POSTPRANDIAL TRIGLYCERIDES AND FIBROBLAST GROWTH FACTOR-19 AS POTENTIAL SCREENING TOOLS FOR PEDIATRIC NON-ALCOHOLIC FATTY LIVER DISEASE

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2019

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May 2022

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ACKNOWLEDGEMENTS

I would like to thank my Major Professor and Chair, **Dr. Sam Emerson**, for his continuous support, guidance, encouragement, and outstanding mentorship throughout this process. It is through these qualities that I was able to truly engage, grow, and challenge myself throughout the scientific process. Thank you, Dr. Emerson, for leading by example, being an exceptional mentor, and for truly facilitating my education, growth, and personal development. I could not have accomplished what I have without you!

I would also like to thank **Dr. Kevin Short** for always being willing to answer my questions and teach me new skills. Dr. Short provided thoughtful and perceptive feedback and mentorship throughout the study duration, that of which was meaningful and an uncommon effort and display of mentorship.

Additionally, I would like to thank **Dr. Stephen Clarke**, NSCI Department Head and Committee Member. Dr. Clarke went above and beyond to ensure my success at Oklahoma State University. I could not have accomplished what I have without his support and mentorship.

Dr. Edralin Lucas, and **Dr. Misty Hawkins**, your different perspectives and insightful feedback throughout this process facilitated my growth and success. Thank you for always challenging me constructively.

Bryant Keirns – the best lab mate ever! Thank you for being such a reliable, hard-working, and pleasant co-pilot in LANES, and for always (willing or not) listening to my rants and stories. And to everyone who has been a part of **LANES** and **NSCI** – fellow students, professors, and staff - without you all, life in the lab and classroom would be difficult. I have spent most of my waking hours with you all and I couldn't have asked for a better team to work with every day. Thank you for all of the support and hard work.

Shane Eachus, thank you for your love and encouragement and for bringing so many smiles and laughs to my life throughout this process. You've always pushed me to achieve and believed in me even when I doubted myself. I am so grateful to have had you as a partner during my time at Oklahoma State University.

To my **friends and family**, your encouragement and support is unmatched, and I am so grateful for each and every one of you.

Name: Christina Sciarrillo

Date of Degree: MAY 2022

Title of Study: POSTPRANDIAL TRIGLYCERIDES AND FIBROBLAST GROWTH FACTOR-19 AS POTENTIAL SCREENING TOOLS FOR PEDIATRIC NON-ALCOHOLIC FATTY LIVER DISEASE

Major Field: NUTRITIONAL SCIENCES

Abstract:

Background: Screening tools for pediatric NAFLD are lacking. We tested the hypothesis that the postprandial triglyceride (TG) and FGF19 response to an abbreviated fat tolerance test (AFTT) could discriminate children with NAFLD from obese and normal weight peers.

Methods: In this cross-sectional study, 15 normal weight controls (NW; 6M/9F; age: 17 ± 2y; BMI: 49 ± 24 %ile), 13 controls with obesity without NAFLD (OB; 5M/8F; age: 17 ± 2y; BMI: 98 ± 1 %ile), and 9 patients with NAFLD (7M/2F; age: 15 ± 2y; BMI: 99 ± 0 %ile) completed an AFTT. Following an overnight fast, participants consumed a high-fat meal (73% fat; 9 kcal/kg) and TG and FGF19 were measured at baseline and 4h post-meal. Liver steatosis (controlled attenuation parameter (CAP)) and fibrosis (stiffness) were measured via Fibroscan.

Results: There were no group differences in fasting TG ($p > 0.05$). NAFLD and OB exhibited greater 4h TG (197 ± 69 mg/dL; 157 ± 72 mg/dL, respectively) than NW (105 ± 45 mg/dL; $p < 0.05$), but NAFLD did not differ from OB control ($p > 0.05$). Within the entire sample, when 4h TG were stratified by high (CAP ≥ 220 dB/m) and low (CAP ≤ 220 dB/m) steatosis, children with high steatosis had 98% greater 4h TG (170 ± 69 mg/dL) compared to children with low steatosis (86 ± 25 mg/dL; $p = 0.0004$). Fasting and 4h FGF19 did not differ across groups ($p > 0.05$). Across all groups, fasting FGF19 and 4h postprandial FGF19 were 226% and 258% higher in children with low steatosis compared to high steatosis, respectively ($p < 0.05$). Using ROC, the only outcome with diagnostic accuracy for NAFLD was 4h TG (0.77 [95% CI: 0.60-0.94; $p = 0.02$]).

Conclusions: These findings suggest that the postprandial TG response in NAFLD is greater in children with NAFLD compared to NW peers without NAFLD but does not differ between children with NAFLD and children with obesity, which is likely related to steatosis detected in the OB group. Despite no differences across groups, the postprandial rise in FGF19 was blunted in children with high liver steatosis.

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CHAPTER I

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by liver fat accumulation, accompanied by different degrees of fibrosis, inflammation, and necrosis. As such, NAFLD is an array of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). NAFLD is a serious public health concern, as it has been recognized as the most prevalent chronic liver disease worldwide and can progress to cirrhosis and increase risk for hepatocellular carcinoma (HCC). One of the hallmarks of the development of NAFLD is insulin resistance. Not surprisingly, NAFLD has been described as the liver's display of metabolic syndrome and medical conditions including type 2 diabetes, dyslipidemia, and obesity commonly parallel a NAFLD diagnosis.

Although the pathophysiology of NAFLD is not fully understood, recent research has revealed a liver-central, but not autonomous, role in the pathophysiology of NAFLD. Specifically, the adipose tissue, gut-liver axis, genetics, and environmental factors, including dietary habits and physical activity, are key modulators in the transition from simple steatosis to NASH. These key modulators all contribute to the imbalance of the use and supply of

triglycerides (triglycerides) and free fatty acids (FFA) in the liver, the initiating step of NAFLD. Despite much recent advancement in understanding the pathophysiology and clinical representation of NAFLD, screening tools for early stages of triglycerides accumulation are lacking. Currently, screening for NAFLD often relies on liver biochemistries, which are non-specific to NAFLD, so much so that the American Gastroenterological Association advises against their use for screening for NAFLD [1]. Liver biopsy is the gold standard for the diagnosis of NAFLD, however liver biopsy is expensive and invasive, thus it is an unfeasible screening tool. Alternatives to liver biopsy include ultrasonography and MRI, but these methods still require cost and skill, and lack the sensitivity of liver biopsy.

Children and adults with NAFLD have a dissimilar pathophysiology. For example, children have a unique fibrosis pattern, characterized by portal fibrosis and inflammation, that is distinctive and seldom seen in adults, and, at the point of diagnosis, 15% of children have stage 3 or 4 fibrosis [2]. Thus, pediatric NAFLD is characterized by a distinctive pathophysiology that may be more aggressive and severe when compared to adults [1, 3]. Because children will carry this burden of risk from childhood into adulthood, early detection tools for NAFLD in children are needed. One potential avenue for the early detection of liver triglycerides infiltration is the assessment of postprandial triglycerides. Specifically, an impaired postprandial triglycerides response has been observed in adults and children with NAFLD and may serve as one etiological mechanism leading to triglycerides accumulation in the liver. Adults and children with NAFLD exhibit a steep increase in triglycerides in response to a high-fat meal (HFM) and this rise is persistent throughout the postprandial period, lasting longer than those without NAFLD. This characterizes the hypertriglyceridemia observed in those with NAFLD – increased postprandial triglycerides, or triglyceride-rich lipoproteins (TRL). The metabolism of these TRL leads to a

more pro-atherogenic lipid profile characterized by increased remnant lipoproteins (RLP), small-dense LDL, and decreased HDL-C. In addition, postprandial hypertriglyceridemia in the context of NAFLD is closely related to insulin resistance and its subsequent metabolic dysfunctions and hypertriglyceridemia is associated with the degree of hepatic steatosis, fibrosis, and inflammation [4-6].

Research has focused on identifying secondary factors connected to hepatic triglycerides metabolism that contribute to the transition from simple steatosis to NASH. The endocrine compound fibroblast growth factor 19 (FGF19) may be one factor related to postprandial dyslipidemia in the context of NAFLD, where it has emerged as an important intestinally derived regulator of hepatic triglyceride metabolism. In fact, FGF19 has been proposed as a novel biomarker for steatosis in those with NAFLD. This is partly because FGF19 production has been shown to be impaired in those with NAFLD and is closely related to hepatic triglyceride metabolism [7, 8]. Specifically, FGF19 regulates key features of triglyceride metabolism that contribute to the progression of NAFLD, including fatty acid oxidation, de novo lipogenesis (DNL), export of triglycerides from the liver, inflammation, cytotoxicity, and steatotic endoplasmic reticulum (ER) stress [5, 15]. Differences in postprandial FGF19 may enhance our understanding of why some children with obesity do not develop NAFLD. It has been demonstrated that children with obesity and NAFLD exhibit lower fasting FGF19 when compared to children with obesity without NAFLD [8]. Despite these findings, postprandial FGF19 has not been studied in obese children with and without NAFLD.

Therefore, the measurement of postprandial triglycerides and FGF19 has the potential to be a sensitive, convenient and child-friendly screening tool for hepatic triglyceride accumulation in NAFLD. Additionally, postprandial FGF19 may explain some of the pathophysiological differences

underlying the various body composition phenotypes in adult and pediatric NAFLD. Accordingly, the **overall objective** of this dissertation was to evaluate the effectiveness of postprandial triglycerides and FGF19, in the context of an AFTT, as sensitive and specific screening tools for NAFLD in children. We plan to meet our objective by achieving the following aims:

Aim 1. Determine the difference in the postprandial triglyceride response to an abbreviated fat tolerance test (AFTT) in obese children with NAFLD compared to normal weight and obese children without NAFLD. We hypothesized that postprandial triglycerides would be higher in children with NAFLD compared to obese and normal weight peers.

Aim 2. Determine the difference in the postprandial FGF19 response to an AFTT in obese children with NAFLD compared to normal weight and obese children without NAFLD. We hypothesized that postprandial FGF19 would be lower in children with NAFLD compared to obese and normal weight peers.

Aim 3. Evaluate the sensitivity and specificity of postprandial triglycerides and FGF19 in the context of an AFTT as a screening tool for NAFLD. We hypothesized that postprandial triglycerides and FGF19 would have moderate diagnostic accuracy for NAFLD.

Aim 4. Explore the relationship between postprandial triglycerides, FGF19, metabolic markers (e.g. adiponectin, insulin), and steatosis/fibrosis to advance understanding of pediatric NAFLD.

CHAPTER II

LITERATURE REVIEW

Prevalence of Non-alcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease has been recognized as the most prevalent chronic liver disease worldwide [9] and is a growing health problem for children and adults in the United States. Approximately 15-25% of individuals that develop NAFLD will develop NASH, 30-40% of NASH patients will develop liver fibrosis, 15-20% of individuals with fibrosis will progress to cirrhosis, and ~2-3% of NASH cirrhosis patients will develop HCC each year [10]. Non-alcoholic fatty liver disease includes a wide spectrum of disorders; it can be categorized by non-alcoholic fatty liver, which is described as the accumulation of triglycerides in the liver (simple steatosis), NASH, which is characterized by steatosis, inflammation, and fibrosis, or cirrhosis, which significantly increases the risk for HCC. As NAFLD progresses, it can lead to fibrosis, which is irreversible and more severe. In fact, one of the most predictive features of NAFLD-related mortality in adults is fibrosis stage, with the worst mortality among those with stage 3 or 4 fibrosis. NAFLD is a growing health problem for children, too. At the point of diagnosis, 15% of children have stage 3 or 4 fibrosis and their fibrosis pattern is distinctive and seldom seen in adults. Thus, pediatric NAFLD is characterized by a unique pathophysiology that may be

more aggressive and severe when compared to adults [1, 3].

The prevalence of NAFLD – as high as 40% in adults and 19% in children – parallels sedentary lifestyles, poor dietary habits, and obesity rates [11]. Unfortunately, obesity is present in roughly 51% and 82% of adults with NAFLD and NASH, respectively and half the U.S. population is expected to be obese by 2030 [12, 13], making NAFLD a major public health concern. Components of the metabolic syndrome including insulin resistance, excess visceral and total adiposity, hyperglycemia, and dyslipidemia, are among the strongest risk factors for NAFLD. In fact, NAFLD has been described as the liver's display of metabolic syndrome and often occurs in the context of insulin resistance [9]. Thus, although once considered a benign liver condition, it is now understood that NAFLD significantly increases risk for a number of conditions including cirrhosis, liver cancer, cardiovascular disease (CVD), and type 2 diabetes.

General Pathophysiology of Non-alcoholic Fatty Liver Disease

The pathophysiology of NAFLD was once widely supported by a "two-hit hypothesis" proposed nearly 20 years ago [14]. This "first hit", characterized by hepatic triglyceride accumulation, predisposed the liver to a second hit, characterized by oxidative stress and inflammation, leading to the development of NASH. As our understanding of hepatic metabolism has advanced, the once widely accepted "two-hit hypothesis" may be too simplistic and not adequately capture the complexity of NAFLD. Specifically, emerging research has described a "multiple-hit hypothesis" and has revealed a liver central, but not autonomous, role in the pathophysiology of NAFLD. The multiple-hit hypothesis has been modified and illustrates that the accumulation of hepatic triglycerides may be a protective mechanism and relatively benign, whereby triglycerides accumulation protects the liver from the potentially damaging influx of fatty acids derived from the diet, DNL, or adipose tissue. More, other organs including the

adipose tissue, gut microbiota, and muscle, as well as environmental factors, including the diet, play key roles in the progression of simple steatosis to NAFLD. These complex interactions may promote simple steatosis, immune activation, inflammation, cell death, or serious liver damage. As such, NAFLD is largely viewed as a multi-organ metabolic disease.

The Multi-factorial Pathophysiology of Non-alcoholic Fatty Liver Disease

NAFLD is the result of an imbalance in use and supply of triglycerides and FFA in the liver, leading to the accumulation of triglycerides in the liver. Triglyceride accumulation in the liver is the initiating step of the pathogenesis of NAFLD and could conceivably occur due to one or more of the following: 1) increased FFA influx from the diet or adipose tissue lipolysis, 2) impaired triglycerides export in the form of VLDL, 3) reduced FFA beta-oxidation, and 4) increased DNL. Thus, put simply, triglycerides accumulation in the context of NAFLD is the result of an imbalance between DNL and lipid clearance (via FFA oxidation or export of triglycerides) [15]. Tracer data suggest that triglycerides that accumulate in the liver are generally derived from FFA from adipose tissue lipolysis (60%), DNL (25%), and the diet (15%) [16]. Regardless of the source, mechanisms responsible for triglycerides accumulation in the liver are still being explored, however insulin resistance, obesity with adipocyte dysfunction, and dysregulation of the gut-liver axis likely contribute to the development of NAFLD. Moreover, what drives the development of NASH, a form of NAFLD characterized by fibrosis, inflammation, and hepatocellular damage, is thought to be mediated by multiple factors. It is thought that when compensatory changes (FFA oxidation or export of triglycerides) are not sufficient enough to keep up with the challenge of FFA influx, NAFLD may progress to NASH. At this time, common pathogenic mechanisms linking steatosis to NASH include oxidative stress, mitochondrial dysfunction, lipotoxicity, and ER stress.

A few potential and over-lapping mechanisms have been explored related to driving factors for the progression from NAFLD to NASH. Chronic hepatic inflammation may be the key driving force for the development of NASH. Specifically, mechanisms to handle the increased influx of FFA in the liver often become diminished or overwhelmed in those with NASH, leading to lipotoxicity and mitochondrial dysfunction. This mitochondrial dysfunction generates reactive oxygen species (ROS), oxidative stress, and induces ER stress, leading to the activation of the innate immune system [17]. This favors the production of pro-inflammatory mediators, including pro-inflammatory cytokines and chemokines. These pro-inflammatory mediators activate macrophages in the liver, known as Kupffer cells, leading to the stimulation of hepatocytes and stellate cells. Together, Kupffer cells and stellate cells are largely responsible for driving fibrogenesis and inflammation, both of which are key pathological characteristics of NASH [18]. More recently, the role of adaptive immune activation has been implicated in the progression of NASH, but much is still unknown [18]. Together, these changes can interfere with hepatic FFA metabolism, driving lipotoxicity, hepatocellular death and inflammation, and enhancing the progression to NASH.

Lipotoxicity

Lipotoxicity describes cellular injury, death, and inflammation as a result of a toxic build-up of FFA, their metabolites, and triglycerides, although triglyceride accumulation is viewed as relatively benign [19]. In fact, the accumulation of triglycerides in lipid droplets in the liver and adipose has been consistently associated with NAFLD, however a causal role for triglyceride accumulation in lipid droplets initiating lipotoxicity has not yet been established. For example, blocking microsomal transfer protein (MTP), a protein that assists in triglyceride loading onto ApoB-100, leads to impaired triglyceride secretion and retention of triglycerides in the liver, without liver injury [20]. However, inhibition of diacylglycerol O-acyltransferase 2 (DGAT2) expression, an enzyme important for triglyceride formation, leads to decreases in hepatic

triglycerides and therefore increases in FFA oxidation, accompanied by worsening of NASH in mice models [21]. Therefore, the perspective that triglyceride accumulation in the liver may be protective has become plausible but is not fully validated.

As previously mentioned, FFA and their metabolites are inherently toxic to cells. Free fatty acids are derived from fats and carbohydrates in the diet, excess endogenous synthesis, and/or adipose tissue lipolysis. Once within the hepatocyte, FFA are activated to fatty acyl-CoA via fatty acyl-CoA synthetase and can then be used in beta oxidation pathway or can undergo esterification [22]. Interestingly, some research has shown that the formation of acyl-CoA from fatty acids, rather than just fatty acids, is required for lipotoxic liver injury [23]. Thus, it is poorly understood if fatty acids per se or their metabolites are responsible for liver injury. Nonetheless, there is sufficient data demonstrating that a surplus of fatty acids or their metabolites in hepatocytes play protective and causative roles in lipotoxicity.

Fatty acids serve as ligands for the fatty acid sensors, peroxisome proliferator-activated receptor (PPAR)α and PPARγ. Upon activation, fatty acid signaling via PPARα and PPARγ leads to the disposal of fatty acids through oxidative and storage pathways, respectively. However, individuals with NASH have been found to have reduced hepatic expression of PPARα, indicating a potentially impaired ability to oxidize FFA [24]. As such, if FFA delivered to the liver are in too high of quantities, the hepatocytes' ability to store FFA or oxidize them may be overwhelmed, predisposing it to lipotoxicity. Further, when mitochondria are overwhelmed and are not able to handle the FFA influx through oxidative pathways, mitochondrial damage and dysfunction ensues. Although mitochondrial damage is a hallmark of NAFLD progression, the mechanism and degree of mitochondrial dysfunction remains poorly identified. What is known is that the mitochondria make adaptations as NAFLD progresses, whereby the increase in nutrient overload leads to an early increase in oxidative capacity of the mitochondria. This chronic activation of mitochondria eventually leads to a high oxidative stress burden and mitochondrial

mass, followed by mitochondrial dysfunction and failure [25]. It is also characterized by mitochondrial anaplerosis, leading to an increase in gluconeogenesis and oxidative stress [26]. Lastly, it is hypothesized that mitochondrial dysfunction leads to the toxic formation of lipid metabolites that impair insulin signaling in a protein kinase C (PKC)-dependent fashion [27]. Despite this, much is still unknown how this mitochondrial damage occurs, including where the mitochondrial damage originates (muscle, adipose, or liver), however mitochondrial dysfunction is consistently associated with NASH. This may be a result of a genetic predisposition, FFA damage, or a combination of both. An alternative route for fatty acids when mitochondria may be dysfunctional or overwhelmed is peroxisomal beta oxidation or cytochrome P450 (CYP) enzymes, both of which are similarly regulated by PPARα [28].

Collectively, mitochondrial, peroxisomal, or CYP oxidation of fatty acids may inherently promote reactive oxygen species (ROS) and enhance oxidative stress [28]. Oxidative stress and ROS can stimulate lipotoxic stress in the ER, which later leads to hepatocellular apoptosis, a key feature of hepatocellular changes in NAFLD [29]. Kupffer cell activation is a major characteristic of NASH and is closely related to lipotoxicity. Stimuli related to lipotoxicity that activate Kupffer cells include peroxidized lipids, increased cholesterol uptake in the liver, saturated fatty acids, gut microbiota material, and ROS [30]. Kupffer cell activation is mediated largely by toll-like receptors (TLRs), a class of pattern recognition receptors that activate inflammatory pathways and cytokine production, such as the activation of nuclear factor-kappa beta (Nf-κβ) and the production of tumor necrosis factor-α (TNFα) [31]. Stellate cells are the major contributors to fibrosis development in NASH via the secretion of collagen and extracellular matrix proteins [32]. Stellate cell activation is not fully understood, but it is thought that this is largely mediated by the inflammatory stimuli released by Kupffer cells and by hepatocyte-derived products of peroxidized lipids (thiobarbituric acid reactive substances (TBARS)) [30]. Interestingly, stellate cells can directly respond to stimuli, too, because they express TLRs on their membranes [30].

Thus, although the origin of lipotoxic intermediates and the mechanism involved in lipotoxicity is not fully understood, it is well-characterized that lipotoxic intermediates promote the activation of immune cells responsible for promoting inflammation, fibrogenesis, and perpetuating altered hepatic lipid metabolism.

De novo lipogenesis

Excess endogenous synthesis of lipids, also known as DNL, is modified by several complex and intricate pathways. Dietary factors can contribute to DNL, such that excess carbohydrates, especially fructose and its derivatives, amino acids, and saturated fat, can stimulate the formation of new fatty acids. Fructose enters the glycolytic pathway and bypasses phosphofructokinase-1 (PFK1), the rate-limiting step in glycolysis. Considering that PFK1 activity is inhibited by increasing concentrations of its by-products, fructose then bypasses a key negative feedback loop and provides an unregulated source of the main substrate for DNL, acetyl-CoA. In addition, considering that insulin resistance often parallels NAFLD, impaired peripheral glucose uptake and disposal then represents another mechanism by which excess glucose is presented to the liver, enhancing DNL.

De novo lipogenesis is activated when the transcription factors sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate response element binding protein (ChREBP), and PPARγ are stimulated. ChREBP is largely regulated by carbohydrates, including glucose and fructose, but works in conjunction with SREBP-1c to regulate DNL. SREBP-1c regulates genes involved in DNL, including fatty acid synthase and acetyl-CoA carboxylase (ACC), and is positively regulated by the liver x receptor (LXR), insulin and dietary fat, particularly saturated fat. Thus, in the context of insulin resistance or diets high in saturated fat, DNL is upregulated through a SREBP-1c-mediated effect. More, considering that insulin resistance often parallels NAFLD, this SREBP-1c-mediated stimulation of DNL is clinically relevant. Interestingly, in

those with NAFLD it is thought that differential expression patterns of IRS-1 and IRS-2 contributes to hepatic insulin resistance and leads to the up-regulation of SREBP-1c and the subsequent increase in triglyceride synthesis [33]. Specifically, in the context of hepatic insulin resistance, IRS-1 signaling through SREBP-1c remains active while IRS-2 signaling to suppress gluconeogenesis is deactivated. Importantly, SREBP-1c is down-regulated upon bile acid stimulation of the farnesoid x receptor (FXR) and this is mediated by FGF19. FGF19 has been shown to inhibit insulin-mediated DNL and promote fatty acid oxidation. Thus, in the context of hyperinsulinemia, FGF19 may serve as a protective mechanism by inhibiting SREBP-1c activity and protecting the liver from hepatic fat accumulation [35].

Adipose-tissue insulin resistance

It is increasingly evident that adipose-derived insulin resistance plays a central, but not entirely autonomous, role in hepatic insulin resistance and NAFLD [34]. Obesity with adipocyte dysfunction is a common feature of NAFLD, and insulin resistance contributes to adipose tissue dysfunction, especially in the context of obesity. During obesity, and as a consequence of over nutrition, the adipose tissue is saturated and the ability to store excess fat is limited. As a result, adipose tissue hypertrophy is overwhelmed, and hypoxia and death of expanding adipocytes ensues [35]. The anti-lipolytic effects of insulin are impaired and increased FFA are released from adipose and delivered to the liver. Adipose-derived inflammation and FFA release increase, perpetuating adipose-derived insulin resistance and adipocyte dysfunction. This is characterized by decreased anti-inflammatory adipokines from the adipose tissue, namely adiponectin, and increased pro-inflammatory adipokines from the adipose tissue, namely leptin, contributing to intrahepatic fat accumulation. Adiponectin is also important for the regulation of hepatic fatty acid beta-oxidation through adenosine monophosphate kinase (AMPK) and ACC signaling. Further, adipocyte death and inflammatory stimuli lead to the recruitment of M1 macrophages (pro-inflammatory) and the proliferation of resident macrophages, resulting in crown-like

structures, or the clustering of immune cells around dead adipocytes. This characterizes the hallmark of adipose tissue inflammation – an increase in the ratio of M1 (pro-inflammatory) to M2 macrophages – and is associated with insulin resistance and metabolic disease [36]. Hypoxia concomitantly leads to adipocyte stress that is characterized by the up-regulation of genes involved in inflammation, however it is unknown if hypoxia is a consequence of adipocyte expansion or precedes and is causally linked to adipose tissue dysfunction. Nonetheless, adipose-derived inflammation is an important source of inflammatory stimuli including TNF-α, IL-6 and IL-1β that function by activating immune cells in the liver, including Kupffer cells and stellate cells, driving hepatic insulin resistance and NASH.

In the liver, insulin resistance contributes to up-regulation of DNL and inhibition of beta-oxidation of FFA, further promoting hepatic triglyceride accumulation [37]. The increase in delivery of FFA to the liver as a result of adipose-derived insulin resistance has an array of metabolic effects, most notably the progression of ectopic lipid storage in hepatic tissues. Simultaneously, increased inflammatory signals as a result of adipose tissue dysfunction result in exacerbating immune cells in the liver and driving fibrosis and hepatic insulin resistance. Lipotoxicity drives the progression further, where it perturbs mitochondrial function, oxidative stress, and the activation of inflammatory pathways. This interferes with normal liver lipid metabolism, which involves an intimate balance of three mechanisms: liver uptake and de novo synthesis of FFAs, energy expenditure of FFAs via oxidation or de novo triglyceride synthesis, and export of triglycerides from the liver as very low-density lipoproteins (VLDL). Taken together, this increase in FFA delivery to the liver over-burdens the liver, leading to further triglyceride accumulation (steatosis), the hallmark of NAFLD, and muscle, hepatic, and whole-body insulin resistance begins to develop.

The Gut-Liver Axis

The gut-liver axis represents another mechanism contributing to the pathogenesis of NAFLD. Blood drains from the intestines through the portal vein to the liver, thus the liver receives a considerable amount of blood from the intestines, making the liver one of the most exposed organs to intestinally derived immune modulators. Lipopolysaccharide (LPS) is a component of gram-negative bacteria that originates in the intestines and can initiate an inflammatory response when present in systemic circulation. As such, LPS influences hepatic triglyceride accumulation and NASH progression by way of a few mechanisms. First, via it's interaction with TLR-4 on Kupffer cells and the subsequent activation of inflammatory pathways, including Nf-κβ, leading to the production of pro-inflammatory cytokines. Second, LPS interacts with TLR4 on Kupffer cells, leading to the ROS-dependent activation of X-box binding protein-1 (XBP-1), a transcription factor that is activated in response to ER stress, leading to the subsequent up-regulation of genes involved in adipogenesis and lipogenesis[38, 39]. Lastly, patients with NAFLD have increased intestinal permeability and small intestine bacterial overgrowth [40, 41].

Another important pathway by which the gut-liver axis contributes to the pathogenesis of NAFLD is via the bile acid-FXR-FGF19 axis. Recently, bile acids have been identified as signaling molecules through their interaction with nuclear receptors and are important compounds regarding liver damage observed in NAFLD. Specifically, those with NAFLD tend to have impaired bile acid synthesis and higher circulating and fecal bile acids [42, 43]. Dysregulated bile acid metabolism in those with NAFLD leads to bile acid accumulation in the liver, which can cause hepatic toxicity [42]. Bile acid damage to the liver may be due to NAFLD individuals exhibiting higher hepatic expression of cholesterol 7-alpha hydroxylase (CYP7α1), the rate-limiting enzyme in bile acid synthesis pathway, and more total bile acids when compared to those without NAFLD [42]. Interestingly, FGF19's primary function is to directly repress bile acid synthesis via inhibiting CYP7α1 [44]. As such, recent studies suggest that impaired bile acid

homeostasis observed in those with NAFLD may be related to lower FGF19 levels. Specifically, individuals with NAFLD exhibit lower circulating FGF19 than those without NAFLD, and low FGF19 may precede NAFLD development [45-48]. The etiology of reduced FGF19 in those with NAFLD has not been clearly explained; however, it may be due to an adverse bile acid composition. Specifically, the secondary bile acid deoxycholic acid (DCA) is disproportionately higher in concentration compared to other bile acids in those with NAFLD. DCA is an FXR antagonist, therefore greater DCA concentration may lead to reduced activation of FXR, leading to impaired intestinally derived FGF19 production and FGF19 signaling in the liver. Under normal conditions, bile acids signal through FXR in the enterocyte, leading to FGF19 production and FGF19 later activates FGFR4 on hepatocytes. The activation of SHP and signaling via β-klotho receptor, FGF19's cognate receptor, is important for the FGF19-FXR activity and repressing bile acid synthesis via CYP7α1 expression in the liver. Not surprisingly, those with NAFLD do not exhibit greater expression of SHP when compared to healthy controls, suggesting that bile acid accumulation in NAFLD may be related to defective FGF19 signaling [42]. Jiao *et al.* observed that while individuals with NAFLD exhibited increased expression of FGFR4, expression of β-klotho receptor was not elevated [42]. Therefore, in those with NAFLD, it may be a combination of lower FGF19 levels and impaired FGF19 signaling that leads to dysregulated bile acid homeostasis.

FGF19 also modulates hepatic triglyceride metabolism via several mechanisms, including promoting fatty acid oxidation and repressing hepatic DNL, thus protecting the liver from steatosis [31, 32]. Specifically, transgenic mice overexpressing FGF19 exhibited reduced hepatic triglyceride accumulation, body weight, and fat mass, and increased expression of enzymes involved in DNL [11]. These effects were largely mediated by FGF19's inhibitory effect on SREBP-1c and stearoyl CoA desaturase 1 [14, 33]. FGF19 has been shown to inhibit SREBP-1c via several cellular mechanisms, including the inhibition of peroxisome proliferator-activated

receptor y coactivator-1β (PGC-1β), increased expression of signal transducer and activator of transcription 3 (STAT3), and the activation of SHP, a negative regulator of CYP7α1. Inhibition of PGC-1β has recently been explored as a therapeutic target for insulin resistance in the context of NAFLD, specifically as it relates to dietary components (i.e. high-fructose containing diets) [34].

Although the etiology of impaired bile acid homeostasis and dysregulated FGF19 in the context of NAFLD has not been clearly elucidated, a role for FGF19 in regulating energy homeostasis, stimulating glycogenesis, inhibiting gluconeogenesis, preventing insulin-mediated lipogenesis, promoting fat oxidation, and reducing hepatic fat accumulation has been discovered [49]. In addition, there is now a clinical course for "lean NAFLD", where some individuals with NAFLD are normal weight by BMI, but display high visceral adiposity, insulin resistance, and metabolic abnormalities consistent with NAFLD. Lean NAFLD is characterized by a unique genetic profile and alterations in the BA and gut microbiota profile. Specifically, those with lean NAFLD exhibit elevated BA with concomitant increased FGF19 concentrations [50]. This observation is the foundation for a unique pathophysiology in those with lean NAFLD, which is characterized by "obesity resistance", such that those with "lean NAFLD" possess an early metabolic adaptation where BA production is increased in response to an obesogenic environment (i.e. excess dietary cholesterol), leading to increases in FGF19. Taken collectively, the gut-liver axis represents an important component of the multi-organ pathophysiology of NAFLD, however much remains unknown regarding its contribution to the development of NAFLD.

Non-alcoholic Fatty Liver Disease Screening Tools

NAFLD is treatable via lifestyle in its early stages, but more advanced liver pathologies (i.e. NASH and cirrhosis) are more difficult to reverse. Steatosis is reversible with lifestyle modification, but there are currently no FDA-approved medications for NASH. Therefore, if

steatosis is not reversed, NAFLD is of strong concern in children because the increased risk for advanced liver conditions will persist over many years – from childhood to adulthood. Unfortunately, screening for early stages of liver triglyceride accumulation is imperfect and often relies on clinical experience and judgment. Commonly used biomarkers of liver defects (e.g. ALT, AST, and other enzymes) are inadequate screening tools because they are non-specific for NAFLD. In addition, liver enzymes are often not elevated in the early stages of liver triglyceride accumulation and can frequently be in normal ranges even in patients with NAFLD and NASH, therefore leading to false negatives [1]. In fact, a recent meta-analysis observed a significantly lower prevalence of NAFLD when using ALT as the determinant of NAFLD compared to studies that used more robust diagnostic methods, such as biopsy, ultrasound, and magnetic resonance imaging (MRI). Specifically, research has shown that approximately 80% of adults with NAFLD would be missed if diagnosis was based on liver enzymes [51].

Moreover, roughly 34% of children with obesity have NAFLD, which is the most common cause for liver transplantation in children [52]. Because obesity is the largest risk factor for NAFLD, current screening methods focus on children with obesity [1]. Currently, children with obesity are screened for NAFLD using liver enzymes, however approximately 70% of children with NAFLD have been found to have normal alanine transaminase (ALT) levels [53]. Thus, utilization of liver biochemistries is so uncertain that the American Gastroenterological Association advises against their use [1]. In addition, liver biochemistries can increase for reasons unrelated to NAFLD, such as hemochromatosis, celiac disease and in response to acetaminophen and certain prescription medications. Further, although NAFLD prevalence is common in children with obesity, it is by no means concurrent. Specifically, the prevalence of NAFLD in children with obesity and without obesity is estimated to be 26% and 5-20%, respectively [2, 52, 54]. Little research has been done to understand why some children with obesity develop NAFLD and why some do not. In addition, there is now a clinical course for “lean NAFLD”, where some

with NAFLD are normal weight by BMI, but display high visceral adiposity, insulin resistance, and metabolic abnormalities consistent with NAFLD. This lack of understanding has concerns for public health guidelines, including developing screening guidelines for children with obesity with NAFLD [52]. Considering these limitations, NAFLD is understood to be underdiagnosed in both adults and children.

Collectively, liver enzymes may not be the most effective approach to detecting early stages of liver fat accumulation. The gold standard for diagnosis of NAFLD is liver biopsy, however this approach is expensive and invasive, leaving it impractical for use as a screening tool. Ultrasonography and MRI are useful alternatives, but they still require cost and skill, and lack the sensitivity of liver biopsy. Thus, there is a critical need to develop a child- and adult-friendly but sensitive screening tool for early detection of NAFLD. In the absence of such a development, efforts to detect, treat, and reverse NAFLD in children and adolescents will likely remain difficult. Thus, a more precise screening tool for early detection of NAFLD is needed, especially in children and adolescents.

It is well known that the standard criterion for diagnosing NAFLD is hepatic triglyceride accumulation of >5% of hepatocytes. Thus, triglyceride accumulation is central to the development of NAFLD and fasting triglycerides have been explored as a potential screening tool for NAFLD. Specifically, fasting triglycerides have been a component of several indices that have been developed as screening tools for NAFLD, including the hepatic steatosis index (HSI) and fatty liver index (FLI) [55]. However, a number of additional biomarkers, including liver biochemistries, which are non-specific to NAFLD, are required in order for these indices to possess clinical diagnostic utility, rendering fasting triglycerides an inapt option for NAFLD screening. One potential early screening tool is the assessment of postprandial triglycerides and FGF19 in the context of an AFTT.

Postprandial Triglyceride-rich Lipoprotein Metabolism and Non-alcoholic Fatty Liver Disease

Postprandial lipemia is increased circulating triglycerides following a meal and can occur via increased triglyceride appearance, reduced catabolism of triglyceride-rich lipoproteins, or some combination [56]. The majority of meals, except breakfast, are likely consumed before the body has returned to post-absorptive values and therefore induce summative lipemic increases throughout the day [56]. Thus, although fasted triglycerides are more commonly assessed in clinical settings, postprandial triglycerides are arguably a more appropriate measure of CVD risk when compared to fasting triglycerides, owing to the fact that postprandial triglycerides represents most individuals' metabolic state during daily living [57]. Evidence has revealed the role that postprandial triglycerides play in atherogenesis [58-60] and elevated triglycerides, despite the use of LDL-C lowering statin therapy, represents residual risk for atherogenesis [61]. The potentially causal role of elevated triglycerides in CVD has not been fully elucidated; however, elevated levels of postprandial triglycerides signify the presence of pro-atherogenic triglyceride-rich lipoproteins (apolipoprotein-B (ApoB) containing lipoproteins), including very-low density lipoprotein (VLDL), chylomicrons, and their lipoprotein remnants.

After the consumption of a high-fat meal, chylomicrons, or ApoB48-containing particles, rich in dietary triglycerides accumulate in circulation. This accumulation of chylomicrons in the postprandial period leads to the inhibition of VLDL metabolism or clearance by the liver, mediated via substrate competition for LPL. More specifically, in the context of a high-fat meal, LPL can become saturated and may lead to VLDL accumulation and impaired clearance of triglyceride-rich lipoproteins by the liver [62, 63]. In fact, the majority of the increase in triglycerides after a high-fat meal are found in ApoB48-containing lipoproteins. However, because of the substrate competition between chylomicrons and VLDL, the majority of lipoproteins in circulation in the postprandial period are ApoB100-containing lipoproteins because chylomicrons are cleared rapidly from circulation postprandially. Nonetheless,

postprandial VLDL accumulation is also due in part to the fact that chylomicron lipolysis results in an increased delivery of FFA to the liver, leading to up-regulation of VLDL production.

As the CM and VLDL are cleaved by LPL at peripheral tissues, they become chylomicron remnants (CM-R) and VLDL remnants, otherwise known as remnant lipoproteins. These remnant lipoproteins can penetrate the vascular endothelium and contribute to foam cell development, a hallmark of atherogenesis [64]. For example, VLDL in particular can be taken up by macrophages directly without modification and contribute to foam cell development in the vasculature [65, 66]. In addition, remnant lipoproteins may be preferentially retained in the endothelium due to their larger size, when compared to LDL [67, 68]. More, although LDL contains more cholesterol as a proportion of lipids when compared to remnant lipoproteins, remnant lipoproteins contain considerably more absolute cholesterol. Thus, increased circulating remnant lipoproteins represent an atherogenic phenomena. Lastly, high levels of postprandial triglycerides may serve as a proxy for insulin resistance, visceral adiposity, NAFLD, all of which significantly increase risk for CVD [69-72].

Dyslipidemia is a common feature in individuals with NAFLD [73]. Importantly, increased fasting and postprandial triglycerides is a common observation in those with NAFLD and is associated with the degree of hepatic steatosis, fibrosis, and inflammation [6]. Dyslipidemia in NAFLD is commonly characterized by increased postprandial triglycerides, or triglyceride-rich lipoproteins. The metabolism of these triglyceride-rich lipoproteins leads to a more pro-atherogenic lipid profile characterized by increased remnant lipoproteins, small-dense LDL, and decreased HDL-C. In addition, hypertriglyceridemia in the context of NAFLD is closely related to insulin resistance and its subsequent metabolic dysfunctions, and although insulin resistance and obesity are closely linked, insulin resistance in the context of NAFLD occurs even in the absence of diabetes mellitus and obesity [4, 5].

As previously mentioned, the mechanism for hepatic triglyceride accumulation in NAFLD involves an imbalance between hepatic triglyceride production, export or oxidation. Considering that a major role of VLDL is to export triglycerides out of the liver, hepatic secretion and metabolism of VLDL then represents an important avenue for the removal of triglycerides from the liver. However, the relationship between NAFLD and VLDL kinetics is unclear. Recent evidence demonstrates a fundamental defect in VLDL metabolism in those with NAFLD, such that those with NAFLD demonstrate over-production of large, triglyceride-rich VLDL particles ($VLDL_1$), leading to consequential pro-atherogenic lipoprotein changes, such as higher circulating remnant lipoproteins, smaller LDL, and reduced HDL-C [74, 75].

The question remains, if VLDL secretion is increased in those with NAFLD, then why do triglycerides accumulate in the liver and perpetuate steatosis? The regulation and production of VLDL is a complex system. The over-production of $VLDL_1$ may not be enough to prevent hepatic triglyceride accumulation. It is well-documented that individuals with NAFLD exhibit increased FFA release from adipose into the plasma and delivery to the liver and this is coupled with increased secretion of VLDL-triglycerides when compared to those without NAFLD. Interestingly, studies have found that the concentrations and secretion rate of ApoB-100 and ApoB-48 is unchanged in the fasting and postprandial period in those with NAFLD compared to obese controls without NAFLD, despite the presence of greater hepatic triglyceride content [76, 77]. This may indicate that VLDL particles produced by NAFLD patients contain more triglycerides and are larger than those in people with NAFLD. Additionally, it appears that those with NAFLD have increased FFA delivery to the liver and increased triglyceride production, yet a stunted capacity to export the triglycerides from the liver related to unchanged production of ApoB-100 [76, 77]. Considering that ApoB-100 synthesis is the chief route of hepatic triglyceride export, it is conceivable that those with NAFLD may not produce adequate amounts of ApoB-100 to compensate for the hepatic triglyceride infiltration and this may limit triglyceride export,

perpetuating steatosis. Notably, this is also related to insulin sensitivity. Specifically, under normal conditions, insulin down-regulates VLDL secretion from the liver. However, in those with insulin resistance, a common feature of NAFLD, the over secretion of VLDL can ensue due to the inability of insulin to suppress VLDL secretion [74]. Insulin resistance has also been shown to drive DNL, leading to greater hepatic triglyceride content [37]. Much research is still needed to understand VLDL kinetics and metabolism in the context of NAFLD.

In addition to the importance of VLDL metabolism and kinetics for understanding NAFLD, the origin of FFA delivered to the liver is of importance, as this has been shown to modulate VLDL-triglycerides and VLDL secretion. Much research demonstrates that triglycerides derived from DNL increase the size of VLDL secreted by the liver, but not the number of VLDL [78]. In other words, DNL may stimulate the secretion of larger, triglyceride-rich VLDL, but not the total number of VLDL. In contrast, FA in circulation taken up by the liver may directly stimulate ApoB-100 secretion, presumably leading to an increased total VLDL number [79]. Nonetheless, increased delivery of FFA to the liver and hepatic export of VLDL-triglycerides contribute to fasting and postprandial hypertriglyceridemia and are thought to be intimately related to insulin resistance and liver fat. In fact, liver fat is closely correlated with serum triglycerides, thus serum triglycerides may serve as a viable proxy of liver fat [80] and the postprandial period is of clinical relevance for improved hepatic triglyceride accumulation and removal.

It was historically thought that increased postprandial triglycerides were a result of interrupted lipolysis of VLDL due to chylomicron competition combined with increased VLDL secretion by the liver. While this still remains partially true, it is understood now that increased ApoB-48 production from the intestine occurs basally in the context of hyperinsulinemia and hypertriglyceridemia [81], both of which are key features of NAFLD. Lastly, the “spillover” of dietary FA from the hydrolysis of chylomicrons and VLDL represents a potential mechanism by

which the postprandial period is an important modulator of hepatic lipid metabolism. For example, even modest quantities of FA as a result of chylomicron hydrolysis at target tissues, that escape re-esterification at the adipose tissue, are taken up by the liver and could stimulate ApoB-100 secretion [15]. Therefore, fasting triglycerides may not capture these physiological processes that are clinically relevant and may help to distinguish those at risk for NAFLD.

Postprandial Triglycerides as a Screening Tool for Non-alcoholic Fatty Liver Disease

A major underlying origin of triglyceride accumulation in NAFLD relates to the failed ability of the liver to regulate lipogenesis that normally occurs when switching from the fasted to fed state. As such, it has been shown in both children [77] and adults [73] that individuals with NAFLD exhibit an elevated postprandial triglyceride response to an high-fat meal. More specifically, adults and children with NAFLD display a sharp increase in triglycerides in response to a high-fat meal and this elevation is sustained throughout the postprandial period. This increase in postprandial triglycerides is coupled by an increase in large VLDL particles, suggestive of pro-atherogenic behavior. The greater postprandial triglyceride response is likely a result of excessive triglyceride accumulation in the liver, leading to impaired hepatic triglyceride uptake and clearance or perhaps excessive VLDL-triglyceride release, however the exact explanation for this greater postprandial triglyceride response in those with NAFLD is not clearly defined. Despite this, NAFLD has recently been described as a multi-organ disease of insulin resistance. More specifically, adipose, muscle, and hepatic insulin resistance are closely related to hepatic triglyceride content [4]. Hepatic insulin resistance results in impaired insulin-mediated suppression of gluconeogenesis, resulting in an increase in non-lipid precursors for DNL, as well as increased VLDL-triglycerides. More, adipose tissue insulin resistance results in an impaired suppression of lipolysis, resulting in an increase in FFA delivery to the liver, leading to increased substrate for triglyceride synthesis and VLDL-triglyceride secretion.

Children and adults with NAFLD demonstrate concurrent rises in insulin and triglycerides in response to a high-fat meal and this may be partly related to adiponectin concentrations [77]. Adiponectin, an anti-inflammatory adipokine, is closely linked to insulin resistance, such that adiponectin can offset the effects of hyperinsulinemia by facilitating FFA oxidation and limiting DNL [82]. Specifically, in adults without NAFLD, a postprandial rise in triglycerides elicits a compensatory adiponectin increase, which results in FFA beta-oxidation and triglyceride-rich lipoprotein catabolism in target tissues [83]. However, this compensatory mechanism does not occur in adults with NAFLD and is persistently associated with insulin resistance. For example, Musso *et al.* found that when compared to those without NAFLD, adults with NAFLD had higher postprandial triglycerides and this was independently correlated with adiponectin concentrations [84]. In other words, adults with NAFLD with higher postprandial triglycerides had lower adiponectin concentrations and adults with NAFLD did not demonstrate a compensatory increase in adiponectin in response to a high-fat meal. Conversely, in adults without NAFLD, adiponectin increased postprandially, as would be expected.

Alterations in adiponectin often precede metabolic complications and administration of adiponectin has been shown to reverse NASH mediated partly by inhibitory effects on TNFα [85]. Therefore, lower circulating adiponectin in those with NAFLD may represent one potential explanation for elevated postprandial triglycerides and enhanced steatosis in those with NAFLD. This may differ between children and adults, though. For example, fasting and postprandial adiponectin concentrations did not differ between children with NAFLD and lean and obese children without NAFLD [77]. This dissimilarity between children and adults may indicate that metabolic aberrations are not yet fully present in children, despite apparent dysfunction in postprandial lipid handling in both children and adults. In addition, Mager *et al.* included children primarily with simple steatosis, compared to Musso *et al.* who included adults with diagnosed

NASH. Considering that simple steatosis and NASH present with differing levels of inflammation, this may largely explain differences observed in adiponectin concentrations.

It has also been observed that adults with NAFLD have decreased ApoB production, despite increased VLDL-triglycerides, which is required for hepatic production of VLDL [76]. Hence, export of triglycerides from the liver in the form of VLDL is dependent upon ApoB-100. Therefore, in the absence of sufficient ApoB-100, excess lipids are likely retained in the liver, perpetuating steatosis. In addition, VLDL production is strongly correlated with liver triglyceride content, such that those with higher liver fat have higher production of large, triglyceride-rich VLDL [86]. However, this appears to plateau when hepatic triglyceride content is ≥10%, indicating that there may be a certain threshold whereby hepatic triglycerides fail to be exported as a component of VLDL and are retained in the liver [76]. Musso *et al.* found that adults with NASH had higher postprandial total triglycerides and VLDL-triglycerides when compared to age- and BMI-matched controls [84]. However, when compared to controls that exhibited concurrent rises in ApoB-100 and ApoB-48 with triglycerides, adults with NASH exhibited relatively unchanged ApoB-100 and ApoB-48 concentrations, indicative of an impairment in VLDL secretion and potentially larger, less-dense, triglyceride-rich ApoB particles [84]. In fact, it has been observed that adults with NAFLD have an increased triglyceride-ApoB ratio and increased particle size [73]. Additionally, these larger, less-dense, triglyceride-rich ApoB particles suggest an impaired enzymatic activity of LPL, which is a common finding in those with insulin resistance and could explain some of the exaggerated postprandial triglyceride response in those with NAFLD [87]. The relationship between large, less-dense, triglyceride-rich ApoB particles and LPL is likely related to ApoC-III concentrations as well, where ApoC-III inhibits LPL activity and interferes with the interaction between triglyceride-rich lipoproteins and LPL [88, 89].

Conversely, children with NAFLD have a unique and dissimilar postprandial response with regard to ApoB-48. Mager *et al.* demonstrated that unlike adults who demonstrate stagnant or diminished ApoB-48 in the postprandial period, children with NAFLD exhibit elevated ApoB-48 in the postprandial period when compared to obese and lean children without NAFLD [77]. More, elevations in ApoB-48 were sustained throughout the postprandial period, indicating that children with NAFLD may have an exaggerated response to high-fat meal coupled with delayed clearance of ApoB-48 containing particles. So far, this is a unique finding to pediatric NAFLD and demonstrates some of the unique pathophysiology of pediatric NAFLD. Despite this finding, Mager *et al*. did not find differences in fasting or postprandial ApoB-100 in children with NAFLD when compared to obese and lean children without NAFLD or throughout the postprandial period, which is consistent with findings in adults with NAFLD [77]. Importantly, though, this unique response observed in children, characterized by exaggerated ApoB-48 production in the postprandial period, may be undetected if using fasting triglycerides alone. Lastly, as previously mentioned, ApoB-48 production rates by the intestines may be largely related to the degree of insulin resistance.

The insulin-mediated clearance of triglyceride-rich lipoproteins is partly regulated by ApoC-III, which is a component of triglyceride-rich lipoproteins and works to inhibit LPL. Specifically, children with NAFLD display elevated ApoC-III concentrations in the fasting state and suppressed NEFA in the fed state [77]. Considering that LPL is essential for the removal of triglycerides from VLDL, higher ApoC-III in children with NAFLD likely relates to the exaggerated postprandial triglyceride response in children with NAFLD, and may contribute to exacerbating steatosis [87]. During the fasting state, NEFA are usually higher, signifying their use for fuel during fasting. During the fed state, NEFA are initially suppressed and then usually increase, due to enhanced lipolysis of triglycerides from meal intake. Thus, suppressed NEFA

throughout the postprandial period is likely partly related to insulin resistance and reduced ApoC-III levels.

Mechanisms that explain why those with NAFLD have reduced ApoB production is of clinical relevance. The apparent inactivity in ApoB-100 secretion from the liver in those with NAFLD could be related to a reduction in MTP or ER stress, leading to greater triglyceride enrichment of VLDL, providing one plausible link between elevated fasting and postprandial triglycerides and NAFLD. Specifically, increased FFA delivery to the liver can induce ER stress which can inhibit ApoB-100 assembly and secretion from the ER [90]. Lastly, it is well known that those with NAFLD have increased VLDL production and impaired insulin-mediated suppression of VLDL. VLDL compete with chylomicrons for clearance via LPL. Therefore, larger VLDL particles enriched in triglycerides observed in those with NAFLD may also be a result of impaired clearance and result in longer residence time. Longer residence time of these triglyceride-rich VLDL has been found to be a result of an impaired ability of the liver to clear these particles, further contributing to increased accumulation. As such, postprandial triglyceride assessment may represent a proxy for detection of this phenomenon.

FGF19 as a Screening Tool for Non-alcoholic Fatty Liver Disease

Research has focused on identifying secondary factors connected to hepatic lipid metabolism that contribute to the detrimental transition from simple steatosis to NASH. The endocrine compound FGF19 may be one factor related to postprandial dyslipidemia in the context of NAFLD, where it has emerged as an important intestinally derived regulator of bile acid homeostasis. As previously mentioned, bile acids and bile acid receptors have been implicated in the pathogenesis of NAFLD and are potential therapeutic targets for the treatment of NAFLD. Several studies have found that individuals with NAFLD have dysregulated bile acid metabolism, where upregulated bile acid synthesis leads to elevated circulating and fecal bile acids [19, 42, 44,

47, 91, 92]. Increased circulating bile acids leads to the toxic accumulation of bile acids in hepatocytes, which propagates inflammation, oxidative stress, and the worsening of NAFLD.

In addition to FGF19's central role in bile acid homeostasis and gallbladder refilling [44], FGF19 is a nutritionally regulated postprandial hormone capable of inhibiting gluconeogenesis and stimulating glycogenesis and protein synthesis, without stimulating lipogenesis [42, 93-97]. FGF19 regulates key features of triglyceride metabolism closely linked to NAFLD including fatty acid oxidation, DNL, export of triglycerides from the liver, inflammation, lipotoxicity, and steatotic ER stress [19, 70]. Importantly, individuals with NAFLD tend to have lower circulating FGF19 and individuals with insulin resistance in the context of NAFLD display hepatic resistance to FGF19, which may exacerbate NAFLD progression [45-48].

Recently, FGF19 has been proposed as a novel biomarker for steatosis in those with NAFLD. This is partly because FGF19 production has been shown to be impaired in those with NAFLD and insulin resistance and is intimately related to hepatic triglyceride metabolism [7, 8]. In fact, Nobili *et al.* investigated the relationship of FGF19 with the Pediatric NAFLD Histological Score and found that FGF19 was inversely and independently associated with NASH [7]. Additionally, adults with NAFLD with severe fibrosis have lower fasting FGF19 when compared to those with mild-moderate fibrosis [50]. However, the majority of research regarding FGF19 and NAFLD has used fasting FGF19. Considering that FGF19 is a postprandial hormone, fasting FGF19 is likely a less sensitive and specific indicator of steatosis and fibrosis in children with NAFLD when compared to postprandial FGF19.

What is more, differences in postprandial FGF19 may enhance our understanding of why some children with obesity do not develop NAFLD. For example, obese and overweight children with NAFLD exhibit lower postprandial FGF19 when compared to normal weight children without NAFLD [47]. However, these differences in postprandial FGF19 may be confounded by

overweight/obesity and it is unclear whether the difference in postprandial FGF19 is attributed to overweight/obesity or NAFLD. In fact, Wojcik *et al.* found that children with obesity and NAFLD exhibited lower fasting FGF19 when compared to children with obesity without NAFLD [8]. Despite these findings, postprandial FGF19 has not been studied in obese children with and without NAFLD. Lastly, other risk factors for NAFLD, including insulin resistance, are related to FGF19, where FGF19 is impaired in adults with insulin resistance with NAFLD when compared to adults with NAFLD without insulin resistance [98].

CHAPTER III

METHODS

Pediatric Participants

In a case-control pilot study, we recruited 3 groups of children (13.0-20.9 years): obese NAFLD cases (n = 9), obese controls (n = 13), and normal weight controls (n = 15). NAFLD cases were recruited from Oklahoma Children's Hospital (Department of Pediatrics, Section of Gastroenterology; Oklahoma City, Oklahoma). NAFLD cases were children with confirmed presence of NAFLD via liver biopsy, elevated liver enzymes, and BMI $\geq$ 30 kg/m^2 or $\geq$ 95th percentile (depending on their age). A diagnosis of NAFLD was made after the exclusion of other competing liver diseases, including autoimmune or metabolic liver diseases, Wilson's disease, and viral hepatitis. Further, patients with diabetes mellitus, genetic syndromes, kidney disease, hematologic disease, and CVD were excluded. Due to the unethical nature of subjecting non-referred children to liver biopsy, liver steatosis and fibrosis were determined in all study participants via Fibroscan (Fibroscan 502 Touch; EchoSens; Paris, France). Obese and

Normal weight controls had not been diagnosed with NAFLD. Participants were not engaging in sports or other regimented exercise 3 days per week. For participants <20 years old, BMI percentile was calculated using age- and sex-specific growth curves for children developed by the United States Centers for Disease Control. For participants 20.0-20.9 years old, BMI was based on the adult BMI formula. Normal weight was defined by BMI of 18.5-24.5 kg/m^2 or within 5th to 84th percentile and obese was defined by BMI of ≥ 30 kg/m^2 or ≥ 95th percentile. A thorough medical and social history was conducted with all participants prior to enrollment to rule out excessive use of alcohol or tobacco. Written and informed consent was obtained from participants if ≥ 18 years and/or from parent/guardians prior to study initiation. This study was approved the Institutional Review Board of Oklahoma State University (HS-19-58).

Overview of Protocol

Participants completed one visit to the laboratory for testing. On the day prior to the study visit, participants were instructed to avoid vigorous physical activity, begin fasting 10 hours prior to their study visit, and accrue ≥ 7 hours of sleep. On the morning of the study visit, participants arrived at the laboratory fasted. Participants provided their informed consent prior to beginning the study in accordance with the university's Institutional Review Board. Once consent was obtained, participants' weight and height were measured via calibrated stadiometer and balance, respectively, after which participants underwent a Fibroscan (Echosens, Fibroscan 520 Touch). Anthropometry measurements were performed including height, body mass, and waist and hip circumference. Body composition was measured using dual energy x-ray absorptiometry (DEXA) (GE/Lunar iDXA, GE-Healthcare, Fairfield, CT) to determine total body and regional fat and lean tissue. Participants then underwent resting metabolic rate (RMR), pulse-wave velocity (PWV), and heart rate variability (HRV) measurements. A saline-lock IV catheter was then placed, and a baseline blood draw was collected, after which the participant consumed a high-fat meal. At 1- and 3-hours post-meal consumption, participants underwent RMR, PWV, and HRV. During the

session, detailed questionnaires assessing the participants' physical activity (BS-BAQ) and dietary habits (BS-FJV-FFQ) were administered. A postprandial blood draw was collected four hours after completing the high-fat meal.

AFTT

The AFTT is a metabolic test that is a simplified version of a standard oral fat tolerance test and is used to quantify postprandial triglycerides. We have validated this AFTT, revealing that it is valid and reliable for determining postprandial triglycerides [99, 100]. Participants arrived after a 10-hour overnight fast, and a saline-lock IV catheter was placed by a registered nurse and a baseline blood draw (15 mL) was collected into EDTA-coated, lithium heparin-coated, and serum separating vacutainers. Participants then consumed a high-fat meal and a 4-hour follow-up blood draw was collected to quantify the postprandial triglyceride response. The high-fat meal consisted of coconut cream, chocolate syrup, and vegan protein powder and was scaled to body weight (9 kcal/kg; 73% fat, 26% carbohydrate, 1% protein). This meal has been used in previous studies from our laboratory [100-102].

Measurements

Fibroscan uses controlled attenuation parameter (CAP) technology to measure attenuation in the liver with signals obtained by a transient elastography probe to measure liver fibrosis and steatosis. For RMR, HRV and PWV measurements, participants rested in a supine position for ~40 minutes at baseline, and then again at 1-, and 3-hours post-high-fat meal. RMR was measured using an indirect calorimetry system with a flow-through canopy (TrueOne 2400, ParvoMedics, Sandy, UT). For each RMR measurement, the duration of the measurement was approximately 25 minutes. The last 15 minutes of each measurement was used to calculate substrate oxidation. Data for HRV were collected using a chest-strap heart rate monitor that communicated with a Polar V800 wristwatch recorder (Polar Electro USA, Lake Success, NY).

HRV was measured to quantify parasympathetic to sympathetic balance regarding the regulation of heart rate. HRV data were collected for 20-25 minutes during each REE measurement. Data were uploaded to Kubios HRV v2.2 software (University of Eastern Finland, Kuopio, Finland). This software generates many HRV measurements. Specifically, we included representative variables for parasympathetic activity from the time-domain indices: standard deviation of normal-to-normal RR intervals (SDNN), root mean square of successive differences (RMSSD), and proportion of successive NN intervals that differ by more than 50 ms relative to the total number of NN intervals (pNN50); from the frequency-domain indices: low-frequency (LF) power, high- frequency (HF) power, and total power; and from the non-linear indices: dispersion of points from line of identity (SD1).

PWV was measured after the REE and HRV tests at each time point. Carotid-femoral PWV was measured using applanation tonometry with the SphygmoCor XCEL instrument (AtCor Medical, Inc., Itasca, IL). Carotid and femoral artery pulses were measured simultaneously using a hand-held tonometer over the carotid artery and a blood pressure cuff placed around the thigh. Brachial blood pressure was assessed with the same instrument, using a standard blood pressure cuff on the upper arm. Analyses of brachial waveforms were used to calculate central systolic and diastolic blood pressure, using a generalized transfer function, and augmentation index (AIx) of pressure waves. PWV and pulse wave analyses were measured in triplicate at each measurement time, with the average of the acquired values used for data analysis.

Laboratory Values

At baseline and 4-hours post-meal consumption, 15 mL of venous blood was collected. Whole blood was collected for the assessment of metabolic outcomes at baseline and 4-hours (triglycerides, Total-C, VLDL-C, LDL-C, HDL-C) and liver outcomes at baseline (albumin,

alkaline phosphatase (ALP), ALT, amylase, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), bilirubin, and total protein) using a Piccolo Xpress clinical chemistry analyzer (Piccolo, Abbott, Princeton, NJ). Whole blood from the baseline blood draw was used to measured hemoglobin A1c (HbA1c) (Siemens DCA Vantage Analyzer, Malvern, PA). A portion of each blood sample was collected into EDTA-coated tubes and stored on ice and another portion was collected into uncoated tubes and allowed to clot. These samples were subsequently centrifuged at 4 degrees Celsius for 15 minutes at 2200 xg and the resulting 1.5 mL of plasma and 1.5 mL of serum were stored at -80 degrees Celsius for later analysis. Those measurements included fasting and 4-hour serum FGF19 (Invitrogen, Human FGF19 ELISA), fasting serum insulin (Alpco, Insulin ELISA), and fasting serum adiponectin (Invitrogen, Human Adiponectin ELISA).

Statistical Analyses

Data were checked for normality prior to analyses (skewness ≤ 2, kurtosis ≤ 7). When data were not normally distributed, corresponding non-parametric analyses were used or the ROUT method was used to identify and remove outliers as appropriate based on examination of the data. Descriptive statistics were calculated for all variables and presented as mean ± standard deviation (SD). A general linear model was used to compare differences in participant characteristics across groups. Post-hoc analyses using Tukey's HSD test were used to determine group differences. A general linear model with repeated measures was used to compare the postprandial response for triglycerides and FGF19 between NAFLD cases, obese controls, and normal weight controls. Time point was set as the within-subjects factor and group was the between-subjects factor. When significant interactions or main effects were present, post-hoc analyses using Tukey's HSD test were used to determine pairwise differences. The same analyses were used to compare augmentation index, PWV, and RMR measurements across groups.

A general linear model was used to compare the total area under the curve (tAUC) and change from baseline to 4h postprandial (Δ) for triglycerides and FGF19 across groups; post-hoc analyses using Tukey's HSD test were used to determine group differences. An unpaired t-test was used to determine differences in fasting and postprandial triglycerides and FGF19 and fasting insulin between participants with and without steatosis and/or fibrosis, respectively. Pearson's correlations or Spearman's correlations were used to determine the relationships between independent and dependent variables. The diagnostic accuracy of fasting and postprandial triglycerides and FGF19 was determined by evaluating the area under the receiver operating characteristic (ROC) curve (equivalent to c-statistic). The dichotomous categorical variable classified participants into two groups: participants with diagnosed NAFLD and participants free of NAFLD. Effect sizes were calculated for postprandial triglycerides and FGF19. Odds ratio and corresponding 95% confidence intervals were calculated to ascertain the odds of NAFLD by various measures of dietary intake. A p-value of <0.05 was considered statistically significant for all statistical tests. Analyses were conducted using GraphPad Prism (GraphPad Prism Inc.; La Jolla, CA, USA).

CHAPTER IV

RESULTS

Participant Anthropometrics, Demographics, and Characteristics

Participant characteristics are displayed in **Table 1**. NAFLD and obese controls exhibited significantly greater body mass, BMI, body fat %, android body fat, gynoid body fat, and trunk fat compared to normal weight ($p < 0.05$), although NAFLD and obese controls did not differ for any of these measures. Systolic blood pressure was greater in obese than normal weight controls ($p = 0.01$); there were no other group differences in blood pressure ($p > 0.05$). NAFLD participants displayed a significantly higher fibrosis score than both the obese controls (mean difference 4.8 kPa, $p = 0.0008$) and normal weight groups (mean difference 5.1 kPa, $p = 0.0003$). There were no significant differences in fibrosis scores between obese and normal weight controls ($p = 0.94$). NAFLD patients had a significantly higher CAP score (i.e. steatosis) than normal weight (mean difference = 137.7 dB/m, $p < 0.0001$), but NAFLD did not differ from obese controls (mean difference = 43.5 dB/m, $p > 0.05$). There was a significant difference in CAP score between obese and normal weight controls (mean difference = 94.2 dB/m; $p < 0.0001$).

Metabolic and Liver Biochemistries

Fasting metabolic and liver parameters are displayed in **Table 2**. There were no group differences in total cholesterol or LDL-C ($p > 0.05$). NAFLD participants exhibited lower HDL-C than normal weight controls (mean difference = -19 mg/dL; $p = 0.003$), as did obese controls (mean difference = -18 mg/dL; $p = 0.001$). There was no significant difference in HDL-C between NAFLD and obese ($p = 0.96$). NAFLD participants had significantly higher VLDL-C than normal weight controls (mean difference = 10 mg/dL; $p = 0.03$). There was no significant difference in VLDL-C between NAFLD and obese control or obese and normal weight ($p > 0.05$). The obese controls had a higher HbA1c compared to normal weight controls ($p = 0.03$), however NAFLD did not differ from obese or normal weight control ($p > 0.05$). NAFLD and obese control displayed significantly higher fasting insulin compared to normal weight ($p < 0.0001$), however NAFLD and obese control did not differ ($p > 0.05$). Across groups, there was no difference in adiponectin concentration ($p = 0.19$). and adiponectin concentration was not correlated with steatosis (i.e. CAP score; r = , $p = 0.66$) or fibrosis (kPa; r = , $p = 0.05$).

NAFLD participants displayed significantly higher ALT, AST, ALP and GGT than both the obese and normal weight control groups ($p < 0.05$). ALT, AST, ALP and GGT did not differ between obese and normal weight control ($p > 0.05$). NAFLD and obese control participants exhibited significantly lower ALB levels when compared to normal weight controls ($p < 0.05$).

There were no group differences in postprandial total cholesterol or LDL-C ($p > 0.05$). NAFLD had higher postprandial HDL-C compared to normal weight ($p = 0.01$) but did not differ from obese controls ($p = 0.99$). The only group difference observed for postprandial VLDL-C was between NAFLD vs. normal weight controls, where NAFLD had higher postprandial VLDL-C compared to normal weight ($p = 0.006$).

Dietary and Physical Activity Habits

NAFLD consumed fewer servings of vegetables/week than the normal weight and obese control group ($p < 0.02$). When participants were divided into tertiles based on vegetable intake, the odds of NAFLD were 83% and 96% lower in people in the second and third tertiles (OR 0.17 [95% CI: 0.03, 1.11]; OR 0.04 [95% CI: 0.002, 0.70]) when compared to children in the first tertile. There were no group differences in sugar sweetened beverage (SSB) intake, fruit juice intake, or fruit intake ($p > 0.05$). Similarly, there were no group differences in active or sedentary hours/week ($p > 0.05$). No dietary or physical activity variable was associated with steatosis ($p > 0.05$).

Fasting and Postprandial Triglyceride Measures

Fasting and postprandial triglyceride measures are presented in **Table 3** and **Figure 1A-E**. Results of a two-way ANOVA revealed a time x group interaction ($p = 0.04$), group effect ($p = 0.006$), and time effect ($p < 0.0001$) for triglycerides. In post-hoc analyses, there were no group differences in fasting triglycerides ($p > 0.05$). However, NAFLD and obese control exhibited greater 4h postprandial triglycerides than normal weight control ($p < 0.05$), but NAFLD did not differ from obese control ($p > 0.05$). To evaluate differences in triglyceride parameters across groups, tAUC and Δtriglycerides were analyzed. Triglyceride tAUC was 75% greater for NAFLD compared to normal weight control ($p = 0.01$) but did not differ between NAFLD and obese control ($p > 0.05$). NAFLD exhibited greater Δtriglycerides (70 ± 37 mg/dL) than normal weight (26 ± 35 mg/dL; $p = 0.03$). Δtriglycerides did not differ between obese control and NAFLD ($p = 0.24$) or normal weight and obese controls ($p = 0.08$). There was a large effect size for the difference in postprandial triglycerides between the obese control and NAFLD groups (d = 0.63), normal weight and obese control groups (d = 0.84), and normal weight and NAFLD groups (d = 1.48).

Fasting triglycerides and 4h postprandial triglycerides were correlated with steatosis (i.e. CAP score; $p = 0.0002$, $r = 0.58$; $p = 0.0001$, $r = 0.60$, respectively) (**Figure 2A-B**). Neither fasting ($p = 0.73$, $r = 0.06$) nor postprandial triglycerides ($p = 0.18$, $r = 0.24$) were correlated with fibrosis (i.e. kPa).

Within the entire study sample, participants were stratified by high (CAP ≥ 220 dB/m) and low (CAP ≤ 220 dB/m) steatosis; fasting triglycerides were 32% higher in children with high steatosis (114 ± 45 mg/dL) compared to children with low steatosis (77 ± 32 mg/dL; $p = 0.02$) (**Figure 3A**). Similarly, children with high steatosis had 98% greater 4h postprandial triglycerides (170 ± 69 mg/dL) compared to children with low steatosis (86 ± 25 mg/dL; $p < 0.0004$) (**Figure 3B**).

Fasting and Postprandial FGF19

FGF19 parameters are displayed in **Figure 4A-E** and **Table 4**. Neither fasting nor postprandial FGF19 differed across groups ($p > 0.05$). Similarly, FGF19 tAUC and ΔFGF19 did not differ across groups ($p > 0.05$). There was a medium effect size for differences in postprandial FGF19 between the obese control and NAFLD groups (d = 0.57) and the normal weight and obese control groups (d = 0.47) and a large effect size between the normal weight and NAFLD groups (d = 0.80).

Fasting and postprandial FGF19 were not associated with steatosis or fasting and postprandial triglycerides ($p > 0.05$). However, fasting (i.e. kPa; $p = 0.03$, $r = -0.36$) and postprandial FGF19 ($p = 0.03$, $r = -0.37$) were inversely associated with fibrosis (**Figure 5A-B**). When participants were stratified by high and low steatosis, fasting FGF19 and 4h postprandial FGF19 were 226% and 258% higher in children with low steatosis compared to high steatosis, respectively ($p < 0.05$) (**Table 4**, **Figure 6A-B**). However, when participants were stratified by no

fibrosis (kPa <6) and fibrosis (kPa >6), fasting FGF19 and 4h postprandial FGF19 did not differ between children with no fibrosis and children with evidence of fibrosis ($p > 0.05$; **Figure 6C-D**).

Fasting Insulin, Steatosis, and Postprandial Triglycerides

NAFLD participants exhibited higher fasting insulin than normal weight controls (mean difference = 43 µIU/mL; $p < 0.0001$) and obese controls (mean difference = 33 µIU/mL; $p = 0.0005$) (**Figure 7**). Fasting insulin did not differ between obese and normal weight controls (mean difference = 10 µIU/mL; $p = 0.30$).

Correlations between fasting insulin and steatosis and triglycerides are presented in **Figure 8A-C**. Fasting insulin was associated with steatosis (i.e. CAP score; $p < 0.0001$; $r = 0.80$). Similarly, fasting insulin was associated with fasting triglycerides ($p = 0.0002$, $r = 0.59$) and 4h postprandial triglycerides ($p = 0.0004$, $r = 0.57$). Within the entire sample, when fasting insulin was stratified by high (CAP ≥ 220 dB/m) and low (CAP ≤ 220 dB/m) steatosis, children with high steatosis had 4x greater fasting insulin (28 ± 21 µIU/mL) compared to children with low steatosis (7 ± 4 µIU/mL; $p = 0.003$) (**Figure 9**).

Diagnostic Accuracy of Metabolic and Liver Outcomes for NAFLD

Figure 10A-D show ROC plots for fasting triglycerides, 4h postprandial triglycerides, fasting FGF19, and 4h postprandial FGF19. The pooled sensitivity and specificity for fasting triglycerides, 4h postprandial triglycerides, fasting FGF19, and 4h postprandial FGF19 was 0.71 (95% confidence interval [CI]: 0.50-0.91; $p = 0.08$), 0.77 (95% CI: 0.60-0.94; $p = 0.02$), 0.60 (95% CI: 0.38-0.82; $p = 0.41$), 0.62 (95% CI: 0.40-0.83; $p = 0.33$), respectively.

Arterial Stiffness Measures

Arterial stiffness results are displayed in **Figure 11A-B**. There were no differences in PWV between groups or over time ($p > 0.05$). Two-way ANOVA of augmentation index

(normalized to a HR of 75 bpm) revealed that NAFLD had a higher augmentation index at baseline (mean difference = 22.63; $p = 0.049$) and 1h post-meal (mean difference = 21.17; p = 0.03) compared to normal weight control, suggesting a greater degree of arterial stiffness in NAFLD compared to normal weight control. No other group or time differences in augmentation index were observed ($p > 0.05$).

Energy Expenditure and Substrate Oxidation

For energy expenditure, although there was no group x time interaction ($p = 0.80$), there were significant group ($p < 0.0001$) and time ($p < 0.0001$) effects (**Figure 12**). Energy expenditure was lower in normal weight than obese control and NAFLD at baseline, 1h, and 3h post-meal ($p < 0.05$). Energy expenditure increased from baseline to 1-hour post-meal in NAFLD ($p = 0.03$) and normal weight control ($p = 0.0001$). Similarly, energy expenditure increased from baseline to 3h post-meal in NAFLD ($p = 0.04$). There were no other changes in energy expenditure within groups over time. There were no significant group x time interactions, group effects or time effects observed for RER, fat oxidation, or carbohydrate oxidation ($p > 0.05$).

CHAPTER V

DISCUSSION

Postprandial Triglycerides were Greater in Children with Steatosis

NAFLD is the leading cause of liver transplantation in children, therefore screening for the early detection of NAFLD in children is particularly important [52]. Unfortunately, child-friendly screening tools for NAFLD are lacking. Research has demonstrated that people with NAFLD tend to have an exaggerated postprandial triglyceride response to a high-fat meal compared to people without NAFLD, leading to pro-atherogenic elevations in postprandial triglycerides (i.e. postprandial lipemia) [4-6]. Additionally, signals that originate in the gut, including intestinally derived FGF19, play a key role in regulating hepatic lipid metabolism and have been proposed as a novel biomarker of NAFLD [103]. Therefore, the purpose of this study was to evaluate the use of postprandial triglycerides and FGF19 within the context of an AFTT as sensitive and specific candidate screening tools for pediatric NAFLD. In support of our hypothesis, the first key finding of this work is that we observed a greater postprandial triglyceride response in children with NAFLD compared to normal weight peers without NAFLD. This occurred even though there were no group differences in fasting triglycerides and children with NAFLD had clinically normal fasting triglycerides (<150 mg/dL). In agreement with the present study, previous research has demonstrated

that fasting triglycerides are not always elevated in children with NAFLD though high TG in children are most likely in the presence of obesity and insulin resistance [52, 77]. Metabolic challenges (e.g., AFTT) can reveal responses encountered in daily living that would otherwise be undetected if relying on fasting indices. Thus, the finding that children with NAFLD had a greater postprandial triglyceride response than normal weight peers without NAFLD agrees with previous research and is significant because current screening for the early detection of triglyceride infiltration in the liver utilizes fasting triglycerides (amongst other clinical biomarkers), which are not sensitive or specific to NAFLD. There are several factors that contribute to this observed exaggerated postprandial triglyceride response in children with NAFLD, including obesity, increased de novo lipogenesis, altered lipoprotein secretion and metabolism, insulin resistance, and imbalances in circulating adipokines. Notably, these are all also key pathophysiological mechanisms involved in NAFLD. Herein we discuss the present study's findings related to these factors and expand upon their contribution to postprandial lipemia and NAFLD.

Obesity with adipocyte dysfunction is a complex, yet common feature of NAFLD, such that obesity contributes to insulin resistance and insulin resistance contributes to adipose tissue dysfunction, both of which perpetuate hepatic steatosis [35]. During obesity, the ability to store excess fat is limited and adipose tissue hypertrophy is overwhelmed, leading to hypoxia within expanding adipose tissue [35]. Adipose-derived inflammation and FFA release increase, perpetuating insulin resistance and adipocyte dysfunction. Adipose tissue insulin resistance leads to impairments in the anti-lipolytic effects of insulin and increased FFA release from adipose depots, leading to an increased delivery of FFA to the liver. However, although obesity is a strong risk factor for NAFLD, not all people with obesity go on to develop NAFLD. This is likely related to the fact that it is adipose tissue dysfunction, and not merely excess adiposity, that drives NAFLD [41]. Nonetheless, adults with obesity have an exaggerated postprandial triglyceride

response to a high-fat meal compared to adults without obesity [104]. A similar finding has been observed in NAFLD, such that adults with NAFLD have an exaggerated postprandial triglyceride response to a high-fat meal compared to adults without obesity [104]. Therefore, because it is estimated that ~60-95% of adults with NAFLD have obesity, determining if the exaggerated postprandial triglyceride response observed in NAFLD can be attributed to the obesity or the NAFLD is critical for determining the efficacy of postprandial triglycerides as a screening tool for NAFLD [105]. Accordingly, a second key finding of the present study was that there were no differences in postprandial triglycerides between children with NAFLD and children with obesity without NAFLD, likely related to the fact that >90% of children in the obese control group had CAP scores suggestive of stage 1 or greater steatosis. This was a somewhat expected observation considering that intra-hepatic triglyceride content increases proportionately with adiposity [52, 106]. However, we were not able to determine the true difference in postprandial triglycerides between children with obesity without NAFLD and children with obesity with NAFLD because the obese control group was confounded by CAP scores suggestive of steatosis.

A third key finding of our study was that when we stratified the study sample by high and low steatosis ($\geq$ 220 dB/m vs. $\leq$ 220 dB/m) we found that children with high steatosis, regardless of NAFLD diagnosis, had 97% higher postprandial triglycerides than children with low steatosis. While fasting triglycerides were also higher in children with high steatosis, this was to a lesser degree (i.e. 32%) and is not clinically meaningful because neither group had fasting triglycerides above the clinical threshold (i.e. 150 mg/dL), agreeing with previous findings of clinically normal fasting triglycerides in children with NAFLD and children with obesity without NAFLD [77]. Thus, the finding that participants in the NAFLD and obese control groups had similar postprandial triglyceride responses may be due to the fact that both groups had elevated steatosis.

Little previous research exists comparing the postprandial triglyceride response between children with NAFLD and children with obesity without NAFLD. Using a design similar to the

current investigation, Mager *et al.* conducted a study in children with NAFLD, children with obesity without NAFLD, and normal weight children without NAFLD to investigate whether a high-fat meal (43% fat, 422 kcal) would evoke postprandial dyslipidemia and hyperinsulinemia [77]. The NAFLD and obese control group exhibited a greater postprandial triglyceride response when compared to lean controls, however postprandial triglycerides did not differ between the NAFLD and obese control group. The similarity between groups may have been related to the method for determining NAFLD. In this study by Mager *et al.*, NAFLD was ruled out in the obese group via abdominal ultrasound, but abdominal ultrasound is only sensitive for NAFLD when liver fat exceeds 30% of hepatocytes [107]. Therefore, like our findings, children in the obese control group could have presented with steatosis suggestive of NAFLD despite a negative ultrasound. Hence, it is conceivable that the obese control group likely had a confounding amount of liver steatosis, similar to our study.

Another central factor related to an exaggerated postprandial triglyceride response is insulin resistance. Epidemiological and cross-sectional data demonstrate that hepatic steatosis is consistently associated with insulin resistance, where insulin resistance can be both a cause and consequence of NAFLD [108-110]. As such, NAFLD has been described as the liver's display of metabolic syndrome [4-6]. In fact, the presence of insulin resistance is a nearly ubiquitous feature of NAFLD and has been found to drive de novo lipogenesis and steatosis [111]. This relationship is complex, such that hepatic insulin resistance manifests as an impaired ability to inhibit gluconeogenesis but a preserved ability to stimulate lipogenesis. This is termed selective insulin resistance of the liver and the increased production of non-lipid precursors (e.g., glucose) serve as substrate for de novo lipogenesis. Accordingly, insulin resistance promotes an exaggerated postprandial triglyceride response and steatosis in three key ways: 1) persisting adipose tissue lipolysis leading to increased FFA delivery to the liver; 2) impaired fasting and postprandial

glucose leading to increased substrate for de novo lipogenesis; and 3) activation of de novo lipogenesis machinery in the liver (e.g., SREBP1c).

There are several key findings related to insulin in the present study. We observed a strong association between fasting insulin and hepatic steatosis (i.e., CAP score), suggesting that fasting hyperinsulinemia likely exacerbates steatosis. Further, we found that NAFLD participants exhibited higher fasting insulin compared to normal weight and obese controls, but obese and normal weight controls did not differ, suggesting some degree of insulin resistance in the NAFLD group. So far, this is a unique finding considering that a previous study by Mager *et al.* that found that fasting insulin was greater in children with NAFLD compared to lean controls but did not differ from obese controls. Moreover, we found that participants with high steatosis displayed 4x greater fasting insulin than participants with low steatosis, regardless of NAFLD diagnosis. We also observed that both fasting and postprandial triglycerides were moderately associated with fasting insulin. Considering that this same pattern was observed for postprandial triglycerides (i.e., participants with high steatosis exhibited higher postprandial triglycerides than participants with low steatosis) and that postprandial triglycerides were associated with fasting insulin, these findings imply that fasting hyperinsulinemia may be one mechanism linking postprandial triglycerides to hepatic triglyceride accrual in pediatric NAFLD.

Another key factor related to the exaggerated postprandial triglyceride response in children with high steatosis is dysregulated lipoprotein and lipid metabolism. Mager *et al.* demonstrated that children with NAFLD have elevated ApoB-48 in the postprandial period when compared to obese and lean children without NAFLD [77]. In contrast, a study of adults with NAFLD reported that the ApoB-48 and ApoB-100 respose in the postprandial period was blunted or even declined [84]. This occurred despite increased postprandial triglycerides, indicative of an impairment in VLDL and chylomicron secretion and potentially larger triglyceride-rich ApoB particles [84]. These differences in lipoprotein metabolism between adults and children with

NAFLD could be related to the stage of NAFLD (i.e. NAFLD vs. NASH), but may underscore a fundamental difference in pediatric and adult NAFLD. Children with NAFLD may experience decreased clearance of triglyceride-rich lipoproteins, leading to postprandial hypertriglyceridemia. For example, Schreuder *et al.* demonstrated that adults with NAFLD display an impaired clearance of triglyceride-rich lipoproteins, as evidenced by a greater area under the curve during the descending phase of postprandial testing (i.e. when clearance of triglyceride-rich lipoproteins prevails, 3-6h post-meal) [98]. Thus, the observed greater postprandial response in children with NAFLD is partially explained by the increased production of ApoB48-containing lipoproteins and could plausibly be related to decreased clearance of triglyceride-rich lipoproteins in the postprandial period, however the latter has yet to be determined in a pediatric NAFLD population.

The exaggerated postprandial triglyceride response in NAFLD is also related to increased de novo lipogenesis, or dysregulation in de novo lipogenesis when switching from the fasted to fed state, which has been identified as a prominent mechanism for hepatic triglyceride accrual in NAFLD. For example, Lambert *et al.* conducted a study utilizing isotope analyses to compare de novo lipogenesis in adults with high and low levels of liver fat [112]. While the majority of FFA incorporated into hepatic triglycerides originated from adipose depots (~60%) [113], Lambert demonstrated that de novo lipogenesis was 2-fold higher in adults with high liver fat compared to adults with low liver fat (23% vs. 10%), despite no differences in the amount of FFA originating from adipose tissue lipolysis [112]. In other words, de novo lipogenesis contributes a greater quantity of FFA to triglyceride-rich lipoproteins in adults with high liver fat when compared to adults with low liver fat, even though the majority (~60%) of FFA originate from adipose tissue lipolysis and adults with high and low liver fat do not have differing contributions of FFA from adipose tissue lipolysis. Additionally, the amount of time that it takes for adults with NAFLD to suppress lipogenesis to baseline levels was significantly greater compared to adults without

NAFLD, suggesting that adults with NAFLD struggle to regulate de novo lipogenesis when transitioning between the fasted and fed state. This is an important finding because a major intermediate of lipogenesis is malonyl-CoA, which is known to attenuate FFA oxidation. The build-up of malonyl-CoA and resulting inhibition of FFA oxidation would lead to greater postprandial triglycerides in circulation, promote lipotoxicity and inflammation via the build-up of FFA and FFA intermediates, and promote steatosis by impairing FFA oxidation and thus preventing triglyceride export from the liver. While this provides one logical physiological factor related to the observed exaggerated postprandial triglyceride response in children with high steatosis in the present study, no study to our knowledge has determined the sources (i.e. adipose tissue, de novo lipogenesis, and diet) of FFA in children.

Lastly, impaired postprandial triglyceride metabolism has been linked to hypoadiponectinemia, especially in the context of NAFLD and insulin resistance. Adiponectin is an anti-inflammatory adipokine that promotes FFA oxidation and suppresses de novo lipogenesis, therefore a decreased concentration of adiponectin likely promotes hepatic triglyceride accrual. Contrary to our hypotheses, we did not find that adiponectin concentrations differed between groups and adiponectin concentrations were not related to postprandial triglycerides, steatosis, or fibrosis. However, this may be due to the time of measurement of adiponectin in the present study (e.g., fasting adiponectin). Adiponectin typically rises following meal consumption, concurrently with the rises in postprandial triglycerides, leading to enhanced catabolism of triglyceride and their metabolites (i.e. FFA). Previous research has demonstrated that adults with NAFLD have an attenuated rise in adiponectin postprandially and this attenuation may partially explain the exaggerated postprandial triglyceride response [6]. For example, Musso *et al*. [6] found that adults with NASH exhibited lower adiponectin concentrations throughout the postprandial period when compared to adults without NASH. Additionally, adiponectin concentrations decreased postprandially in NASH and increased postprandially in adults without NASH. The postprandial

triglyceride response was also strongly related to fasting adiponectin concentrations. Findings in children are slightly conflicting, though. For example, Louthan *et al.* found that children with NAFLD (suspected via elevated liver enzymes) had lower adiponectin concentrations than their normal weight and overweight peers [114]. Conversely, Mager *et al.* [77] found no differences in fasting or postprandial adiponectin between children with NAFLD (diagnosed primarily via liver biopsy) and their normal weight and obese peers. One reason for this discrepancy in the literature could be related to the fact that Louthan *et al.* recruited children with NAFLD suspected via elevated ALT while Mager *et al.* recruited children with NAFLD diagnosed primarily via liver biopsy [6]. While liver enzymes are the status quo for early screening for NAFLD, liver enzymes frequently fall within normal ranges in people with NAFLD and can be elevated for reasons beyond NAFLD. Despite the conflicting evidence in pediatric NAFLD, our findings appear to agree with Mager *et al.*, suggesting that children with NAFLD may not exhibit derangements in fasting adiponectin.

Postprandial FGF19 was Lower in Children with Steatosis

Identifying secondary factors connected to hepatic lipid metabolism that contribute to the development of NAFLD may lead to the advancement of early screening tools for pediatric NAFLD. The intestinally derived endocrine compound FGF19 regulates hepatic lipid metabolism by promoting FFA oxidation and inhibiting de novo lipogenesis. As such, FGF19 has been linked to postprandial dyslipidemia and hepatic triglyceride accrual in the context of NAFLD and has gained recent interest as a novel screening tool for NAFLD. Despite this, little previous research has investigated postprandial FGF19 metabolism in pediatric NAFLD and has instead largely centered on fasting FGF19 in pediatric NAFLD. This is important because FGF19 is a hormone produced following meal intake and bile acid entry into the small intestine, thus FGF19 acts principally in the post-absorptive state. Therefore, an additional aim of the present study was to evaluate FGF19, in the context of an AFTT, as a sensitive and specific screening tool for pediatric

NAFLD. Accordingly, a fourth key finding in the present study is that while we did not observe a difference in fasting or postprandial FGF19 between groups, fasting and postprandial FGF19 were 226% and 258% higher in children with low steatosis compared to high steatosis, respectively. However, neither fasting nor postprandial FGF19 were associated with any triglyceride parameter or steatosis. To our knowledge, this is among the first studies to investigate differences in postprandial FGF19 between children with obesity and NAFLD, children with obesity without NAFLD, and normal weight children.

Work from others agree with the present study's finding that children with high steatosis exhibited lower FGF19 compared to children with low steatosis. For example, one study found that children with obesity and NAFLD exhibited lower fasting FGF19 when compared to children with obesity without NAFLD [8]. However, to our knowledge, postprandial FGF19 has not been compared in obese children with and without NAFLD. Research in adults with NAFLD may provide some insight. Adults with NAFLD exhibit altered FGF19 signaling, sometimes referred to as FGF19 resistance. Friedrich *et al.* investigated the postprandial FGF19 and bile acid synthesis response (i.e. C4) to an oral fat tolerance test in overweight and obese NAFLD adults and observed reduced baseline FGF19 concentrations and a blunted postprandial FGF19 increase to meal intake in obese and overweight adults with NAFLD [47]. The postprandial FGF19 response was coupled by no change in C4 levels in obese and overweight adults with NAFLD. FGF19's primary function is to directly repress bile acid synthesis via inhibition of the rate-limiting enzyme for bile acid synthesis, CYP7α1 [44]. Therefore, under normal conditions, the increase in FGF19 postprandially would lead to changes (e.g., reduction) in bile acid synthesis (i.e., C4). Thus, Friedrich *et al.* demonstrated that there may be an alteration in both FGF19 production and FGF19 signaling in NAFLD. Schreuder *et al.* revealed a similar finding, where adults with NAFLD exhibited no change in C4 levels despite an increase in FGF19 postprandially. Therefore, people with NAFLD may experience reduced postprandial FGF19

production and signaling, leading to reduced triglyceride disposal in the liver, and increased postprandial triglycerides. Since a major role of FGF19 is to promote FFA oxidation (e.g. via inhibiting acetyl-CoA carboxylase) and inhibit triglyceride synthesis in the liver via suppressing SREBP1c, our finding that children with high steatosis had lower fasting and postprandial FGF19 than children with low steatosis may provide insight on how hepatic fat accrues in pediatric NAFLD, such that impairments in FGF19 production or signaling at the site of the liver could lead to on-going triglyceride synthesis. So far, this is a novel finding since we are one of the first to investigate differences in fasting and postprandial FGF19 in a pediatric NAFLD population compared to children without NAFLD [98].

FGF19 is also related to fibrosis development in NAFLD. In fact, phase 2 clinical trials have recently been conducted investigating the efficacy of a synthetic FGF19 analog for improving fibrosis in adult NAFLD [115]. Briefly, FXR stimulation in the ileum results in FGF19 production, suppressing CYP7α1. The suppression of CYP7α1 is clinically relevant because bile acid accumulation intensifies hepatic stellate cell activation, leading to liver injury and histological changes that are characteristics of NASH. In the present study, we did not find that fasting or postprandial FGF19 differed between children with and without fibrosis, however we did find that fasting and postprandial FGF19 was inversely associated with fibrosis. This partially disagrees with a study conducted by Chen *et al.* who demonstrated that adults with NAFLD with stage 2-4 fibrosis had lower fasting FGF19 when compared to those with stage 0-2 fibrosis; postprandial FGF19 was not measured [50]. Children and adults with NAFLD have dissimilar liver histology and fibrosis patterns, though. For example, children have a unique fibrosis pattern, characterized by portal fibrosis and inflammation, that is distinctive and seldom seen in adults, and, at the point of diagnosis, 15% of children have stage 3 or 4 fibrosis [2]. Thus, the finding that children with NAFLD and fibrosis did not experience differing fasting or postprandial FGF19 from their normal weight and obese peers, but that fasting and postprandial FGF19 were

inversely associated with fibrosis, is novel and one of the first examinations of this relationship in pediatric NAFLD and may elaborate on the dissimilar liver pathology between children and adults with NAFLD.

Postprandial Triglycerides, not FGF19, had Moderate Diagnostic Accuracy for NAFLD

Screening for pediatric NAFLD first consists of ruling out non-NAFLD conditions that can promote steatosis, including genetic diseases, excessive alcohol intake, infections, and certain medications [3]. Thereafter, the North American Society of Pediatric Gastroenterology, Hepatology, and Nutrition recommends screening in children with obesity and additional risk factors including insulin resistance, diabetes, dyslipidemia, central adiposity, or family history of NAFLD [116]. Historically, liver enzymes have been the gold standard for screening in children suspected of NAFLD. Once abnormal liver enzymes are detected and other co-morbidities or liver diseases have been ruled out, children with persistently elevated ALT often undergo an imaging-related screening tool, including either ultrasound, elastography, or MRI [117]. This is problematic for the early detection of NAFLD, though, because liver enzymes can fall within normal ranges in up to 70% of children with NAFLD and can increase for reasons beyond NAFLD, including medication, stress, and other competing liver diseases [53]. More, ultrasound is not recommended for diagnosing NAFLD because it lacks sensitivity and specificity for steatosis [118]. Elastography and MRI are expensive, not widely accessible, and require trained personnel. A few indices have been developed as screening tools for NAFLD, including the hepatic steatosis index (HSI) and fatty liver index (FLI) [55]. However, additional biomarkers, including liver biochemistries, which are non-specific to NAFLD, are required for these indices to possess clinical diagnostic utility. Therefore, a third aim of the present study was to investigate the diagnostic accuracy of triglyceride and FGF19 measures for pediatric NAFLD. Fasting triglycerides, fasting FGF19, and postprandial FGF19 all had low diagnostic accuracy (i.e., could not discriminate between children with and without NAFLD). Of the measures used to assess

diagnostic accuracy, postprandial triglycerides had the strongest diagnostic accuracy, where they were moderately accurate. To our knowledge, we are the first to assess the diagnostic accuracy of both fasting and postprandial triglycerides and FGF19 in pediatric NAFLD.

No previous research has investigated the diagnostic accuracy of postprandial triglycerides or FGF19 for pediatric NAFLD. However, the FLI, consisting of BMI, waist circumference, fasting triglycerides, and GGT, and the HSI, consisting of BMI, the presence of diabetes, and ALT/AST ratio, has been examined in an adult NAFLD population. Dehnavi *et al.* examined the diagnostic accuracy of the FLI in an adult Iranian population and found that the AUC of FLI was 0.85, suggesting that FLI had strong diagnostic accuracy for detecting steatosis, however FLI did not have better diagnostic accuracy for steatosis than waist circumference [119]. Another study conducted by Lee *et al.* in Korean adults found that the HSI had moderate diagnostic accuracy for detecting steatosis (AUC = 0.81) [55]. A few fundamental issues remain in these previous studies, though. In these examinations, NAFLD has largely been diagnosed via imaging tools, which are not the gold standard for NAFLD. Additionally, these investigations have not been conducted in an ethnically or racially diverse cohort. This is important because significant racial and ethnic disparities exist in NAFLD; NAFLD prevalence is highest in Hispanics and Whites and lowest in Black and African Americans [120]. More, the pathophysiology and clinical presentation of NAFLD differs in people of Asian ethnicity when compared to other ethnic cohorts, such that people of Asian ethnicity have a higher prevalence of lean NAFLD (i.e. visceral adiposity and central obesity) despite having lower BMI [120]. Children and adults share dissimilarities in the pathophysiology of NAFLD, therefore extrapolating these findings to a pediatric NAFLD population is not justified. Lastly, relying on anthropometric measurements, including BMI and waist circumference, in pediatric NAFLD is not recommended because children are in a constant and dynamic state of growth and maturation, such that weight and body composition changes can be transient and volatile. Therefore, our

finding that postprandial triglycerides have moderate diagnostic accuracy for pediatric NAFLD is novel, but given the modest diagnostic accuracy of postprandial triglycerides, combining postprandial triglycerides with other biomarkers may increase the diagnostic accuracy of these screening tools. Future examinations are warranted in pediatric NAFLD populations to determine biomarkers that are uniquely accurate for diagnosing pediatric NAFLD.

Children with NAFLD exhibited Arterial Stiffness but not Alterations in Whole-body Substrate Metabolism

NAFLD is associated with increased cardiometabolic disease risk that begins in childhood and adolescence [121]. The increased risk for cardiometabolic disease in children with NAFLD is largely related to the dyslipidemia that characterizes NAFLD (e.g. high fasting and postprandial triglycerides, high LDL-C, and low HDL-C) [73]. This dyslipidemic profile is also related to markers of subclinical atherosclerosis, including vascular dysfunction and arterial stiffness, both of which are risk factors for cardiovascular mortality [122]. This is important because both vascular dysfunction and arterial stiffness have been independently associated with NAFLD [122].

Augmentation index is a measure of arterial stiffness and vascular dysfunction and can serve as a proxy for aortic pressure. Augmentation index is an independent risk factor for CVD and is usually higher in patients with cardiometabolic conditions than those without [123]. Similarly, PWV is a measure of arterial stiffness and has been associated with cardiac dysfunction [123]. In the present study, augmentation index was higher in children with obesity and NAFLD at baseline and 1h post-meal compared to their normal weight and obese peers. Although there are few prior studies that have reported values for augmentation index in pediatric NAFLD, our findings are largely in agreement with is known during fasting. A cohort study of pediatric NAFLD participants enrolled subjects defined as “high metabolic risk” and “low metabolic risk” according to measurements including systolic blood pressure, homeostatic model

assessment of insulin resistance, serum triglycerides, and BMI [124]. Children in the high metabolic risk profile had greater PWV and augmentation index. Therefore, the higher augmentation index in the present study may be indicative of early signs of CVD risk and arteriosclerosis. This is important because it highlights that the risk of NAFLD is beyond that which is attributable to metabolic disturbances in the liver and highlights the risk of NAFLD as a multi-organ disease and a serious concern for the pediatric population.

Metabolic flexibility is a concept defined by the ability to switch between substrates (e.g., glucose, fatty acids) during periods of feeding, fasting, and exercise. The concept is best illustrated by the skeletal muscle's ability to switch between using fatty acids as fuel during fasting and carbohydrate as fuel following consumption of mixed meal or with a high level of carbohydrate (i.e. insulin-stimulated conditions) in people with a high degree of insulin sensitivity. Under insulin resistant conditions (e.g. type 2 diabetes, metabolic syndrome, NAFLD), lipid oxidation is reduced in the fasting period while the ability to suppress lipid oxidation and increase carbohydrate oxidation in the fed state is diminished, indicating that a higher proportion of energy expenditure is derived from carbohydrate during fasting [125]. It is not surprising then that people with cardiometabolic disease typically have less metabolic flexibility than people without [125, 126]. In the present study, children with NAFLD and children with obesity without NAFLD had a greater energy expenditure than their normal weight peers, but children with NAFLD and children with obesity without NAFLD did not differ. This is to be expected, considering that children within the NAFLD and obesity groups had a significantly greater body mass than their normal weight peers and lean mass is a key determinant of energy expenditure. The NAFLD participants were the only group that demonstrated significantly elevated energy expenditure from baseline at 3h post-meal. Importantly, the observed differences in energy expenditure may be confounded by differences in lean mass, as lean mass is also known to increase proportionately with increasing body size and is a major

determinant of energy expenditure [127]. Nonetheless, in addition to body mass and body composition, key determinants of energy expenditure include food intake and physical activity [127]. Our findings indicate that children with NAFLD were not more active than their normal weight and obese peers. Therefore, the observed differences in energy expenditure are not likely attributable to differences in physical activity. More, children with NAFLD consumed less vegetables than their normal weight and obese peers, however this difference in food intake is unlikely to produce clinically relevant differences in energy expenditure.

Despite the observed differences in energy expenditure, we did not find that children with NAFLD had a higher RER at fasting compared to normal weight and obese peers, indicating that children with NAFLD did not derive a greater proportion of energy from carbohydrates during fasting than children without NAFLD. Moreover, while RER in the NAFLD group did not differ from fasting to postprandial, this lack of change in RER postprandially was observed for all groups, thus the change in RER throughout the postprandial period did not differ between NAFLD and obese and normal weight peers. Lastly, we observed that NAFLD did not affect fat and carbohydrate oxidation. Our findings disagree with others. For example, research has demonstrated that children with NAFLD have a stifled increase in carbohydrate oxidation in the postprandial period, such that RER does not change throughout the postprandial period when compared with children without NAFLD [126]. Lee *et al.* observed higher fasting glucose oxidation and lower postprandial glucose oxidation in obese children with NAFLD compared to obese children without NAFLD [126]. Additionally, RER increased from fasting in both groups, however the increase from fasting was lower in children with NAFLD compared to children without NAFLD.

There are a few potential explanations for our findings. Firstly, children are in a dynamic state of weight gain governed by growth and pubertal maturation, therefore measuring fluxes in energy expenditure and substrate oxidation may be too heterogeneous. Secondly, patients in our

study may not have had insulin resistant NAFLD. Thus, the insulin bolus following high-fat meal consumption may have been enough to overcome the rise in substrate (e.g. postprandial lipids and glucose) following the high-fat meal. This is related to the fact that insulin resistance impairs glucose uptake, leading to an inability to switch to using carbohydrate as fuel in the fed state. Indeed, children with NAFLD in the present study had higher fasting insulin than their normal weight peers. Lastly, unlike the present study that measured substrate oxidation and RER via indirect calorimetry within an AFTT, Lee *et al.* measured substrate oxidation and RER during a 3h hyperinsulinemic euglycemic clamp. This could explain the disagreement in findings between Lee *et al.* and the present study. More specifically, macronutrient distribution can alter RER and whole-body substrate metabolism. For example, high-carbohydrate diets decrease fat oxidation and therefore increase RER, while high-fat feeding increases fat oxidation and decreases RER [128]. In the present study, 73% of energy was derived from dietary fat in the test meal, while Lee *et al.* utilized a drink containing ~100% of energy from glucose. The small amount of energy from carbohydrate and large amount of energy from fat in the present study meal may not have been sufficient to produce changes in carbohydrate oxidation and may have also blunted the increase in glucose postprandially, masking differences across groups.

Strengths and Limitations

There are several strengths of the present study. First, to our knowledge, our study is the first examination of postprandial triglycerides and FGF19, within the context of an AFTT, as screening tools for pediatric NAFLD. This is clinically relevant because screening tools for pediatric NAFLD are urgently needed and metabolic challenges, like the AFTT, can reveal differences in metabolism that would be otherwise undetected if using fasting values alone. Second, NAFLD was confirmed in the pediatric NAFLD group via liver biopsy, which is the gold standard for assessing and diagnosing NAFLD. Third, the use of Fibroscan for measuring hepatic steatosis in all groups allowed for a novel, non-invasive, and robust assessment of liver health

status. Fourth, the inclusion of a control group with obesity without NAFLD theoretically allowed for proper inference regarding whether postprandial triglyceride and FGF19 responses were unique to NAFLD alone, considering that ~90% of children with NAFLD have obesity. However, the obese control group was confounded by high CAP scores. As such, the present study is not without limitations. Further, FGF19 usually peaks around 2-3 hours post-meal intake. Our present study collected blood samples at baseline and 4h post-meal, therefore we may not have captured the true FGF19 peak, and this could explain the lack of statistical difference in FGF19 measures across groups. However, we did observe medium-large effect sizes between both NAFLD and normal weight and between NAFLD and obese control for both postprandial triglycerides and FGF19, suggesting that an increase in group size may reveal true statistical differences between groups. Moreover, including a measurement of serum glucose would allow for the estimation of insulin resistance using HOMA-IR method. And lastly, the present study did not measure C4 levels and thus cannot infer that FGF19 signaling was altered in children with NAFLD.

Conclusions

These preliminary findings demonstrate that the postprandial triglyceride response in NAFLD is greater in children with NAFLD compared to normal weight peers without NAFLD but does not differ between children with NAFLD and children with obesity. This was likely because ~92% of children in the obese control group exhibited evidence of stage 1 or greater steatosis. As such, we found that children with high steatosis experienced greater postprandial triglycerides than children with low steatosis and postprandial triglycerides had moderate diagnostic accuracy for NAFLD, suggesting that postprandial triglycerides may be a valuable screening tool for pediatric NAFLD. Despite this, we were unable to determine if children with obesity and NAFLD have a greater postprandial triglyceride and FGF19 response than children with obesity with NAFLD because children in the obese group were not free of steatosis. More, the postprandial rise in FGF19 was blunted in children with higher liver steatosis, however

FGF19 measures did not have diagnostic accuracy for NAFLD. A major role of FGF19 is to promote fatty acid oxidation and inhibit triglyceride synthesis in the liver, thus our findings provide insight on how hepatic fat accrues in pediatric NAFLD but suggest that FGF19 may not be a valuable screening tool for pediatric NAFLD. Future research should investigate the diagnostic accuracy of postprandial triglycerides and FGF19 in a larger, more ethnically diverse, sample of pediatric NAFLD. Additionally, future research should focus on screening for steatosis prior to study enrollment to avoid improper allocation of children with obesity but undetected steatosis. Additionally, determining factors that contribute to the heterogeneity in triglyceride and FGF19 responses is clinically relevant when considering their potential use as screening tools for pediatric NAFLD. Further, determining what lifestyle factors increase risk for pediatric NAFLD and how these can modify postprandial metabolism in NAFLD may allow for better intervention and diagnostic accuracy. Finally, determining when peak concentrations of postprandial triglycerides occur in children with NAFLD and capturing the true peak postprandial FGF19 concentration would more accurately determine the diagnostic accuracy of postprandial triglycerides and FGF19.

	NAFLD	OB Control	NW Control	*p*-value
Participants (n)	9	13	15	–
Sex (M/F)	7/2	5/8	6/9	–
Age (years)	15 ± 2	17 ± 2	17 ± 2	0.11
Height (in)	66 ± 3	68 ± 3	66 ± 5	0.59
Weight (lb)	238 ± 62[a]	237 ± 39[a]	135 ± 23[b]	**<0.0001**
BMI (kg/m^2)	37 ± 8[a]	37 ± 6[a]	21 ± 3[b]	**<0.0001**
Systolic BP (mmHg)	124 ± 11[a]	128 ± 7[ab]	117 ± 9[a]	**0.01**
Diastolic BP (mmHg)	67 ± 8	73 ± 8	67 ± 7	0.09
Body fat (%)	48 ± 6[a]	48 ± 6[a]	31 ± 10[b]	**<0.0001**
Android body fat (%)	56 ± 7[a]	55 ± 7[a]	28 ± 12[b]	**<0.0001**
Gynoid body fat (%)	46 ± 5[a]	49 ± 6[a]	34 ± 11[ab]	**0.0001**
Trunk fat (kg)	28.9 ± 13.3[a]	26.9 ± 6.6[a]	7.9 ± 3.3[b]	**<0.0001**
Fibrosis score (kPa)	10.1 ± 5.5[a]	5.3 ± 0.9[b]	5.0 ± 0.7[b]	**0.0002**
Steatosis score (CAP, dB/m)	343 ± 34[a]	300 ± 59[a]	205 ± 36[b]	**<0.0001**

Table 1. Participant characteristics. Data are mean ± SD. The *p*-value column reflects the results of a one-way ANOVA. Within rows, groups with shared letters indicate no statistically significant difference between groups in post-hoc analyses. **BP** blood pressure, **BMI** body mass index, **CAP** controlled attenuation parameter, **NAFLD** non-alcoholic fatty liver disease, **OB** obese, **NW** normal weight. $p < 0.05$ is considered statistically significant.

	NAFLD	OB Control	NW Control	*p*-value
Cholesterol (mg/dL)	153 ± 19	156 ± 28	164 ± 30	0.59
HDL-C (mg/dL)	41 ± 7[a]	42 ± 14[a]	60 ± 13[b]	**0.0004**
LDL-C (mg/dL)	87 ± 16	91 ± 19	88 ± 21	0.92
VLDL-C (mg/dL)	25 ± 8[a]	23 ± 9[ab]	16 ± 6[b]	**0.02**
HbA1c (%)	5.4 ± 0.3[ab]	5.4 ± 0.4[a]	5.0 ± 0.4[b]	**0.02**
Insulin (µIU/mL)	51 ± 32[a]	18 ± 8[b]	8 ± 4[b]	**<0.0001**
Adiponectin (ng/mL)	2268 ± 356	2388 ± 727	2853 ± 900	0.19
ALT (U/L)	108 ± 92[a]	26 ± 11[b]	20 ± 12[b]	**0.0001**
AST (U/L)	71 ± 51[a]	28 ± 8[b]	31 ± 12[b]	**0.0009**
ALB (g/dL)	3.9 ± 0.3[ab]	3.9 ± 0.2[a]	4.1 ± 0.3[b]	**0.02**
ALP (U/L)	180 ± 96[a]	79 ± 25[b]	81 ± 34[b]	**0.0001**
AMY (U/L)	38 ± 12	44 ± 22	57 ± 22	0.07
GGT (U/L)	42 ± 25[a]	20 ± 7[b]	12 ± 4[b]	**<0.0001**

Table 2. Fasting metabolic and liver biochemistries. Data are mean ± SD. The *p*-value column reflects the results of a one-way ANOVA. Within rows, groups with shared letters indicate no statistically significant difference between groups. **HDL-C** high-density lipoprotein cholesterol, **LDL-C** low-density lipoprotein cholesterol, **VLDL-C** very low-density lipoprotein cholesterol, **HbA1c** hemoglobin A1c, **ALT** alanine aminotransferase, **AST** aspartate aminotransferase, **ALB** albumin, **ALP** alkaline phosphatase, **AMY** amylase, **GGT** gamma-glutamyl transferase, **NAFLD** non-alcoholic fatty liver disease, **OB** obese, **NW** normal weight. $p < 0.05$ is considered statistically significant.

	NAFLD	OB Control	NW Control	*p*-value
Fasting TG (mg/dL)	127 ± 42	115 ± 47	79 ± 32	>0.05*
Postprandial TG (mg/dL)	197 ± 69[a]	157 ± 72[a]	105 ± 45[b]	<0.05*
tAUC TG (mg/dL)	647 ± 217[a]	544 ± 230[ab]	369 ± 139[b]	0.006
ΔTG (mg/dL)	70 ± 37[a]	42 ± 40[ab]	27 ± 35[b]	0.04

Table 3 Triglycerides. Data are mean ± SD. The *p*-value column reflects the results of either a one-way or two-way ANOVA. In the *p*-value column, rows with a *indicate results of two-way ANOVA. Within rows, groups with shared letters indicate no statistically significant difference between groups in post-hoc analyses. **tAUC** total area under the curve, **TG** triglycerides, **NAFLD** non-alcoholic fatty liver disease, **OB** obese, **NW** normal weight. $p < 0.05$ is considered statistically significant.

	NAFLD	OB Control	NW Control	*p*-value
Fasting FGF19 (pg/mL)	539 ± 382	518 ± 646	2703 ± 3025	>0.05
Postprandial FGF19 (pg/mL)	465 ± 330	679 ± 648	1809 ± 2086	>0.05
tAUC FGF19 (pg/mL)	2007 ± 1418	3927 ± 5402	7293 ± 8318	0.21
ΔFGF19 (pg/mL)	-17 ± 175	257 ± 555	-112 ± 409	0.14
	CAP ≤ 220	**CAP ≥ 220**	------	***p*-value**
Fasting FGF19 (pg/mL)	1881 ± 2240[a]	577 ± 499[b]	------	0.03
4h Postprandial FGF19 (pg/mL)	2203 ± 2236[a]	616 ± 672[b]	------	0.008
	kPa <6	**kPa >6**	------	***p*-value**
Fasting FGF19 (pg/mL)	1713 ± 1997	597 ± 561	------	0.11
4h Postprandial FGF19 (pg/mL)	1809 ± 2111	393 ± 279	------	0.04

Table 4. FGF19 Measurements. Data are mean ± SD. The *p*-value column reflects the results of a one-way, two-way ANOVA or unpaired t-test. Within rows, groups with shared letters indicate no statistically significant difference between groups. **tAUC** total area under the curve, **TG** triglycerides, **NAFLD** non-alcoholic fatty liver disease, **OB** obese, **NW** normal weight. $p < 0.05$ is considered statistically significant.

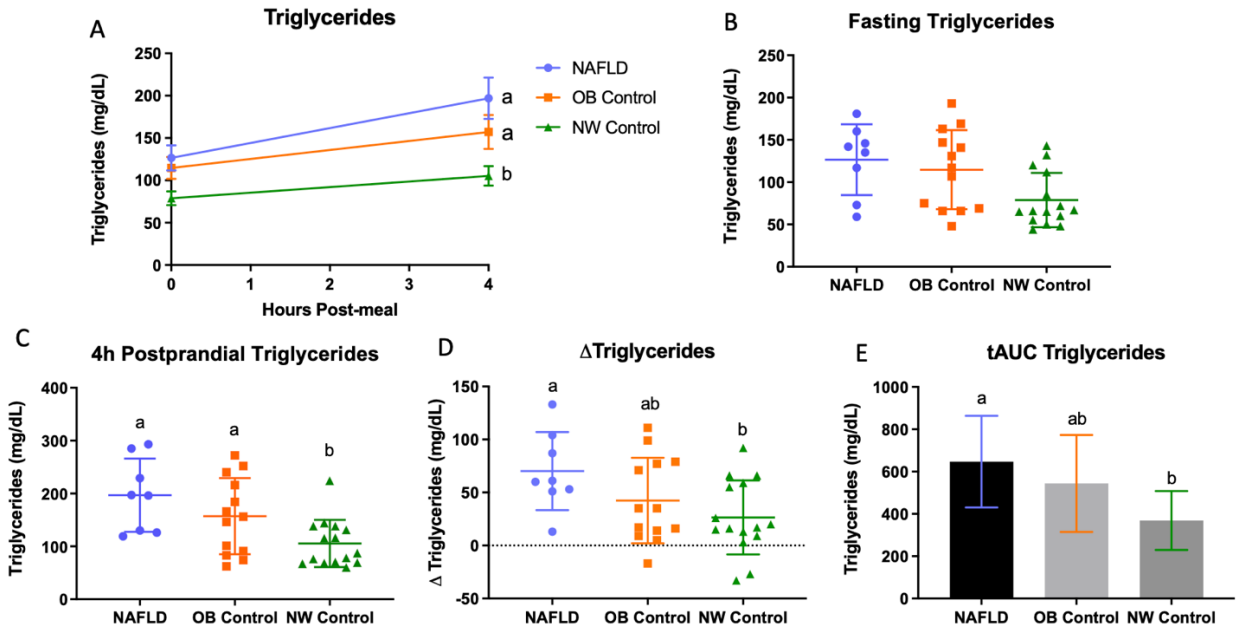

Figure 1A-E. Triglyceride parameters. Data are mean ± SD. Data points represent individual participant measurements. Groups with a shared letter indicate no statistically significant difference between groups. $p < 0.05$ is considered statistically significant.

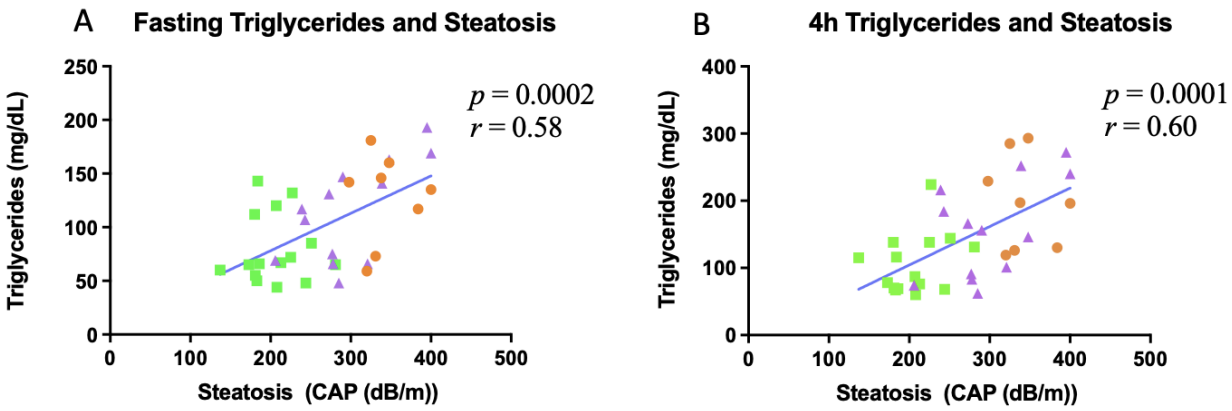

Figure 2A-B. Fasting and Postprandial Triglyceride Correlations with Steatosis. Data points represent individual participant measurements. Statistical results are for Pearson's correlation. $p < 0.05$ is considered statistically significant.

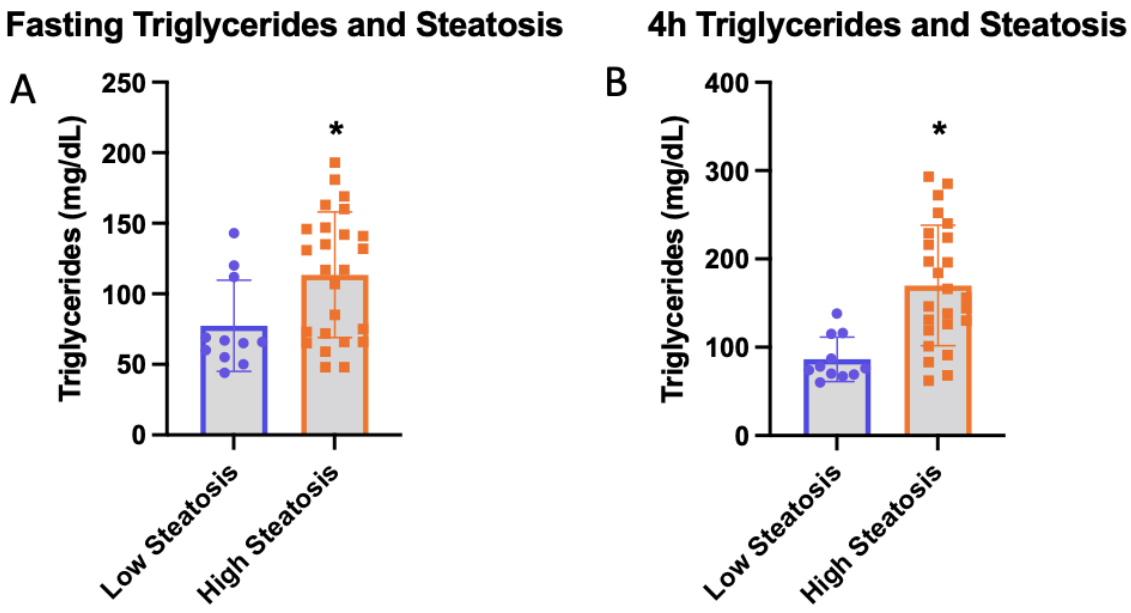

Figure 3A-B. Fasting and Postprandial Triglycerides and Steatosis. Data are mean ± SD. *indicates a statistically significant difference between groups. Data are based on results of an unpaired t-test. $p < 0.05$ is considered statistically significant.

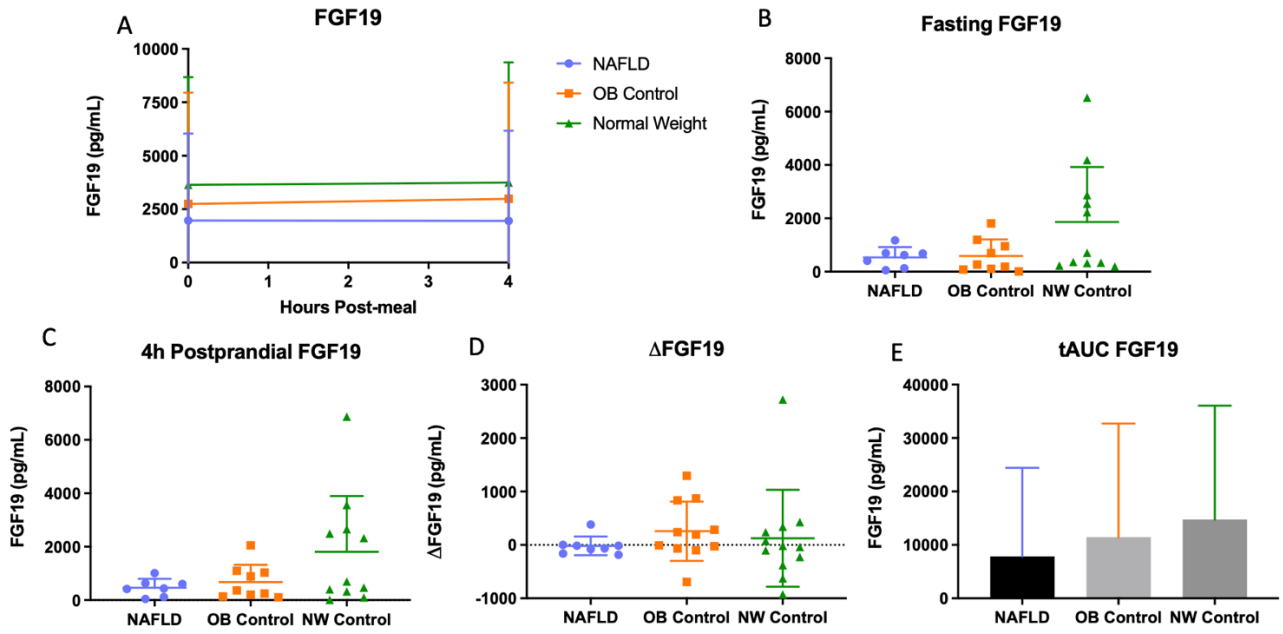

Figure 4A-E. FGF19 Parameters. Data are mean ± SD. Data points represent individual participant measurements. There were no significant differences in all analyses. $p < 0.05$ is considered statistically significant.

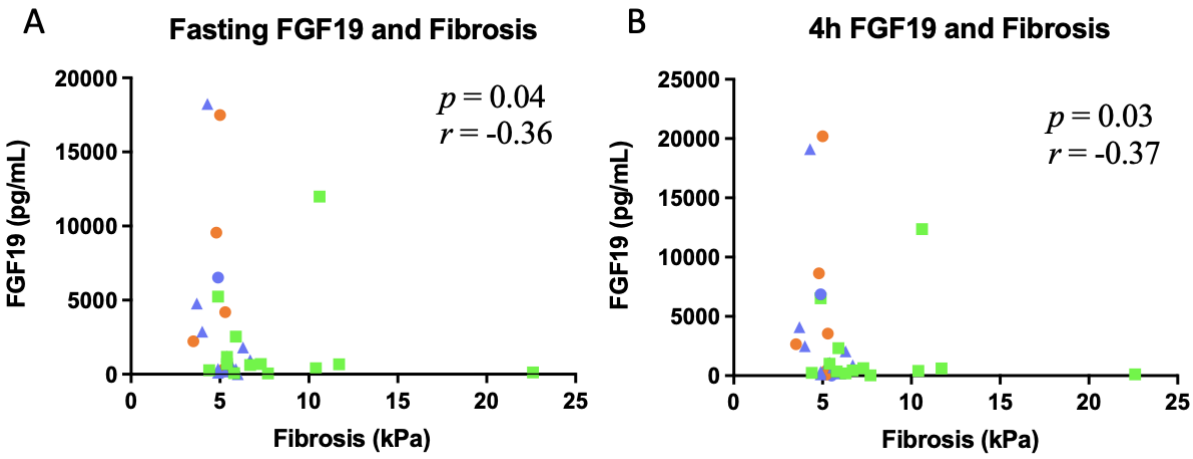

Figure 5A-B. Correlation between fasting and 4h FGF19 and fibrosis. Data points represent individual participant measurements. Results are based on results of Spearman's correlation. $p < 0.05$ is considered statistically significant.

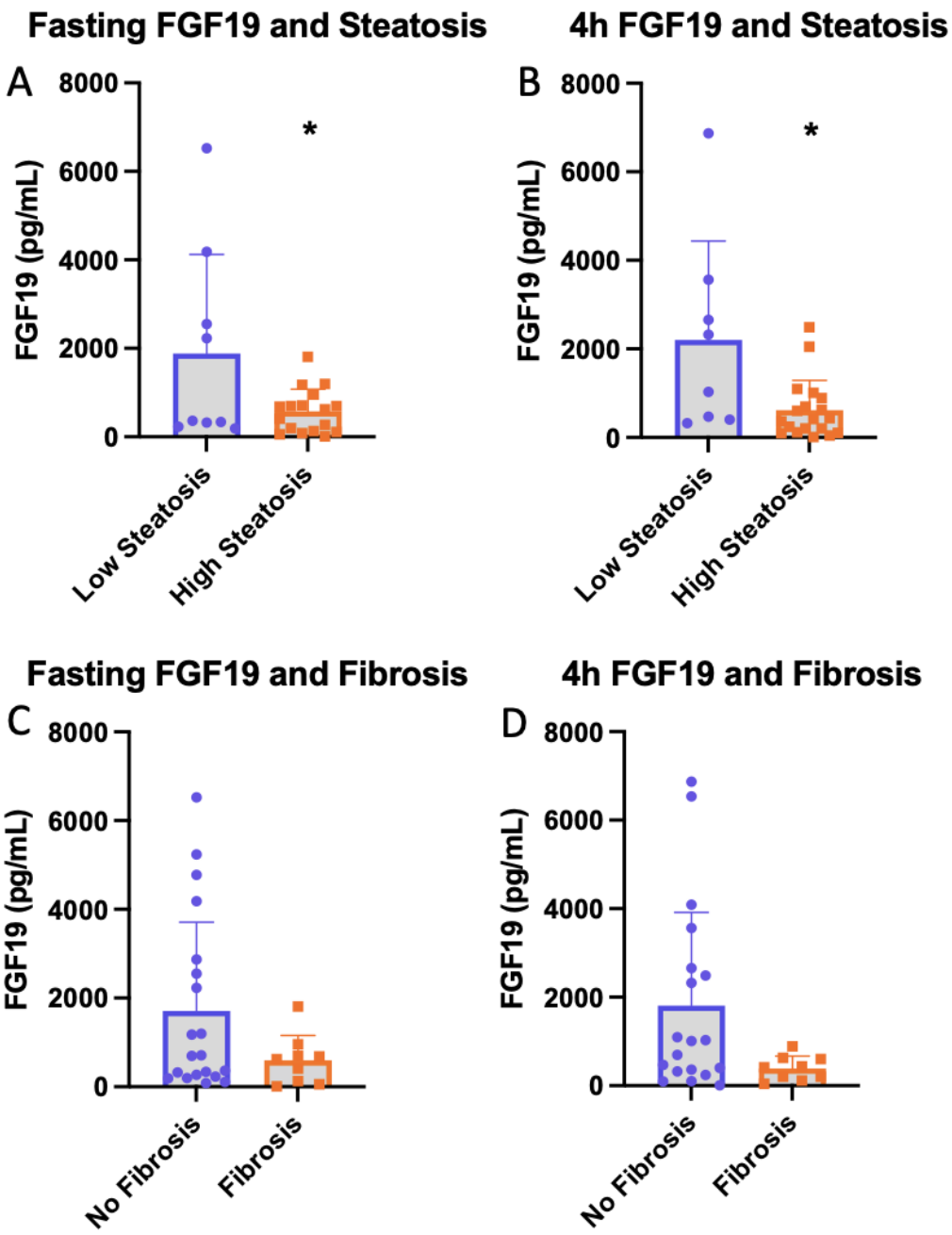

Figure 6A-D. Differences in FGF19 Parameters Between Fibrosis and Steatosis Status. Data are mean ± SD. *indicates a statistically significant difference between groups. Groups were compared with an unpaired t-test. $p < 0.05$ is considered statistically significant.

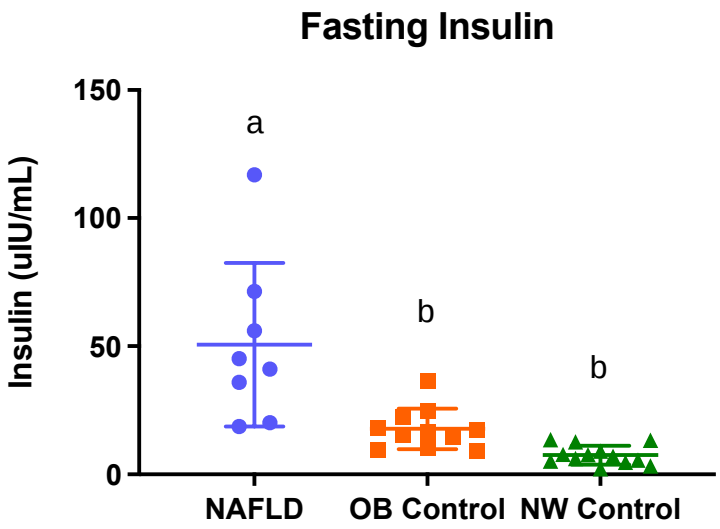

Figure 7. Fasting Insulin. Data are mean ± SD. Data points represent individual participant measurements. Groups were compared with a one-way ANOVA and Tukey's post-hoc test. $p < 0.05$ is considered statistically significant.

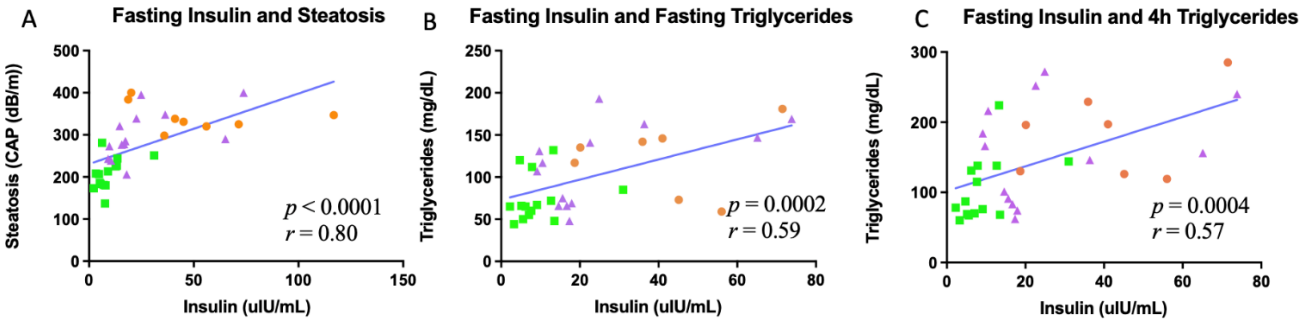

Figure 8A-C. Triglycerides and Steatosis Correlations with Fasting Insulin. Data points represent individual participant measurements. Statistics are for Spearman's correlation. $p < 0.05$ is considered statistically significant.

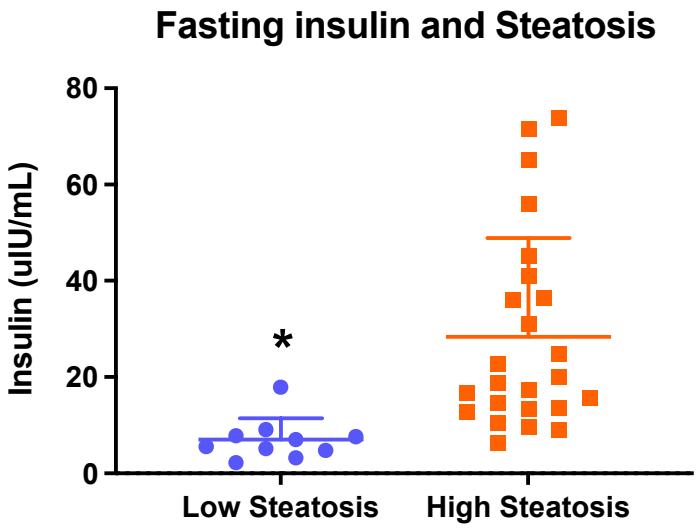

Figure 9. Fasting Insulin and Steatosis. Data are mean ± SD; data points represent individual participant measurements. Groups were compared with an unpaired t-test. $p < 0.05$ is considered statistically significant.

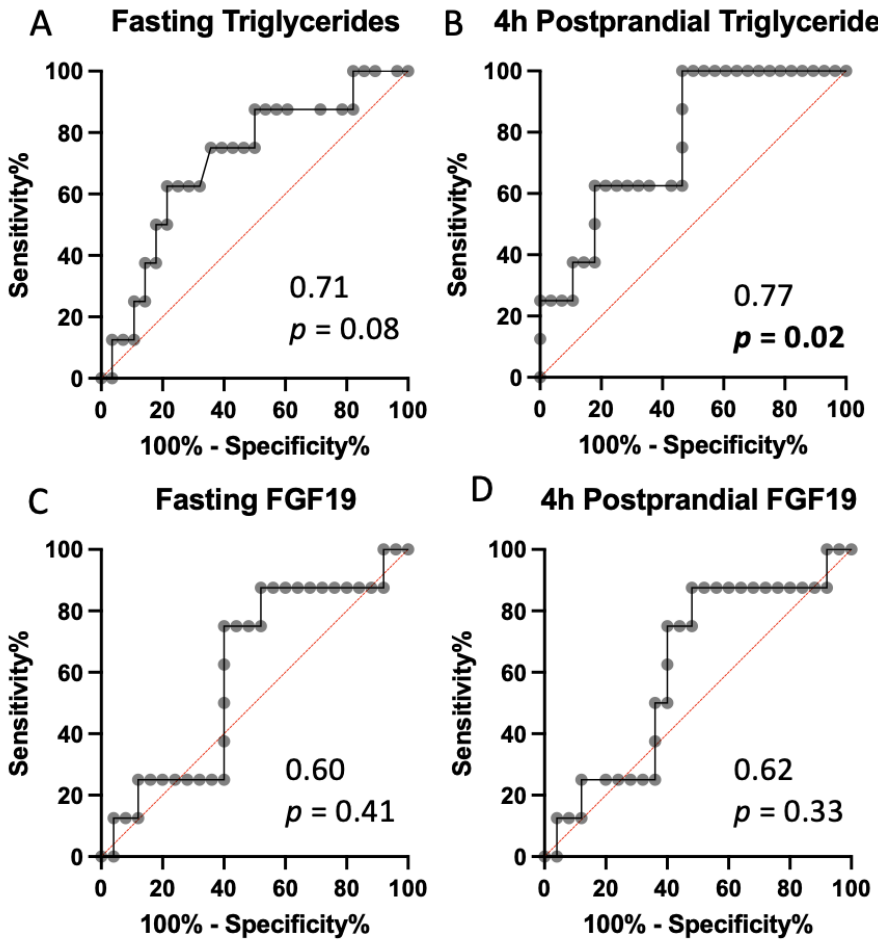

Figure 10A-D. ROC plots. The red line denotes the line of no discrimination (area under the curve of 0.5, meaning a worthless test). A *p*-value > 0.05 means that the area under the ROC curve is not significantly different from 0.5 area under the curve and therefore there is not enough evidence to conclude that the test has diagnostic accuracy to distinguish between people with and without NAFLD. $p < 0.05$ is considered statistically significant.

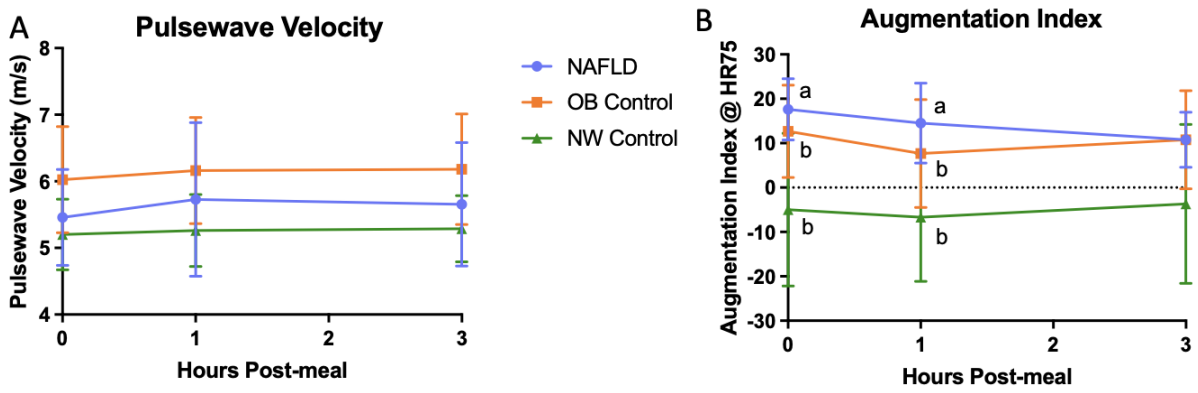

Figure 11A-B. Arterial Stiffness. Data are mean ± SD. Time x group comparisons were made with a two-way ANOVA. Groups with a shared letter indicate no statistically significant difference between groups. $p < 0.05$ is considered statistically significant.

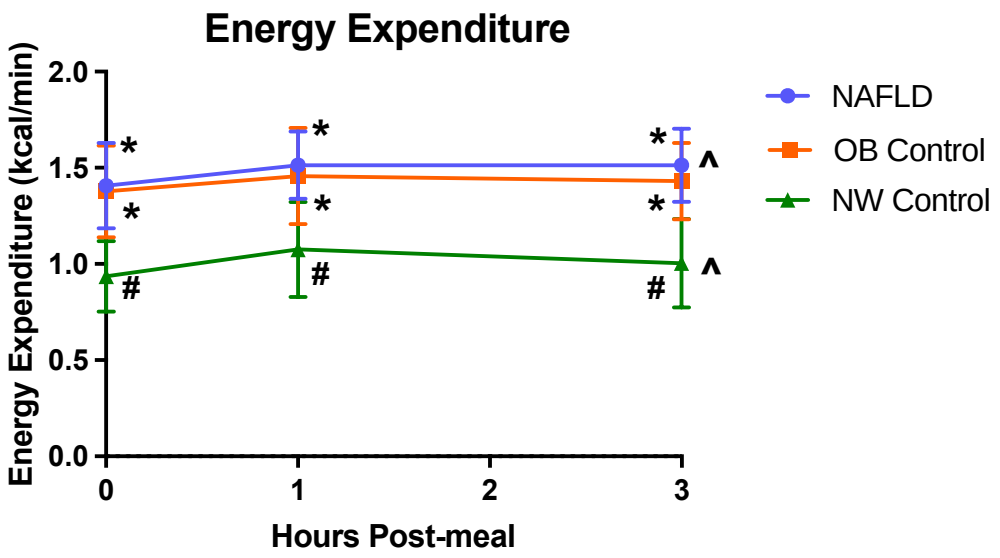

Figure 12. Energy Expenditure. Data are mean ± SD. Data are based on results of two-way ANOVA. Groups with a shared symbol indicate no statistically significant difference between groups; groups with ^ indicated significant time-course changes within groups. $p < 0.05$ is considered statistically significant.

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APPENDICES

Oklahoma State University Institutional Review Board

Date:	11/22/2019
Application Number:	HS-19-58
Proposal Title:	Fat Tolerance Testing as a Screening Tool for NAFLD
Principal Investigator:	Sam Emerson
Co-Investigator(s):	Christina Sciarrillo
Faculty Adviser:	Sam Emerson
Project Coordinator:	
Research Assistant(s):	Kara Poindexter, Madison Dixon, Nick Koemel
Processed as:	Expedited
Expedited Category:	

Status Recommended by Reviewer(s): Approved
Approval Date: 11/21/2019

The IRB application referenced above has been approved. It is the judgment of the reviewers that the rights and welfare of individuals who may be asked to participate in this study will be respected, and that the research will be conducted in a manner consistent with the IRB requirements as outlined in section 45 CFR 46.

This study meets criteria in the Revised Common Rule, as well as, one or more of the circumstances for which continuing review is not required. As Principal Investigator of this research, you will be required to submit a status report to the IRB triennially.

The final versions of any recruitment, consent, and assent documents bearing the IRB approval stamp are available for download from IRBManager. These are the versions that must be used during the study.

As Principal Investigator, it is your responsibility to do the following:

1. Conduct this study exactly as it has been approved. Any modifications to the research protocol must be approved by the IRB. Protocol modifications requiring approval may include changes to the title, PI, adviser, other research personnel, funding status or sponsor, subject population composition or size, recruitment, inclusion/exclusion criteria, research site, research procedures and consent/assent process or forms.
2. Submit a status report to the IRB when requested
3. Promptly report to the IRB any harm experienced by a participant that is both unanticipated and related per IRB policy.
4. Maintain accurate and complete study records for evaluation by the OSU IRB and, if applicable, inspection by regulatory agencies and/or the study sponsor.
5. Notify the IRB office when your research project is complete or when you are no longer affiliated with Oklahoma State University.

If you have questions about the IRB procedures or need any assistance from the Board, please contact the IRB Office at 405-744-3377 or irb@okstate.edu.

Sincerely,
Oklahoma State University IRB

VITA

Christina Marie Sciarrillo

Candidate for the Degree of

Doctor of Philosophy

Dissertation: POSTPRANDIAL TRIGLYCERIDES AND FIBROBLAST GROWTH FACTOR-19 AS POTENTIAL SCREENING TOOLS FOR PEDIATRIC NON-ALCOHOLIC FATTY LIVER DISEASE

Major Field: Nutritional Sciences

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