

**The Role of Hepatic Mitochondrial Function and Mitochondrial
Turnover in the Development and Progression of Nonalcoholic Fatty
Liver Disease and Lifestyle Modifications to Treat the Disease**

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THE ROLE OF HEPATIC MITOCHONDRIAL FUNCTION AND MITOCHONDRIAL
TURNOVER IN THE DEVELOPMENT AND PROGRESSION OF NONALCOHOLIC
FATTY LIVER DISEASE AND LIFESTYLE MODIFICATIONS TO TREAT THE DISEASE

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ABBREVIATIONS

NAFLD nonalcoholic fatty liver disease

NASH nonalcoholic steatohepatitis

IRB institutional review board

NAS NAFLD activity score

TC total cholesterol

TG triglycerides low-density

LDLc lipoprotein cholesterol

HDLc high-density lipoprotein cholesterol

AST aspartate transaminase

ALT alanine aminotransferase

ALP alkaline phosphatase

FCCP Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

β -HAD β -hydroxyacyl-CoA dehydrogenase

TEM Transmission electron microscopy

IPA Ingenuity Pathway Analysis

GO Gene Ontology

BMI body mass index

CPT1A carnitine palmitoyltransferase 1A

SOD superoxide dismutase

NRF2 nuclear factor erythroid 2-related factor 2

GPx glutathione peroxidase

OXPHOS oxidative phosphorylation

PGC1 α peroxisome proliferative activated receptor gamma coactivator 1 α

SIRT sirtuin

AMPK α 5'-AMP-activated protein kinase α

PPAR α peroxisome proliferative activated receptor α

ULK1 Unc-51 like autophagy activating kinase 1

ATG autophagy related protein

LC3 microtubule associated protein 1 light chain 3 A/B

BNIP3 BCL2 interacting protein 3

PINK1 PTEN induced kinase 1

DRP1 Dynamin-related protein 1

MFN Mitofusin

SREBP-1c sterol regulatory element binding protein-1c

VLDL very low density lipoprotein

ROS reactive oxygen species

NIX Nip3-like protein X

CaMK calcium/calmodulin-dependent kinase

TFB transcription factor B protein

TFAM mitochondrial transcription factor A

ERR estrogen-related receptors

eNOS endothelial nitric oxide synthase

NRF nuclear factor erythroid 2-related factor

MFF mitochondrial fission factor

FIS1 mitochondrial fission 1

DNL de novo lipogenesis

FAO fatty acid oxidation

MRS magnetic resonance spectroscopy

MRI magnetic resonance imaging

HIIT high intensity interval training

MICT moderate intensity continuous training

H&E hematoxylin and eosin

HbA1C hemoglobin A1C

ECG electrocardiogram

FUNDC1 FUN14 domain-containing protein 1

IHL intrahepatic lipid

ETC electron transport chain

FFA free fatty acid

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) and its more advanced form steatohepatitis (NASH) is associated with obesity and is an independent risk factor for cardiovascular, liver-related, and all-cause mortality. Available human data examining hepatic mitochondrial fatty acid oxidation and hepatic mitochondrial turnover in NAFLD, and NASH are scant. Further, the mechanistic effects of weight loss induced by diet and exercise on histological outcomes of NAFLD/NASH are poorly understood. Data from this dissertation demonstrates a reduction in hepatic mitochondrial fatty acid oxidation and the rate limiting enzyme in β -oxidation (β -hydroxyacyl-CoA dehydrogenase activity) by ~50% in individuals with moderate [NAFLD activity score (NAS) = 3-4]; and severe (NAS \geq 5) NAFLD compared with no disease (NAS = 0). This coincided with increased hepatic mitochondrial reactive oxygen species production, as well as significant reductions in markers of mitochondrial biogenesis, autophagy, mitophagy, fission and fusion. Active treatment consisting of caloric restriction and exercise training to induce weight loss resulted in significant reductions in total NAS, intrahepatic lipid content, and hepatocellular ballooning. Changes in NAS significantly correlated with reduced body fat mass and increased aerobic capacity. Interestingly, hepatic fatty acid oxidation did not change at follow-up in response to weight loss induced by diet and exercise and did not correlate with changes in NAS or its components. The findings presented here suggest that compromised hepatic mitochondrial fatty acid oxidation and mitochondrial turnover are intimately linked to increasing NAFLD severity in humans with obesity. Further, lifestyle changes aimed at promoting weight loss through dietary caloric restriction and exercise leads to histological improvement in NAFLD outcomes that are linked to improvements in body composition and aerobic capacity.

CHAPTER 1 – BACKGROUND AND AIMS

NONALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) is a progressive disease of the liver that ranges on a pathological spectrum from simple hepatic steatosis to a more severe nonalcoholic steatohepatitis (NASH; steatosis + inflammation) phenotype, which can progress to fibrosis and cirrhosis [1]. NAFLD is the most prevalent liver disease in the U.S., with reports in the general population ranging from 10-30% and as high as 80-100% in obese populations [1-4]. Hence, there is a serious need to understand its progression to the advanced state, NASH. NAFLD progression is the most rapidly increasing indication for liver transplantation in the United States [5], and now can be considered a multisystem disease affecting many extrahepatic organs. Indeed, NAFLD is an independent risk factor for cardiovascular, liver-related, and all-cause mortality [6, 7], and doubles the risk of type 2 diabetes development [8]. Currently, there are no FDA-approved pharmacological treatments for NAFLD, and further research is required to elucidate the pathological mechanisms of NAFLD/NASH.

MECHANISMS OF NAFLD DEVELOPMENT

Despite its alarming prevalence, the pathophysiology of NAFLD progression is not well understood. The mechanisms of NAFLD and NASH development are complex and multifactorial. Currently, NAFLD/NASH is understood to develop through a 'multiple-parallel hits' hypothesis: consisting of a number of insults acting together to induce disease [9]. Such hits include the interaction between dietary, environmental, and genetic factors as well as inter-organ cross talk which all contribute to the pathogenesis of NAFLD. Loss of insulin sensitivity at the level of the adipose tissue disrupts insulin-

mediated suppression of lipolysis, resulting in circulating free fatty acids (FFA) being taken up ectopically by the liver and converted to triglycerides. At the same time, hepatic insulin resistance disrupts the insulin-stimulated suppression of hepatic glucose output. This combination of hepatic and adipose tissue insulin resistance results in elevated serum insulin and glucose - potent stimulators of *de novo* lipogenesis (DNL), where lipids are synthesized from dietary carbohydrates. Up to 25% of hepatic triglyceride content can be attributed to DNL in NAFLD in humans [10], while other sources that contribute to liver triglyceride accumulation include dietary fat and adipose release of FFAs. The increasing levels of triglycerides in the liver contributes to hepatic lipotoxicity and central insulin resistance, promoting the activation of hepatic pro-inflammatory pathways. Additionally, dysbiosis of the gut microbiota has been linked with NAFLD development and progression [11, 12], with gut-derived endotoxins and altered bile acids further exacerbating hepatic inflammation [13]. The mechanisms behind NAFLD development have not been fully elucidated, though convincing evidence suggests that hepatic mitochondrial dysfunction is tightly linked to disease progression [14-16]. Strong evidence has implicated a loss in mitochondrial function with more advanced NAFLD/NASH with rodent data from our lab [17-21] and others [14, 22-25], and data from human studies [26-28] to support this.

HEPATIC MITOCHONDRIAL DYSFUNCTION AND NAFLD

Evidence from experimental models highlights the hallmarks of hepatic mitochondrial dysfunction in the setting of NAFLD/NASH progression, including decreased electron transport chain (ETC) content, abnormal morphology, and reduced respiration and β -oxidation [20, 29-32]. Further, increasing hepatic steatosis and loss in mitochondrial function is coupled with the generation of reactive oxygen species and inflammatory cytokine production, exacerbating NAFLD/NASH pathology [33]. Despite increases in

mitochondrial structural and functional abnormalities being clearly implicated in exacerbating disease progression in humans [15, 34, 35], there are a lack of studies employing direct (i.e., high resolution respirometry) approaches to assess changes in mitochondrial ETC and fatty acid oxidation in patients with NAFLD/NASH.

In vivo assessments of hepatic ATP production, using NMR labelling patterns [36] and ³¹P MRS [37, 38], demonstrate that TCA cycling increases linearly with NAFLD score. Recent publications, highlight the inherent challenges of inferring TCA flux using these indirect assessment tools (i.e., possible influence of cytosolic ATP), particularly when biopsies have not been taken, since the severity of disease will impact the results [39-41]. Direct assessment of mitochondrial bioenergetics in isolated hepatic mitochondria, using high resolution respirometry, bypasses the challenges of these indirect assessment tools and also allows for us to determine whether dysregulation exists within specific sites of the mitochondrial oxidative phosphorylation cascade. In a recent study by Lund et al [39], the authors reported similar in vitro mitochondrial oxidative capacity related to fat oxidation, despite increases in liver lipid content in individuals with obesity vs non-obese controls. Conversely, Koliaki et al [42], found that compared to lean controls, hepatic mitochondrial oxidative capacity related to fat oxidation was first increased in a graded fashion, in obese and NAFLD subjects [NAFLD activity score (NAS) =3] and then waned in NASH patients with advanced disease (NAS=7). Indeed, these findings challenge the prevailing conceptual models of mitochondrial dysfunction as a causal event in the onset and progression of NAFLD/NASH and highlight the need for future conciliatory investigations to assess hepatic mitochondrial function *in vitro* in humans across the spectrum of NAFLD/NASH. Additionally, a decline in hepatic mitochondrial fatty acid oxidation is linked to NAFLD development in rodent models [15, 43]. Whether a similar decline is present in humans with NAFLD is less clear. Indirect

measures of fatty acid oxidation in the liver of patients with NAFLD, such as plasma ketone body concentrations, suggest that hepatic FAO is either normal or increased [44-46]. However, given their unstable nature, ketone body concentrations are an unreliable marker of hepatic fatty acid oxidation. Thus, such studies are strongly warranted.

In Figure 1 we present preliminary data in C57BL6J male mice fed a high-fat, high-sucrose Western diet for 16 weeks to induce NAFLD/NASH (Fig. 1.1A). Mice fed a Western diet for 16 weeks had significantly lower FAO rates in whole liver (Fig. 1.1B). Further, reductions in isolated hepatic mitochondria complete palmitate oxidation to CO₂ correlated significantly with increasing NAFLD severity (Fig. 1.1C, P < 0.05). These changes in mitochondrial function were observed despite no changes in citrate synthase activity, a surrogate measure of mitochondrial mass with NAFLD/NASH (Fig. 1.1D). Indeed, this preliminary data demonstrates that there is a loss in mitochondrial function with a worsening NAFLD/NASH phenotype in rodents. Whether similar effects on *ex vivo* FAO is observed in human liver with NAFLD/NASH progression requires exploration.

Indeed, it is clear that loss in normal mitochondrial function increases with NAFLD/NASH severity in experimental models, and it is essential that they be further explored in a human population. These data add significant clarification to the relationships between hepatic mitochondrial bioenergetics, fatty acid oxidation and NAFLD severity. *Hence, the present dissertation interrogated this gap in knowledge by determining whether NAFLD/NASH progression is associated with alterations in hepatic mitochondrial ETC and fatty acid oxidation in biopsied liver tissue from patients with obesity (Aim 1a, Fig. 1.4A).*

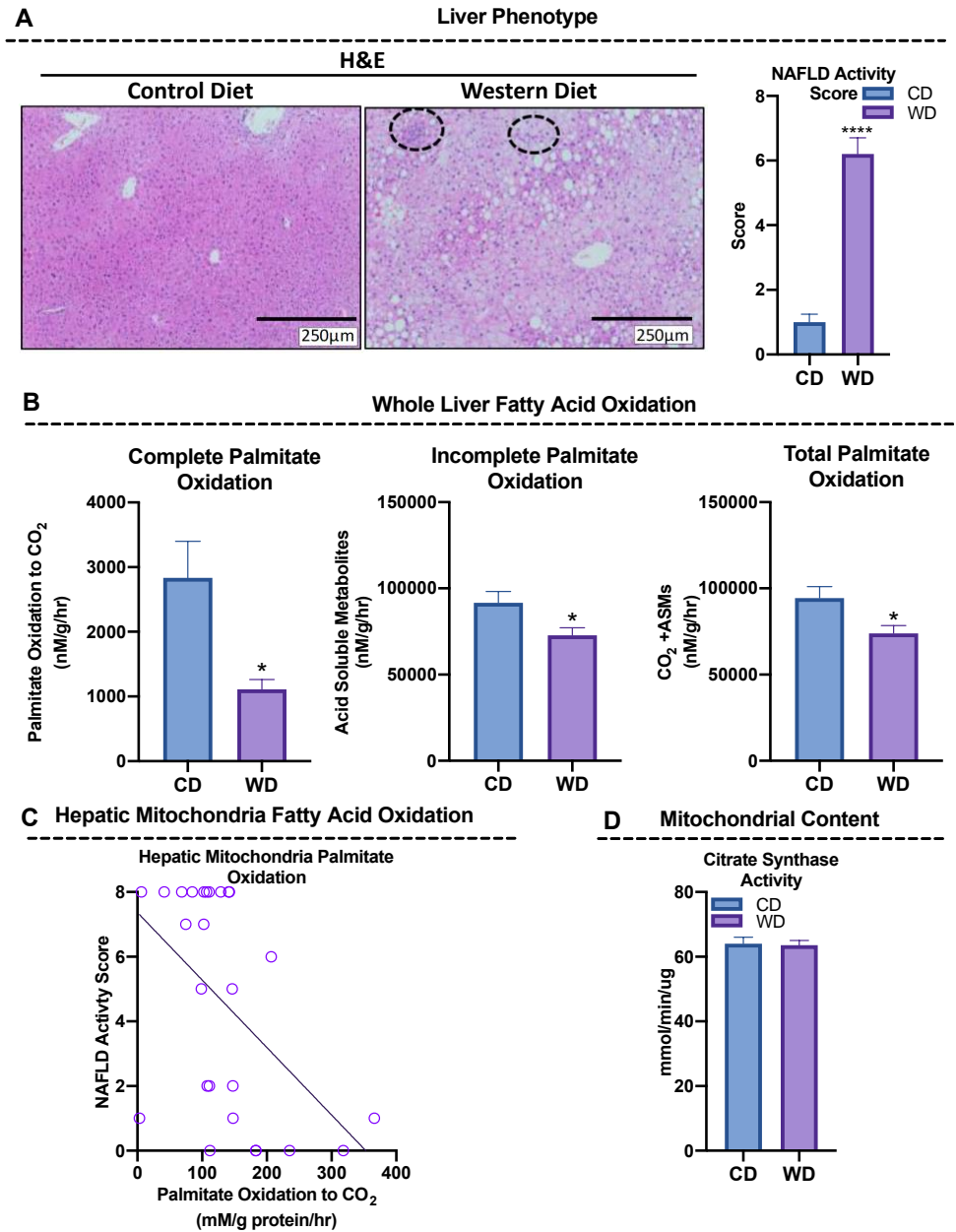


Figure 1.1. Reduced Mitochondrial fatty acid oxidation in sedentary, male, C57BL6J mice fed a western diet for 16 weeks versus control diet fed mice.

A) H&E staining and NAFLD activity score, B) whole liver fatty acid oxidation, C) complete liver palmitate oxidation in whole liver correlated with NAFLD activity score, D) mitochondrial mass. All data presented as means \pm SE (n= 9-11). * indicates significant difference for control diet versus western diet fed mice ($p \leq 0.05$). CD, Control Diet; WD, Western Diet.

MITOCHONDRIAL TURNOVER IN NAFLD/NASH

Mitochondrial health and quality control hinges on the intimately linked processes of biogenesis, the production of new healthy mitochondria, and mitophagy, where dysfunctional mitochondria are removed via selective autophagy, in an effort to maintain mitochondrial homeostasis within the cell [47]. Perturbations in mitochondrial quality control are linked with mitochondrial dysfunction in a number of tissues, including the liver. Peroxisome proliferator activator receptor γ coactivator α (PGC-1 α), the master regulator of mitochondrial biogenesis, is reduced in the liver of patients with obesity and NASH [42, 48]. Further, our group has previously shown that hepatic PGC-1 α overexpression in rats increases hepatic mitochondrial function and reduces steatosis *in vivo* and *in vitro* [17, 49]. Indeed, these data highlight the important role of hepatic mitochondrial biogenesis in the regulation of mitochondrial function and susceptibility to NASH. As highlighted previously, mitochondrial health is also dependent on mitophagy - the turnover and degradation of low functioning mitochondria via the lysosome [50]. Impairments in autophagy have been linked to NAFLD in rodent models. Genetic ablation of autophagy-related (ATG) proteins necessary for autophagy function have been linked to elevations in hepatic steatosis [51-53], depletion of hepatic ATP production, and trigger mitochondrial apoptotic signaling [54, 55]. BCL-2/adenovirus E1B 19-kDa interacting protein (BNIP3) is a highly conserved mitophagy protein in the liver and is a well-established critical regulator of receptor-mediated mitophagy. Loss of hepatic BNIP3 is thought to result in increased mitochondrial mass within the cell and is associated with reduced membrane potential, reduced mitochondrial respiration, increased H₂O₂ emission, defective mitochondrial fatty acid oxidation, and hepatic steatosis [56]. Rodent models of obesity induced NAFLD have been shown to exhibit reduced hepatic protein content and gene expression of markers of autophagy including cleaved microtubule-associated protein 1A/1B-light chain 3 (LC3), Beclin 1, Atg5/Atg12,

and Atg7 [51, 57]. Here we present preliminary data in C57BL6J male mice fed a Western diet for 30 weeks to induce advanced NASH (Fig. 1.2A). Mice with NASH displayed significant reductions in markers of mitochondrial content (OXPHOS), mitochondrial biogenesis (PGC-1 α), and mitophagy (BNIP3) compared to control diet fed mice (Fig. 1.2B, $P < 0.05$). Indeed, the role of reduced biogenesis and mitophagy in NAFLD/NASH development and progression is intriguing but has not been fully investigated in a human NAFLD/NASH population and so will be interrogated in this proposal (Aim 1b, Fig.1.4B).

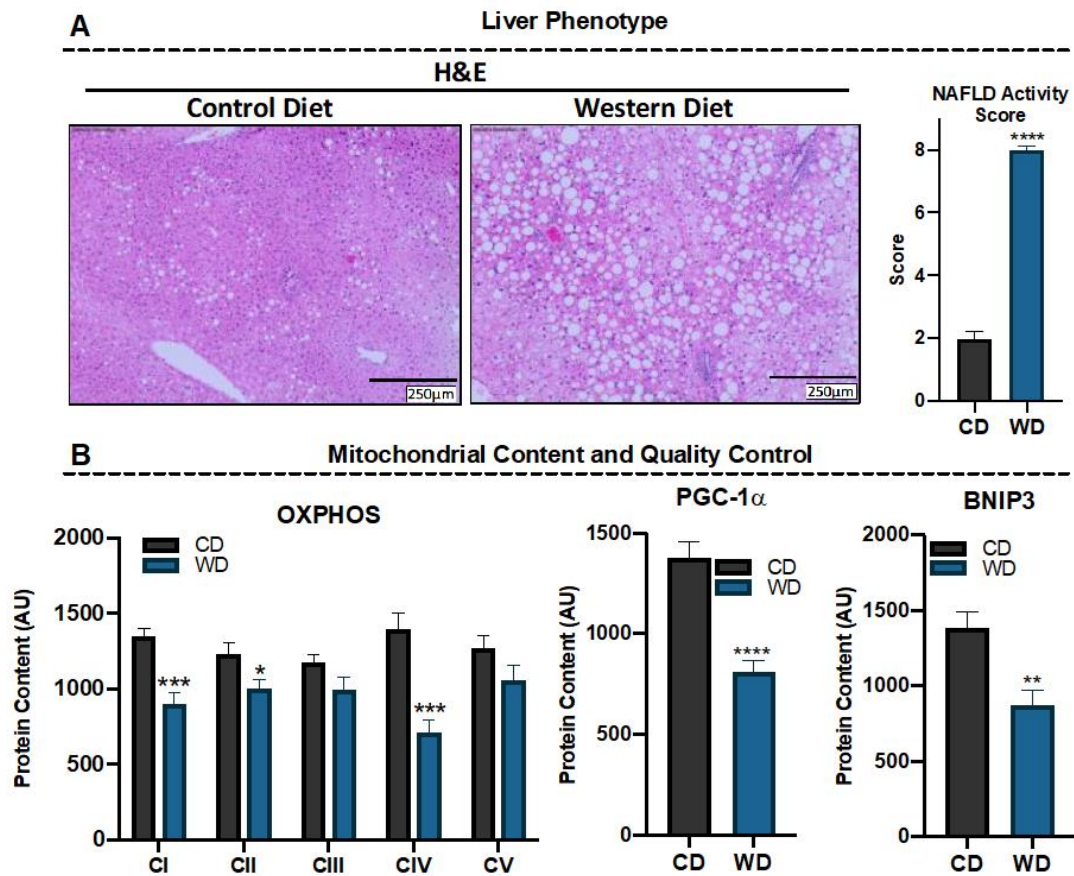


Figure 1.2. Reduced Mitochondrial content and quality control/turnover in sedentary, male, C57BL6J mice fed a western diet for 30 weeks versus control diet fed mice. A) H&E staining and NAFLD activity score, B) markers of mitochondrial content (OXPHOS), biogenesis (PGC1 α), and mitophagy (BNIP3). All data presented as means \pm SE (n= 9-11). * indicates significant difference for control diet versus western diet fed mice ($p \leq 0.05$). CD, Control Diet; WD, Western Diet; PGC1 α , PPAR-gamma coactivator-1 α ; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.

LIFESTYLE MODIFICATIONS VIA CALORIC RESTRICTION AND EXERCISE

REVERSES NAFLD

Given the lack of a pharmaceutical drug that specifically treats NAFLD, lifestyle modifications such as caloric restriction and exercise, which have been shown to prevent or reverse NAFLD, remain the cornerstone of NAFLD therapy [58-62]. A number of reviews [63-67] highlight the current evidence for the use of exercise with weight loss [62, 68-74] and at weight stability to ameliorate intrahepatic lipid content [measured by magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI)] [75-78]. Caloric restriction and/or exercise training lessens the nutrient burden on the liver, improves peripheral insulin sensitivity [31, 59-62], and promotes fuel uptake at the periphery. Indeed, it is clear that combining caloric restriction and exercise regimens results in the greatest reductions in IHL and improvements in substrate metabolism [79-85].

With regards to lifestyle modifications (caloric restriction and exercise) to treat NAFLD in humans, most of the research discussed above has relied on noninvasive imaging techniques (MRS, MRI, ultrasonography) to measure intrahepatic lipid content in the absence of histological interrogation. However, biopsy studies assessing the effects of behavioral lifestyle modifications on histological outcomes pre- and post-intervention are limited. Some evidence demonstrates that body weight loss of 7-10%, via dietary caloric restriction is efficacious in regressing histological steatosis, hepatocellular inflammation and fibrosis [86, 87]. While histological improvements have also been observed with as little as 3-5% weight loss [23, 88, 89]. In patients who lost $\geq 10\%$ of their body weight, >90% had complete resolution of NASH [85, 86], or a reduction in NAS of ≥ 3 [90]. Indeed, existing literature supports the benefits of weight loss via caloric restriction to prevent the development of [91] and treat [1, 92-97] NAFLD/NASH. While these studies

provide important knowledge on the potential clinical utility of lifestyle modifications to treat NAFLD, the mechanisms by which weight loss via caloric restriction and exercise improve liver histology remains unknown. Further, when discovered this knowledge can support the development of new therapies for this disease.

In recent years, high intensity interval exercise training (HIIT) gained popularity as a safe [98, 99] and even superior form of aerobic exercise, compared to moderate intensity continuous exercise training (MICT) in patients with cardiovascular disease [100-102]. Emerging evidence highlights the role of HIIT to be as effective as MICT in the treatment of NASH and a more time efficient strategy [69, 103, 104]. In fact, a recently published study from our lab, demonstrates that energy-matched HIIT and MICT resulted in similar marked reductions in IHL content in obese adults [105]. Individuals (n=18) were randomized to either 4 weeks of HIIT (4 min 80%VO₂ peak/ 3 min, 50% VO₂ peak) or MICT (55% VO₂ peak, ~60 min), matched for energy expenditure (~400 kcal/session), 4 times per week. There was a 20.1±6.6% and 37.0±12.4% reduction in IHL for HIIT and MICT, respectively (Fig. 1.3A & B). However, the change in IHL was not different between exercise conditions. Of further note, 81% of subjects exhibited >5% exercise induced reduction in IHL. While 18% of subjects displayed a complete resolution of steatosis (i.e., IHL < 5.5%) and 50% of participants had <10% IHL content after 4 weeks of aerobic exercise training, independent of exercise conditions (Fig. 1.3C). Furthermore, these reductions in IHL content occurred despite no significant changes in body mass, abdominal adiposity, liver enzyme levels, or biomarkers of hepatic function (data not shown). These findings highlight that energy-matched HIIT and MICT are effective at decreasing intrahepatic lipid and NAFLD risk.

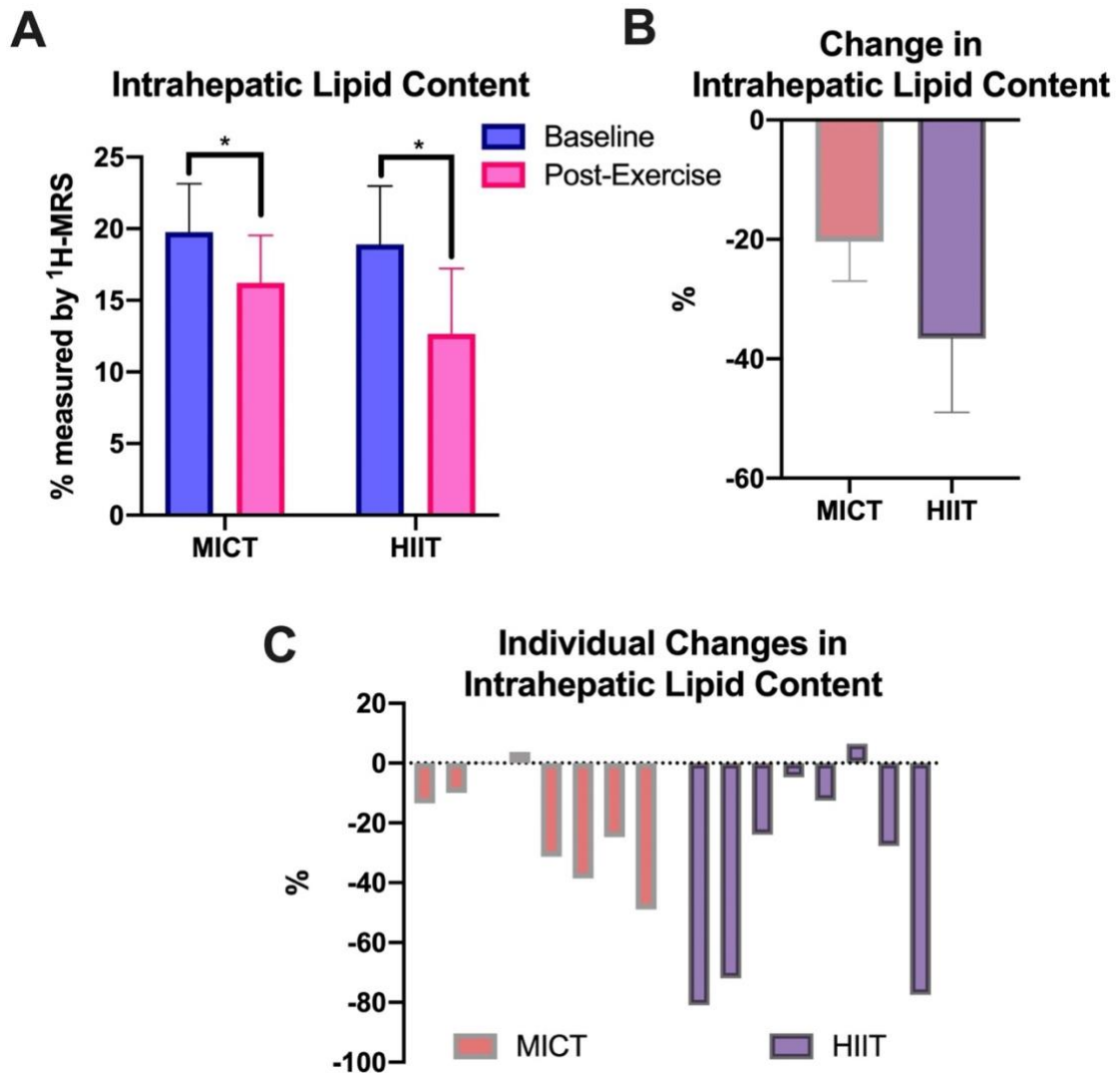


Figure 1.3. Exercise training decreased IHL in both MICT and HIIT in obese adults. A) Baseline and post-exercise training IHL; B) mean changes in IHL, and C) individual subject changes in IHL levels after 4 weeks of no exercise MICT or HIIT. Data are mean \pm SE. * indicates significant difference baseline vs post-exercise ($P < 0.05$). IHL, intrahepatic lipid; MICT, continuous moderate exercise; HIIT, high intensity interval exercise. Adapted from Winn et al, [105].

While the exact mechanisms of caloric restriction and exercise on hepatic outcomes remain elusive and are likely multifaceted, our lab and others have shown that exercise is an effective approach in improving NAFLD outcomes via improved lipid metabolism, decreased oxidative stress, inflammation, and fibrosis in rodent models [106, 107]. Further, our group has previously shown that exercise resulted in additional benefits

beyond caloric restriction alone in a rodent model of NAFLD/NASH [108]. Our group and others have also demonstrated that exercise increases hepatic mitochondrial content/function [17, 32, 108, 109] and mitochondrial quality control [110, 111]. Whether similar improvements in hepatic health are observed in humans following a lifestyle intervention remain to be explored.

In summary, the preliminary data presented here, and the existing literature highlight that calorie restriction and exercise are beneficial in the treatment of NAFLD. *However, studies with pre- and post-liver biopsies examining the effect of lifestyle modifications, via caloric restriction and exercise, on reversing NASH are limited and are examined here (Aim 2, Fig. 1.4BC).* Further, *elucidating the mechanisms by which caloric restriction and exercise reverse NASH and improves hepatic fatty acid metabolism has important implications and holds promise for future discovery of novel therapeutic targets for management of this disease (Aim 2).*

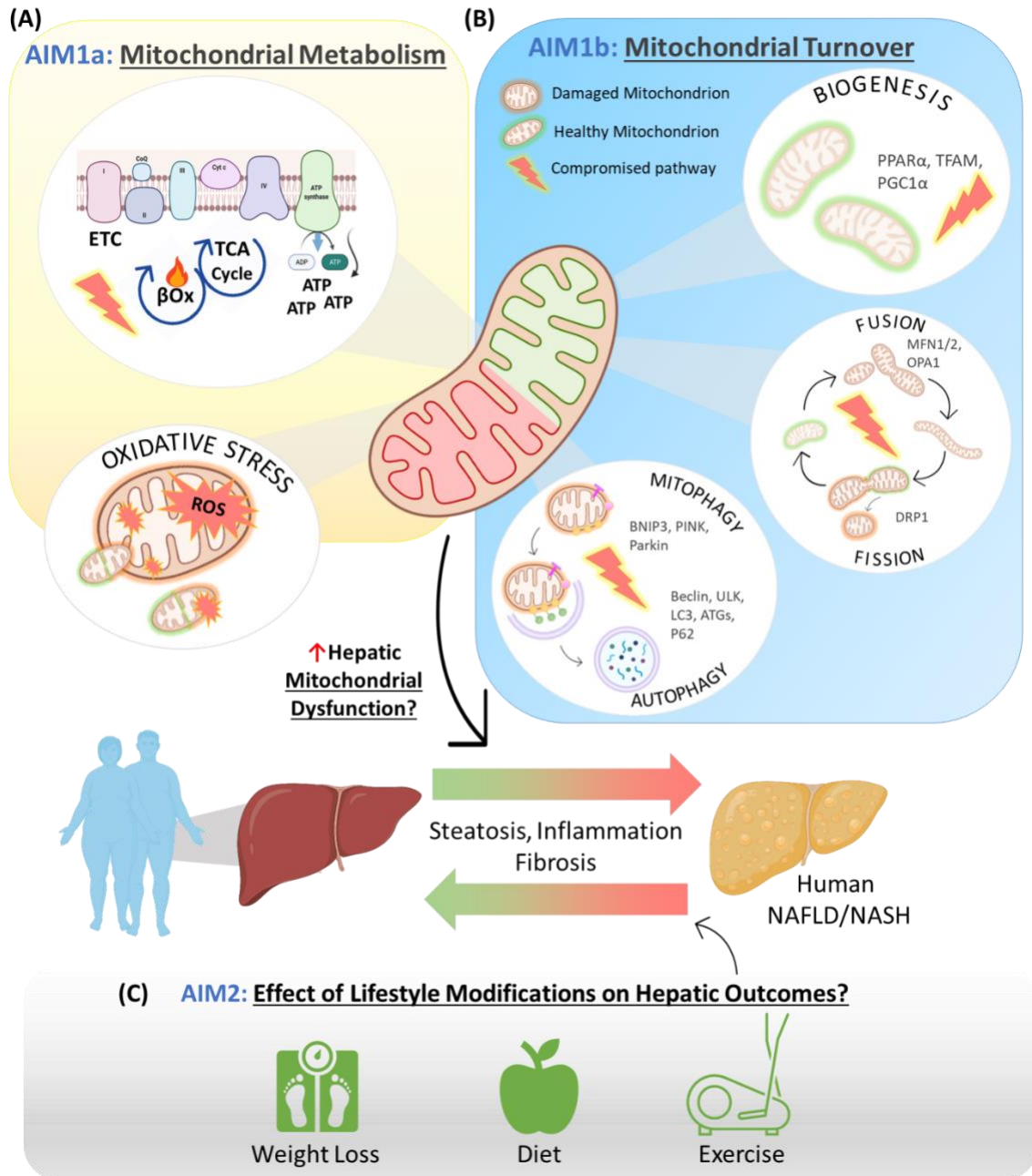


Figure 1.4. Schematic illustration of the potential role of mitochondrial function and turnover in the development and progression of nonalcoholic fatty liver disease (NAFLD) / nonalcoholic steatohepatitis (NASH). (A) Losses in mitochondrial fatty acid oxidation and compromised mitochondrial biogenetics have been linked to worsening NAFLD/NASH. Whether reduced hepatic mitochondrial function is linked to NAFLD/NASH development and progression in humans requires further exploration (Aim 1a). (B) Rodent studies highlight that compromization in mitochondrial turnover (biogenesis, fission/fusion, autophagy, and mitophagy) play a role in NAFLD/NASH development and progression. Whether a loss in mitochondrial turnover is linked to NAFLD/NASH development and progression in humans requires further study (Aim 1b). (C) Studies assessing the therapeutic effects of lifestyle modifications (weight loss, diet, and exercise) on biopsy-determined NASH in humans pre- and post-intervention

remains understudied and requires investigation (Aim 2). Abbreviations: ETC, electron transport chain; TCA cycle, tricarboxylic acid cycle; β Ox, fatty acid β -oxidation; ATP, adenosine triphosphate; ROS, reactive oxygen species; PPAR α , Peroxisome proliferator-activated receptor- α ; PGC1 α , PPAR-gamma coactivator-1 α ; TFAM, Mitochondrial Transcription Factor A; MFN1/2, Mitofusin-1/2; DRP1, dynamin-related protein 1; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; Pink, Phosphatase and tensin homolog (PTEN)-induced kinase 1; ULK1, Unc-51 like autophagy activating kinase; LC3, Microtubule-associated protein 1A/1B-light chain; ATG, Autophagy-related protein.

IMPACT AND INNOVATION OF THIS PROPOSAL

1) This project is translational in nature. Our group have previously comprehensively studied the role of mitochondrial dysfunction in NAFLD/NASH development and progression in rodents. The data garnered from Aim 1 will add significant clarification to the relationships between hepatic mitochondrial metabolism and turnover, and disease severity and will further drive the need to target mitochondrial function for the treatment of NAFLD/NASH. 2) This project is technically innovative. I will use advanced technologies to investigate and combine direct measures of hepatic metabolism, with gene expression and protein abundance of markers related to mitochondrial quality control as well as mitochondrial structure and morphology. 3) This project is conceptually innovative and translational in nature. Our lab has previously studied the effects of exercise and caloric restriction in animal models. The present proposal translates our findings from animal models to humans. 4) The information on mitochondrial dysfunction as a mediator of cellular stress garnered from Aim 1 and the role of lifestyle modifications via dietary caloric restriction and exercise on NASH (Aim 2) will be pertinent not only to NAFLD/NASH treatment clinically but will also benefit several NAFLD related diseases including diabetes and cardiovascular risk conferred through oxidative stress and inflammation in the setting of NAFLD/NASH.

APPROACH

Specific Aim 1a: To determine whether reduced hepatic mitochondrial function is linked to worsening NAFLD/NASH progression in humans.

Specific Aim 1b: To determine whether a loss in mitochondrial turnover (mitochondrial biogenesis and autophagy/mitophagy) is linked to worsening NAFLD/NASH progression in humans.

SA1. Rationale: Previous data in rodents [14, 22-25], and data from human studies [26-28] implicates the loss of mitochondrial function with advancing NAFLD/NASH, however, further research is needed to integrate changes in *ex vivo* hepatic mitochondrial fatty acid oxidation and bioenergetics with NAFLD/NASH progression in liver biopsies in humans. Additionally, the relationship between losses in normal hepatic mitochondrial function and mitochondrial morphology and quality control is yet to be established in a human population. *Hence, the present dissertation interrogated this critical gap in knowledge.* The preliminary data demonstrates a clear link between a loss in mitochondrial metabolic function with advancing NAFLD/NASH, which is linked to changes in mitochondrial morphology and quality control. ***I hypothesize that mitochondrial metabolic function ex vivo will be highest in patients with no disease. FAO and bioenergetics will be lower with simple steatosis (NAS 1-2) and will progressively decline with moderate disease (NAS 3-4; steatosis + some lobular inflammation) and severe disease (NAS 5-8; steatosis, inflammation, and ballooning degeneration).*** *Coincident with this I hypothesize that markers of mitochondrial turnover will decline in a similar manner with disease progression.*

Specific Aim 2: To determine the therapeutic effects of lifestyle modifications (via caloric restriction and exercise) on biopsy-determined NASH and hepatic fatty acid metabolism in humans.

SA2 Rationale: It is clear that lifestyle modifications, in the form of dietary caloric restriction and exercise, beneficially lower IHL content [1], but, studies investigating the effects of lifestyle modifications using biopsied-liver to assess histological outcomes of NASH pre- and post-intervention are limited. Most of the research listed above has relied on imaging (MRS, MRI, ultrasound, etc.) to measure steatosis without histological evaluation. While these studies suggest potential clinical utility, the mechanisms by which the treatments improve liver histology remains unknown. The abilities of calorie restriction and exercise training to improve hepatic fatty acid oxidation have not been examined in patients with NASH. Whether mitochondrial function can be improved in advanced disease is a critical question in the field. Identifying the mechanisms by which these effects can be reversed in human subjects with NASH has important implications and holds promise for future discovery of novel therapeutic targets for NAFLD/NASH. ***I hypothesize that weight loss via caloric restriction and exercise training will significantly improve liver metabolism and reduce steatosis, inflammation thus promoting the resolution of the histological features NASH.***

**CHAPTER 2 - COMPROMISED HEPATIC MITOCHONDRIAL FATTY ACID
OXIDATION AND REDUCED MARKERS OF MITOCHONDRIAL TURNOVER IN
HUMAN NAFLD**

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ABSTRACT

Background and Aims - Nonalcoholic fatty liver disease (NAFLD) and its more advanced form steatohepatitis (NASH) is associated with obesity and is an independent risk factor for cardiovascular, liver-related, and all-cause mortality. Available human data examining hepatic mitochondrial fatty acid oxidation and hepatic mitochondrial turnover in NAFLD and NASH are scant. To investigate this relationship, liver biopsies were obtained from patients with obesity undergoing bariatric surgery (n=110) and data clustered into four groups based on NAFLD activity score [(NAS); (No Disease, NAS = 0, n = 13; Mild NAS = 1-2, n = 28; Moderate NAS = 3-4, n = 41, and Advanced NAS \geq 5, n=28)].

Results - Hepatic mitochondrial fatty acid oxidation and the rate limiting enzyme in β -oxidation (β -hydroxyacyl-CoA dehydrogenase activity) were reduced by ~50% with Moderate and Severe NAS compared with NAS = 0 ($p < 0.05$). This coincided with increased hepatic mitochondrial reactive oxygen species production, as well as significant reductions in markers of mitochondrial biogenesis, autophagy, mitophagy, fission and fusion ($P < 0.05$).

Conclusions -These findings suggest that compromised hepatic mitochondrial fatty acid oxidation and mitochondrial turnover are intimately linked to increasing NAFLD severity in patients with obesity.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) and its progressive form of steatohepatitis (NASH) is the most prevalent liver disease in the U.S. [1] and is an independent risk factor for cardiovascular, liver-related, and all-cause mortality [112]. Moreover, there are no established, validated pharmacological therapies for NAFLD and NASH. This is, in part, attributed to poorly understood pathological mechanisms of the disease process. An increasing body of evidence highlights hepatic mitochondrial dysfunction as an important pathological discovery in NAFLD/NASH in both rodent models [18, 20, 32, 113], and humans [39, 42, 44-46, 114-117]. Recently, Koliaki et al. described the adaptation of hepatic mitochondrial function to increase bioenergetic needs in the setting of obesity and NAFLD, which was then lost with NASH [42]. Additionally, indirect measures of hepatic substrate oxidation have been shown to either be normal or increased in patients with NAFLD compared to no disease [44-46, 114-117]. Whether perturbations in hepatic mitochondrial long chain fatty acid oxidation occur in patients with increasing NASH severity is unknown. Moreover, despite being well-established in rodent literature [49, 57, 118], very little is known about alterations in markers of hepatic mitochondrial turnover, mitophagy, fission and fusion in humans with increasing NAFLD severity.

Here, we simultaneously assessed *ex vivo* fatty acid oxidation in isolated hepatic mitochondria and whole liver using radiolabeled [$1\text{-}^{14}\text{C}$] palmitate, isolated mitochondria respiration and measures of mitochondrial biogenesis, mitophagy, and dynamics in patients with varying degrees of liver disease severity. Our findings from multiple, independent lines of investigation demonstrate a dramatic decline in hepatic fatty acid oxidation and markers of mitochondrial biogenesis, autophagy, mitophagy, fission and fusion with increasing liver disease. These findings provide mechanistic insights into the

potential role of compromised mitochondrial function and turnover in the etiology of NAFLD/NASH.

EXPERIMENTAL METHODS

Study Participants - Liver samples were obtained from adults with clinical obesity undergoing elective bariatric surgery at the University of Missouri Hospital, Columbia MO. Before inclusion, all participants gave written informed consent to the protocol, which was approved by the Institutional Review Board (IRB) of University of Missouri (protocol #2008258) and conducted according to World's Medical Association Declaration of Helsinki. This study is registered at ClinicalTrials.gov (Identifier: NCT03151798). Degree of NAFLD severity was determined using the NAFLD activity score (NAS) [119], by a blinded hepatopathologist. Participants were clustered into four groups based on NAS - No Disease (NAS = 0), Mild (NAS = 1-2), Moderate (NAS = 3-4), and Severe (NAS \geq 5) from a larger group of enrolled participants. NAS inclusion criteria for NAFLD patients were based on an alcohol intake lesser than 20 g/day and histologically confirmed steatosis with/without necroinflammation and/or fibrosis. Other causes of liver disease were excluded based on history, laboratory data, and histological features.

Blood Metabolic Panels - Blood samples were drawn prior to metabolic surgery following an overnight fast and were immediately processed for biochemical measurements by a CLIA-standardized laboratory (Quest Diagnostics, St. Louis, MO, Lic.#26D0652092), according to standard procedures. Lipid measurements [total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDLc), high-density lipoprotein cholesterol (HDLc)] were performed via auto-analyzer (Roche Cobas 8000 System, CV 0.6-0.9%, Indianapolis, IN) using electrochemiluminescent

immunoassay. Liver enzymes [aspartate transaminase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)] were measured using UV Absorbance (Roche Cobas 8000 System, CV 0.5-3.2% for AST and 0.5-3.1% for ALT, Indianapolis, IN).

Liver Biopsies - Wedge liver biopsies (200–700 mg tissue) were obtained by surgeons ~30 minutes after initiation of anesthesia according to standardized protocols.

Approximately 200 mg was placed in ice-cold mitochondrial isolation buffer (220mM Mannitol, 70 mM sucrose, 10mM tris-base, 1mM EDTA; pH 7.4) for high resolution respirometry and fatty acid oxidation, ~10 mg was fixed in 1% formaldehyde for histological examination, 1-2mg was fixed in 2% paraformaldehyde, 2% glutaraldehyde in 100 mM sodium cacodylate buffer pH=7.35 for transition electron microscopy, and the remaining tissue was snap-frozen in liquid nitrogen and stored at –80°C. According to standard techniques, liver histology was performed by an experienced pathologist using hematoxylin-eosin and Masson's trichrome staining.

Hepatic Mitochondrial Isolation - Hepatic mitochondria were prepared by differential centrifugation as previously described [19, 113]. The mitochondrial pellet was resuspended in MiPO₃ buffer (0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH₂PO₄, 20mM HEPES, 110 mM Sucrose, 1g/l BSA, 20 mM Histidine, 20 μM vitamin E succinate, 3 mM glutathione, 1 μM leupeptine, 2 mM glutamate, 2 mM malate, 2 mM Mg-ATP) for mitochondrial respiration. Protein concentration was determined via bicinchoninic acid reaction using Pierce bicinchoninic acid protein assay (no. 23225, ThermoFisher Scientific).

Hepatic Mitochondrial Respiration - Mitochondrial respiration was assessed using high-resolution respirometry (Oroboros Oxygraph-2k; Oroboros Instruments; Innsbruck,

Austria) and values were corrected to total mitochondrial protein loaded, as previously described [120]. Isolated mitochondria (100-150 µg protein) were placed in respiration chambers in respiration media (MiR05; sucrose, 100 mM; K-lactobionate, 60 mM; EGTA, 0.5 mM; MgCl₂, 3 mM; taurine, 20 mM; KH₂PO₄, 10 mM; HEPES, 20 mM; adjusted to pH 7.1 with KOH at 37°C; and 1 g/L fatty acid free BSA) for assessment of basal respiration at 37°C. State 2, State 3, maximal coupled and uncouple O₂ flux rates were assessed upon sequential exposure to mitochondrial substrates and titrating ADP concentrations, using three separate protocols – malate glutamate stimulated, malate palmitoylcarnitine stimulated, and malate octanoylcarnitine stimulated. Due to limited sample availability all protocols were not ran in all samples. For details on substrate concentrations used and subject numbers see Supplementary Table 2.

Mitochondrial Content and structure - Hepatic citrate synthase activity was determined as previously described [20]. Briefly, liver homogenates were incubated in the presence of oxaloacetate, acetyl-CoA, and DTNB. Spectrophotometric detection of reduced DTNB at a wavelength of 412 nm served as an index of enzyme activity. Transmission electron microscopy (TEM) was used to visually assess mitochondrial content and ultrastructural differences. All tissue preparation and imaging were performed at the Electron Microscopy Core Facility, University of Missouri (Columbia, MO). See supplemental material for extended methods.

Fatty Acid Oxidation and β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity -

Fatty acid oxidation was measured with radiolabeled [1-¹⁴C] palmitate (American Radiochemicals) in fresh liver homogenate and isolated hepatic mitochondria, using methods from [20] and as previously described by [121, 122]. Hepatic β-HAD activity was assessed as previously described by our group [20].

Hepatic Oxidative Stress - H₂O₂ emission in isolated hepatic mitochondria was measured via oxygen consumption and the addition of AmplexTM UltraRed reagent (#A36006, Thermo Fisher Scientific), reflecting reactive oxygen species production from complexes I and III at basal levels and following stimulation with palmitoyl-CoA (at increasing titrations 20 – 70 μM), using the Oroboros and as described previously [123]. Due to small sample sizes data from NAS = 0 and NAS = 1-2 were pooled for H₂O₂ emission (NAS0-2, n = 25; NAS3-4, n = 32; NAS≥5, n = 22). Superoxide dismutase (SOD) activity was measured in hepatic tissue lysates according to manufacturer's instructions (Cayman Chemical Company).

Western Blotting - Western blot analysis was completed in whole liver homogenate. Western blot sample preparation and methods have been described previously by our group [124]. For a list of primary antibodies used see supplemental information.

Gene Expression - RNA and cDNA samples from whole liver tissue were prepared as previously described [120]. Quantitative real-time PCR was completed with the ABI 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, CA) using iTaq Universal SYBR Green Supermix (Bio-Rad). Results were quantified using the ddC_T technique relative to the GAPDH housekeeping gene. All data are normalized to expression levels of the group with a NAS = 0. Primer pairs were obtained from Sigma (St. Louis, MO) and primer sequences are listed in Supplementary Table 1.

Ingenuity Pathway Analysis - For examination of the top up- and downregulated proteins and the corresponding top pathway network, protein markers obtained from Western blot analysis that were significantly different between NAS ≥ 5 vs NAS = 0

following a one-way ANOVA and follow up post-hoc analysis were included in Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood, CA). Additionally, the Gene Ontology (GO) Consortium database was used to generate transcript lists of changes in relevant biological processes, cellular components, and molecular functions.

Statistical Analysis - Analyses were performed with SPSS 21.0. Data are presented as means \pm SEM. Data was grouped based on NAS: NAS0, n = 13; NAS1-2, n = 28; NAS3-4, n = 41, and NAS \geq 5, n = 28. Comparisons were performed with one-way ANOVA using a Tukey post hoc. Correlations were analyzed using Spearman correlation. Data were also grouped based on histological Fibrosis Scores: F0-F1, n = 94; F2-F4, n = 12. Comparisons were performed using a Two-Tailed t-test. Differences were considered statistically significant at $P \leq 0.05$. Data was graphed using GraphPad Prism 8.1. All data are presented as means \pm standard error.

Contact for Reagent and Resource Sharing - Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, R. Scott Rector (rectors@health.missouri.edu).

RESULTS

Group Characteristics - NAFLD severity was determined using NAS [119] based on the degree of histological hepatic steatosis, inflammation, and hepatocellular ballooning present. Participants (n =110) were clustered into four groups based on NAS: No Disease (NAS = 0, n = 13), Mild (NAS = 1-2, n = 28), Moderate (NAS = 3-4, n = 41), and Advanced (NAS \geq 5, n = 28) disease (**Fig. 2.1A & C**). Individuals with NAS \geq 5 presented with significantly greater histological fibrosis compared with all other groups (**Fig. 2.1B & D**; $P \leq 0.05$). Groups did not differ in age, body mass, body mass index (BMI), or by fasting serum TG, LDL-cholesterol or total cholesterol (**Table 2.1**; $P > 0.05$). Individuals with NAS \geq 5 had higher fasting serum glucose and glycosylated hemoglobin (HbA1c) concentrations compared to NAS = 0 (**Table 2.1**; $P \leq 0.05$). Further, NAS \geq 5 was associated with significantly higher AST and ALT serum concentrations compared to all other groups (**Table 2.1**; $P \leq 0.05$).

Reduced hepatic mitochondrial fatty acid oxidation and NAFLD - In rodent models, we have previously shown that NAFLD development and progression corresponds with impaired hepatic fatty acid oxidation [20]. Here, we report complete, incomplete, and total [$1\text{-}^{14}\text{C}$] palmitate oxidation in whole liver samples were reduced in NAS \geq 5 (**Fig. 2.2A**; $P \leq 0.05$). In addition, isolated hepatic mitochondrial complete [$1\text{-}^{14}\text{C}$] palmitate oxidation to CO_2 was significantly reduced with increasing NAS (NAS 3-4 and NAS \geq 5) compared to no disease (NAS = 0; **Fig. 2.2D**, $P \leq 0.05$). However, hepatic mitochondrial incomplete and total palmitate oxidation was significantly elevated in these groups relative to NAS = 1-2 (**Fig. 2.2D**; $P \leq 0.05$). Furthermore, fat oxidation in isolated hepatic mitochondria, compared to F0-F1 fibrosis, increases in hepatic fibrosis (F2-F4) were also accompanied by a significant reduction (~50%) in complete palmitate oxidation (**Fig. 2.2F**, $P \leq 0.05$). To compliment these findings, whole liver and isolated mitochondrial β -

HAD activity, the rate limiting enzyme in fatty acid oxidation, was also reduced by ~50% across all levels of NAS vs NAS = 0 (**Fig. 2.2B & E**, $P \leq 0.05$).

NAFLD, hepatic mitochondrial respiration, and increased oxidative stress -

Previous studies have shown reduced hepatic mitochondrial respiration and OXPHOS complex activity with NASH in humans [42, 125]. Here we demonstrate that malate + glutamate and malate + palmitoylcarnitine-stimulated hepatic mitochondrial respiration did not differ across NAS groups (**Fig. 2.3A**, $P > 0.05$). However, malate + octanoylcarnitine-stimulated mitochondrial respiration was significantly elevated for NAS ≥ 5 compared to NAS = 1-2 and NAS = 3-4 (**Fig. 2.3A**, $P \leq 0.05$). These differences occurred in the absence of differences in carnitine palmitoyltransferase 1A (CPT1A) mRNA expression or protein content in whole liver (data not shown). Furthermore, hepatic mitochondrial H₂O₂ emission was significantly elevated (>30%) with NAS ≥ 5 relative to NAS = 3-4 and NAS = 0-2 (**Fig. 2.3B**, $P \leq 0.05$). Hepatic mitochondrial H₂O₂ emission was also significantly elevated with increases in hepatocellular inflammation and ballooning (**Fig. 2.3B**, $P \leq 0.05$), but not with elevated histological steatosis ($P > 0.05$, data not shown). Elevated mitochondrial H₂O₂ emission was not related to differences in superoxide dismutase (SOD) activity or markers of oxidative stress [nuclear factor erythroid 2-related factor 2 (NRF2), catalase, glutathione peroxidase (GPx)-1, GPx-4, SOD1, and SOD2] in whole liver (**Supplementary Fig. 2.1A-C**, $P > 0.05$).

Mechanisms underlying reduced mitochondrial fatty acid oxidation with elevated NAS were tested through investigation of markers of hepatic mitochondrial content and biogenesis. Hepatic citrate synthase activity, a surrogate measure of mitochondrial mass/content, was not significantly different across NAS groups (**Fig. 2.3C**, $P > 0.05$).

Similarly, protein content of hepatic oxidative phosphorylation (OXPHOS) complexes did differ not across NAS groups (**Supplementary Fig. 2.1D**, $P > 0.05$). However, increasing histological fibrosis did correspond with a loss in OXPHOS complex CI and CIV (F0-F1 versus F2-F4; **Fig. 2.3D**, $P \leq 0.05$).

Assessment of mitochondrial biogenesis markers revealed a dramatic attenuation in peroxisome proliferative activated receptor gamma coactivator 1 α (*PGC1 α*) mRNA expression in NAS = 3-4 and NAS ≥ 5 compared with NAS = 0 (**Fig. 2.3E**, $P \leq 0.05$). In addition, *PGC1 α* mRNA expression was significantly reduced with elevated liver fibrosis (F0-F1 versus F2-F4; **Fig. 2.3F**, $P < 0.05$). Similarly, sirtuin 1 (*SIRT1*), 5'-AMP-activated protein kinase α (*AMPK α*), and peroxisome proliferative activated receptor α (*PPAR α*) mRNA expression were significantly lower by 20-50% in NAS ≥ 5 vs NAS = 0. (**Fig. 2.3E**, $P \leq 0.05$).

Worsening NAFLD is linked to abnormalities in mitochondrial structure and morphology - Mitochondrial morphology was determined via TEM and revealed that patients with NAS ≥ 5 presented with more round, swollen, hypodense mitochondria with loss of cristae, compared to NAS = 0 (**Fig. 2.4**). These findings correspond with previous analyses of biopsied liver from patients with NASH showing abnormal mitochondrial ultrastructure compared to controls [14, 126].

NAFLD is linked to a loss in markers of hepatic mitochondrial turnover and dynamics - Given the dramatic reduction in markers of hepatic mitochondrial biogenesis, we next examined the potential influence of increasing liver disease severity on markers of autophagy and mitophagy. Hepatic markers of macro-autophagy, including Unc-51 like autophagy activating kinase 1 (ULK1), serine phosphorylated

ULK1 (pULK1^{S555}), Beclin1, autophagy related protein (ATG) 5, ATG12:5 and P62, were significantly lower in NAS ≥ 5 to NAS = 0 (**Fig. 2.5A**, $P \leq 0.05$). In addition, increasing fibrosis (F0-F1 versus F2-F4) was associated with a loss in P62 protein content (**Fig. 2.5C**, $P \leq 0.05$). On the other hand, the protein content of microtubule associated protein 1 light chain 3 A/B (LC3) I and II was significantly elevated with NAS ≥ 5 compared with NAS = 0 (**Fig. 2.5A**, $P \leq 0.05$). Despite these differences, increasing NAS was not associated with significant changes in LC3II/LC3I ratio, a marker of LC3 activation (**Fig. 2.5A**, $P > 0.05$); however, LC3II/LC3I ratio was significantly upregulated with increased hepatic fibrosis (F0-F1 versus F2-F4; **Fig. 2.5B**, $P \leq 0.05$).

In rodent models, reduced mitophagic processes correspond with NAFLD development and progression [50], but these processes have not been previously explored in humans. Remarkably, hepatic BCL2 interacting protein 3 (BNIP3) protein content was 35-40% lower across all stages of NAS compared with NAS = 0 (**Fig. 2.5D**, $P \leq 0.05$). Similar reductions were exhibited with increasing fibrosis score (**Fig. 2.5E**, $P \leq 0.05$). Interestingly, hepatic Parkin and PTEN induced kinase 1 (PINK1) protein content did not differ across NAS groups (**Fig. 2.5D**, $P > 0.05$). Markers of mitochondrial dynamics revealed significantly lower hepatic mitochondrial fission [Dynamin-related protein 1 (DRP1) and serine 616 phosphorylated DRP1 (pDRP^{S616})] and fusion [mitofusin (MFN)1, and MFN2] with increased NAS (**Fig. 2.5F**, $P \leq 0.05$).

Mitochondrial functional measures are correlated with markers of mitochondrial

quality control - The important role of mitochondrial quality control in the maintenance of hepatic mitochondrial metabolism was evident in the data (**Fig. 2.6**). Of note, lower markers of autophagy, mitophagy, mitochondrial biogenesis, fission, and fusion correlated with worsening ex vivo complete, incomplete and total FAO in whole liver ($P \leq$

0.05). Further, lower mitochondrial respiration was significantly related to lower markers of autophagy, mitochondrial biogenesis and fission, and significantly negatively related to markers of oxidative stress ($P \leq 0.05$). Finally, higher palmitoyl-CoA stimulated H_2O_2 emission significantly correlated with lower markers of mitochondrial health (oxidative stress, autophagy, and mitochondrial fission) ($P \leq 0.05$).

Ingenuity Pathway Analysis (IPA) of protein markers related to mitochondrial biogenesis, autophagy/mitophagy and mitochondrial dynamics - To determine how elevated NAS is related to hepatic mitochondrial health, differentially expressed proteins were entered into IPA software. The significant downregulation in hepatic protein content of autophagy (ULK1, SQSTM1, ATG5), mitophagy (BNIP3), mitochondrial dynamics (MFN1, MFN2), and mitochondrial biogenesis (PPAR α) markers reveal a reduction in processes related to 'cellular homeostasis, formation of autophagosomes, macroautophagy of cells, autophagy of mitochondria, and inflammation of organ' with increasing NAFLD/NASH (**Fig. 2.7**; NAS ≥ 5 versus NAS = 0). Although not presented here, similar outcomes were observed for NAS = 3-4 versus NAS = 0 and NAS = 1-2 versus NAS = 0 (data not shown), thus highlighting the intricate role these pathways play in early NAFLD and its more advanced form NASH.

DISCUSSION

Human data investigating the role of hepatic mitochondrial fatty acid oxidation and markers of hepatic mitochondrial turnover in liver disease progression are scant. We provide novel evidence that hepatic mitochondrial fatty acid oxidation and markers of mitochondrial biogenesis, autophagy, mitophagy, fission and fusion are reduced with increasing NAFLD severity and hepatic fibrosis in humans with obesity.

Whole liver tissue and hepatic mitochondria long chain complete fatty acid oxidation and β -HAD activity were dramatically reduced in humans with increasing NAS. These decrements were notable even early in the disease process and persisted in advanced NAFLD and fibrosis, findings supported by our previous work in rodent models [20]. Malate + glutamate and malate + palmitoylcarnitine-stimulated mitochondrial respiration did not differ across NAS; whereas, malate + octanoylcarnitine-stimulated mitochondrial respiration was significantly higher with NASH (NAS \geq 5). These findings are somewhat in contrast to work by Roden and colleagues [42] who found a compensatory upregulation in malate + octanoylcarnitine-stimulated hepatic mitochondrial respiration (per mitochondria) in patients with NAFLD and obesity that was largely lost in patients with NASH. On the other hand, ^{13}C -octanoate oxidation, a measure of hepatic mitochondrial medium chain fatty acid oxidation, was increased in patients with NASH [127], which was consistent with the current data. It is unclear the reason for differences in the findings [42], but perhaps could be related to greater NAFLD severity (NAS = 7) in their NASH group compared to patients in our study (NAS = 5.5). Another explanation for the discrepancy between the two studies may be differences in mitochondrial mass. Here we show no differences in mitochondrial mass across groups occurred, while Roden *et al* reported ~25% higher mitochondrial mass in individuals with NASH compared to individuals with obesity, NAFL, and lean controls. Indeed, the difference

between the two studies is puzzling and further experiments examining changes in mitochondrial respiration and mass in patients with increasing NAS are required. It appears that hepatic mitochondrial medium chain fatty acid oxidation may be intact or even upregulated in advanced liver disease. This would also mirror the majority [36, 128, 129] but not all [115] studies showing elevated in vivo hepatic TCA cycle flux in humans with increased intrahepatic triglyceride content compared with lean controls. Collectively, our data collected simultaneously in the same patient population suggest that long chain fatty acid oxidation and the rate limiting step in β -oxidation are downregulated with increasing liver disease severity. The possibility of these reductions playing a causal role in advancement of liver disease warrants future investigation.

Reduced hepatic mitochondrial fatty acid oxidation with increasing NAS was not accompanied by prominent differences in assessed markers of hepatic mitochondrial content. Existing literature has either reported no change or an increase in hepatic mitochondrial content with NAFLD/NASH [42, 130], thus further supporting the notion that worsening NAFLD is not likely explained simply by a reduction in mitochondrial content/mass but rather is associated with impaired mitochondrial function and quality control. In support, mitochondrial ultrastructural abnormalities also were noted with increasing NAFLD severity in the current study (NAS \geq 5), supporting existing literature of deterioration in hepatic mitochondria ultrastructural integrity with increasing NAFLD severity [14, 126]. Worsening mitochondrial morphology may be indicative of increasing bioenergetic or oxidative stress, and perturbations in machinery that regulate mitochondrial biogenesis, mitophagy, fission, and fusion [131]. Indeed, the perturbations in mitochondrial function shown here likely explain the higher mitochondrial H₂O₂ emission observed with advanced NAFLD (NAS \geq 5) and increasing histological lobular inflammation and hepatocellular ballooning. Taken together, the data presented here

highlight that NAFLD and its more advanced form NASH, is linked to increasing loss of hepatic mitochondrial function, reduced mitochondrial health and elevations in hepatic oxidative stress.

Mitochondrial health and content are governed by the production of new healthy mitochondria (mitochondrial biogenesis) and the degradation of poorly functioning mitochondria (mitophagy) [50]. Multiple markers of hepatic mitochondrial biogenesis (*PGC1 α* , *SIRT1*, *AMPK* and *PPAR α*) were dramatically downregulated with elevated NAS, indicating a clear impairment in these processes with worsening disease. PGC-1 α is considered the master transcriptional regulator of mitochondrial biogenesis and is reduced in patients with obesity and NASH [42, 132]. Furthermore, we have previously demonstrated that liver-specific PGC-1 α overexpression increases hepatic mitochondrial function and reduces hepatic lipid accumulation *in vivo* and *in vitro* in rats [17, 49]. Indeed, present observations point to the importance of hepatic mitochondrial biogenesis in the regulation of a healthy mitochondrial pool and in susceptibility to NAFLD/NASH in humans.

To our knowledge, we demonstrate for the first time that early NAFLD (NAS \geq 1) and more advanced NASH is associated with a loss in hepatic protein content of markers related to autophagy (ULK1, pULK1, Beclin1, ATG5, ATG12:5 and P62) compared to no disease (NAS = 0) in patients with obesity. Reductions in autophagy and mitophagy processes are associated with an increasing pool of dysfunctional mitochondria in the liver [133]. Indeed, rodent models of diet-induced NAFLD/NASH exhibit reduced autophagy [51, 134], with direct deletion of autophagy related proteins causing NAFLD and NASH [51, 53]. Moreover, our findings also highlight that onset of NAFLD, more advanced NASH, and fibrosis corresponded with a loss in hepatic BNIP3 protein

content, a key regulator of mitophagy. BNIP3 is known to play a functional role in the regulation of lipid metabolism and fatty liver disease in rodent models [56, 123]. Further, the adaptor proteins, BNIP3 and P62 - which play a role in LC3 facilitated mitophagy, were downregulated with increasing fibrosis, despite increases in LC3II/I ratio. Together, these data highlight that autophagy/mitophagy is interrupted in the early onset of NAFLD and even preceded losses in hepatic mitochondrial fatty acid oxidation, and may also play a role in fibrogenic processes with more advanced disease. To further evaluate this point, the downregulation in several of these proteins correlated with reduced functional outcomes of mitochondrial fatty acid oxidation and respiration (**Fig. 2.6**), thus supporting the importance of these processes in maintaining hepatic mitochondrial health and metabolism.

Mitochondria are dynamic organelles that constantly undergo fusion and fission processes in order to maintain a healthy mitochondrial pool, and changes in mitochondrial dynamics are associated with adaptations to cellular apoptotic, autophagic and bioenergetic processes [135, 136]. Here we show that pDRP^{S616} protein content, a marker of mitochondrial fission activation, was significantly lower with NAFLD onset and NAS elevation. DRP1 is reduced in rodent models of diet-induced NASH [137], and liver-specific deletion causes mitochondrial enlargement and loss of fatty acid oxidation [118]. Similarly, markers of hepatic mitochondrial fusion (MFN1 and MFN2) were significantly reduced with NAFLD onset and worsening NAS, in line with previous studies in rodents with NAFLD [138, 139]. Additionally, the inhibition of repeated fission/fusion cycles results in mitophagic arrest and the subsequent accumulation of damaged mitochondria and decreased function [140, 141]. These findings collectively highlight that a loss in mitochondrial quality control likely contributes to NAFLD onset and

worsening NASH phenotype, and future mechanistic studies in human NAFLD pathogenesis are warranted.

Despite the cross-sectional nature of this study, we demonstrate through multiple, independent lines of evidence that increasing NAFLD severity corresponded with a loss in hepatic fatty acid oxidation and an impairment in mitochondrial biogenesis, mitophagy, and dynamics, likely resulting in a stagnant dysfunctional hepatic mitochondria pool. Lower markers of mitochondrial turnover were present prior to reduced mitochondrial fatty acid oxidation and increased ROS production, suggesting that these reductions could trigger further deterioration of mitochondria and subsequent NASH. These data collectively highlight a critical and novel role for mitochondrial fatty acid oxidation and turnover in the liver and indicate their potential as a future target for prevention and treatment of NAFLD/NASH in humans.

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AUTHOR CONTRIBUTIONS

M.P.M., R.S.R., E.J.P., J.A.I. were involved in the study concept and design. M.P.M., R.P.C., G.M.M., S.A.J., V.J., L.B., A.A.W., A.D., R.R.G, N.M.S., J.B.P., A.I.A.S., J.A.I.,

E.J.P., M.M.S.A., J.A., and R.S.R. helped with acquisition of data. M.P.M., G.M.M., A.D., J.A.I., E.J.P., and R.S.R analyzed and interpreted results. M.P.M. and R.S.R. provided statistical analysis of the data. M.P.M. and R.S.R. drafted the manuscript, while R.P.C., G.M.M., S.A.J., A.A.W., A.D., R.R.G, N.M.S., J.B.P., A.I.A.S., J.A.I., E.J.P., and R.S.R. revised the manuscript and provided important intellectual content and approved final version of manuscript.

DECLARATION OF INTERESTS

No conflicts of interest, financial or otherwise, are declared by the authors.

FIGURES AND FIGURE LEGENDS

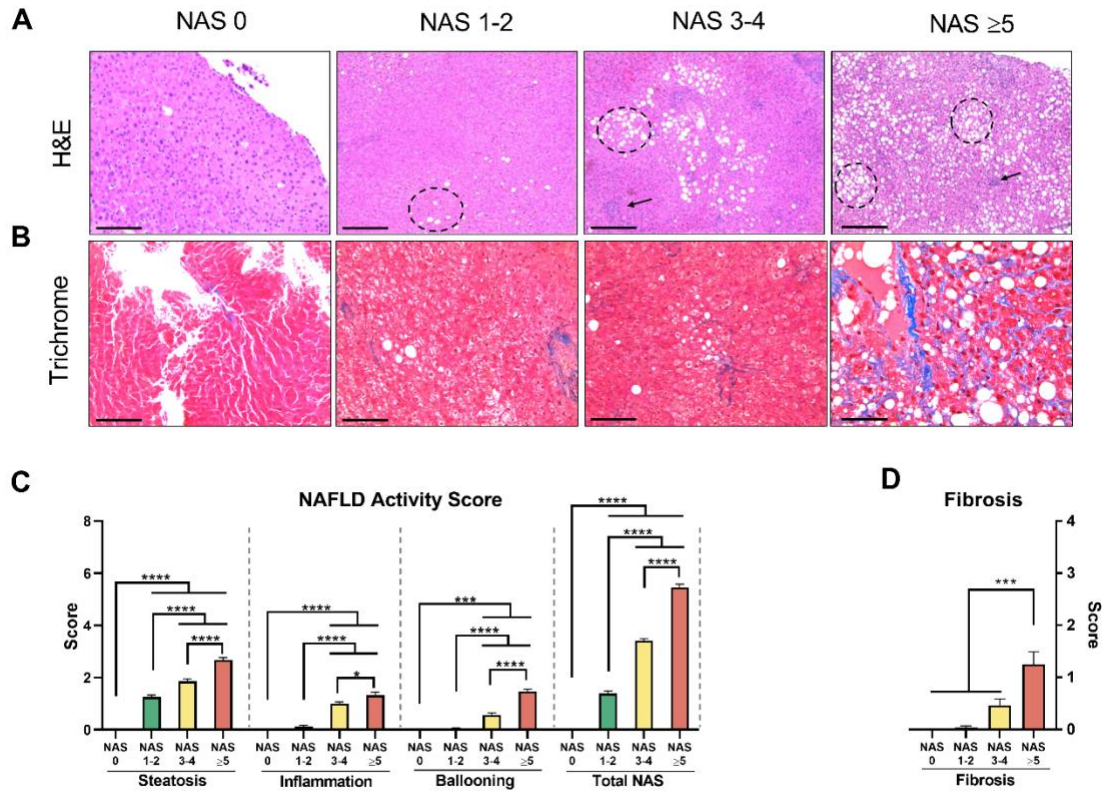
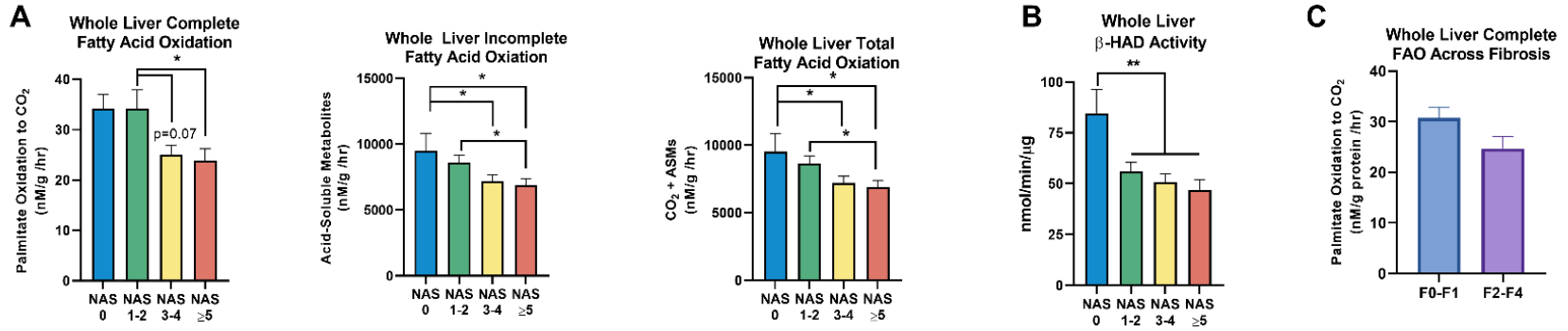


Figure 2.1. Liver phenotype and measures of oxidative stress in humans. A) Representative liver H&E and B) trichrome staining, C) histological NAFLD activity score, and D) histological fibrosis score. Values are presented as mean \pm SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Whole Liver Fatty Acid Oxidation



Hepatic Mitochondrial Fatty Acid Oxidation

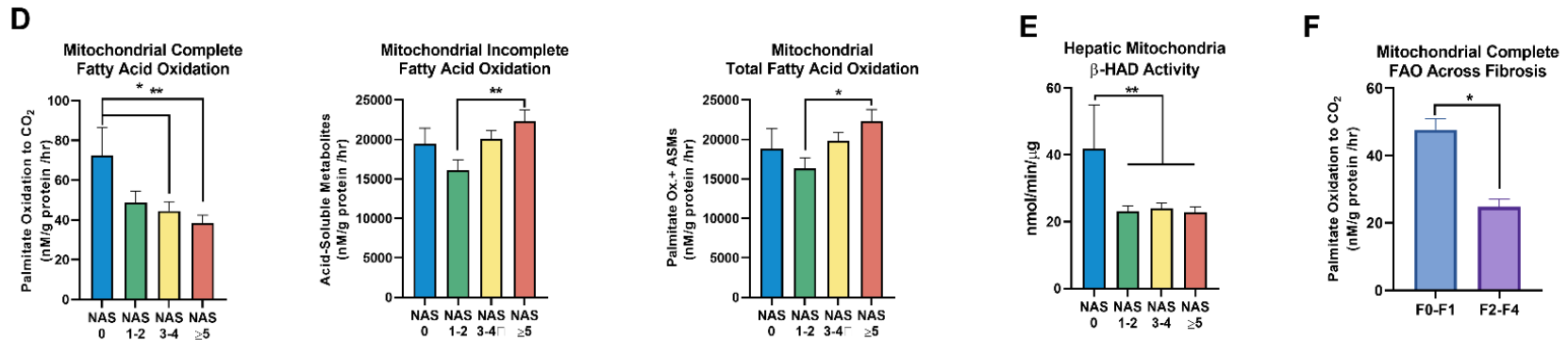
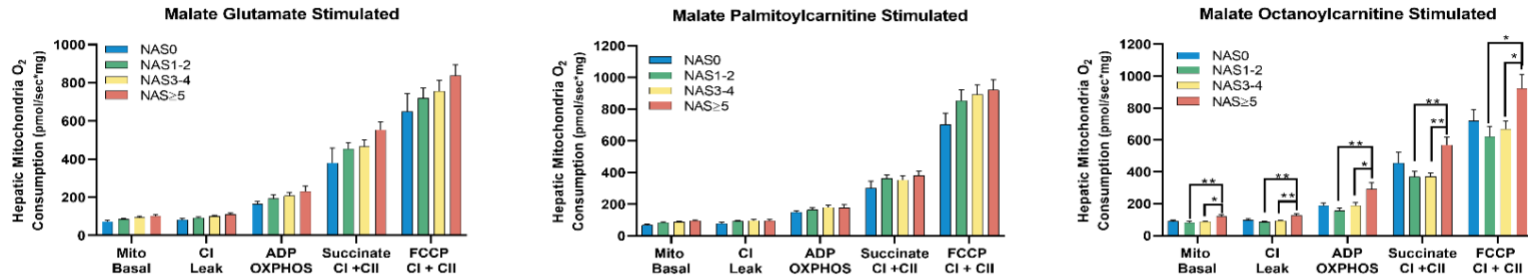
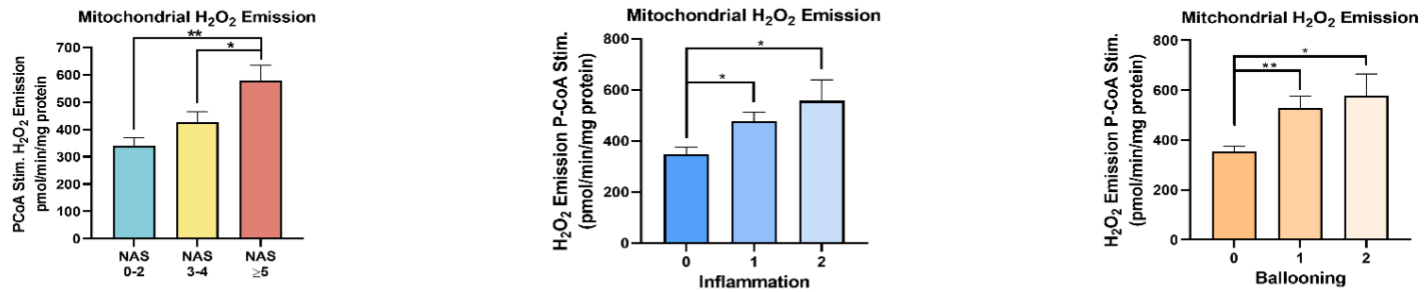


Figure 2.2. Whole liver and isolated hepatic mitochondria ex-vivo fatty acid oxidation in humans. Whole liver A) complete oxidation to CO₂, incomplete, and total [1-¹⁴C] palmitate oxidation. B) Whole liver β-HAD activity and C) Whole liver complete palmitate oxidation across hepatic fibrosis scores. Isolated hepatic mitochondria D) complete oxidation to CO₂, incomplete, and total [1-¹⁴C] palmitate oxidation. E) Isolated hepatic mitochondria β-HAD activity and F) Isolated hepatic mitochondria complete palmitate oxidation across hepatic fibrosis scores. Values are presented as mean±SE. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Hepatic Mitochondrial Respiration

A


Hepatic Mitochondrial Oxidative Stress

B


Hepatic Mitochondrial Content and Biogenesis

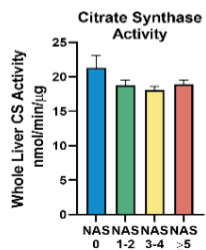
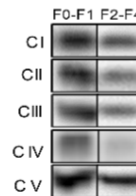
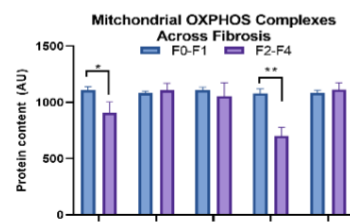
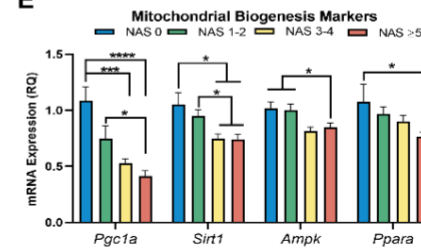
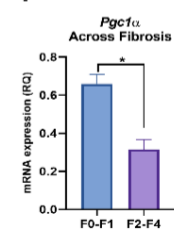
C

D

E

F


Figure 2.3. Measures of hepatic mitochondrial function, content and dynamics in humans with NAS0, NAS 1-2, NAS3-4, and NAS≥5. O₂ consumption in isolated hepatic mitochondria A) malate + glutamate-stimulated, malate + palmitoylcarnitine-stimulated, and malate + octanoylcarnitine-stimulated; followed by the addition of adenosine diphosphate (ADP), succinate, and carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP). B) Palmitoyl-CoA stimulated H₂O₂ emission in isolated hepatic mitochondria across NAFLD and comparisons across histological inflammation and histological ballooning. C) Whole liver citrate synthase activity. D) Mitochondrial OXPHOS complexes protein content across hepatic fibrosis scores measured in whole liver, and representative Western blot images. E) Mitochondrial biogenesis markers mRNA expression in whole liver. F) *Pgc1α* mRNA expression across fibrosis scores. All Western blots were run on continuous gels. Values are presented as mean±SE. P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

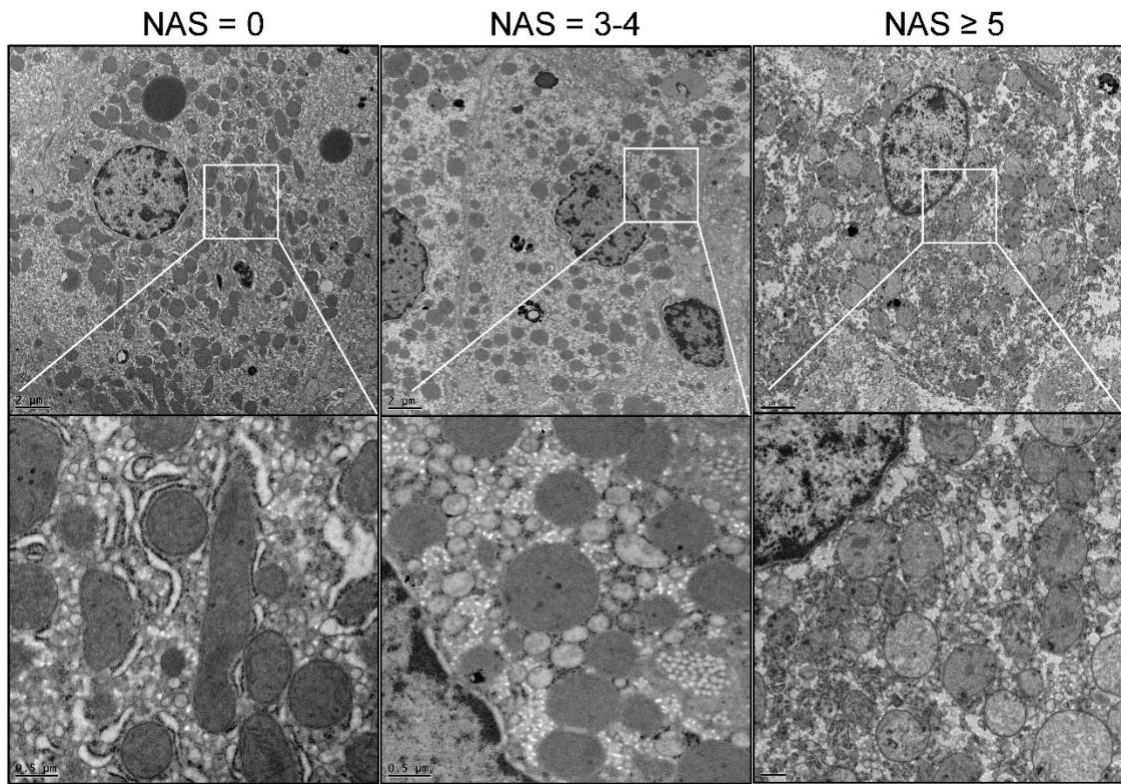
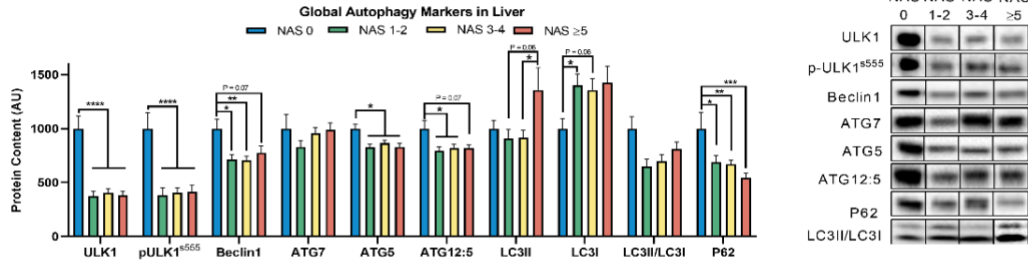


Figure 2.4. Mitochondrial structure and morphology with increasing NAFLD/NASH.

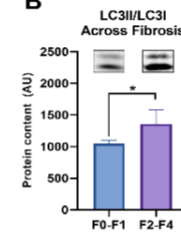
Representative transmission electron microscopy images of whole liver in patients NAS = 0, NAS= 3, and NAS = 6 at A) 5,000x and B) 10,000x magnification.

Hepatic Autophagy Markers

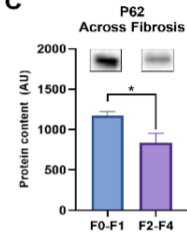
A



B

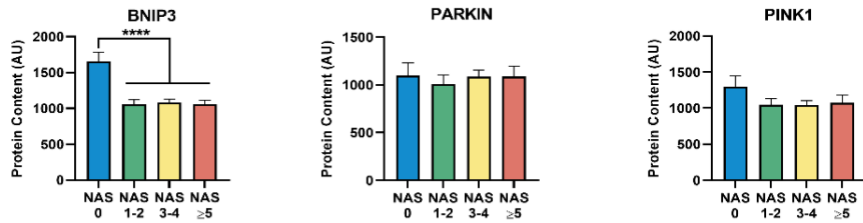


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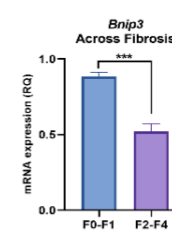


Hepatic Mitophagy Markers

D



E



Hepatic Mitochondrial Dynamics Markers

F

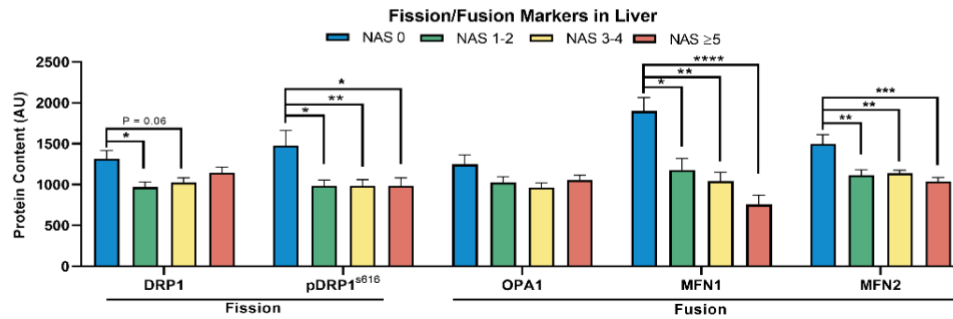


Figure 2.5. Measures of autophagy and mitophagy in whole liver in humans. A) Protein content of autophagy markers in whole liver across NAS and representative Western blot images. B) LC3II/LC3I ratio across fibrosis scores and C) P62 protein content across fibrosis scores. D) BNIP3, PARKIN, and PINK1 protein content in whole liver and representative Western blot images. E) *Bnip3* mRNA expression across fibrosis scores. F) Markers of mitochondrial fission and fusion protein content in whole liver and representative Western blot images. All Western blots were run on continuous gels. Values are presented as mean±SE. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

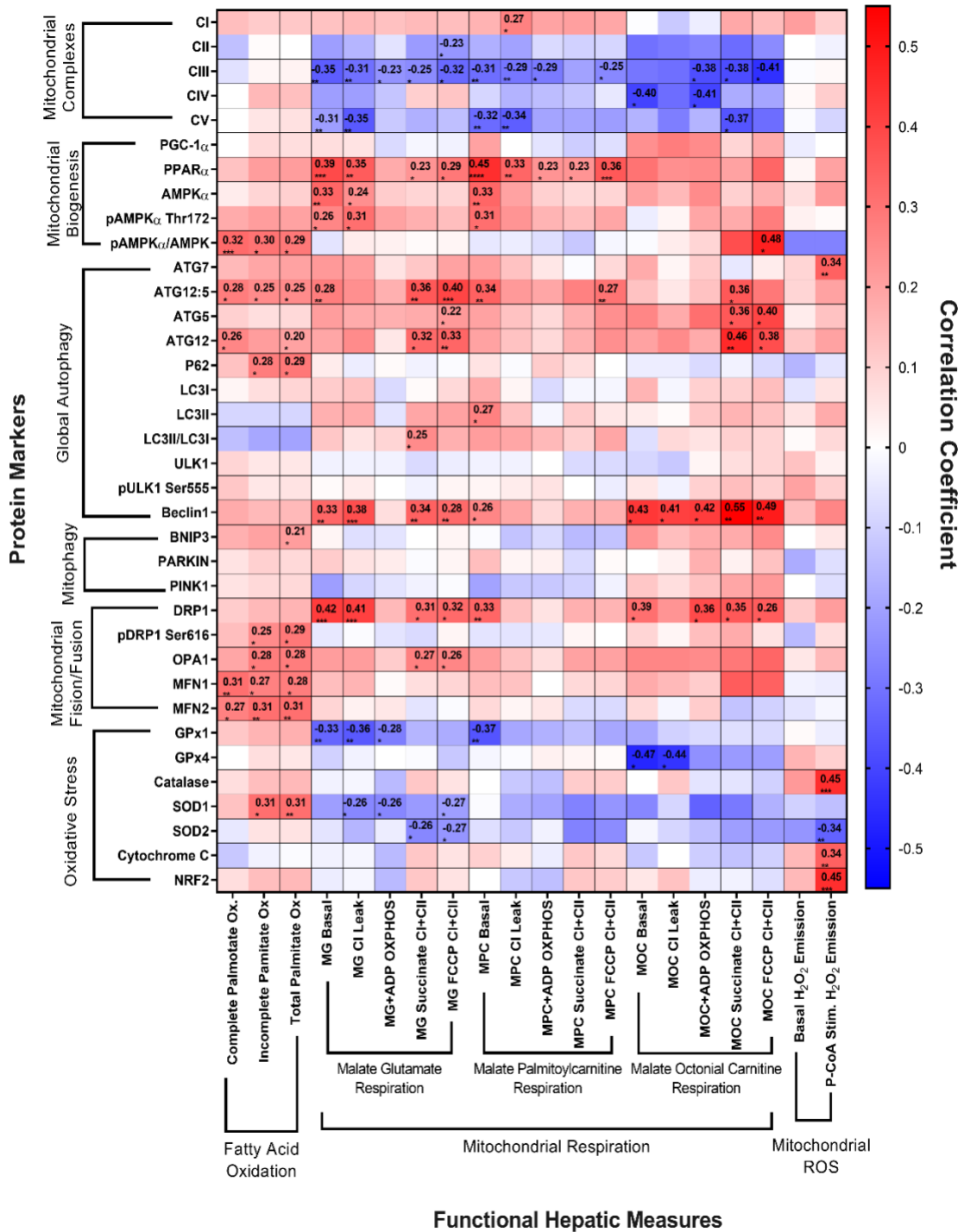


Figure 2.6. Correlational and Ingenuity Pathway Analysis

Correlation Heat map - showing Pearson's correlation coefficients of functional *ex vivo* measures of hepatic fatty acid oxidation, O₂ respiration, and ROS with protein content of markers related to mitochondrial content, quality control and oxidative stress. Protein content was measured via Western blot. Significant correlations are indicated on heat map with r value and significance level. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Regulated Diseases and Biological Functions NAS ≥ 5 vs NAS = 0

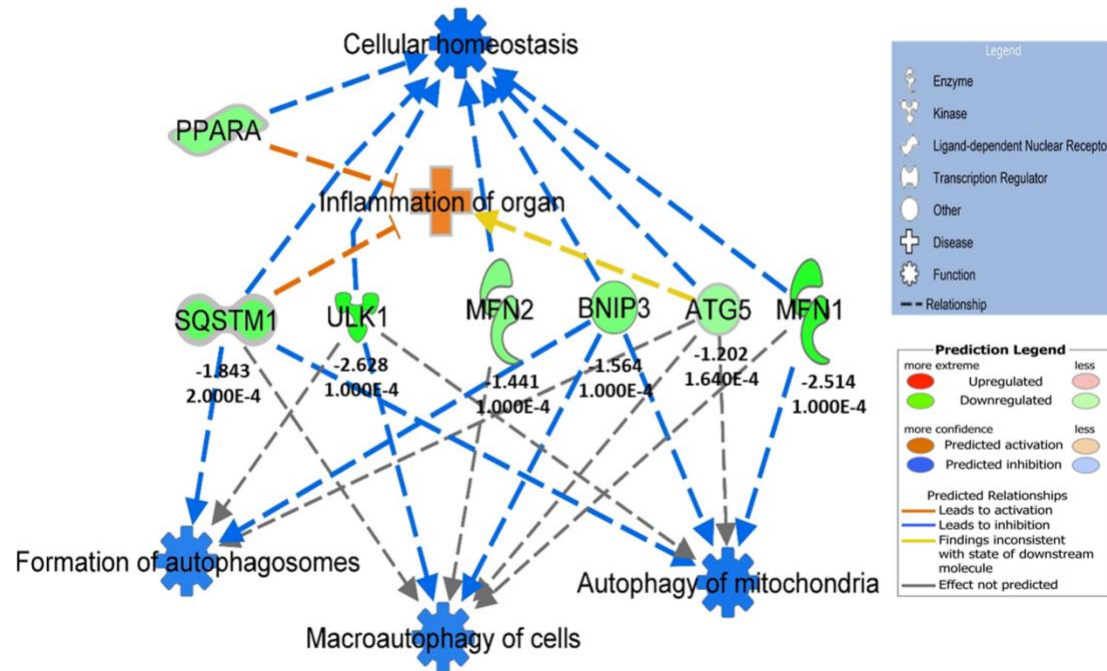


Figure 2.7. Ingenuity Pathway Analysis network revealing changes in the regulation of disease and biological functions for NAS ≥ 5 versus NAS = 0. Protein content of markers related to mitochondrial content, quality control and oxidative stress were measured via Western blot. Significant differences were determined using a one-way ANOVA, with significance level set a $P \leq 0.05$, and followed up with a Tukey post-hoc analysis. Values shown in Figure 7 are fold change and p-value of the individual proteins.

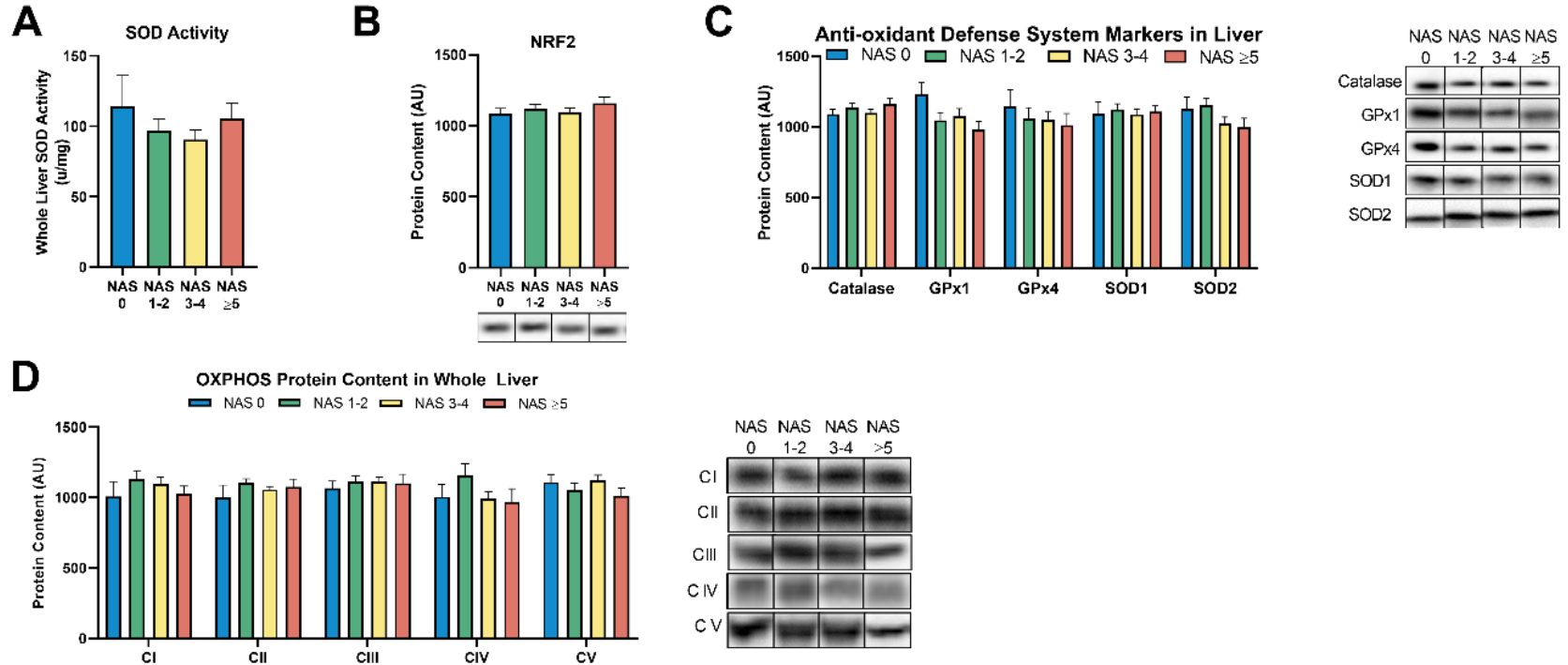
TABLES AND TABLE LEGENDS

Table 2.1. Subject Characteristics (Mean±SE)

	Overall (n=110)	NAS0 (n=13)	NAS1-2 (n=28)	NAS3-4 (n=41)	NAS≥5 (n=28)	ANOVA p-value
Age (years)	48±1	39±2	44±2	47±2	45±2	0.178
Sex (M/F)	19/91	0/13	5/23	11/30	3/25	-
Weight (kg)	136.3±2.4	131.0±8.8	132.3±3.8	139.9±3.9	137.6±5.2	0.552
BMI (kg/m ²)	48.7±0.7	47.1±2.6	48.6±1.2	49.2±1.1	48.7±1.5	0.859
-	-	-	-	-	-	-
Glucose (mg/dL)	104.1±3.2	84.8±4.2	98.8±5.3	106.5±6.0	*114.3±6.0	0.042
HbA1C (%)	6.2±0.1	5.5±0.2	6.0±0.2	6.0±0.2	*6.9±0.3	0.011
AST (U/L)	35.6±3.1	24.1±1.6	28.0±2.3	30.5±1.8	*,\$,&55.4±10.6	0.001
ALT (U/L)	41.0±3.4	26.2±2.7	32.8±4.0	36.8±3.7	*,\$,&61.7±10.6	0.002
-	-	-	-	-	-	-
Cholesterol (mg/dL)	169.8±3.6	172.1±9.9	171.6±7.8	163.6±5.0	176.0±8.5	0.591
Triglycerides (mg/dL)	129.1±5.5	109.7±9.3	117.7±11.3	139.5±9.7	133.4±10.4	0.254
HDLc (mg/dL)	41.8±1.2	50.8±4.0	41.9±2.9	*39.4±1.5	41.2±1.8	0.026
LDLc (mg/dL)	102.1±3.6	99.4±9.1	106.1±7.8	96.3±5.5	108.1±8.0	0.572

Post Hoc analysis: *p<0.05 vs NAS0, \$p<0.05 vs NAS1-2, &p<0.05 vs NAS3-4

SUPPLEMENTAL INFORMATION



Supplemental Figure 2.1

A) Whole liver SOD activity. B) Whole liver NRF2 protein content and representative Western blot image. C) Whole liver antioxidant defense system markers protein content and representative Western blot images. D) Whole liver mitochondrial OXPHOS complexes protein content and representative Western blot images. All Western blots were run on continuous gels. Values are presented as mean±SE *P < 0.05, **P < 0.01, ***P < 0.001.

Supplemental Table 2.1

Primers	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
<i>gapdh</i>	AACAGCCTCAAGATCAGCAA	CAGTCTGGGTGGCAGTGAT
<i>ppara</i>	CCTAAAAAGCCTAAGGAAACC	GATCTCCACAGCAAATGATAG
<i>prkaa1</i>	TGTGATGGGATCTTCTATACC	CCCTGATATCTTTGATTGTGG
<i>ppargc1a</i>	GCAGACCTAGATTCAAACCTC	CATCCCTCTGTCATCCTC
<i>sirt1</i>	AAGGAAAACCTACTTCGCAAC	GGAACCATGACACTGAATTATC

EXTENDED METHODS

Isolated Mitochondria O₂ Respiration Protocol and Subject Numbers

Supplemental Table 2.2 Malate Glutamate Stimulated O₂ flux Analysis				
Basal	State 2	State 3	ADP OXPHOS	Max Uncoupled Respiration
-	Malate 2 mM Glutamate 10 mM	ADP 100-250 μM	Succinate 10 mM	FCCP 0.25 μM
NAS 0 n=7	NAS1-2 n=28	NAS3-4 n=30	NAS≥5 n=22	
Malate Palmitoylcarnitine Stimulated O₂ flux Analysis				
Basal	State 2	State 3	ADP OXPHOS	Max Uncoupled Respiration
-	Malate 2mM Palmitoylcarnitine 20 μM	ADP 100-250 μM	Succinate 10 mM	FCCP 0.25 μM
NAS 0 n=8	NAS1-2 n=28	NAS3-4 n=31	NAS≥5 n=22	
Malate Octanoylcarnitine Stimulated O₂ flux Analysis				
Basal	State 2	State 3	ADP OXPHOS	Max Uncoupled Respiration
-	Malate 2mM Octanoylcarnitine 20 μM	ADP 100-250 μM	Succinate 10 mM	FCCP 0.25 μM
NAS 0 n=5	NAS1-2 n=12	NAS3-4 n=12	NAS≥5 n=10	

Transmission Electron Microscopy - To assess structural differences in the mitochondria via transmission electron microscopy (TEM), a small section of fresh liver (~1-2mg) was immediately fixed in 2 % paraformaldehyde, 2 % glutaraldehyde in 100 mM sodium cacodylate buffer pH=7.35. Next, fixed tissues were rinsed with 100 mM sodium cacodylate buffer, pH 7.35 containing 130 mM sucrose. Secondary fixation was performed using 1 % osmium tetroxide (Ted Pella, Inc. Redding, California) in cacodylate buffer using a Pelco Biowave (Ted Pella, Inc. Redding, California) operated at 100 Watts for 1 minute. Specimens were next incubated at 4°C for 1 hr, then rinsed with cacodylate buffer and further with distilled water. En bloc staining was performed using 1% aqueous uranyl acetate and incubated at 4°C overnight, then rinsed with distilled water. A graded dehydration series was performed using ethanol, transitioned into acetone, and dehydrated tissues were then infiltrated with Spurr resin for 48 hr at room temperature and polymerized at 60°C overnight. Sections were cut to a thickness of 75 nm using an ultramicrotome (Ultracut UCT, Leica Microsystems, Germany) and a diamond knife (Diatome, Hatfield PA). Images were acquired with a JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA) at 80 kV on a Gatan Ultrascan 1000 CCD (Gatan, Inc, Pleasanton, CA). Post primary fixation, all tissue preparation and imaging were performed at the Electron Microscopy Core Facility, University of Missouri.

Western blotting primary antibodies -The primary antibodies used are as follows: Light chain 3 (LC3) A/B (no. 12741, Cell Signaling Technologies, Danvers, MA, USA), PTEN Induced Kinase 1 (PINK1; no. 6946, Cell Signaling Technologies), BCL2 Interacting Protein 3 (BNIP3; no. 44060, Cell Signaling Technologies), autophagy-related gene 12 (ATG12, no. 4180, Cell Signaling Technologies) ATG5 (no. 12994, Cell Signaling Technologies), SQSTM1/p62 (p62; no. 5114, Cell Signaling Technologies), ATG7 (no. 8558, Cell Signaling Technologies), Parkin RBR E3 Ubiquitin Protein Ligase

(PARKIN; no. 4211, Cell Signaling Technologies), Beclin1 (no. 4122, Cell Signaling Technologies), Unc-51 Like Autophagy Activating Kinase 1 (ULK1; no. 8054, Cell Signaling Technologies), Ser555 phosphorylated ULK1 (pULK1; no. 6888, Cell Signaling Technologies), Total Oxidative phosphorylation (OXPHOS; no.110413, Abcam, Cambridge, MA, USA), Peroxisome Proliferator Activated Receptor α (PPAR α ; no. 398394, Santa Cruz Biotechnologies), peroxisome proliferator activated receptor-1 (PGC-1 α , no. 3242, MilliporeSigma, St. Louis, Mo., USA), nuclear factor E2-related factor 2 (NFE2L2/NRF2; no. 137550, Abcam), Dynamin-related protein 1 (DRP1; no. 5319, Cell Signaling Technologies), Ser616 phosphorylated DRP1 (pDRP1^{S616}; no. 3455, Cell Signaling Technologies), OPA1 Mitochondrial Dynamin Like GTPase (OPA1; no. 80471, Cell Signaling Technologies), Superoxide dismutase 1 (SOD1; no. 13498, Abcam), SOD2 (no. 13194, Cell Signaling Technologies), glutathione peroxidase 1 (GPx1-; no. 22604, Abcam), GPx-4 (no. 166570, Santa Cruz Biotechnologies), Catalase (no. 14097, Cell Signaling Technologies), Mitofusin 1 (MFN1; no. 204274, Abcam), MFN2 (no. 9482, Cell Signaling Technologies).

**CHAPTER 3 - IMPACT OF LIFESTYLE MODIFICATIONS ON HISTOLOGICAL
OUTCOMES AND HEPATIC FATTY ACID OXIDATION IN THE SETTING OF
NONALCOHOLIC STEATOHEPATITIS**

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KEYWORDS

Nonalcoholic fatty liver disease, fatty acid oxidation, lifestyle intervention, nonalcoholic steatohepatitis

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ABSTARCT

Backgrounds and Aims: Nonalcoholic fatty liver disease (NAFLD) and its more advanced form steatohepatitis (NASH) is associated with obesity and is an independent risk factor for cardiovascular, liver-related, and all-cause mortality. Available human data examining the effects of weight loss induced by diet and exercise on histological outcomes of NAFLD/NASH and the mechanisms involved are scant. To investigate this relationship, we performed a clinical trial to examine the effects of a 9-month Active Treatment (n = 9) phase using a combination of dietary caloric restriction and exercise to induce weight loss. Liver biopsies were obtained pre and post treatment to examine the effect of Active Treatment on histological outcomes of NAFLD/NASH and hepatic fatty acid oxidation. **Results:** After 9-months of intervention, participants assigned to Active Treatment lost an average of ~9% body weight compared to baseline ($P < 0.05$). Active Treatment resulted in a significant reduction in NAFLD activity score ($P \leq 0.05$), intrahepatic lipid content, and hepatocellular ballooning ($P \leq 0.05$). Reductions in hepatic fibrosis trended toward significance at follow-up ($P = 0.08$). Changes in NAS significantly correlated with reduced fat mass ($r = .726$, $P < 0.05$) and increased aerobic capacity ($r = -0.675$, $P \leq 0.05$). Interestingly, hepatic fatty acid oxidation did not change at follow-up in response to weight loss induced by diet and exercise and did not correlate with changes in NAS or its components ($P > 0.05$). **Conclusion:** Weight loss achieved through dietary caloric restriction and exercise leads to histological improvements in NAFLD outcomes that are linked to improvements in body composition and aerobic capacity.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) and its progressive form of steatohepatitis (NASH) is the most prevalent liver disease in the U.S. [1]. NAFLD is tightly linked with obesity, insulin resistance, and type 2 diabetes and is considered an independent risk factor for cardiovascular, liver-related, and all-cause mortality [112].

Given the lack of a pharmaceutical drug that specifically treats NAFLD, lifestyle modifications to induce weight loss, including caloric restriction and exercise, remain the cornerstone of NAFLD therapy to prevent the development of [91] and treat [1, 92-97] the disease. Evidence suggests there are histological improvements in NASH with as little as 3% body weight loss, while weight loss of 7-10% is efficacious in regressing histological steatosis, hepatocellular inflammation, and fibrosis [23, 86-90, 142, 143]. Caloric restriction and/or exercise training lessens the nutrient burden on the liver, improves peripheral insulin sensitivity and promotes fuel uptake at the periphery. However, the majority of research examining the metabolic effects of weight loss on hepatic health has relied on noninvasive imaging techniques [measured by magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), ultrasonography] to measure IHL content in the absence of histological interrogation [75-78]. Biopsy studies assessing the mechanisms by which weight loss via caloric restriction and exercise improve liver histology pre- and post-intervention are limited. While current studies provide important knowledge on the potential clinical utility of lifestyle modifications to treat NAFLD, the mechanisms by which weight loss via caloric restriction and exercise improve liver histology remains unknown. Further, when discovered this knowledge can support the development of new therapies for this disease.

One such way in which lifestyle interventions may reverse NAFLD/NASH is via improvements in hepatic fatty acid oxidation. As shown in Chapter 2 of this dissertation, more advanced NAFLD and NASH is associated with compromised hepatic fatty acid oxidation (~50% reduction), increased hepatic mitochondrial reactive oxygen species production and reduced mitochondrial turnover. Our lab and others have shown that lifestyle modifications such as caloric restriction and exercise is an effective approach in improving histological NAFLD outcomes via improved lipid metabolism, decreased oxidative stress, inflammation, and fibrosis in rodent models [17, 32, 106-111]. Whether similar improvements in hepatic fatty acid oxidation are observed in humans following a lifestyle intervention remain to be explored.

This project examined the effect of lifestyle modifications, via a combination of dietary caloric restriction and exercise, on reversing histological outcomes of NASH using pre- and post-liver biopsies. Further, this project also investigated whether lifestyle modifications can be used to improve hepatic fatty acid metabolism in advanced NAFLD/NASH. It was hypothesized that a dietary intervention to induce weight loss, via caloric restriction and exercise, could result in the histological reversal or resolution of NAFLD/NASH and improved hepatic fatty acid oxidation.

EXPERIMENTAL DESIGN AND METHODS

Experimental design - This study was comprised of a Phase 1 and Phase 2. Detailed inclusion and exclusion criteria are outlined below and are listed in **Table 3.1**. In Phase 1, adult subjects scheduled for a medically indicated, diagnostic liver biopsy for suspected NAFLD were recruited to participate in Phase 1. Participants consented to allow a portion of their liver biopsy samples to be used for research purposes. Liver samples were scored histological according to standard practice guidelines [119], by a

blinded pathologist. A fasting baseline blood draw was also obtained at the time of biopsy. Patients with a NAS ≥ 4 and whom consented, were recruited to participate in the Phase 2 (treatment phase) of the study. Baseline intrahepatic lipid content measured via MRI and aerobic exercise capacity (VO_2 peak) measured via an exercise tolerance test were completed. Following baseline measures, participants were randomized to one of two treatment arms (Active Treatment or Standard Care) and then followed for nine months. Following completion of the 9-month treatment phase, repeat measurements were made. See **Fig. 3.1** for experimental design. All liver samples collected pre- and post- treatment underwent histological assessment for the degree of NAFLD present and in vitro assessments of hepatic fatty acid oxidation. Data from these tests were compared with the scores obtained through histologic examination.

Participant Recruitment - Participants were recruited via the Liver Service at the Digestive Health Center and the Division of Gastroenterology and Hepatology at University of Missouri Columbia. Following histological confirmation of NASH [NAFLD Activity score (NAS) ≥ 4], participants who consented to the treatment phase of the study were randomized to Active Treatment (n = 9) or Standard of Care (n = 2).

Phase 2 - Treatment Phase - Following the initial liver biopsy, each participant who consented to the treatment phase completed a baseline graded exercise tolerance test, DEXA, and MRS/MRI imaging of the liver. All visits occurred within 2 weeks of each other. Following completion of baseline testing, the participant was randomized to either treatment group (Active Treatment or Standard of Care) and remained in these groups until the completion of the study. In month 4 of the treatment phase, participants completed an interim visit in which a fasting blood draw was obtained for biochemistries and body composition (DEXA), and a graded exercise tolerance test were also

performed (Data shown in **Supplementary Table 3.1**). Baseline measures were repeated at 9-month follow-up. Active Treatment - Patients in the Active Treatment group received weekly counselling from the study dietician throughout the study (described below). Participants also underwent a supervised exercise training program at the Clinical Research Center Exercise Facility at University Hospital or at McKee Gym for the duration of the study. A high intensity interval training (HIIT) protocol was used and is described briefly in the following section. Standard Care - Participants assigned to the Standard Care group received a single dietician consult to learn about weight loss. They were also given information on current physical activity guidelines for adults [144], and encouraged to be physically active. Those receiving standard of care did not meet with study staff again until their interim visit, and then again at follow-up. Standard Care participants who completed the study were offered weekly lifestyle counseling after their participation was completed.

Liver Biopsy - Biopsies were performed in a clinical, outpatient setting by the same hepatologist, according to standard clinical practice guidelines. A total of 6 needle passes were made to obtain liver tissue. A portion of the liver biopsy went to pathology, where it was stained with hematoxylin and eosin (H&E), Masson's trichrome stain, reticulum and iron stains and scored for NASH according to the procedures of the NASH Clinical Network Scoring System [119]. All biopsies were graded and evaluated for inclusion or exclusion on a case-by-case basis. The second portion of the biopsy went to *ex vivo* assays/fresh tissue studies to assess hepatic fatty acid oxidation.

Hepatic Fatty Acid Oxidation Assay - *Ex vivo* whole liver palmitate oxidation studies were completed in fresh whole liver homogenate preparations in pre- and post-liver biopsies as described in Chapter 2 (Page 21).

MRS/MRI – MRS/MRI was used to assess intrahepatic lipid content as previously reported by our group [105]. Localized ¹H-MRS spectra of the liver was acquired with subjects in the supine position using a Phillips 3T Siemens Trio Scanner with head, neck, spine and body coils, using standard techniques. Liver volume was assessed via the MRI data, obtained during abdominal visceral fat measurement. IDEAL sequence analysis was also used to calculate hepatic proton density fat fraction [145].

Blood Draw and Biochemical Measures – A fasting blood draw (30ml whole blood) was collected prior to the liver biopsy, on the same day. Patients were instructed to fast for 12 hours prior to the blood draw. Plasma concentrations of total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDLc), high-density lipoprotein cholesterol (HDLc), liver enzymes [(aspartate aminotransferases (AST), alanine aminotransferases (ALT)], Alkaline phosphatase, plasma glucose concentrations, and Glycated hemoglobin (HbA1C) were measured by a CLIA standardized laboratory using standardized commercially available kits (#26D0652092, Quest Diagnostics, St. Louis, MO). Plasma insulin was measured using an enzyme-linked immunosorbent assay (#EZHI-14K, Human Insulin, CV 7.2%, EMD Millipore, Billerica, MA). Insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) equation [146].

Exercise Tolerance Testing and Physical Activity - Each subject completed a maximal cardiorespiratory exercise (VO₂ peak) test at baseline, midpoint (4.5 months into intervention), and after the 9-month intervention to determine initial fitness and changes in fitness status. This data was used to determine the maximal exercise heart rate and appropriate exercise intensity for HIIT. A modified Bruce protocol was used on a treadmill or bike with gas analysis (TrueOne® 2400, Parvo Medics, Sandy, UT) and

12-lead ECG as previously described by our group [105, 147]. Resting heart rate (Polar USA) and blood pressure (Welch Allyn) was also be recorded at the time of the exercise test. Physical activity was tracked throughout the treatment phase by providing each participant with a Fitbit to track daily step count.

Anthropometrics - Body composition was measured on a Hologic A, S/N 100158 DEXA (Analysis Version 13.5.2, Auto Whole-Body Fan Beam). Body weight was measured to the nearest 0.1 kg and height to the nearest 0.1 cm.

Dietary Counselling and Dietary Recalls - Participants met with a trained dietician/nutritionist once a week for the first 8 weeks, then bimonthly for months 3-5, then monthly for months 6-8. In the 9th month, patients met with the dietician/nutritionist twice to attain weight stability prior to follow-up testing. Participants were encouraged to achieve a caloric deficit of ~500 kcal/d with a goal of ~12% reduction in body weight within the 36-wk intervention, based on current treatment guidelines [1, 143]. Emphasis was placed on reducing sugar intake to <5% of energy and ensuring adequate consumption of sufficient energy and protein (1g/kg/d) to reduce muscle loss. Compliance to the dietary regimen was assessed by weekly phone calls/texts and measurement of body weight during the in-person visits. Three-day dietary recalls were collected prior to baseline, interim and follow-up visits. Dietary recalls were analyzed using Nutrition Data System for Research analysis software. There was no significant differences in daily caloric intake, dietary carbohydrate or protein content in the Active Treatment groups diet at baseline compared to follow-up (Supplementary Table 3.3, P > 0.05). There was a significant increase in dietary fat intake content in the Active Treatment groups diet at baseline compared to follow-up (Supplementary Table 3.3, P < 0.05). To control dietary factors and promote weight stability participants were provided

with packed-out food three days prior to each testing visit. Caloric and macronutrient content of the packed-out meals were based on three-day dietary recall records.

Exercise Training - The HIIT sessions, are described previously [108, 109]. HIIT consisted of four, 4-min intervals at 90-95% of heart rate max on a treadmill, bicycle, or elliptical. Each interval was separated by 3-min active pauses (low intensity walk, ~50% heart rate max). The exercise intensity was monitored during each session using heart rate monitors. The exercise training followed a ramped-up progression over the first 3-4 weeks to reach the above targets. Each exercise session was supervised by graduate and undergraduate students in exercise physiology.

Statistical Analysis - Statistical analyses was completed using GraphPad Prism version 9.1.0 (216) with $P \leq 0.05$ used to determine statistical significance of all comparisons. For Phase 1 analysis significant statistical differences were determined for NAS = 0-3 versus NAS ≥ 4 using a two-tailed, unpaired sample t-test. In Phase 2 - Due to the small sample size in the Standard Care group (n=2) it was not possible to determine differences between groups (Active Treatment v Standard Care) using a one-way analysis of variance. The Standard Care group was excluded from the baseline versus follow-up analysis. For the Active Treatment group (n =9), significant statistical differences were determined for baseline versus follow-up using a two-tailed, paired sample t-test. For all participants (n =11), Spearman Rank or Pearson's correlations were used to examine the association between functional measures (fatty acid oxidation, aerobic capacity) and the full range of NAS scores.

RESULTS

Phase 1

Patient characteristics - NAFLD severity was determined using NAS [119] based on the degree of histological hepatic steatosis, inflammation, and hepatocellular ballooning present. Participants (n = 59) were clustered into two groups based on NAS: NAS = 0-3 (n = 8) and NAS \geq 4 (n = 51) (**Fig. 3.2A** and **C**). Individuals with NAS \geq 4 presented with significantly greater histological fibrosis compared with to the NAS = 0-3 group (**Fig. 3.2B** and **D**; $P \leq 0.05$). Groups did not differ in age, body weight, body mass index (BMI), or by fasting serum triglycerides, glucose, HbA1c, LDL-c, HDL-c or total cholesterol (**Table 3.2**; $P > 0.05$). Further, NAS \geq 4 was associated with significantly higher AST and ALT serum concentrations compared to all other groups (**Table 3.2**; $P \leq 0.05$).

Reduced hepatic fatty acid oxidation and NASH - As shown in Chapter 2, advanced NAFLD corresponds with impaired hepatic fatty acid oxidation in humans, and we have also shown similar effects in rodent models with diet induced NAFLD [20]. Here, we report significantly lower complete, incomplete, and total [1- 14 C] palmitate oxidation in whole liver samples in patients with NAS \geq 4 (**Fig. 3.3A-C**; $P \leq 0.05$). Correlational analysis also reveals that reduced hepatic fatty acid oxidation corresponded with increasing lobular inflammation, hepatocellular ballooning, NAFLD activity, and hepatic fibrosis (**Fig. 3.4**). Of note, lower incomplete and total [1- 14 C] palmitate oxidation was significantly related to increasing hepatic lobular inflammation (**Fig. 3.4B** and **C**; $r = -0.286$ and $r = -0.283$, respectively, $P \leq 0.05$), while lower complete [1- 14 C] palmitate oxidation correlations with increased lobular inflammation displayed a similar relationship but was not significant (**Fig. 3.4A**; $r = -0.229$, $P = 0.089$). Similarly, increased hepatocellular ballooning negatively corresponded with worsening complete ($r = -0.317$), incomplete ($r = -0.327$) and total ($r = -0.325$) [1- 14 C] palmitate oxidation (**Fig. 3.4A-C**; $P \leq 0.05$). Finally, increasing histological fibrosis scores corresponded with worsening,

incomplete and total [$1\text{-}^{14}\text{C}$] palmitate oxidation (**Fig. 3.4B-C**; $r = -0.296$ and $r = -0.291$, respectively, $P \leq 0.05$).

Phase 2

Active Treatment promoted the resolution of NASH Characteristics - Studies from our lab [17, 31, 105, 108, 142, 148] and others [23, 86-90, 143], have shown that lifestyle interventions aimed at caloric restriction via diet and exercise ameliorate NAFLD in both human and rodent models. Here we show that Active Treatment significantly reduced NAS from baseline (NAS = 6) versus follow-up (NAS = 4; **Fig. 3.5A and C**; $P \leq 0.05$). Active Treatment significantly reduced intrahepatic lipid content by $7 \pm 3\%$ (mean \pm SE) at follow-up versus baseline (**Fig. 3.5E**; $P \leq 0.05$), while reductions in histological hepatic steatosis was not significantly different (-0.6 ± 0.3 -point reduction, mean \pm SE, **Fig. 3.5D**; $P = 0.09$). Interestingly, Active Treatment was accompanied by significant reductions in hepatocellular ballooning (-1.1 ± 0.3 -point reduction, mean \pm SE, **Fig. 3.5G**; $P \leq 0.05$), but not lobular inflammation (-0.4 ± 0.4 -point reduction, mean \pm SE, **Fig. 3.5F**; $P > 0.05$) at follow-up. Additionally, active treatment corresponded with a non-significant lowering in hepatic fibrosis staging at follow-up versus baseline (-0.8 ± 0.4 -point reduction, mean \pm SE, **Fig. 3.5F**; $P = 0.088$). Minimal changes in NAFLD activity and fibrosis scoring were observed for the Standard Care group for baseline versus follow-up.

Impact of lifestyle modifications on anthropometrics in individuals with NASH -

Patients who underwent active treatment significantly reduced body weight and BMI by $9 \pm 2\%$ (mean \pm SE) at follow-up compared to baseline (**Fig. 3.6A and B**, $P \leq 0.05$), while reductions in fat mass trended toward significance at follow-up (60.2 ± 5.1 kg versus 54.5 ± 5.1 kg, mean \pm SE, **Fig. 3.6C**; $P = 0.078$). There was no significant change in lean mass following the active treatment period (baseline versus follow-up, **Table 3.3**, $P =$

0.194). In a similar manner, the Standard Care group displayed a 7±9% reduction in body weight and BMI at follow-up when compared to baseline (**Table 3.3**). Interestingly, reductions in fat mass corresponded positively with reductions in hepatic steatosis, lobular inflammation, and NAS ($r = 0.726$, $r = 0.646$, and $r = 0.678$, respectively; $P \leq 0.05$), but not hepatocellular ballooning or hepatic fibrosis ($r = 0.236$, and $r = 0.299$, respectively $P > 0.05$, values are absolute change from baseline, **Fig. 3.7C**). Changes in body weight and BMI did not significantly correlate with histological outcomes (**Fig. 3.7A and B**; $P > 0.05$). Active Treatment did significantly lower fasting glucose, insulin, HOMA-IR, triglycerides or HDLc at follow-up when compared with baseline (**Table 3.3**; $P > 0.05$). Individuals who underwent Active Treatment exhibited significantly lower LDLc concentrations (**Table 3.3**, $P \leq 0.05$), and lower total cholesterol and HbA1C concentrations that trended toward significance at follow-up versus baseline (**Table 3.3**, $P = 0.075$ and $P = 0.071$, respectively). Absolute change in total cholesterol and LCLc corresponded positively with a lowering in NAS ($r = 0.751$ and $r = 0.66$, respectively) and lobular inflammation ($r = 0.888$ and $r = 0.816$, respectively $P \leq 0.05$; **Table 3.4**). Finally, the Active Treatment group had significantly lower AST and ALT serum concentrations at follow-up compared with baseline concentrations (75.3 ± 14.2 vs 26.8 ± 3.3 and 74.4 ± 12.3 vs 32.2 ± 4.3 , respectively; mean \pm SE; **Table 3.3**; $P \leq 0.05$). The Standard Care group also displayed reductions in AST and ALT serum concentrations at follow-up compared with baseline (82.0 ± 25.0 vs 29.5 ± 4.5 and 126.0 ± 62.0 vs 43.5 ± 11.5 , respectively; mean \pm SE, **Table 3.3**).

Impact of lifestyle modifications on aerobic fitness and Physical Activity in

individuals with NASH - The Active Treatment group displayed significant increases in VO₂ peak and exercise time to exhaustion for baseline versus follow-up (**Fig. 3.8A, B, and C**; $P \leq 0.05$). Patients receiving standard care displayed minimal changes in VO₂

peak for baseline versus follow-up (**Fig. 3.8A, B, and C**). Interestingly, favorable increases in absolute VO₂ peak also inversely correlated with reduced hepatocellular ballooning and NAFLD activity score (**Fig. 3.8D**; $r = -0.675$ and $r = -0.796$, respectively, $P \leq 0.05$) and trended toward significance for hepatic steatosis ($r = -0.574$, $P = 0.09$) and lobular inflammation ($r = -0.577$, $P = 0.07$; **Fig. 3.8D**). Patients in the Active Treatment group tended to increase average monthly step count (**Supplemental Table 3.2**), which corresponded with greater weight loss (**Supplemental Figure 3.1**).

Impact of lifestyle modifications on hepatic fatty acid oxidation in individuals with NASH - We have previously shown that lifestyle modifications such as caloric restriction and exercise increases whole liver fatty acid oxidation in rodents with diet induced NAFLD (30, 34). Interestingly, active treatment did not result in changes in complete, incomplete, and total [1-¹⁴C] palmitate oxidation in whole liver for baseline versus follow-up (**Fig. 3.9A, B, and C**; $P > 0.05$). Similar outcomes were observed for Standard Care (**Fig. 3.9A, B, and C**). Additionally, absolute changes in complete, incomplete, and total [1-¹⁴C] palmitate oxidation in whole liver did not correlate significantly with absolute changes in histological steatosis, lobular inflammation, hepatocellular ballooning, NAS or fibrosis (**Figure 3.10A, B and C**; $P > 0.05$). Remarkably, increased absolute VO₂ peak (absolute change) inversely corresponded with reductions in complete [1-¹⁴C] palmitate oxidation ($r = -0.740$, $P \leq 0.05$; **Table 3.5**). Further, reductions in HOMA-IR also corresponded with a lowering in incomplete, and total [1-¹⁴C] palmitate oxidation in whole liver ($r = 0.767$ and $r = 0.766$, respectively, $P \leq 0.05$; **Table 3.5**). Changes in whole liver fatty acid oxidation did not correspond with changes in biometric measures such as fasting glucose, lipids, AST, and ALT (**Table 3.5**, $P > 0.05$). Changes in dietary composition did not correspond with changes in fatty acid oxidation (data not shown).

Sub-analysis within the Active Treatment group comparing histological outcomes in those that lost < 10% versus ≥10% bodyweight and across patients- Given that NAFLD resolution has been shown to occur with weight loss of ≥ 10% body weight [86, 87], further sub-analysis of the Active Treatment group comparing individuals who lost < 10% versus ≥ 10% bodyweight at follow-up was completed. Statistical analysis using a two-way repeated measures ANOVA was completed. There was no significant group*time interaction for histological outcomes such as NALD activity score, steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis (data not shown). A lack of significant differences between groups may be due to the small sample size (< 10% n = 5, ≥ 10% n = 4) and variability within groups. Alternatively, histological NAFLD activity outcomes for each individual are presented in **Table 3.6**. Of the patients in the Active Treatment group (n=9), 78% of patients (n = 7/9) reduced their NAS by ≥ 1 point, 22% of patients (n = 2/9) had NASH resolution (NAS < 4 at follow-up), and one patient had complete resolution of NAFLD at follow-up (NAS = 0). Two patients did not improve NAS from baseline versus follow-up. Reasons for the lack of change in NASH severity may be due to smaller body weight loss (≤ 4%) compared to others and poor compliance to exercise (≤ 85% of exercise sessions completed). Overall, Active Treatment resulted in the stabilization or regression of the degree of NASH present. The greatest reduction in NASH was observed with 21% body weight loss, while improvements in NAFLD activity score were observed with a little as 3% weight loss. Similarly, Active Treatment was associated with fibrosis regression in 55% (n = 5/9) of patients. Three patients stabilized fibrosis staging (no change), while one patient increased their fibrosis score by one point.

Case study - Individual responses to active treatment in subjects with the most weight lost - To tease out individual differences in liver disease outcomes in response to active

treatment, two patients were compared, Patient A and Patient B, who shared similar characteristics and NASH disease severity at baseline (**Fig. 3.11**). Further, both patients had similar compliance to exercise sessions (>99% exercise session completed) and degree of weight lost at follow-up (-20% and -17% body weight loss for Patient A and Patient B, respectively; **Fig. 3.11**). Patient A was a 46-year-old, non-diabetic woman who presented with advanced NASH (NAS = 7/8), bridging fibrosis (Fibrosis score = F3/4), and elevated AST and ALT serum concentrations (55U/L and 38U/L, respectively). Patient A completed 99% of her exercise sessions and lost 20% of her body weight. At her follow-up liver biopsy, Patient A had complete resolution of histological NASH (NAS = 0/8), reduced fibrosis staging to F1 (periportal/perisinusoidal fibrosis), and normalized AST and ALT serum concentrations (18U/L and 16U/L, respectively). Patient B was a 57-year-old, non-insulin dependent type 2 diabetic woman who presented with advanced NASH (NAS = 6/8), bridging fibrosis (F = 3/4), and elevated AST and ALT concentrations (55U/L and 38U/L, respectively). Patient B completed 100% of her exercise sessions and lost 17% of her body weight. At her follow-up liver biopsy, Patient B reduced her liver disease by 2 points to moderate NASH (NAS = 4/8). However, fibrosis staging (bridging fibrosis, F = 3/4) and AST and ALT serum concentrations remained relatively unchanged compared with baseline. The lack of changes in NASH severity occurred despite reductions in fasting blood glucose and HbA1C concentrations at baseline compared to follow-up. Reasons for differing outcomes in disease severity between Patient A and Patient B in response to active treatment are unknown. Indeed, factors such as age difference (11 years), presence of type 2 diabetes in Patient B, hormonal factors, genetics, and environmental variables may have played a role. Nonetheless it is interesting to note individual responses to active treatment in patients with similar compliance and degree of weight lost.

DISCUSSION

A major problem in the management of NASH is the lack of effective therapy [149]. Weight reduction through diet and exercise has been promoted as initial therapy for NASH. However, human data examining the mechanisms by which lifestyle modifications reverse histological outcomes of NASH using pre- and post-liver biopsies are limited. We provide novel evidence that an active treatment phase, consisting of weight loss (-9%), resulted in significant reductions in histological outcomes of disease severity in humans with well characterized NASH (NAS \geq 4). Further, favorable reductions in histological outcomes of NAFLD corresponded with a lowering in body fat mass (-8%) and increases in aerobic fitness (17%, absolute VO₂ peak). Intriguingly, improvements in NAFLD activity occurred despite no change in hepatic fatty acid oxidation. Importantly, the results from this study thus far suggest that lifestyle modifications focusing on caloric restriction and exercise can successfully lead to improvements in overall NASH histologic activity, degree of steatosis and liver chemistry and provides insight into the mechanisms by which the treatments improve liver histology.

Rigorously conducted randomized controlled trials addressing the efficacy of weight reduction, via diet and/or exercise, in patients with well characterized NASH and mechanisms involved are limited [90, 93, 143]. Additionally, several studies used outcomes such as serum aminotransferases concentration, sonographic, and/or MRI/MRS findings as primary NAFLD/NASH outcomes that presently are not well accepted [81, 150-152]. Further, no studies to date have paired fresh tissue analysis of hepatic fatty acid oxidation with histological outcomes of NASH pre- and post-lifestyle intervention.

Undoubtedly, combining caloric restriction and exercise regimens results in the greatest reductions in hepatic steatosis and improvements in whole body substrate metabolism [68-74][79-82, 85, 90]. Limited studies assessing the effects of lifestyle modifications on NASH outcomes using liver biopsies demonstrate that caloric restriction improves inflammation and fibrosis [86, 87], and of the patients who lost $\geq 10\%$ of their weight, $>90\%$ had either resolution of NASH [85, 143], or a reduction in NAS of 3-points or more following a 12-month intervention [90]. Similarly, here we observed that on average patients reduced disease severity by 2-points (of NAFLD activity score, 33% reduction) in response to a 9-month active treatment phase. Of the patients in the Active Treatment group, 78% of patients reduced their NAS by ≥ 1 point, 22% of patients had NASH resolution (NAS < 4 at follow-up), and one patient had complete resolution of NAFLD at follow-up (NAS = 0). Magnitude of body fat mass lost correlated strongly with improvements in histological steatosis, inflammation, and NAS. Interestingly, magnitude of weight lost did not correspond significantly with improvements in histological outcomes of NAFLD activity. This outcome is in contrast to previous studies demonstrating strong correlations with improvements in disease markers of NAFLD/NASH and magnitude of weight lost [86, 90, 143]. Reasons for differing outcomes are likely due to the lower subject size in our dataset (n=11). Promat et al reported in their study that participants who achieved $\geq 7\%$ body weight loss had significantly greater improvements in all aspects of NASH histologic activity including steatosis, lobular inflammation, ballooning injury. Indeed, if we visually examine individual outcomes of NAFLD activity (**Table 3.5**) in this study we can appreciate that in all patients who lost $\geq 7\%$ body weight, NAFLD activity was improved, while some patients also exhibited histological improvements with as little as 3% weight loss. These findings are in line with what has previously been shown [86, 90, 143].

We have previously shown that lifestyle modification such as moderate intensity treadmill exercise reduced markers of hepatic fibrosis and fibrosis score in a rodent model of diet induced NAFLD/NASH [153]. In contrast here, we did not observe a significant reduction ($P = 0.08$) in the degree of hepatic fibrosis at 9-month follow-up. Human studies examining the effects of caloric restriction on hepatic fibrosis staging outcomes are mixed. Indeed, one previous study has demonstrated significant improvements in fibrosis following a 12-month weight loss intervention in patients who lost $\geq 10\%$ bodyweight [143]. Conversely, others have reported no beneficial effects of $> 7\%$ body weight loss via caloric restriction on hepatic fibrosis following a 48-week intervention [90]. Given that patients on average lost $\sim 9\%$ body weight in the current study, this highlights that weight loss of $< 10\%$ body weight may not be effective in treating hepatic fibrosis. Further, given that our study was of shorter duration (9-months), a longer treatment period maybe required to detect changes in fibrosis score. In addition, our current dataset may be underpowered to detect meaningful changes in fibrosis in response to active treatment. Also, previous studies have included patients with relatively low fibrosis score at baseline [90]. Future clinical trials in NASH patients should consider a patient enrollment scheme to include subjects with a full spectrum of NASH and fibrosis severity.

It is well accepted that aerobic exercise training is an established cornerstone of NAFLD/NASH disease management through the reduction of intrahepatic lipid content and the attenuation of nutrient overload in the liver by improving substrate metabolism [154-156]. However, studies examining the effects of improvements in aerobic fitness on histological outcomes of NAFLD/NASH in humans are lacking. To our knowledge, this study demonstrates for first time that improvements in aerobic fitness (absolute VO_2 peak) correspond strongly with total NAFLD activity score, steatosis, lobular

inflammation, and hepatocellular ballooning in humans. In rodent models of NAFLD, we [32, 105, 107-109, 148] and others [157-159], have shown that exercise training and genetic predisposition for a higher aerobic capacity protects against liver disease onset and progression and restores hepatic function via improved lipid metabolism, decreased oxidative stress, inflammation, and fibrosis. In particular, our recent studies in rodent models of NAFLD/NASH established that the therapeutic effect of exercise on liver histology is mediated through increases in both hepatic mitochondrial content and function [17, 32, 109, 148]. Further, our group has previously shown that exercise brought about additional benefits in reducing hepatic de novo lipogenesis not observed with caloric restriction alone in a rodent model of NAFLD/NASH [108]. Future studies examining the effects of exercise training on hepatic mitochondrial function in the setting NASH are warranted.

In patients referred for a liver biopsy to clinically diagnose NAFLD, we demonstrate that advanced NASH (NAS ≥ 4) is associated with ~50% lower complete oxidation of long chain fatty acid oxidation in whole liver, and ~25% lower incomplete and total long chain fatty acid oxidation compared with no or mild disease (NAS = 0-3). Indeed, these data support our findings in Chapter 2, where we report that long chain fatty acid oxidation and the rate limiting step in β -oxidation are downregulated (~50%) early in the disease onset and remained lower with increased liver disease severity in humans with obesity. Further, these findings in a human population support our previous work in rodent models [20] and these data highlight the possibility of perturbations in hepatic long chain fatty acid oxidation playing a causal role in advancement of liver disease and warrants future investigation.

Our lab and others have shown that lifestyle modifications such as caloric restriction and exercise is an effective approach in improving NAFLD outcomes via improved lipid metabolism and mitochondrial function [106, 107]. Intriguingly, improvements in histological outcomes of NASH in the Active Treatment group occurred despite no change in long chain fatty acid oxidation at follow-up. Indeed, the exact mechanisms of caloric restriction and exercise on hepatic outcomes are likely multifaceted. We and others have demonstrated that exercise improves hepatic mitochondrial health in the setting of NAFLD, in rodent models [17, 32, 108, 109]. Whether similar improvements in hepatic mitochondrial health and markers of content and quality control are observed in humans and are linked to or independent of changes in long chain fatty acid metabolism following a lifestyle intervention remain to be explored. *This analysis will likely be investigated at a later date in this data set when more participants have been added to each group.*

One could speculate as to why we see a lack of change in hepatic long chain fatty acid oxidation in the Active Treatment group. NAFLD is characterized by elevations in intrahepatic lipid content [151, 160-164], resulting from excess consumption of 1) dietary fats, 2) dietary carbohydrates, which are made into fatty acids via the process of de novo lipogenesis, DNL, and 3) from poorly controlled release of adipose FFA [10, 165], which clear to the liver in proportion to their concentration in blood [166, 167]. Eventually, this nutrient excess in the liver causes steatosis which can lead to progression from NAFLD to NASH. Further, this transition is also characterized by poor insulin action on the liver, skeletal muscle, and adipose tissue [168-172]. Active treatment, combining caloric restriction and exercise would lessen the nutrient burden on the liver and improve peripheral insulin sensitivity [58-62], thus reducing nutrient flux through the liver and decreasing de novo lipogenesis. Reductions in steatosis, dietary FFA, de novo

lipogenesis, FFA release from adipose, and increased FFA uptake at the muscle may result in less substrate availability within the liver and less of a requirement for fatty acid oxidative machinery in the liver [107]. This theory may explain the strong relationship we observe with reductions in hepatic fatty acid oxidation and serum total cholesterol and LDLc. Further, the inverse relationship we observed with increased VO_2 peak and HOMA-IR corresponding with reductions in hepatic fatty acid oxidation may indicate the diversion of nutrients toward the now more insulin sensitive periphery away from the liver. Additionally, little is known about the effects of lifestyle modifications such as diet and exercise on hepatic mitochondrial health and content in a human population. Changes in hepatic mitochondrial content in response to active treatment may also explain why we observe a lack of change in fatty acid oxidation at follow-up. While these observations are speculative, they lend reason to the potential mechanisms at play and further investigation is required.

In conclusion, an intensive weight loss regime of dietary caloric restriction coupled with high intensity interval exercise training produces beneficial effects in ameliorating histological NASH related features, particularly hepatocellular ballooning steatosis and overall NAFLD activity score. Additionally, a dose response relationship is evident with improvements in body composition and aerobic fitness, with the greatest reduction in NAFLD activity observed in those with the greatest decrease in body fat mass and in those who improved aerobic capacity the most. Modest weight losses (~3%) also produced important benefits on NAFLD activity score. Furthermore, we demonstrated that hepatic long chain fatty acid oxidation is significantly lower in a clinical NASH population ($NAS \geq 4$) compared to mild disease ($NAS = 0-3$). Intriguingly, favorable changes in NASH outcomes occurred despite no change in hepatic long chain fatty acid oxidation at follow-up in the active treatment group, however further studies are

warranted. Taken together, these findings are the first to demonstrate that an active treatment phase of dietary caloric restriction and exercise produces favorable changes in disease severity. Further, these findings support the current recommendation for weight loss using lifestyle modifications as the first step in the management of patients with NASH.

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AUTHOR CONTRIBUTIONS

M.P.M., R.S.R., E.J.P., J.A.I. were involved in the study concept and design. M.P.M., R.P.C., G.M.M., S.A.J., V.J., L.B., A.A.W., A.D., R.R.G, N.M.S., J.B.P., A.I.A.S., J.A.I., E.J.P., M.M.S.A., J.A., J.M.M, M.S., and R.S.R. helped with acquisition of data. M.P.M., G.M.M., A.D., J.A.I., E.J.P., and R.S.R analyzed and interpreted results. M.P.M. and R.S.R. provided statistical analysis of the data. M.P.M. and R.S.R. drafted the manuscript, while R.P.C., G.M.M., S.A.J., A.A.W., A.D., R.R.G, N.M.S., J.B.P., A.I.A.S., J.A.I., E.J.P., and R.S.R. revised the manuscript and provided important intellectual content and approved final version of manuscript.

DECLARATION OF INTERESTS

No conflicts of interest, financial or otherwise, are declared by the authors.

Suspected NAFLD:
Liver biopsy referral for clinical diagnosis

Informed Consent

Baseline:
Liver biopsy and blood draw

PHASE I **NAS \geq 4** →

Informed Consent

Baseline:
MRS/MRI, DEXA, ETT

TREATMENT PHASE

Interim:
Blood draw,
DEXA, ETT

Follow-up:
Blood draw, DEXA,
ETT, MRS/MRI,
liver biopsy



Figure 3.1. Experimental Design

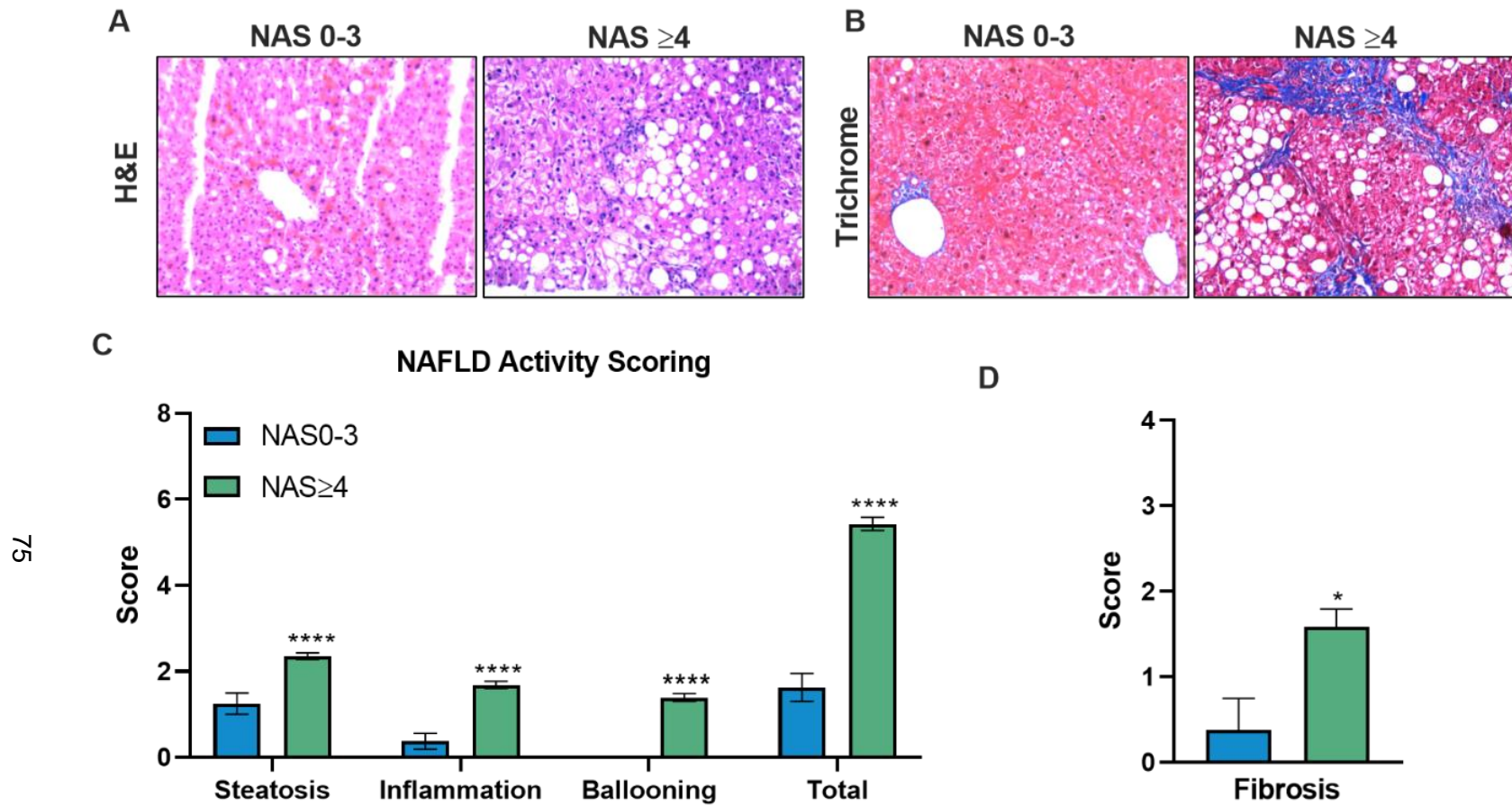
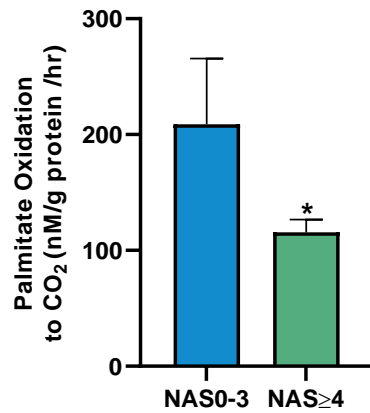
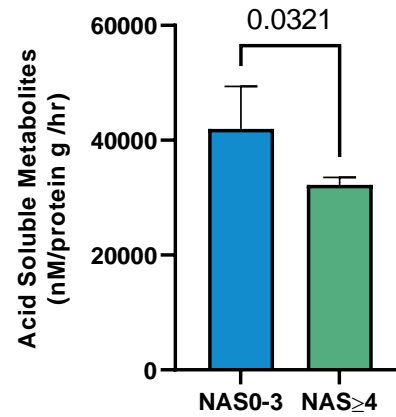


Figure 3.2. Phase 1 Liver Phenotype. A) Representative liver H&E, B) trichrome stain, C) NAFLD activity score, and D) fibrosis score. Values are presented as mean \pm SE. *P \leq 0.05, ****P \leq 0.0001.

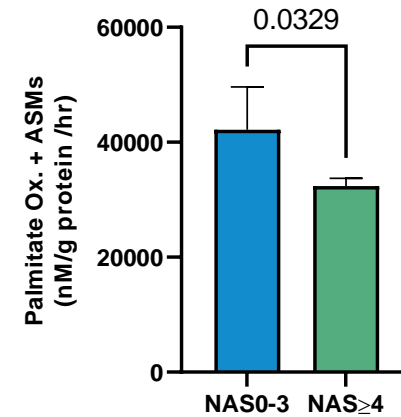
A Whole Liver Complete Fatty Acid Oxidation



B Whole Liver Incomplete Fatty Acid Oxidation



C Whole Liver Total Fatty Acid Oxidation



76 **Figure 3.3. Phase 1 whole liver ex-vivo fatty acid oxidation.** Whole liver A) complete palmitate oxidation to CO₂, B) incomplete, and C) total [1-¹⁴C] palmitate oxidation. Values are presented as mean±SE. *P ≤ 0.05.

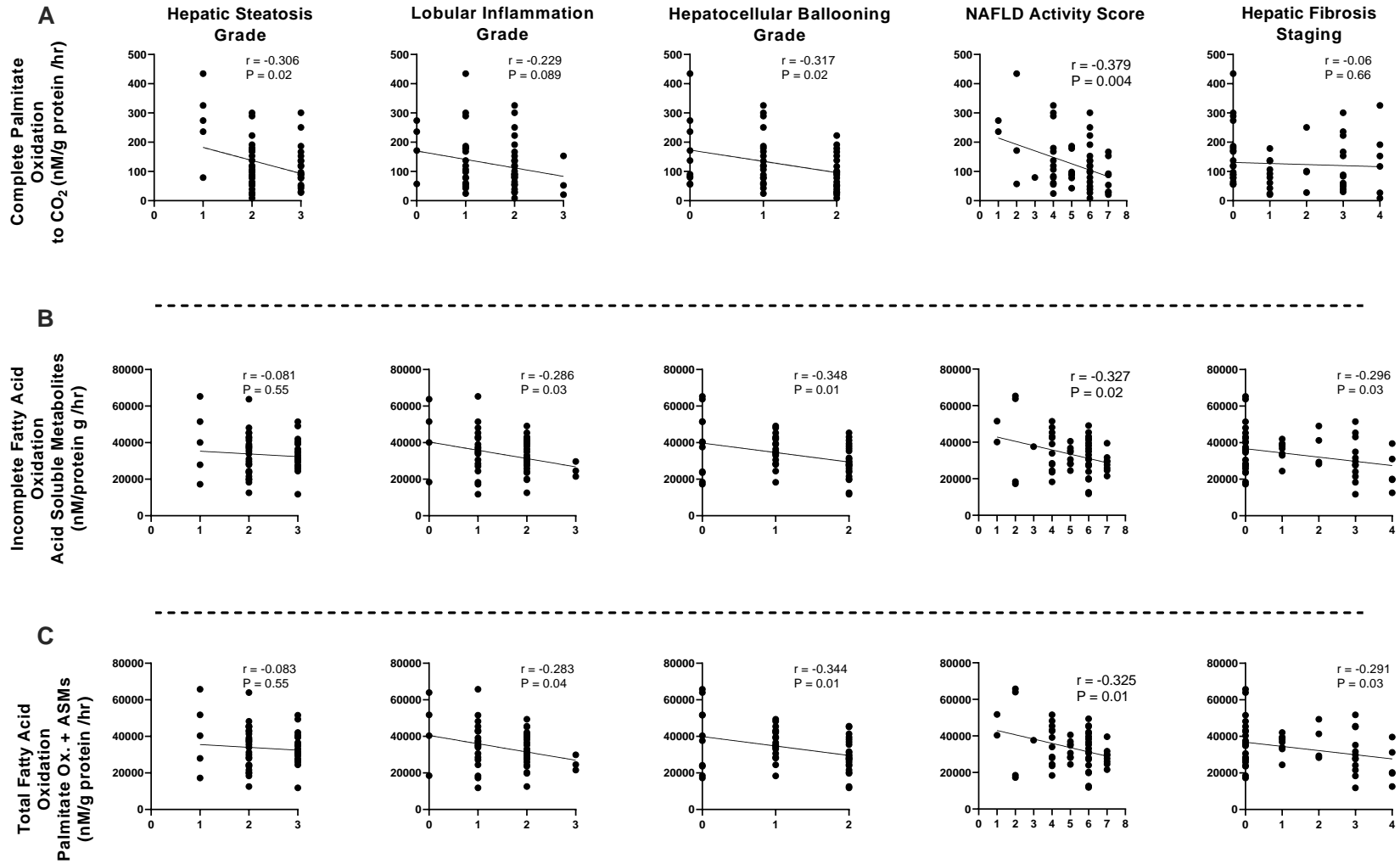


Figure 3.4. Phase 1 whole liver ex-vivo fatty acid oxidation correlations with histological outcomes of NAFLD. Whole liver A) complete palmitate oxidation to CO₂, B) incomplete, and C) total [1-¹⁴C] palmitate oxidation correlations with histological steatosis, lobular inflammation, hepatocellular ballooning, NAFLD activity score, and fibrosis.

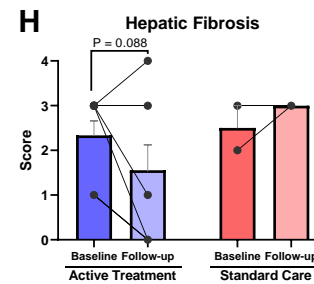
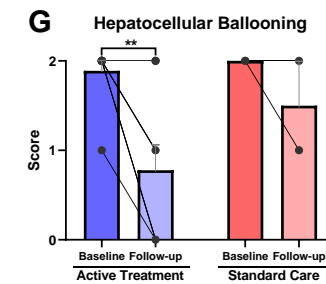
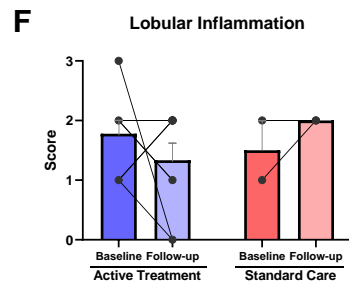
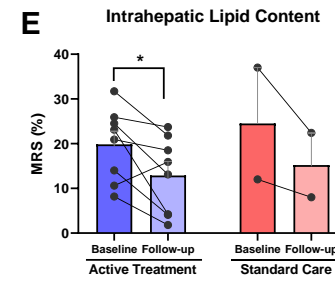
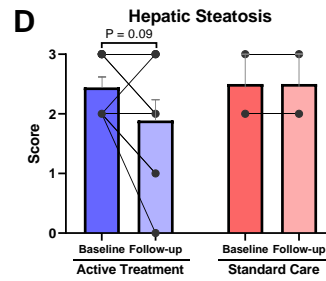
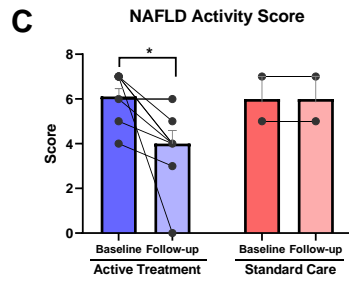
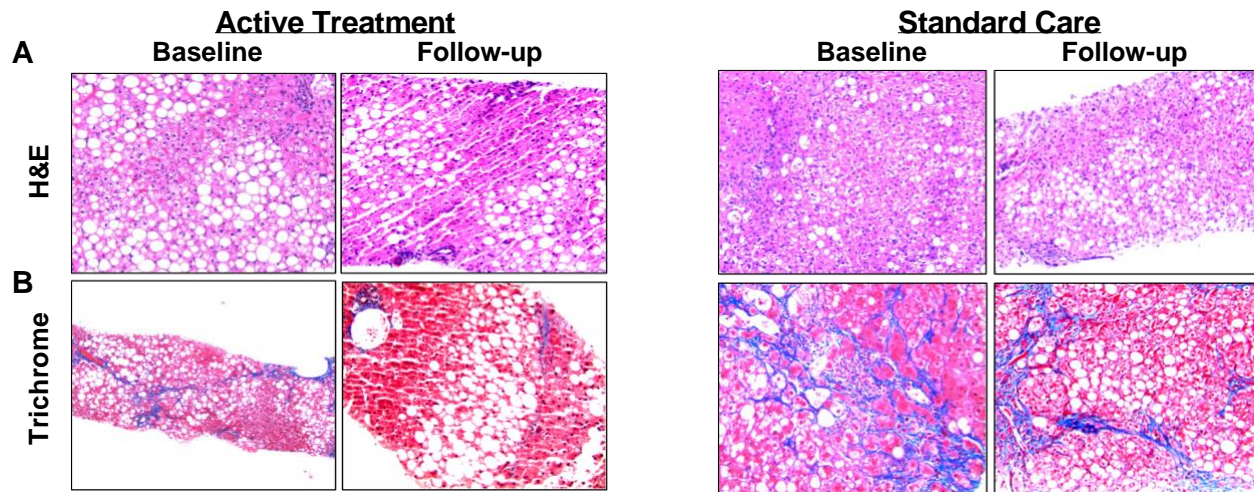


Figure 3.5. Phase 2 Liver Phenotype at baseline and follow-up. Representative liver H&E and trichrome staining for A) Active Treatment and B) Standard Care groups. C) Total NAFLD activity score, D) Hepatic steatosis, E) Intrahepatic Lipid content, F) Lobular Inflammation, G) Hepatocellular Ballooning, and H) fibrosis score. Mean±SE, *P ≤ 0.05, **P ≤ 0.01.

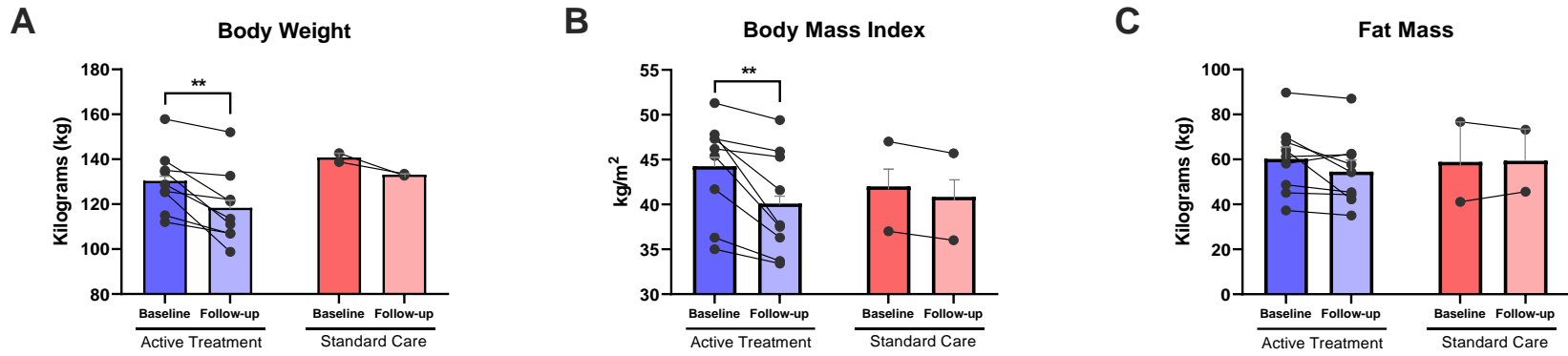


Figure 3.6. Phase 2 Body weight and composition at Baseline and Follow-up. A) Body weight, B) Body mass index, and C) Fat mass. Mean±SE, **P ≤ 0.01.

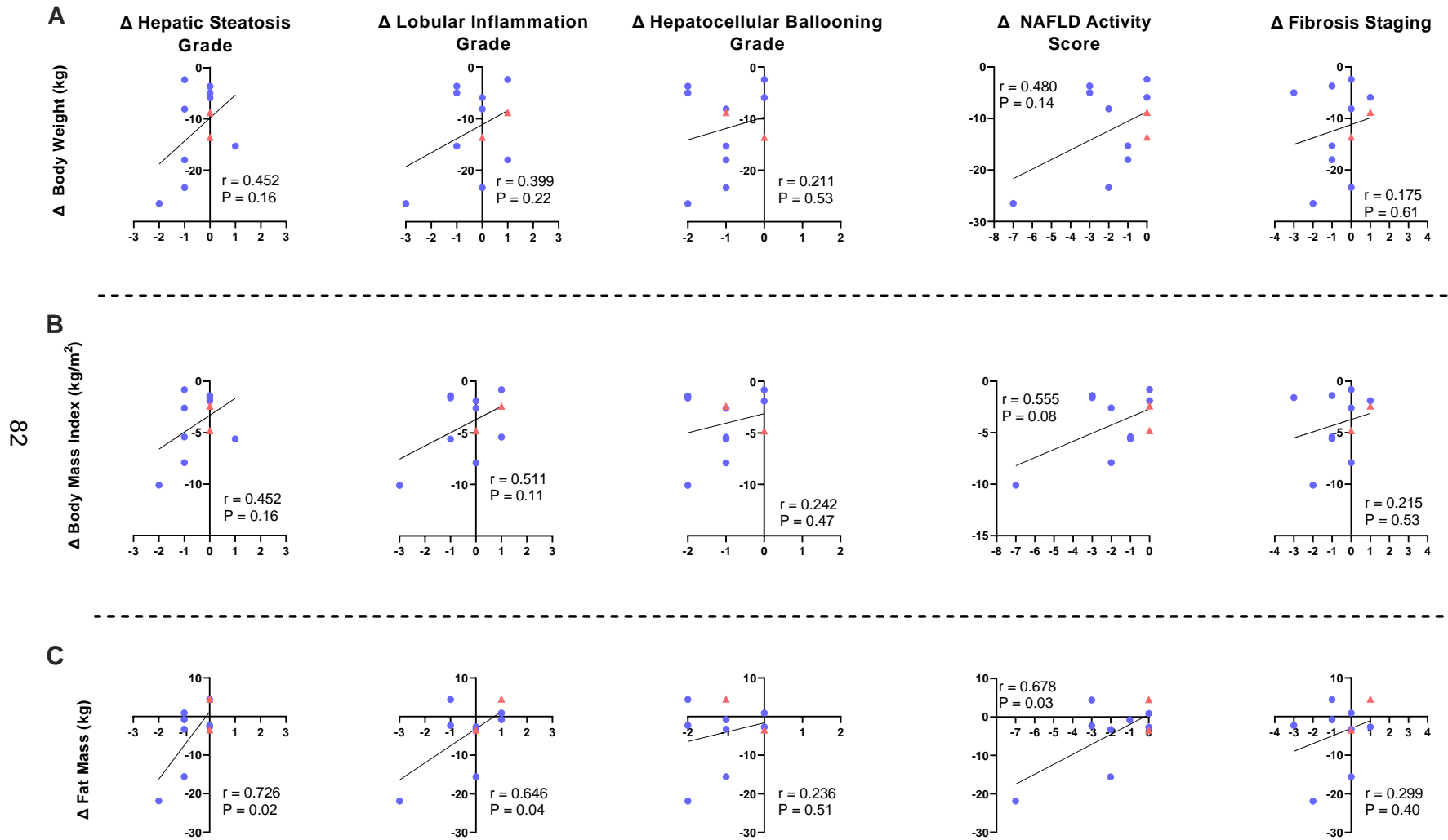


Figure 3.7. Phase 2 Change in body weight and composition with change in histological outcomes of NAFLD at Baseline vs Follow-up. Change in A) body weight, B) body mass index, and C) at mass correlations with histological steatosis, lobular inflammation, hepatocellular ballooning, NAFLD activity score, and fibrosis.

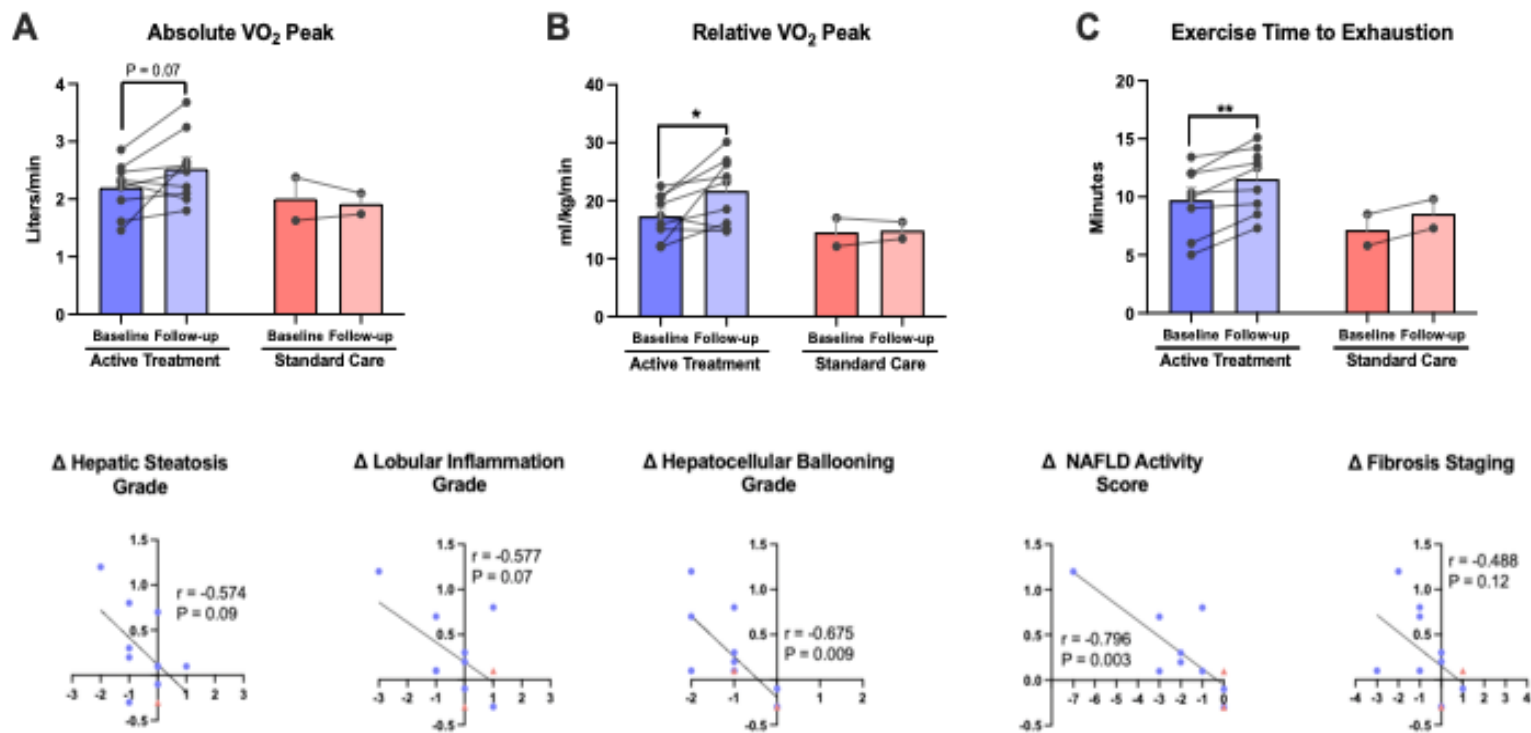


Figure 3.8. Phase 2 Aerobic Capacity at Baseline and Follow-up. A) Absolute VO₂ Peak, B) Relative VO₂ Peak, and C) Exercise Time to Exhaustion. D) Change in absolute VO₂ Peak correlated with changes in with histological steatosis, lobular inflammation, hepatocellular ballooning, NAFLD activity score, and fibrosis. Mean±SE, *P ≤ 0.05, **P ≤ 0.01.

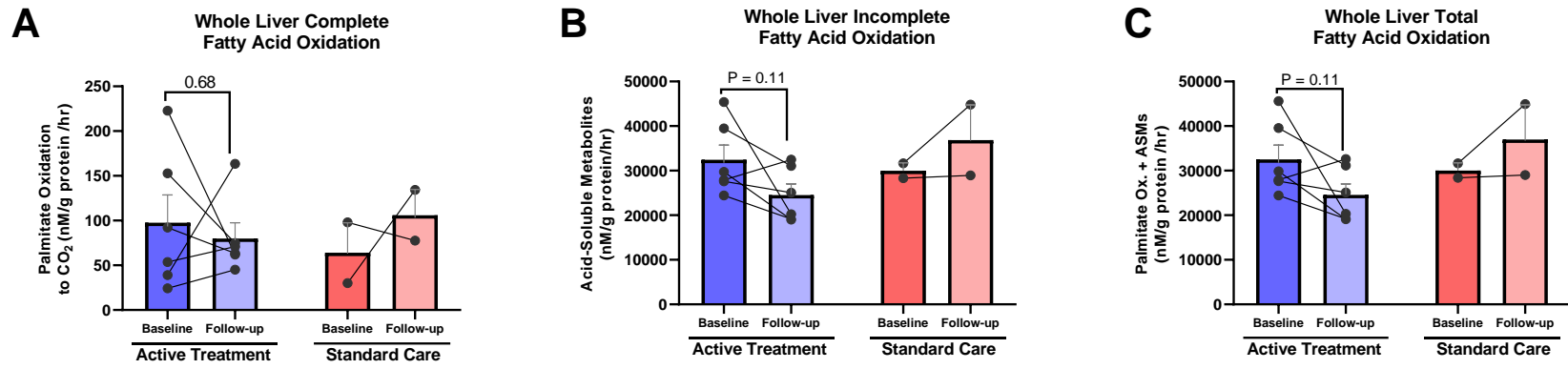


Figure 3.9. Phase 2 whole liver ex-vivo fatty acid oxidation at Baseline and Follow-up. Whole liver A) complete palmitate oxidation to CO₂, B) incomplete, and C) total [1-¹⁴C] palmitate oxidation.

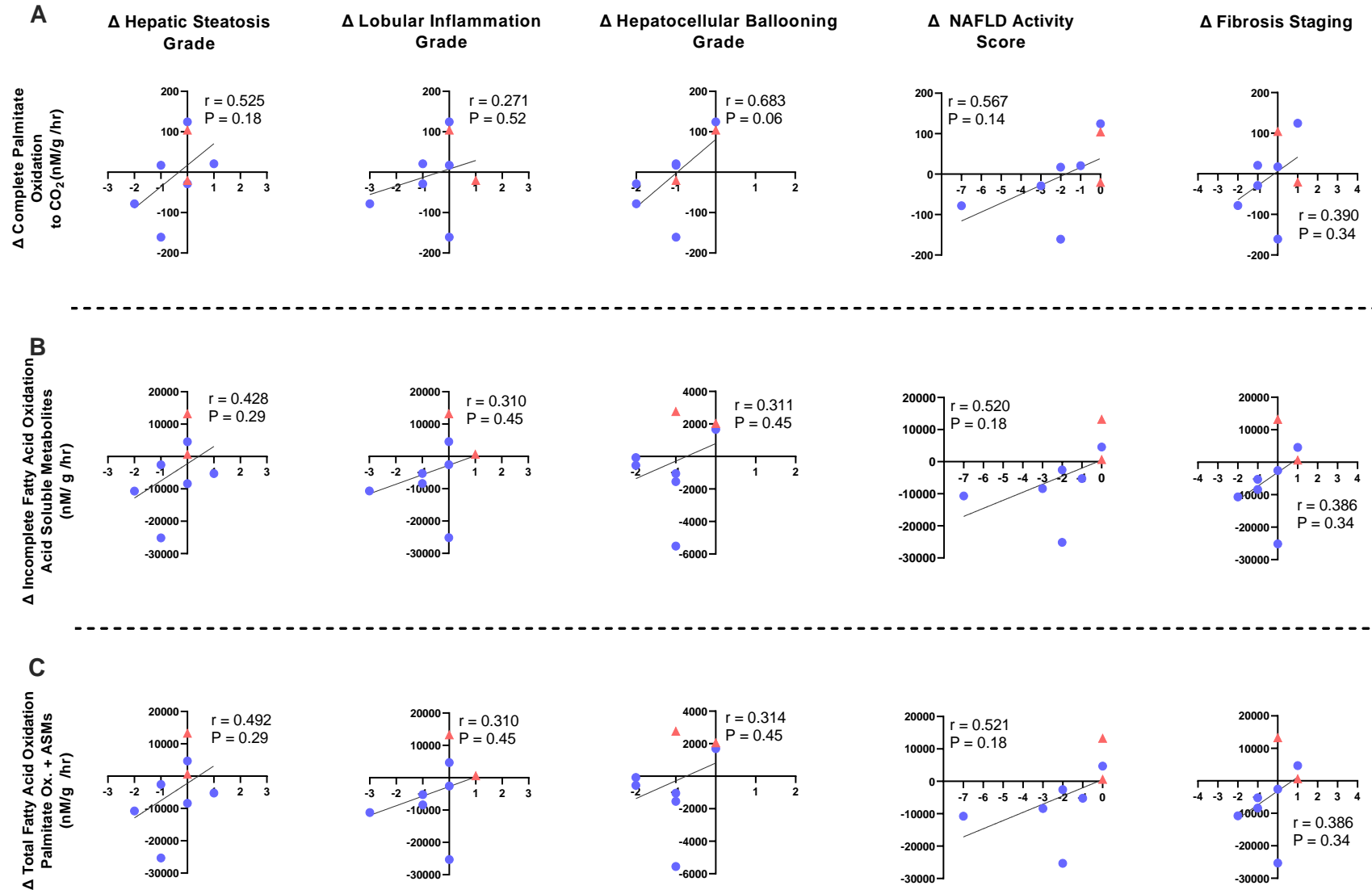
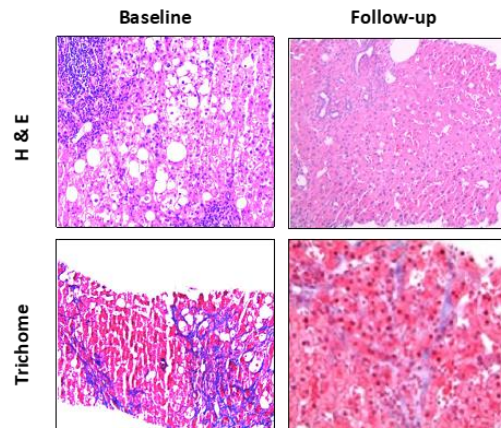


Figure 3.10. Phase 2 change in whole liver ex-vivo fatty acid oxidation correlations with change in histological outcomes of NAFLD at Baseline vs Follow-up. Change in whole liver A) complete palmitate oxidation to CO₂, B) incomplete, and C) total [1-¹⁴C] palmitate oxidation correlations with histological steatosis, lobular inflammation, hepatocellular ballooning, NAFLD activity score, and fibrosis.

Patient A

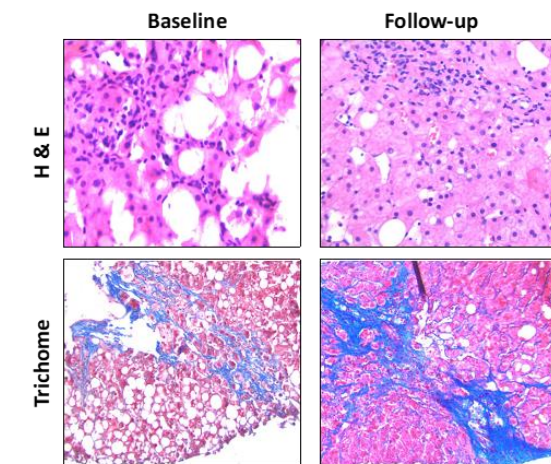
(Female, 46 years, 99% compliance to exercise)



Baseline	Follow-up
Advanced steatohepatitis	NASH resolution
Bridging fibrosis	Perisinusoidal/Periportal fibrosis
NAS = 7/8	0/8
Steatosis = 2/3	1/3
Lobular Inflammation = 3/3	2/3
Hepatocellular Ballooning = 2/2	1/2
Fibrosis Stage = 3/4	1/4
Body weight = 124kg	99kg (-20%)
VO₂ Peak = 1.46 L/min	2.65 L/min
ALT = 38 U/L	16
AST = 55 U/L	18
Fasting BG = 87mg/dL	83mg/dL
HbA1C = 5.5%	5.6%
T2D = No	

Patient B

(Female, 57 years, 100% compliance to exercise)



Baseline	Follow-up
Advanced steatohepatitis	Moderate NASH
Bridging fibrosis	Bridging fibrosis
NAS = 6/8	4/8
Steatosis = 2/3	1/3
Lobular Inflammation = 2/3	2/3
Hepatocellular Ballooning = 2/2	1/2
Fibrosis Stage = 3/4	3/4
Body weight = 134 kg	111 kg (-17%)
VO₂ Peak = 1.61 L/min	1.8 L/min
ALT = 34 U/L	34
AST = 38 U/L	34
Fasting BG = 223 mg/dL	86 mg/dL
HbA1C = 9.8%	5.1%
T2D = Yes	

Figure 3.11. Case Study: Patient A and Patient B comparison

Table 3.1 Inclusion and Exclusion Criteria

Inclusion Criteria

- Men and Women (pre- and post-menopausal)
- overweight/obese (BMI \geq 25.9 or \leq 40.0 kg/m²)
- characteristics of the metabolic syndrome (fasting glucose 100-125 mg/dL or 2h glucose 140-200 mg/dL) or diabetes type II
- 22-65 years of age
- sedentary (\leq 60 minutes/week of structured physical activity)
- alcohol intake < 20g/d

Exclusion Criteria

- Acute disease or advanced cardiac or renal disease, anticoagulation therapy, or any severe co-morbid condition limiting life expectancy < 1 year,
 - other causes of hepatitis (hepatitis B & C, autoimmune hepatitis, hemochromatosis, celiac disease, Wilson's disease, alpha-1-antitrypsin deficiency, medication induced hepatitis, any clinical or biochemical evidence of decompensated liver disease),
 - use of medications that interfere with lipid, protein, or CHO metabolism (steroids, niacin, etc.) pregnant or trying to become pregnant,
 - alcohol consumption > 20 g/d,
 - inability to exercise on a bike, treadmill or elliptical,
 - contraindications of MRS/MRI.
-

Table 3.2. Phase 1 Subject Characteristics (Mean±SE)

	Overall (n=59)	NAS0-3 (n=8)	NAS≥4 (n=51)	t-test p-value
Age (years)	46±0.1	48±5.8	46±1.5	0.580
Sex (M/F)	21/38	3/5	18/33	-
Weight (kg)	117.1±3.1	115.1±11.3	117.4±3.2	0.800
BMI (kg/m ²)	40.9±1.1	38.0±3.0	41.4±1.1	0.285
-	-	-	-	-
Glucose (mg/dL)	127.2±6.6	100.9±5.3	131.4±7.4	0.112
HbA1C (%)	6.6±0.2	5.6±0.2	6.9±0.3	0.110
AST (U/L)	43.2±3.5	23.3±3.3	*46.3±3.8	0.021
ALT (U/L)	57.5±4.9	30.0±7.0	*61.8±5.3	0.023
-	-	-	-	-
Cholesterol (mg/dL)	168.0±5.2	150.8±18.3	170.7±5.3	0.188
Triglycerides (mg/dL)	178.3±17.1	105.0±10.4	190.0±19.3	0.087
HDLc (mg/dL)	41.3±1.7	44.4±2.6	40.8±1.9	0.464
LDLc (mg/dL)	91.0±4.8	85.4±17.0	91.9±5.0	0.645

*p<0.05 vs NAS0-3

Table 3.3. Phase 2 Subject Characteristics (Mean±SE)

	Active Treatment (n=9)		t-test p-value	Standard Care (n=2)		
	Baseline	Follow-up		Baseline	Follow-up	
Age (years)	45±3	-	-	40±9	-	-
Sex (M/F)	3/6	-	-	1/1	-	-
Weight (kg)	130.4±4.6	118.3±5.4**	0.004	140.8±2.0	129.6±0.5	-
BMI (kg/m²)	44.2±1.8	40.1±1.9**	0.005	44.5±6.1	40.9±4.9	-
Fat Mass (kg)	60.2±5.1	54.5±5.1	0.078	58.9±17.8	59.4±13.8	-
Lean Mass (kg)	65.4±4.3	62.1±2.6	0.194	74.5±16.2	67.9±11.4	-
-	-	-	-	-	-	-
Glucose (mg/dL)	152.0±50.7	119.8±39.9	0.178	152.0±21.0	109.5±36.5	-
HbA1C (%)	7.3±2.6	6.4±0.4	0.072	7.8±1.2	6.9±1.1	-
Insulin (mg/dL)	21.4±4.8	16.2±3.1	0.486	30.7±12.5	13.3±1.8	-
HOMA-IR	21.4±4.8	4.5±0.8	0.341	10.3±1.5	3.8±1.7	-
AST (U/L)	75.3±14.2	26.8±3.3**	0.004	82.0±25.0	29.5±4.5	-
ALT (U/L)	74.4±12.2	32.2±4.3**	0.003	126.0±62.0	43.5±11.5	-
-	-	-	-	-	-	-
Cholesterol (mg/dL)	180.9±23.6	153.3±13.5	0.057	161.5±8.5	153.5±3.5	-
Triglycerides (mg/dL)	172.0±23.6	164.2±40.8	0.989	286.0±139.0	168.0±90.0	-
HDLc (mg/dL)	41.1±3.9	38.3±1.9	0.371	32.5±4.5	32.0±3.0	-
LDLc (mg/dL)	105.3±8.4	82.2±8.0*	0.022	71.8±31.8	87.9±11.5	-

*P<0.05, **P<0.01 Active Treatment Baseline vs Follow-up

Table 3.4. Phase 2 Absolute Δ in Histological Outcomes with absolute Δ in Anthropometrics and Biometric measures

		Δ NAFLD Activity Score	Δ Steatosis	Δ Lobular Inflammation	Δ Hepatocellular Ballooning	Δ Fibrosis
Δ Lean mass (kg)	R	-0.276	0.034	-0.473	-0.060	-0.156
	P	0.44	0.93	0.17	0.87	0.67
Δ AST (U/L)	R	0.155	0.106	-0.049	0.383	0.373
	P	0.65	0.76	0.89	0.24	0.26
Δ ALT (U/L)	R	-0.065	-0.037	-0.312	0.343	0.126
	P	0.85	0.91	0.35	0.30	0.71
Δ Glucose (mg/dL)	R	-0.237	0.113	-0.405	-0.140	-0.112
	P	0.48	0.74	0.22	0.68	0.74
Δ HbA1C (%)	R	0.556	0.150	0.535	0.526	0.311
	P	0.10	0.68	0.11	0.12	0.38
Δ Insulin (mg/dL)	R	-0.372	0.115	-0.569	-0.378	-0.579
	P	0.36	0.79	0.14	0.36	0.13
Δ HOMA-IR	R	0.113	0.327	-0.048	0.018	-0.321
	P	0.79	0.43	0.91	0.97	0.44
Δ Cholesterol (mg/dL)	R	0.751	0.577	0.666	0.376	0.157
	P	0.01	0.08	0.04	0.28	0.66
Δ Triglycerides (mg/dL)	R	-0.395	0.037	-0.418	-0.474	-0.599
	P	0.26	0.92	0.23	0.17	0.07
Δ HDL (mg/dL)	R	0.622	0.285	0.604	0.445	0.094
	P	0.05	0.43	0.06	0.20	0.80
Δ LDL (mg/dL)	R	0.888	0.517	0.816	0.587	0.542
	P	0.00	0.13	0.00	0.07	0.11

Table 3.5 Phase 2 Absolute Δ in FAO correlations with absolute Δ in biometric measures

		Δ Palmitate Oxidation to CO ₂ (nM/g/hr)	Δ Incomplete Fatty Acid Oxidation (nM/g/hr)	Δ Total Fatty Acid Oxidation (nM/g/hr)
Δ NAFLD Activity Score	R	0.693	0.346	0.348
	P	0.06	0.40	0.40
Δ AST (U/L)	R	0.647	0.153	0.157
	P	0.08	0.72	0.71
Δ ALT (U/L) (U/L)	R	0.446	-0.073	-0.069
	P	0.27	0.86	0.87
Δ VO₂ Peak (L/min)	R	-0.740	-0.259	-0.261
	P	0.04	0.54	0.53
Δ Glucose (mg/dL)	R	0.471	0.605	0.607
	P	0.24	0.11	0.11
Δ Insulin (mg/dL)	R	-0.136	-0.274	-0.274
	P	0.77	0.55	0.55
Δ HOMA-IR	R	0.426	0.767	0.766
	P	0.34	0.04	0.04
Δ HbA1C (%)	R	0.511	-0.086	-0.086
	P	0.24	0.85	0.85
Δ Cholesterol (mg/dL)	R	0.622	0.278	0.279
	P	0.14	0.55	0.54
Δ Triglycerides (mg/dL)	R	-0.176	-0.579	-0.577
	P	0.71	0.17	0.18
Δ HDL (mg/dL)	R	0.674	0.467	0.469
	P	0.10	0.29	0.29
Δ LDL (mg/dL)	R	0.515	0.419	0.418
	P	0.24	0.35	0.35

Table 3.6 Individual improvements in histological outcomes at the end of treatment

Subject	A	B	C	D	E	F	G	H	I
Weight Loss (%)	-21%	-17%	-13%	-12%	-7%	-4%	-4%	-3%	-2%
Exercise Sessions Completed	99%	100%	98%	87%	95%	89%	70%	91%	85%
Resolution in Steatohepatitis	Regression	Regression	Regression	Regression	Regression	Regression	Stabilized	Regression	Stabilized
Change in NAS from Baseline	-7	-2	-1	-1	-2	-3	0	-3	0
% change in NAS from baseline	-100%	-33%	-20%	-25%	-29%	-43%	0%	-43%	0%
Δ Steatosis	-2	-1	-1	1	-1	0	0	0	-1
Δ Lobular Inflammation	-3	0	1	-1	0	-1	0	-1	1
Δ Hepatocellular Ballooning	-2	-1	-1	-1	-1	-2	0	-2	0
Fibrosis Status	Regression	Stabilized	Regression	Regression	Stabilized	Regression	Worsened	Regression	Stabilized
Δ Fibrosis	-2	0	-1	-1	0	-3	1	-1	0

Supplementary Table 1. Phase 2 Baseline, Interim, and Follow-up Anthropometric and Biometric Data

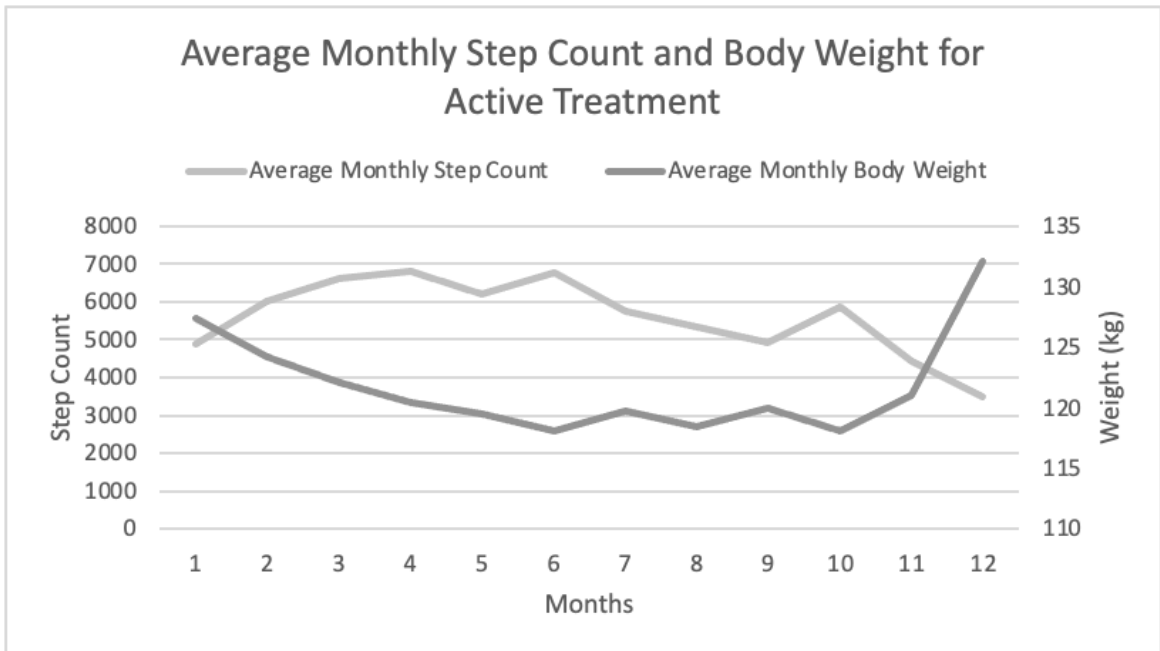
	Active Treatment (n=9)				Standard Care (n=2)			
	Baseline	Interim	Follow-up	ANOVA p-value	Baseline	Interim	Follow-up	
Age (years)	45±3	-	-	-	40±9	-	-	-
Sex (M/F)	3/6	-	-	-	1/1	-	-	-
Weight (kg)	130.4±4.6	117.8±4.6 ****	118.3±5.4 **	0.001	140.8±2.0	133.1±0.4	129.6±0.5	-
BMI (kg/m ²)	44.2±1.8	39.9±1.6***	40.1±1.9**	0.001	44.5±6.1	42.0±5.0	40.9±4.9	-
Fat Mass (%)	44.9±3	53.5±4.8	45.3±9.8	0.255	42.9±12.5	-	45.4±9.8	-
Lean Mass (kg)	65.4±4.3	62.4±3.0	62.1±2.6	0.228	74.5±16.2	-	67.9±11.4	-
AST (U/L)	75±14	27±2.86**	26±3**	0.004	82±25	58±13	29±4	-
ALT (U/L)	74±12	32±**	32±4**	0.002	126±62	88±18	43.5±11.5	-
Glucose (mg/dL)	152.0±50.7	115.7±11.2	119.8±39.9	0.125	152.0±21.0	149.5±59.5	109.5±36.5	-
HbA1C (%)	7.3±2.6	6.0±0.2*	6.4±0.4 [§]	0.053	7.8±1.2	7.6±1.0	6.9±1.1	-
Cholesterol (mg/dL)	180.9±23.6	166.4±12.5	153.3±13.5	0.078	161.5±8.5	183.5±8.5	153.5±3.5	-
Triglycerides (mg/dL)	172.0±23.6	127.8±17.1	164.2±40.8	0.179	286.0±139.0	191.5±35.5	168.0±90.0	-
HDLc (mg/dL)	41.1±3.9	42.8±2.7	38.3±1.9	0.292	32.5±4.5	36.0±5.0	32.0±3.0	-
LDLc (mg/dL)	105.3±8.4	98.1±10.3	82.2±8.0*	0.021	71.8±31.8	109.2±6.4	87.9±11.5	-
Absolute Peak VO₂ (L/min)	2.2±0.1	2.7±0.2**	2.5±0.8*	0.007	2.0±0.4	1.9±0.2	1.9±0.2	-
Relative Peak VO₂ (ml/kg/min)	23.4±2.0	21.7±1.9***	21.7±1.9*	0.003	14.5±2.4	14.2±1.4	14.8±1.4	-
METs	4.9±0.4	6.7±0.6***	6.2±0.5*	0.003	4.2±0.7	4.1±0.4	4.2±0.4	-
Exercise Time to Exhaustion (minutes)	9.7±1.0	11.7±1.0**	11.5±0.9**	0.001	7.1±1.3	8.1±2.0	8.5±1.3	-
Exercise adherence (%)	-	-	91±3	-	-	-	-	-

*P<0.05, **P<0.01 Active Treatment Baseline vs Follow-up § p= 0.07

Supplementary Table 2. Phase 2 Active Treatment Patient Step Counts

Month:	1	2	3	4	5	6	7	8	9	10	11	12	Overall Patient Average	Standard Deviation	Standard Error
Patient A	3036	4849	6359	5803	6353	6364	5742	5524	6177	5485	4210	5096	5416	1003	289
Patient B	2028	2450	3920	6352	7060	7808	7438	7271	7012	6478	6170	-	5817	2045	617
Patient C	6047	8144	7181	9481	7388	6867	6637	7068	8007	9503	-	-	7632	1154	365
Patient D	5342	6588	8586	6936	6519	6609	5328	4774	4798	5170	5560	3007	5768	1401	404
Patient E	6487	6158	6563	6548	5529	6258	5346	5174	5161	4497	-	-	5772	724	229
Patient G	4881	4205	4716	5937	5387	4857	4689	3862	3234	4064	3184	2257	4273	1029	297
Patient F	4057	8119	8569	7984	7319	5662	3970	-	-	-	5115	4403	6133	1869	623
Patient H	8899	9255	9923	8947	6778	9792	7003	7003	2186	-	-	-	7754	2422	807
Patient I	3291	4226	3820	3350	3497	-	-	2047	2754		2439	2605	3114	703	234
Monthly Average	4896	5999	6626	6815	6203	6777	5769	5340	4916	5866	4446	3474			
Standard Deviation	2093	2246	2171	1841	1239	1491	1186	1809	2090	1967	1438	1220			
Standard Error	698	749	724	614	413	527	419	639	739	803	587	545			

Grey cells indicate COVID19 Lockdown months



Supplementary Figure 3.1 Average Monthly Step Count and Body Weight for Active Treatment

Supplementary Table 3.3 Dietary Recall Records at Baseline and Follow-up for Active Treatment Group

	Active Treatment (n=6)		t-test p-value
	Baseline	Follow-up	
Total Kilocalories	2422±199	2091±185	0.218
Carbohydrate (% of kcals/day)	43.8±4.8	33.6±4.6	0.211
Fat (% of kcals/day)	39.0±5.2	49.2±3.4*	0.040
Protein (% of kcals/day)	16.4±0.9	19.2±3.4	0.480
Fiber (grams/day)	22.2±3.6	25.2±5.9	0.680
Sugar (grams/day)	69.8±29.0	28.2±10.5	0.225
Polyunsaturated Fat (grams/day)	22.9±2.9	32.0±6.5	0.285
Monounsaturated Fat (grams/day)	36.2±1.9	41.3±3.8	0.229

*P<0.05 Active Treatment Baseline vs Follow-up

CHAPTER 4 - SUMMARY LIMITATIONS, AND FUTURE DIRECTIONS

SUMMARY

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease in the U.S., and its increasing prevalence comes at a tremendous cost to the individual and the medical system. Hence there is a serious need to understand its progression to the advanced state, nonalcoholic steatohepatitis (NASH). Further, this condition affects >75% of people with overweightness or obesity [173], which is thought to confer risk by causing increasing hepatic nutrient burden and lipotoxicity, leading to the production of reactive oxygen species, lipid peroxidation, and impaired mitochondrial activity. Due to its association with many other life-threatening diseases and lack of approved pharmacological treatments studies investigating the molecular mechanisms of NAFLD are vital. The link between mitochondrial dysfunction and disease progression remains a critically unanswered question in NASH pathophysiology and the present dissertation has attempted to fill this critical gap in knowledge. Firstly, this project interrogated the link between mitochondrial dysfunction and increasing disease severity. Secondly, we have investigated the effects of weight loss on hepatic fatty acid oxidation paired with histopathological outcomes of NAFLD.

Hepatic mitochondrial dysfunction, compromised structure, and quality control is a striking feature of NAFLD/NASH development; to such an extent that NAFLD has been considered a mitochondrial disease [16]. However, the mechanisms behind the decline in hepatic mitochondria during disease progression remains unresolved, particularly in humans. The studies completed here extend previous work by our group and others demonstrating a link between compromised hepatic mitochondrial function with

increasing disease severity. Further, employing weight loss interventions, using caloric restriction and exercise, to assess whether improvements in histological outcomes of NASH in response to weight loss are coupled to changes in hepatic fatty acid oxidation (see **Figure 1.4**).

The first study provided mechanistic insights into the potential role of compromised mitochondrial function and turnover in the etiology of NAFLD/NASH in a human population. We demonstrate a dramatic decline in whole liver and hepatic mitochondria long chain complete fatty acid oxidation and β -HAD activity and markers of mitochondrial biogenesis, autophagy, mitophagy, fission and fusion with increasing liver disease severity (Aim 1). Further, we also demonstrate dramatic decline were observed in whole liver long chain fatty acid oxidation in a clinical NASH population (Aim 2). Decrements in fatty acid oxidation were notable even early in the disease process and persisted in advanced NAFLD and fibrosis. Our group has previously demonstrated that NAFLD onset and progression is associated with a loss in hepatic mitochondrial fatty acid oxidation that even precedes insulin resistance and steatosis in an obese rodent model [20]. Taken together, our data collected simultaneously in the same patient population suggest that long chain fatty acid oxidation and the rate limiting step in β -oxidation are downregulated with increasing liver disease severity. The potential of these reductions playing a causal role in advancement of liver disease highlights possible avenues for therapeutic targets for NAFLD prevention and treatment in the future.

In the current project, mitochondrial respiration did not differ across groups or was upregulated (medium chain fatty acid oxidation) with increasing NAS severity (Aim1). These findings are somewhat in contrast to previous *ex vivo* work by others [42], who found a compensatory upregulation in hepatic mitochondrial respiration (per

mitochondria) in patients with NAFLD and obesity that was lost in patients with NASH. Indeed, the difference between the current findings and other studies is puzzling and further experiments examining changes in mitochondrial respiration and mass in patients with increasing NAFLD activity scores are required.

Increasing mitochondrial ultrastructural abnormalities were noted with greater NAFLD severity in the current study (NAS \geq 5), despite no differences in assessed markers of hepatic mitochondrial content. Further, greater NAFLD severity was paired with a loss in markers of mitochondrial biogenesis, autophagy, and dynamics. Existing literature has either reported no change or an increase in hepatic mitochondrial content with NAFLD/NASH [42, 130], thus further supporting the notion that worsening NAFLD is not likely explained simply by a reduction in mitochondrial content/mass but rather is associated with impaired mitochondrial function and quality control. Worsening mitochondrial morphology may be indicative of increasing bioenergetic or oxidative stress, and perturbations in machinery that regulate mitochondrial biogenesis, mitophagy, fission, and fusion [131]; and has been shown here for the first time in a human population with varying degrees of NAFLD. Further, these perturbations in mitochondrial function likely explain the higher mitochondrial oxidative stress (H_2O_2 emission) observed with advanced NAFLD (NAS \geq 5) and increasing histological lobular inflammation and hepatocellular ballooning. Taken together, the data presented in this dissertation highlight that NAFLD and its more advanced form NASH, is linked to increasing loss of hepatic mitochondrial function, reduced mitochondrial health and elevations in hepatic oxidative stress.

Despite the cross-sectional nature of this Study (Aim 1), it demonstrates through multiple, independent lines of evidence that increasing NAFLD severity corresponded

with a loss in hepatic fatty acid oxidation and an impairment in mitochondrial biogenesis, mitophagy, and dynamics, likely resulting in a stagnant dysfunctional hepatic mitochondria pool. Lower markers of mitochondrial turnover were present prior to reduced mitochondrial fatty acid oxidation and increased reactive oxygen species production, suggesting that these reductions could trigger further deterioration of mitochondria and subsequent NASH. These data collectively highlight a critical and novel role for mitochondrial fatty acid oxidation and turnover in the liver and indicate their potential as a future target for prevention and treatment of NAFLD/NASH in humans.

A major problem in the management of NASH is the lack of effective therapy [149]. Weight reduction through diet and exercise has been promoted as initial therapy for NASH, however, human data examining the mechanisms by which lifestyle modifications reverse histological outcomes of NASH using pre- and post- liver biopsies are limited. This study provides novel evidence that an active treatment phase (caloric restriction + exercise), consisting of weight loss (-9% body weight), resulted in significant reductions in histological outcomes of disease severity in humans with well characterized NASH (NAS \geq 4). Further, favorable reductions in histological outcomes of NAFLD corresponded with a lowering in body fat mass (-8%) and increases in aerobic fitness (17%, increase in absolute VO_2 peak). In line with this, studies assessing the effects of lifestyle modifications on NASH outcomes using liver biopsies pre- and post-intervention demonstrate that dietary caloric restriction improves inflammation and fibrosis [86, 87] and of the patients who lost \geq 10% of their weight, >90% had either resolution of NASH [85, 143], or reduction in NAS of 3-points or more following a 12-month intervention [90].

Intriguingly, improvements in NAFLD activity occurred despite no change in hepatic fatty acid oxidation at follow-up. Previous work has shown that lifestyle modifications such as

caloric restriction and exercise is an effective approach in improving NAFLD outcomes via improved lipid metabolism and mitochondrial function in pre-clinical models [106, 107]. Indeed, the exact mechanisms of caloric restriction and exercise on hepatic outcomes are likely multifaceted. Combining caloric restriction and exercise has been shown to reduce intrahepatic lipid content and improve peripheral and hepatic insulin sensitivity [58-62], thus reducing nutrient flux through the liver and decreasing de novo lipogenesis. However, such studies examining the effects of improved peripheral and hepatic insulin sensitivity on histological outcomes are limited and is currently being investigated by our group. Additionally, we and others have demonstrated that exercise improves hepatic mitochondrial health in the setting of NAFLD, in rodent models [17, 32, 108, 109]. Whether similar improvements in hepatic mitochondrial health and markers of content and quality control are observed in humans and are linked to or independent of changes in long chain fatty acid metabolism, following a lifestyle intervention remain to be explored. Importantly, the results from this project (Aim 2) thus far suggest that lifestyle modifications focusing on weight loss via caloric restriction and exercise can successfully lead to improvements in overall NASH histologic activity, degree of hepatic steatosis and liver chemistry and provides insight into the mechanisms by which the treatments improve liver histology.

LIMITATIONS

The present dissertation has allowed us to translate our findings from animal models to humans, and then uses reverse translation to test hypotheses *in vitro* using human samples. Further this study is the first of its kind to prospectively evaluate lifestyle treatment effects on liver histology paired with measures of hepatic function in patients with NASH. However, there a few limitations.

Study 1 (Aim 1) benefits from the simultaneous assessment of multiple features of mitochondria in intensively phenotyped patients from the same population. However, its cross-sectional nature does not provide conclusions on casual relationships. Furthermore, there is currently no gold standard for relating mitochondrial function to mitochondrial content with previous studies expressing respiration rates either per protein or additionally by citrate synthase activity, a surrogate measure of mitochondrial mass. Thus, differing techniques between labs makes it difficult to directly compare the same outcomes from other studies. Finally, in study 1 we did not control for factors such as patients with established diabetes in study, hence an effect of chronic hyperglycemia on mitochondrial dysfunction, structural abnormalities or markers of quality control cannot be excluded.

Study 2 (Aim 2) benefits from the use of pre- and post- liver histological outcomes paired with functional measures of hepatic metabolism. However, given the small cohort number in the Active Treatment group (n = 9) and Standard Care group (n = 2) the study lacks statistical power to compare each group. Reasons for certain observations such as a normalization in liver enzymes despite still having elevated NAFLD activity scores (NAS = 4) or a lack of change in fatty acid oxidation in the Active Treatment group may be related to changes in eating habits or dietary components, or changes in physical activity that are difficult to quantify and have not been completed here.

FUTURE DIRECTIONS

Targeting mitochondria for treatment of NASH - Indeed, the increasing health care and socioeconomic cost of NAFLD and its more advanced form NASH highlights the demand

for adequate treatment strategies to prevent progression and treat the advanced disease. Improvement of mitochondrial function within NAFLD has come into the focus for NAFLD treatment. Indeed, data generated in this dissertation combining direct measurements of mitochondrial function with gene expression and protein abundance highlight key areas of mitochondrial function and quality control that can potentially be targeted therapeutically. Treatment of hepatic mitochondria impairment in early stages of NAFLD could prevent disease progression to NASH. For instance, increasing mitochondrial biogenesis, mitophagy, protection from uncoupling of mitochondrial respiration with ATP turnover, including reactive oxygen species production and preservation of antioxidant defense, could be major treatment targets. Indeed, we have previously shown that liver-specific PGC-1 α overexpression increases hepatic mitochondrial function and reduces hepatic lipid accumulation *in vivo* and *in vitro* in rats [49]. Alternative approaches could aim to enhance β -oxidation, improve resistance toward increasing availability of fatty acids in NASH, preserve or increase mitochondrial respiration without risk of greater uncoupling of mitochondrial respiration causing reactive oxygen species production leading to more injury. Indeed, this dissertation alongside previous literature provides novel insight into potential therapeutic targets we can test pre-clinically. These examples for modification of mitochondrial function might reflect the broad spectrum of treatment approaches to choose from when attempting to treat NAFLD.

More robust mitochondrial content and turnover measurements - As we have suggested with these studies, impaired mitochondrial biogenesis and a dysfunctional mitochondrial pool is linked to increasing NASH severity. Assessment of mitochondrial content is often based on transmission electron microscopy or quantification of selected biomarkers such as citrate synthase activity. However, transmission electron microscopy is an

inconvenient approach in terms of time-expenditure and cost of analysis when working with large data sets. Further, quantification of selected biomarkers such as citrate synthase activity possess limited utility for capturing changes in mitochondrial content with physiological intervention and for estimating mitochondrial content in certain pathological states. The use of deuterated water for the measurement of protein synthesis has recently been popularized as a potential tool to measure synthesis of mitochondrial protein in humans [174, 175]. The greatest asset of deuterated water labeling is that it facilitates the measurement of synthesis rates over prolonged periods of time from single proteins through integrated tissue-based measurements. Because the ease of administration, the method is amenable for use in a variety of models and conditions. This may be a potential tool to further interrogate the mitochondrial related protein synthesis rates and degradation across the NAFLD spectrum in human liver samples. Further, such a measure could also be used to examine changes in mitochondrial proteins in response to lifestyle intervention.

More robust measurements of autophagic/mitophagic flux - As shown here, impaired markers of autophagy/mitophagy are linked to worsening NAFLD activity scores, but, measuring autophagic/mitophagic flux may be a better approach to assessing perturbations in these processes with increasing disease. Preclinical studies have demonstrated that reductions in autophagic flux are related to metabolic disease. To date, translation of this knowledge has been hampered because there has been no way to directly measure autophagic flux in humans. To overcome this issue a recent study proposed a novel method to measure autophagy processes [176]. The authors detailed a method whereby human macroautophagic/autophagic flux can be directly measured from human blood samples. Whole blood samples can be treated with the lysosomal inhibitor chloroquine, and peripheral blood mononuclear cells isolated from these

samples could be used to measure autophagic machinery (LC3B-II protein a marker of end stage autophagy/mitophagy). Further, these methods could also be used to assess factors that alter autophagic flux in humans, such as weight loss, caloric restriction, and exercise, and better aid their translation in the clinic. With further research, it could also be used as a novel biomarker for risk of disease or assessing disease state. Another approach to measuring autophagic in the human liver may be to culture fresh liver tissue slices obtained using precision cut liver slice methodologies and treat with the lysosomal inhibitor chloroquine or other autophagy/mitophagy reporters.

Future studies – More rigorous studies exploring the effects of caloric restriction and/or exercise, and exercise at weight stability are warranted to further understand the impacts of such lifestyle modifications on hepatic fatty acid oxidation and mitochondrial health in patients with NAFLD. Additionally, such studies should include both moderate intensity aerobic exercise training and high intensity aerobic exercise training groups to explore potential impacts of various modalities of exercise on hepatic fatty acid metabolism. In a similar study design to chapter 3, patients with clinically diagnosed NASH would be recruited for a lifestyle intervention. Participants would undergo the same baseline and follow-up testing as outlined in chapter 3 in addition to been given deuterated water to consume prior to the baseline and follow-up liver biopsy to assess mitochondrial turnover. Blood will be drawn to assess autophagic flux. Participants would then be randomized to one of the following groups: 1) weight loss via caloric restriction alone, 2) weight loss with moderate intensity aerobic exercise training, 3) moderate intensity aerobic exercise training at weight stability, 4) weight loss with high intensity interval exercise training, and 5) high intensity interval exercise training at weight stability.

CHAPTER 5 – EXTENDED LITERATURE REVIEW

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) and its progression from steatosis to steatohepatitis (NASH) is the most prevalent liver disease in the U.S. [1], and is an independent risk factor for cardiovascular, liver-related, and all-cause mortality [7, 112].

Numerous factors are involved in the progression of NAFLD, including changes in lipid metabolism, insulin resistance, inflammatory processes, and oxidative stress [177].

NAFLD has a high incidence rate, with reports in the general population ranging from 10-30% and as high as 80-100% in obesity and morbid obesity [1, 178]. NAFLD progression is the most rapidly increasing indication for liver transplantation in the United States and currently, there are no FDA-approved pharmacological treatments for NAFLD [5]. Indeed, obesity, hyperglycemia, type 2 diabetes and hypertriglyceridemia are important risk factors, while genetic factors undoubtedly predispose to NAFLD, as supported by higher prevalence of steatosis in Hispanics than Caucasians and African-Americans. If left untreated, NAFLD can potentially progress to hepatocellular carcinoma or liver failure, both events that ultimately lead to early death.

DEFINITION AND CHARACTERISTICS OF NAFLD

NAFLD can be defined as excess fat deposition in the liver in which at least $\geq 5\%$ of hepatocytes display lipid droplets [179, 180] in patients who do not consume significant amounts of alcohol - 140 g ethanol/week for men and 70 g ethanol/week for women [181]. Histologically, hepatic steatosis manifests as the accumulation of macrovesicular or microvesicular fat droplets within hepatocytes. Hepatic steatosis is reversible or can progress to NASH. NASH represents steatosis, inflammation, fibrosis, ballooning hepatocytes, apoptotic cells and Mallory's hyaline. Inflammation arises from Kupffer cell activation, resident hepatic macrophages, and the infiltration of peripheral lymphocytes

and leukocytes. Apoptotic ballooned and Mallory hyaline hepatocytes also indicates the onset of NASH. The extent of inflammatory and hepatocellular ballooning varies considerably and does not always correlate with the degree of hepatic steatosis. NAFLD onset and progression is also associated with elevations in specific liver enzymes measured in circulation including elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), with ALT levels being greater than AST levels [182, 183].

HEPATIC METABOLISM

Carbohydrate Metabolism - The liver maintains blood glucose within a narrow concentration range through its ability to store glucose as glycogen and produce glucose via glycogenolysis or gluconeogenesis and is also a major site for insulin clearance [184]. In a normal healthy metabolic state glycogenolysis and gluconeogenesis are stimulated in the fasted state and inhibited in the postprandial state as a result of rapid insulin action [185]. In periods of plasma insulin and glucose elevations, the liver metabolizes glucose via TCA cycle/oxidative phosphorylation, glycogen storage, and *de novo* lipogenesis of free fatty acids. Under normal conditions, excess glucose is directed toward a prolipogenic fate in hepatocytes resulting in the production of free fatty acids (FFA) and triacylglycerols (TAGs), primarily through activation of the sterol regulatory element binding protein-1c (SREBP-1c) pathway and subsequent *de novo* lipogenesis. Considerable evidence from both human [10, 186], and animals models documented the involvement of increased *de novo* lipogenesis in the development of fatty liver disease [20, 31, 43, 187-193].

Hepatic Lipid Metabolism - *Liver mitochondria* represent the major orchestrator of hepatocellular lipid metabolism, since they are the primary site of fatty acid oxidation and

ATP synthesis [194]. Several sources contribute to hepatic levels of free fatty acids including - (a) hydrolysis of intestinal chylomicrons; (b) *de novo* synthesis from acetyl-CoA in the liver; (c) FFA influx into the liver from lipolysis of adipose tissue; (d) diminished export of lipids from the liver; and (e) reduced oxidation of fatty acids [195]. The metabolism of Hepatic free fatty acids in the liver occurs through either mitochondrial β -oxidation or esterification into triglycerides. Hepatic triglycerides in turn either accumulate within hepatocytes as cytoplasmic lipid droplets or are secreted as very low-density lipoprotein (VLDL) particles into blood circulation [195-198]. Hepatic β -oxidation also fuels gluconeogenesis and the synthesis of ketone bodies, 3-hydroxybutyrate and acetoacetate, which are utilized as alternative sources of energy by extrahepatic organs, such as the brain, when blood glucose levels are low.

Fatty acid oxidation occurs in three subcellular organelles, β -oxidation in mitochondria and peroxisomes, and ω -oxidation in the endoplasmic reticulum [199, 200]. Under normal physiologic conditions, mitochondrial β -oxidation is the dominant oxidative pathway for the disposal of fatty acids and is primarily involved in the oxidation of short-chain ($< C_8$), medium-chain (C_8 - C_{12}), and long-chain (C_{12} - C_{20}) fatty acids. While short-chain and medium-chain FFAs can freely enter the mitochondria, long-chain FFAs mitochondrial entry is regulated by the activity of the enzyme carnitine palmitoyl transferase-I (CPT-I). CPT-I mediates the conversion of acyl-CoA into acyl-carnitine, allowing for its transport into the mitochondrial matrix. Subsequent β -oxidation produces an acetyl-CoA, which can enter the TCA cycle, resulting in production of NADH to drive oxidative phosphorylation or can be converted to ketone bodies for nonoxidative disposal.

Glycolysis results in the production of pyruvate, which is then transformed into acetyl-CoA in the mitochondria which is then cycled through the citric acid cycle for the production of reducing agents and ATP. In the presence of high glucose and energy levels, acetyl-CoA is converted to citrate which can be transported out of the mitochondrial matrix into the cytosol via the tricarboxylate carrier where it is regenerated to acetyl-CoA and then converted to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA is the initial constituent for fatty acid synthesis and is an important regulatory factor in hepatic fatty acid oxidation and lipid synthesis. During periods of caloric excess energy supply, high malonyl-CoA levels reduce fatty acid oxidation by reducing the rate of fatty acid entry into the mitochondria via CPT-I inhibition. Conversely, in the fasted state, hepatic malonyl-CoA levels are low, allowing extensive mitochondrial import of long-chain FFAs and high rates of β -oxidation. During the process of mitochondrial β -oxidation, FFAs undergo a dehydrogenation, followed by hydration, a second dehydrogenation, and finally thiolytic cleavage, resulting in the release of one 2-carbon acetyl-CoA molecule and a shortened fatty acid. Successive cycles of β -oxidation split the fatty acid into acetyl-CoA subunits which then enter the citric acid cycle to produce reducing agents which are ultimately converted to ATP in the electron transport chain. Under conditions of fasting, after undergoing β -oxidation, acetyl-CoA moieties can be converted into ketone bodies (acetoacetate and β -hydroxybutyrate) via the ketogenic pathway, which are exported from the liver to be oxidized in peripheral tissues for energy.

MITOCHONDRIAL STRUCTURE

Serving as the cellular powerhouse, mitochondria generate ATP or heat from substrates derived from fat and glucose. Hepatocytes are rich in mitochondria with each containing up to 800 mitochondria per cell and occupying ~18% of the hepatocellular space. Given that mitochondria are the primary site for fatty acid oxidation and oxidative

phosphorylation, hepatic mitochondria play a central role in the maintenance of hepatic and whole-body metabolism. A mitochondrion contains an inner and outer membrane composed of phospholipid bilayers and proteins, which regulates the import and export of metabolites. The highly impermeable inner mitochondrial membrane also contains proteins involved in the oxidative reactions of the electron transport chain, ATP synthase, and transport proteins that regulate the passage of metabolites into the mitochondrial matrix. The inner mitochondrial matrix is highly concentrated with enzymes required for pyruvate oxidation, fatty acid oxidation and the citric acid cycle. The matrix also contains mitochondrial DNA (mtDNA), which encode for some of the respiratory chain polypeptides. Due to its close proximity to the inner membrane, the absence of protective histones, and the incomplete DNA repair mechanisms, mtDNA is extremely sensitive to oxidative damage. Hence, factors that damage or cause harm to mitochondrial integrity will impact mtDNA and cause reduced mitochondrial function. As mitochondria play an important role in energy supply, fat and glucose metabolism, antioxidant defense, calcium homeostasis, and apoptosis, mitochondrial function is critical to cellular physiology [201].

ENERGY HOMEOSTASIS

Fuel oxidation into subunits of acetyl-CoA and its subsequent oxidation by the TCA cycle results in the reduction of oxidized coenzymes, NADH and FADH₂, which are in turn re-oxidized by the mitochondrial respiratory chain [194, 198]. During their re-oxidation, NADH and FADH₂ donate their electrons to the polypeptide complexes of the mitochondrial respiratory chain. Electron transfer down the respiratory chain couples with a transfer of protons from the mitochondrial matrix to the intermembrane space, thus creating an electrochemical gradient across the inner mitochondrial membrane, acting as an energy reservoir. Re-entry of protons to the matrix via ATP synthase

(complex V), results in the production of ATP. Mitochondrial ATP is then exchanged for cytosolic ADP, and cytoplasmic ATP is used to power hepatocellular energy-requiring metabolic processes [194].

REACTIVE OXYGEN SPECIES

Mitochondria are a major site for reactive oxygen species (ROS) generation, accounting for about 90% of cellular ROS. The excessive flow of electrons down the electron transport chain can result in the production of ROS [194, 202]. Under normal metabolic conditions, about 1%–2% of mitochondrial oxygen consumption results in ROS production [203]. However, excessive ROS production beyond the cell's antioxidant capacity can result in damage to components of the cell including lipids, proteins, and nucleic acids leading to oxidative stress and subsequent apoptosis. Increased ROS production and oxidative stress is observed in steatohepatitis in the setting of increased energy availability [204].

MECHANISMS OF NAFLD DEVELOPMENT

Despite its alarming prevalence, the pathophysiology of NAFLD progression is not well understood. The mechanisms of NAFLD and NASH development are complex and multifactorial. Currently, NAFLD/NASH is understood to develop through a 'multiple-parallel hits' hypothesis: consisting of a number of insults acting together to induce disease [9]. Such hits include the interaction between dietary, environmental, and genetic factors as well as inter-organ cross talk all contribute to the pathogenesis of NAFLD. Loss of insulin sensitivity at the level of the adipose tissue disrupts insulin-mediated suppression of lipolysis, resulting in circulating FFAs being taken up ectopically by the liver. These FFAs challenge the liver to enhance esterification of TAG, increase oxidation, or both. Tracer approaches in human patients with hepatic steatosis

(measured in postabsorptive state) revealed that ~60% of intrahepatic lipids were derived from circulating FFAs emerging from adipose depots via lipolysis [10].

At the same time, hepatic insulin resistance disrupts the insulin-stimulated suppression of hepatic glucose output. This combination of hepatic and adipose tissue insulin resistance results in elevated serum insulin and glucose - potent stimulators of *de novo* lipogenesis. Up to 25% of hepatic triglyceride content can be attributed to *de novo* lipogenesis in NAFLD in humans [10], while other sources that contribute liver triglyceride accumulation include the dietary fat and adipose. Indeed, excess energy balance, hormonal, and substrate-driven systemic factors (increasing levels of TAGs and glucose) push susceptibility for increased hepatic steatosis and lipotoxicity that can ultimately drive NAFLD/NASH. Hepatic-specific cellular mechanisms also likely impact risk, promoting the activation of hepatic pro-inflammatory pathways. Additionally, dysbiosis of the gut microbiome has been linked with NAFLD development and progression [11, 12], with gut-derived endotoxins and altered bile acids further exacerbating hepatic inflammation [13]. The mechanisms behind NAFLD development have not been fully elucidated, though convincing evidence suggests that hepatic mitochondrial dysfunction is tightly linked to disease progression [14-16]. Strong evidence has implicated a loss in mitochondrial function in more advanced NASH with rodent data from our lab [17-21] and others [14, 22-25], and data from human studies [26-28] to support this.

HEPATIC MITOCHONDRIAL DYSFUNCTION AND NAFLD

The hallmarks of hepatic mitochondrial dysfunction in the setting of NAFLD/NASH progression include decreased electron transport chain content, abnormal morphology, and compromised respiration and β -oxidation. Additionally, increasing hepatic steatosis

and loss in mitochondrial function is coupled with the generation of ROS and inflammatory cytokine production, further exacerbating NAFLD/NASH pathology [33]. The magnitude to which hepatic mitochondrial fatty acid oxidation can increase in response to greater lipid availability in the hepatocyte may play a role. Current literature in both rodent models and humans using *in vivo* and *ex vivo* approaches demonstrate that hepatic steatosis, induced by obesity and high-fat diet feeding contributes to compensatory increases in measures of mitochondrial oxidative capacity including tricarboxylic acid cycle flux, fatty acid oxidation, and mitochondrial respiratory capacity [42, 128, 205]. Conversely, we have shown that a decline in *ex vivo* hepatic mitochondrial long chain fatty acid is linked to NAFLD development in rodent models [15, 43]. Whether a similar decline is present in humans with NAFLD is less clear. Indirect measures of fatty acid oxidation in the liver of patients with NAFLD, such as plasma ketone body concentrations, suggest that hepatic fatty acid oxidation is either normal or increased [44-46]. However, given their unstable nature, ketone body concentrations are an unreliable marker of hepatic fatty acid oxidation. Further, rodents and humans with hepatic steatosis have shown that ketogenesis is increased initially in high-fat diet-induced hepatic steatosis [129, 205, 206]. However, this diet-induced upregulation in ketogenesis transitions and becomes suppressed with prolonged high-fat feeding [205, 206]. Indeed, recent human data show that patients with elevated intrahepatic lipid content have lower hepatic ketogenic capacity [129], thus linking ketogenic insufficiency to worsening hepatic steatosis and metabolic dysfunction [129, 206, 207]. Despite increases in mitochondrial structural and functional abnormalities being clearly implicated in exacerbating disease progression [15, 34, 35], there are a lack of studies employing direct approaches to assess changes in hepatic fatty acid oxidation in patients with NAFLD/NASH. Thus, such studies are strongly warranted.

Patients with NASH have been shown to present with abnormal mitochondrial structure and alterations in hepatic respiration [14, 42, 126]. In a recent study by Kolaki et al [42], the authors interrogated hepatic mitochondrial function *ex vivo* using liver biopsies from patients with obesity, NAFLD or NASH versus lean controls [42]. The findings demonstrated that mitochondrial respiration initially increased in a graded fashion with obesity and NAFLD compared to lean subjects. However, with the progression to more advanced NASH mitochondrial function and markers of mitochondrial biogenesis declined and was coupled to increases in hepatic oxidative stress. Indeed, it is clear that there is a link between increasing mitochondrial fatty acid oxidation dysfunction and NAFLD/NASH pathophysiology and, it is essential that this be confirmed in a human population which will add significant clarification to the relationships between hepatic long-chain fatty acid oxidation and disease severity (Aim 1a)

MITOCHONDRIAL TURNOVER AND QUALITY CONTROL

Factors regulating Mitochondrial Content and Quality - Mitochondrial health and quality control hinges on several intimately linked processes including mitochondrial biogenesis, dynamics, and autophagy [47]. Increasing evidence suggests that mitochondrial biogenesis and mitophagy play an important role in maintaining overall hepatic and hepatic mitochondrial health, and losses in their function may therefore play a role in NAFLD [208, 209].

Autophagy - Autophagy is a highly conserved process across all eukaryotic organisms from yeast to humans which involves the self-degradation of damaged and/or superfluous cellular components. This process occurs in order to eliminate deleterious material (e.g., damaged organelles and microbial invaders) or because the resulting breakdown products are needed to support metabolism and survive. The word stems

from the Greek “auto” meaning oneself, and “phagy” meaning to eat. Autophagy function/dysfunction has gained attention as an important contributor to health and disease. There are several forms of autophagy, each resulting the delivery of intracellular cargo to the lysosome for degradation. Macroautophagy (referred to as autophagy hereafter), involves the production of vesicles called autophagosomes that capture and deliver cytoplasmic material to lysosomes [210, 211]. In the absence of stress, basal autophagy serves a housekeeping function. It is the only mechanism to degrade large structures such as organelles and protein aggregates, preventing toxicity from a buildup of damaged proteins. In starvation, autophagic processes provide a nutrient source to sustain survival. Autophagy can also be induced by a broad range of stressors resulting in the subsequent degradation of protein aggregates, oxidized lipids, damaged organelles, and even intracellular pathogens. Indeed, autophagy plays an important role in maintaining cellular homeostasis and any deficiency in autophagy can lead to deleterious effects in cellular and systemic health. Defects in autophagy have been linked to a myriad of diseases; neurodegenerative diseases [212], cancer [213], aging [214], and obesity and metabolism related disorders [211], including NAFLD, NASH, and liver cancer [208, 215].

There are several forms of autophagy, each resulting in the delivery of intracellular cargo to the lysosome for degradation. Macroautophagy, involves the production of double membraned vesicles called autophagosomes that capture and deliver cytoplasmic material to lysosomes for enzymatic degradation [210]. Microautophagy relates to the direct engulfment of cytoplasmic content by the lysosome without the requirement of autophagosome formation. Chaperone-mediated autophagy requires chaperone-dependent selection of soluble cytosolic proteins that targeted to the lysosome and translocated across the lysosomal membrane for subsequent degradation.

Macroautophagy (referred to as autophagy hereafter), is comprised of selective and non-selective autophagy. Selective autophagy serves as a quality control process in the cell targeting an array of different organelles for degradation such as ribosomes (ribophagy), lipids (lipophagy), glycogen (glycophagy), and mitochondria (mitophagy), among others. Non-selective autophagy occurs during times of extreme nutrient deprivation to supply the cell of metabolic building blocks and energy until homeostasis is reached [216].

Regulation of Autophagy - Autophagy occurs in basal conditions to serve as a homeostatic function for cells but is also significantly induced in certain physiological states (starvation, intracellular remodeling, etc.) when required. In pathological conditions where cellular debris/organelles require removal, such as oxidative stress, infection or protein aggregate accumulation, also promotes the upregulation of autophagy. Other factors including temperature, acidity, oxygen concentrations, hormonal factors, and cellular density also play intricate roles in autophagy regulation and have been discussed elsewhere [211, 217].

Mechanisms of Autophagy - Autophagic mechanisms gives rise to nascent autophagosome membranes, which subsequently these membranes assemble around and encapsulating cargo in a vesicle that later fuses with a lysosome, generating an autolysosome. The lysosomal contents are then degraded by proteases, lipases, nucleases, and glycosidases. The breakdown products—amino acids, lipids, nucleosides, and carbohydrates— are released by lysosomal permeases into the cytosol, where they are available for synthetic and metabolic pathways. A series of protein complexes composed of AuTophagy (atg) regulatory proteins coordinate the formation of the autophagosome. In the setting of nutrient abundance, the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibits autophagy. mTORC1 is a

multi-complex protein that phosphorylates ULK1 to maintain autophagy inhibition. The Atg1/ULK1 complex (Atg1 in yeast and ULK1 in mammals) is an essential positive regulator of autophagosome formation [210]. During periods of starvation, mTORC1 dissociates from the ULK1 complex, freeing it to trigger autophagosome nucleation and elongation. Autophagosome nucleation requires a complex containing Beclin 1 (Atg6), that recruits the class III phosphatidylinositol 3-kinase VPS34 to generate phosphatidylinositol 3-phosphate [218]. Subsequent autophagosome membrane expansion requires two ubiquitin-like molecules, ATG12 and LC3, and two associated conjugation systems. Atg7 and Atg10 covalently links Atg12 with Atg5, which together bind Atg16 to form pre-autophagosomal structures. In the second ubiquitin-like reaction, cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II) and requires Atg7 and Atg3. This lipidated LC3-II is then remains on mature autophagosomes to act as a 'docking station' for organelles targeted for degradation until after fusion with lysosomes and is commonly used to monitor autophagy.

Mitophagy - In an effort to maintain mitochondrial homeostasis and quality in cells, the evolutionarily conserved degradative process of mitophagy is invoked. The mitophagic process removes damaged and dysfunctional mitochondria in order to regenerate the mitochondrial pool and preserve energy production [219, 220]. Several external stimuli, including hypoxia, nutrient deprivation, and mitochondrial uncoupling, have been demonstrated to trigger stress-induced mitophagy to promote the clearance of dysfunctional mitochondria [221-226]. Conversely, deregulation of mitophagy has been shown to impair the synthesis of new healthy mitochondria and leads to the accumulation of defective mitochondria. Further, compromised mitophagy has been implicated in the pathogenesis of a wide spectrum of human diseases such as cancer,

neurodegenerative diseases, cardiovascular diseases, tissue injury, metabolic disorders, and autoimmune diseases [227]. Hence, modulation of mitophagy has emerged as a potential therapeutic for developing new treatment strategies to combat human diseases (reviewed elsewhere) [227].

The phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1)–Parkin pathway is the regulator for ubiquitin-dependent mitophagy [118]. During mitochondrial injury/loss of membrane potential, PINK1 is cleaved from the inner mitochondrial membrane and is stabilized on the outer mitochondrial membrane. Its auto-phosphorylation results in cytosolic Parkin translocating to the mitochondrial surface, triggering and Parkin's E3 ligase activity and causes poly-ubiquitination. These poly-ubiquitinated chains are phosphorylated by PINK1 and serves as signal for these dysfunctional mitochondria to be targeted by autophagy proteins associated with the autophagosome. These adapter proteins, such as p62, recognize these ubiquitinated mitochondria and facilitate their binding to the autophagosome through LC3. Additionally, several LC3 receptors are located on the mitochondria and can directly bind to LC3 ultimately resulting in the recruitment of damaged mitochondria to the autophagosome. Nip3-like protein X (NIX), BCL2/Adenovirus E1B 19 kDa Interacting Protein 3 (BNIP3), and FUN14 domain-containing protein 1 (Fundc1) are proteins present on the outer mitochondrial membrane that can promote Parkin-independent mitophagy in mammalian cells by binding to LC3 [228-233]. There are also examples of crosstalk between these pathways, as BNIP3 helps stabilize PINK1 to the outer mitochondrial membrane during loss of membrane potential [118].

Mitochondrial Biogenesis - The multistep process of mitochondrial biogenesis requires the coordinated transcription of both mitochondrial and nuclear-originated transcripts and

recruitment of newly synthesized proteins and lipids. There are several known regulators of mitochondrial biogenesis including - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), adenosine monophosphate (AMP)-activated protein kinase (AMPK), Sirtuin (SIRT)1 and SIRT3, endothelial nitric oxide synthase (eNOS), nuclear receptor factors (NRFs), mitochondrial transcription factor A (TFAM). In particular, PGC-1 α plays an integral role in the regulation of mitochondrial biogenesis by coordinating the activity NRFs and estrogen-related receptors (ERRs) [234]. NRF1 and NRF2 regulate the transcription of mitochondrial transcription factor A (TFAM) and transcription factor B proteins (TFBs) which subsequently mitochondrial DNA transcription and replication. *PGC-1 α* expression has been linked to *improvements* in mitochondrial respiration suggesting a major role for PGC-1 α in mitochondrial biogenesis and function [235]. PGC-1 α itself is the subject of modulations through several metabolic sensors including AMPK, SIRT1, and SIRT3. AMPK senses cellular energy demands and also promotes SIRT1 activity by increasing cellular NAD⁺ levels. SIRT1 deacetylates PGC-1 α and promotes mitochondrial content and oxidative metabolism. Increased *SIRT3* expression is important for PGC-1 α mediated induction of mitochondrial biogenesis, while its deletion inhibits PGC-1 α -induced mitochondrial biogenesis [236]. Additionally, deletion of PGC-1 α has been linked to reduced expression of SIRT3 and reductions in mitochondrial biogenesis [236, 237]. Other factors that can also modulate PGC-1 α activity and subsequent mitochondrial biogenesis includes cytosolic calcium concentration through the activation of p38 mitogen-activated kinase and calcium/calmodulin-dependent kinase (CaMK) [238, 239].

Mitochondrial Dynamics - Mitochondria are a dynamic pool of organelles that change their shape and structure constantly in response to changes in energy demand and supply using fusion and fission processes. Changes these fission and fusion process

are associated with cell viability, apoptosis, and bioenergetic adaptations [240]. When mitochondria are subjected to damage due to factors such as oxidative stress, fission occurs in order to separate damaged mitochondria from healthy mitochondria. These damaged mitochondria and mitochondrial fragments are then tagged for mitophagy. During the last decade, we have gained significant insights into the molecular basis of mitochondrial dynamics in relation to several biological processes such as apoptosis, autophagy, metabolism, development, and aging. Mammalian orthologs of key proteins involved in mitochondrial fission include mitochondrial fission 1 (Fis1), dynamin-related protein 1 (DRP1), mitochondrial fission factor (MFF), mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MiD51) [241-244].

In particular, activated DRP1 oligomerizes at the fission sites of the outer mitochondrial membrane after being recruited from the cytosol, it interacts with other mitochondrial-resident proteins to drive the fission process before returning back to the cytosol upon completion of the fission process [245-249]. Other proteins including MFF, Fis1, Mid49, and Mid51 have been shown to contribute to Drp1 recruitment and oligomerization at the outer mitochondrial membrane [250-255]. Dysregulation of the proteins involved in mitochondrial fission have been demonstrated to significantly alter the mitochondrial morphology ultimately impairing mitochondrial function [256, 257].

In mammalian cells, Mitochondrial fusion is primarily mediated by mitofusin-1 (MFN1) and mitofusin-2 (MFN2) [258-260]. MFN1 tethers opposing mitochondria, while MFN2 oligomerizes with either other Mfn2 proteins or with Mfn1 proteins to promote mitochondrial fusion. The fusion of the inner mitochondrial membrane is regulated by optic atrophy 1 (Opa1), which plays a critical role in maintaining the balance between mitochondrial fusion and fission [261]. The inhibition of mitochondrial has been shown to

induce severe growth defects accompanied by changes in the mitochondrial membrane potential and decreased respiration [262, 263]. Fusion allows for the restoration of mitochondrial function by joining damaged mitochondria with oxidized lipids, proteins, and mutant mitochondrial DNA, and aberrant mitochondrial membrane potential with healthy ones. This process restores mitochondrial functions and maintain cellular homeostasis. However, mitochondria that have suffered significant loss of membrane potential do not fuse and are subsequently tagged for mitophagy [264-266].

Perturbations in Mitochondrial Quality Control are linked to NAFLD - Perturbations in mitochondrial quality control are highly linked with mitochondrial dysfunction in a number of tissues, including the liver. PGC-1 α , the master regulator of mitochondrial biogenesis, is reduced in patients with obesity and NASH [42, 48]. Further, our group has previously shown that hepatic PGC-1 α overexpression in rats, increases hepatic mitochondrial function and reduces steatosis *in vivo* and *in vitro* [17, 49].

Mice with liver specific deletion of PGC1a present with hepatic steatosis due at least in part to impaired mitochondrial oxidative capacity and mitochondrial dysfunction Further, our group has previously shown that hepatic PGC-1 α overexpression in rats, increases hepatic mitochondrial function and reduces steatosis *in vivo* and *in vitro* [17, 49].

Similarly, high-fat diet feeding in sedentary WT mice reduces hepatic PGC-1 α levels resulting in reduced hepatic mitochondrial respiration [267]. PGC-1 α overexpression in rat primary hepatocytes results in an increase in markers of mitochondrial content and function (citrate synthase, mitochondrial DNA, and electron transport system complex proteins) and an increase in fatty acid oxidation [49]. Voluntary running wheel exercise also increases hepatic PGC-1 α levels in rat models [113].

As highlighted previously, mitochondrial health is also dependent on mitophagy - the turnover and degradation of low functioning mitochondria in the lysosome [50]. Impairments in autophagy have been linked to NAFLD in both human and rodent models. Genetic ablation of ATG proteins necessary for autophagy have been linked to elevations in hepatic steatosis [51-53], depletion of hepatic ATP production, and trigger mitochondrial apoptotic signaling [54, 55]. Further, rodent models of obesity induced NAFLD have been shown to exhibit reduced hepatic protein content and gene expression of markers of autophagy including cleaved microtubule-associated protein 1A/1B-light chain 3 (LC3), Beclin 1, Atg5/Atg12, and Atg7. However, little is known about the role of mitophagy compared with global macro-autophagy in the regulation of hepatic lipid metabolism, particularly in humans. High-fat diet fed mice with NAFLD presented with lower expression of PINK1 and Parkin compared to control mice, which was associated with activation of the mitochondria-related apoptotic pathway and mPTP opening [268]. BCL-2/adenovirus E1B 19-kDa interacting protein (BNIP3) is a highly conserved mitophagy protein in the liver and is a well-established critical regulator of receptor-mediated mitophagy. Loss of hepatic BNIP3 is thought to result in increased mitochondrial mass within the cell and is associated with reduced membrane potential, reduced mitochondrial respiration, increased H₂O₂ emission, defective mitochondrial β -oxidation capacity, and hepatic steatosis [56] inflammation, and steatohepatitis-like features [56]. These results suggest that BNIP3-related mitophagic processes are required to maintain mitochondrial integrity in the liver via reductions in mitochondrial mass, and also plays a significant role in the regulation of lipid metabolism.

Fission (DRP1 and FIS1) have been shown to be reduced in the Western diet induced NASH and is accompanied by hepatic inflammation and liver fibrosis [269]. Conversely, greater uncontrolled mitochondrial fission triggers hepatic fibrosis and liver inflammation

resulting in increased hepatocyte death through the caspase-9-related apoptotic pathway [270]. Mitochondrial fission was shown to be abolished in response to nutrient stimuli in mice fed a high fat diet to induce NASH, through the down regulation of Drp1 at Ser616 and BNIP3 in the hepatocytes which may cause defects in mitochondrial quality control [271]. Furthermore, mitochondrial fission has been shown to play an important role in inducing mitophagy in the liver hepatocytes and other cell types [264, 272]. However, excessive mitochondrial fission triggers hepatocyte death by inducing mitochondrial damage. Therefore, the relationship between mitochondrial fission and mitophagy in NAFLD needs to be further investigated.

In relation to fusion processes, reduced MFN2 levels have been observed in the liver biopsies from patients with NASH and in the mouse models of steatosis and NASH. Liver-specific deletion of Mfn2 induces inflammation, triglyceride accumulation, fibrosis, and liver cancer whereas Mfn2 re-expression ameliorates the disease in the NASH model mice [273-275].

Indeed, this inverse relationship between Mfn2 and fatty liver disease progression suggests that mitochondrial fusion is involved in the pathophysiology of fatty liver disorders. Further, mice fed a high-fat diet also show reduced expression of Opa1 [276]. Hepatic-specific ablation of Opa1 increases the risk of HFD-induced NAFLD. These molecular alterations are associated with lipid accumulation, decreased gluconeogenesis, and extensive liver damage. However, re-restoration of Opa1 via adenovirus delivery restores the function of mitochondria in the hepatocytes and protects against NAFLD [277]. Re-activation of mitochondrial fusion protects against fatty liver disease. Exercise counteracts NASH by improving Mfn1 and Mfn2 expression, which

promotes mitochondrial fusion and helps maintain mitochondrial homeostasis in the high-fat diet fed Sprague-Dawley rats [268].

THERAPEUTIC APPROACHES TO TREAT NAFLD

As previously highlighted NASH, the severe form of NAFLD, is now one of the most common reasons for liver transplants, in the United States. While NASH therapies may be on the horizon, there is an urgent need to better understand the pathology of NAFLD/NASH and how to treat it. Currently, there are no proven pharmacological therapies for the effective treatment of NAFLD. Excess energy intake, overweightness, obesity and related comorbidities is a leading risk factor for NAFLD [173]. There is a consensus that gradual weight reduction achieved by caloric restriction, with or without increased physical activity [278], leads to an improvement in serum liver enzymes, liver fat, degree of hepatic inflammation and fibrosis and benefits the underlying risk factors like diabetes, hyperlipidemia, obesity and other comorbidities [143].

The American Association for the study of Liver Disease and the European Association for the Study of the Liver Clinical Practice Guidelines recommend that in overweight/obese NAFLD patients, a 7–10% weight loss is the target of most lifestyle interventions, and results in an improvement of liver enzymes and histology [1, 278]. To date, only a few studies have evaluated the impact of lifestyle modifications on NAFLD. Reductions in intrahepatic lipid content and improvements in histological NAFL/NASH outcomes, have been shown to result from caloric restriction [87, 90, 151, 279], and from exercise training, both with weight loss [62, 70, 71, 73] and exercise training at weight stability [77]. Further combining both caloric restriction and exercise training regimens results in the greatest reductions in intrahepatic lipid content and improvements in substrate metabolism [90, 280]. The majority of studies conclude that at least 7–10% of

weight loss is required to induce an improvement in NAFLD activity score and its components (steatosis, lobular inflammation and ballooning) [8]. However, lifestyle changes that produce even modest, sustained weight loss of about ~3-5% of initial body weight, can reduce steatosis, [82] liver enzymes [281] and produce clinically meaningful reductions in triglycerides, blood glucose, haemoglobin A1c, and the risk of developing type 2 diabetes [282].

In a small-randomized controlled trial, Promrat *et al.*, examined the effects of a lifestyle intervention via a combination of diet, exercise, and behavior modification on histological parameters of NASH [90]. At 48 weeks, 67% of those in the lifestyle intervention group had significant improvements in NAFLD activity scores. These improvements included reductions in steatosis, lobular inflammation, and ballooning in the intervention group; but results showed no improvement in fibrosis in either group. In contrast, a clinical retrospective study in patients who lost weight from a variety of means (diet, bariatric surgery) study demonstrated higher rates of fibrosis regression in NASH patients who lost 10% or more of total body weight, compared to those who lost < 10% (63% vs. 9%) [283]. A large prospective study by Vilar-Gomez *et al.*, investigated the impact of lifestyle changes through a hypocaloric diet combined with exercise on histological features of NASH during routine clinical practice over 12-months [143]. Specifically, the authors demonstrated a dose-response relationship with degree of weight loss and improvements in NAFLD activity score. Weight reductions of $\geq 10\%$ of their weight, resulted in resolution of NASH in >90% of patients and improvements in fibrosis by at least 1 point in 81% of patients. Similar to lifestyle interventions, weight loss induced by drugs such as orlistat or bariatric surgery appear to have the same positive impact on NASH resolution and fibrosis regression [86, 284].

Mechanisms by which weight loss improves, caloric restriction, and exercise

reverses NAFLD - Indeed, the overwhelming evidence that improvements in energy balance reduce liver lipid supports a consensus on the benefits of lifestyle interventions to prevent the development of NAFLD [91] and to treat the condition [97, 285]. While these studies suggest potential clinical utility for histological outcomes, the mechanisms by which the treatments improve liver histology remains poorly understood, and when discovered, this knowledge can support the development of new therapies for this disease.

There are a handful of studies coupling non-invasive approaches to assess hepatic and metabolic outcomes, particularly insulin resistance and free fatty acid uptake, in response to weight loss using caloric restriction alone and in combination with exercise. These studies have shown a correlation of weight loss and improved metabolic health with decreased intrahepatic lipid content following a hypocaloric diet and/or exercise [286-290]. Reduced intrahepatic lipid content was associated with decreased hepatic FFA uptake as well as an improvement in insulin mediated suppression of hepatic glucose production and skeletal muscle insulin sensitivity [286, 289, 290]. More specifically, weight loss induced by a low-calorie diet resulted in a 32% improvement in whole-body insulin sensitivity and 60% decrease in hepatic insulin resistance on average in patients with obesity. Further, these improvements were paired with an 11% reduction in liver volume, 60% decrease in liver triglyceride content as determined by magnetic resonance studies [286]. In a study by Kirk *et al.* [291], subjects underwent MRS and hyperinsulinemic euglycemic clamps to determine insulin sensitivity in liver and skeletal muscle before and after subjects achieved 7% using calorie restriction (either low fat or low carbohydrate). Both calorie restricted diets (low-fat versus low-carbohydrate) lead to equivalent reductions in intrahepatic lipid content. Insulin resistance (HOMA-IR), hepatic

insulin sensitivity index and fasting serum insulin concentrations were also improved in both groups but showed more of an improvement in the low carbohydrate group.

Studies combining both exercise and dietary caloric restriction make it difficult to ascertain whether exercise modifies hepatic outcomes through altering energy balance or through systemic or hepatic-specific metabolic mechanisms. Indeed, we and others have shown that exercise with weight loss and at weight stability produce beneficial effects on hepatic outcomes. Specifically, we have recently shown that 4 weeks of moderate-intensity continuous training or high-intensity interval training is effective in reducing intrahepatic lipids independent of changes in abdominal adiposity or body mass [105]. High-intensity exercise (80% VO_{2peak}) lowered intrahepatic lipids by 37% compared with only a 20% reduction with moderate-intensity exercise (55% VO_{2peak}). the majority of studies performed in participants with clinically defined hepatic steatosis (>5.5%, quantified by MRI/MRS) have shown that aerobic and resistance exercise training without weight loss is effective in reducing intrahepatic lipids by 20–40% (see excellent reviews [292-295]).

Exercise is a powerful tool that increases whole body insulin sensitivity via increased skeletal muscle glucose uptake, inhibition of adipose tissue lipolysis, reduced hepatic glucose output. Further, exercise can also reduce hepatic lipid synthesis pathways via improvements in fasting and fed insulin homeostasis. Together, these systemic benefits reduce excess nutrient delivery to the liver which can contribute to lipid synthesis and hepatic steatosis. Furthermore, one of the most consistent effects of aerobic exercise is to promote the downregulation of genes and proteins in the *de novo* lipogenesis pathway [108]. However, whether similar effects are observed in humans are unknown. Further, it is unknown whether these effects are solely due to insulin or due to exercise-specific

signaling within the liver. Indeed, there are a number of studies in humans demonstrating beneficial effects of exercise on hepatic and whole-body insulin sensitivity and glucose homeostasis [59-61]. In a well-controlled study, Coker et al compared the effects of exercise training at weight stability, exercise training with weight loss, and weight loss via dietary caloric restriction only on hepatic and peripheral insulin sensitivity in individuals with obesity. Both weight loss groups lost similar amounts of body weight (~6% each). Interestingly, the improvements in insulin-stimulated glucose disposal were similar between the caloric restriction and exercise-induced weight loss groups, but not the exercise at weight stability group. Furthermore, significant insulin-stimulated suppression of glucose production was observed in the exercise group ($+12 \pm 2\%$), caloric restriction group ($+10 \pm 2\%$) and 3 times greater in the exercise-induced weight loss group ($+27 \pm 2\%$) [296]. The findings from this study illustrates Exercise-induced weight loss in this context produced the most benefits on systemic insulin sensitivity despite similar weight loss between groups. Whether similar effects would be observed in a NAFLD/NASH population and would be paired with favorable histological outcomes remains to be explored.

Additionally, we have repeatedly shown that exercise and intrinsic high aerobic capacity is linked to increased hepatic long-chain fatty acid oxidation, improved mitochondrial respiration, and increases in other associated mitochondrial outcomes (citrate synthase activity, β -HAD activity, cytochrome c content, etc.) in rodent models [19, 32, 148]. Moreover, the liver also likely increases the breakdown of intrahepatic lipids to fuel fatty acid oxidation in order to fuel exercise. One could speculate that regular exercise increases turnover and storage patterns of hepatic lipid pools. Indeed, it appears exercise that provides multiple benefits that may be beneficial in ameliorating NAFLD/NASH including increasing hepatic oxidative pathways, improving hepatic insulin

sensitivity and promoting glucose disposal in the muscle diverting nutrients away from the liver. Thus, both exercise and aerobic capacity upregulate hepatic oxidative pathways due to the energy demands of exercise pulling on hepatic metabolism, responses that are associated with improved metabolic health. Enhanced glucose disposal in muscle would lead to reduced insulin levels and would also reduce oxidative stress that has been found with steatosis and hyperglycemia in conditions of excess energy consumption.

CONCLUSION

In summary, strong evidence exists to support the rationale that mitochondrial dysfunction and impairments in mitochondrial quality control are linked with worsening NAFLD pathology in rodent models. Whether similar outcomes are observed in humans with NAFLD remains to be explored. Further, the abilities of weight loss via caloric restriction and exercise to reverse disease remain the cornerstone of therapy for patients with NAFLD/NASH. Identifying the mechanisms by which these therapies reverse NASH has important implications and holds promise for future discovery of novel therapeutic targets for management of this increasingly common disease.

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APPENDIX A – CURRICULUM VITAE

CURRICULUM VITAE Mary Moore, PhD Candidate



PERSONAL INFORMATION

Mary P. Moore
Laboratory of R. Scott Rector, PhD
Department of Nutrition and Exercise Physiology and Truman VA Hospital
204 Gwynn Hall, Hitt St, University of Missouri, Columbia, MO 65211
Cell: +1(312) 536-0825
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ORCID ID: <https://orcid.org/0000-0002-6365-7718>
CV Current as of June 2021

EDUCATION

<u>Degree</u>	<u>Institution</u>		<u>Field</u>	
PhD	University of Missouri	Columbia, MO, USA	Nutrition and Exercise Physiology GPA 3.9/4.0	2017-present
MSc	Central Michigan University	Mount Pleasant, MI, USA	Exercise Physiology GPA 4.0/4.0	2015-2017
BSc	Dublin City University	Dublin, Ireland	Sports Science and Health 1 st Class Honors	2011-2015

DOCTORAL COMMITTEE

R. Scott Rector, PhD (Chair); Jill A. Kanaley, PhD; Jaume Padilla, PhD; Frank Booth, PhD

PhD Dissertation: The role of mitochondrial function and turnover in NAFLD/NASH pathophysiology in human and rodent models. Dr. Scott Rector, Mentor.

SCIENTIFIC TRAINING

2017-present	Graduate Research Assistant, Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, MO, USA. Supervisor: R. Scott Rector, PhD
2015-2017	Graduate Research Assistant, Department of Health Sciences, Central Michigan University, Mount Pleasant, MI, USA. Supervisor: Micah Zuhl, PhD
MAY-AUG 2016	Graduate Research Assistant, Department of Health Sciences, Central Michigan University, Mount Pleasant, MI, USA. Supervisors: Micah Zuhl, PhD and Naveen Sharma, PhD
2013-2015	Undergraduate Research, School of Health and Human Performance, Dublin City University, Dublin, Ireland. Supervisor: Donal O’Gorman MD, PhD (see description of projects under ‘Research Projects’)

TEACHING EXPERIENCE

Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, MO
NEP2222 Landscape of Obesity – Guest lecture: Exercise and Weight Loss

Department of Exercise Science, Central Michigan University, Mount Pleasant, MI

HSC 600 Advanced Exercise Physiology – Laboratory Instructor (Fall 2016)

HSC 310 Exercise Physiology – Teaching Assistant (Fall 2016)

HSC 215 Human Physiology – Laboratory Instructor (Fall/Spring 2015-2017)

HSC 411 Pathophysiology – Teaching Assistant (Fall 2015)

PREVIOUS WORK EXPERIENCE

2017 Internship in Cardiac Rehab, Henry Ford Medical Center, Detroit, MI
2014-2015 Fitness and Community-Based Wellness Coach, Dublin City University Sport Centre, Dublin, Ireland
2014 Undergraduate Summer Research Internship Program, Dublin City University, Dublin, Ireland
2014 Internship, Sport Ireland Institute - High Performance Centre, Dublin, Ireland
2013 Summer Sports Camp, Cavan, Ireland
2012 Research Assistant, High 5 Sports Supplement Company, Dublin, Ireland

CURRENT RESEARCH INTERESTS

- The role of mitochondrial function, mitophagy, and dynamics in the pathophysiology of insulin resistance, diabetes and non-alcoholic fatty liver disease.
- Impact of lifestyle interventions (i.e. diet and exercise) on obesity, diabetes and non-alcoholic fatty liver disease.
- Understanding the molecular mechanisms by which exercise and diet impacts metabolic disease.

CERTIFICATIONS

2017 ACSM Certified Clinical Exercise Physiologist
2018 American Heart Association - First Responders Training
2015 Irish Training and Educational Centre - Level 2 Gym Instruction
2015 Irish Training and Educational Centre - Level 2 Studio Instruction
2015 Irish Training and Educational Centre - Level 2 Personal Training
2015 Irish Training and Educational Centre - Spin Instruction
2015 National Rescue Award for Swimming Teachers and Coaches – Lifeguard Certification
2015 Triggerpoint - Foam Rolling Principles and Practices Level 1
2014 Gymnastics Ireland – GymEdge Course - Foundations of Functional Movement

PROFESSIONAL AFFILIATIONS

2016-Present American College of Sports Medicine (ACSM)
2017-Present American Society for Nutrition (ASN)
2017-Present American Physiologic Society (APS)

PROFESSIONAL SERVICE

2021 - Reviewer for Applied Physiology, Nutrition, and Metabolism
2017-2021 - Co-reviewer under R. Scott Rector, PhD

- *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*
- *American Journal of Physiology - Endocrinology*
- *Applied Physiology, Nutrition, and Metabolism*
- *Experimental Physiology*
- *Frontiers in Endocrinology*

Session co-chair - The Physiological Society – Physiology of Obesity Webinar Series, August 18th 2020

UNIVERSITY SERVICE

- 2019-2021 Exercise is Medicine on Campus Organization. Co-founder and Vice President, University Missouri
2019-2020 Graduate Professional Council Representative – Department Representative, University of Missouri
2019-2020 Graduate Professional Council Finance Committee, University of Missouri
2019-2021 Chancellor's Standing Committee on the Status of Women – Graduate Rep, University of Missouri
2019-2020 Graduate Student Association Treasurer, Nutrition and Exercise Physiology, University of Missouri
- Applied for travel funding on behalf of the organization
 - Received ~\$4000 for graduate student travel over two years
- 2018-2019 Graduate Student Association President, Nutrition and Exercise Physiology, University of Missouri
2018-2021 Exercise is Medicine Strategic Committee - Graduate Representative, University of Missouri
2015-2017 Exercise is Medicine Committee - Graduate Representative, Central Michigan University

UNDERGRADUATE STUDENT MENTORING

- Nicole Weischhaus Undergraduate student, University of Missouri
Corey Diemer Undergraduate student, University of Missouri
Vivien Jepkemoi Undergraduate student, University of Missouri
Luigi Boccardi Undergraduate student, University of Missouri
Alyssa Morley Undergraduate student, Central Michigan University

LAY/VOLUNTEER CONTRIBUTIONS

- 2019-2021 Writing contributor to The Food Medic Educational Hub
2013-2015 Diet and Exercise for Life (DEXLIFE), lifestyle intervention-based study; Dublin, Ireland
2013-2015 DCU Sport, Exercise and Wellness Community-based Program, Fitness Coach; Dublin, Ireland
2013-2017 Special Olympics Volunteer; Dublin, Ireland and Michigan, USA
2012-2017 Youth sports coach; Cavan, Ireland

ACADEMIC AND PROFESSIONAL HONORS/AWARDS

- 2021 James L. McGregor Scholarship in Health and Exercise Sciences (\$500)
2021 Distinguished Graduate Research Award, MU College of Human Environmental Sciences (\$500)
2021 The Donald K. Anderson Award - MU Graduate School Graduate Research Assistant Award (\$1000)
2021 M. Harold Laughlin Scholar Award for outstanding research in physical inactivity and chronic disease (\$500)
2020 Annual Research & Creative Activities Forum Presentation Award, University of Missouri (\$100)
2020 Ben R. Londree/Tom R. Thomas Award for Outstanding Graduate Student in Exercise Physiology, MU (\$500)
2020 Keystone Symposia Travel Scholarship: National Institute on Aging Grant, #1R13AG066282-01- (\$1200)
2019 Truman VA Research Poster Symposium Award, Columbia, MO (\$500)
2019 School of Medicine Dean's Award for Outstanding Student Research, University of Missouri (\$500)
2019 Experimental Biology Annual Meeting – American Physiological Society Travel Award (\$500)
2019 Graduate Professional Council, Professional Development Travel Award, University of Missouri (\$200)
2019 NEP Graduate Student Association Travel Award, University of Missouri (\$200)
2018 NEP Graduate Student Association Travel Award, University of Missouri (\$200)
2017 Herbert H. & Grace A. Dow College of Health Professions Student Research Award, CMU (\$500)
2017 Graduate Student Research & Creative Endeavors Award, Research and Graduate Studies, CMU (\$500)
2016 Herbert H. & Grace A. Dow College of Health Professions Student Economic Assistance Fund, CMU (\$1800)
2016 Graduate School Conference Travel Award, Central Michigan University (\$500)
2015 Health and Fitness Internship Award, Dublin City University Sports Center, Dublin, Ireland (€1800)
2014 Undergraduate Student Summer Research Fellowship, Dublin City University, Dublin, Ireland (€3000)

GRANT WRITING CONTRIBUTIONS

Graduate Student Research & Creative Endeavors Grant, Office of Research and Graduate Studies, Central Michigan University (\$1000 received)

American College of Sports Medicine Pre-Doctoral Grant. Title: *Combining exercise and dietary ketone ester supplementation in the prevention of western diet induced NASH*. Submitted: Spring 2020.

LECTURES AND PRESENTATIONS

"The impact of nutritional ketogenesis on hepatic outcomes" Kansas University Medical Center, KS. January 2021

"Hepatic Mitochondrial Metabolism and Turnover is Linked to increasing NASH severity in Humans" 4th Annual UCLA Mitochondria Symposium, University of California, Los Angeles, CA. November 2020

"Reduced Hepatic Mitochondrial Metabolism and Markers of Mitochondrial Biogenesis/Turnover are Linked to increasing NAFLD/NASH severity in Humans" 37th Annual Research & Creative Activities Online, University of Missouri, Columbia, MO. November 2020

"The impact of nutritional ketosis on hepatic outcomes" Nutrition and Exercise Physiology Seminar Series Spring 2020. University of Missouri, Columbia, MO. September 2020

"Reduced Hepatic Mitochondrial Metabolism and Markers of Mitochondrial Biogenesis/Turnover are Linked to NAFLD Progression in Humans" Integrative Physiology—Muscling in on Liver. American Diabetes Association 80th Scientific Sessions. Virtual Conference. June 2020

"A Dietary Ketone Ester Reduces Markers of Hepatic Stellate Cell Activation and Fibrogenesis in High-Fat Diet Fed Mice." Health Sciences Research Day. School of Medicine Dean's Award Presentation. University of Missouri, Columbia, MO. November 2019

"Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female Wistar rats." Environmental and Exercise Physiology Session. Experimental Biology, Orlando Florida. April 2019

"Exercise Prescription Techniques in Cardiac Rehabilitation Centers in the Midwest" Michigan Chapter ACSM Meeting, Gaylord, MI. February 2017

"Exercise is Medicine – Exercise Prescription for the Busy Physician" Central Michigan University School of Medicine Seminar Series, Mount Pleasant, MI. February 2017

"Exercise is Medicine – Exercise as a Pill" Central Michigan University School of Medicine Seminar Series, Mount Pleasant, MI. December 2016

RESEARCH PROJECTS

2017- Present **Graduate Research Assistantship** – Laboratory of Scott Rector, Department of Nutrition and Exercise Physiology, and Harry S. Truman VA Hospital, University of Missouri, Columbia, MO

1. *Nutrient overload, insulin resistance, and hepatic mitochondrial dysfunction*

Project: #2008258

Sponsor: NIH R01 DK113701

Investigators: E Parks, PhD, RS Rector, PhD, JA Ibdah, MD, PhD

Dates: 05/01/17 – 04/30/22

Role: Graduate Researcher

Goal: The goal of this project is to examine the role of hepatic mitochondrial dysfunction in the development and progression of NASH and to determine whether hepatic mitochondria function is modifiable with lifestyle intervention therapies

2. *Hepatic eNOS and Mitochondrial Function in NASH*

Project: BX003271-01

Sponsor: VA Merit Grant 1 I01

Investigators: R. Scott Rector, PhD

Dates: 4/1/2017-3/31/2021

Role: Graduate Researcher

Goal: The goal of this project is to examine the role of hepatocellular endothelial nitric oxide synthase in the regulation of NRF2 and BNIP2 in the progression of NASH.

3. *Impact of ketosis on hepatic outcomes in the setting of NASH*

Sponsor: University of Missouri

Investigators: R. Scott Rector, PhD

Role: Graduate Researcher

Goal: The goal of these projects are to examine the roles of nutritional and exercise-induced ketosis on hepatic outcomes of steatosis, inflammation, and fibrosis.

2015-2017

Graduate Research Assistantship – Exercise Physiology Laboratory, Department of Exercise Science, School of Health Professions, Central Michigan University, Mount Pleasant, MI

1. *Oral glutamine supplement reduces subjective fatigue ratings during repeated bouts of firefighting simulations*

Sponsor: Central Michigan University

Investigators: Micah Zuhl, PhD

Dates: 11/01/16 – 04/30/17

Role: Graduate Researcher

Goal: The goal of this project is to examine the role of acute low-dose glutamine supplementation on fatigue and inflammation during exercise under heat stress

2. *The role of heat shock proteins in insulin sensitivity*

Sponsor: Central Michigan University

Investigators: Micah Zuhl, PhD; Naveen Sharma, PhD

Dates: 01/01/15 – 01/01/17

Role: Graduate Researcher

Goal: The goal of this project was to examine how heat shock proteins respond to stresses, including caloric restriction which has been shown to enhance insulin sensitivity in cell culture models

2012-2015

Undergraduate Research - School of Health and Human Performance, Dublin City University, Dublin, Ireland.

1. *Diet and Exercise for Life (DEXLIFE) Study*

Sponsor: European Union FP7 collaborative project

Supervisor: Donal O’Gorman

Role: Undergraduate Researcher

Goal: To explore mechanisms of prevention of type 2 diabetes by lifestyle intervention in subjects with pre-diabetes or at high-risk for progression and to identify novel biomarkers that better predict the onset of type 2 diabetes.

PUBLICATIONS

Peer-Reviewed Publications

- 1) Syed-Abdul MM, Moore MP, Wheeler A, Ganga RR, Diaz-Arias A, Rector RS, Ibdah JA, Parks EJ. Improvements in nonalcoholic fatty liver disease (NAFLD) after metabolic surgery is linked to an increased hepatic fatty acid oxidation—a case report. *AME Surg J* 2021. doi: 10.21037/asj-20-5.
- 2) Moore, M.P., Davis, R.A.H., Deemer, S.E., Plaisance E.P., and Rector, R.S. 2019. A Dietary Ketone Ester Reduces Markers of Hepatic Stellate Cell Activation and Fibrogenesis in High-Fat Diet Fed Mice. *AJP- Gastrointestinal and Liver Physiology*. 2021 320:4, G564-G572
- 3) Cunningham, R.P., Moore, M.P., Meers, G.M., Jepkemoi, V., Boccardi, L., and Rector, R.S. Deletion of Hepatocellular eNOS Impairs Hepatic Mitochondrial Function. In revision. *Diabetes*
- 4) Moore, M.P., Cunningham, R.P., Dashek, R.J., Mucinski, J., and Rector, R.S.; A fad too far? Dietary strategies for the prevention and treatment of NAFLD. *Obesity*. 2020. 28(10), 1843-1852.
- 5) Moore M.P. and Mucinski J.M., 2020. Impact of nicotinamide riboside supplementation on skeletal muscle mitochondria and whole-body glucose homeostasis: Challenging the current hypothesis. *The Journal of Physiology*, 598(16), 3327-3328.
- 6) Moore, M.P., Cunningham, R.P., Kelty, T.J., Boccardi, L.R., Nguyen, N., Booth, F.W., and Rector, R.S. 2020 Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female Wistar rats. *Applied Physiology, Nutrition, and Metabolism*, 45(1), 35-44.
- 7) Moore, M.P., Moriarty, T. A., Connolly, G., Mermier, C., Amorim, F., Miller, K., & Zuhl, M. (2019). Oral Glutamine Supplement Reduces Subjective Fatigue Ratings during Repeated Bouts of Firefighting Simulations. *Safety*, 5(2), 38.
- 8) Cunningham, R.P., Moore, M.P.*(*co - first author), Moore, A.N., Healy, J.C., Roberts, M.D., Rector, R.S. and Martin, J.S., 2018. Curcumin supplementation mitigates NASH development and progression in female Wistar rats. *Physiological reports*, 6(14), p.e13789.
- 9) Cunningham, R.P., Moore, M.P., Meers, G.M., Rueggsegger, G.N., Booth, F.W. and Rector, R.S., 2018. Maternal Physical Activity and Sex Impact Markers of Hepatic Mitochondrial Health. *Medicine and science in sports and exercise*, 50(10), pp.2040-2048.
- 10) O'Neil, S., Thomas, A., Pettit-Mee, R., Pelletier, K., Moore, M.P., Thompson, J., Barton, C., Melson, R., and Zuhl, M, 2018. Exercise Prescription Techniques in Cardiac Rehabilitation Centers in Midwest States. *Journal of Clinical Exercise Physiology*, 7(1),pp.8-14.
- 11) Roth J, Szczygiel T, Moore M.P., O'Connor P, Edwards J, Sharma N, Pettit-Mee R, Zuhl M. Profiling inflammatory markers during the competitive season and post season in collegiate wrestlers. *The Journal of Strength & Conditioning Research*. 2019;33(8):2153-61.
- 12) Szczygiel T, Moore M.P., Pettit-Mee R, Slezsak A, Zuhl M. Low dose glutamine supplementation preserves the biological stress response among US university students. *Journal of Human Nutrition and Food Science*. 2017;5:1114.
- 13) Lossing A, Moore M.P., Zuhl M, 2016. Dance as a treatment for neurological disorders. *Body Movement and Dance in Psychotherapy* 12(3):1-15.

Manuscripts in preparation

- 1) Moore M.P., Cunningham R.P., Meers G.M., Johnson S.A., Wheeler A.A., Ganga R.R. Spencer N.M., Pitt J.B., Diaz-Arias A., Swi A. I. A., Ibdah J.A., Parks E.J., Rector R.S., Reduced Hepatic Mitochondrial Metabolism and Markers of Mitochondrial Turnover in Humans Are Linked to NAFLD Progression. Under Review.

- 2) Cunningham, R.P., **Moore, M.P.**, Meers, G.M., Jepkemoi, V., Boccardi, L., Dashek, R., Vieria-Potter, V.J., Kanley, J. A., Booth, F.W., and Rector, R.S. Deletion of hepatocellular eNOS impairs exercise-induced improvements in hepatic mitochondrial function and autophagy markers. In Revision.
- 3) Syed-Abdul MM, **Moore M.P.**, Wheeler A, Ganga RR, Diaz-Areas A, Rector RS, Ibdah JA, Parks EJ. Contribution of de novo lipogenesis in the progression of fatty liver disease. In preparation.
- 4) Slezsak, A. Nelson, R., McKenna, Z., O'Connor, P., **Moore, M.P.**, Connolly, G. and Zuhl, M., The impact of oral sucralose ingestion during exercise on markers of gastrointestinal discomfort and damage among healthy adults. Under review.

Abstracts/Conference Presentations

- 1) JM Mucinski, NS Nallapeta, **MP Moore**, JA Ibdah, RS Rector, EJ Parks. Lifestyle treatment-induced improvements in nonalcoholic steatohepatitis (NASH). American Diabetes Association 81st Virtual Scientific Sessions, June 2021. Abstract #: 2021-A-5162-Diabetes; Presentation #: 285-OR.
- 2) Ryan Dashek, Rory Cunningham, **Mary Moore**, Vivien Jepkemoi, Grace Meers, Bysani Chandrasekar, Scott Rector. Hepatocellular RECK Overexpression Attenuates NASH Susceptibility. Presented at National American Association of Laboratory Animal Science Annual Meeting 2020.
- 3) **Moore M.P.**, Cunningham R.P., Meers G.M., Johnson S.A., Wheeler A.A., Ganga R.R. Spencer N.M., Pitt J.B., Diaz-Arias A., Swi A. I. A., Ibdah J.A., Parks E.J., Rector R.S., Reduced Hepatic Mitochondrial Metabolism and Markers of Mitochondrial Turnover in Humans Are Linked to NAFLD Progression. American Diabetes Association, Chicago; 2020. Abstract #: #2020-A-4368-Diabetes; **Presentation #: 311-OR**
- Also presented at: 4th Annual UCLA Mitochondria Symposium - Los Angeles, CA Nov 5th-6th 2020.
- 4) Mucinski J.M., Sharma N., **Moore M.P.**, Ibdah J.A., Rector R.S., Parks E.J. Role of glucose flux to elevate hepatic lipogenesis in biopsy-proven NASH. American Diabetes Association, Chicago; 2020. Abstract #: #2020-A-4368-Diabetes; Presentation #: 310-OR
- 5) **Moore, M.P.**, Davis, R.A.H., Deemer, S.E., Plaisance E.P., and Rector, R.S. 2019. A Dietary Ketone Ester Reduces Markers of Hepatic Stellate Cell Activation and Fibrogenesis in High-Fat Diet Fed Mice. Presented at:
 - Keystone Symposia: New Insights into the Biology of Exercise. Keystone, CO. Poster #3003. March 4th, 2020.
 - ACSM South West Chapter, Newport Beach, CA, Abstract #59. Oct. 26, 2019.
 - Health Sciences Research Day, University of Missouri, Columbia, MO, Poster #170. Nov. 11th, 2019
 - Truman VA Research Poster Symposium, Columbia, MO. Abstract #14. Dec 13th, 2019
- 6) Cunningham, R.P., **Moore, M.P.**, Dashek, R., Jepkemoi, V., Meers, G.M., Chandrasekar, B., and Rector, R.S. 2020. Deletion of Hepatocellular eNOS Impairs Exercise-Induced Improvements in Hepatic Mitochondrial Function. Keystone Symposia: New Insights into the Biology of Exercise. Keystone, CO. March 5th, 2020.
- 7) Syed-Abdul MM, **Moore MP**, Wheeler A, Ganga RR, Diaz-Arias A, Rector RS, Ibdah JA, Parks EJ. 2020. Contribution of de novo lipogenesis (DNL) in progression of nonalcoholic fatty liver disease. Keystone Symposia: Obesity and NAFLD: Mechanisms and Therapeutics. Fairmont Banff Springs, Alberta, Canada. February 23rd-27th, 2020. Poster#3025.
- 8) Syed-Abdul MM, **Moore MP**, Wheeler A, Ganga RR, Diaz-Arias A, Rector RS, Ibdah JA, Parks EJ. A case study demonstrating the impact of metabolic surgery on liver disease. *The Missouri Physiological Society Annual Meeting*, University of Missouri. Abstract #8. Oct. 26, 2019.
- 9) Cunningham, R.P., **Moore, M.P.**, Nguyen, N., Dashek, R., Boccardi, L.R., Jepkemoi, V., Meers, G.M., Chandrasekar, B., and Rector, R.S. 2019. Hepatic Knockdown of RECK Increases NASH Susceptibility. *The FASEB Journal*, 33(1_supplement), 582-5.
- 10) Cunningham, R.P., **Moore, M.P.**, Meers, G.M., Nguyen, N., Boccardi, L.R., Jepkemoi, V., Takahashi, T., and Rector, R.S. 2019 Hepatocyte-Specific Deletion of eNOS Impairs Mitochondrial Function and Exacerbates Hepatic Steatosis. *The FASEB Journal*, 33(1_supplement), 582-2.

- 11) **Moore, M.P.**, Cunningham, R.P., Kelty, T.J., Boccardi, L.R., Nguyen, N., Booth, F.W., and Rector, R.S. 2019 Combining a ketogenic diet with voluntary exercise impacts markers of hepatic metabolism in male and female Wistar rats. Cell Symposia: Exercise Metabolism. Sitges, Spain. May 6th, 2019.
- 12) **Moore, M.P.**, Cunningham, R.P., Kelty, T.J., Boccardi, L.R., Nguyen, N., Booth, F.W., and Rector, R.S. 2019 Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female rats. *The FASEB Journal*, 33(1_supplement), 699-4. Presentation at EB, March 2019.
- 13) **Moore, M.P.**, Cunningham, R., Moore, A.N., Healy, J.C., Roberts, M.D., Rector, S. and Martin, J.S., 2018. Curcumin Supplementation Mitigates Nash Development and Progression in Female Wistar Rats: Board# 209 June 13. *Medicine & Science in Sports & Exercise*, 50(5S), p.724. Presented at:
 - American College of Sports Medicine, Annual Meeting, Minneapolis, MA. Abstract#209. June 1st, 2018.
 - Midwest Liver Symposium, University of Kansas medical Center, KS. April 13th, 2018
 - Life Sciences Week University of Missouri, Columbia, MO, April 10th, 2018
- 14) **Moore, M.P.**, Roth, J. Szczygiel, T, Pettit-Mee, R., & Zuhl, M., (2017). Profiling Inflammatory Markers During The Competitive Season And Post Season In Collegiate Wrestlers. 2017. 2599 Board #119 June 2 2017. *Medicine & Science in Sports & Exercise*. 49. 735.
- 15) Roth, J; **Moore, M**; Szczygiel, T; Pettit-Mee, R; and Zuhl, M. Profiling inflammatory markers during the competitive season and post season in division I collegiate wrestlers. *Submitted Poster Presentation: Central Michigan University SRCEE, Mt. Pleasant, MI. April 1st, 2017.*
- 16) Moriarty, T., Nava, R., **Moore, M.P.**, Connolly, G., Thompson, J., Amorim, F., Mermier, C., and Zuhl, M. The Effect of Acute Glutamine Supplementation on Markers of the Heat Shock Protein Response After Repeated Firefighting Simulations. ACSM South West Chapter, Annual Meeting, Long Beach, CA. Board# 62. Oct 20th, 2017.
- 17) **Moore, M.P.**, Connolly, G., Wagner, B., Slezsak, A., Thompson, J., and Zuhl, M. "The effect of acute glutamine supplementation on markers of immune function and stress after repeated firefighting simulations." Poster. Central Michigan University Student Research and Creative Endeavors Exhibition, Mt. Pleasant, MI. April 1st, 2017.
- 18) **Moore, M.P.**, Thompson, J., Pettit-Mee, R., Thomas. A., Pelletier, K., and Zuhl, M. "Exercise prescription techniques in cardiac rehabilitation centers among two mid-west states" Poster. Central Michigan University Student Research and Creative Endeavors Exhibition, Mt. Pleasant, MI. April 1st, 2017.
- 19) Szczygiel, T., **Moore, M.P.**, Pettit-Mee, R., Slezsak, A., and Zuhl, M. "The effect of 8-weeks of low dose oral glutamine supplementation on markers of stress, immune, and gastrointestinal function in healthy college-age subjects." Poster. Central Michigan University Student Research and Creative Endeavors Exhibition, Mt. Pleasant, MI. April 2nd, 2016.

Books and Book Chapters

- 1) Zuhl, M., Slezsak, A., Hasanaj, K., Thompson, J., **Moore, M.P.**, Workbook and Study Guide for Electrocardiogram Assessment, published at Central Michigan University, 2016

VITA

Mary Moore was born on August 9th, 1993, in Cavan, Ireland. She completed her undergraduate degree in Sports Science and Health at Dublin City University in Ireland focusing on lifestyle interventions in pre-diabetics. In her Master's, in Exercise Physiology at Central Michigan University, Mary was involved in studies examining responses to environmental heat stress in wildland firefighting. During this time she also completed an internship in clinical exercise physiology at the Henry Ford Health System, Detroit, MI. It was this foray into clinical research led her to pursue a PhD in Exercise Physiology at the University of Missouri, under the mentorship of Dr. Scott Rector. Mary obtained her doctoral degree in 2021. She plans to pursue a post-doctoral degree at Columbia University studying macrophage function in the setting of fatty liver disease. In Dr. Ira Tabas' laboratory.