health. These elements must be maintained through homeostatic regulation; when there is a disturbance of this regulation, such as obesity, pathologies ranging from anemia to neurotoxicity can occur. The importance of trace element homeostasis is seen in system organs, liver, spleen, and adipose tissues where dysregulation has been thought to be associated with liver disease, such as non-alcoholic fatty liver disease. The following study aimed to expand previous work by examining the effect of high fat diet-induced obesity (DIO) on systemic trace element concentrations of iron, copper, manganese, and zinc in a mouse model with sex and strain as contributing factors. C57BL/6J and DBA/2J male and female mice were fed either a high-fat (60% Kcal from fat) or a control diet (10% Kcal from fat) for 16 weeks. Food intake and body mass were measured weekly. At the end of the 16 weeks, blood, liver, and spleen tissues were collected. Iron, copper, manganese, and zinc concentrations were measured using graphite furnace atomic absorption spectroscopy. Further, hepatic gene expression for IRP-1, DMT-1, CTR-1, and CP were altered between strains and sexes. HAMP was the only hepatic gene expression to decrease in C57 males. HIF-1 α

was the only hepatic gene to remain relatively unaffected. These results expand previous data by showing strain and sex differences not only in iron, but copper, manganese, and zinc systemic concentrations. For females, the impact of DIO on iron was more significant in the C57 strain whereas in males the effect of DIO was more significant in the DBA strain.

Introduction

Essential trace elements such as Iron (Fe), Copper (Cu), Manganese (Mn) and Zinc (Zn) are critical for many cellular and physiological process which require homeostatic control for optional health. (1,8) Some of these processes are respiration, catalytic reactions, regulatory functions, DNA synthesis and repair, signaling cascades, and antioxidant health. (1,9,14) When there is a disturbance of this tight regulation such as obesity various pathologies from anemia to neurotoxicity can occur. (2) According to data from the World Health Organization (WHO), there are roughly 1.6 billion overweight and 400 million obese adults worldwide and expected to rise. (15,16) An association between obesity and iron deficiency has been seen in children and adults in both developed and underdeveloped countries. (36,69) Obesity can increase the risk factor for other chronic diseases such as diabetes, cardiovascular disease, and stroke. (15) Inflammation from obesity is thought to induce hepcidin expression impairing iron absorption and retention of iron in liver and spleen macrophages leading to iron deficiency. (2)

Tight regulation of these trace elements are important to systemic health, the liver is one organ which is greatly impacted in numerous ways by both obesity and

dysregulation of trace elements. (2) In obesity as well as insulin resistance, the liver is characterized by accumulation of lipids, resulting in nonalcoholic fatty liver disease and potentially increasing via inflammation and severity from nonalcoholic steatohepatitis to cirrhosis. There is varying amounts of evidence suggesting that obesity plays an important role in altering iron, copper and manganese homeostasis and chronic disease, inflammation and even neurodegeneration processes. (18–20) More specifically there is some evidence which proposes potential links between obesity and dysregulation of iron (in mice) and copper and manganese (in humans, specifically children) (20,28,36–39) One such suggestion is obesity being associated with a chronic low-state of inflammation on systemic organs leading to dysregulation of trace elements. (32) Inflammation of the adipose tissue and release of inflammatory molecules like cytokines or adipokines is considered to be a hallmark of obesity which is often in varying degrees in individuals. (34,35) Over time with increased adipose tissue in the liver, steatosis can be observed. (50) This fat accumulation can cause additional obesity-related disorders which can cause exacerbation of inflammation potentially leading to liver cirrhosis. When the liver is impaired, dysregulation of trace elements can occur such as the excretion of iron or copper which only increases liver injury. Obesity and subsequently the inflammation that increased adipose tissue produces is thought to induce gene expression which can alter homeostasis. It is thought to induce hepcidin expression impairing iron absorption and retention of iron in liver and spleen macrophages leading to iron deficiency. (2) Here we sought to examine strain (C57BL/6J and DBA/2J) and sex differences in the distribution of systemic trace elements due to diet-induced obesity (DIO). We hypothesize that high fat

DIO causes significant systemic alterations of iron, copper, and manganese distributions compared to low fat diet with sex and strain as contributing factors. Additionally, we sought to identify alterations in the expression of both trace element and inflammatory related genes in liver, spleen, and adipose tissue due to diet-induced obesity. We hypothesize that high fat DIO causes significant changes in iron, copper, and manganese, and inflammatory related genes in a sex and strain dependent manner.

Materials and Methods

Animals

Prior to initiation of the study, approval for all animal care and procedures was obtained from the University of North Carolina at Greensboro's Animal Care and Use Committee. Male/female weanling (post-natal day 21) C57BL/6J and DBA/2J mice (n=72) were purchased from Jackson Laboratory (Bar Harbor, ME). They were randomly divided into dietary treatment of 36 DBA 2J mice (9 per group X 2 sexes X 2 diets=36) and 36 C57BL/6J mice (9 per group X 2 sexes X 2 diets=36). Ear notching was used to identify mouse number and housed in triplicates from beginning of the study. The housing environment was temperature controlled (25 ± 1° C) with automatic lights, which cycle off between 1800 h and 600 h. The mice were visually examined and weighed weekly throughout the study.

Diet

Mice, housed by sex and dietary group, were provided free access to diet and water 24 hours/day for 16 weeks. The control group was fed a low-fat diet (LFD; 10% kcal

from fat, D12450B; Research Diets) and the experimental group received a high-fat diet (HFD; 60% kcal from fat, D12492; Research Diets) according to previous research. The source of the fat in the diets was lard. The diets included the same mineral mix (S10026; Research Diets). See figure 1 for pictorial breakdown of animal and diet groups.

Hematocrit and Plasma

Blood from each mouse was collected in heparinized tubes at the time of sacrifice and stored on dry ice until processed. Hematocrit was determined by centrifugation of heparinized micro-hematocrit capillary tubes (Fisher Scientific; Waltham, MA). Remaining whole blood samples were centrifuged for 15 minutes at 1000 x g to separate plasma for metal analysis. The plasma was stored at -80°C.

Tissue Collection

Liver, spleen and adipose tissues were collected and placed in pre-labeled RNAse free collection tubes. All tissues were flash frozen in liquid nitrogen and kept on dry ice for transport to a -80° C freezer where they were stored until processing.

Protein analysis and Trace Element Analysis

The tissue samples were sonicated in cold radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors to disrupt the tissue degradation. Aliquots of the homogenate from each tissue sample were used to determine protein concentration using the Pierce Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL). Separately, tissues were digested in ultra-pure nitric acid for 24 hours in a sand bath (60°C). Aliquots (50 µl) of digested homogenate were further diluted in 2%

nitric acid for metal analysis. Metal concentrations were measured using graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) 184 µg Fe/g was digested in ultrapure nitric acid and used as an internal standard for analysis as well as sample internal standards were used when possible. All samples and controls were run in duplicate with %RSD <10%. Iron Concentrations were ran in triplicate and outlier readings were excluded. Metal concentrations are expressed as µg (Fe, Cu, Mn, or Zn) / mg protein.

Gene Expression Analysis

RNA was isolated from a <30mg cut sample of liver tissue. The RNeasy Plus Mini Kit (Qiagen Inc., Valencia, Ca) was used to extract the RNA from all tissue samples according to the manufacturers protocol, with the optional step 10 membrane drying step being utilized; and 50µL of RNase-free water was used to elute RNA from filter, with optional second elution of 30µL. Initial and secondary elution were placed into separate collection tubes and labeled appropriately. Immediately following the RNA extraction, RNA concentration and purity was determined by spectrophotometric analysis with the nanodrop. An aliquot of RNA containing 2µg of RNA (20µL total Volume) was taken and used for reverse transcription using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (with RNase inhibitor) (Life Technologies, Carlsbad, Ca) per the manufacturers protocol. Sample cDNA was used immediately or stored at -20° C.

Fast Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR gene expression assays were conducted with 20 ng of cDNA using TaqMan™ Fast Advanced Master Mix (Life Technologies, Carlsbad, Ca) and gene assays according to the manufacturer's protocol. The following trace element metabolism related TaqMan® gene expression assays (Life Technologies, Carlsbad, Ca) were evaluated: DMT-1 (Mm00435363_m1), CTR-1 (Mm00558247_m1), HIF-1α (Mm00468869_m1), IRP (Mm00801417_m1), Ceruloplasmin (Mm01289313_m1), and Hepcidin (Mm00519025_m1). 18s gene assay (Hs99999901_s1) was selected as the appropriate endogenous control. All assays were confirmed as appropriate for use in a murine species. Control and target assays were tested and validated on excess sample tissue. Relative gene expression was quantified using the delta-delta Ct method.

Statistical Analysis

Body weight, metal concentration, plasma, and gene expression were analyzed using the statistical software package SPSS, Version 26 (Chicago, IL). Analysis of variance (ANOVA) was used to evaluate interaction effects and main effects of strain, sex, and diet for both metal concentration and gene expression. Student's t-test (independent t-tests) was used to evaluate the significant in mRNA expression fold change due to diet for each strain and sex. For gene expression fold change data, error is represented using the upper and lower 95% confidence intervals. All other data, mean ± standard error of the mean was used. The significance level was set at $p < 0.05$ for all analysis.

Results

Descriptive Data

Table 1: Body Weight and Whole Blood Hematocrit by Diet

Strain	Sex	Diet	N	Body Weight	Hematocrit
C57	Male	LF	5	28.7 ± 0.41	49.5 ± 0.74
		HF	5	48.2 ± 0.36	49.0 ± 0.68
	Female	LF	5	21.6 ± 0.21	49.8 ± 0.91
		HF	5	41.6 ± 1.38	47.8 ± 0.81
DBA	Male	LF	5	28.4 ± 0.78	44.6 ± 0.81
		HF	5	43.9 ± 1.81	44.6 ± 0.81
	Female	LF	5	23.3 ± 0.74	45.6 ± 0.81
		HF	5	34.5 ± 1.45	45.8 ± 0.81

Whole blood hematocrit in LF verses HF diets in C57 and DBA, male/female mice seen above in TABLE 1. Hematocrit differed significantly between strains (C57 and DBA groups) at ($p<0.001$). There was n=44 mice available to use from initial study for the Erikson lab. The other n=28 was used in collaboration with another lab for additional preliminary data. N=5 mice were used in each group, with extra four mice from C57 male (2 from LF and 2 HF) being banked or used as test samples.

Mice fed a high-fat diet (HF) weighed significantly more than the low-fat diet (LF) mice. There was a strain main effect on hematocrit levels with DBA mice having 5% lower hematocrit compared to C57 mice. However, there was no significant differences in hematocrit due to diet induced obesity. All mice were approximately over 45% Hematocrit; no anemia was present in this sample set.

Food Efficiency was also calculated on weekly measures as seen in the Table 2, below. Fresh chow was fed, feed consumption, and weights were taken 3-times weekly to

calculate the mean weekly rate gains and efficiency. The * indicates potential sites for statistical significance.

Table 2: Food Efficiency

Strain	*Sex*	*Diet*	*Week 2*	*Week 4*	*Week 6*	*Week 8*	*Week 10*	*Week 12*	*Week 14*	*Week 16*
C57	M	L	2.47 ± 0.13	6.47 ± 1.21	14.97 ± 1.96	14.83 ± 34.15	21.1 ± 23.9	38.1 ± 39.18	14.2 ± 12.2	23.67 ± 46.53
		H	2.03 ± 0.13	5.8 ± 1.21	*5.4 ± 1.96	5.37 ± 34.15	*5.27 ± 3.94	8.57 ± 39.18	12.2 ± 12.2	16.77 ± 46.53
	F	L	4.23 ± 0.13	9.63 ± 1.21	21.73 ± 1.96	82.2 ± 34.15	16.07 ± 3.97	-15.97 ± 39.18	-2.37 ± 12.2	-75.46 ± 46.53
		H	3.0 ± 0.13	10.73 ± 1.21	*7.67 ± 1.96	*6.4 ± 34.15	*7.9 ± 3.97	10.23 ± 39.18	8.4 ± 12.2	9.3 ± 46.53
DBA	M	L	2.73 ± 0.17	6.17 ± 6.41	-5.5 ± 10.09	14.03 ± 1.57	16.27 ± 8.66	-121.63 ± 49.55	45.03 ± 30.03	56.7 ± 29.35
		H	2.00 ± 0.17	3.77 ± 6.41	6.07 ± 10.09	*7.4 ± 1.57	7.3 ± 8.66	*36.27 ± 49.55	11.97 ± 30.03	34.23 ± 29.35
	F	L	3.3 ± 0.17	17.63 ± 6.41	10.27 ± 10.09	9.73 ± 1.57	25.03 ± 8.66	-1.27 ± 49.55	23.4 ± 30.03	-45.1 ± 29.35
		H	2.6 ± 0.17	*4.2 ± 6.41	11.67 ± 10.09	*6.33 ± 1.57	8.93 ± 8.66	25.1 ± 49.55	3.9 ± 30.03	-4.93 ± 29.35

Sex was referenced as Male (M) or female (F) and Diet as low-fat diet (L) and high fat diet (H).

Plasma Trace Element Concentrations

Plasma trace element concentrations presented mixed results between diet, strain, and sex. There was a significant difference between C57 and DBA groups for plasma iron concentration (p=0.038) (FIGURE 2) Further analysis revealed that diet and sex drove this trend for DBAs showing an insignificant increase in DBA male plasma iron concentrations (p=0.055). There were deficits in plasma manganese concentrations in the HF group compared to the LF groups, showing decreased amounts in male DBA mice (p=0.025) whereas female DBA mice insignificantly increased in plasma Mn. Plasma

zinc concentrations were significantly elevated in HF male C57 mice (p=0.020) compared to LF groups. Plasma copper concentrations were not affected by HFD.

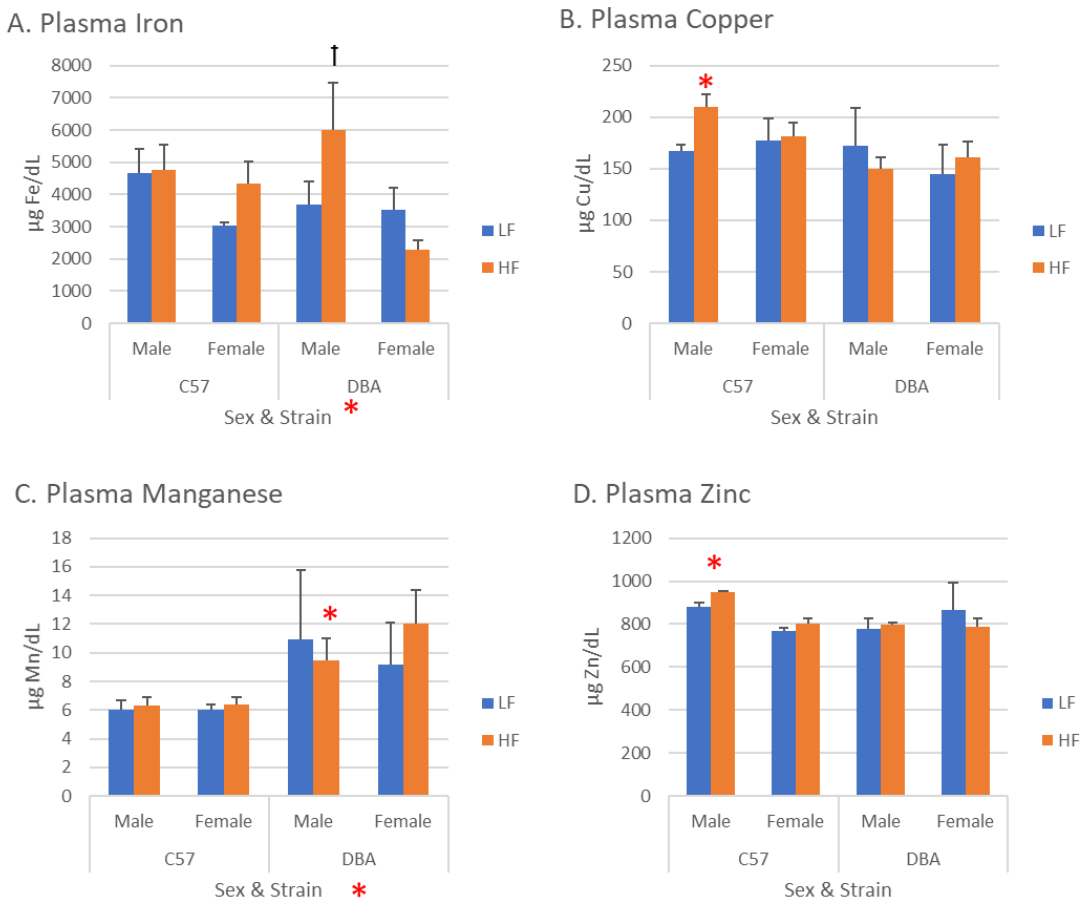

Figure 2: Plasma Trace Element Concentration by Strain, Sex, and Diet. LF (blue; left bar) HF (orange; right bar). Concentration was determined by Atomic Absorption Spectrometry and expressed in µg of element/dL of plasma. Metals included: **A.** Plasma Iron showed a statistical significance between strain and HF diet (*p=0.039) with male DBA mice *trending increased Fe concentration* († p=0.055). **B.** Plasma Copper showed no statistical significance; however, HF diet significantly increased Cu in C57 males (*p=0.015) **C.** Plasma manganese showed a statistically significant loss of Mn concentration in male DBA mice due to HF diet (*p=0.025). **D.** Plasma zinc concentrations were statistically elevated in HF male C57 mice (*p=0.020). The asterisk (*) indicates statistical significance at $p<0.05$ between LF and HF groups or by C57 and DBA strains.

Liver Trace Element Concentration

Trace element concentrations in the liver also varied by strain and sex due to HFD. Hepatic Iron and Copper were lower while hepatic Manganese and Zinc tended to be higher in the HFD fed mice compared to LFD. There was a statistically significant loss of Fe concentration in both HF male and female C57 mice ($p<0.001$ and $p<0.001$, respectively) compared to HF DBA mice. A two-way ANOVA split by strain showed another statistically significant deficit of Cu concentration but in male DBA mice ($p<0.001$) (FIGURE 3).

Using a two-way interaction for sex and diet separated by strain showed statistically significant increased liver Mn concentration for female DBA mice ($p<0.001$). Liver Zn concentrations significantly gained in concentration in both male and female C57 mice ($p<0.001$ and $p=0.005$, respectively), which is the inverse of iron.

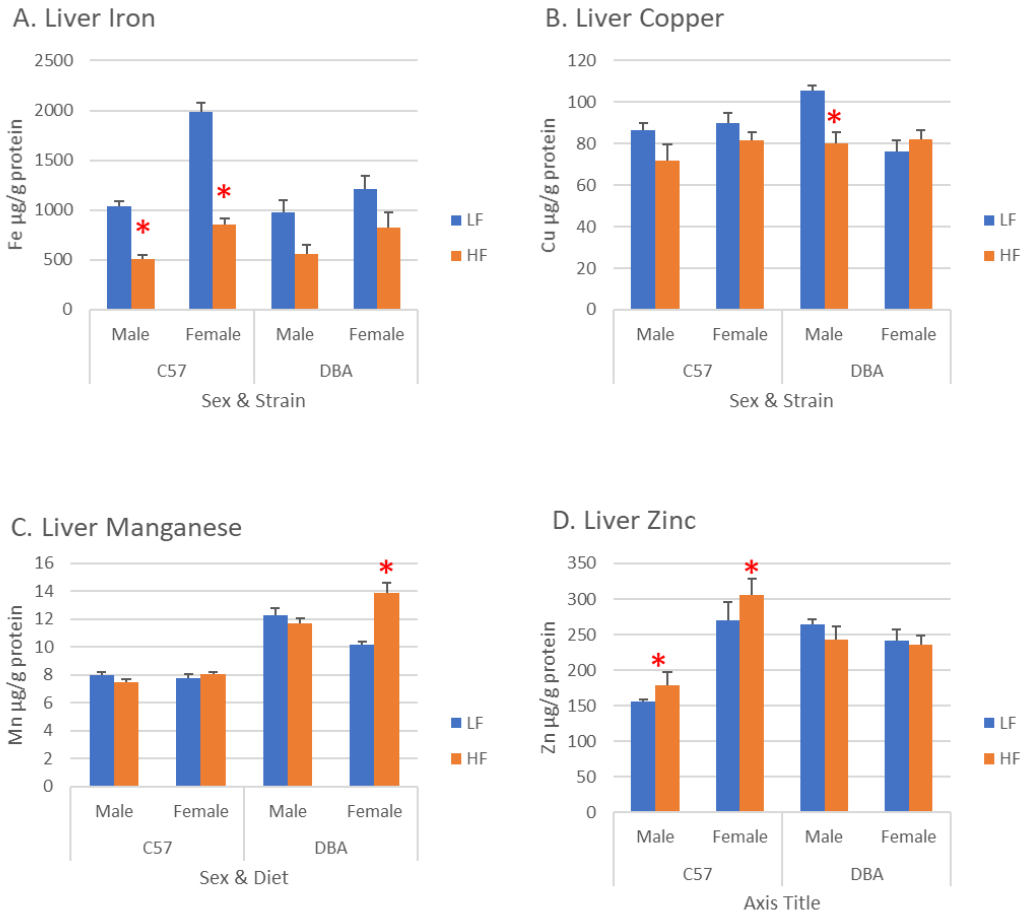

Figure 3: Liver Trace Element Concentration by Strain, Sex, and Diet. LF (blue; left bar) HF (orange; right bar). Concentration was determined by Atomic Absorption Spectrometry and expressed in µg of element/dL of Liver. Metals included: **A.** Liver Iron showed a statistical significance between sex, strain, and diet. Both C57 male and female mice statistically reduced liver iron (* $p<0.001$). **B.** Liver Copper was decreased statistically significant in male DBA mice (*$p<0.001$) **C.** Liver manganese showed a statistically significant increase of Mn concentration in female DBA mice (*$p<0.001$). **D.** Liver zinc concentrations were statistically elevated in both male and female C57 mice (*$p<0.001$ and $p=0.005$). The asterisk (*) indicates statistical significance between LF and HF groups.

Spleen Trace Element Concentration

Splenic trace element concentrations presented a loss in each element measured, except for Zinc for which was not analyzed due to limited funds. There was a statistically significant two-way interaction for C57 mice ($p<0.001$) (FIGURE 4). A t-test showed statistically significant lower splenic Fe concentrations for both HF male and female C57 mice ($p=0.028$ and $p=0.001$, respectively) compared to the LF c57 groups, with female C57 losing over half of their splenic Fe.

Splenic Cu presented a statistically significant simple two-way interaction on diet and sex for both C57 and DBA mice ($p=0.009$ and $p<0.001$, respectively). Further analysis showed statistically significance deficits for C57 Female and DBA male mice on splenic copper concentration ($p=0.006$ and $p<0.001$, respectively).

Similarly, to that of Cu, splenic Mn presented a statistically significant strain by sex interaction for both C57 and DBA mice ($p=0.001$ and $p=0.040$, respectively). However, there were no statistical significance found in either sex in either strain. T-test showed that C57 male mice trended toward a significant loss ($p=0.091$).

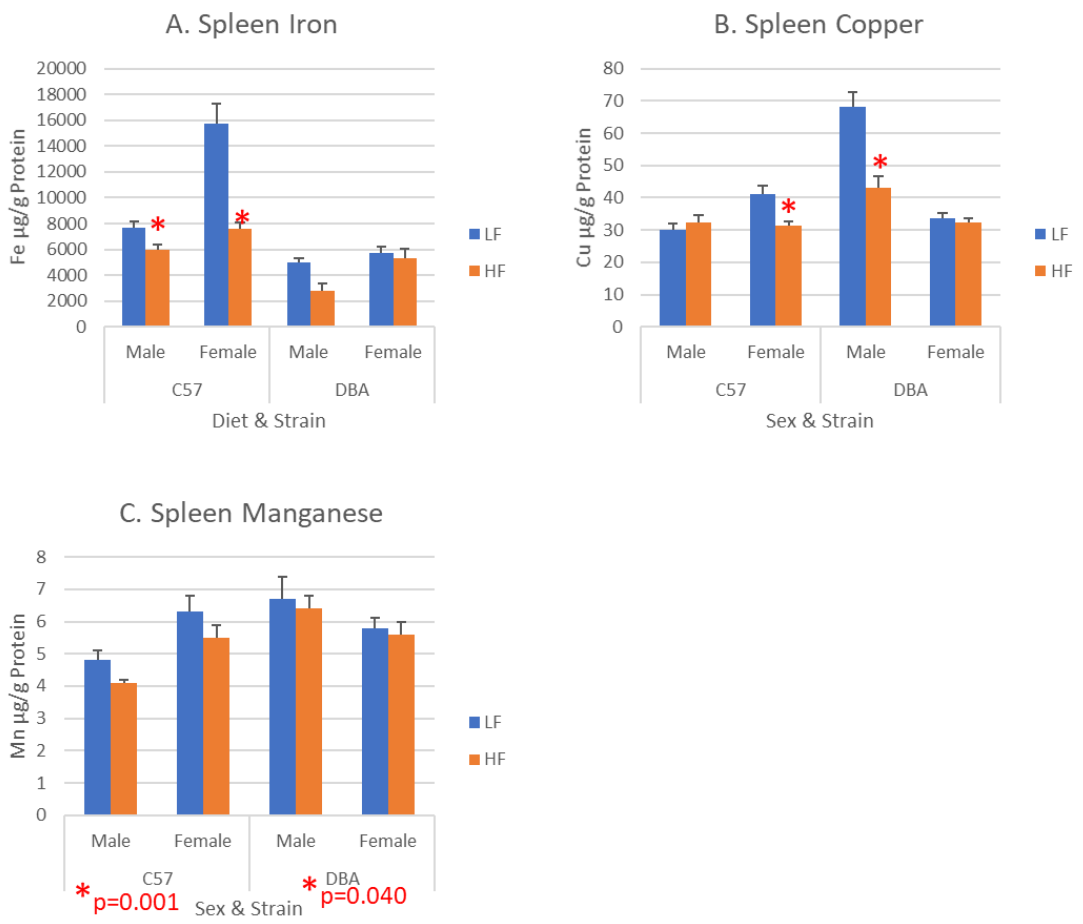

Figure 4: Spleen Trace Element Concentration by Strain, Sex, and Diet. LF (blue; left bar) HF (orange; right bar). Concentration was determined by Atomic Absorption Spectrometry and expressed in µg of element/dL of Spleen. Metals included: **A.** Spleen Iron showed statistically significant reduction in Spleen iron in both C57 male and female mice (* p=0.028 and p<0.001). **B.** Spleen Copper was decreased statistically significant in C57 female and DBA male mice (*p=0.006 and p<0.001) **C.** Spleen manganese showed a statistically significant interaction in both stains C57 and DBA (*p=0.001 and p=0.040). There was no statistical significance found in either sex in either strain, however t-test showed that C57 male mice trended toward significance.

Adipose Trace Element Concentration

Adipose trace element concentration also presented mixed results. There was a statistically significant three-way interaction between diet, strain, and sex for iron levels in adipose ($p=0.005$) specifically showing a sex by diet interaction for C57 mice ($p=0.006$) (FIGURE 5). Within these mice, there was a statistically significant loss of adipocyte Fe for female C57 mice ($p=0.002$). For adipose Cu concentrations there was only a statistically significant main effect of strain ($p=0.001$). There were no statistical interaction effects, such as effect of diet, on Cu concentrations. Using a two-way ANOVA split by strain, there was a statistically significant deficit in adipocyte Mn due to diet for females in both C57 and BDA strains ($p=0.039$ and $p=0.016$, respectively). There were no statistically significant interactions effects on Adipocyte Zn concentrations.

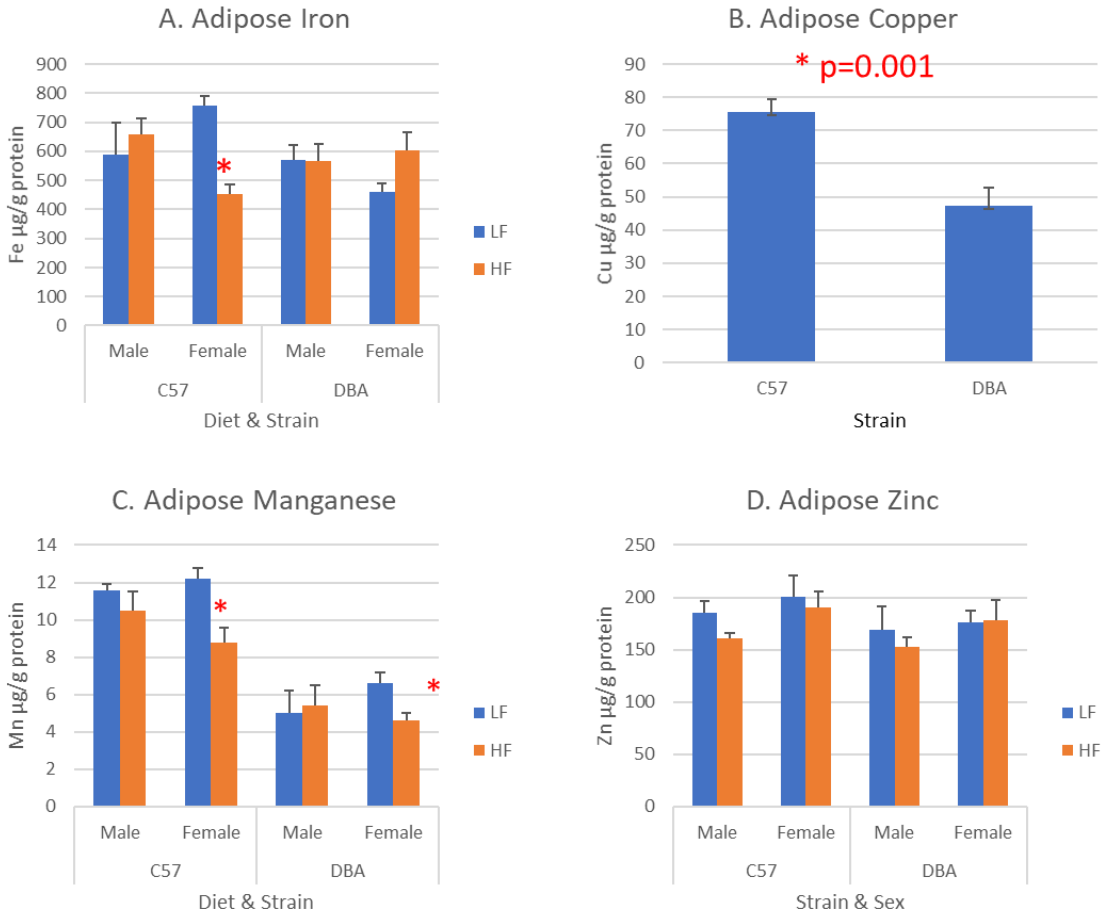

Figure 5: Adipose Trace Element Concentration by Strain, Sex, and Diet. LF (blue; left bar) HF (orange; right bar). Concentration was determined by Atomic Absorption Spectrometry and expressed in µg of element/dL of Adipose. Metals included: **A.** Adipose Iron showed statistically significant reduction in Adipose iron in C57 male mice (*p=0.002). **B.** There was a statistically significant main effect of strain on copper concentration (*p=0.001), but no interaction effects, such as Diet. **C.** Adipose manganese showed a statistically significant decrease in manganese concentration in both C57 and DBA female mice (*p=0.016 and p=0.039). **D.** There were no strain, sex, or sex interactions effects for zinc in adipose tissue.

Trace Element Ratios

Cu/Zn ratios for both plasma and liver tissue presented mixed results than anticipated. In the plasma there were no interactions or main effects of diet. Male C57 mice were trending toward significance at p=0.062. In liver tissue the Cu/Zn ratio was a statistically significant simple main effect of sex within C57 strain but not in the DBA Strain. There was an effect of diet in this ratio. (FIGURE 6)

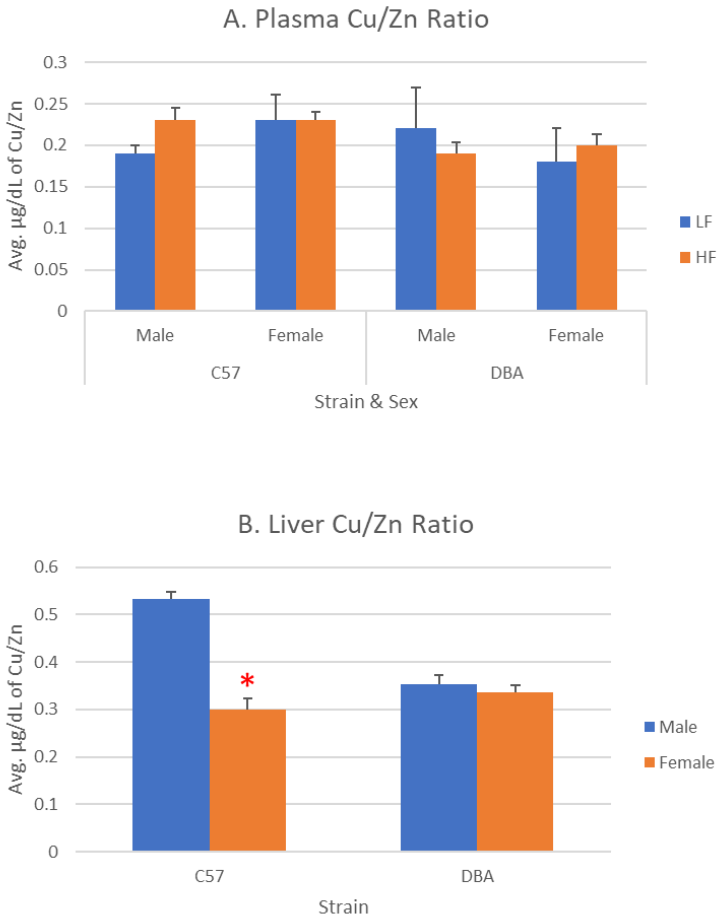

Figure 6: Plasma and Liver Cu/Zn Ratio

Concentration was determined by Atomic Absorption Spectrometry; ratios were determined and ran for statistical analysis. **A.** The Cu/Zn ratio in the plasma presented no interactions or main effects of diet. Male C57 mice approached significance at p=0.062. **B.** The Cu/Zn ratio in liver tissue presented a statistically significant simple main effect of sex in the C57 strain, p<0.001, but not in the DBA Strain. No effect of diet was seen.

The asterisk (*) indicates statistical significance at $p<0.05$ between sexes within C57 and DBA strains.

mRNA Expression of Trace Element Homeostasis Proteins Fast RT-PCR

Liver gene expression presented mixed results. For IRP-1 there was a three-way interaction between strain, sex, and diet. There was a significant increase in IRP-1 for C57 female mice ($P<0.001$) and DBA male mice ($p<0.001$) (FIGURE 7). As for HAMP expression, there was loss in gene expression among all mice, but there was a statistically significant decrease in HAMP gene expression for C57 male mice ($p<0.001$). HIF-1α gene expression remained near normal levels; strain was significant in male mice ($p=0.001$). Both C57 and DBA male mice were approaching significance ($p=0.073$ and $p=0.055$, respectively). DMT-1 gene expression was significantly increased in both C57 male and female mice ($p=0.005$ and $p=0.004$ respectively). CTR-1 was significantly and highly expressed in C57 female mice ($p=0.004$). Cp presented mixed results, Both C57 male and DBA female mice significantly increased in Cp gene expression, ($p=0.007$ and $p=0.035$ respectively). There was no change in Cp C57 females and an insignificant loss in DBA males.

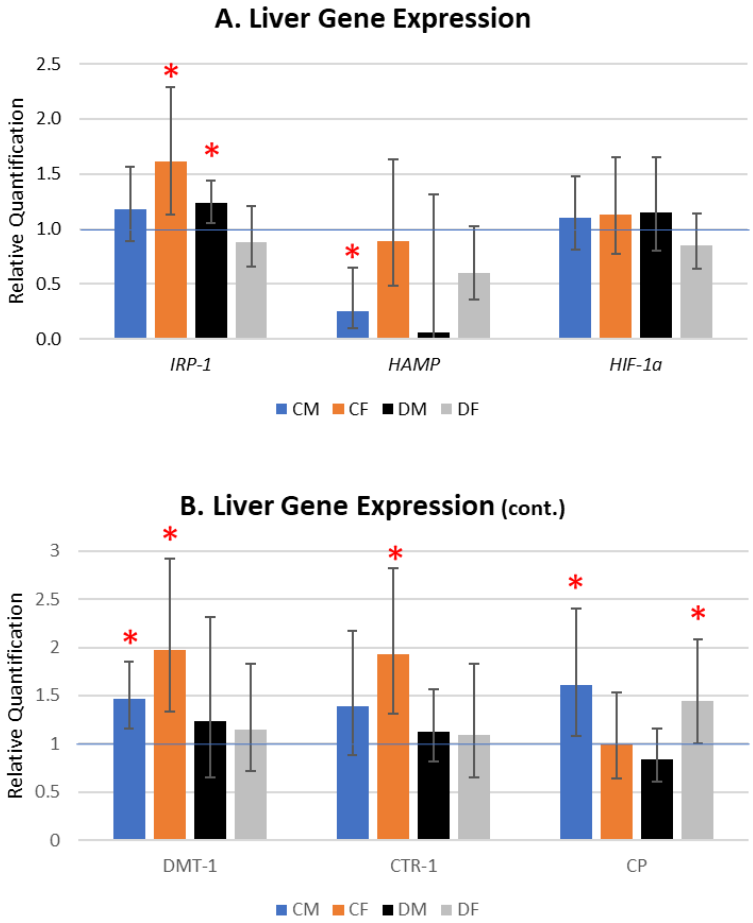

Figure 7: HF diet Liver tissue mRNA gene expression on various genes, represented in fold change, compared to LDF.

Gene expression of HFD mice was determined by RT-PCR and graphs are expressed in Relative Quantification (RQ) values and fold change compared to LF values which are represented via the blue bar at the value of one (1). Genes included: Iron Regulatory Protein-1 (IRP-1), Hepcidin (HAMP), Hypoxia-inducible Factor-1 alpha (HIF-1α), Divalent Metal Transporter-1 (DMT-1), High-affinity Copper-uptake Protein-1 (CTR-1), and Ceruloplasmin (Cp) **A.** HF diet liver gene expression of IRP-1, HAMP, and HIF-1α. IRP-1 had a statistically significant increase in both C57 female and DBA male mice (*P<0.001). HAMP gene expression was significantly decreased for C57 male mice (*p<0.001) due to HF diet. There were no significant changes in HIF-1α gene expression due to HF diet. **B.** HF diet significantly increased DMT-1 gene expression in both C57 male and female mice (*p=0.005 and *p=0.004). CTR-1 was only significantly increased in C57 female mice, (*p=0.004). Lastly, Cp was significantly increased in both C57 Male and DBA female mice (*p=0.007 and *p=0.035) due to HF diet.

Discussion

While the effects of obesity on systemic trace elements have been studied in both animals and humans, this study included both female mice and the DBA/2J strain providing a novel approach. Further, these data add to and corroborate previous studies done in the Erikson lab. (70) The C57BL/J6 has been a traditional mouse model to reflect that of the human metabolic syndrome because it develops obesity, hyperinsulinemia, hyperglycemia and hypertension on high fat ad libitum diets. (71) When fed a low-fat chow diet they remained lean and physically normal paralleling that of normal disease progression of that in human disease progression. It has been noted that DIO in these mice are characterized by deposition of fat in the mesentery and increased feed efficiency, which is relative to that of abdominal obesity in humans. Model characterization and gender are important when using a DIO mouse model. (72,73) C57BL/6J and DBA/2J mice are responsive to a high-fat diet, while other strains such as C57BL/KsJ and Balb/cJ mice are resistant. This study used both C57BL/J6 and DBA/2J strains which are established models of diet-induced obesity. (71,74,75) With DIO rodent models feeding can induce short-term inflammation in adipose tissue, however, this phase is generally termed the early phase in the inflammation response and does not involve immune cells. (34) Gender usually plays an important role; male mice are typically affected by diabetes than female mice and are typically used more often in DIO studies. (72) While the effects of diet-induced obesity have been studied in female DBA/2J mice and hypothalamic infertility, DIO and systemic trace element status in both male and female C57BL/J6 and DBA/2J mice have yet to be elucidated.

We found that both strains C57 and DBA models developed obesity-induced iron deficiency without anemia, which corroborates with both animal and human epidemiological studies. (37,76,77) These models show that obesity is sufficient in affecting systemic trace element status. (37,78) Chronic inflammation associated with obesity is known to generate cytokines, which perpetuate inflammation and increase hepcidin expression and sequestration of iron within the cells. (79) These data present decreased hepatic hepcidin expression in the HF group. Similar results were seen in a study by Chung et al. where both liver iron concentrations and hepcidin gene expression were decreased by 16 weeks of study. (37) These data were similarly corroborated in a recent Erikson lab study. (70) Han et al. described an increase in liver hepcidin gene expression in a HF group at 6 weeks of study but decreased by 20 weeks of study. Another aspect of inflammation is IRP regulation in iron hemodynamics and can be a sign of cellular oxidative stress; at decreased iron levels, IRP-1 binding activity increases which regulates iron absorption and by controlling HIF-1α. (67,80) Hepcidin, a peptide hormone, decreases iron efflux by causing the degradation of ferroportin, the only known iron export protein. (79,81) This inhibition of ferroportin decreases intestinal absorption of iron and causes endogenous iron to be utilized for systemic homeostasis. In collaboration with this study, low systemic iron concentrations were seen in liver, spleen, and adipose tissues of HF diet mice. Decreased iron concentrations in these tissues are suggestive of iron deficiency. With insignificant changes to hematocrit, anemia was not present.

The etiology of obesity is complex and multifactorial, with links between copper status, oxidative stress, and inflammation with mixed and inconsistent results. (82) Some studies show significantly higher copper concentrations in obese individuals while others report no correlation. One suggestion is that increased serum copper, increases ceruloplasmin, which lead to increased inflammation and oxidative stress. Other reports describe decreased hepatic copper concentrations in people who are suffering from liver disease, like NAFLD or NASH. (55,83) Our study here showed marked decreases in copper levels other than an increase in plasma levels in C57 male mice. Other studies are present similar findings; Aigner et al describes that patients with NAFLD had significantly lower copper concentrations in liver biopsy samples. (83) They also reported that both copper concentrations as well as ceruloplasmin levers were also lower. Additionally, this research group reports a copper-deficient diet fed to rats develop hepatic steatosis and insulin resistance. Conversely, Yang et al. describes that serum levels, along with tissue levels, of copper concentrations are elevated in obesity. (84) The majority of copper in serum is bound to cuproproteins such as ceruloplasmin which are higher in obese patients. While copper results were mixed to less desirable in the present study, there were mixed results of increase cp in mice fed a high fat diet (obese). Ceruloplasmin is an abundant plasma protein which oxidize both iron and copper. Ceruloplasmin plays an important roles in iron mobilization such as uptake and efflux, works as an acute phase protein, and has been considered to have antioxidant properties. (68) Similarly to that of iron, ceruloplasmin has been proposed to have redox capabilities with manganese. Jursa and Smith describe the role of aceruplasminemic humans who

genetically are unable to make functional ceruloplasmin. These individuals accumulate iron in the liver and brain and show signs of neurodegeneration. They go on to describe studies involving aceruplasminemic mice models have reported abnormal iron metabolism and manganese neurotoxicity in aged animals. These mice here show mixed significant ceruloplasmin changes as well as altered iron homeostasis and the potential of manganese toxicity in the liver.

In addition to the effects of altered trace element biology due to DIO on liver functioning or inflammation, macrophage biology has also been implicated. During obesity, there is a shift in macrophages in adipose tissue from a M2-like phenotypic state (antioxidant state) to a M1 like phenotypic state (a pro-oxidant state). (85) Traditionally, this polarization occurs when there is an immune response or microbial agents which is associated with increased reactive oxygen species and other pro-inflammatory cytokines are present. M2 and M1 polarized macrophages handle iron homeostasis and handling very differently. M1 state macrophages are iron-sequestering and are secret inflammatory cytokines while M2 is iron efflux and express very low levels of ferroportin. There is also a direct result by both intracellular and extracellular iron availability. (85,86) There is also some evidence that hypoxia has an influence in adipose tissue and increasing HIF-1α in adipose tissues and is controversial in human research. (87) Hypoxic conditions promote saturated fatty acid-induced mRNA expression of IL-6, an inflammatory cytokine. It is suggested that this is not the sole response of hypoxia, but is a factor in IL-6 production. Hypoxia and promotion of IL-6 exacerbates macrophage-mediated inflammation and self-perpetuates this process. This current study was the first from this

lab to look at diet induced obesity and HIF-1α levels in liver tissues. However, these values were not significant, but are valuable in preliminary research in determining what is the root cause of systemic trace element dyshomeostasis. Reversing or preventing hypoxic conditions in adipose tissues could lead to a reduction in tissue inflammation and a change in macrophage polarization. Therefore, it could be necessary to examine these changes in adipocyte macrophages and their effects on inflammation and biochemical markers associated with obesity.

In conclusion, we see that high fat diet induced obesity has systemic effects on liver, spleen, and adipose tissue trace element concentration in a sex-dependent manner on both C57BL/6J and DBA/J2 (male and female) mice. In the liver, there is a decrease in Mn concentration in male mice while HFD causes significant increases in females. There was an overall reduction in systemic Fe concentration in DBA female mice (plasma), C57 male and female mice (liver), and C57 female mice for adipose tissue. Additionally, we see a reduction in systemic Cu concentrations (male DBA mice) which are pathologically similar to that of non-alcoholic fatty liver disease. While both plasma and liver tissue Cu/Zn ratios presented mixed results. We see that male C57 mice are approaching significance in plasma Cu/Zn ratios. This implies there could be a vulnerability to oxidative stress in the HF diet mice, specifically males of this strain. Liver Cu/Zn ratios presented were low, with no effect of diet on the strain. However, sex was a factor. There could be implications that since females have a lower liver Cu/Zn ratio there could be a sex protective effect on oxidative stress, whereas males with diet as a factor could be more vulnerable to a high fat diet induced oxidative stress. We see key

changes in expression of various metal related genes which are also implicated in the etiology of non-alcoholic fatty liver disease, obesity, and other comorbidities such as diabetes and inflammation. This study also provided relevant data regarding systemic zinc concentrations under conditions of obesity as well as genes related to obesity such as HIF-1α. Measurements of decreased iron concentrations and increased ceruloplasmin levels in some mice further implicate iron and manganese related oxidative stress and the potential roles or copper and ceruloplasmin as an antioxidant which may delay or prevent the progression of liver damage and cirrhosis. Additional studies should evaluate Cu/Zn ratios as well as liver histology to determine if in a DIO mouse model there is a correlation between Cu and liver damage.

In this assessment, we see that both of our initial aims were met; high fat DIO has altered trace element concentrations in systemic organs as well as trace element and inflammatory related gene regulation. We hypothesized that high fat DIO causes significant alterations in iron, copper, manganese, and zinc distributions with sex and strain as factors. There were decreases in hepatic manganese concentration in male mice while HFD causes significant increases in females. DBA female mice have reduced plasma iron levels, while both C57 male and female mice had lower hepatic iron concentrations. Female C57 mice adipose tissue had decreased iron concentrations. There was also a reduction in systemic Cu concentrations in male DBA mice. As for gene expression, we hypothesized that high fat DIO causes significant changes in iron, copper, manganese, and inflammatory related genes in a sex and strain dependent manner. Trace element related protein gene expression was mainly increased in iron and copper related

genes in both C57 and DBA, male and female strains. IRP gene expression was elevated in both C57 female and DBA male mice. DMT-1 was increased in C57 male mice while there was nearly a 2-fold increase in C57 female mice in both DMT-1 and CTR-1 gene expression. Cp showed increased gene expression in both C57 male mice and DBA female mice. HIF-1α remained largely unchanged but with data approaching significant reduction. HAMP expression was significantly decreased in C57 male mice. Furthermore, we added novel data to this lab with the additional DIO strain (DBA) as well as valuable data between sexes of these strains. Additional organ analysis and methods of assessment such as through liver histology or specific protein quantification and analysis of these proteins could help to clarify the role or mechanism obesity effects system organs related to both strains and sexes.

CHAPTER IV

EPILOGUE

The Erikson lab has traditionally focused on understanding how altered homeostasis of divalent trace elements including iron, copper, and manganese affect brain health and neurodegeneration such as Parkinson's disease. Current and future research is looking at unpublished preliminary data on expanding research to include the trace element zinc and looking at the system as a whole, not just the liver and brain. Previous published data indicated that chronic high fat diet induced obesity has a regional effect on brain iron biology and markers of neurodegeneration in male C57BL/6J mice. Han et al. reported abnormal midbrain iron concentrations, increased α-synuclein expression, and increased lipid peroxidation, which fit into the pathology of Parkinson's disease. (70) Our study sought to confirm and expand these results by looking at four vital and essential trace elements, additional system organs, and associated genes as well as expanding on strain and gender effects to strengthen generalizability. The primary goal of this study was to: 1) collect critical data on the effects of diet-induced obesity on system trace element biology with a focus on strain and sex dependencies, 1a) acquire preliminary data on trace element zinc biology, and 2) identify alterations in the expression of trace element and inflammatory related gene expression in the liver due to diet-induced obesity.

The model that this study was built on was on the knowledge that obesity causes systemic alterations in trace element status as expanded upon the previous work within this lab, which focused on neurodegenerative disease process, by utilizing an additional mouse strain and both genders. (36,70,71,74–77) The Erikson lab sought to address gaps in the literature by determining what effects diet-induced obesity has on trace element status in system organs outside of the brain, which could elucidate obesity and disease progression of organ damage. We hypothesized, based on previous data, that a high fat diet-induced obesity causes significant systemic alterations on only in Fe, but Cu, Mn, and Zn distributions compared to low fat diet with both sex and strain main effects. To assess the effects of a high fat diet on system trace elements we used well-characterized animal models of obesity (C57BL/6J and DBA/2J), either fed a control low fat diet (10% fat) or high fat diet (60% fat) on either male or female mice. (8,74,75) The intervention of high fat diet was started after arrival at 21d old and lasted for 16w; 44 mice were euthanized after 16wk treatment (mice were 18wk old). At this point in time HFD mice were significantly heavier. We saw diet caused general increases in plasma metals, with significant increases in zinc and significant decreases with manganese concentrations. In the liver there was significant reductions in liver iron in C57 strain (both) and liver copper in DBA (male). Inversely there were significant increases in liver manganese in DBA (females) and liver zinc in C57 mice (both). In the spleen there were significant decreases in both iron for C57 (both) and copper in C57 (female) and DBA (male). Lastly there were decreases in both adipose iron and manganese in C57 (females).

A strength of this study was the use of graphite furnace atomic absorption spectrometry (GFAAS) , which will atomize samples at very high temperatures (up to 3000 K), and then analyze the fundamental atomic parts/elements by interaction with electromagnetic radiation absorption. (88) This measure of detection provides an access to the lowest limit of detection, which is currently available in routine analytical chemistry. (The Erikson lab is greatly appreciative of the UNCG Chemistry department for the use the GFAAS). Graphite furnace compared to flame AAS, allows for higher atomization temperatures and 10-100 better detection limits. On the other hand, this technique is still essentially a single-element analysis method, despite having the capability of adding additional elements to the method protocol, due to dilutions required for specific metals and organs. Additionally, relatively small quantities, both solid and liquid, may be analyzed. The addition of background correction and matrix modification can strengthen element analysis if interferences between elements arise. Additional strengths to this study were the use of both male and female mice which addresses the NIH's call to use both sexes and the use of multiple DIO strains to assess genetic differences. (7,71,74,75)

Some limitations to this study previously mentioned were the limited resources and supplies that the Erikson lab had. Initially the goal was to analyze Zn in each systemic organ analyzed for Fe, Cu, and Mn. Due to the high cost of running the GFAAS, spleen was omitted to from this preliminary data collection. Additionally, gene expression was limited to priority only the liver as the focus of gene expression was between two different studies from the Erikson lab. Attempts were made at gene

expression and RNA isolation on extra adipose tissues, but results failed or yielded unusable amounts of RNA concentrations, with suspicions that a lipid specific RNeasy Rapid Kit would be required to conduct gene expression on adipose tissue. For future research, verification of most appropriate RNA isolation kit should be done prior to the analysis.

In conclusion these findings support previous work from the Erikson lab indicating that obesity alters system trace element biology and advances our understanding of the effect of diet-induced obesity on organ health as it relates to the liver and liver damage. These results also utilize 2 models of DIO (C57 and DBA) which support epidemiological association between obesity and dysregulation of trace elements. Future studies may be conducted to elucidate mechanisms which drive this dysregulation such as macrophage morphology and expression of HIF-1α in obese adipose tissue which would fundamentally contribute to the understating of systemic element homeostasis under chronic inflammation and other disease states such as in liver cirrhosis. Another path of future research could focus on various animal models, such as DIO vs. genetic obese/resistant models to tease out underlying causes of obesity or genes that promote/resist induction of obesogenic genes. Since copper accumulation and depletion is easily identifiable in vivo using existing imaging techniques, serum collection, or even biopsy, the continuation of this work has the potential to develop diagnostic biomarkers or characteristics that could identify prognosis and progression of liver disease in an obese population.

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