Effect of Sex on the Development of Non-Alcoholic Fatty Liver Disease During the Progression of Metabolic Syndrome

Noah C. Wallace

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Effect of Sex on the Development of Non-Alcoholic Fatty Liver Disease During the Progression of Metabolic Syndrome

By: Noah Cy Wallace

A Thesis Defense

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Major: Nutrition

The University of Memphis

May 2024
Abstract

Background: There is an association between sex and the prevalence of Metabolic Syndrome (MetS) and Non-Alcoholic Fatty Liver Disease (NAFLD). Objective: Determine the effects of sex and high fat/sucrose diet on the development of MetS and NAFLD. Methods: 30 male and 30 female C57BL/6 mice were place on one of three diets: High Fat (45% Fat), High Sugar (60% Sucrose), and Control. Results: Male mice on a High Fat diet showed the greatest weight gain amongst all the group as well as demonstrating earlier signs of glucose intolerance. Only those placed on a high sugar diet had indications of liver fibrosis as apparent with Masson’s Trichrome staining. Sex affected protein expression of SREBP-1c and ChREBP. Fasn and Glut4 showed no difference. Conclusion: There are diet and sex differences in the signaling for liver de novo lipogenesis that may contribute to the dimorphic development of NAFLD and MetS.
# Table of Contents

<table>
<thead>
<tr>
<th>Content</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Abbreviations</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Methods</td>
<td>4</td>
</tr>
<tr>
<td>Animals and Experiment Design</td>
<td>4</td>
</tr>
<tr>
<td>Histology</td>
<td>5</td>
</tr>
<tr>
<td>RNA isolation and qPCR</td>
<td>5</td>
</tr>
<tr>
<td>Western Blot</td>
<td>6</td>
</tr>
<tr>
<td>Triglyceride Assay</td>
<td>6</td>
</tr>
<tr>
<td>Insulin Assay</td>
<td>7</td>
</tr>
<tr>
<td>Homeostatic Model Assessment of Insulin Resistance</td>
<td>7</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>7</td>
</tr>
<tr>
<td>Results</td>
<td>8</td>
</tr>
<tr>
<td>Body Weigh and Composition</td>
<td>8</td>
</tr>
<tr>
<td>Development of Insulin Resistance</td>
<td>9</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>9</td>
</tr>
</tbody>
</table>
# List of Abbreviations

<table>
<thead>
<tr>
<th>Definition</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Phosphoinositide-dependent protein kinase 1</td>
<td>PDK1</td>
</tr>
<tr>
<td>Acetyl-CoA Carboxylase</td>
<td>ACC</td>
</tr>
<tr>
<td>Agouti-Related Peptide</td>
<td>AgRP</td>
</tr>
<tr>
<td>AMP-Activated Protein Kinase</td>
<td>AMPK</td>
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<tr>
<td>Carbohydrate Response Element-binding Protein</td>
<td>ChREBP</td>
</tr>
<tr>
<td>Carnitine Palmitoyltransferase 1</td>
<td>CPT1</td>
</tr>
<tr>
<td>Cytokine Signaling 3</td>
<td>SOCS3</td>
</tr>
<tr>
<td>Damage-Associated Molecular Pattern Molecules</td>
<td>DAMPs</td>
</tr>
<tr>
<td>De novo lipogenesis</td>
<td>DNL</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>DAG</td>
</tr>
<tr>
<td>Fibroblast Growth Factor 21</td>
<td>FGF21</td>
</tr>
<tr>
<td>Forkhead Box 01</td>
<td>Fox01</td>
</tr>
<tr>
<td>Glucose-6-Phosphatase</td>
<td>G6Pase</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>GS</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3</td>
<td>GSK3</td>
</tr>
<tr>
<td>High-Density Lipoprotein</td>
<td>HDL</td>
</tr>
<tr>
<td>Insulin Receptor Substrate 1</td>
<td>IRS-1</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
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<td>--------------</td>
</tr>
<tr>
<td>Insulin Receptor Substrate 2</td>
<td>IRS-2</td>
</tr>
<tr>
<td>Interleukin-1β</td>
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<tr>
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<tr>
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</tr>
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<td>IL-6</td>
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<tr>
<td>Interleukin-8</td>
<td>IL-8</td>
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<tr>
<td>Janus Kinase 2/ Signal Transducer and Activator of Transcription</td>
<td>JAK2/STAT3</td>
</tr>
<tr>
<td>Leptin Receptor</td>
<td>LepRs</td>
</tr>
<tr>
<td>Mammalian Target of Rapamycin</td>
<td>mTOR</td>
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<tr>
<td>Metabolic Syndrome</td>
<td>MetS</td>
</tr>
<tr>
<td>Neuropeptide Gamma</td>
<td>NPY</td>
</tr>
<tr>
<td>Non-Alcoholic Fatty Liver</td>
<td>NAFL</td>
</tr>
<tr>
<td>Non-Alcoholic Fatty Liver Disease</td>
<td>NAFLD</td>
</tr>
<tr>
<td>Non-Alcoholic Steatohepatitis</td>
<td>NASH</td>
</tr>
<tr>
<td>P 38 Mitogen-activated protein Kinases</td>
<td>MAPK</td>
</tr>
<tr>
<td>Peroxisome Proliferator-activated receptor Alpha</td>
<td>PPARα</td>
</tr>
<tr>
<td>Phosphatidylinositol 3 Kinase</td>
<td>PI3k</td>
</tr>
<tr>
<td>Phosphodiesterase 3B</td>
<td>PDE3B</td>
</tr>
<tr>
<td>Phosphoenolpyruvate Carboxyl kinase</td>
<td>PEPCK</td>
</tr>
<tr>
<td>Platelet-Derived Growth Factor</td>
<td>PDGF</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>Proliferator-Activated Receptor</td>
<td>PPAR</td>
</tr>
<tr>
<td>Pro-Opiomelanocortin/Cocaine-amphetamine-regulated Transcript</td>
<td>POMC/CART</td>
</tr>
<tr>
<td>Protein Kinase B</td>
<td>AKT</td>
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<td>Protein Kinase c-ε</td>
<td>PKCε</td>
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<tr>
<td>Pyruvate Carboxylase</td>
<td>PC</td>
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<tr>
<td>RAC-beta Serine/Threonine-Protein Kinases</td>
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</tr>
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<td>Transforming Growth Factor Beta</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Tumor Necrosis Factor Alpha</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Very Low-Density Lipoprotein</td>
<td>VLDL</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Body weights over 12 weeks</td>
<td>22</td>
</tr>
<tr>
<td>2. MRI fat mass over 12 weeks</td>
<td>22</td>
</tr>
<tr>
<td>3. MRI lean mass over 12 weeks</td>
<td>23</td>
</tr>
<tr>
<td>4. Glucose tolerance test, area under the curve for glucose tolerance test, fasting insulin, fasting glucose, and Homeostatic Model Assessment of Insulin Resistance</td>
<td>24</td>
</tr>
<tr>
<td>5. Plasma triglycerides</td>
<td>25</td>
</tr>
<tr>
<td>6. Epidydimal fat pad weights, Mesenteric fat pad weights, and representative H&amp;E staining of adipocytes</td>
<td>26</td>
</tr>
<tr>
<td>7. Liver weights, representative H&amp;E staining of liver, Masson’s Trichrome staining of liver</td>
<td>27</td>
</tr>
<tr>
<td>8. Quantification of Western Blot analysis, Western blot images</td>
<td>30</td>
</tr>
<tr>
<td>9. Quantification of Fasn and Glut4 gene expression</td>
<td>31</td>
</tr>
</tbody>
</table>
Introduction

In 1988, endocrinologist Gerald M. Reaven created the term “Metabolic Syndrome” (MetS) in response to noticing a trend with insulin resistance and other metabolic syndromes (Reaven, 1988). This term would be used to describe a cluster of metabolic abnormalities that increase the risk for cardiovascular disease as well as diabetes mellitus rather than a disease itself (Fahed, et al., 2021). The most current criteria for MetS are stated as have three or more of the following: Insulin resistance, Hypertension, Dyslipidemia, and Obesity with a focus on central adiposity (Alberti, et al.). While we do know that factors such as genetics and lifestyle contribute to MetS, we still lack a solid understanding of the mechanisms that cause this phenomenon (Fahed, et al., 2021). Those exhibiting a “pear shape” body will have less central adiposity and more subcutaneous fat than those classified as having an “apple shape” body (Fahed, et al., 2021). Rates for obesity have doubled since 1980 in most countries with trends being higher in adolescence (Saklayen, 2018). Increases in adiposity as well as a surplus in caloric intake results in elevated free fatty acids as well as a reduction in glucose uptake due to the development of insulin resistance (Fahed, et al., 2021). Insulin resistance further drives the viscous cycle by interfering with regulators of both lipolysis and de novo lipogenesis causing an increase in adiposity (Li, Chi, Setrerrahmane, Xie, & Xu, 2022). Increased hepatic uptake of free fatty acids results in reduced storage of glycogen and increases in gluconeogenesis which can lead to steatosis of the liver (Carr, Oranu, & Khungar, 2016).

Non-alcoholic Fatty Liver Disease (NAFLD) is currently the most common chronic liver disease that exposes individuals to future hepatic complications (Flisiak-Jackiewicz, Bobrus-Chociej, Wasilewska, & Lebensztejn, 2021). NAFLD has been described as the hepatic
manifestation of MetS and can also be seen to mirror the increased rates with obesity and type 2 diabetes mellitus (Wong, Ekstedt, Wong, G. L. H., & Hagström, 2023). This has led it to become the one of the leading causes for chronic liver disease globally (Gadiparthi, et al., 2020).

Diagnosis of NAFLD requires the presence of >5% lipid accumulation in the liver in the absence of other causes such as alcohol, viruses, or autoimmune disease (Byrne & Targher, 2015). The advanced form of NAFLD, Non-Alcoholic Steatohepatitis or NASH, requires a liver biopsy for the histological presence of steatosis, hepatocellular ballooning, and lobular inflammation (Friedman, Neuschwander-Tetri, B. A., & Sanyal, 2018). NASH can further progress to more detrimental conditions such as cirrhosis, liver failure, hepatocellular carcinoma as well as increased risk for cardiovascular disease and malignancy (Friedman, Neuschwander-Tetri, B. A., & Sanyal, 2018). Interestingly, the prevalence of NAFLD is significantly higher in the male population than in the female population (Balakrishnan, et al., 2021). De novo lipogenesis (DNL) has been reported to account for 15% to 26% of intrahepatic triglyceride formation in the liver of NAFLD patients (Smith, et al., 2020). Increases in circulating insulin and glucose appears to play a role in the stimulation of DNL in the liver due to their ability to activate Sterol regulatory element-binding protein 1c (SREBP-1c) and Carbohydrate response element-binding protein (ChREBP) respectively (Smith, et al., 2020). These proteins transcriptionally activate genes such as fatty acid synthase (FASN) and others that are involved in DNL (Smith, et al., 2020).

While women have higher rates for both obesity and impaired glucose tolerance, men have been shown to have higher instances of MetS as well as NAFLD (Rochlani, Pothineni, & Mehta, 2015). Studies have shown the prevalence for both MetS and NAFLD are the lowest during their fertile years with increased prevalence postmenopausal demonstrating a role for sex
hormones (Ballestri, et al., 2017). While estrogen does appear to play an integral part in both protecting or promoting both conditions, sex hormones are not fully the culprit for the sex discrepancies due to hormone replacement therapies showing a lack of benefit in lowering MetS as well as cardiovascular disease (Rochlani, Pothineni, & Mehta, 2015). With increasing strain on the global health system due to MetS and NAFLD as well as an increase in a western diet and a sedentary lifestyle, it is imperative to try to elucidate the mechanisms behind this dimorphic difference to create more targeted and individualized treatment strategies. In order to attempt to fill these gaps, investigated this sexually dimorphic progression in Metabolic Syndrome and Non-alcoholic Fatty Liver Disease in response to different diet treatments and time points. With this, our overall hypothesis is that female mice will not only exhibit less severe diet-induced obesity and insulin resistance but will also show better liver metabolic flexibility to their respected diet when compared to male mice on the same diets.
Methods

Animals and Experiment Design:

All experimental and housing protocols were approved by the Institutional Animal Care and Use Committee of the University of Memphis protocol number 0877. C57BL/6 male (30) and female (30) mice, 7 weeks of age, were purchased through Envigo. All animals were housed in a 12:12 hour light-dark cycle and were given ad libitum access to food and water during the study. After a week of acclimation, baseline measurements of bodyweight were taken as well as a glucose tolerance test (GTT) after being fasted overnight as well as MRI. Mice were then randomized to either a control, high fat, or high sugar diet (Table 1). These groups were further divided into either being part of a six-week diet plan or 12-week diet plan in order to view progressions of metabolic differences in each group. Bodyweight, food consumption, and mouse health was closely monitored during the duration of the experiment. Bodyweight was measured weekly with food consumption being recorded every three days. MRI scans were performed at weeks 0, 2, 4, 6, 8, 10, and 12 in order to assess body composition (ECO MRI-100, Houston, TX). GTT were performed at weeks 0, 6, 9, and 11 after being fasted overnight. Blood glucose was measured from collecting blood from the tails of the mice at time points of 0, 15, 30, 60, and 90 minutes after an intraperitoneal injection of a 20% glucose solution (2g/kg) using a Bayer Contour glucometer. Mice were sacrificed and tissues harvested at either the 6-week or 12-week time point. Mice were fasted overnight for both conditions prior to being euthanized by CO₂ and secondarily by cervical dislocation.
Table 1. Composition of High Fat, High Sugar, and Control Diets

<table>
<thead>
<tr>
<th></th>
<th>Carbohydrate</th>
<th>Protein</th>
<th>Fat</th>
<th>Calories</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Fat</td>
<td>35%</td>
<td>20%</td>
<td>45%</td>
<td>4.7 kcal/g</td>
</tr>
<tr>
<td>High Sugar</td>
<td>60% (Sucrose)</td>
<td>20%</td>
<td>20%</td>
<td>4.1 kcal/g</td>
</tr>
<tr>
<td>Control</td>
<td>60%</td>
<td>20%</td>
<td>20%</td>
<td>4.1 kcal/g</td>
</tr>
</tbody>
</table>

Histology:

Liver and epididymal white adipose tissue were fixed in a 10% neutral buffered formalin solution. The samples were then dehydrated with graded ethanol solutions, cleared with histoclear, and embedded in paraffin. Five μm sections were stained with hematoxylin and eosin (H&E). Liver was also stained using Trichrome stain (Masson) (Sigma, St. Louis, MO) in order to assess liver fibrosis. An Imager M7000 microscope (Invitrogen, EVOS, M7000 Imaging system, Waltham, MA) was used for histological analysis. Representative images for both liver and epididymal white adipose tissue were taken at 20x magnification.

RNA isolation and qPCR:

Liver samples were homogenized in 1 mL of Trizol (TRIzol Reagent, life Technologies, Carlsbad, CA) and RNA isolated using the Clean and Concentrator Kit-5 (Zymo Research, Irvine, CA) RNA was quantified using a nanodrop (ThermoFisher Scientific, Waltham, MA). Following quantification, 1 μg of RNA was reverse transcribed to cDNA (Applied Biosytem High Capacity RNAs to cDNAs kit, ThermoFisher Scientific, Waltham, MA). Forward and reverse primers (Table 2) for intended genes and SYBR Green qPCR master mix (PowerUp
SYBR Green Master Mix, ThermoFisher Scientific, Waltham, MA)) was added with the cDNA. Gene expression was measured using a QuantStudioTM 6 flex system Real-Time PCR software (ThermoFisher Scientific, Waltham, MA). Analysis was carried out using the 2-\(\Delta\Delta CT\) method.

Table 2. Gene primers for qPCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasn</td>
<td>GAT GAC AGG AGA TGG AAG GC</td>
<td>GAG TGA GGC TGG GTT GAT AC</td>
</tr>
<tr>
<td>Glut4</td>
<td>GTA ACT TCA TTG TCG GCA TGG</td>
<td>TGC TCT AAA AGG GAA GGT GTC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CCC TTA AGA GGG ATG CTG CC</td>
<td>TAC GGC CAA ATC CGT TCA CA</td>
</tr>
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</table>

Western Blot:

Evaluation of protein expression levels in liver samples was performed through western blot analysis. Liver samplers were homogenized in a 10x Mueller buffer with protein concentrations being measured using the Bradford Assay using the BioRad Protein Assay kit (Bio-Rad Laboratories, Inc, Hercules, California, USA). Twenty µg of homogenates were loaded on 10% SDS-polyacrylamide gels, separated, and transferred to a polyvinylidene difluoride membrane using a Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc, Hercules, California, USA). Ponceau staining was used to confirm gel transfer as well as equal loading.

Triglyceride Assay:

Plasma samples underwent a triglyceride assay to assess variations in triglyceride concentrations. The Free Glycerol Reagent and Triglyceride Reagent (Sigma, St. Louis, MO) were prepared as per the manufacturer's guidelines. Initial absorbance readings for blank,
standard, and samples were taken at 540 nm using a spectrophotometer (BioTek, Synergy2, Winooski, VT). Each sample was then mixed with Triglyceride Reagent and incubated at room temperature for 5 minutes. Subsequent absorbance readings for blank, standard, and samples were measured at 540 nm. Triglyceride concentrations were computed following the manufacturer's instructions.

Insulin Assay:

Fasting plasma insulin was measured using a commercial insulin ELISA assay (Crystal Chem, Elk Grove Village, Illinois). The wide range assay was conducted according to the manufacturer’s instructions using 5ul of plasma sample.

Homeostatic Model Assessment of Insulin Resistance:

HOMA-IR was calculated using the following equation:

\[(\text{Fasting Glucose mg/dl}) \times (\text{Fasting Insulin } \mu \text{U/ml}) / 405\] (Mathews, et al., 1985).

Statistical Analysis:

All data are represented as means ± SE. A three-way ANOVA was used to determine the effects of diet, sex, and time using GraphPad Prism 8. Repeated measures ANOVA was used to analyze changes over time. Two-way ANOVA was used to analyze the effects of sex and diet within the 6 week and 12-week time points for protein analysis. Tukey post hoc analysis was used to examine interactions. Significance was set at \(p \leq 0.05\).
Results

Body Weight:

Analysis of body weight showed there was a significant effect for both time (p<0.000001) and group (p<0.000001) (Figure 1). The effect of sex was seen at baseline when comparing the same diet with the opposite sex (p<0.000001). Males on a high fat diet began to be significantly heavier than all other groups around the two-week mark from baseline (p≤0.0081). Males on a high fat diet weighed significantly more than their female high fat counterparts at this time point (p<0.000001). This increased weight for the male high fat group continued for the remainder of the study (p≤0.01). Females on a high sugar diet showed a trend of losing weight beginning around the 9-week time point; however, there was no significant difference between the Female High Sugar and High Fat (p=0.5987) or between the Female High Sugar and Control (p=0.9999). There was a trend at this time point for lower body mass between female high sugar and male high sugar groups (p=0.0567). Analysis of lean mass data showed there was significance with regards to both time (p<0.000001) and group (p<0.000001) (Figure 3). Starting at 8 weeks Male High Fat had significantly more lean mass than the Male High Sugar group (p=0.0126), and this continued through the end of the study. Females in all groups were not significantly different throughout the study (p≥0.8823).

Prior to being placed on their respective diets, females had just over two times as much fat mass as males. By week two, Male High Fat were significantly higher than Male Control and Male High Sugar groups (p=0.0010 and p=0.0096, respectively) (Figure 2). There is a significant increase in fat mass for the Male High Fat group at week 4 that continues to greatly increase until
the end of the 12-week study (Figure 2). The Male High Fat group also continues to be significantly higher when compared to all Female groups from week 4 until week 12. While female fat mass did increase throughout the 12-week study, there were no significant differences in the female population for either diet or time (p≥0.9999).

Insulin Resistance:

Glucose tolerance was measured by glucose tolerance test after an overnight fast. There was a main effect of time (p<0.000001) and diet (p<0.000001) (Figure 4a-d). Males on a high fat diet were significantly higher than males on a high sugar diet at the 6- and 9-week time point (p<0.000001 for both) (Figure 4b and c). At week 11 males on a high fat diet had significantly larger AUC than both high sugar and control males (p<0.000001 for both) (Figure 4e). Females did not show any glucose intolerance until much later at week 11 with female high group being significantly greater than both control and high sugar females (p<0.000001 for both) (Figure 4e).

Fasting insulin was measured at 6 and 12 weeks. There was no difference in fasting insulin among groups at 6 weeks (Figure 4f). At 12 weeks males on the High Fat diet had significantly elevated fasting insulin compared to all other groups (p< 0.0001) (Figure 4f). To further assess insulin resistance Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was used calculated. There was a significant difference between the 12-week High Fat Diet male group and all others (p>0.000001) with the 12-week High Fat males having higher HOMA-IR values (Figure 4h). Male 6-week High Fat had significantly higher HOMA-IR values when compared to Male 6-week High Sugar (p=0.023942) (Figure 4h). Females did not show any significant changes in HOMA-IR values with regard to diet or time.

Plasma Triglyceride:
Interestingly, there was no significant differences in plasma triglyceride levels between sexes, time points, or diets. Though not significant, triglyceride levels decreased for all 12-week male diets when compared to male 6-week diets (p≥0.1676) (Figure 5). Females on the 12-week High Fat and High Sugar showed decreased levels when compared to their 6-week counterpart.

Adipose Tissue and Histology:

Epidydimal and Mesenteric fat pads were weighed upon euthanasia. Male 12-week and 6-week High fat diets weights were significantly elevated compared to all other groups (p≥0.0015) (Figure 6a and b). Histological analysis showed there were differences adipocyte size for males when compared to females regardless of both time and diet (Figure 6c). For both sexes, a high fat diet resulted in discrepancies in adipocyte size when compared to the control diet. Adipocyte size did not appear to become larger when comparing the 6 and 12-week high sugar female diets. For males, there did appear to be an increase in adipocyte size when comparing 6 and 12-week high sugar images (Figure 6c).

Metabolic Liver Signaling:

Analysis of liver weights showed 12-week High Fat Males had significantly high liver weights when compared to all other groups (p>0.000001) (Figure 7a). 6-week High Fat Males had significantly larger liver mass than all female groups (p≥0.0169) (Figure 7a). Hematoxylin and Eosin staining showed greater steatosis in both Male High Fat and High Sugar 12-weeks (Figure 7b). While all Female groups showed greater lipid accumulation for all three diets at the 6-week time point when compared to 6-week control males, liver steatosis did not appear to progress any further. Histological analysis using Masson Trichrome stain indicated the presence
of fibrosis for Males on a 12-week High Sugar diet and Females on the 6-week High Sugar diet (Figure 7c). Fibrosis was not seen in the other groups.

AMP-activated protein kinase (AMPK) is a protein kinase acts as a regulator for cellular and whole-body energy homeostasis through coordination of multiple metabolic pathways. Total AMPK (tAMPK) and phosphorylated AMPK (pAMPK) was measured in the liver at 6 and 12 weeks. There was a main effect of Sex for tAMPK at the 6-week time point (p=0.0069) and the 12-week time point (0.0019) (Figure 8a and b). Similarly, there was a main effect of sex at 12-week (P≥0.2277) for pAMPK (p=0.0015) (Figure 8c and d).

The ratio for pAMPK to tAMPK showed there was no significance with either diet (p=0.0819) or sex (p=0.2881) for the 6-week group (Figure 8e). Likewise, pAMPK to tAMPK ratio for the 12-week groups showed no significance for diet (p=0.9174) or sex (p=0.4996) (Figure 8f).

Sterol Regulatory Element-Binding Protein 1c (SREBP-1c) and Carbohydrate response element binding protein (ChREBP) are both proteins that regulate de novo lipogenesis in the liver. A main effect of sex was significant for SREBP-1c for both 6-week (P=0.00001) and 12-week (p=0.0013) (Figure 8g and h). Multiple comparison showed that Male control 6-week mice had significantly higher protein levels for SREBP-1c when compared to all female groups (p≤0.0009) (Figure 8g). Interestingly, at 12-weeks male control group was not significantly higher when compared to all female groups (p≥0.09) (Figure 8h).

ChREBP also had a significant main effect of sex at 6-week (p=0.0006) and 12-week (p=0.00001) with males expressing higher levels of ChREBP than females (Figure 8i and j). Multiple comparisons indicated again that the male control 6-week group had significantly
higher levels of protein when compared to all female groups (p≤0.0465) (Figure 8i). Unlike SREBP-1c, the male control group was significantly higher than all females at the 12-week point (p≤0.005) (Figure 8i and j). 12-week male high fat group also showed significantly higher levels of protein when compared to all female groups (p≤0.006) (Figure 8i).

Fatty Acid Synthase (FASN) and Glucose Transporter 1 (GLUT4) have been shown to be involved in NAFLD development by increasing de novo lipogenesis and glucose impairment, respectively. Fasn gene expression showed no significance effects of sex (p=0.9068) or group (p=0.3476) (Figure 9a). Time also appeared to not have a significant effect on Fasn gene expression (p>0.9999). Glut4 expression showed similar results with there being no significance for the effects of sex (p=0.2808) or group (p=0.2895) (Figure 9b). Again, time did not have a significant effect on Glut4 expression (P>0.9999) (Figure 9b).

Discussion:

Rates for both obesity and diabetes are currently on the rise globally. No longer are these ailments a problem for more affluent countries with prevalence being shown to be drastically increasing for developing countries. The likely cause for this increase is due to a sedentary lifestyle and the cheap and accessible westernized food. The westernized diet is high in both sugar and fat which has been shown to induce not only obesity and MetS, but also NAFLD (Pompili, et al., 2020).

High fat diet feeding is widely associated with increases in body mass and fat mass (Lin, Thomas, Storlien, & Huang, 2000) (Yang, Smith Jr, Keating, Allison, & Nagy, 2014). Similar to our study, significant weight gain was evident for mice on a high fat diet after only two weeks of feeding with the trend continuing for the remainder of the study (Lin, Thomas, Storlien, &
Huang, 2000). Similar to our study, male mice on a high fat diet showed significant weight gain earlier than their female counterparts (Yang, Smith Jr, Keating, Allison, & Nagy, 2014). Studies looking at carbohydrate induced obesity found diets with sucrose levels similar to our study (~60%) resulted in significant weight gain in male mice (Fortino, 2007). Maho, Masahiro, and Yoshiyuki showed that mice fed a high fat diet culminated the most weight gain when compared to a high sucrose diet. Our current study found similar results with the high fat males gaining the most weight in the study as well as being significantly higher than all females (Figure 1).

Interestingly, female high sugar diet decreased weight from week 9 until the end of the study (Figure 1). This too was observed in a study comparing both high fat and high carbohydrate diets (Pompili, et al., 2020). This could be due to degeneration of liver health as seen in other studies (Tessitore, et al., 2017).

Studies have shown that a high fat diet had the effect of increasing fat mass when compared to a control diet in male mice as well as female (Gallou-Kabani, et al., 2007) (Nilsson, et al., 2016). Our study showed similar results with both male and female mice showing the greatest increase in fat mass gained over the course of the study (Figure 2 and 6). The increase in fat mass was associated with larger adipocytes as have previously been demonstrated (Oliveira, et al., 2013). Nilsson et al. also found that with a high fat diet, female mice experienced a loss in lean mass while Yang, Smith Jr, Keating, Allison, & Nagy saw fat free mass increase with a high fat diet. Our data mimics the later with both males and females accruing lean mass on the high fat diet (Figure 3).

Despite the accumulation of lean mass on the high fat diet, males showed insulin resistance after only two weeks on the high fat diet. Others have shown insulin resistance after only one week of high fat feeding in male mice (Mosser, et al., 2015). The same was found for
female mice after three weeks of consuming a high fat diet with nearly no glucose elimination seen between 15 and 60 minutes (Winzell & Ahrén, 2004). Our results are consistent with studies that have compared high fat and high sugar in male mice and found that high fat diets resulted in a greater decrease in glucose clearance when compared to a high sugar diet (Sumiyoshi, Sakanaka, & Kimura, 2006) (Figure 4a-d). Further, comparison of fasting glucose and insulin has been shown to be elevated in a high fat diet and slightly decreased in a high sugar diet in male mice (Sumiyoshi, Sakanaka, & Kimura, 2006). While we did see a significant increase in glucose and insulin in our male high fat group, we did not see any changes in our male high sugar group (Figure 4f-g). Males on a high fat diet have been shown to have higher levels of glucose and insulin when compared to diet matched females (Hwang, et al., 2010). Our results also mimic this sex difference (Figure 4f-g). Our study found that there was no significant difference found for plasma triglyceride levels for both sexes and all three diets (Figure 5). This is consistent with previous findings when comparing a high fat and control diet between males and females (Hwang, et al., 2010).

Studies comparing the effects of a high fat diet to a high sugar diet on liver steatosis showed that chronic consumption of a high sugar diet was just as detrimental as a high fat diet in male mice (Pompili, et al., 2020). In figure 7b, we show these same results when comparing our high fat and high sugar males at both the 6 and 12-week time points. Multiple studies have suggested that C57BL/6 female mice are more susceptible to NAFLD with histological analysis showing greater steatosis in female mice consuming a high sugar diet when compared to male mice on the same diet (Spruss, et al., 2012); (Choi, Abdelmegeed, & Song, 2017). Interestingly, our female mice on a high sugar diet did not exhibit progression of steatosis between the 6 and
12-week time points; however, when comparing 6-week male and female groups, females showed earlier signs of steatosis (Figure 7b).

While a high fat diet showed greater steatosis, only those on a high sugar diet showed signs of fibrosis in the liver for both male and females (Figure 7c). Ishimoto et al. was able to show this in male mice when comparing a high fat diet to a diet with the same fat content but with added sucrose. This suggesting sugar may be of greater importance in the development of NASH than dietary fat.

SREBP-1c and ChREBP are two crucial regulators of lipogenic genes in DNL in the liver. Insulin and glucose stimulation activate these regulators, respectively (Zhu, et al., 2021). Translocation of both of these proteins results in transcription of Fasn (Zhu, et al., 2021). This ultimately results in accumulation of triglycerides in the hepatocytes and progresses into NAFLD (Zhu, et al., 2021). AMPK has been shown to inhibit both SREBP1-c and ChREBP while in a fasted state resulting in shut down of the lipogenic pathways in healthy livers (Marcondes-de-Castro, Reis-Barbosa, Marinho, Aguila, & Mandarim-de-Lacerda, 2023). In a diseased state, the AMPK pathway is depressed due to inflammation, steatosis, lipogenesis, and DNL which can ultimately lead to increase in pro-inflammatory cytokines, AMPK pathway dysregulation, and possibly insulin resistance (Marcondes-de-Castro, Reis-Barbosa, Marinho, Aguila, & Mandarim-de-Lacerda, 2023).

Studies investigating sex differences in pAMPK and tAMPK in mice on a high fat diet were able to find a significant effect of sex in terms of individual protein expression and pAMPK to tAMPK ratio in renal tissue (Juszczak, et al., Sex differences in obesity-induced renal lipid accumulation revealed by lipidomics: a role of adiponectin/AMPK axis, 2023). Our study showed similar results with regards to pAMPK, tAMPK, and pAMPK/tAMPK protein
expression in the liver of male and female mice not only on a high fat diet but also a high sugar diet showing there does lie an effect of sex but not diet (Figure 8a-f).

Protein expression of SREBP-1c and ChREBP has been shown to be significantly elevated in male and female mice feeding on a high fat diet when compared to a control diet (Zhu, et al., 2021). Previous research also was able to find sex differences in SREBP-1c with the liver in female mice showing higher protein expression (Améen, et al., 2004). Intriguingly, we did not find these same results. Quantification of protein expression showed that control males had significantly higher protein levels when compared to females at 6-week which was not maintained at the 12-week mark (Figure 8g-h). One possible reason for these results may lie in IRS 1 and 2. SREBP-1c expression has been associated with increased expression for IRS-1 with no association to IRS-2 (Kohjima, et al., 2008). Diabetic mice have shown increased degradation for IRS-1 resulting in impaired GLUT4 mobilization and reduced glucose uptake in adipose tissue with others finding the same but with IRS-2 becoming the main receptor (Wang, Nishina, & Naggert, 2009); (Rondinone, et al., 1997). Male and female mice have been shown to have differences in IRS-1 and IRS-2 in aortic tissue (Akther, et al., 2021). Female mice may primarily be utilizing IRS-2 resulting in the lower expression of SREBP-1c when compared to male mice which could explain this sex difference. The differences in diet may have to do with male mice on the high fat and high sugar diets exhibiting impaired glucose earlier than others and quicker degradation of IRS-1 and thus lower SREBP-1c. This too could explain why we see ChREBP being higher in males than females. The insulin signaling cascade for IRS-2 in hepatocytes ultimately culminates with upregulation of gluconeogenic genes (Valverde, et al., 2003). Through these gluconeogenic genes as well as incoming glucose, ChREBP protein can be upregulated resulting in increases in lipogenic genes. Though Fasn was found to not be significant, other
lipogenic genes in DNL more than likely were and will need to be measured. A lack of significance for Glut4 is not surprising due to the liver generally lacking Glut4. One study had demonstrated that Glut4 was involved in the infiltration and proliferation of hepatocellular carcinoma (Huang, et al., 2022). Future plans will involve looking into Glut2 expression instead.

**Conclusion**

Studies have shown that a westernized diet consisting of high fat and/or sugar results in not only obesity and MetS, but also NAFLD. Epidemiological studies have also pointed out there lies sex differences in the prevalence for NAFLD with DNL being the primary pathway. The exact mechanism for these differences and inter workings of DNL are not fully understood. Our study was able to show that high fat diets were able to significantly increase body weight when compared to all others with males gaining the most weight as well as earlier development for glucose intolerance. Our study further found there to be sex differences in the DNL protein regulators SREBP-1c and ChREBP with no differences in downstream Fasn and Glut4.

**Limitations and Future Directions**

Future directions involve looking into gene expression for liver fibrosis such as Thbs2, Col4a2, Itgav, Lamb1, or Pdgfra. Due to most studies using fructose for their NAFLD model, validation of our results through future studies utilizing sucrose is important. This too highlights a limitation in our study due to our choice of sugar.
References


Choi, Y., Abdelmegeed, M. A., & Song, B. J. (2017). Diet high in fructose promotes liver steatosis and hepatocyte apoptosis in C57BL/6J female mice: Role of disturbed lipid homeostasis and increased oxidative stress. Food and Chemical Toxicology, 103, 111-121.


Figure 1. Body weight of mice over time from baseline to the end of their 12-week diet intervention. Data is represented as mean± SEM. Significance was set at p<0.05.

Figure 2. Fat mass over the course of 12 weeks in all mice groups collected via EchoMRI. Data is represented as mean± SEM. Significance was set at p<0.05.
Figure 3. Lean mass over the course of 12 weeks in all mice groups collected via EchoMRI. Data is represented as mean± SEM. Significance was set at p<0.05.
Figure 4. (a) Baseline measurements for GTT. (b) 6-week GTT test results (c) 9-week GTT results (d) 11-Week GTT results (e) Area Under the Curve for all male and female groups
throughout the study (f) Fasting insulin (g) Fasting glucose (h) HOMA-IR results. Data is represented as mean± SEM. Significance was set at p<0.05.

Figure 5. Plasma triglyceride concentrations. Data is represented as mean± SEM. Significance was set at p<0.05.
Figure 6. (a) Gonadal fat pad weights (b) Mesenteric fat pad weights (c) Representative H&E staining of adipocytes. Data is represented as mean± SEM. Significance was set at p<0.05.
Liver Weight

a. p<0.05 vs all other comparison
b. p<0.05 vs Male 6wk High Fat

b. 6WK(M) 12WK(M) 6WK(F) 12WK(F)

CON

HF

HS
Figure 7. (a) Liver weights taken upon euthanasia for all groups (b) Representative H&E staining of liver (c) Masson’s Trichrome staining of liver: Collagen is stained blue, nuclei are stained dark brown, and cytoplasm is stained red. Data is represented as mean± SEM. Significance was set at p<0.05
c. pAMPK 6wk

- Male Female
- pAMPK (Fold Change)
- Control
- HF
- HS

d. pAMPK 12wk

- Male Female
- pAMPK (Fold Change)
- Control
- HF
- HS

e. pAMPK/tAMPK 6wk

- Male Female
- pAMPK/tAMPK (Fold Change)
- Control
- HF
- HS

f. pAMPK/tAMPK 12wk

- Male Female
- pAMPK/tAMPK (Fold Change)
- Control
- HF
- HS

g. SREBP-1c 6wk

- Male Female
- SREBP-1c (Fold Change)
- Control
- HF
- HS

h. SREBP-1c 12wk

- Male Female
- SREBP-1c (Fold Change)
- Control
- HF
- HS

i. ChREBP 6wk

- Male Female
- ChREBP (Fold Change)
- Control
- HF
- HS

j. ChREBP 12wk

- Male Female
- ChREBP (Fold Change)
- Control
- HF
- HS
Table 3. Relative quantification of Western Blot analysis for the following proteins: (a) tAMPK 6 weeks (b) tAMPK 12 weeks (c) pAMPK 6 weeks (d) pAMPK 12 weeks (e) pAMPK/tAMPK 6 weeks (f) pAMPK/tAMPK 12 weeks (g) SREBP-1c 6 weeks (h) SREBP-1c 12 weeks (i) ChREBP 6 weeks (j) ChREBP 12 weeks (k) 6-week western blot images (l) 12-week western blot images.

Con: Control, HF: High Fat, HS: High Sugar. Data is represented as mean± SEM. Significance was set at p<0.05.
Figure 9. Quantification for qPCR (a) FASN (b) GLUT4. Data is represented as mean± SEM.

Significance was set at p<0.05.
Appendix 1:

Extended Literature Review

Metabolic Syndrome Intro:

Metabolic Syndrome (MetS) was first postulated in 1988 by endocrinologist Gerald M. Reaven (Reaven, 1988). Reaven proposed that insulin resistance is not only involved in the development of Type 2 diabetes mellitus, but as well as the pathogenesis of hypertension and cardiovascular disease (Reaven, 1988). Reaven also noticed that in conjunction with insulin resistance, various metabolic abnormalities are also usually present (Reaven, 1988). Initially, Reaven created the umbrella term “ Syndrome X” for this anomaly but later added “metabolic” in order to distinguish it from the already existing syndrome x in cardiology (Kemp Jr., 1973). It is important to note that MetS is not a disease per se. Rather, it is a term used to encapsulate those metabolic abnormalities that are associated with increased risk of cardiovascular disease as well as diabetes mellitus (Fahed et al., 2022). The metabolic abnormalities included in MetS include waist circumference, glucose levels, triglyceride levels, high-density lipoprotein levels, cholesterol levels, and blood pressure (Fahed et al., 2022). The most recent parameters used for each abnormality comes from a joint statement given by the National Heart, Lung, and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity (Alberti et al., 2009).

Due to blood insulin requiring too many resources and time to measure larger scale screenings, waist circumference is used instead due to the correlation between waist circumference and insulin resistance (Fahed et al., 2022). This inclusion of waist circumference is also important since body mass index alone is inadequate due to individuals who may be
classified as “metabolically healthy obesity” (Ross et al., 2020). Waist circumference complements body mass index in helping show the distribution of adiposity in individuals (Ross et al., 2020). Those who exhibit a “pear shape” have less abdominal or central adiposity and more subcutaneous fat than those who are classified as having an “apple shape” body (Fahed et al., 2022). It is also important to note that for a given body mass index, waist circumference is increasing suggesting a change in obesity to that of increasing abdominal adiposity (Ross et al., 2020). This trend can be seen in various countries across the world since the 1980s (Albrecht et al., 2015). Not only does this trend in increasing visceral adiposity contribute to insulin resistance, but also lead to lipid infiltration of hepatocytes which causes non-alcoholic fatty liver (NAFL) and can progress to cirrhosis or hepatocellular carcinoma (Fahed et al., 2022).

**Epidemiology MetS:**

Since the formation of the term “metabolic syndrome x” in 1988, MetS has increased by more than 35% in the US adult population (Moore, Chaudhary, and Akinyemiju, 2017). The prevalence of MetS is comparable to obesity and type 2 diabetes mellitus (Fahed et al., 2022). Center for Disease Control data from 2017 indicated that 30.2 million adults had type 2 diabetes mellitus with a quarter of these individuals being unaware of their condition (Saklayen, 2018). The rates for prediabetes or MetS was almost three times more meaning a third of the US population has MetS (Saklayen, 2018). Rates for obesity have doubled since 1980 in several countries with increasing trends in most others with even higher increases in childhood obesity (Saklayen, 2018). Globally we now see the highest rates of obesity in countries that rank low on the socioeconomic index (Saklayen, 2018). Bangladesh, one of the poorest countries in the world, experienced the highest change in age-standardized body mass index related deaths.
signifying that obesity is no longer a problem for those who come from more affluent parts of the world (Saklayen, 2018).

As of 2015, the global rate for diabetes mellitus is 8.8% of the world population which accounts for 415 million individuals (Ogurtsova et al., 2017). These numbers are estimated to increase to 10.4% which will account for 642 million people (Ogurtsova et al., 2017). Currently, the highest prevalence of diabetes is in North American and Caribbean regions (Ogurtsova et al., 2017). While the continent of Africa currently has the lowest rates for diabetes, it is expected to experience the largest growth of those with diabetes with an expected increase of 140.7% by the year 2040 (Ogurtsova et al., 2017). In 2015, an estimated 5.0 million deaths were attributed to diabetes (Ogurtsova et al., 2017).

Despite women outnumbering men in prevalence of obesity and having more fat mass, women have lower rates of MetS when compared to age matched males (Regitz-Zagrosek, Lehmkuhl, and Mahmoodzadeh, 2007). When comparing pre- and postmenopausal women, we see an increase in incidence of MetS with postmenopausal women (Regitz-Zagrosek, Lehmkuhl, and Mahmoodzadeh, 2007). The likely cause of this being the shift in sex hormones during this transitional period in womanhood as well as explaining the discrepancy seen between woman and men (Gerdts and Regitz-Zagrosek, 2019).

Due to the complex nature and multiple working components of MetS, we lack a solid understanding of what causes this phenomenon to happen. There are still discussions about whether the individual components of MetS themselves form different pathologies or a broader process (Fahed et al., 2022). Currently, we do know that genetic and epigenetic factors along with lifestyle environmental factors contribute to the development of MetS (Fahed et al., 2022). Caloric surplus and sedentary lifestyle are important instigators of the different pathways for
MetS due to increase in visceral adiposity and its downstream targets (Matsuzawa, Funahashi, and Nakamura, 2011). These downstream targets include insulin resistance, hormonal activation, and chronic inflammation which lead to MetS and ultimately Type 2 diabetes mellitus and cardiovascular disease (Fahed et al., 2022).

**Insulin Resistance:**

Insulin is a peptide hormone that is secreted by beta cells of pancreatic islet cells (Wilcox, 2005). The role of insulin is to maintain normal blood glucose levels by promoting glucose uptake as well as regulating carbohydrate, lipid, and protein metabolism along with inducing cell division and growth (Wilcox, 2005). Insulin resistance can be defined as a normal or elevated insulin level produces an attenuated biological response which usually refers to impaired insulin sensitivity to insulin mediated glucose disposal (Wilcox, 2005). Insulin resistance is considered a key component in the progression on MetS as well as other components found in the syndrome (Wang et al., 2020). When insulin resistance is present in fat tissue, the mediated inhibition of lipolysis is impaired causing an increase in free fatty acids and dysregulating insulin signaling cascade in other tissues (Fahed et al., 2022). Free fatty acids impair insulin uptake to the muscles by inhibiting insulin receptor substrate (IRS-1) associated PI3k activity which leads to GLUT-4 translocation to the surface to be reduced and inhibit glucose uptake (Fahed et al., 2022). Free fatty acids will promote gluconeogenesis as well as lipogenesis in the liver causing overcompensation of insulin to keep normal levels of glucose (Fahed et al., 2022). These free fatty acids are able to achieve this hepatic dysregulation by directly entering the liver through the portal vein (Patel and Abate, 2013).

The increase in free fatty acids to the liver cause an elevation in the accumulation of hepatic triglycerides (Klop, Elte, and Cabezas, 2013). This hypertriglyceridemia is not only a
characteristic of MetS but also an independent risk factor for cardiovascular disease (Ebbert and Jensen, 2013). Very Low-Density Lipoprotein (VLDL) serves the role of exporting excess energy from the liver in the form of triglycerides (Ebbert and Jensen, 2013). This hypertriglyceridemia seen in insulin resistance is due to this increased presence of free fatty acids and hepatic triglycerides as well as overproduction of VLDV (Ebbert and Jensen, 2013). Due to this increase in triglyceride saturated VLDV, an increase in cholesterol ester transfer protein will promote the transfer of triglycerides from the VLDV to High-Density lipoproteins (HDL) increasing their clearance and lowering overall HDL concentrations (Fahed et al., 2022). VLDL may use atherogenic clearance pathways such as macrophages and smooth muscles increasing the risk of cardiovascular (Ebbert and Jensen, 2013).

Elevated levels of free fatty acids due to insulin resistance have also been shown to increase hypertension due to the loss of insulin’s vasodilation effect and vasoconstriction as a result of reactive oxygen species and loss of nitric oxide (Tripathy et al., 2003). This increase of pro-inflammatory cytokines due to free fatty acids also increases the risk of cardiovascular disease and type 2 diabetes mellitus (Tripathy et al., 2003).

**Adipose Tissue’s Role as an Endocrine Organ:**

White adipose tissue is the primary storage site for excess energy and largest endocrine organ that can release adipokines and pro-inflammatory cytokines systematically (Kawai, Autieri, and Scalia, 2021). These adipose tissue-secreted molecules play important roles in energy homeostasis through the regulation of glucose and lipid metabolism, immunity, and neuroendocrine systems (Ahima and Lazar, 2008). Two well known adipokines associated with metabolic regulation and health are adiponectin and leptin (Fahed et al., 2022).
Adiponectin:

Adiponectin, also referred to as adipQ, ACRp30, apM1, and GBP28, is involved in the regulation of glucose levels and lipolysis (Gunturiz Albarracin and Forero Torres, 2020). In the presence of nutrient depravation, adiponectin is released from white adipose tissue in order to signal a shift in metabolism toward fatty acid oxidation (Wensveen et al., 2019). This in turn allows for the decrease in circulating fatty acid concentrations as well as reduction in triglyceride content in liver and muscle (Gunturiz Albarracin and Forero Torres, 2020). Adiponectin achieves through adipoR1 activation of AMP-activated protein kinase (AMPK) and Peroxisome proliferator-activated receptor (PPARα) (Stern et al., 2016). Through AMPK, adiponectin suppresses acetyl-CoA Carboxylase (ACC) through phosphorylation (Nguyen, 2020). In typical fatty acid synthesis, ACC converts acetyl-CoA to malonyl-CoA which is an inhibitor of Carnitine palmitoyltransferase 1 (CPT1) (Nguyen, 2020). CPT1 transports fatty acids to the mitochondria of muscle cells where they can be degraded in β-oxidation due to adiponectin lifting this inhibition (Nguyen, 2020). Adiponectin further regulates lipolysis by the expression of PPARα (Nguyen, 2020).

The same pathway can be seen in the liver with regards to promoting lipid catabolism and inhibiting gluconeogenesis (Gamberi et al., 2018). A second receptor for adiponectin, adipoR2, stimulates PPARα that works in conjunction with activated AMPK to promote fatty acid combustion (Gamberi et al., 2018). AMPK further phosphorylates Sterol regulatory binding protein 1c (SREBP1c) that leads to its inhibition and further reduces fatty acid synthesis (Gamberi et al., 2018).

Adiponectin levels are reduced in the presence of obesity, metabolic syndrome, and type 2 diabetes mellitus (Nguyen, 2020). There is also a relationship between adiponectin
and insulin resistance with regards to lower levels of adiponectin associated with higher rates of insulin resistance (Nguyen, 2020). Adiponectin enhances the uptake of glucose through stimulation of GLUT 4 in muscle cells and adipocytes following the phosphorylation of AMPK (Nguyen, 2020). The production of new glucose molecules and metabolism of glycogen are suppressed due to a decrease in the glucose-6-phosphatase and PEPCK enzymes due to adiponectin (Combs et al., 2001). Loss of glucose and lipid metabolism regulation due to decreased levels of adiponectin enhances the pathogenesis of obesity-linked disorders (López-Jaramillo, 2014).

**Leptin:**

Similar to adiponectin, leptin plays important roles in energy homeostasis and metabolism as well as neuroendocrine function (Kelesidis et al., 2011). Also, like adiponectin, leptin is synthesized and secreted from white adipose tissue though acts on the brain to regulate energy homeostasis (Park and Ahima, 2014). Unlike adiponectin, leptin levels are proportional to the amount of adipose tissue one carries and shows it relationship to long term energy storage (Park and Ahima, 2014). Nutrient depravation sees a significant decrease in circulating leptin levels whereas obese individuals have increased levels (Park and Ahima, 2014).

Leptin binds to leptin receptors (LepRs) in both the brain and peripheral tissue in order to exhibit its effects on the body (Park and Ahima, 2014). Leptin directly targets two neuronal populations in the arcuate nucleus in the hypothalamus that co-express pro-opiomelanocortin (POMC)/cocaine-amphetamine-regulated transcript (CART), and agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Park and Ahima, 2014). The increase expression of POMC/CART and inhibition of AgRP/NPY expression results in reduced food intake, increased energy usage, and decreased body weight (Park and Ahima, 2014). Obese individuals having elevated levels of leptin
expression and circulating levels without reduction in adiposity suggest a state of leptin resistance (Park and Ahima, 2014).

Binding of leptin to its long isoform of leptin receptor (LepRb) activates the Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT3) pathway through phosphorylation of JAK2/STAT3 at the site of the receptor (Park and Ahima, 2014). Phosphorylated STAT3 promotes expression of POMC and inhibition of AgRP (Park and Ahima, 2014). Both leptin and insulin pathways meet on phosphatidylinositol 3 kinase (PI3K) where its activation phosphorylates Forkhead box O1 (FoxO1) through Protein kinase B (AKT) which may also activate mammalian target of rapamycin (mTOR) (Park and Ahima, 2014). In activation of FoxO1 allows STAT3 to bind to POMC and AgRP promoters as well as increases the expression of suppressor of cytokine signaling 3 (SOCS3) allow for feedback inhibition of leptin and insulin signaling (Park and Ahima, 2014). Inhibition of AMPK through leptin stimulates ACC and suppresses calorie intake while activation of AMPK blocks leptin’s appetite suppressing effect (Park and Ahima, 2014).

While leptin acts primarily in the brain, research suggests that leptin can act directly in surrounding tissues (Park and Ahima, 2014). Leptin stimulates oxidation of fatty acids and glucose uptake in skeletal muscle through AMPK (Park and Ahima, 2014). While leptin expresses an inhibitory effect in the hypothalamus, leptin activates AMPK and consequently inhibits ACC in skeletal muscle and prevents steatosis and lipotoxicity (Park and Ahima, 2014). In models with disrupted PI3K activity in POMC, hepatic insulin sensitivity as well as glucose homeostasis is altered negatively suggesting leptin’s effect of improving insulin sensitivity in skeletal muscle depends on PI3K/Akt modifying AMPK/ACC signaling (Park and Ahima, 2014). Leptin exhibits an inhibitory effect on hepatic glucose and stimulation of peripheral glucose uptake possibly
through POMC and AgRP neurons as well as changes in the autonomic nervous system (Park and Ahima, 2014). Studies have shown leptin directly affects glucose metabolism in hepatocytes through PI3K-dependent activation of phosphodiesterase 3B (PDE3B) (Park and Ahima, 2014). Research has also shown stimulation of fatty acid oxidation by p38 mitogen-activated protein kinases (MAPK)-dependent mechanism when leptin was administered in peripheral tissue (Park and Ahima, 2014). Inhibition of de novo lipogenesis and stimulation of lipolysis due to leptin are lost when PI3K signaling is disrupted in the hypothalamus (Park and Ahima, 2014).

As previously mentioned, obese individuals appear to have significantly elevated levels of leptin without the anorexigenic effects and reduction in adiposity (Park and Ahima, 2014). This blunted effect on leptin targeted organs with higher levels of leptin suggest a type of leptin resistance (López-Jaramillo, 2014). Insulin resistance has been shown to indirectly contribute to the raised levels of leptin due to the increase in obesogenic gene expression (López-Jaramillo, 2014). This association between insulin resistance and leptin levels likely reflects the size of adipose tissue and could be explained due to this leptin resistance (López-Jaramillo, 2014). Chronically high levels of leptin resistance in obese individuals have been shown to decrease the responsiveness of pancreatic β-cell receptors and in turn increase insulin secretion which may further exacerbate obesity and leptin levels causing a vicious cycle (López-Jaramillo, 2014). With increased leptin, enhanced levels of tumor necrosis factor (TNF) and interleukin-6 (IL-6) are also present due to the JAK2/STAT3 pathway (Agrawal et al., 2011).

Liver Dysfunction in MetS:

Insulin resistance, hormones, and low-grade inflammation can all contribute to liver dysfunction in individuals with MetS (Carr et al., 2016). As previously mentioned, the role of insulin is to primarily increase the uptake of circulating glucose as well as promote esterification
of fatty acids and storage in adipose tissue (Carr et al., 2016). In hepatocytes, insulin’s role is to promote glycogen storage as well as inhibit gluconeogenesis while regulating key activators of de novo lipogenesis (Carr et al., 2016). With the presence of insulin resistance, there is an increase in lipolysis in adipocytes that results in elevated levels of free fatty acids that become readily available for hepatic take up along with reduced storage of glycogen in the liver and increased gluconeogenesis (Carr et al., 2016). This results in an accumulation of intra-hepatic lipids and increased triglyceride secretion in the form of very-low density lipoproteins (Carr et al., 2016). The elevated levels of circulating lipids further exacerbate the problem since adipocytes already experience reduced abilities to store lipids (Carr et al., 2016). Hepatocytes are then exposed to lipotoxic lipids that can further dysregulate insulin signaling and result in oxidative damage, inflammation, and fibrosis in liver tissue (Carr et al., 2016).

The adipokines adiponectin and leptin also appear to play a role in the dysregulation of liver function. In obese individuals, adiponectin levels are significantly reduced resulting in impaired metabolism as well as hepatic insulin sensitivity and increase in body weight (Carr et al., 2016). While adiponectin levels are lowered in obesity, leptin levels are elevated in the same individuals resulting in leptin resistance. This resistance of leptin inhibits leptin’s ability to protect the liver from lipotoxicity from triglyceride accumulation and thus further promotes impaired liver function (Chitturi et al., 2002).

NAFLD:

Non-alcoholic fatty liver disease (NAFLD) is currently the most common chronic liver disease in the world that exposes individuals for serious hepatic complications (Flisiak-Jackiewiz et al., 2021). NAFLD is said to be the hepatic manifestation of MetS and exist with the different criteria of MetS that includes obesity, dyslipidemia, and insulin resistance (Loomba and Arun,
NAFLD can range in severity from simple non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), the most severe form on the range (Pouwels et al., 2022). NAFL can be used interchangeably with hepatic steatosis. Diagnosis of NAFLD requires medical imaging to show the presence of >5% lipid accumulation in the liver in the absence of other causes such as alcohol, viruses, drugs, or autoimmunity (Byrne and Targher, 2015). A liver biopsy is required for the diagnosis of NASH using histological techniques to find the presence of steatosis, hepatocellular ballooning, and lobular inflammation (Friedman et al., 2018). NASH can further be divided into four categories depending on the severity of the disease. Those diagnosed with NASH typically progress one stage every 7 years with those diagnosed with NAFL progress half as fast (Friedman et al., 2018). NASH can progress to more serious conditions such as cirrhosis, liver failure, and hepatocellular carcinoma and increased outcomes associated with cardiovascular disease and malignancy (Friedman et al., 2018).

**NAFLD Epidemiology:**

NAFLD has grown drastically in the past decades in parallel with the obesity and type 2 diabetes mellitus epidemic (Wong et al., 2023). Due to this increase in obesity, NAFLD has become the one of the leading causes for chronic liver disease globally (Gadiparthi et al., 2020). The global prevalence of NAFLD has increased by more than 50% over the past 30 years from 25.3% to 38% (Wong et al., 2023). Estimates put the global prevalence of NAFLD as high as one billion individuals with populations in the USA between 80-100 million (Gadiparthi et al., 2020). While 25% of those affected by NAFL will progress to NASH, the true prevalence is not truly known due to most NAFL patients not having a liver biopsy (Gadiparthi et al., 2020). It also hard to truly find the prevalence of NASH due to errors in diagnostic testing such as ultrasound failing.
to find mild forms of NAFL, patients may exhibit normal liver enzymes, and liver biopsy suffers sampling errors (Gadiparthi et al., 2020).

The highest rates of NAFLD can be found in the Middle East and South America with the lowest being in Africa (Friedman et al., 2018). While NAFLD is typically associated with central obesity in Europe and North America, large populations in Asia experience “lean NASH” with a normal body mass index despite the criteria for obesity in Asian countries being lower than those in Europe and North America (Friedman et al., 2018). While men have a higher prevalence for NAFLD when compared to women, the rate of NASH is not significantly different with advanced fibrosis even being higher in women than men (Balakrishnan et al., 2021).

**Progression of NAFLD:**

The first step in the progression of NAFLD is the accumulation of lipids in the liver which is associated with features of MetS as previously stated (Pierantonelli and Svegliati-Baroni, 2019). Three mechanisms have been identified in the progression of NAFLD including increased adipose tissue, hepatic de novo lipogenesis, and overnutrition or a high fat diet (Pierantonelli and Svegliati-Baroni, 2019). The contribution of each of these mechanisms towards progression in NAFLD from greatest to least are as they appear in the previous sentence respectively (Pierantonelli and Svegliati-Baroni, 2019). Further accumulation of hepatic free fatty acids promotes a lipotoxic environment in the liver that is associated with lipid dysregulation as well as chronic inflammation that leads to NASH (Pierantonelli and Svegliati-Baroni, 2019).

**Fatty acids:**
Insulin resistance is the key driver for development of steatosis due to its effects on adipose tissue (Friedman et al., 2018). Resistance to insulin results in adipose tissue being resistant to antilipolytic effects of insulin resulting in triglyceride breakdown and formation of free fatty acids and glycerol (Pierantonelli and Svegliati-Baroni, 2019). This increase in free fatty acids results in increased hepatic uptake and increases triglyceride storage in the liver (Pierantonelli and Svegliati-Baroni, 2019). Higher levels of insulin also result in elevated levels of hepatic triglyceride synthesis (Pierantonelli and Svegliati-Baroni, 2019).

De novo lipogenesis contributes to further fat storage in the liver (Pierantonelli and Svegliati-Baroni, 2019). The process primarily takes place in the liver and adipose tissue (Ameer et al., 2014). A byproduct of glycolysis, citrate, is converted to acetyl-CoA by ATP-citrate lyase (Ameer et al., 2014). ACC converts this acetyl-CoA to malonyl-CoA that is used as a substrate for 16-carbon saturated palmitate by the enzyme fatty acid synthase (Ameer et al., 2014). Glycerol-3-P is used from glucose and fructose metabolism in hepatocytes for energy or the synthesis of triglycerides (Paglialunga and Dehn, 2016). Expression of enzymes in hepatic de novo lipogenesis depends on the nutritional state of the individual (Heeren and Scheja, 2021). After food consumption, increases in glucose metabolism and insulin signaling increases the transcription factors Carbohydrate response element binding protein (ChREBP) and SREBP1 (Heeren and Scheja, 2021). Long term consumption of food in excess and hyperinsulinemia results in both transcription factors to be active and in turn de novo lipogenesis as well resulting in increased levels of triglycerides (Heeren and Scheja, 2021). ChREBP and Fibroblast Growth Factor-21 (FGF21) also appear to have a signaling axis as well as a role in NAFL and its progression with regards to inflammation and fibrosis, though these mechanisms are still not
fully known as well as FGF21’s full role in NAFLD and MetS (Szczepańska and Gietka-Czenerl, 2022).

Consumption of a Western diet is associated with increase prevalence of insulin resistance, dyslipidemia, MetS, as well as NAFLD (Pierantonelli and Svegliati-Baroni, 2019). Increased intake of sugar-sweetened food and beverages have also been identified as factors contributing to NAFLD. Fructose, unlike glucose, can impair hepatic lipid metabolism either by direct activation of SREBP1C or ChREBP and decreases mitochondrial beta oxidation through de novo lipogenesis (Caligiuri et al., 2016).

**Hepatic Insulin Resistance:**

Patients with NAFLD among those with the highest risk of developing type 2 diabetes mellitus and MetS with a twofold increase in risk over a five-year period (Sakurai et al., 2021). It has also been shown that nearly all individuals who have type 2 diabetes mellitus also have NASH regardless of liver enzyme levels (Sakurai et al., 2021). This alone suggesting that steatohepatitis may be representative of liver damage in patients with type 2 diabetes mellitus (Sakurai et al., 2021). Due to the comorbidities of MetS having their underlying cause in insulin resistance, NAFLD is also considered a phenotype associated with the pathophysiological conditions of insulin resistance (Sakurai et al., 2021).

Hepatic insulin resistance is thought to occur due to proinflammatory cytokines, endoplasmic reticulum stress, and reactive oxygen species causing an overactivation of JNK in hepatocytes through inhibitory serine phosphorylation of insulin molecules Insulin receptor substrate 1 and 2 (IRS-1, IRS-2) (Sakurai et al., 2021). Short-term feeding of a high fat diet in
mice resulted in hepatic fat accumulation without peripheral accumulation along with a threefold increase in hepatic triglycerides and total fatty ACC content (Samuel et al., 2004). As previously stated, this was attributed to impaired stimulation of IRS-1 and IRS-2 with activation of Protein kinase C – ε (PKCε) and JNK1 (Samuel et al., 2004). Since these findings, it has been generally accepted that this is the molecular mechanism that results in hepatic insulin resistance (Sakurai et al., 2021).

Elevated levels of diacylglycerol (DAG) in the liver results in PKCε activation and inhibits insulin signaling at the cell membrane (Perry et al., 2014). This reduction in phosphorylation of IRS-2 and PI3K impairs activity of RAC-beta serine/threonine-protein kinases (AKT2) due to reduction in 3-phosphoinositide-dependent protein kinase 1 (PDK1) and suppresses glycogen synthase kinase-3 (GSK3) phosphorylation which results in reduced insulin-stimulated liver glycogen production through reduced glycogen synthase (GS) activity (Perry et al., 2014). Dysfunction of AKT2 also reduces insulin suppression of gluconeogenesis in the liver through promoting FOXO1 translocation to the nucleus due to reduced phosphorylation and increased expression of gluconeogenic proteins such as pyruvate carboxylase (PC), Phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) (Perry et al., 2014). While hepatic content of DAG is the best predictor of hepatic insulin resistance, studies have shown there are no association between these markers and hepatic inflammation but rather inflammation is a result of hepatic insulin resistance (Perry et al., 2014).

**Hepatic Inflammation:**

As previously mentioned, NAFLD is a spectrum of a liver disease ranging from simple steatosis to the more severe NASH which is the more progressive form of the disease (Liu et al., 2010). The development of NAFL to NASH is said to be the result of a “two hit” theory where
the “first hit” is lipid accumulation which results in liver sensitivity to a variety of “second hits” that include inflammation that progress to NASH (Liu et al., 2010). The “second hits” likely due to adipose tissue secreting proinflammatory cytokines that include TNF-α, Interleukin (IL)-6, and IL-8 (Byrne et al., 2009).

While hepatocytes play a crucial role in metabolism, they also are involved in the immune response to infectious or noninfectious injury (Peiseler and Tacke, 2021). In NASH, hepatocytes use pattern-recognition receptors to detect excess metabolites and elevated levels of endotoxins that result in local inflammation and cell death (Peiseler and Tacke, 2021). The liver also contains multiple innate immune cells that include Kupffer cells, neutrophils, dendritic cells, mast cells, resident lymphocytes, hepatocytes, and liver sinusoidal endothelial cells (Cai et al., 2019). Kupffer cells, mast cells, hepatocytes, and liver sinusoidal endothelial cells detect endogenous damage-associated molecular pattern molecules (DAMPs) and excessive metabolites through pattern-recognition receptors (Cai et al., 2019). This in turn results in release of inflammatory cytokines and chemokines including Interleukin (IL)-1β, IL-1B, IL-6, and TNF-α (Cai et al., 2019). This increase in stressors results in increased recruitment of neutrophils that initiate liver damage by either enzymes or reactive oxygen species (Cai et al., 2019). Kupffer cells, mast cells, and hepatocytes also increase expressions of matrix metalloprotease, angiotensin 2, transforming growth factor, and hepatic growth factor in order to stimulate the activation of hepatic stellate cells and liver fibrosis (Cai et al., 2019). The innate immune signals further drive lipogenesis and insulin resistance as well as apoptosis, proptosis, or necrosis in hepatocytes (Cai et al., 2019). All of these events result in not only liver steatosis but also hepatic inflammation and fibrosis (Cai et al., 2019).

Fibrogenesis:
The formation of fibrotic tissue is important for the progression of NAFLD in response to chronic injury and macrophage activation (Ota, 2021). The accumulation of this extracellular matrix in the liver is the major cause for liver-related deaths in individuals with NAFLD (Friedman et al., 2018). Generation of fibrotic tissue is driven by the stress signaling as mentioned before and the resulting activation of the resident hepatic stellate cells into myofibroblast to generate matrix proteins faster than they can be degraded (Friedman et al., 2018).

As mentioned, the inflammation caused in NASH results in hepatocyte death and apoptosis (Heyens et al., 2021). These dying hepatocytes release a danger signal through DAMPs to surrounding cells that activates hepatic progenitor cells (Heyens et al., 2021). On the contrary, apoptosis will result in lower levels of DAMPs due to the cell being mostly retained in an apoptotic body (Heyens et al., 2021). Hepatic stellate cells and Kupffer cells will phagocytose the apoptotic cell bodies and induce a pro-fibrogenic response (Heyens et al., 2021).

Hepatic stellate cells are activated and differentiate from a quiescent phenotype to contractile myofibroblasts in the presence of liver injury (Heyens et al., 2021). During this differentiation, hepatic stellate cells will transform from their typical star-like shape and into a droplet from (Heyens et al., 2021). Under normal conditions, anti-fibrotic mechanisms like Kupffer cells would keep this in check and result in inactivation or apoptosis of myofibroblasts and scar correction (Heyens et al., 2021). In NAFLD, this balance is lost and results in abundance of extracellular matrix products that will destroy the physiological architecture of the liver (Heyens et al., 2021). Activation of hepatic stellate cells can occur through either transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), inflammasome (NLRP3)- caspase 1, and WNT/β-catenin (Heyens et al., 2021).
Dimorphism in MetS and NAFLD:

As mentioned previously, rates for MetS and NAFLD appear to a discrepancy with regards to males having higher prevalence of both conditions (Rochlani et al., 2015). All components of MetS excluding obesity sees higher levels in the male population rather than female (Rochlani et al., 2015). Men have shown to have a sharp increase in blood pressure during and after adolescence while females tend to see an increase only after menopause (Rochlani et al., 2015). While a study had shown women to have higher systolic blood pressure when compared to men and was an indicator of cardiovascular disease risk, the female population of the study were comprised of 82% postmenopausal women (Rochlani et al., 2015).

It has been noted that the anatomical structure between males and females for the cardiovascular system are different with females having stiffer hearts and arteries (Rochlani et al., 2015). It is thought that sex hormones during the reproductive years, mainly estrogen, is responsible for tempering this feature (Rochlani et al., 2015). Estrogen is responsible for an increase in the synthesis of angiotensinogen and expression of angiotensin type 2 receptor while inhibiting synthesis of renin, angiotensin converting enzyme, and decreasing angiotensin type 1 receptor (Rochlani et al., 2015). While angiotensin 2 that is mediated by angiotensin type 1 receptor is associated with hypertension, those mediated by angiotensin type 2 receptor has been linked with a drop in blood pressure (Rochlani et al., 2015). These affects though are lost with aging and can help explain for this post-menopausal rise in hypertension.

While men typically have a 1.5-3 times higher impaired fasting glucose measurement, impaired glucose tolerance is more prevalent in women (Rochlani et al., 2015). Though women have higher percentages of adipose tissue, it is typically stored as subcutaneous adipose tissue in the abdominal and gluteofemoral locations of the body with men having larger visceral fat stores.
in the abdomen or upper body (Rochlani et al., 2015). Despite these differences in fat percentage, women still appear to be more sensitive to insulin (Rochlani et al., 2015). The mechanism for these sex specific differences in insulin sensitivity are not fully understood (Varlamov et al., 2015). Some studies have demonstrated there are no sex differences in insulin signaling while others have shown that females have increased first-phase insulin secretion when compared to males (Varlamov et al., 2015). It was also shown that white adipose tissue in females had a greater response to insulin as well as a greater increase in Akt, extracellular signal-related kinase phosphorylation, and lipogenesis when compared to male white adipose tissue (Varlamov et al., 2015). This was reversed when males were castrated and females were ovariectomized (Varlamov et al., 2015).

Women have been shown to have higher levels of Large HDL, higher levels of large HDL to total HDL ratio, and less small HDL compared to men (Rochlani et al., 2015). Large HDL comprises 65% of HDL in women where men it is only 45% (Rochlani et al., 2015). Large HDL cholesterol is shown to have athero-protective qualities leading to reduced risk of cardiovascular disease in women (Rochlani et al., 2015). Mechanisms have been proposed in order to explain this dimorphic difference that differences in hepatic lipase and lipoprotein lipase and hormones (Rochlani et al., 2015). Women have been shown to have higher levels of lipoprotein lipase per unit of adipose tissue when compared to men (Rochlani et al., 2015). This increase allows for greater uptake of triglycerides and a mover cholesterol metabolism (Rochlani et al., 2015). The effect of hormones with regard to lipid metabolism is more complicated (Rochlani et al., 2015). Postmenopausal women have higher levels of total and small dense LDL cholesterol (Rochlani et al., 2015). Hormone replacement in postmenopausal women was shown to help alleviate LDL levels was not able to increase HDL levels (Bittner, 2005).
While women globally have higher rates of obesity at 38% when compared to men at 36.9%, we see men having higher rates of being overweight and obese in developed countries while developing countries have women at higher rates (Rochlani et al., 2015). Genetic, hormonal, and social factors all influence this data (Rochlani et al., 2015). The expectation of a sedentary and physically inactive female trait in various cultures can help explain some this disparity and has even led to the concept of “sex inequality” (Rochlani et al., 2015). Differences in function of visceral and subcutaneous adipose tissue also plays a role in the cardiovascular risk associated with obesity (Rochlani et al., 2015). Visceral adipose tissue produces proinflammatory cytokines and is associated with insulin resistance and increased risk in cardiovascular disease (Rochlani et al., 2015). Due to men typically storing visceral fat, there is stronger association of cardiovascular disease in obese men as opposed to obese premenopausal women (Rochlani et al., 2015). Men also exhibit hypertrophy of adipose cells resulting in increased cell size and greater proinflammatory adipokines (Rochlani et al., 2015).

Postmenopausal women start to see an increase in visceral adipose storage (Rochlani et al., 2015). While hormone therapy has been promising in animal studies, clinical studies have yet to show any benefit with some demonstrating possible harmful effects of estrogen supplementation (Rochlani et al., 2015).

Prevalence of NAFLD has been shown to be higher in men with ranges of 4.3-42% than women with ranges of 1.6-24% (Burra et al., 2021). Men typically have the highest rates of NAFLD in adulthood with reduced rates occurring around the ages of 50-60 years old (Ballestri et al., 2017). Women typically experience the lowest rates for NAFLD during their fertile years due to the protective effects of estrogen and experience an increase in prevalence at age 50 and peaks around 60-69 years of age (Ballestri et al., 2017). Because of this, postmenopausal women
will have similar or even higher rates of NAFLD when age matched (Ballestri et al., 2017). This estrogen deficiency can lead to increased risk for NAFLD and fibrosis of the liver of women experiencing menopause and is associated with insulin resistance, hypertriglyceridemia, and central obesity (Ballestri et al., 2017). Hormone replacement treatment has been shown to consistently protect against NAFLD development (Ballestri et al., 2017). This has been supported by studies showing that fibrosis severity was increased in those with higher body mass index, advanced steatosis, and menopause in Hepatitis C infection but decreased in those receiving hormone replacement therapy (Ballestri et al., 2017). While younger women generally have decreased risk for NAFLD, those of reproductive age and those exposed to synthetic hormones in the form of oral contraceptives and hormone replacement therapy have more severe hepatocyte injury and inflammation (Ballestri et al., 2017).

When ovariectomized, rats showed increased intrahepatic steatogenesis due to decreased synthesis of peroxisome proliferator-activated receptor (PPAR) and increased SREBP1 (Ballestri et al., 2017). The negative effects of the ovariectomy were prevented when 17β- estradiol, a potent estrogen found in humans, was administered suggesting estrogen’s role in regulating hepatic fat accumulation (Ballestri et al., 2017). While the hedgehog signaling pathway has been shown to play a key signaling role in the recruitment of hepatic stellate cells for the differentiation into myofibroblasts, more studies are needed in order to fully understand the role sex hormones play in fibrosis and more severe NASH (Ballestri et al., 2017).

Diet in MetS and NAFLD:

While many factors including an increasing sedentary lifestyle have been attributed to the obesity epidemic, the increased consumption of a “Western Diet” has been one of the major contributors (Rakhra et al., 2020). A western diet is characterized by low consumption of fruits
and vegetables and high in sodium and fat (Rakhra et al., 2020). This diet also consists of large portions with high calories and excess amounts of sugar (Rakhra et al., 2020). Most of these excess sugars come from sweetened beverages (Rakhra et al., 2020). The western diet is also comprised of increased consumption of saturated and trans fats that lead to increased levels of low-density lipoproteins and risk of atherosclerosis (Rakhra et al., 2020). Symptoms of Metabolic syndrome have been linked to the western diet due to the high intake of carbohydrates and fats (Panchal et al., 2011). Models utilizing rats on either a high-fat or high-carbohydrate diet have been used to mimic the diet-induced effects of obesity that is seen with consumption of a western diet (Panchal et al., 2011). High-fat and high-sugar diets have also been shown in the development of Non-Alcoholic Fatty Liver Disease (Nakamura and Terauchi, 2013). More specifically, a diet with high levels of fructose has been shown to increase the rate of Non-Alcoholic Fatty Liver Disease due to its metabolism in the liver (Inci et al., 2023).

Gaps:

It is currently known that western diets promote the prevalence of both MetS and NAFLD through modification of lipogenic and glucogenic pathways. Additionally, research has shown the prevalence for both MetS and NAFLD appear to be higher in males rather than premenopausal females. The exact mechanism behind this has yet to be fully elucidated. Comparison of a high fat to a high sugar diet in MetS and NAFLD has yet to be undertaken. It has also not been shown what pathophysiological changes occur at different time points between a high-fat diet and a high-sugar. The need for further research in these two areas are crucial to the development of targeted therapies in terms of sex and time point of diagnosis.
Specific Aims:

Globally, 39% of men and 40% of women of the adult population are classified as overweight with 11% of men and 15% of women being obese. This trend in the worldwide prevalence of obesity has nearly tripled between the years 1975 and 2016. Metabolic Syndrome, a term used to encompass multiple metabolic abnormalities, has also seen a steady increase along with obesity with the highest rates now being seen in developing countries whereas it was originally thought to be a condition of affluence.

This trend is also seen in Non-Alcoholic Fatty Liver Disease. Global prevalence of Non-Alcoholic Fatty Liver Disease has increased from 26% in 2005 to 38% from 2016 and beyond. Rates are even as high as 40% in the Americas as well as South-East Asia. Non-Alcoholic Fatty Liver Disease has become the global leading cause of liver disease with its more severe form, Non-Alcoholic Steatohepatitis, being the fastest-rising cause for hepatocellular carcinoma.

There exists a dimorphic difference the Prevalence for both Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease. Men typically have a higher rate for both conditions when compared to women. This difference becomes less as women age and experience menopause. While there appears to be a link between sex hormones and the development and progression of both ailments, the mechanism behind it has not been fully elucidated. Studies have yet to compare diet, sex, and time as well in order to try to find an explanation for this difference. **In order to attempt to fill these gaps, we propose to investigate this sexually dimorphic progression in Metabolic Syndrome and Non-alcoholic Fatty Liver Disease in response to different diet treatments and time points.** With this, **our overall hypothesis is that in investigating these variables with relation to Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease could shed light on potential biomarkers as well as insights into the sex and diet specific pathways in order to treat and prevent metabolic diseases.**

**Specific Aim 1: Examine obesity and insulin resistance in relation to sex and diet composition in the progression of Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease.**

*Hypothesis:* Female mice will exhibit less severe diet-induced obesity and insulin resistance when compared to male mice for both diets.

**Specific Aim 2: The effects of sex and diet on liver metabolic flexibility.**

*Hypothesis:* Female mice on a high sugar diet will exhibit higher markers of glycolysis while male mice on a high sugar diet will show higher markers for lipogenesis. Female mice on a high fat diet will utilize lipolysis pathways while high fat fed male mice will show impaired fat oxidation and increased fat accumulation in the liver.
Methods:

Mice:

30 C57BL/6J Mice will be used. These mice be housed 5 to a cage. 15 female and 15 male mice will be placed on one of three diets: high fat, high sugar, and a standard chow diet. The high fat diet will consist of 45% fat, 20% protein, and 35% carbohydrate. High sugar will consist of 60% carbohydrate with 60% coming from sucrose, 20% protein, and 20% fat. Standard chow diet will consist of 20% fat and protein and the remaining 60% consisting of carbohydrates. Mice will be weighed every three days to monitor health and keep track of weight gain. MRI testing will be used to determine body composition biweekly. Glucose tolerance test (GTT) will be conducted once every month to monitor for insulin resistance. Mice in each group will either be sacrificed at 6 or 12 weeks in order to visualize the progression of MetS and NAFLD. At both time points, mice will be euthanized by CO2 and then followed by cervical dislocation. Tissues harvested will include liver, spleen, kidney, intestine, adipose tissue, skeletal muscle, and heart that will be frozen in liquid nitrogen or formalin immediately upon after euthanasia.

Western Blot:

Western blot analysis will be performed on liver tissue to evaluate protein expression levels. Portions of liver tissue will be homogenized in a 10x Mueller buffer solution and protein concentrations will be determined using a Bradford Assay. Total (T) and Phosphorylated (P) AMPK antibodies will be used. Antibodies for SREBP1c and ChREBP will be used as well.

RNA Isolation and qPCR:
Tissue will be homogenized using 3-5mL of Trizol. Total RNA will be extracted from this Trizol solution with chloroform. Extracted RNA will be washed using 75% ethanol and then dissolved in water in order to be quantified using a Nanodrop. qPCR will be used to measure mRNA transcripts from cDNA. These transcripts will include fatty acid synthase (Fasn) and GLUT4.

**Histological Analysis:**

The use of hematoxylin and eosin staining will be used to quantify hepatocyte and adipocyte size in paraffin embedded tissue samples. Trichrome staining will be used in order to visualize fibrosis in liver samples.
References:


47. Pierantonelli, I., & Svegliati-Baroni, G. (2019). Nonalcoholic fatty liver disease: basic pathogenetic mechanisms in the progression from NAFLD to NASH. *Transplantation, 103*(1), e1-e13.


Appendix 2:

IACUC Approval
IACUC Protocol
For Use of Live Vertebrae for Research, Teaching or Demonstration
University of Memphis

Date submitted to Attending Veterinarian for pre-review: 7-19-2021

IACUC Protocol #0877 Date Submitted to IACUC 8-12-2021

Dates Protocol will be in effect from 9-1-2021 to 8-31-2022 (not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes □ No X

If yes, complete the following:

Agency: ____________________________ Date Submitted ____________________________

Grant #: ____________________________

University account for Animal Care Facility per diem charge: index 210655

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: ____________________________

Project Title: (If project relates to a grant or contract application, give that title; if multiple protocols relate to one grant, give unique titles for each protocol; if the project is related to a class, give the course name and number):

Elucidating mechanisms for the sexually dimorphic response to diet induced obesity and metabolic syndrome

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