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**Identification of novel biomarkers and
treatments for non-alcoholic fatty liver disease
and associated co-morbidities**

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A thesis submitted for the degree of Doctor of
Philosophy on 1st April 2022

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is characterised by impaired lipid metabolism, hepatic fibrosis and linked to systemic insulin resistance, which is partly mediated by remodelling of liver-secreted proteins or “hepatokines.” There are no effective screening tools or approved pharmacotherapies for NAFLD and NAFLD-associated fibrosis. Proteomic studies estimate that ~25% of circulating proteins are liver-derived, suggesting that hepatokines could be biomarkers and, due to well described impacts on energy homeostasis, effective treatments for NAFLD and NAFLD-associated fibrosis.

This thesis aimed to discover novel biomarkers and treatments for NAFLD, non-alcoholic steatohepatitis (NASH), and NAFLD with significant fibrosis (NAFLD F2-3). For biomarker discovery, bariatric patients were prospectively recruited, plasma and a liver wedge biopsy were procured, livers were precision-cut to assess hepatokine secretion and were grouped based on histological confirmation of NAFLD, NASH, and NAFLD F2-3. Chapter 3 identified 3333 liver-secreted proteins, of which, 107 and 5 hepatokines were remodelled with NASH and NAFLD F2-3, respectively. EMILIN1 secretion was increased in NASH, NAFLD F2-3, and in the plasma of NASH patients. However, plasma EMILIN1 poorly stratified patients NASH. In Chapter 4, we speculated that hepatokine secretion may not correspond to changes in plasma levels. Notably, highly abundant plasma proteins corresponded to ~62% of the proteins detected in the human liver-secreted proteome. Plasma proteomics showed few changes in patients with NASH and NAFLD F2-3. Proteins APOF and FCN3 stratified patients with NASH and IGKV2-28 and APCS outcompeted existing non-invasive scores to stratify patients with NAFLD F2-3. Together, this provides a novel resource

characterising human liver-secreted and highly abundant plasma proteins and identified IGKV2-28, APCS, APOF, and FCN3 as biomarkers for NAFLD-associated fibrosis and NASH.

In Chapters 5 and 6, we exploited hepatokine remodelling to uncover novel treatments for NAFLD and hepatic fibrosis. In Chapter 3, we detected EMILIN1, which was previously reported to reduce TGF β signalling. Hepatocyte-specific overexpression of EMILIN1 with severe NASH and advanced fibrosis reduced TGF β signalling and hepatic fibrosis, while overexpression of EMILIN1 mice in a mouse model of mild NASH did not impact fibrosis or metabolic co-morbidities, suggesting that EMILIN1 could be a therapeutic target for advanced, but not mild, fibrosis.

Physical activity can resolve NAFLD and improve its metabolic co-morbidities, however, the effects of exercise-training on hepatokine secretion and the metabolic impact of exercise-regulated hepatokines in NAFLD remain unresolved. Chapter 6 assessed whether exercise-training could remodel hepatokine secretion to mediate improvements in NAFLD and associated co-morbidities. Mass spectrometry proteomics analysis detected 2657 intracellular and 1593 secreted proteins from isolated mouse hepatocytes. Exercise-training remodelled the hepatocyte proteome, with differences in 137 intracellular and 35 secreted proteins. Hepatocyte-secreted factors from exercise-trained mice improved insulin action in skeletal muscle and increased hepatic fatty acid oxidation. Hepatocyte-specific overexpression of SDC4 reduced hepatic steatosis, which was associated with reduced fatty acid uptake, and blunted pro-inflammatory and pro-fibrotic gene expression. Treating hepatocytes with recombinant SDC4 recapitulated these effects.

Together, the work in this thesis addresses an unmet clinical need to discover novel biomarkers and therapeutics to treat NAFLD and co-morbidities.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The thesis comprises only original work, within the word limit and is submitted towards the Doctor of Philosophy except where indicated.

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Student	Role	Date (Year)	Project	Year level	Mark
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Anagi Wickremasinghe	Mentor	2018	The Physiology of the gastric sleeve	BSc (Hons)	82
Rachith Jayawardana	Mentor	2019	Characterising fatty acid metabolism in human liver slices	BMedSci (Hons)	85
Daniel Wallace	Mentor	2021	Assessment of PXDN in human and murine NAFLD	BSc (Hons)	84
Joshua Goundar	Co-supervisor	2022	EMILIN1 improves glucose tolerance in obese mice	BSc (Hons)	TBD

Description of collaborative work and contribution

Thesis chapter	Publication / chapter title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution
3	Characterisation of human liver-secreted proteins	Unpublished, not submitted	85% Assisted in experimental design Performed all experiments and data analysis. Wrote and edited the chapter.	1) Paul Burton, assisted with experiments, 3% 2) Wendy Brown, assisted with experiments, 1% 3) Paula Miotto, assisted with experiments 1% 4) Magda Montgomery, designed experiments, 3% 5) Matthew Watt, designed experiments, data analysis, input into manuscript 7%
4	Plasma proteomics discovers biomarkers that effectively stratify NASH and hepatic fibrosis	Unpublished, not submitted	90% Designed all experiments. Performed experiments, data analysis, wrote and edited the chapter.	1) Mark Larance, assisted with experiments 2% 2) Matthew Watt 8%, designed experiments, data analysis, input into chapter
5	Characterisation of a novel hepatokine EMILIN1 in NASH and NAFLD-associated fibrosis	Unpublished, not submitted	90% Designed all experiments. Performed all experiments and data analysis. Wrote and edited the chapter	1) Jackie Bayliss, assisted with experiments 1% 2) Stacey Keenan, assisted with experiments 1% 3) Magda Montgomery, assisted with experiments 1% 4) Matthew Watt, designed experiments, data analysis, input into manuscript 7%
6	Proteomic analysis reveals exercise training induced remodelling of hepatokine secretion and uncovers syndecan-4 as a regulator of hepatic lipid metabolism	Accepted 29/3/22	80% Designed all experiments. Performed all experiments and data analysis. Wrote and edited the manuscript. Submitted 23.3.22	1) Paula Miotto, assisted with experiments 1% 2) Jackie Bayliss, assisted with experiments 1% 3) Shuai Nie, assisted with experiments 1% 4) Stacey Keenan, assisted with experiments 1% 5) Magda Montgomery, assisted with experiments 6%, 6) Matthew Watt, designed experiments, data analysis, input into manuscript 10%

I have re-numbered sections of the submitted paper to generate a consistent presentation within this thesis.

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Chapter 1: Introduction

1.1. Non-alcoholic fatty liver disease

In the absence of alcohol consumption and other factors that cause lipid storage, non-alcoholic liver disease (NAFLD) is clinically characterised by lipid accumulation that exceeds 5% of the total liver weight [6; 7]. NAFLD is the most prevalent liver disease worldwide [8-10] and is a broad term describing two histologically distinct disease stages. Non-alcoholic fatty liver (NAFL) also termed steatosis has long perceived to be a benign manifestation where excess lipids are stored within lipid droplets inside hepatocytes, without the presence of inflammation or fibrosis. However, it is now functionally linked to many pathological conditions such as the development of insulin resistance (IR) [11; 12]. Over time, non-alcoholic fatty livers can become inflamed and progress to non-alcoholic steatohepatitis (NASH), which is histologically defined by the presence of NAFL along with inflammation, and hepatocyte ballooning [13], also termed hepatocyte apoptosis or necrosis [14], with or without fibrosis [15] (Figure 1.1). Subsequently, NASH can lead to liver failure or hepatocellular carcinoma (HCC) [15], the fourth leading cause of liver cancer related death worldwide [16].

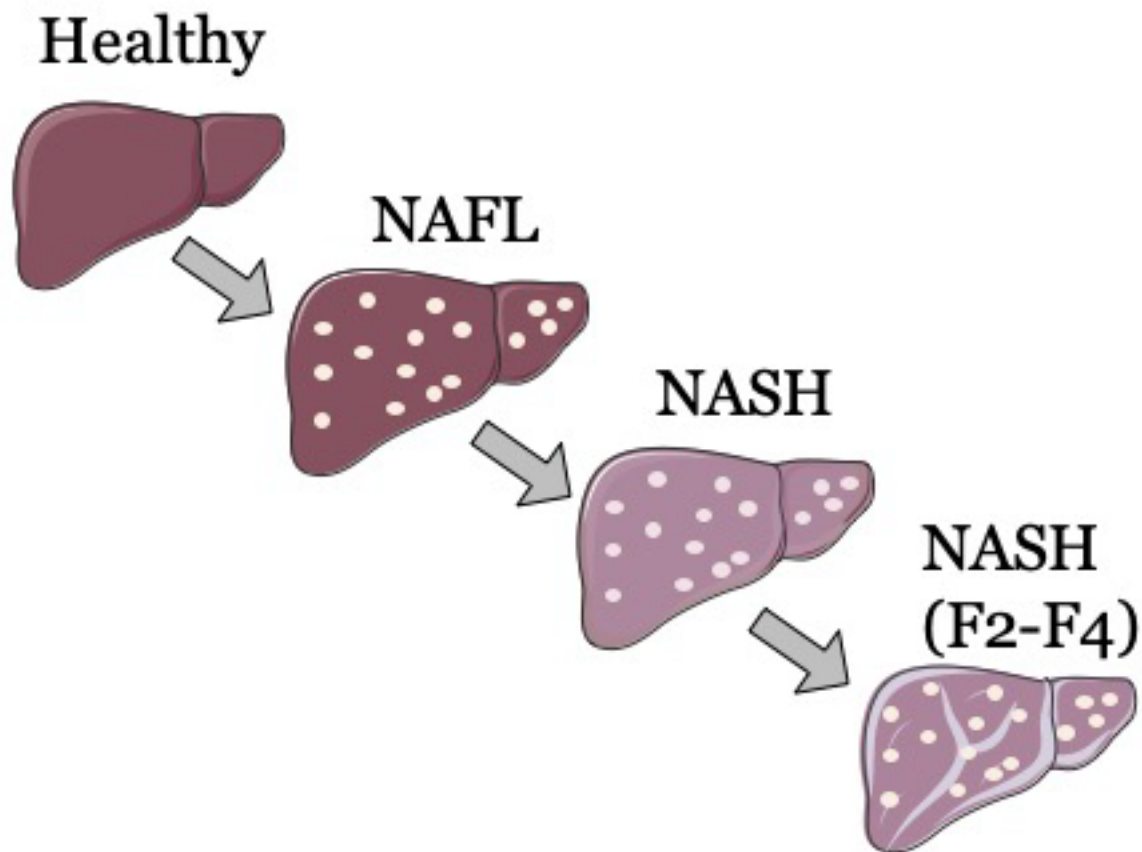


Figure 1.1: Pathogenesis of non-alcoholic fatty liver disease (NAFLD). Excessive lipid storage in the liver can lead to non-alcoholic fatty liver (NAFL). Over time, the NAFL can become inflamed and lead to hepatocyte damage, the disease stage termed non-alcoholic steatohepatitis (NASH). NASH can progress to the onset of fibrotic scars graded in severity from F1 to F4 (cirrhosis). F2-F4 denoted as significant fibrosis. Image is authors own.

1.1.1. Epidemiology

NAFLD is a global health issue. First described in the early 1980s [17], NAFLD has rapidly become the most common liver disease worldwide [18] affecting approximately 25% of the global [19] and up to 40% of the Australian population [20], respectively. Of these NAFLD cases, approximately 20% are diagnosed as NASH [9; 19]. The rise of NAFLD to pandemic proportions is concurrent with obesity [19; 21; 22], sedentary lifestyle [7], and the high fat, high sugar diets [23] which have made it recognised as a disease of the western world. Utilising cohorts of patients undergoing bariatric surgery, the prevalence of NAFL ranges from 70-96% [24; 25] with NASH present in up to 17%

[24; 25] of obese individuals, with these proportions being significantly greater compared to the general population. However, the increasing prevalence of NAFLD in developing countries [26-28] is becoming much more apparent. Furthermore, given the rise in childhood obesity and its associated comorbidities in the United states of America (USA) [29], Australia [30] and globally [31], it is not surprising that estimates suggest that by 2030, NAFLD and its more progressive and severe form, NASH, will increase in prevalence by 21% and 63%, respectively [9]. Coupled with this rising prevalence, paired biopsy studies demonstrate that regardless of the stage of NAFLD, approximately 37% of all NAFL and NASH patients will develop progressive fibrosis [32]. This is of great clinical concern as fibrosis is a major predictor of liver-related mortality [33]. Moreover, given the lack of reliable, non-invasive diagnostic capabilities for individuals at risk, the exact number of individuals with NAFLD or those likely to develop it, is undoubtedly underestimated [6; 9; 10; 28; 34; 35].

1.1.2. NAFLD & insulin resistance

NAFLD is strongly associated with other metabolic comorbidities commonly featured in the metabolic syndrome such as IR, type 2 diabetes (T2D), dyslipidaemia and hypertension. Particularly, T2D and NAFLD are strongly associated with up to 75% of patients with T2D having NAFLD [36-39]. Functional studies in lean and obese non-diabetic humans have demonstrated that, regardless of excess body weight, NAFL is associated with insulin resistance in the liver, muscle, and adipose tissue [12; 40-42]. These association studies indicate that NAFL is a not a benign condition and it is intrinsically linked to the development of metabolic disease. Importantly, murine studies have highlighted that NAFL occurs before hyperglycaemia and hyperinsulinemia [43;

44] and subsequent IR, suggesting that the NAFL may be impacting and/or controlling insulin action in peripheral tissues to drive IR.

1.2. The pathogenesis of NAFLD

The exact mechanisms that detail NAFLDs stepwise progression from a healthy liver to steatosis and NASH are not yet fully understood. In this section we will describe the most recent understanding of the interplay of risk factors and/or metabolic changes that propel the liver to accumulate lipids. This net increase in hepatic lipids can occur through (1) increased lipid uptake and/or (2) reductions in lipid export, (3) decreased oxidation, and or (4) elevated *de novo* lipogenesis (DNL).

1.2.1. Increased hepatic lipid uptake

The liver is sensitive to changes in energy balance that occur because of increased caloric intake and reduced energy expenditure. Thus it is influenced by changes in diet such as an increase in dietary lipids that can drive hepatic lipid accumulation [45]. Given that the hepatic portal vein delivers ~75% of the liver's blood flow which collects nutrients from key digestive sites such as the stomach, small and large intestines [46; 47], it is not surprising that an increase in lipid uptake can be readily stored in the liver with current estimates indicating that up to 20% of fatty acids derived from the diet are stored intra-hepatically [48-50]. Furthermore, not only is delivery increased, but in obese individuals, the expression of fatty acid translocase (FAT) encoded by the gene (CD36), a transmembrane protein that facilitates fatty acid transport into the cell, is also increased in the liver [51], thus further fuelling hepatic lipid storage (Figure 1.2). Additionally, upon initiating a low fat, hypocaloric diet, hepatic lipid content is reduced by 40-80% [52], highlighting the liver's flexibility in storing dietary lipids.

Adipose tissue is the first site of excess energy to be stored [53]. This process can occur either by adipogenesis (formation of new adipocytes) or by increased internalisation of lipids in existing adipocytes [54]. While these changes are intended to induce safe expansion of the adipose tissue, chronic dietary lipid overload can lead to metabolic dysfunction within adipocytes, particularly in hypertrophic adipocytes, which results in increased lipolytic capacity [55]. Adaptations in angiogenesis, and therefore in oxygen and nutrients supply, are insufficient to meet the demands of the increased size of the adipose depots, leading to hypoxia and subsequently inflammation [56]. This increase in inflammation leads to the activation of serine kinase c-Jun N-terminal kinase (JNK) that functions to not only mediate the pro-inflammatory signalling cascade in resident immune cells [57], but also functions as a downstream negative regulator of insulin signalling [58]. This is important as hindering insulin action in adipose tissue can lead to decreased phosphatidylinositol 3-kinase (PI3K) signalling and a consequential impairment in the suppression of hormone sensitive lipase and adipose triglyceride lipase [59], enzymes involved in the basal rate of adipose tissue lipolysis. Thus, this consequential increase in lipolysis regardless of the prandial state and insulin action induces higher secretion of fatty acids into the blood stream [60]. This is fundamental because adipose tissue lipolysis directly influences NAFLD, and human *in vivo* studies have shown that 60% of the liver triglyceride content originates from free fatty acids released by adipose tissue [61]. Visceral fat is considered the strongest culprit as it has the greatest lipolytic capacity and the free fatty acids are delivered directly into the hepatic portal vein [12; 62] where it can be directly internalised by the liver (Figure 1.2).

Despite this wealth of evidence, increased lipolysis in individuals with obesity and NAFLD remains controversial. Dual isotope tracing in lean and obese individuals demonstrate a reduction in adipose tissue derived fatty acids [63]. However, this finding was normalised to adipose tissue mass, not protein content, which is largely influenced by the increased volume of lipids stored and not truly reflective of the enzymatic machinery and the capacity liberates fatty acids from triglyceride molecules.

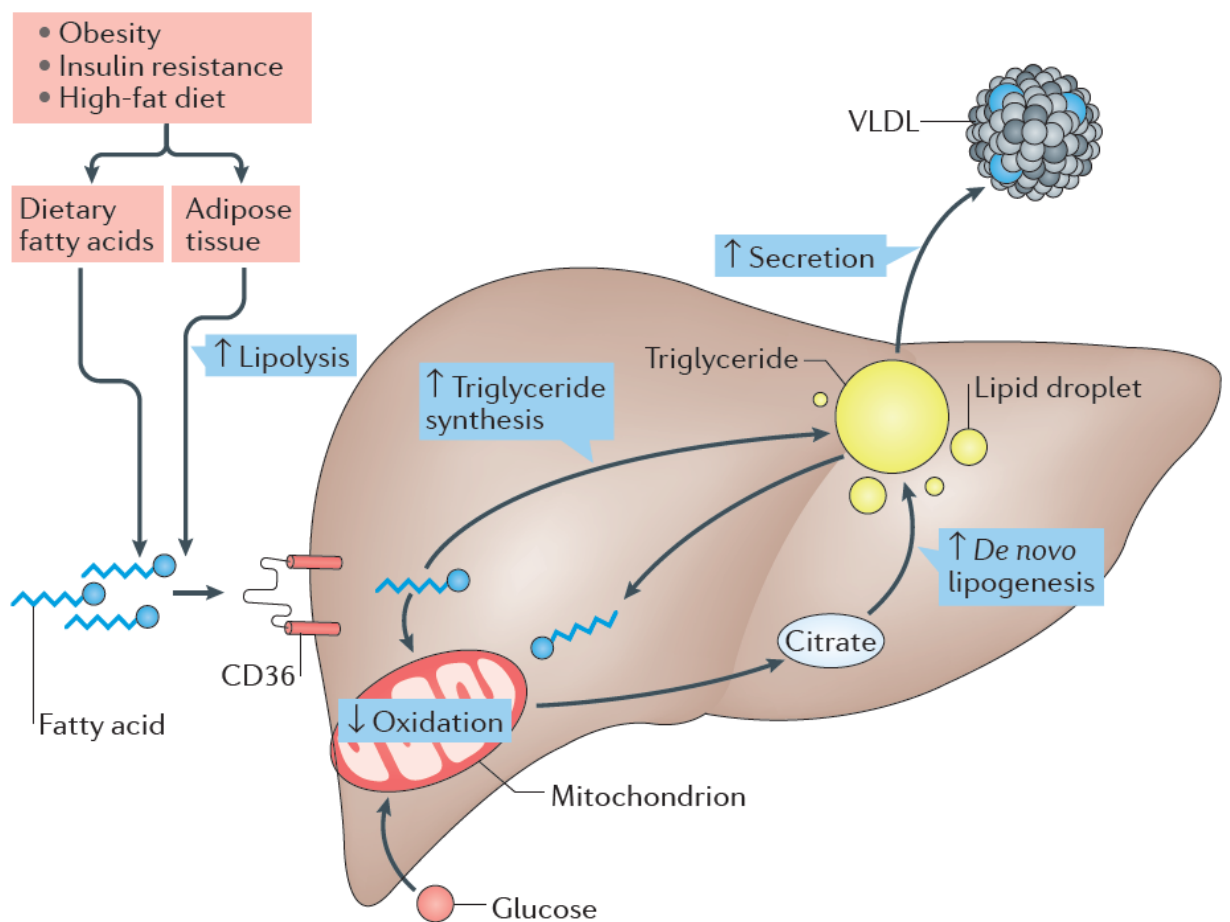


Figure 1.2: Alterations in lipid metabolism that aid in the development of hepatic steatosis. Image taken from Meex et al 2016 [59]. CD36, Cluster differentiation factor 36; VLDL, very low density lipoprotein.

1.2.2. Increased lipid secretion

To transport lipids to peripheral tissues, the liver packages hydrophobic lipids including triglycerides, cholesterol, phospholipids, as well as apolipoproteins into very low-density lipoproteins (VLDLs) to allow for hydrophilic transport through the blood. While the exact mechanism of how this process occurs and is regulated remains incompletely described, human tracer studies have shown that in individuals with NAFLD the secretion of triglyceride (TG) rich [64; 65] and apolipoprotein B100 enriched [65; 66] VLDL is much greater compared to healthy controls. This observation is supported by a positive linear relationship between the intra-hepatic triglyceride (IHTG) content and plasma VLDL, with the relationship reaching a plateau when the IHTG reaches 10% of total liver weight in humans [64]. This may reflect maximal cholesterol secretion rates and suggests that increased VLDL secretion is insufficient to overcome the increased fatty acid burden leading to lipid accumulation and consequent NAFLD.

1.2.3. Fatty acid oxidation

Once fatty acids enter hepatocytes with the help of FAT/CD36 and fatty acid transporter proteins (FATP) such as FATP1, 2 and 5 [67; 68], a proportion is converted to acyl-carnitines by carnitine *O*-palmitoyltransferase 1 (CPT1) allowing their translocation to the mitochondrial matrix by carnitine translocase and carnitine *O*-palmitoyltransferase 2 (CPT2). Mitochondrial fatty acid oxidation (also termed β -oxidation) occurs in a four-step sequential process, utilising straight chain acyl-CoA dehydrogenases. These enzymes continually cleave an acetyl group off the carbon chain, which can fuel the Krebs cycle in the form of acetyl-CoA to drive oxidative phosphorylation (OXPHOS), which produces adenosine triphosphate (ATP). Defects in the β -oxidation pathway have been linked to the development of NAFL [69; 70]. Mice with defective very long and

medium chain acyl-CoA dehydrogenases that are involved in the shortening of the fatty acid chain, were found to develop NAFL [69]. Additionally, mice heterozygous for a mutation in mitochondrial trifunctional protein, which catalyses three out of the four steps in β -oxidation, also developed steatosis [70]. Isotopic tracer studies in humans after weight gain and increase in IHTG content have shown an overall decrease in fat oxidation [65]. The findings highlight the importance of β -oxidation in the maintenance of the healthy liver.

The exact mechanism that impairs mitochondrial function and consequential β -oxidation that gives rise to steatosis is not yet fully understood. Obese individuals exhibit increased fatty acid oxidation compared to their lean counterparts [71]. While this study did not assess for NAFLD or NASH, it is highly probable that the obese individuals have some form of NAFLD given that obesity is a major risk factor. The surplus fatty acid delivery to the liver (in settings of chronic dietary lipid oversupply) and subsequent increase in fatty acids shuttled towards mitochondrial oxidation, can result in increased levels of reactive oxygen species (ROS) production [59; 72; 73]. The accumulating ROS may induce lipid peroxidation (*i.e.* oxidative damage to lipids) [74] and may deplete mitochondrial DNA [74], impair mitochondrial proteins through protein carbonylation (*i.e.* oxidative damage to proteins) and ultimately reduce the efficiency of oxidative respiration, leading to steatosis and potentially NASH [59; 72]. However, isotopic tracer studies in humans show that NAFLD livers exhibit 80% greater TCA cycle flux and show that mitochondrial oxidative metabolism is markedly increased [75]. Congruently, high-resolution respirometry studies of isolated mitochondria from human NAFLD livers demonstrate an ~4.5 fold increase in maximal respiration when compared to livers from lean individuals and this is lost in patients

with NASH [74]. Further, NASH patients exhibited increase in ROS, lipid peroxides, inflammation, and reductions in the activity of antioxidant defence protein catalase [74; 76]. These findings suggests that the liver adapts to excess lipid accumulation by increasing fatty acid oxidation which is subsequently blunted with NASH potentially due to ROS mediated impairments in oxidative phosphorylation. However, none have directly assessed fatty acid metabolism intact human livers using radiometric tracers. To address this uncertainty, Moore and colleagues radiometrically assessed human liver homogenates and isolated mitochondria and show a reduction in fatty acid oxidation in NASH and patients with significant fibrosis (stages F2-4) compared to patients without pathology or NAFL [76]. Contrasting previous findings, they show no difference in fatty acid oxidation between NAFL and livers without pathology [76]. Collectively, whether NAFLD and or NASH remodels fatty acid oxidation remains controversial. Nonetheless, fatty acid oxidation is insufficient to overcome the increase in uptake and de novo synthesis of fatty acids. Future studies should assess fatty acid metabolism on intact liver tissues to adequately reflect the liver in its resident cellular architecture.

1.2.4. Hepatic de novo lipogenesis

De novo lipogenesis (DNL) is the incorporation of carbons derived from carbohydrates and amino acids into fatty acids. Typically, DNL is increased in the post prandial state to mediate nutrient clearance. In a healthy fasted individual, DNL contributes to approximately 5% of the total liver fatty acid pool [77]. This contribution is drastically increased in patients with NAFLD, with DNL contributing 20-25% of the total hepatic free fatty acid (FFA) pool [61; 78]. The increase in DNL with NAFLD observed, was inferred from changes in plasma total triglyceride-rich particles, VLDLs, and non-esterified fatty acid pools not direct measures of human hepatic DNL [61; 78].

Nonetheless, this highlights that DNL may be a critical mediator in the development of steatosis.

In response to a rise in blood glucose or from a meal, insulin is secreted by the beta-cells of the pancreas. Insulin signalling in the liver induces the expression, nuclear translocation, and transcriptional activity of transcription factors such as sterol response element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) [79; 80]. These transcription factors can induce the expression of lipogenic genes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearyl-CoA desaturase 1, and mitochondrial glycerol 3-phosphate acyltransferase [81] that facilitate the conversion of glucose into lipids and are upregulated in the livers of obese patients [82]. Importantly, ChREBP stimulates the expression of pyruvate kinase within the liver, which allows for more glucose derived pyruvate to be incorporated into lipids [83]. Hyperinsulinemia, which is typically present in individuals with steatosis and NAFLD [12; 62; 84], can induce chronic over-expression of hepatic SREBP-1c and ChREBP [85] and thereby increase the rate of DNL [86] even under IR conditions [61; 87]. Additionally, fructose directly activates SREBP-1c and ChREBP in the liver regardless of insulin action and can promote DNL [88]. Hyperinsulinemia independent of NAFLD, is associated with increased risk of NAFLD development [89], and posited increased CD36 mediated fatty acid uptake may drive increased lipid accumulation [90], suggesting that either NAFLD and hyperinsulinemia can drive the development of the other. On the other hand, hyperinsulinemia is may be consequent of reduced insulin clearance which is evidence in obesity [91], and obese individuals with NAFLD compared to patients without NAFLD [92]. However, patients with NAFLD and obesity in this study were not matched for BMI compared to no NAFLD group and the changes

observed may not be solely attributed to NAFLD pathology [92]. Nonetheless, hyperinsulinemia or reduced insulin clearance may explain why in obesity [65; 86; 93], T2D [94; 95] and NAFLD [92] that DNL is profoundly higher and likely to accelerate hepatic steatosis.

1.3. Non-alcoholic steatohepatitis (NASH)

NASH is characterised by hepatic inflammation and hepatocyte ballooning or cell death within the steatotic liver [96]. While not all patients who have steatosis develop NASH (approx. 5% of patients with steatosis develop NASH), NASH development is increased in patients with metabolic diseases such as hypertension [32; 97; 98], T2D [97], dyslipidaemia [99] and obesity [97]. However, the definitive pathogenesis of NASH remains elusive and the mechanisms that drive its progression appears to be a combination of multiple pathogenic conditions. In the following section, the pathophysiology of NASH will be reviewed.

1.3.1. Lipotoxicity

Lipotoxicity is a term that describes excessive ectopic lipid storage (i.e., outside of adipose tissue) that can hinder cellular homeostasis, initiate inflammatory pathways and induce apoptosis (Figure 1.3) [1], the distinguishing features of NASH. Ectopic lipid deposition typically occurs via two mechanisms (1) dietary lipid oversupply [44; 100] and (2) an increase in adipose tissue lipolysis and excessive FFA release into the circulation [101]. Lipotoxic lipids such as FFAs, lysophosphatidyl choline (LPC), diacylglycerol (DAG), and ceramides are mediators of cell dysfunctions and/or apoptosis [102]. Palmitate, a saturated free fatty acid has the capacity to bind to toll like receptor 4 (TLR4) on the resident immune cells (e.g., Kupffer cells), leading to subsequent nuclear factor kappa- B (NFkB) and Interferon regulatory factor (IRF) activation and expression of pro-inflammatory cytokines [103] (Figure 1.3). Additionally, when palmitate-loaded primary macrophages are exposed to lipopolysaccharide (LPS), it is sufficient to induce inflammasome activation and consequent apoptosis [104]. This is important because LPS is increased in individuals

with NAFLD and NASH [105] and thus may drive NASH progression. Furthermore, palmitate also has indirect lipotoxic effects. Once internalised, palmitate can be metabolised to lysophosphatidyl choline (LPC) and promote cytotoxicity [106; 107]. LPC has been shown to be lipotoxic and induce ER stress, but it can also induce the expression of p53 upregulated modulator of apoptosis (PUMA) [106], that can bind to and inhibit all members of the anti-apoptotic B-cell lymphoma 2 protein family, leading to apoptosis [108]. This is important because LPC and palmitate are increased in the serum [109] and/or liver [107] in patients with NASH. However, one must be cautious in concluding the cytotoxic role of LPC, as human studies have associated LPC with a protective role in distinguishing insulin sensitive versus insulin resistant NAFL [110]. Given that NASH intersects both increased lipid storage and hepatic inflammation and cell death, it is likely that lipotoxicity is a significant driver of NAFLD progression. DAGs are involved in signal transduction and can act as precursors for the formation of phospholipids and triglycerides, and are increased in NASH [111]. DAGs can directly activate various protein kinase C (PKC) isoforms, with PKC ϵ being the major hepatic isoform [112], leading to insulin receptor substrate inhibition and subsequent hepatic insulin resistance [113; 114]. Other lipid species such as ceramides have also been implicated in lipotoxicity mediated dysfunctions [115-117] (Figure 1.3). Pre-clinical models have shown that inhibition of ceramide synthesis can prevent hepatic insulin resistance [118], and an increase of hepatic ceramides coincides with the onset of NASH [119]. However, few studies have examined ceramide metabolism in patients with NAFLD and NASH. Recent work by Ooi and colleagues examined the human liver lipidome and demonstrated substantial increase in ceramide and sphingolipids in the liver with steatosis and this corresponded to changes in plasma ceramide species [120]. Further, ceramide and sphingolipid content was increased in NASH livers compared to

patients with NAFL [120] and considering murine findings [118; 119], warrant the development of novel ceramide inhibiting therapies. Indeed, lipotoxicity plays a role in mediating hepatic inflammation allowing steatosis to progress to NASH, but such assertions require more mechanistic studies combined with human findings to identify how individual lipid species can or cumulatively drive such progression.

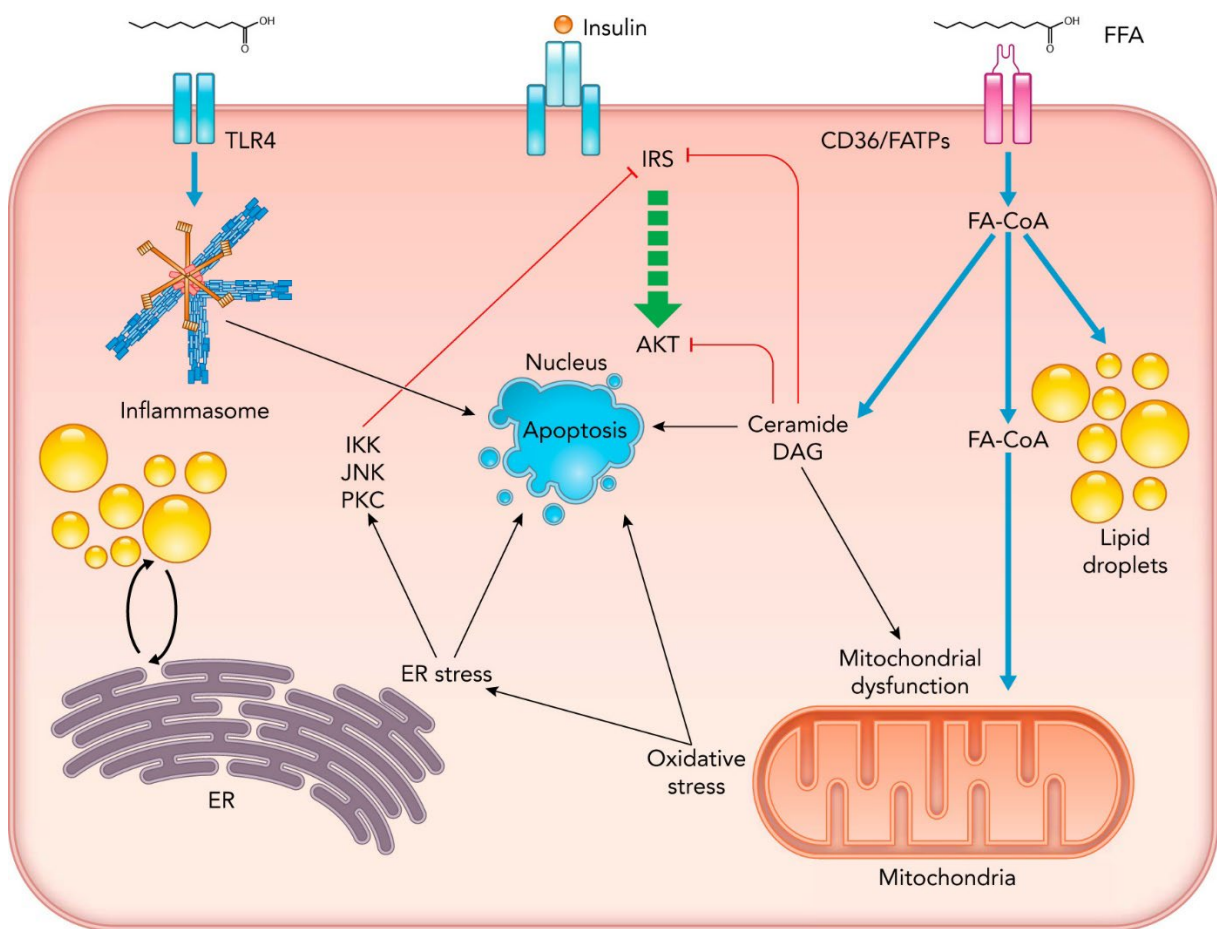


Figure 1.3: The impact lipotoxicity on intracellular pathways. Schematic of how free fatty acids (FFA), diacylglycerols (DAG), and ceramides can induce mitochondrial dysfunction, endoplasmic reticulum (ER) and oxidative stress that can in turn activate the inflammasome and cause apoptosis. CD36, cluster of differentiation 36/fatty acid translocase; FATPs, fatty acid transport proteins; IKK, I κ B kinase; IRS, insulin receptor substrate; JNK, c-Jun NH₂-terminal kinase. Figure adapted from Montgomery et al 2019 [1].

1.3.2. Gut microbiome

Gut microbiota may play a significant role in the onset of NASH. In obese individuals, a dysbiotic microbiome is frequently observed [121] and is postulated to be a major risk factor for NAFLD development [122]. A greater proportion of gram-negative bacteria in the intestinal microbiome are associated with both obesity and NASH [123]. This is important because gram-negative bacteria have the capacity to produce alcohol that can

increase the permeability of the gut by widened tight junctions[124]. As the gut is more 'leaky' this may explain the increase in gram-negative bacteria present in the liver of patients with biopsy proven NAFLD and NASH compared to healthy controls [105]. As the portal vein delivers blood directly from the gut to the liver, it consistently exposes the liver to microbes, food antigens and environmental toxins. LPS is an endotoxin found on the outer membrane of gram-negative bacteria and can bind to TLR4 on leukocytes and likely Kupffer cells, leading to the activation of pro-inflammatory signalling cascade and subsequent release of pro-inflammatory cytokines [125]. Such signalling can also induce inflammasome activation [126] (Figure 1.4). Thus, it may be possible that gut microbiota instigates inflammation, however, more detailed studies are required to support this assertion.

1.3.3. Immune activation

The liver is an essential immune organ. The liver has a number of specialised leukocytes to aid in its defence, such as cells including Kupffer cells, bone marrow derived monocytes/macrophages, liver sinusoidal endothelial cells, natural killer (NK) cells, NKT cells and hepatic stellate cells (HSCs). Kupffer cells have been implicated in NASH progression. High fat diet (HFD) fed mice lacking Kupffer cells demonstrate decreased TG storage and interleukin-1 beta (IL-1 β) expression [127]. This was coupled with an increased expression of PPAR α , fatty acid transport protein (FATP) 2, and CPT1 α , all of which are involved in fatty acid oxidation and are negatively regulated by IL-1 β [127]. Furthermore, IL-1 β , can also induce HSC activation and secretion of tissue inhibitor of metalloproteinase-1 [128], which is a potent promoter, but not initiator, of hepatic fibrosis [129]. Additionally, genetic deletion of the interleukin-1 receptor (IL-1R) induced a marked reduction in both steatohepatitis and fibrosis severity [128]. This

suggests that Kupffer cells can communicate with hepatocytes to drive hepatic lipid accumulation while concurrently promoting the progression of fibrosis in HSCs. Once liver damage and consequent apoptosis occurs, endogenous DNA or mitochondrial DNA (mtDNA) released by the apoptotic cell can be recognised by toll like receptor 9 (TLR9), that is expressed by HSCs and Kupffer cells, and lead to consequential activation of HSCs and fibrosis deposition [128]. Further, humans and mice with NASH secrete more microparticles containing hepatocyte-derived mtDNA that activates TLR9 and consequently Kupffer cells [130]. Notably, when TLR9 is eliminated, the severity of steatohepatitis and fibrosis severity was reduced [128], highlighting the importance of this inflammatory pathway to drive NASH. Previous findings have demonstrated that activation of two distinct TLR pathways (i.e., TLR4 and TLR9) can synergise and amplify the immune response [131], which may accelerate liver injury and consequential apoptosis, further demonstrating the importance of the immune system in the development of NASH.

1.3.4. Mitochondrial dysfunction mediated cell death

Mitochondrial dysfunction is central in the progression of NASH. A common hallmark of NASH histology is megamitochondria, whereby mitochondria containing paracrystalline inclusions lead to structural alterations [132]. An oversupply of fatty acids, evidenced in NAFLD, that are subsequently trafficked to the mitochondria and oxidised for energy in conjunction with continual Krebs cycling to drive oxidative phosphorylation produces large amounts of ROS. This can damage both mtDNA and the protein subunits of the OXPHOS chain [133-135], leading to mitochondrial dysfunction. This is central to the development of NASH as oxidised mtDNA is released by the mitochondria into the cytosol where it can activate the inflammasome

and subsequently lead to cell death [136]. Thus, an oversupply of lipids in the liver can lead to increased levels of ROS that can impair the efficacy of mitochondrial energy forming pathways whilst also damaging the mtDNA leading to cell death.

1.4. Diagnosis of NAFLD and NASH

NAFLD is clinically characterised by lipid accumulation that exceeds 5% of the total liver weight [6; 7; 137]. The presence of NAFLD rarely manifests systemic symptoms and thus diagnosis is frequently incidental [138; 139]. This is not surprising given the lack of reliable, simple and cost-effective tests to diagnose NAFLD. Diagnosis and screening for NAFLD is important. Simple and effective modes to diagnose and stage NAFLD is paramount to allow clinicians to recognise patients who have a greater risk of long-term adverse outcomes along with patients who need treatment (diagnosed with NASH and/or advanced fibrosis). Additionally, diagnosis of fibrosis at any stage is associated with treatment complications, liver-related and overall mortality [33; 140; 141]. Furthermore, since inflammation is a predictor of fibrosis progression [142], simple measures to predict or diagnose NASH is of paramount importance to stratify at risk patients to identify those at most need for monitoring and treatment. Thus, the ability to readily diagnose individuals with NAFLD and their degree of fibrosis will not only facilitate large scale clinical trials for treatment modalities, but it will also allow accurate stratification of individuals in need of more advanced care. In this section, we will discuss the approaches used to diagnose NAFLD in all its pathological stages.

1.4.1. Liver biopsy

The current gold standard of NAFLD diagnosis is through a liver biopsy. This is the only reliable means to discriminate between steatosis from NASH with or without

fibrosis [143]. The most common histological grading and staging system for NAFLD and NASH is the NAFLD activity score (NAS) which assigns a score based on the severity of steatosis, inflammation and ballooning to create a NAS score (Table 1.1) [144]. Fibrosis is also graded in this scoring system however it does not contribute to the overall NAS score (Table 1.1). The NAS score provides a simple output to determine the severity of NAFLD. However, given that NAFLD / NASH is present in approximately 25% of the global population [9], the use of a liver biopsy as a screening tool is not feasible. Furthermore, liver biopsies are an invasive procedure with a 0.01% risk of mortality and an incidence of 0.1% for severe complications [145]. In addition, the widespread use of liver biopsies is hindered by sampling error, inter-observer variability (histopathological assessment) and cost [146]. This highlights the need for an improved non-invasive and accurate test to detect NAFLD at each of its pathogenic stages to allow for routine use in the clinic.

Table 1.1. Histological features, scoring and definition of the NAS score and fibrosis staging [147].

Histological feature	Score	Definition
Steatosis	0	<5% of area
	1	5-33% of area
	2	34-66% of area
	3	>66% of area
Lobular inflammation	0	None
	1	1-2 foci per 20 X field
	2	2-4 foci per 20 X field
	3	>4 foci per 20 X field
Hepatocyte ballooning	0	None
	1	Few
	2	Many
NAFLD activity score (NAS)	0-8	Sum of steatosis + inflammation + and hepatocyte ballooning score
Fibrosis stage	0	No fibrosis
	1	Perisinusoidal or periportal
	1a	Mild, zone 3, perisinusoidal
	1b	Moderate, zone 3, perisinusoidal
	1c	Portal or periportal
	2	Perisinusoidal and Portal or periportal
	3	Bridging fibrosis
	4	Cirrhosis

1.4.2. Non-invasive tests

Fibrosis stage is strongly correlated with both liver-related [148] and all-cause mortality [149], and thus many simple scores have been developed (Table 1.2). These simple scores are comprised of routine biochemical and clinical measures to aid in the diagnosis of NAFLD. Some commonly used non-invasive scores include the aspartate aminotransferase to platelet ratio index (APRI), the BARD score, the Fibrosis-4 (FIB-4) score, the Forn index and the NAFLD fibrosis score (recently reviewed [150]). The liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are clinically used to assess liver damage and are utilised in all the non-invasive simple score tests [150]. This is important because AST and ALT do not necessarily correlate with stages or progression of NAFLD. For example, normal ALT values have been seen in individuals at any stage of the NAFLD spectrum and even low-normal values have been observed in the presence of NAFLD or advanced fibrosis severity [139; 151]. Additionally, validation studies have generally been sourced from patient cohorts with abnormal liver function tests, such as those with established NAFLD and many studies have been at least partially retrospective in nature [141; 142; 152-156], suggesting that their validation may not hold true as a screening tool for the general population.

More complex fibrosis panels have been developed such as the enhanced liver fibrosis (ELF) score that utilises serum markers for fibrogenesis and fibrolysis with strong diagnostic accuracy (area under receiver operating curve (0.92, 0.98, 0.99 for F1, F2 and F3, respectively). However, the ELF score is patented and utilises serum markers that are not typically assessed in the clinical setting, causing its use to be rare in practice [145]. Furthermore, when assessed in obese only populations such as performed in Ooi et al 2018 [145], its accuracy for diagnosis of F1-F4 considerably decreased (Table 1.2).

Moreover, previous work has shown that on one hand these risk scores perform very poorly in obese individuals [157] and on the other hand, non-invasive tests to measure early stages of fibrosis and NASH lack early detection (Table 1.2). The lack of early detection of NAFLD is a substantial clinical problem, as even ‘mild’ fibrosis (F1) has been shown to be associated with long term liver-related and overall mortality [140]. Thus, simple measures to diagnose NAFLD/NASH in obese individuals, especially at the early stages of disease progression, are of significant importance to stratify patients at risk for those at most need for monitoring and treatment.

Table 1.2. Sensitivity, specificity, and heterogeneity (I^2) of non-invasive approaches to diagnose or stratify fibrosis in NAFLD-associated fibrosis in an obese only population. Sensitivity and specificity represented as a proportion. Adapted from Ooi et al 2018 [145].

	Sensitivity	I^2	Specificity	I^2
NFS (Low Threshold)	0.73	87.6%	0.50	96.2%
NFS (high threshold)	0.35	90.5%	0.88	0.0%
BARD	0.50	88.9%	0.57	96.5%
FIB4	0.45	95.7%	0.91	93.9%
Fibrotest (low threshold)	0.66	74.8%	0.75	0.0%
Fibrotest (high threshold)	0.13	4.1%	0.98	36.3%
ELF	0.70	0.0%	0.81	0.0%
TE	0.74	85.5%	0.77	88.6%
MRE	0.64	0.0%	0.93	22.7%

NFS, NAFLD fibrosis score; FIB-4, fibrosis -4, ELF, enhanced liver fibrosis; I^2 , heterogeneity between studies; TE, transient elastography; MRE magnetic resonance elastography.

1.4.3. Elastography techniques

Elastography techniques assess the elastic properties of the liver to give an indication of liver stiffness. Magnetic resonance elastography utilises mechanical waves to determine liver stiffness, with decreased stiffness indicating increased fat deposition [158].

Transient elastography using FibroScan® is a rapid and effective means to assess the magnitude of fibrosis. The technique detects elastic shear waves from the liver tissue sent from an ultrasound transducer probe [159]. Despite ease of use, transient elastography is not widely available but it also has high equipment costs [160]. It is usually used after a diagnosis of NASH to identify more severe forms of fibrosis. While elastography techniques are accurate in identifying fibrosis [161; 162] they are impractical as a large population screening tool due to accessibility, high equipment costs, time consuming and non-reimbursable service costs to the patient (through government rebates) rendering them unsuitable for most patients. Additionally many of these imaging techniques have been shown to perform poorly in obese individuals (Table 1.2) [163]. This is due to the obese habitus and increased density of adipose tissue between the liver and skin altering the mechanical waves detected by the receiver [163]. Given that those primarily affected by NAFLD and NASH related fibrosis are obese [19; 25; 157], the use of elastography techniques for diagnosis are ineffective due to cost or inaccuracy highlighting the need for diagnostic capabilities that are accurate in all body sizes.

1.5. Approved NAFLD treatments

While treatment of NAFL or NASH is of utmost priority, it is important to recognise that both conditions come with comorbidities such as IR and obesity and there is no approved pharmacological therapies specifically designed for the treatment of NAFLD in any of its forms [96]. Therefore, lifestyle modification such as dietary changes and/or exercise are encouraged to reduce IR, adiposity and hepatic lipid accumulation in the liver to potentially regress the NAFLD [96].

1.5.1. Exercise

Exercise is an effective treatment in maintaining glucose homeostasis and body weight [164; 165]. In obese individuals, a 10% reduction in body weight is effective in regressing NASH in 90% of patients [166]. In addition, exercise can improve peripheral insulin sensitivity in individuals with NAFLD [167]. However, the majority of patients involved in exercise clinical trials with the aims of resolving NAFLD and NASH are unable to achieve 10% weight loss [168]. Somewhat counter-intuitively to the clinical trial set points [168], exercise-induced weight loss is not always required to improve NAFLD [169; 170]. Following 3 months of exercise training, obese men exhibited a 9.5% reduction in liver steatosis, plasma ALT, fasting glucose, triglyceride, and non-esterified free fatty acids [169]. Similarly, resistance exercise reduces liver lipid accumulation by 13% without changes in adiposity [170]. Thus, despite exercise and dietary interventions providing a simple mode to treat NAFLD and NASH, adherence to such lifestyle is a practical barrier in the resolution of the disease. While commonly prescribed as a treatment, the exact molecular mechanism of how exercise impacts NAFLD is not well established. Hallmarks of exercise training have been focused on adaptations within skeletal muscle, such as mitochondrial biogenesis, increased oxidative capacity and myocyte hypertrophy, all of which are consistent with a training response [171]. In mice, exercise induces secretion of exosomes from the muscle to the liver, however no follow up studies on the impact on the liver were determined [172]. To the best of our knowledge, only one study has examined how exercise altered the liver proteome [173], which show a marked alterations in proteins regulating redox homeostasis. Given that the liver has an altered secretory profile in NAFLD [59], whether or not exercise is sufficient to alter liver secretion and/or induce a metabolically 'healthy' secretory profile is yet to be explored.

Liver, the endocrine organ

The liver is a major secretory organ. Recent quantitative proteomics have identified over 10,000 proteins in the human [174] and mouse [175] liver, of which 40% encode secreted proteins [176]. The liver is composed of multiple cell types, including parenchymal cells such as the hepatocytes which occupy approximately 80% of the liver volume. The hepatocyte proteome composes the largest proportion of the total liver proteome suggesting that hepatocytes are most likely the potent source of secreted proteins from the liver [175]. Furthermore, proteomic profiling of the mouse serum and liver indicates that approximately 25% of the plasma proteins may originate from the liver [177], accentuating the notion that the liver is an abundant source of proteins and is important in the control of systemic energy homeostasis.

1.6.1. Mechanisms of protein secretion

To communicate with adjoining cells or distant tissues, cells can secrete proteins by two mechanisms (Figure 1.4). Conventional (*i.e.*, classical) secretion occurs with proteins containing an NH₂ terminal signal peptide being transported within the endoplasmic reticulum to the Golgi apparatus by vesicular transport. Subsequently, proteins are re-packaged into vesicles that fuse with the plasma membrane for release directly into the extracellular environment (Figure 1.4). Proteins can also be secreted by unconventional means whereby proteins that do not have a NH₂ terminal signal peptide can be transported from the endoplasmic reticulum (ER) to the Golgi by vesicular transport and are then shuttled to the early endosome where they fuse with the endosome (Figure 1.4) [1]. Once inside the endosome, the membrane folds to create multi-vesicular bodies (MVB) and the delivered proteins are sorted and packaged inside the MVB (Figure 1.4) [1]. The MVB then fuses with the plasma membrane to release endosomes into the

interstitium as exosomes and these can be transported into the circulation and exert a myriad of metabolic and pathological functions[178-180].

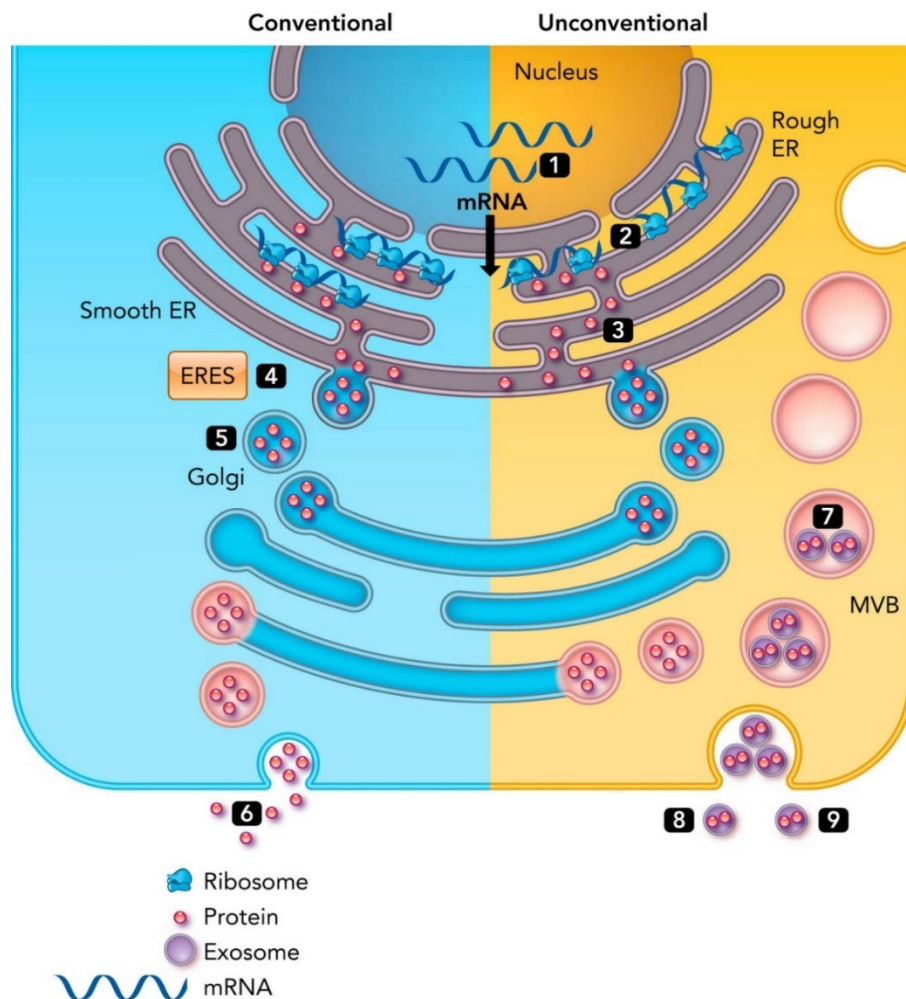


Figure 1.4: Distinct modes of protein secretion. Either conventional (blue shading) or unconventional (yellow shading) protein secretion. mRNA is transcribed in the nucleus and trafficked to be translated by ribosomes on the endoplasmic reticulum (ER). Proteins destined for conventional secretion are transported from the ER to the golgi via ER exit sites (ERES) (4) to the Golgi apparatus within COPII-coated vesicles, where post translational modifications can occur (5). Proteins are then trafficked through the golgi and shuttled in vesicles to the plasma membrane whereby they are secreted into the extracellular space. For unconventional secretion, exosomes develop through intracellular budding of the membrane within multivesicular bodies (MVB), depicted by 7. These MVBs fuse to the with the plasma membrane, releasing the intraluminal vesicles into the interstitium as exosomes. Each number represents sites where lipotoxic states can alter such process. Figure adapted from Montgomery et al., 2019 [1].

1.6.2. Liver secreted proteins & metabolic homeostasis

Liver secreted factors or ‘hepatokines’ are well described [59; 181; 182] with the current knowledge about liver secreted proteins and their impact on distal tissues described in Figure 1.5 and in Table 1.3. Since the liver is intrinsically involved in the regulation of whole-body energy homeostasis (Figure 1.5), it is not surprising that hepatokines are considered attractive targets for T2D therapies. A critical example of this is fibroblast growth factor 21 (FGF21). FGF21 is secreted by the liver with circulating levels being increased in individuals with NAFLD [183; 184]. FGF21 has a variety of endocrine metabolic functions including the reduction of adipose tissue lipolysis [185] and thus reduced fatty acids available for hepatic uptake. FGF21 also dose dependently reduces body weight, hepatic TG content and improves insulin sensitivity [186]. It also acts in an autocrine / paracrine manner to increase hepatic β -oxidation [187]. Thus, it logically follows that FGF21 analogues have been developed and show therapeutic utility in the treatment of dyslipidaemia and hyperinsulinemia in randomised clinical trials [188]. Another example is the hepatokine Fetuin B. The secretion of Fetuin B is increased from steatotic hepatocytes and is increased in the plasma of mice and humans with NAFLD [11; 189; 190] and T2D [11; 191] and recombinant administration of fetuin B impairs glucose tolerance in mice [11]. This demonstrates that hepatokines exhibit crucial roles in the regulation of metabolic homeostasis (Figure 1.5).

In addition to FGF21 and fetuin B, the serine protease dipeptidyl peptidase-4 (DPP4) has been described as a hepatokine that is secreted at greater levels from NAFLD livers (Figure 1.5). It acts by inhibiting the incretin hormones glucagon like peptide-1 and gastric inhibitor peptide that promote insulin secretion and suppress glucagon secretion

[192]. Further, overexpression of DPP4 in the liver of mice fed a HFD exacerbates steatosis and NAFLD severity and insulin resistance [193]. Thus, it is not surprising that sitagliptin, a pharmacological DPP4 inhibitor, is in clinical use for the treatment of T2D [194] although did not improve hepatic steatosis [195].

Hepatokine secretion is remodelled in NAFLD and to date, only two studies have investigated global changes in liver-secreted proteins. Hepatic steatosis without inflammation, hepatocyte ballooning or hepatic fibrosis altered the secretion rate of 20% of all detected hepatokines [11]. This implies that in NAFLD's most primitive stage (steatosis) there are potentially identifiable markers that can be exploited for clinical tests. In the more severe form of NAFLD, mice fed two NASH inducing diets exhibited remarkable remodelling of hepatokine secretion [5]. Given that the correlation between NASH and other metabolic diseases such as T2D is more robust compared to steatosis alone [196; 197], it holds true that NASH secreted proteins are markedly different from both the steatosis and healthy hepatocytes and these changes may be driving features of metabolic syndromes such as T2D.

Ultimately, hepatokines exert a myriad of effects impacting systemic insulin sensitivity, inflammation, as well as glucose and lipid metabolism. The ability to assess the liver secreted proteome in NAFL and NASH (with or without fibrosis secretome) will provide insight into the liver's involvement in peripheral metabolic crosstalk, as well as allowing a unique opportunity to exploit changes in potential biomarkers for the presence and staging of NAFLD / NASH, and the degree of fibrosis.

Table 1.3. List of hepatokines associated with NAFLD and glycaemic control adapted from Watt et al 2019 [2]

Hepatokine	Expression in NAFLD	Contribution in NAFLD	Relationship to glycaemic control
Activin E	Increased	Reduces steatosis [198].	Reduces glucose intolerance in mice [199].
Adropin	Decreased	Suppresses lipogenesis [200].	Improves insulin sensitivity [200].
Cathepsin S	Increased	Secretion increased with NAFLD [201].	Stimulates GLP1 secretion [201].
DPP4	Increased	Exacerbates steatosis [193].	Reduces incretin levels and impairs insulin secretion [192].
Ectodysplasin	Increased	Unknown	Induces insulin resistance in skeletal muscle [202]
Fetuin A	Increased	Unknown	Induces hepatic insulin resistance [203].
Fetuin B	Increased	Unknown	Promotes insulin resistance in myocytes and hepatocytes [204].
FGF21	Increased	Increase fatty acid oxidation [187].	Improves insulin sensitivity [186].
HFREP1	Increased	Increases lipogenesis [205] and NAFLD [206].	Induces skeletal muscles insulin resistance [205].
LECT2	Increased	Unknown	Promotes insulin resistance in skeletal muscle [207].
PEDF	Increased	Increases lipolysis, ablations increase steatosis [208].	Induces skeletal muscle insulin resistance [209].
SeP	Increased	Unknown	Causes insulin resistance in the liver and skeletal muscle [210].
SHBG	Decreased	Suppresses lipogenesis in the liver [211].	Unknown
TSK	Increased	Promotes steatosis to NASH [212; 213].	Associated with insulin resistance [213] however loss does not alter glucose tolerance [214].

DDP4, dipeptidyl peptidase 4; FGF21, fibroblast growth factor 21; HFREP1, hepatocyte-derived fibrinogen-related protein 1; LECT2, leukocyte cell-derived chemotaxin 2; PEDF, pigment derived epithelium factor; SeP, selenoprotein P; SHBG, sex hormone-binding globulin TSK, tsukushi.

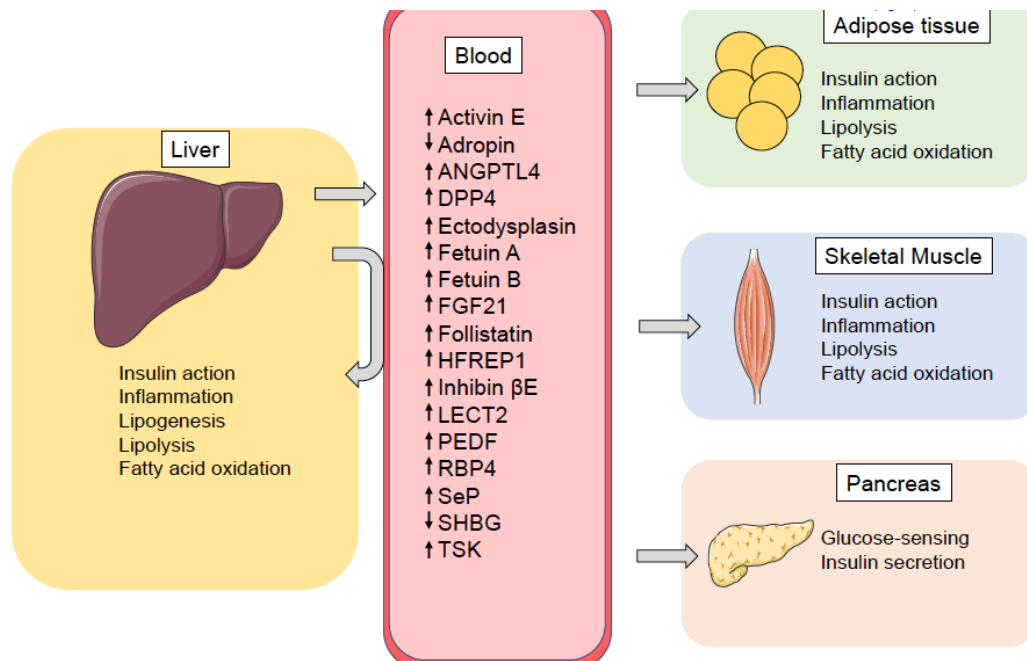


Figure 1.5: Liver-secreted proteins or hepatokines and their impact on metabolic processes in distal tissues. Proteins depicted in the blood are all hepatokines with arrows indicating the hepatokines that are altered with NAFLD. The hepatokines that are increased are typically associated with insulin resistance while those that are downregulated with NAFLD exert positive metabolic effects on such tissues such as the promotion of fatty acid oxidation. ANGPTL4, angiopoietin-like protein 4; DPP4, dipeptidyl peptidase 4; FGF21, fibroblast growth factor 21; HFREP1, hepatocyte-derived fibrinogen-related protein 1; LECT2, leukocyte cell-derived chemotaxin 2; PEDF, pigment derived epithelium factor; RBP4, retinol binding protein 4; SeP, selenoprotein P; SHBG, sex hormone-binding globulin TSK, tsukushi. Figures adapted from Watt et al., 2019 [2].

1.6. Summary and Aims

There is a paucity of non-invasive tests and pharmacological therapies to effectively stratify, and treat patients with NAFLD, NASH and significant fibrosis. Based on the current state of knowledge, hepatokines are dysregulated in NAFLD and can act in an autocrine, paracrine, and endocrine manner to govern energy homeostasis. Whether hepatokine secretion is altered in humans with NAFLD and if these changes can be exploited for a blood test remains to be determined. Further, how hepatokine secretion is altered with the front-line therapy, exercise training, and if these changes can reduce NAFLD severity are yet to be described.

Specifically, the overarching aims of this thesis are:

1. To characterise hepatokine secretion in obese patients with and without NASH, NAFLD and significant fibrosis for biomarker discovery.
2. To exploit changes in hepatokine secretion with NAFLD and or exercise training to identify potential hepatokines that regulate for hepatic steatosis, inflammation, and liver fibrosis.

Chapter 2: General Methods

2.1. Introduction

This chapter describes the methods and materials used throughout the thesis.

2.2. Human Studies

2.2.1. Ethics & patient recruitment

Ethics for the prospective cohort study was obtained from the University of Melbourne Human Ethics Committee (ethics ID 1851533) and approved by The Avenue Hospital Human Research Ethics Committee (Ramsay Health; ethics ID WD00006, HREC reference number 249). Additional patients were recruited with the Alfred Hospital Human Research Ethics Committee (ethics ID GO00005) and by the Cabrini Hospital Human Research Ethics Committees (ethics ID 09-31-08-15), and the study was registered with the Australian Clinical Trials Register (ACTRN12615000875505). Eligible patients with obesity scheduled for primary or secondary sleeve gastrectomy, gastric bypass or the insertion of a laparoscopic-adjustable band were screened to participate and subjects provided informed written consent, and a detailed medical history was taken. All patients were fasted for ~12 h overnight. Exclusion criteria included: Age <18 years, previous gender reassignment, other causes of chronic liver disease and/or hepatic steatosis including Wilson's disease, alpha-1-antitrypsin deficiency, viral hepatitis, human immunodeficiency virus, primary biliary cirrhosis, autoimmune hepatitis, genetic iron overload, hypo- or hyperthyroidism, coeliac disease, as well as recent (within three months of screening visit) or concomitant use of agents known to cause hepatic steatosis including corticosteroids, amiodarone, methotrexate, tamoxifen, valproic acid and/or high dose oestrogens. Further exclusion criteria included potential for alcohol induced liver disease, which was assessed through a

modified version of the alcohol use disorders identification test (AUDIT) [215] where, patients were asked (1) how much alcohol they would drink per day and on average per week, and (2) if they had alcohol free days, (3) if they consumed more than 20 or 30g per day without alcohol free days for females and males, respectively.

2.2.2. Blood and liver biopsy collection

During the bariatric procedure, an anaesthetist collected approximately 25 mL of blood before anaesthesia was induced. The blood was then transferred to 2 x K2E Ethylenediaminetetraacetic acid (EDTA), 2 x SST™ II Advance and 1 x FX 5 mg bio-containers for subsequent storage or clinical/biochemical assessment. All blood samples except for one bio-container of EDTA were sent to Melbourne Pathology™ (Victoria, Australia) for standardised measurement of biochemical and metabolic variables (Table 2.1). During surgery, a 1cm³ wedge liver biopsy was collected from the left lobe of the liver. The liver was cut into two portions. A portion of the liver biopsy was placed in formalin and transported to TissuPath™ (Mount Waverley, Victoria), paraffin embedded and processed for histological analysis. All human histology samples were graded by a single pathologist according to the Clinical Research Network (CRN) NAFLD activity score (NAS) [147] and Kleiner classification of liver fibrosis [216]. The remainder of the liver wedge was placed in oxygenated M199 media (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (P/S; Gibco, USA), and along with the blood sample, were transported to the University of Melbourne for further processing. The blood was centrifuged at 8000 x g for 10 min at 4 °C, and the serum was collected and snap-frozen in liquid nitrogen. Patients were grouped based on histopathology. Patients that were grouped as NASH had a steatosis, inflammation, and ballooning score of ≥ 1 for each parameter; those in the NAFL group

had a steatosis of ≥ 1 and either an inflammation or ballooning score ≥ 1 . Patients diagnosed as No pathology were grouped as those with a steatosis, inflammation, and ballooning score of 0. When grouped to assess NAFLD associated fibrosis, patients were grouped based on the presence of steatosis and the degree of fibrosis. Requirements to be grouped with NAFLD F2-3, required a steatosis ≥ 1 and fibrosis ≥ 2 . For NAFLD F1, steatosis ≥ 1 and fibrosis =1; and NAFLD F0, steatosis ≥ 1 and fibrosis =0 was required; and those with No pathology, had a steatosis =0 and fibrosis =0.

Table 2.1. List of biochemical parameters assessed on blood samples by Melbourne Pathology™ (Victoria, Australia).

Blood Tests performed	
Liver & kidney function tests	ALT, AST, total bilirubin, albumin, eGFR, ALP, GGT, urea, serum creatine,
Glucose homeostasis	Fasting glucose and insulin, HbA1c, C-peptide,
Full blood examination	Hb, WCC, platelets, MCV, MCH, MCHC
Lipid profile	Total cholesterol, HDL, LDL, Non-HDL, and TG
Iron Studies	Serum iron, ferritin
Other liver pathology Screen	Viral hepatitis (Hepatitis B & C) and HIV serology
Vitamins	Vitamin D and B12
Thyroid Function test	TSH

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; GORD, gastro-oesophageal reflux disease; GGT, gamma-glutamyl transferase; Hb, haemoglobin; HbA1c, glycosylated haemoglobin; HDL, High-density lipoprotein; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TG, Triglycerides; TSH, Thyroid-stimulating hormone; WCC, white cell count.

2.2.3. Non-invasive algorithms to stratify NAFLD and NAFLD-associated fibrosis.

Current non-invasive tests to diagnose NAFLD-associated fibrosis perform poorly in obese populations and have not been extensively validated [145; 157]. Therefore, to reinforce the need for novel biomarkers, we assessed the efficacy of current non-invasive scores to effectively stratify patients with NAFLD and significant fibrosis (NAFLD F2-3) and those with NAFLD. The patients' blood test results (Table 2.1 and section 2.1.1.) were used for calculation of non-invasive algorithms to stratify patients in respect to absence/presence of NAFLD and NAFLD-related fibrosis, as listed in Table 2.2.

Table 2.2. List of non-invasive algorithms that stratify NAFLD and NAFLD related fibrosis.

Score	Indices	Calculation	Interpretation fibrosis or NAFLD
APRI score [217]	AST, platelets	$(\text{AST [U/L]}/\text{upper limit normal})/\text{platelet count [10}^9\text{/L]} \times 100$	≥ 0.7 = high risk of fibrosis
BARD [97]	BMI; AST/ALT ratio; T2D	Weighted sum: 1. BMI ≥ 28 = 1 point. 2. AST/ALT ratio ≥ 0.8 = 2 point 3. T2D = 1 point.	Sum of ≥ 2 = High risk of fibrosis
FIB-4 Score [218]	Age AST ALT	$\text{Age} \times \text{AST (IU/L)}/\text{platelet count (}\times 10^9\text{/L)} \times \sqrt{\text{ALT (IU/L)}}$	≥ 3.25 = High risk ≤ 1.3 = Low risk of fibrosis
Forn index [157; 219]	Platelets, GGT, age, cholesterol	$7.811 - 3.131 \times \log_e(\text{platelet [10}^9\text{/L)}) + 0.781 \times \log_e(\text{GGT [U/L)}) + 3.467 \times \log_e(\text{age [years]}) - 0.014 \times \text{cholesterol [mg/dl]}$	≥ 6.9 = High risk ≤ 4.2 = Low risk of fibrosis
Hepatic steatosis index [220]	ALT, AST, BMI, T2D, sex	$8 \times \text{ALT/AST ratio} + \text{BMI (+2, if T2D; +2, if female)}$	≥ 36 = High risk ≤ 30 Low risk of steatosis
NAFLD fibrosis score [221]	Age, hyperglycaemia, BMI, Platelet count, Albumin, AST/ALT ratio	$-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2) + 1.13 \times \text{IFG or diabetes (yes=1, no=0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet (}\times 10^9\text{/L)} - 0.66 \times \text{albumin (g/dL)}$	≥ 0.676 = high risk ≤ -1.455 = low risk of fibrosis
NAFLD liver fat score [222]	Metabolic syndrome, Type 2 diabetes, insulin, ALT and AST	$-2.89 + 1.18 \times \text{metabolic syndrome (yes = 1 / no = 0)} + 0.45 \times \text{type 2 diabetes (yes=2/no=0)} + 0.15 \times (\text{serum insulin (mU/L)} + 0.04 \times \text{ALT (U/L)} - 0.94 \times \text{AST/ALT})$	Score ≥ -0.640 = high risk of steatosis

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; Fib-4, Fibrosis-4; GGT, gamma-glutamyl transferase; T2D, type 2 diabetes; WCC, white cell count. a denotes the prediction of fibrosis. b indicates the prediction of NAFLD. Upper limit normal for AST = 35.

2.2.4. Liver slicing and culture

The liver wedge was dissected into 3 x 15 mg pieces which were snap-frozen in liquid nitrogen for subsequent analysis. The remainder of the liver was embedded in 3% SeaPlaque agarose (Lonza BioScience, USA) using a Tissue Embedding Unit (Alabama Research and Development, USA) and sliced on the Alabama R&D Tissue Slicer (Alabama Research and Development, USA) in 500 mL of oxygenated phenol red-free Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) with 1% P/S at 300 μ m thickness. The liver slices were washed in Phosphate Buffered Saline (PBS; Gibco USA) and cultured in oxygenated Medium 199 (M199) containing 1% P/S for 1 h. Subsequently, the liver slices were washed with PBS and cultured in 1 mL EX-CELL 325 protein free medium (Sigma-Aldrich, Australia) for 16 h. The following day, the liver slices were weighed and snap-frozen in liquid nitrogen, while the conditioned media was collected and centrifuged at 300 x g at 4 °C for 10 min. Finally, the supernatant was transferred to a new tube, snap-frozen and stored at -80 °C for subsequent proteomics analysis.

2.2.5. Processing of human conditioned media for proteomic analysis

For the assessment of liver-secreted proteins contained within the conditioned media, the media was concentrated using Amicon Ultra-4 Centrifugal Filters (Merck, USA) at 4000 x g for 45 min, the concentrated sample washed in 2 mL 50 mM Tris-HCl, pH 8.0 + 150 mM NaCl at 4000 x g for 45 min, transferred to Eppendorf tubes and the protein content determined as per 2.5.14. Protein disulphide bonds were reduced through the addition of 10 mM TCEP (Tris(2-carboxyethyl) phosphine) at 65 °C for 20 min. The sample was mixed with 6.6x (v:v) of 5 M Urea, added to Microcon-30kDa Centrifugal Filter (Sigma Aldrich, Australia), and centrifuged at 14,000 x g and 10 °C for 15 min.

This was followed by the addition of 200 μ L of 5 M urea, centrifugation at 14,000 x g at room temperature for 15 min, and the flow-through was discarded. Chloroacetamide was added to a final concentration of 10 mM for the centrifugal filters to alkylate proteins, and samples incubated for 20 min in the dark, and centrifuged at 14,000 x g. Proteins were washed 3 x with 100 μ L of 5M urea and 3x with 100 μ L 50 mM ammonium bicarbonate (pH 8.5), each wash step followed by centrifugation at 14,000 x g and 10 °C for 15 min and discarding of flow-through. After the last wash step, a digestion solution was added to the centrifugal filter (1 μ g Lys-C/100 μ g protein in 75 μ L of 50 mM ammonium bicarbonate for 2 h in a wet chamber (sealed plastic box with water rising 0.5cm from the bottom of the box) at 37 °C. Subsequently, 2 μ g trypsin/100 μ g protein was added, and the samples were incubated in a wet chamber at 37 °C for 16 h to allow for trypsin-mediated protein digestion. The following day, peptides were eluted from the centrifugal filters through the addition of 2 x 40 μ L of 50 mM ammonia bicarbonate (pH 8.5) and 1 x 50 μ L of 0.5 M NaCl; with each step being followed by centrifugation at 14,000 x g at room temperature for 15 min. The eluted peptides were acidified with 10% trifluoroacetic acid (TFA) to a pH of 3, dried in a SpeedVac concentrator (Eppendorf Concentrator Plus, Germany) and stored at -80 °C. SDB-RPS (polystyrene-divinylbenzene, reversed-phase sulfonate discs) (Sigma-Aldrich, Australia, Cat#66886- U) were doubled stacked and punctured with an 18-gauge needle and mounted in 200 μ L tips (Eppendorf, Germany) to make SDB-RPS Stage tips. The filter tips were washed followed by the addition of 1 x 50 μ L of acetonitrile (ACN), 1x 50 μ L of 30% methanol, 1 x 50 μ L 0.2% TFA and 1 x 1% TFA to the top of the tip and centrifugation at 1000 x g for 2 min, with the flow through discarded at each step. The dried-down peptides were resuspended in 50 μ L of ACN, added to the top of the equilibrated filter tip and centrifuged at 1000 x g for 2 min. The peptides were washed

once with 100 μ L of 1% TFA and 99% ethyl acetate, and once with 100 μ L of 5% ACN and 0.2% TFA in MilliQ-water. To elute the peptides 60 μ L of 80% ACN / 5% ammonium hydroxide (w:v) in water was added to each tip, the tips centrifuged at 1000 x g for 2 min and the eluted samples dried in a SpeedVac concentrator (Eppendorf Concentrator Plus, Germany). Dried peptides were resuspended in 30 μ L of 2% ACN and 0.1% TFA and sent to the Charles Perkins Centre (The University of Sydney, Australia) for liquid chromatography tandem mass spectrometry (LC-MS/MS).

2.2.6. Liquid chromatography tandem mass spectrometry of liver conditioned medium

Three microliters of peptides were injected and separated by Dionex 3500 ultra-high performance liquid chromatography (UHPLC, (Thermo fisher, USA)) coupled to a Q-Exactive HF-X mass spectrometer (ThermoFisher, USA) in the positive polarity mode as previously described [223]. Peptides were resolved on a gradient set at 2 – 35% ACN containing 0.1% formic acid over 60 min at 800 nL/min, and separated on an in-house 100 μ m x 20 cm column with an integrated emitter using a Sutter laser puller (1.9 μ m particle size, C18AQ; Dr Maisch). Peptides were ionised by electrospray ionisation at 2.3 kV. Data was acquired in positive ionization mode and the instrument was operated in data-independent acquisition mode (DIA). The DIA-MS method consisted of an MS1 scan that is acquired between 350 – 1650 m/z at a resolution of 30,000 and 3×10^6 automatic gain control (AGC), with a 50 ms injection time. Subsequently, using automatic injection and step normalized collision energies of 22.5, 25 and 27.5 at 15,000 resolution and 1×10^5 AGC, 3×10^6 AGC with higher-energy C-trap dissociation (HCD), 20 variable window sized DIA isolation and fragmentation occurred [224].

2.2.7. Human proteomic data analysis

All DIA data was processed using Spectronaut v14 (Biognosys, Schlieren, Switzerland) with default settings employing retention time and mass recalibration. The default settings included retention time prediction type set to dynamic indexed retention time. Mass calibration was set to local mass calibration. Methionine oxidation and cysteine carbamidomethylation were set as variable modifications and fixed modifications, respectively. As previously described, interference correction on the MS1 and MS2 scans were enabled to remove fragments from quantification based on interfering signals, keeping at least three fragments per scan for quantification [224]. The data was searched against the human uniprot database (canonical and isoform – downloaded 16/03/2021) and the quantification was assessed on the MS2 extracted ion chromatograms. This was set at 3-6 fragment ions with >450 m/s. Labelling was set to label-free quantification with a minimum detection rate of 2. All other search parameters were default settings. The false discovery rate of the spectral peptide match to Uniprot proteins was set at 1%. The result output was further processed with Perseus (Version 1.6.6.0) [225], a module from the MaxQuant suite.

Statistical analysis of proteomic data and differential expression analysis of NAFLD-associated fibrosis or NASH, stratifications were Log₂ transformed and median normalised. Differential expression analyses of proteins by NAFLD-associated fibrosis or NASH severity (e.g. NAFLD F2-3 vs No pathology or NASH vs no pathology) was conducted using NormalyserDETM software [226] and assessed with a multi-group comparison by using median normalisation and robust linear regression and linear models for microarray with a one-way analysis of variance (ANOVA) and 2-way comparisons against each group using the program. This allowed the estimation of

variance of each protein and reporting of the differential expression (or amounts) in secreted proteins between all groups. Subsequently, students 2-way t-tests with Benjamini-Hochberg false discovery rate was performed and significant proteins were assigned based on $q < 0.05$. Data visualisation was performed in Prism 9.0 (GraphPad, USA).

2.2.8. Plasma proteomic studies

Protein extraction, digestion, and clean-up of human plasma were performed as previously described [227]. Briefly, one microliter of plasma was added to 24 μL of 1% sodium deoxycholate, 10 mM TCEP, 40 mM chloroacetamide, and 100 mM Tris-HCl pH 8.5) in a 96-well plate (Eppendorf, Germany). Plates were sealed with a silicone mat (Eppendorf, Germany), and proteins were denatured, reduced, and alkylated by mixing on a Thermomixer-C (Eppendorf, Germany) with a ThermoTop (Eppendorf, Germany) at 95 °C for 10 min at 1,000 rpm. Samples were cooled to room temperature and diluted to 10-fold with water. Proteins were digested into peptides with the addition of endoproteinase Lys-C and trypsin (both 1:100 ratio, $\mu\text{g}/\mu\text{g}$ protein) and incubated at 37 °C for 16 h at 1,000 rpm in an Eppendorf Thermomixer-C with a ThermoTop (heated lid). Peptides were diluted 2-fold with 99% ethyl acetate/ 1% TFA to form a 49.5% ethyl acetate/ 0.5% TFA solution.

SDB-RPS tips were generated as described in 2.2.5. Tips were washed with 1 x 100 μL of 100% ACN and centrifuged at 1000 x g for 1 min, and the flow-through was discarded. This was followed by addition of 1x 100 μL of 0.1% TFA in water and 1x 30% methanol/1% TFA in water, each wash step followed by centrifugation at 1000 x g for 3 min and discarding of flow through. Next, tips were loaded with $\sim 10\mu\text{g}$ of peptides

and washed 2x with 100 μ L of 99% ethyl acetate/ 1% TFA and 1x with 100 μ L of 0.2% TFA in water. Peptides were eluted by addition of 100 μ L of 5% ammonium hydroxide/80% ACN followed by centrifugation at 1000 x g for 5 min. Samples were dried using a GeneVac EZ-2 (Genevac, UK) at 40 °C for 1 h. Dried peptides were resuspended in 30 μ L of 5% (v:v) formic acid and stored at 4 °C until analysed by LC-MS.

LC-MS was performed as previously described [227; 228]. An Exion LC system (Sciex, USA) was used to analyse peptide samples and was set up with a 5 cm $\times$ 2.1 mm, 1.9 μ m particle, C18 column (Agilent, Australia), which was coupled to a Turbo-V ESI source on a Sciex 6600 mass spectrometer. The digested peptides (10ug) in 5% (v:v) formic acid were directly injected onto the column and resolved over a gradient of 5-40% ACN at a flow rate of 1mL min⁻¹ for 5 min at 40°C. Peptides were ionised by electrospray ionisation at 5.6 kV. The scanning SWATH acquisition was for 8 min per injection and scanned from 400-900 m/z with a 10 ms accumulation time.

2.2.9. Plasma proteomic data analysis

Raw data were processed using DIA-NN 1.8 [229] with a search performed against the whole human proteome (Uniprot – canonical and isoform, downloaded 16/03/2021). A library-free strategy was employed with match between runs enabled. Cysteine carbamidomethylation was set as a fixed modification. N-terminal acetylation and methionine excision were set as variable modifications. Trypsin cleavage was allowed to have 1 missed cleavage event. Statistical analysis was performed on MaxLFQ normalised protein abundances [230] grouped by NASH and NAFLD-associated fibrosis. The data was uploaded into Perseus version 1.6.10.50 [225], a module from the

MaxQuant suite and the data was normalised using Log₂ transformation and median normalisation.

2.2.10. EMILIN1 ELISA

The concentration of EMILIN1 in human plasma was determined by an enzyme-linked immunosorbent assay from Aviva Systems Biology (Cat No: OKCD00659, CA, USA).

2.3. *In vivo* mouse procedures

2.3.1. Animal housing

All procedures conducted complied with the National Health and Medical Research Council guidelines concerning the use and care of experimental animals and were approved by the University of Melbourne Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee (ID 20063 and 2015165). Mice used throughout were 8-10 weeks old male C57BL/6J (obtained from Animal Resources Centre, Canning Vale, Australia). All mice had *ad libidum* access to food and water and were maintained in a temperature-controlled room (22°C ± 1°C) with a 12-h light/dark cycle. Body weight was measured weekly.

2.3.2. Dietary induced models of NAFLD, NASH and fibrosis

To induce NAFLD and NAFLD-associated fibrosis, mice were fed one of three diets: (1) a high-fat (40% calories from lard) high-fructose (30% calories from fructose) high-cholesterol (20g/kg) diet (SF16-033; Specialty Feeds, Australia) fed for 32-40 weeks, hereafter denoted as CHOL; or (2) a methionine and choline deficient (MCD) diet (SF16-035; Specialty Feeds, Australia) fed for 8 or 12 weeks. This diet contains 44% of

calories from fat (lard) and is low in methionine (1g/kg) and choline (70mg/kg). The MCD respective control diet termed methionine choline supplemented (MCS) diet in this study group received the same dietary composition, however with higher levels of methionine (6.1g/kg) and choline (2.6g/kg) (SF16-034; Specialty Feeds, Australia). (3) Lastly, to induce obesity, glucose intolerance and hepatic steatosis, mice were fed a HFD, (High Fat Rodent Diet SF04-001, 43% energy from fat, Specialty Feeds, Australia) for a maximum of 14 weeks. Control mice in this study, received standard rodent chow (5% of energy from fat, Specialty Feeds, Australia). For validation of presence of NAFLD, NASH and fibrosis, a piece of liver was fixed in formalin and histologically processed as described in 2.5.12.

2.3.3. Exercise training studies

Mice fed a HFD for 5 weeks were acclimatized to the treadmill for 3 days prior to experimental testing. This protocol consisted of increasing the intensity and duration of the running on the enclosed treadmill without mice reaching fatigue as previously described [231].

To assess whole body adaptations to exercise, mice were placed on the treadmill and ran at a speed of 10 m/min. Following 2 min of running, the speed was increased to 12 m/min for 2 min. This process was repeated until mice reached volitional exhaustion defined as more than 10 seconds spent at the treadmill's base despite manual encouragement. The running time until exhaustion was recorded.

2.3.3.1. Endurance exercise training

Mice were grouped based on body weight, glucose tolerance and running time until exhaustion, prior to exercise intervention. Mice in the exercise group underwent an endurance exercise running protocol previously described [232] and shown in Table 2.3 for 6 weeks at 5% grade (incline). Mice in the Sedentary group were placed on a stationary treadmill for the same duration as the exercise trained mice.

Following 6 weeks of exercise training, all mice undertook another maximal velocity test to assess improvements in endurance capacity. Two days later, mice were anaesthetised using 4% isoflurane (SomnoSuite, Kent Scientific, USA), the mixed quadriceps muscle was dissected for later analysis (see 2.5.7 and 2.5.8.) and hepatocytes were isolated (see 2.4.1.) and cultured for the proteomic evaluation of the intracellular proteome and hepatokine secretion (see sections 2.5.1.-2.5.3.).

Table 2.3. Schedule, speed and time of the endurance exercise intervention as previously described in Borg et al., 2012. Table taken from Borg et al., 2012 [232].

Week	Day	Speed and Time	Week	Day	Speed and Time
1	Mon	12m/min (30min)	4	Mon	15 m/min (50min)
	Tues	12m/min (30min)		Tues	15 m/min (55min)
	Wed	12m/min (30min)		Wed	15 m/min (60min)
	Thu	13m/min (30min)		Thu	16 m/min (60min)
	Fri	13m/min (30min)		Fri	16 m/min (60min)
	Sat	-		Sat	-
	Sun	-		Sun	-
2	Mon	14 m/min (30min)	5	Mon	16 m/min (60min)
	Tues	14 m/min (30min)		Tues	16 m/min (65min)
	Wed	14 m/min (40min)		Wed	16 m/min (65min)
	Thu	14 m/min (40min)		Thu	16 m/min (70min)
	Fri	15 m/min (40min)		Fri	17 m/min (70min)
	Sat	-		Sat	-
	Sun	-		Sun	-
3	Mon	15 m/min (40min)	6	Mon	17m/min(70min)
	Tues	15 m/min (45min)		Tues	17 m/min (70min)
	Wed	15 m/min (45min)		Wed	18 m/min (70min)
	Thu	15 m/min (50min)		Thu	18 m/min (70min)
	Fri	15 m/min (50min)		Fri	19 m/min (70min)
	Sat	-		Sat	-
	Sun	-		Sun	-

2.3.4. Adeno-associated virus – mediated overexpression

Adeno-associated virus (AAV) was generated at Vector Biolabs (Malvern, PA, USA). For overexpression purposes, an AAV serotype 8 (AAV8) driven by a thyroxine binding globulin (TBG) promoter, containing a cDNA sequence specific for murine *Emilin1* or *Syndecan-4* (AAV8-TBG-m-EMILIN1; AAV8-TBG-m-SDC4), was injected via the tail vein at 0.5×10^{12} or 1×10^{12} genome copies/mouse, respectively. Control mice were injected with the same vector and dosage (AAV8-TBG-Control). Subsequent whole body metabolic assessment was carried out as outlined in sections 2.3.4. - 2.3.7. Eight or 12 weeks following AAV administration for *Sdc4*-AAV or *Emilin1*-AAV, respectively, precision-cut liver slices were generated using the Alabama tissue slicer (Alabama research and development, USA) as described in section 2.2.4, hepatic metabolism was assessed (see section 2.4.2-2.4.7), and immunoblotting (see section 2.5.5, 2.5.6) and gene expression analysis (section 2.5.9.) carried out.

2.3.5. Body composition and energy expenditure

Body composition (fat and lean mass) in awake, constrained mice was assessed by nuclear magnetic resonance (MiniSpec LF50, Bruker, USA), following manufacturer's instructions.

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Whole body energy metabolism was assessed using a 16-chamber Promethion Metabolic Cage System (Sable Systems International, USA), including locomotor activity, energy expenditure, food intake and respiratory exchange ratio (RER). The RER was determined as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2). Mice were housed individually for 48 h and the above outcome measures collected following 12 h of acclimatisation to the metabolic chamber. Flow

rates into all chambers were set at 2000 mL/min and room air (~21% O₂ and 5% CO₂) was passed through into the chambers. Gas sensor calibration was conducted before experiments. Food intake was calculated as the mass of food at the end of the 48-h period subtracted from food mass following the 12 acclimatisation. Chambers had photobeams to assess physical activity and physical movement at speeds ≥ 1 cm/s was considered directed locomotion.

2.3.6. Glucose tolerance test

Mice were fasted for four h (0700-1100 h). Mice received an oral glucose load (2 g/kg body weight), and blood was obtained from a tail nick before and at 15, 30-, 45-, 60- and 90-mins following glucose administration for glucose assessment (Accu-Check, Roche, USA). An additional 20 μ L blood was collected at 0, 15 and 30 min, centrifuged at 4000 x g at 4°C for 10 minutes to separate plasma for later assessment of plasma insulin (2.5.10).

2.3.7. Insulin tolerance test

Mice were fasted for four h (0700-1100 h), then injected i.p. with insulin (Actrapid, 1 U/kg body weight). Blood was obtained from a tail nick before and at 10, 20, 30, 45 and 60 mins for glucose assessment (Accu-Check, Roche, USA).

2.4. Mouse surgical procedures and *ex vivo* assessments

2.4.1. Hepatocyte isolations

Mice were anaesthetised with an intraperitoneal injection of ketamine/xylazine (v:v) that was diluted four fold in saline. A lateral incision, followed by a midline incision

across the lower abdomen was made to expose the liver and other organs. A small liver biopsy (~5mg of tissue) was taken from mice that underwent exercise training or their Sedentary controls to assess liver triglyceride levels (see 2.5.6), all other mice were not biopsied. A 23-gauge catheter (BD vacutainer Safety-Lok) was inserted into the inferior vena cava and the hepatic portal vein was cut. The catheter was connected to peristaltic tubing (Gilson F117946) and a circulating pump which circulated pre-warmed Hank's Buffered Salt Solution (HBSS) (138 mM NaCl, 5.6 mM Glucose, 5.4 mM KCl, 0.4 mM KH_2PO_4 , 50 mM $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$, 0.3 mM Na_2HPO_4 , 0.44 mM Na_2HPO_4 , 0.44 mM NaHCO_3 and 0.5M $\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_{10}$) at a pH of 7.4 and at 37 °C into the liver was infused at a rate of 5 mL/min for 15 min to isolate hepatocytes at the desmosomes. Following this, the tubing pumping the fluid was transferred to another falcon tube containing 50 mL of HBSS as described above, with 1 mg/mL of Collagenase H (Roche, cat: 11 074 032 001) and 2 mM of CaCl_2 , and circulated through the liver for approximately 10 min to facilitate the isolation of hepatocytes from the liver parenchyma. Livers were removed and minced with scissors, passed through a cell strainer (100 μM Nylon) into a falcon tube and washed in cold HBSS buffer containing 2mM CaCl_2 . Hepatocytes were pelleted by centrifugation at 50 g for 3 min and the supernatant was removed with a pasteur pipette. This step was repeated to remove dead cells, debris and to separate hepatocytes from other non-parenchymal cells residing in the liver. Subsequently, hepatocytes were resuspended in 3 mL of cold M199 media containing insulin (100 nmol/L), 100 nmol/L dexamethasone, 10% bovine serum albumin (BSA; Merck, USA) (10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (PenStrep, Gibco, USA).

2.4.1.1. Cell viability and plating

To assess the viability of the isolated hepatocytes, a 10 μ L aliquot was taken and diluted 1 in 10 with 0.1% trypan blue (Gibco, USA) and the number of live and dead cells were counted on each corner of the hemocytometer (Boeco, Germany). The number of viable cells was assessed as per equation 1 considering dilution and the haemocytometer conversion factor, 2.5×10^6 cells were plated per well in a collagen pre-coated with rat tail collagen in a 6 well plate and allowed to adhere to the plate for 4 h. Following this, the hepatocytes were either utilized to assess protein secretion (see 2.4.1.2) or used for recombinant syndecan-4 studies as per 2.4.1.3 and 2.4.1.4.

$$\text{Viable cell count} = (\text{average total cell count} - \text{average dead cell count}) \times 10 \times 10^4$$

Equation 1: Assessment of cell viability.

2.4.1.2. Hepatocyte secretion

Hepatocytes isolated from exercise or sedentary mice were washed 3 x with PBS and cultured in 2 mL of EX-CELL 325 PF CHO Serum-Free medium (Merck, USA) for 24 h, maintained at 37 °C and 5% CO₂. The conditioned media containing all secreted factors was collected, centrifuged at 300 x g for 10 min and stored at -80 °C until proteomic assessment (2.5.1).. Hepatocytes were lysed in 100 mmol/L Tris-HCl + 1% sodium deoxycholate (w:v) (pH 8.1) and snap frozen in liquid nitrogen until proteomic assessment (2.5.2)

2.4.1.3. Acute recombinant SDC4 studies

Hepatocytes isolated from control mice were plated in a 6 well plate and cultured overnight in basal media (Gibco M199 media +100 U Penicillin/Streptomycin, 100 nmol/l Dexamethasone, 1 nmol/l insulin) overnight. Hepatocytes were washed 3x with

PBS and incubated. For 2 h in 1 g/L glucose DMEM media (Gibco, USA) containing 1 µg/mL of recombinant FC-SDC4 (referred to subsequently as rSDC4) (Cat: 50726-M02H, JOMAR Life Research, Australia) and assessed for lipid metabolism (see 2.4.2.).

2.4.1.4. Assessment of lipid droplet remodelling

Hepatocytes isolated from high fat fed mice (section 2.4.1) were maintained as detailed in section 2.4.1.2, with the following amendments. Hepatocytes were plated and cultured on chambered coverslips (Sarstedt, Germany) and treated with or without rSDC4 (1 µg/mL) for 48 hours. Culture medium was then incubated with 100 nM MitoTracker Deep Red (Molecular Probes, USA) and 2µM BODIPY FL C 16 (ThermoFisher Scientific, USA) for 20 min or 15 min, respectively. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific, USA) (1µg/mL) to visualise nuclei. Confocal microscopy was performed as described in section 2.5.11.

2.4.2. Assessment of fatty acid metabolism

Radiometric assays were performed to track the fate of carbons in the metabolic pathways. For all murine experiments, precision-cut liver slices were generated as described in section 2.2.4. Following 1 h of settling in M199 media (Gibco, USA), approximately 20 mg of slices were washed in PBS (Gibco, USA) and moved to a glass vial containing “tracer medium” comprised of oxygenated low-glucose DMEM (1g/L glucose, Gibco, USA), 0.5 mM palmitic acid (Sigma-Aldrich, #110K0301) and 1 µCi/ml [1-¹⁴C] palmitic acid (GE Healthcare, CFA23) conjugated to 2% (w:v) BSA for a total of 2 h at 37 °C in a shaking water bath. A 3 x 50 µL aliquot of this radioactive

medium was placed in a polyethylene scintillation vial with 2 mL of Ultima Gold scintillation fluid and counted on the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA) to determine the specific activity as described in equation 2. To trap $^{14}\text{CO}_2$, the glass vial further contained a 1.7 mL Eppendorf tube filled with 300 μL of 1 M NaOH. After 2 h, liver slices were removed from the glass scintillation vials and washed in ice-cold PBS (Gibco, USA) and stored at $-80\text{ }^{\circ}\text{C}$. Subsequently, 1 mL of 1 M perchloric acid was added to the glass vial, leading to the release of $^{14}\text{CO}_2$ from the acidified assay buffer and subsequent capture in the basic NaOH solution. The vial was immediately capped and left for 12 h at $4\text{ }^{\circ}\text{C}$. Following this, the radioactivity in the NaOH phase, representing complete oxidation of fatty acids, was counted on the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA).

$$\text{Specific Activity (CPM/nmol)} = \frac{\text{Average total radioactivity substrate (CPM)}}{\text{total nmol of substrate (nmol)}}$$

Equation 2: Determination of specific activity. Total reactivity of the assay buffer divided by the total nanomole of the substrate (non-radioactive palmitate) present in the medium. CPM, counts per minute.

To assess the intracellular fate of the radiolabelled palmitic acid, frozen liver slices were homogenised using the Biogen Pro 200 homogeniser (Pro Scientific, USA) in a glass vial containing 1.8 mL of 2:1 (v:v) chloroform: methanol. To induce phase separation, 600 μL of 0.9% NaCl was added, the samples were vortexed and incubated for 30 min at room temperature. The samples were then centrifuged for 10 min at $4\text{ }^{\circ}\text{C}$ and $2000 \times g$ (5810R, Eppendorf, USA), leading to phase separation, with the top phase containing acid-soluble metabolites (ASM; intermediates of β -oxidation representing incomplete fatty acid oxidation) and the bottom organic phase containing lipids. Radioactivity within the ASM fraction was assessed by adding 900 μL of the aqueous phase to 2 mL

of Ultima Gold scintillation cocktail in a polypropylene vial. Radioactivity was measured using the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA) and total oxidation was calculated as described in Equation 3. Following this, 1 mL of the organic phase was transferred to another glass tube, the solvent containing lipids was dried under N₂ stream at 37 °C and the dried lipid stored at -20 °C until further analysis.

$$\text{Oxidation rate (nmol/mg /h)} = \frac{\left(\frac{{}^{14}\text{CO}_2 \text{ (CPM)} + \text{ASM (CPM)}}{\text{Specific activity} \left(\frac{\text{CPM}}{\text{nmol}} \right)} \right)}{\text{Tissue mass (mg)/ time (h)}}$$

Equation 3: Calculation of fatty acid oxidation in tissues. The complete oxidation to CO₂ plus the incomplete oxidation represented as acid soluble metabolites (ASM). This value is normalised to tissue mass and time. CPM, counts per minute. Oxidative rate was calculated the same with hepatocytes except for using total protein instead of tissue mass.

2.4.3. Lipid extraction for incorporation into total lipids

The dried down organic phase was further assessed for incorporation of palmitic into various lipid species. Lipids were resuspended in 200 µL of 95 % ethanol, and 100 µL was transferred to a 2 mL of Ultima Gold scintillation cocktail in a polypropylene vial. Vials were assessed for radioactivity using the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA) as described in equation 3. Fatty acid uptake was calculated by summing fatty acid oxidation and total lipid storage. The remaining lipid suspension was dried under N₂ stream at 37 °C and the dried lipid stored at -20 °C until further analysis.

2.4.4. Thin-layer chromatography & determination of fatty acid incorporation into complex lipids

The incorporation of fatty acids into triglycerides and other lipid classes was assessed by thin-layer chromatography (TLC). A mixture of lipids (used as an internal standard) consisting of 2.5 mg/mL of cholesterol linoleate (Sigma-Aldrich, USA), 1.75 mg/mL of glycerol tripalmitate (Sigma-Aldrich, USA), 875 µg/mL of dipalmitin (Sigma-Aldrich, USA), 1 mg/mL of C-16 ceramide and 2.5 mg/mL of L- α -phosphatidylcholine (Sigma-Aldrich, USA) was dissolved in 2:1 (v:v) chloroform: methanol to permit lipid identification following TLC. Lanes were formed with a width of 1.5 cm on a glass-backed silica gel 60-plate (AnalTech, USA). Dried down lipid samples (from section 2.1.4.1) were resuspended in 30 µL of the lipid mixture, vortexed and spotted with a syringe in their respective lane 2 cm from the base of the plate. The blanks consisted of 60 µL of the standard mixture and were spotted 2cm from the base of the plate. The samples were then subjected to a three-step solvent separation to resolve the lipids, as previously described [233]. Briefly, the TLC plate was placed in chloroform-methanol-water (65:25:4, v/v) until the solvent migrated 10 cm and was thereafter air-dried for 10 min. The plate was then placed in hexane-diethyl ether-acetic acid (75:35:1, v/v) until the solvent migrated 18 cm from the base of the plate and again air-dried for 10 min. The plate was then run again to 18 cm in the hexane-diethyl ether-acetic acid solution and air-dried. The TLC plate was sprayed with 0.02% (w:v) dichlorofluorescein dye (Sigma-Aldrich, USA), and air dried for 10 min to allow visualisation of lipids under UV light on the UV Light Transilluminator (Analytik Jena, USA). Lipid bands corresponding to cholesterol, triglycerides, diglycerides, ceramides, and phospholipids

were then scraped into polyethylene scintillation vials containing 2 mL of Ultima Gold scintillation cocktail. Samples were vortexed, and their radioactivity was measured on the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA). Substrate incorporation into lipids was calculated using equation 4. Total lipid storage was defined as the sum of lipid species assessed in TLC.

For ‘conditioned medium’ experiments, medium containing the secreted products from the hepatocytes of exercise or sedentary mice was applied 1:1 (v:v) with tracer medium. Fatty acid metabolism was assessed as described above. All values were normalised to tissue mass.

For assessment of lipid metabolism in isolated hepatocytes, the method was performed the same with few exceptions. Firstly, lipid metabolism was assessed in a 6 well plate maintained at 37 °C and 5% CO₂. Following the 2 h incubation with oxygenated low-glucose DMEM (1g/L glucose, Gibco, USA), 0.5 mM palmitic acid (Sigma-Aldrich, #110K0301, Australia) and 1 µCi/ml [1-¹⁴C] palmitic acid (GE Healthcare, CFA23, USA) conjugated to 2% (w:v) BSA, the incubation medium was added into the glass vial with 1 mL of 1M perchloric acid and 300 µL of 1M NaOH in an Eppendorf tube to capture ¹⁴CO₂. Hepatocytes were lysed in 250 µL of PBS with 0.01% Triton X100 (Sigma Aldrich, Australia), 50 µL was used to assess protein content (described in 2.5.14). Two hundred microliters of lysed hepatocytes were added to glass vial in 12 x 75 mm glass tubes containing 1.8 mL of 2:1 (v:v) chloroform: methanol to extract lipids. Hepatocytes and precision-cut liver slices were processed the same from

following 2:1 (v:v) chloroform: methanol suspension. Fatty acid metabolism in isolated hepatocytes was normalised to intracellular protein content (determined in 2.5.10).

$$\text{Lipid (nmol/mg /h)} = \frac{\left(\frac{\text{Lipid (CPM)} - \text{Blank (CPM)}}{\text{Specific Activity} \left(\frac{\text{CPM}}{\text{nmol}} \right)} \right)}{(\text{Tissue mass (mg)} / \text{time (h)})}$$

Equation 4: Calculation of substrate incorporation into various lipids after incubation with ^{14}C palmitic acid. CPM, counts per minute. Blank represents the background radioactivity of the Ultima Gold scintillation cocktail.

2.4.5. Assessment of glucose metabolism in precision-cut liver slices

Liver slices were prepared as per sections 2.2.4 and 2.4.2. Glucose metabolism was assessed like fatty acid metabolism 2.4.2-2.4.3. Following 1 h of settling in M199 media (Gibco, USA), approximately 20 mg of slices were washed in PBS (Gibco, USA) and moved to a glass vial containing a 0.5 mL Eppendorf tube filled with 300 μL of 1 M NaOH, oxygenated low-glucose DMEM (1g/L glucose, Gibco, USA), 0.5 mM palmitic acid (Sigma-Aldrich, #110K0301) and 1 $\mu\text{Ci/ml}$ of (D-[$^{14}\text{C}(\text{U})$]-Glucose (GE Healthcare, CFA23) with 2% (w:v) BSA. Total activity and specific activity were determined as 2.4.2 and equation 1. Liver slices were cultured in glass vial was cultured for 2 h at 37 $^{\circ}\text{C}$ in a shaking water bath. After 2 h, liver slices were removed from the glass scintillation vials and washed in ice-cold PBS (Gibco, USA) and stored at -80 $^{\circ}\text{C}$. Complete oxidation was determined as 2.4.2 by trapping $^{14}\text{CO}_2$ in NaOH by the addition of 1 mL of 1 M perchloric acid in a glass vial. Glucose oxidation calculated as per equation 5.

$$\text{Glucose oxidation rate (nmol/mg/h)} = \frac{\left(\frac{{}^{14}\text{CO}_2 \text{ (CPM)}}{\text{Specific Activity} \left(\frac{\text{CPM}}{\text{nmol}} \right)} \right)}{\text{Tissue mass (mg)} / \text{time (h)}}$$

Equation 5: Calculation of glucose oxidation rate. CPM, counts per minute.

To assess de novo lipogenesis, frozen liver slices were homogenised in glass tubes containing 1.8 mL of 2:1 (v:v) chloroform: methanol using the Biogen Pro 200 homogeniser (Pro Scientific, USA). Lipids were extracted as described in 2.4.2. Total lipid storage was assessed as described in 2.4.3 and calculated by equation 3.

2.4.6. 2-Deoxyglucose uptake

On the day of culling, epididymal adipose tissue (eWAT) explants and intact tendon-to-tendon soleus muscles were placed into glass vials containing 1 mL (v:v) of oxygenated Krebs Daily Buffer (120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM Na₂HCO₃ and 5.5 mM glucose) and 0.1% BSA, 8 mM mannitol and 2 mM pyruvate, and with medium containing secreted factors from hepatocytes of exercise or sedentary mice, at pH 7.2 for 30 min at 30 °C in a shaking water bath. Following this, the medium was removed and replaced with oxygenated Krebs buffer containing 0.1% BSA, 8 mM mannitol and 10 µM 2-deoxy-D-glucose, with or without 10 nM insulin for 20 min. The media was removed and replaced with 1 mL of oxygenated Krebs buffer containing 0.1% BSA, 8 mM mannitol, 10 µM 2-Deoxy-D-glucose (Sigma-Aldrich, Australia), 0.5 µCi/mL ³H-2-deoxy-D-glucose (Perkin Elmer, USA Cat# NET328A250UC) and 0.2 µCi/mL of ¹⁴C mannitol (Perkin Elmer, USA Cat #NEC314250UC), with or without 10 nM insulin. Following the last incubation step, tissues were rapidly dipped in ice-cold PBS, blotted on a Kim-wipe tissue, and snap-frozen in liquid nitrogen.

Samples were homogenised in radioimmunoprecipitation assay (RIPA) buffer [65 mM Tris-HCl, 150 mM NaCl, 1% NP-40 (v:v), 0.5% sodium deoxycholate (v:v), 0.1% sodium dodecyl sulfate (SDS) (v:v), and 10% glycerol (v:v)] with 1 mM dithiothreitol

(DTT), 1x protease inhibitor (Complete Protease Inhibitor Cocktail; Roche Diagnostics, Switzerland), and 1 x phosphatase inhibitor (PhosSTOP Phosphatase Inhibitor Cocktail, Roche Diagnostics, Switzerland) using Precellys Evolution homogenizer (Bertin technologies, France) and transferred to a scintillation vial containing a 2 mL of Ultima Gold scintillation cocktail in a polypropylene vial. Vials were assessed for radioactivity using the TriCarb® 2810 TR liquid scintillation analyser (PerkinElmer, USA) and counted on a dual-channel to assess ^3H and ^{14}C .

2.4.7. Hepatic glucose output

Liver slices were prepared as described in section 2.2.4., and then incubated at 37 °C in high-glucose DMEM containing 1 mmol/L sodium pyruvate for 1 h. Liver slices were washed 3 x in PBS and incubated with medium containing liver secreted factors (Exercise or Sedentary) diluted 1:4 with glucose-free DMEM (Gibco, USA; Cat: 11966-025) (v:v) and supplemented with 10 mmol/L sodium pyruvate and 10 mmol/L sodium lactate for 10 min. Slices were washed in PBS then incubated for 1 h in the same pyruvate/lactate-supplemented media containing hepatocyte secreted factors. Glucose content in the medium was assessed using Infinity™ glucose hexokinase reagent (Thermo Fisher, USA), as per the manufacturer's instructions.

2.4.8. Lipolysis

Epididymal adipose tissue explants from a healthy control mouse were cultured in medium containing secreted factors from livers of Exercise or Sedentary mice diluted 1:4 (v:v) with oxygenated phenol red-free low-glucose DMEM (Gibco, USA) and 2% BSA and incubated in a shaking water bath at 37 °C for 4 h. Glycerol content in the

media was assessed by colorimetric assay as per manufacturer's instructions (Free glycerol reagent, Sigma-Aldrich, USA).

2.5. Biochemical analyses

2.5.1. Murine hepatocyte conditioned medium processing for proteomic analysis

For the assessment of exercise-regulated hepatocyte-secreted factors in mice, proteins were extracted, reduced and alkylated and digested with trypsin (1 μ g trypsin/100 μ g protein) from the conditioned medium as described above (section 2.2.5). No Lys-C was added in this digestion. The following day, 40 μ L of 50 mM of ammonia bicarbonate was added and centrifuged at 14000 x g for 15 min. This step was repeated twice. Additionally, 50 μ L of 0.5 M NaCl was added to the centrifugal filter and the samples centrifuged at 14000 x g for 15 min. After the final centrifugation step, the flow-through containing all digested peptides was acidified to a pH3 through the addition of 1 μ L of formic acid. The peptides were purified with Millipore® C18 Ziptips (Sigma Aldrich, USA). This involved activation of the Millipore® C18 Ziptips (Sigma Aldrich, USA) with methanol, washing with 0.1% formic acid, binding of peptides to Ziptips, a second wash step in 0.1% formic acid, and final elution into 200 μ L 50% ACN and 0.1% formic acid solution. The solvent was evaporated by centrifugation on a Speedvac concentrator (Eppendorf, Germany) and the purified peptides were reconstituted in 0.1% Formic acid at a concentration of 2 μ g / μ L. Samples were then stored at 4 °C until liquid chromatography and mass spectrometry was performed.

2.5.2. Murine hepatocyte processing for proteomic analysis

Hepatocytes were heated to 95 °C for 5 min followed by three rounds of sonication for 10 seconds. The protein concentration of the lysate was determined using a using PierceTM (bicinchoninic acid assay) BCA protein assay kit (Thermo Scientific, #23225, USA) as described in section 2.5.14, and 100 µg of protein was reduced with 10 mM TCEP for 20 min at 65 °C and alkylated with 40 mM chloroacetamide and incubated in the dark at room temperature for 20 min. Proteins were digested with 1 µg trypsin at 37 °C in a shaking water bath overnight, the peptides acidified with 100% formic acid to a pH of 3, diluted 1:1 (v:v) with water saturated ethyl acetate, vortexed, centrifuged at 15,000 x g for 5 mins, and repeated twice to remove the sodium deoxycholate. The peptides were purified with a C18 cartridge (Oasis HLB, SKU: WAT094225). This involved activation of the C18 cartridge with the addition of 1 mL of ACN, centrifugation for 1 min at 500 x g at 20 °C. The cartridge was washed 3 x with 1 mL of 0.1% formic acid and centrifuged for 1 min at 500 x g for 20 °C and the flow through was discarded. The peptide sample was loaded into the cartridge, centrifuged for 3 min at 500 x g at 20 °C, and the column washed three times with 0.1 % formic acid, centrifuged at for 5 min 500 g and 20 °C. The column was transferred to a fresh 15 mL falcon tube and the peptides were eluted in 60% ACN in 0.1% formic acid by centrifugation at 100 g for 5 min. The eluted peptides were transferred to an Eppendorf tube, dried by vacuum centrifugation and reconstituted in 0.1% formic acid and 2% ACN to a final peptide concentration of 1 µg /µL. Samples were subsequently sonicated in a water bath for 10 min, centrifuged at 14,000 g for 5 min, the supernatant transferred to a fresh tube and centrifuged again. The supernatant was then collected and transferred into a mass spectrometry vial, and samples were stored at 4 °C until liquid chromatography and mass spectrometry was performed.

2.5.3. Liquid chromatography and mass spectrometry for murine studies

Nano-Electrospray Ionisation (ESI)-LC-MS/MS was used to analyse the peptide samples. The Nano-LC system, Ultimate 3000 RSLC (ThermoFisher Scientific, USA) was set up with an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 µm × 50 cm, Thermo Fisher Scientific, USA) and Acclaim Pepmap nano-trap column (75 µm × 2 cm, C18, 100 Å) at 50°C. Solvent A was 0.1% v/v formic acid and 5% v/v dimethyl sulfoxide (DMSO) in water and solvent B was 0.1% v/v formic acid and 5% DMSO in ACN. The trap column was loaded with the tryptic peptides on an isocratic flow of 3% ACN containing 0.05% TFA at 6 µl/min for 6 mins, followed by the switch of the trap column as parallel to the analytical column. The gradient setting for the LC runs at a flow rate 300 nL/min, were as follows: solvent B 3% to 23% in 89 mins, 23% to 40% in 10 mins, 40% to 80% in 5 mins, maintained at 80% for 5 mins before dropping to 3% in 2 mins and equilibration at 3 % solvent B for 8 mins. A positive mode, Nano-ESI and Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA) was employed to execute the MS experiments with settings of spray voltages, S-lens RF, and capillary temperature level of 1.9 kV, 60 %, 275 °C, respectively. Data-dependent acquisition of mass spectrometry data was performed by acquiring each full scan MS spectra followed by rapid collision induced dissociation (CID)-MS/MS spectra of top 20 most abundant precursor ions. Full scan MS spectra features ions at m/z of 300-1650, a maximum ion trapping time of 200 milliseconds, an auto gain control target value of 1e6, and a resolution of 120,000 at m/z 200. An m/z isolation window of 2, an auto gain control target value of 5e3, a 30 % normalized collision energy, an activation q value of 0.25, an activation time of 10 milliseconds, a maximum ion trapping time of 25 milliseconds

were used to perform data-dependent rapid CID-MS/MS of precursor ions having charge states more than 1. A dynamic exclusion of 30 seconds was applied.

2.5.4. Analysis of murine mass spectrometry data

All generated files were processed with MaxQuant (v1.6.2.10) [234] to identify (at least 2 razor + unique peptide) as well as to report their label free quantitation intensities. Database searching was performed with settings followed here, methionine oxidation and protein N-terminal acetylation as variable modification, cysteine carbamidomethylation as fixed modification, trypsin as enzyme, maximum missed cleavage as 2, UniProt mouse database with 17,082 entries. LFQ was enabled and a minimum of 2 peptides for pair-wise comparison in each protein, false discovery rate at 1% at peptide and protein level, match between run enabled. The MaxQuant output was processed further through Perseus (v1.6.6.0) [235]. The values were Log₂ normalised and underwent two sample t-test (correcting for multiple hypothesis testing using Benjamini-Hochberg set at FDR < 5%) to obtain a list of significantly regulated proteins based on $q < 0.05$. Bioinformatic assessment of the hepatocyte intracellular and secreted proteome data sets was performed using Ingenuity Pathway Analysis (IPA, QIAGEN) [236], and functions assessed included canonical pathways, upstream regulators and disease and biological functions.

2.5.5. Targeted liquid chromatography and mass spectrometry

Liver slices from *Sdc4*-AAV mice were collected, settled for one h in oxygenated M199 media, then cultured in EX-CELL 325 medium (Sigma-Aldrich, Australia) for 16 h. The conditioned medium was collected and frozen at -80 °C until further analysis. Proteins were processed as per the S-TrapTM method (Protifi, USA). This method comprised of

an aliquot of 23 μ L of conditioned medium mixed with 23 μ L of 5% sodium dodecyl sulfate in 50 mM Tris-HCl buffer (pH8.5). Samples were vortexed and debris was removed by centrifugation at 13,000 x g for 10 min and 5 mM TCEP was added for 15 min at 55 °C to denature disulphide bonds. Disulphide bonds were alkylated by the addition of 20 mM chloroacetamide in the dark for 10 min at room temperature. The proteins were acidified with 1% TFA to yield a pH of 1 and vortexed. Subsequently, 1522 μ L of 100 mM Tris-HCl in 90% methanol was added and samples were transferred to an S-trap mini column (Protifi, USA) and centrifuged at 4000 x g for 30 seconds and the flow through was discarded. Samples were washed 3 x 400 μ L of 100 mM Tris-HCl in 90% methanol and centrifuged at 4000 x g for 30 seconds, each time discarding the flow through. The S-trap was centrifuged again for 1 minute at 4000 x g and the column was transferred to a fresh tube. Next, 125 μ L of 50 mM ammonia bicarbonate with 1 μ g trypsin / 50 μ g protein was added and samples were wrapped in parafilm and incubated in a wet chamber at 37 °C overnight. The next day, peptides were eluted by centrifugation at 4000g for 1 min following the addition of 1 x 80 μ L of 50 mM Tris-HCl buffer (pH = 8.5), 1 x 0.2% formic acid, and 1x 50% acetonitrile. Samples were dried on a Speedvac concentrator and resuspended in 2% acetonitrile and 0.05% TFA. Samples were then centrifuged at 14,000 g for 5 min, the supernatant transferred to a mass spectrometry vial, and stored at 4 °C until liquid chromatography and mass spectrometry was performed.

A positive mode, Nano-ESI and Eclipse Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was employed to execute the MS experiments with settings of spray voltages, S-lens RF, and capillary temperature level of 1.9 kV, 30 %, 275 °C, respectively. The mass spectrometry data was acquired with a 3-second cycle

time for one full scan MS spectra and as many data-dependent HCD-MS/MS spectra as possible. Full scan MS spectra features ions at m/z of 300-1500, a maximum ion trapping time of 50 msec, an auto gain control target value of $4e5$, and a resolution of 120,000 at m/z 200. An m/z isolation window of 1.6, an auto gain control target value of $5e4$, a 30 % normalized collision energy, a maximum ion trapping time of 22 ms, and a resolution of 15,000 at m/z 200 were used to perform data-dependent HCD-MS/MS of precursor ions having charge states from 2 to 7. A dynamic exclusion of 30 sec was applied. After each data-dependent acquisition cycle, targeted HCD-MS/MS using identical parameters as that for data-dependent HCD-MS/MS described above was performed for selected SDC4 peptides based on experimentally observed peptide of mouse SDC4 from Peptide Atlas (Table 2.4 for peptide sequence, m/z and charge state). The intensity of the detected SDC4 peptides from the secreted medium was extracted from MaxQuant (Table 2.5). Skyline (20.2.0.343) [237] was used to extract the peak area of fragment ions on the liquid chromatography run for each peptide detected on the MS2 scan (Table 2.5). The peptide sequence that was detected in all samples and is unique to SDC4 (ETEVIDPQDLLEGR) and corresponds to the ectodomain was used to determine liver SDC4 secretion.

Table 2.4. List of SDC4 peptides previously experimentally identified from Peptide Atlas [238] and targeted for in the liver conditioned medium from Sdc4-AAV or Control-AAV mice.

Compound	m/z	z
ETEVIDPQDLLEGR	807.4045	2
ETEVIDPQDLLEGR	538.6054	3
ELEENEVIPK	600.3113	2
ELEENEVIPK	400.5433	3
KDEGSYDLGK	556.2669	2
KDEGSYDLGK	371.1804	3
RAPSDVGDDMSNK	696.3146	2
RAPSDVGDDMSNK	464.5455	3
RAPSDVGDDMSNK	348.6609	4
APSDVGDDMSNK	618.2641	2
APSDVGDDMSNK	412.5118	3
APSDVGDDMSNK	309.6357	4
ESIRETEVIDPQDLLEGR	1050.034	2
ESIRETEVIDPQDLLEGR	700.3587	3
ESIRETEVIDPQDLLEGR	525.5208	4
VPSEPKELEENEVIPK	918.9831	2
VPSEPKELEENEVIPK	612.9912	3
VPSEPKELEENEVIPK	459.9952	4
PFPEVIEPLVPLDNHIPENAQPGIR	1396.243	2
PFPEVIEPLVPLDNHIPENAQPGIR	931.1641	3
PFPEVIEPLVPLDNHIPENAQPGIR	698.6249	4

Table 2.5. Liver secreted SDC4 peptides from Sdc4-AAV or Control-AAV mice and their intensity output from MaxQuant.

Sequence:	Intensity		
	ELEENEVIPK	ETEVIDPQDLLEGR	RAPSDVGDDMSNK
Control-AAV	2588800	1929900	0
Control-AAV	7193500	14704000	0
Control-AAV	8264600	13176000	0
Control-AAV	8019400	8473800	0
Control-AAV	4794600	7383000	0
Control-AAV	4510800	7512600	2334700
Control-AAV	0	7100200	0
Control-AAV	0	623020	0
Control-AAV	10521000	18456000	0
Sdc4-AAV	13772000	19657000	6233600
Sdc4-AAV	11905000	16013000	0
Sdc4-AAV	7533700	16397000	0
Sdc4-AAV	8171300	15111000	0
Sdc4-AAV	9778700	20734000	0
Sdc4-AAV	7731700	11149000	0
Sdc4-AAV	7322400	11402000	2274700

2.5.6. Liver triglyceride content by lipidomics

A liver wedge ~5 mg was resected prior to hepatocyte isolation to assess hepatic triglyceride levels. Lipids were extracted as previously described [239]. Briefly the liver was homogenized in 200 μ L of 60% methanol:water (v:v), containing 10 μ L of SPLASH® II LIPIDOMIX® Mass Spec Standard (330709W, Avanti Polar Lipids Inc, USA) and 10 μ L of Ceramide LIPIDOMIX® Mass Spec Standard (330712X, Avanti Polar Lipids Inc, USA) using a Precellys Evolution Tissue Homogenizer (Bertin Instruments, USA). This was followed by the addition of 120 μ L of water, 420 μ L of 100% methanol, and 260 μ L of 100% chloroform with a vortex after each step. Samples were mixed at 1400 rpm for 30 min at room temperature in an Eppendorf thermomixer C (Eppendorf, Germany), centrifuged at 14,000 rpm for 15 min and the supernatant was collected and dried using a Speedvac concentrator (Eppendorf Concentrator Plus, Germany). Lipids were resuspended in 100 μ L of 1:9 (v:v) chloroform:methanol, mixed on an Eppendorf thermomixer C (Eppendorf, Germany) for 3 min at room temperature and centrifuged at 14000 rpm for 15 min. The supernatant was transferred to glass vials for analysis by ultrahigh performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) employing a Vanquish UHPLC linked to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific), with separate runs in positive and negative ion polarities. Solvent A was 6/4 (v:v) acetonitrile/water with 10 mM ammonium acetate and 5 μ M medronic acid and solvent B was 9/1 (v:v) isopropanol/acetonitrile with 10 mM ammonium acetate. Each sample was injected into an RRHD Eclipse Plus C18 column (2.1 $\times$ 100 mm, 1.8 μ m, Agilent Technologies) at 60 °C at a flow rate of 350 μ L/min for 3 min using 30% solvent B. During separation, the percentage of solvent B was increased from 30% to 70% in 5 min, from 70% to 93% in 9 min and, from 93% to 99% in 7 min, and from 91% to 97% in 31 min.

Subsequently, the percentage of solvent B was increased to 99.5% in 0.1 min and then maintained at 99.5% for 3 min. Finally, the percentage of solvent B was decreased to 30% in 0.1 min and maintained for 3.9 min.

All MS experiments were performed using a heated ESI source. The spray voltages were 3.5 kV in positive ionisation-mode and 3.0 kV in negative ionisation-mode. In both polarities, the flow rates of sheath, auxiliary and sweep gases were 20 and 6 and 1 ‘arbitrary’ unit(s), respectively. The ion transfer tube and vaporizer temperatures were maintained at 350 °C and 400 °C, respectively, and the S-Lens RF level was set at 50%. In the positive ionisation-mode from 3 to 24 min, top speed data-dependent scan with a cycle time of 1 s was used. Within each cycle, a full-scan MS spectra were acquired firstly in the Orbitrap at a mass resolving power of 120,000 (at m/z 200) across an m/z range of 300–2000 using quadrupole isolation, an automatic gain control (AGC) target of $4e5$ and a maximum injection time of 50 milliseconds, followed by higher-energy collisional dissociation HCD-MS/MS at a mass resolving power of 15,000 (at m/z 200), a normalised collision energy (NCE) of 27% at positive mode and 30% at negative mode, an m/z isolation window of 1, a maximum injection time of 22 milliseconds and an AGC target of $5e4$. For the improved structural characterisation of TG lipid cations, the fatty acid + NH_3 neutral loss product ions observed by HCD-MS/MS were used to trigger the acquisition of the top-3 data-dependent CID-MS³ scans in the cycle using a q -value of 0.25 and a NCE of 30%, with other settings being the same as that for HCD-MS/MS.

2.5.6.1 Identification and quantification of lipids and statistical analysis

MS data were processed using LipidSearch software v.4.2.23 (Thermo Fisher Scientific, USA). Key processing parameters were: target database, general; precursor tolerance, 5 ppm; product tolerance, 10 ppm; product ion threshold, 1%; m-score threshold, 2; quantification m/z tolerance, ± 5 ppm; quantification retention time range, ± 1 min; use of main isomer filter and ID quality filters A, B and C; adduct ions, +H and +NH₄, +Na and +H-H₂O for positive ionisation mode, and -H, +CH₃COO, -2H and -CH₃ for negative ionisation mode. The lipid class of TG was selected for the search. The same lipid annotations (within 0.1 min of retention time and 5 ppm of mass error) were merged into the aligned results. Unassigned peak areas were calculated for relative quantification and alignment. The shorthand notation used for lipid classification and structural representation follows the nomenclature proposed previously [240]. A grade A- or B-identification was required in at least one sample. For all lipids, a ratio of MS/MS spectrum peaks assigned to the lipid among all fragmentation product ions (i.e., MainOccupy value) > 40 was required in at least one sample. All lipid LC-MS features were manually inspected and re-integrated when needed.

2.5.7. Immunoblotting

Tissues were homogenised in RIPA buffer [65 mM Tris-HCl, 150 mM NaCl, 1% NP-40 (v:v), 0.5% sodium deoxycholate (v:v), 0.1% SDS (v:v), and 10% glycerol (v:v)] with 1 mM DTT, 1x protease inhibitor (Complete Protease Inhibitor Cocktail; Roche Diagnostics, Switzerland), and 1 x phosphatase inhibitor (PhosSTOP Phosphatase Inhibitor Cocktail, Roche Diagnostics, Switzerland) using Precellys Evolution homogenizer (Bertin technologies, France). Tissue homogenates were centrifuged at 10,000 x g for 10 min at 4 °C, protein content was assessed as described later (section

2.5.14), samples diluted to a final concentration of 1 $\mu\text{g}/\mu\text{L}$ in 2 x Laemmli (Biorad Laboratories, USA), and equal amounts of protein were loaded on to a 10% or 12% gradient stain free gel (Biorad Laboratories, USA). Thereafter, protein was transferred to polyvinylidene difluoride membranes using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories), membranes were blocked for 60 min in Tris buffered saline containing 1% tween 20 (TBST, Sigma-Aldrich, Australia) and 5% BSA, and incubated with the respective primary antibodies overnight, including mouse monoclonal antibody cocktail against EMILIN1 (1:250, catalogue no. 103-M80, Relia Tech, Germany), the five subunits of oxidative phosphorylation complexes one each against CI subunit NDUFB8, CII-30kDa, CIII-Core protein 2, CIV subunit I, and CV alpha subunit, present in the cocktail (cat: ab110413, Abcam, UK) (1:250, catalogue no. MS604-300, Abcam, UK) or proliferator-activated receptor- γ coactivator-1 α (PGC1 α) monoclonal antibody (1:250 catalogue number Ab54481, Abcam, UK). Membranes were subsequently washed 3 x 10 min with TBST and then incubated in 1:10,000 dilution of anti-rat (Merck, USA) or anti-rabbit (Abcam, UK) secondary antibodies, in 5% BSA in TBST for 2 h. Again, membranes were subsequently washed 3 x 10 min with TBST and were visualised following exposure to Clarity Western ECL substrate (Bio-Rad) using Image Lab software version 4.1 (Biorad Laboratories, USA), and density was quantified using ImageJ version 1.8.0_112 (National Institutes of Health, USA).

2.5.8. Citrate synthase activity assay

Approximately 20 mg of tissue was homogenised in 400 μL of 50 mM Tris-HCl, 1 mM EDTA and 0.1% Triton X-100 (pH7.4) using a Precellys Evolution homogenizer (Bertin technologies, France) and centrifuged at 7000 rpm for 10 min at 4 $^{\circ}\text{C}$. The supernatant was added to 2.4 mL of the same homogenising buffer. An aliquot of 10 μL of this

diluted homogenate was added to a 96 well plate and topped with 25 μL of 3.6 mM acetyl-CoA and 215 μL of 100 mM Tris-HCl, 1 mM EDTA, 1 mM of MgCl_2 and 0.25 mM of $\text{C}_{14}\text{H}_8\text{N}_2\text{O}_8\text{S}_2$. Citrate synthase activity was initiated by the addition of 50 μL of 3 mM oxaloacetate and the change in absorbance measured at 412 nm was assessed for 2-3 min on the SpectroStar Nano. Citrate synthase activity was assessed as per equation 7.

$$\text{CS activity } (\mu\text{mol}/\text{min}/\text{g}) = \Delta \text{ absorbance} \times 120 \text{ (dilution)} \times \text{C}_{14}\text{H}_8\text{N}_2\text{O}_8\text{S}_2 \text{ extinction co-efficient}$$

Equation 6: Assessment of citrate synthase (CS) activity, where $\text{C}_{14}\text{H}_8\text{N}_2\text{O}_8\text{S}_2$ extinction co-efficient is 13.6 $\mu\text{mol}/\text{ml}/\text{cm}$.

2.5.9. RNA extraction

30 mg of tissues were homogenised in 1 mL of TriReagent Reagent (Sigma-Aldrich, Australia) using a Precellys Evolution homogenizer (Bertin Technologies, France) and 200 μL of 100% chloroform was added. Samples were subsequently centrifuged at 12,000 x g for 15 min at 4 °C. The clear aqueous phase was taken and added to 500 μL of 100% isopropanol and incubated for 10 min at room temperature. The RNA was then pelleted by centrifugation at 12,000 x g and the supernatant was removed. The pellet was resuspended in 1 mL of 100% ethanol, vortexed and the RNA was pelleted by centrifugation at 10000 g for 15 min at 4 °C. The ethanol supernatant was removed, and the RNA pellet was dried under a nitrogen stream and resuspended in 30 μL of RNase free water. RNA concentration and purity were determined by absorbance at 260 and 280 minus using a LVis plate on the SpectroStar Nano. To remove any DNA contaminant, 10 μg of RNA was mixed with 5 μL of DNase buffer (Invitrogen) and 1 μL of DNase I (Invitrogen) and then incubated at 37 °C for 30 min. The reaction was

stopped by the addition of 5 μ L of the inactivation reagent (Invitrogen), the samples centrifuged for 2 min at 10,000 g, and the supernatant was collected and re-assessed for RNA concentration as described above. To reverse transcribe the RNA into cDNA, 1 μ g of RNA was mixed with 4 μ L of 5x ISCRIPRT reaction mix (Biorad Laboratories, USA), 1 μ L of ISCRIPRT reverse transcriptase (Biorad Laboratories, USA), filled to a volume of 20 μ L with RNase free water and incubated at 25 °C for 5 min, then 42 °C for 30 min and lastly at 85 °C for 5 min.

2.5.9.1. Real Time Polymerase Chain Reaction

To analyse gene expression, 10 ng of cDNA was added to each well of a 384 well hard-shell PCR plate and mixed with 8 μ L of a master mix containing 5 μ L of SYBR Green (QIAGEN), 1 μ L of the respective forward and reverse 20 μ M primers and 2 μ L of water. The PCR was completed on the Biorad CFX384™ Real-Time system driven by a C100 Touch™ Thermal cycler using an initial 10-minute cycle at 95 °C for 10 min then 40 cycles of 5 seconds of 95 °C then 20 seconds at 60 °C and 20 seconds at 72 °C. To assess the melting curve, the plate was heated for 5 seconds at 60 °C then 31 seconds at 65 °C and then followed by 65 °C for 5 seconds, this last step was repeated for 60 cycles with each cycle increasing the temperature by 0.5 °C.

To analyse the results, we used the inverse proportional relationship between the threshold cycle (Ct) and the expression level for the gene of interest denoted as $=2^{-Ct}$. To normalise to the expression of the gene to the human or mouse housekeeping gene Hypoxanthine Guanine Phosphoribosyltransferase (*HPRT*), the level of expression relative to the expression of *HPRT* was assessed as per equation 7.

$$2^{-Ct(\text{gene of interest})} / 2^{-Ct(HPRT)} = 2^{-[Ct(\text{gene of interest}) - Ct(HPRT)]} = 2^{-\Delta Ct}$$

Equation 7: Relative expression of the gene of interest to housekeeping gene HPRT. HPRT, hypoxanthine guanine phosphoribosyltransferase.

To determine the expression of the gene of interest relative to the control group, the experimental sample is divided by the normalised expression of the same gene of interest in the control sample determined from equation 8. Primer sequences used described in Table 2.6.

$$2^{-\Delta Ct \text{ experimental sample}} / 2^{-\Delta Ct \text{ control sample}} = 2^{-\Delta \Delta Ct}$$

Equation 8: Relative expression of gene of interest in intervention group compared to control group.

Table 2.6. Primer sequences

Genes	Forward Sequence	Reverse Sequence
<i>Acta2</i>	CATCTTTCATTGGGATGGAG	TTAGCATAGAGATCCTTCCTG
<i>Cav1</i>	CTCAGTTCTCTTAAATCACAGC	CATACACTTGCTTCTCAGTC
<i>Ccl2</i>	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
<i>Cd36</i>	CATTTGCAGGTCTATCTACG	CAATGTCTAGCACACCATAAG
<i>Colla1</i>	GATCTGTATCTGCCACAATG	TGGTGATACGTATTCTTCCG
<i>Colla2</i>	AGGCAGGTCTGGGCTTTATT	CGTATCCACAAAGCTGAGCA
<i>Col3a1</i>	ACTCAAGAGTGGAGAATACTG	AACATGTTTCTTCTCTGCAC
<i>Dgat2</i>	GTGGCAATGCTATCATCATC	CTGCTTGTATACCTCATTCTC
<i>EMILIN1</i>	GCATCATGTACCGCCGCTTCC	CTGACGGGCTGCCTGCACTG
<i>Emilin1</i>	TGTGCCTACGTGGTGACTC	CGGTACATGATACTTCGGGAAC
<i>F4/80</i>	CCTGGACGAATCCTGTGAAG	GGTGGGACCACAGAGAGTTG
<i>Fatp1</i>	ATGTGCTCTATGACTGCC	TATGTACTGCACTACCGTG
<i>Fatp2</i>	GAGGATACAAGATACCATTGAG	GGAATATTCAGAGCTTCAGAG
<i>Fatp5</i>	CAAACAGGGTTTTTGCATTC	TTGAAGTACAGGTCTCCTAC
<i>HPRT</i>	ATAAGCCAGACTTTGTTGG	ATAGGACTCCAGATGTTTCC
<i>Hprt</i>	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG
<i>Il1b</i>	GGATGATGATGATGATAACCTGC	CATGGAGAATATCACTTGTTGG
<i>Il6</i>	CCAGTTTGCCTTCTTCCCACT	GGTCTGTTGGGAGTGGTATCC
<i>Mmp2</i>	GTCGCCCTAAAACAGACAA	GGTCTCGATGGTGTCTGGT
<i>Mmp13</i>	AGCAGTTCCAAAGGCTACAACCT	GGATGCTTAGGGTTGGGGTC
<i>Mmp14</i>	AGCACTGGGTGTTTGACGAA	CCGGTAGTACTTATTGCCCCG
<i>Sdc1</i>	CAAAGGTGAAGTCTTGTTCTC	CTTCTGTCATCAAAGAGGTTG
<i>Sdc2</i>	CAACTGATTCCAAGAATCCC	CTCAGAAATGTCAACTTCCTC
<i>Sdc3</i>	CAGGAGACAAAAACATACCC	GGACCAATGTGTGTAAAGAG
<i>Sdc4</i>	CAACAAAGTATCCATGTCCAG	TTTGTAAGATGGGTTTCTTGC
<i>Tgfb1</i>	GGATACCAACTATTGCTTCAG	TGTCCAGGCTCCAAATATAG
<i>Tnfa</i>	CTGTGAAGGGAATGGGTGTT	GGTCACTGTCCCAGCATCT
<i>Timp1</i>	CATCCTCTTGTTGCTATCAC	CATGAATTTAGCCCTTATGACC
<i>Timp3</i>	GCTAGAAGTCAACAAATACCAG	TAGTAGCAGGACTTGATCTTG
<i>Tgfb1</i>	GGATACCAACTATTGCTTCAG	TGTCCAGGCTCCAAATATAG

2.5.10. Blood metabolites

Whole blood was collected into Eppendorf tubes and plasma was isolated by centrifugation at $8,000 \times g$ at 4°C for 10 min. Plasma insulin levels were determined using an ultrasensitive mouse Insulin ELISA kit (Catalog #90080; Crystal Chem, Downers Grove, IL), as per manufacturer's instructions. Plasma non-esterified fatty acids and total cholesterol levels were assessed by colorimetric assay as per manufacturer's instructions (Wako Diagnostics, Osaka, Japan). Plasma triglyceride levels were evaluated by colorimetric assay as per manufacturer's instructions (Triglycerides GPOPAP; Roche Diagnostics, Indianapolis, IN). Absorbance was measured using a spectrophotometer (SPECTROstar Nano -BMG LABTECH, Germany). The standards were used to form a scatterplot and a line of best fit was inputted to determine by the linear relationship of protein concentration with absorbance. The equation from the line of best fit was used to determine the blood metabolite concentration.

2.5.11. ALT & AST Assay

To determine the relative levels of plasma ALT, we assessed the enzymatic capacity of ALT to catalyse the transfer of the amino group from alanine to α -ketoglutarate forming pyruvate and glutamate, respectively. Ten microliters of plasma was plated in a 96 well plate and mixed with 200 μL of 134 mM of alanine, 6.64 mM of α -ketoglutarate, 0.24 mM of the reduced form of nicotinamide adenine (NADH) with 5 U/mL of lactate dehydrogenase (Roche, Switzerland) in 50 mM of sodium phosphate buffer (pH 7.4). Absorbance was read at a wavelength of 340 nm for 5-10 min at 37°C . ALT activity was calculated as the time-dependent decrease in NADH at 340 nm using the extinction coefficient of NADH ($\epsilon_{340} = 6.220 \text{ mmol/L/cm}$).

To determine the relative levels of plasma AST, we assessed the enzymatic capacity of AST to catalyse the transfer of the amino group from aspartate to α -ketoglutarate forming oxaloacetate and glutamate, respectively. The activity of plasma AST was performed the same as above with minor exceptions. Instead of alanine and lactate dehydrogenase, 134 mM of aspartate and 1.25U/mL of malate dehydrogenase (Roche, Switzerland), respectively.

2.5.12. Liver histology and hepatocyte microscopy assessment

A piece of liver from *Emilin1*-AAV, *Sdc4*-AAV, or respective Control-AAV mice was fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned at 5 μ m and stained with hematoxylin and eosin (H&E) or Masson trichrome (MT) (University of Melbourne Histology Platform, Melbourne, Australia). To determine fibrosis accumulation, five images per mouse were captured at 20 x magnification and were quantified using Orbit machine learning analysis to determine fibrosis area [241; 242].

To determine lipid droplet distribution, lipid droplets were identified by ilastikTM machine learning image analysis [243], converted to an 8-bit image and quantitation of lipid droplet number and area was assessed with ImageJ version 1.8.0_112 (National Institutes of Health, USA).

Hepatocytes stained as described in 2.4.1.4 were viewed using confocal microscopy (ZEISS LSM 900 with Airyscan 2, Germany) using 63X objective (oil-immersion). Lipid droplet number and area quantitation was measured using Fiji (ImageJ) software version 1.8.0_112 (National Institutes of Health, USA). Edges are detected and local

intensity maxima are determined in the pre-processed image. Edge-defined particles were obtained using thresholding followed by hole-filling and watershedding. An edge-defined particle sharing coordinates with a local maximum is then counted as a “true” lipid droplet. The areas of the segmented particles are measured, and the radius and corresponding volume are calculated assuming that the area describes a circle.

2.5.13. Adenosine triphosphate (ATP) assay

To determine if the liver slices were viable following the 16-h incubation period, a subset of the liver slices were lysed in 400 μ L of EX-CELL 325 protein free medium (Sigma-Aldrich, Australia). Adenosine triphosphate (ATP) content was measured using the CellTiter-Glo 2.0 cell viability assay (Promega, Australia). ATP is present in viable cells, and when combined with luciferin, oxygen and luciferase will luminesce which can be quantified to determined ATP content. Thus, 2 μ L of the liver slice lysate was mixed with 10 μ L of CellTiter-Glo solution (Promega, Australia) in an opaque walled 384 well plate. ATP standards were created at 1 μ M, 0.5 μ M, 0.25 μ M, 0.125 μ M and 0.0625 μ M in EX CELL media and were also added to the plate. Samples were then placed on a shaker for ten min and then luminescence intensity was measured using the SpectroStar Omega (BMG LABTECH, Australia). The ATP standards were used to construct a scatterplot and line of best fit allowing the prediction of the liver lysate ATP content.

2.5.14. Protein assay

The PierceTM BCA protein assay kit (Thermo Scientific, #23225, USA) was employed to determine the total protein content of the liver slices. Samples were diluted 1:25 in MiliQ water and added to a 96 well microtiter plate with BSA standard sample

concentrations of 0, 25, 50, 100, 200, 300, 500, 1000 and 2000 $\mu\text{g/mL}$. Two hundred microliters of the BCA buffer (98:2 Reagents A: B) was added to each well and the plate was incubated at 37 °C for 30 min. The absorbance was determined at 560nm on the SpectroStar Omega (BMG LABTECH, Australia). The protein standards were used to form a scatterplot and a line of best fit was inputted to determine by the linear relationship of protein concentration with absorbance. The equation from the line of best fit was used to determine liver lysate protein content.

2.5.15. Statistical Analysis

Data was assessed for normal distribution using D'Agostino-Person test. Statistical analysis was performed using unpaired Student's *t*-test or one way or two-way ANOVA for normally distributed data. Means were compared using Bonferoni's multiple comparisons test where appropriate. Mann-Whitney test or Kruskal-Wallis test was performed for data that was not normally distributed. Statistical significance was determined when $p < 0.05$. Data are reported as mean $\pm$ standard error of the mean (SEM) or median where appropriate.

Chapter 3: Characterisation of human liver-secreted proteins

3.1. Introduction:

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease worldwide [8; 10; 244; 245], with current estimates that the global and Australian prevalence is 25% and 22.2%, respectively [8; 246]. A significant risk factor for NAFLD is obesity, affecting 31.3% of Australians [247]. This is of utmost concern given that obesity rates are increasing [248; 249], and therefore, NAFLD cases are also projected to rise [9; 10]. This estimated increase in NAFLD incidence is accompanied by an eventual increased progression from NAFL to the more severe NASH [32], and thus, those progressing to end stage disease such as decompensated cirrhosis and HCC [8; 10].

NAFLD is a spectrum of liver disease. When histologically assessed by light microscopy, NAFL is defined by greater than 5% macrosteatosis in hepatocytes [138]. NAFL can progress to its more severe form NASH, characterised by the joint presence of macrosteatosis, lobular inflammation, and hepatocyte ballooning [138]. While liver fibrosis can occur at any stage of the disease spectrum, patients with NASH develop fibrosis at double the rate of those with NAFL [32; 250]. Liver fibrosis is the most important prognostic factor for patients with NAFLD as long-term studies demonstrate that regardless of steatosis or inflammation, fibrosis stage is the only independent histological variable associated with overall and liver-related mortality [141; 153]. Therefore, it is critical that the stages of NAFLD and fibrosis can be differentiated simply and accurately to facilitate appropriate patient treatment.

A liver biopsy and histopathology are the gold standard to diagnose NAFLD and liver fibrosis. However, the risks, cost, time, and sampling problems inherent to this invasive procedure render it unsuitable for routine use on individuals at risk of NAFLD [251]. Therefore, non-invasive tests are increasingly being used to assess NAFLD and fibrosis, with the two primary diagnostic methods being serum biomarkers and elastography techniques [252]. Elastography requires specialised equipment, such as FibroScan [253] rendering these approaches impractical at the population level. Several serum biomarkers tests have been developed and typically utilise routine blood tests and clinical features to predict or exclude NAFLD, NASH, or fibrosis [254-256]. However, most current non-invasive scores use ALT and AST in their algorithm [256], which are not always elevated in obese patients with NAFLD and hepatic fibrosis [151; 157; 257]. Thus, there is an urgent need to discover new markers to develop or enhance non-invasive tests to reliably identify patients with NAFL, NASH, and/or significant fibrosis specific to these liver pathologies.

Identification of liver and pathology specific biomarkers required understanding of complex interplay between the livers' multiple parenchymal and non-parenchymal cells in NAFLD and hepatic fibrosis. The pathogenesis from NAFL to NASH comprises a variety of factors that include a combination of lipotoxicity, mitochondrial dysfunction, innate and adaptive immunity that contribute to inflammation and hepatocyte ballooning [258-261]. Liver fibrosis is an uncontrolled wound healing response, leading to an accumulation of extracellular matrix (ECM) proteins that replaces the parenchymal cells of the liver [262]. Steatotic hepatocytes and other immune cells types are hypothesised to release factors that can polarise the resident macrophages 'Kupffer cells' to an M1 phenotype [263]. The steatotic hepatocytes alongside resident inflammatory cells can

activate the HSC to become ACTA2 expressing, collagen-producing myofibroblasts that initiate and propagate the production of ECM proteins [262; 264; 265]. Deciphering the hepatic inter-cellular crosstalk in NASH and significant fibrosis provides an opportunity to identify secreted factors that may be reflective of hepatic pathophysiology. Previous findings have demonstrated remarkable remodelling of hepatokine secretion from hepatocytes isolated from mice fed a HFD [11] or NASH inducing diet [5]. However, not only is assessment of human hepatokines lacking, but capturing secreted proteins from intact livers containing parenchymal and non-parenchymal cells with an intact ECM has not been explored. Further, identifying alterations in hepatokine secretion implicates a potential change in circulating levels of hepatokines and this provides an opportunity to identify pathology specific biomarkers that can distinguish the stage of disease progression with greater accuracy compared with current approaches.

Therefore, we aim to assess liver secreted proteins with NASH and NAFLD associated fibrosis to identify potential biomarkers to stratify and stage the disease. We hypothesise that protein secretion from the liver will differ between NAFL, NASH, and significant fibrosis compared to ‘healthy’ livers. We further hypothesise that the identification of NASH and fibrosis-induced liver secreted proteins can be used as novel non-invasive biomarkers for these diseases.

3.2. Results

3.2.1. NASH Patient characteristics

A total of 118 obese patients were recruited into the study. However, upon examination of histology and serum biochemistry, two patients were excluded due to newly diagnosed Hepatitis C (n=1) and the other for previous gender reassignment surgery

with medications altering the physiological sex hormone levels (n=1). Other patients were excluded due to presence of a hemangioma (n=1), granulomatous hepatitis (n=1), mesothelial hyperplasia (n=1), or the presence of fibrosis without accompanying steatosis or inflammation (n=6). Additionally, n=11 patients' liver secreted medium was not collected and therefore are not included in the biomarker discovery cohort (Table 3.1).

Patients were grouped as based on histological diagnosis and staging of NASH and NAFLD -associated fibrosis as defined by the NASH CRN (Summarised in Table 1.1) [147]. For NASH, patients were grouped if they obtained a steatosis, inflammation, and ballooning score of ≥ 1 ; and those with NAFL had a steatosis score of ≥ 1 and either ballooning or inflammation of ≥ 1 . Patients without pathology had a steatosis, inflammation, and ballooning score of 0. For fibrosis groupings, patients with NAFLD and a fibrosis score of ≥ 2 were defined as those with significant fibrosis (NAFLD F2-3), those with NAFLD and a fibrosis score of 1 or 0 were grouped as NAFLD F1 or NAFLD F0, respectively. Those without NAFLD and fibrosis were grouped as No pathology. No patients recruited in this prospective study had hepatic cirrhosis (score of F4).

Across all 96 patients, 72.9% of patients were diagnosed with NAFLD and 27.1% of the cohort did not have NAFLD. Rates of type 2 diabetes mellitus (n=25, 26%), hypercholesterolemia were lower than expected [266; 267]. Prevalence of hypertension (n=20, 20.8%) in our cohort was within the expected range [268]. There was a significant difference in males between groups as assessed by a chi square test ($p < 0.05$).

There was no difference in age, weight, or BMI between groups. There was a statistical difference in the prevalence of diabetes, hypertension, GORD and OSA between groups ($p < 0.05$, by chi-square test) (Table 3.1). We observed a significant increase in C-peptide in the NASH cohort compared to those with no pathology, however, there was no differences between these groups for glucose, insulin, or HbA1c. There was also no difference in any other plasma metabolite between groups. Plasma ALT and AST levels were similar between groups and contrasting previous observations using elastography [269] total bilirubin was increased with NASH compared to patients with NAFL or no pathology ($p < 0.05$, Table 3.1). There were no differences in any other parameter measured.

Table 3.1. NAFL & NASH biomarker discovery participant characteristics.

	No pathology (n=26)	NAFL (n=59)	NASH (n=11)
Age (years)	42.2 ± 13.2	41.5 ± 9.3	42.6 ± 9.6
Male (n)	3	17	5
Weight (kg)	116.0 ± 17.3	126.4 ± 24.9	128 ± 21.9
BMI (kg/m ²)	41.9 ± 6.0	44.1 ± 6.9	42.6 ± 6.6
Diabetes Mellitus (n)	1	22	2
Hypercholesterolemia (n)	1	3	1
Hypertension (n)	5	12	3
GORD (n)	2	10	0
OSA (n)	4	9	2
TG (mM)	1.3 ± 0.5	1.6 ± 0.5	1.7 ± 1.2
Total cholesterol (mM)	3.9 ± 0.9	4.2 ± 0.9	4.2 ± 1.2
HDL (mM)	1.1 ± 0.2	0.9 ± 0.3	0.9 ± 0.2
Non-HDL (mM)	2.9 ± 0.8	3.3 ± 0.9	3.2 ± 1.2
LDL (mM)	2.3 ± 0.8	2.5 ± 0.9	2.4 ± 1.0
Fasting Glucose (mM)	4.9 ± 0.9	5.5 ± 2.1	5.2 ± 1.0
HbA1c (%)	5.3 ± 0.3	5.6 ± 0.7	5.8 ± 0.9
Insulin (mU/L)	10.3 ± 7.0	13.4 ± 9.7	17.5 ± 11.8
C-peptide (nM)	0.8 ± 0.4	1.0 ± 0.4	1.4 ± 0.6*
Urea (mM)	4.7 ± 2.4	4.6 ± 1.8	4.7 ± 1.6
Creatine (µM)	67.0 ± 12.3	74.6 ± 25.5	74.7 ± 19.2
eGFR (mL/min/1.73m ²)	86.7 ± 8.5	85.0 ± 11.3	83.5 ± 11.8
Albumin (g/L)	37.7 ± 3.7	39.9 ± 6.2	39.5 ± 4.3
Total Bilirubin (µM)	6.8 ± 3.4	9.6 ± 5.5	20.2 ± 21.1 ↓*
ALT (U/L)	46.7 ± 75.2	50.5 ± 37.3	59.5 ± 27.4
AST (U/L)	33.2 ± 30.7	36.5 ± 21.9	44.2 ± 21.2
GGT (U/L)	27.1 ± 16.7	35.5 ± 21.6	32.3 ± 14.3
ALP (U/L)	76.1 ± 19.8	76.3 ± 22.6	72.1 ± 32.9
Iron (µM)	11.7 ± 4.0	12.8 ± 4.4	13.8 ± 4.9
Ferritin (ng/mL)	98.6 ± 86.4	175.4 ± 146.8	211.7 ± 148.8
Haemoglobin (g/L)	132.3 ± 15.3	140.4 ± 13.0*	142.0 ± 12.9
White Cell Count (*10 ⁹)	7.2 ± 2.0	7.0 ± 2.2	7.5 ± 2.1
Platelets (*10 ⁹ /L)	257.6 ± 60.0	269.5 ± 58.6	251.1 ± 66.5
MCV (fl)	89.2 ± 3.0	88.4 ± 3.9	89.7 ± 5.4
MCH (pg)	28.7 ± 1.5	28.7 ± 1.8	29.5 ± 2.2
MCHC (g/L)	321.8 ± 11.5	325 ± 12.4	328 ± 15.1
TSH (mU/L)	5.3 ± 14.4	2.1 ± 1.8	2.4 ± 1.1
Vitamin B12 (pg/mL)	366.0 ± 124.4	419.1 ± 211.8	519.4 ± 254.4
Vitamin D (ng/mL)	59.8 ± 25.0	57.3 ± 21.2	56.0 ± 22.0

ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; GORD, gastro-oesophageal reflux disease; GGT, gamma-glutamyl transferase; Hb, haemoglobin; HbA1c, glycosylated haemoglobin; HDL, High-density lipoprotein; LDL, low-density

lipoprotein; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; OSA, obstructive sleep apnoea; TG, Triglycerides; TSH, Thyroid-stimulating hormone; WCC, white cell count. * Indicates significant difference ($p<0.05$) compared to no pathology. † denotes significant difference compared to NAFL as determined by one-way ANOVA with Bonferroni's multiple comparisons. Significance set at $p<0.05$.

3.2.2. Current non-invasive scores for NAFLD and fibrosis perform poorly in this cohort

We assessed whether current non-invasive algorithms could adequately stratify NAFLD and NAFLD-associated fibrosis in our cohort. Non-invasive algorithms to stratify liver fat accumulation identified that the NAFLD liver fat score performed adequately to distinguish NASH with 4 incorrectly stratified, however, it performed poorly in patients with NAFL as 24 out of 59 incorrectly distinguished (Figure 3.1A). When comparing patients based on fibrosis severity, the NAFLD liver fat score performed well in patients with NAFLD F2-3, with only 3 out of 26 patients with an incorrect stratification, this did not hold true in other groups with 11 and 10 incorrectly stratified for patients with NAFLD F0 and NAFLD F1, respectively (Figure 3.1B). The hepatic steatosis index (HSI) did not perform well with all patients without NAFLD being incorrectly stratified with NAFLD when grouped by NASH or by NAFLD-associated fibrosis. (Figure 3.1C & D). Therefore, novel markers are required to develop and or enhance current algorithms to effectively stratify those with NAFLD and or significant fibrosis.

We further investigated complex scores to stratify those with NAFLD and significant fibrosis. No difference in the NAFLD fibrosis score (NFS) was observed between all four cohorts. Only 3 individuals with NAFLD F2-3 were stratified as high risk of fibrosis, and 69% of patients were classified as 'intermediate' and unable to be stratified (Figure 3.1E). Similarly, 50% of individuals with No pathology were intermediate and

unable to be stratified by the NFS (Figure 3.1E). The BARD score, defined by BMI, ALT, AST and diabetes showed that 80% of patients with No pathology were incorrectly stratified as high risk of significant fibrosis (Figure 3.1F). No patients were diagnosed with having significant fibrosis with the FIB-4 score with 73% of the NAFLD F2-3 being stratified for low risk (Figure 3.1G). The APRI score while accurately excluding those with No pathology and NAFLD F0, only 15% of patients with NAFLD F2-3 were stratified as high risk (Figure 3.1H). Together, these findings show that current complex panels to stratify those with fibrosis in patients with NAFLD are lacking and necessitates the needs for novel liver-specific biomarkers.

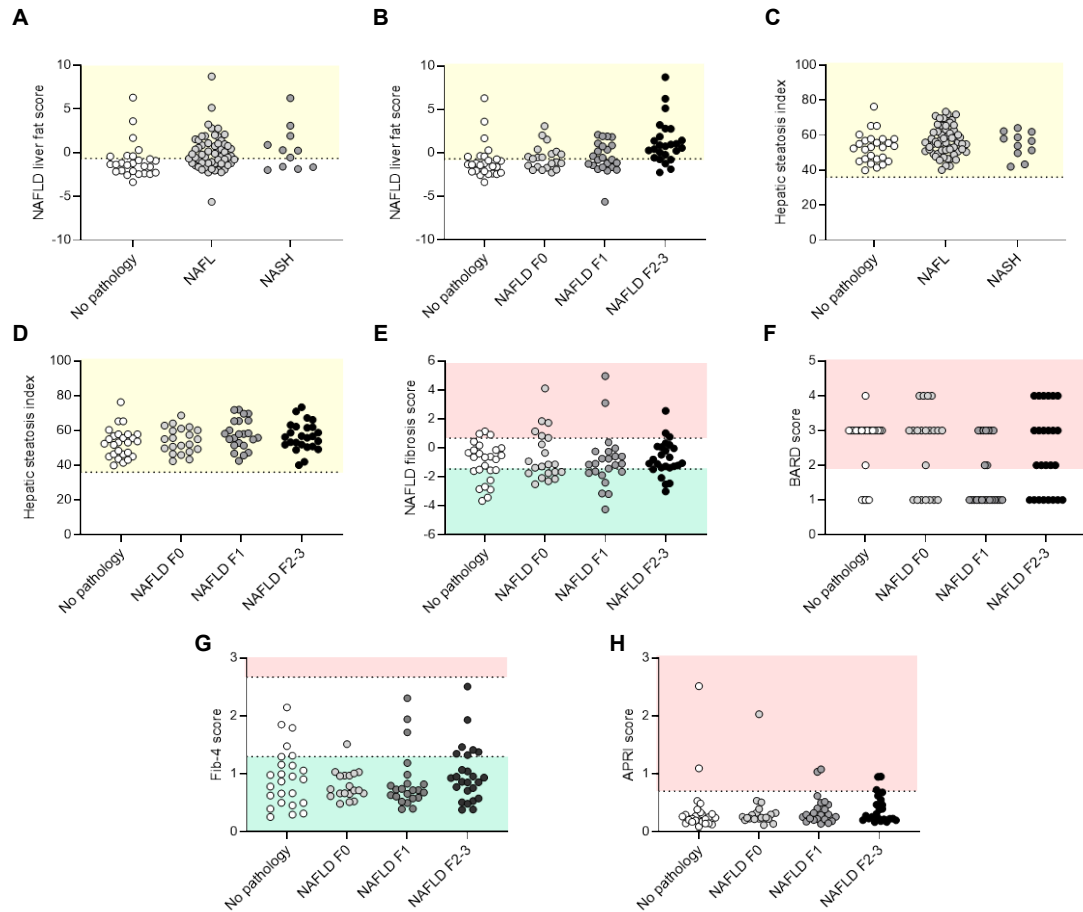


Figure 3.1: Non-invasive scores perform poorly in the obese. (A) Assessment of non-invasive algorithms to stratify NAFLD including the NAFLD liver fat score in patients grouped by NASH or **(B)** NAFLD associated fibrosis. **(C)** The hepatic steatosis index in patients grouped by NASH or **(D)** NAFLD associated fibrosis. **(E)** Assessment of non-invasive algorithms to diagnose fibrosis including the NAFLD fibrosis score, **(F)** BARD score **(G)** Fib-4 score **(H)** APRI score. yellow = high risk of NAFLD. Pink = high risk of fibrosis, green = exclusion of significant fibrosis. Dashed lines indicate the threshold for diagnosis or exclusion. No pathology, n=26; NAFLD F0, n=21; NAFLD F1, n=23; NAFLD F2-3, n= 26, NAFL, n=59; NASH, n=11. Each dot represents an individual patient.

3.2.3. Study design and validation of approach

To identify liver pathology-specific biomarkers, an intraoperative liver wedge biopsy was collected, precision-cut at 300 μm , and incubated in protein-free media for 16 hours, after which secreted proteins were detected using mass spectrometry proteomics and their relative abundance calculated (Figure 3.2A). We initially assessed the viability of the precision-cut liver slice preparation. The concentration of ATP in liver slices was not different following 16 hours of incubation compared to 0 hours (immediately before incubation) (Figure 3.2B) and ATP content was not different between groups (Figure 3.2C & D). Further, we assessed cytotoxicity marker lactate dehydrogenase in the secreted medium and there were no differences in lactate dehydrogenase alpha and beta chain between groups (Figure 3.2E & F).

Time course assessment of secreted proteins revealed a relatively linear increase over the duration of the 16 h incubation period (Figure 3.2G). Total protein secretion from the liver slices was significantly decreased in patients with NAFLD, with or without fibrosis, compared with no pathology (Figure 3.2H). Similarly, total protein secretion was reduced by ~52% in NASH compared with no pathology (Figure 3.2I). There was no significant correlation between the amount of secreted protein and intracellular ATP content, suggesting that the energy state of the liver preparation was not mediating changes in total protein secretion (Figure 3.2J). Together, these data indicate that the precision-cut liver slices are a viable approach to study liver secreted proteins.

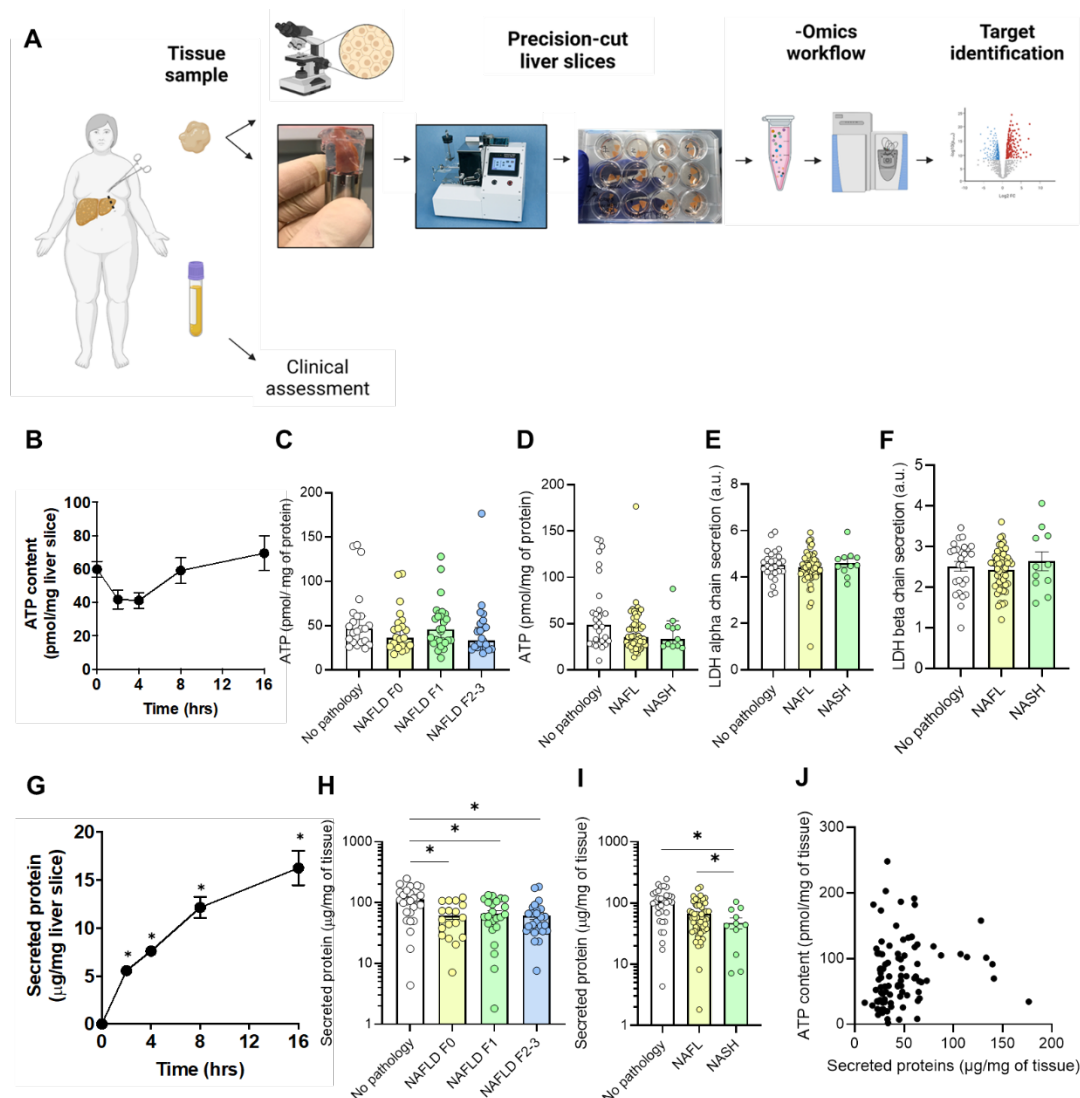


Figure 3.2: Validation of human liver slices for assessing liver-protein secretion. (A) Schematic representation of study design. Patients undergoing bariatric surgery were recruited, and a plasma and liver biopsy was taken. Plasma and liver were assessed by pathology clinics and the remaining liver was precision-cut and cultured in protein free medium to identify secreted proteins and these proteins were identified by mass spectrometry for biomarker identification. The additional plasma was used for mass spectrometry biomarker validation. (B) ATP content in liver slices incubated for 2, 4, 8 and 16 h (n=12). (C) Tissue ATP concentration stratified by severity of NAFLD associated fibrosis or (D) NASH. (E) Lactate dehydrogenase (LDH) alpha chain and (F) LDH beta chain detection in the secreted medium. (G) Proteins content in the secreted medium cultured for 2, 4, 8 and 16 h with liver slices (n=12). (H) Secreted protein concentration stratified by severity of NAFLD-associated fibrosis or (I) NASH. (J) Correlational analysis of tissue ATP levels and secreted protein concentration. No pathology, n=26; NAFLD F0, n=21; NAFLD F1, n=23; NAFLD F2-3, n= 26; NAFL, n=59; NASH, n=11. Where appropriate, significance was determined by two-way (Panel B & G) or one-way (Panel C-F, H & I) ANOVA with Bonferoni's post hoc test or Pearson's correlation co-efficient (Panel J). Asterisks in panel G are relative to time 0. Values are presented as mean $\pm$ SEM. * denotes $p < 0.05$.

3.2.4. Identification of NASH secreted proteins

We assessed global changes in liver protein secretion using mass spectrometry and identified a total of 3333 proteins in the secreted medium. After removal of potential contaminants or proteins identified by reverse, we identified 3281 proteins in total. The average number of proteins identified across 96 individuals and all groupings was 2806 (median=2840) (Figure 3.3A) with MS signals that span six orders of magnitude (Figure 3.3B). Of these 3249 proteins, approximately 9.8% were classically secreted and 90.2% were non-classically secreted (Figure 3.3C) as assessed by the Human Protein Atlas. There were no proteins identified exclusively in any one group and at least 2 peptides were required for protein detection. We detected 63 proteins enriched and 39 proteins less abundant in the secreted medium with NASH than those with no liver pathology (Figure 3.3D). Bioinformatic assessment of the biological functions of significantly altered proteins revealed increased involvement in humoral immune and inflammatory responses, blood vessel morphogenesis, and integrin signalling pathways with NASH (Figure 3.3E). On the other hand, regulation of protein catabolism, endocytic recycling, and endosomal transport process was reduced with NASH secreted proteins (Figure 3.3E). Canonical pathways enriched from NASH secreted proteins reveal upregulation of extracellular matrix pathways such as the core matrisome, basement membrane, and ECM regulator (Figure 3.3F). No canonical pathways were revealed for downregulated NASH proteins assessed by MetascapeTM. Extending on this, when compared to NAFL livers, we identified increased secretion of 25 proteins and a reduction in the secretion of 16 proteins (Figure 3.3G). Enrichment analysis of significantly altered secreted proteins predicts upregulation of cell-substrate adhesion, apoptosis signalling pathway, wounding response, and reduction in protein catabolism, translation, and amide metabolism (Figure 3.3H). Canonical pathways upregulated in NASH secreted proteins compared to NAFL encompass extracellular matrix pathways, including integrin

signalling, ECM regulators, and core matrisomes (Figure 3.3I). To identify NASH regulated proteins, we compared proteins that were significantly altered in NASH compared to No pathology and NAFL and identified that of the 107 proteins altered with NASH, 36 proteins were significantly increased in both comparisons (Figure 3.3J). Further of these 36 proteins 20 were increased and 16 were decreased (Figure 3.3K).

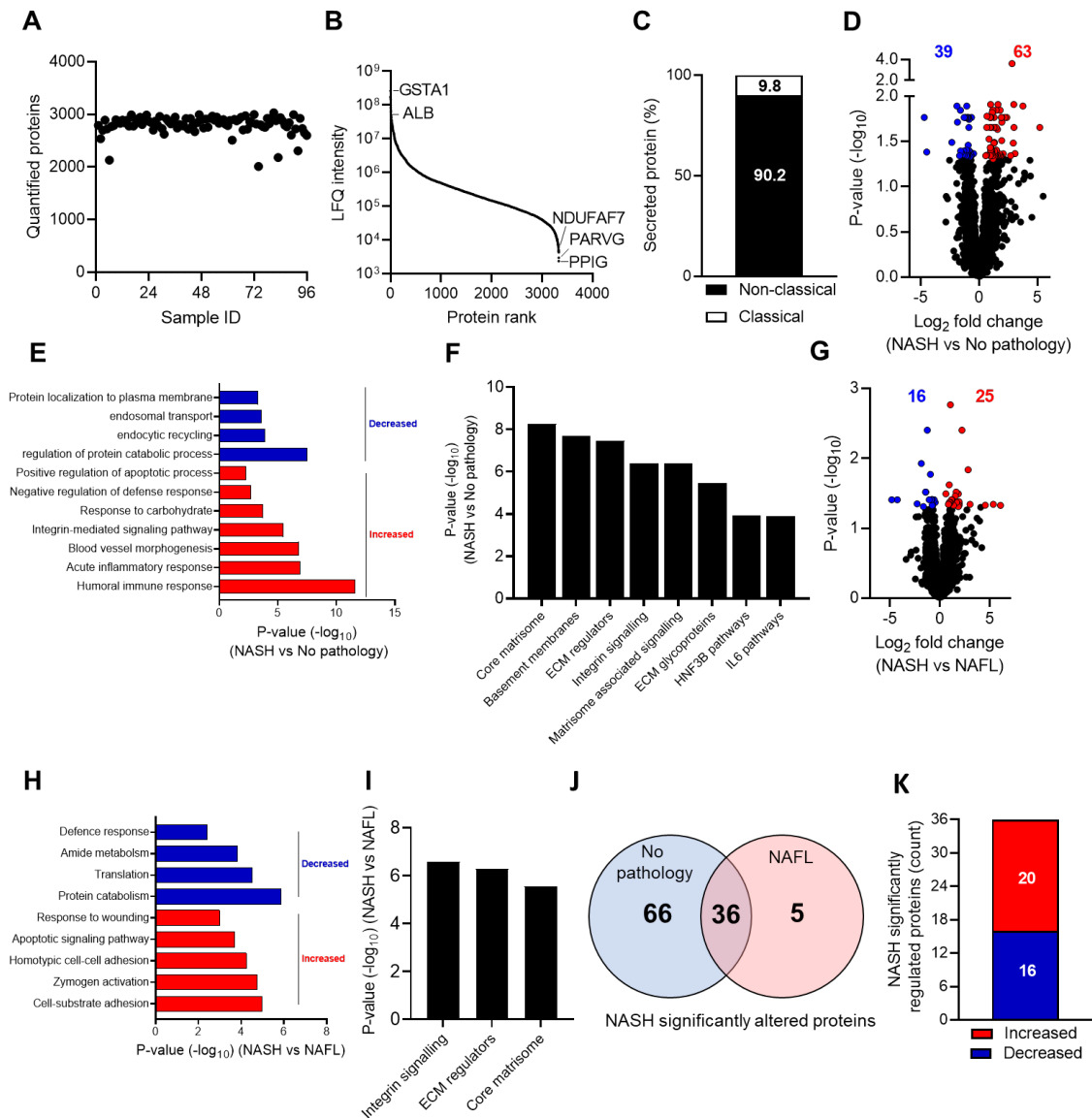


Figure 3.3: Characterisation of the human NASH liver-secreted proteome. (A) Numbers of proteins quantified per patient. **(B)** Dynamic range of quantified proteins (LFQ, label-free intensity; ALB, Albumin; GSTA1, Glutathione S-Transferase Alpha 1; NDUFAF7, NADH: Ubiquinone Oxidoreductase Complex Assembly Factor 7; PARVG, Parvin Gamma; PPIG, Peptidylprolyl Isomerase G. **(C)** Percentage of classically secreted proteins secreted by human liver slices identified by The Human Protein Atlas. **(D)** Volcano plot showing secreted proteins increased (red), decreased (blue), or not different (black) with NASH compared to livers without adverse pathology. **(E)** Metascape enrichment analysis showing biological processes associated with significantly altered proteins. **(F)** Canonical pathways upregulated with secreted proteins. **(G)** Volcano plot showing liver slice showing secreted proteins increased (red), decreased (blue), or not different (black) with NASH compared to livers with NAFL. **(H)** Metascape enrichment analysis showing biological processes associated with significantly altered proteins with NASH vs NAFL and **(I)** upregulated canonical pathways. **(J)** The number of significantly altered proteins in NASH vs NAFL and or NASH vs No pathology comparisons. **(K)** The number of NASH -responsive proteins that were increased (red) or decreased (blue). Significance was determined by students

t-test corrected by permutation-based false discovery rate of $q < 0.05$. No pathology, $n=26$; NAFL, $n= 59$; NASH, $n=11$.

3.2.5. Identification of global changes in hepatokine secretion in NAFLD associated fibrosis

While the identification of NASH is important, clinically there is immense interest in identifying a biomarker for NAFLD-associated fibrosis attributed to its consistent with overall and liver-related mortality [141; 153]. Therefore, we re-stratified patients based on the presence of NAFLD and NAFLD-associated fibrosis.

Patients with NAFLD and a fibrosis score of ≥ 2 were defined as those with significant fibrosis (NAFLD F2-3), those with NAFLD and a fibrosis score of 1 or 0 were grouped as NAFLD F1 or NAFLD F0, respectively. Those without NAFLD and fibrosis were grouped as No pathology. No patients recruited in this prospective study had hepatic cirrhosis (score of F4).

There was a significant increase in weight in those with NAFLD F1 than those without liver pathology ($p < 0.05$). Additionally, there was a tendency for NAFLD F1 individuals to have an increased weight compared to individuals with NAFLD F0 ($p = 0.06$). There was no difference in BMI between any groups regardless of NAFLD or fibrosis severity.

We observed no difference in triglycerides, total cholesterol, LDL, or fasting plasma glucose between groups. Those with NAFLD F2-3 tended to have decreased HDL compared to those without adverse liver pathology ($p = 0.05$) (Table 3.2). Plasma urea

levels were increased in patients with NAFLD F2-3 compared to NAFLD F0 but were not different compared to any other group. Patients with NAFLD F2-3 exhibited greater circulating glycosylated haemoglobin (HbA1c) ($p<0.05$) compared to patients with NAFLD F1 without liver pathology ($p<0.05$). Furthermore, those with NAFLD F2-3 had a corresponding rise in insulin and C-peptide compared to patients without liver pathology. Total bilirubin was increased with NAFLD F2-3 than those without liver pathology ($p<0.05$). Circulating alkaline phosphatase (ALP) was increased in patients with NAFLD F2-3 than in those with NAFLD F1. No differences were observed for ALT or AST between cohorts (Table 3.2) but there was extensive variability in these measures, particularly in the no pathology group. We observed no difference in all other clinical measures (Table 3.2).

Table 3.2. Participant characteristics of patients stratified for significant fibrosis

	No pathology (n=26)	NAFLD F0 (n=21)	NAFLD F1 (n=23)	NAFLD F2-F3 (n=26)
Age (years)	42.2 ± 13.2	39.7 ± 8.8	38.9 ± 7.7	45.7 ± 9.8
Male (n)	3	3	8	11
Weight (kg)	116.0 ± 13.2	117.8 ± 8.7	134.6 ± 28.0*	126.9 ± 23.6
BMI (kg/m ²)	41.9 ± 6.1	42.3 ± 6.6	45.5 ± 7.4	43.7 ± 6.4
NASH (n)	0	3	2	6
Diabetes Mellitus (n)	1	7	5	12
Hypercholesterolemia (n)	1	0	0	4
Hypertension (n)	5	2	3	10
GORD (n)	2	1	2	7
Obstructive sleep apnoea (n)	4	2	3	6
TG (mM)	1.3 ± 0.5	1.6 ± 0.4	1.7 ± 0.9	1.6 ± 0.6
Total cholesterol (mM)	3.9 ± 0.9	4.6 ± 1.0	4.2 ± 0.9	4.0 ± 1.0
HDL (mM)	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
Non-HDL (mM)	2.9 ± 0.81	3.5 ± 0.9	3.3 ± 0.9	3.0 ± 0.9
LDL (mM)	2.2 ± 0.8	2.8 ± 0.9	2.5 ± 0.8	2.3 ± 0.9
Fasting glucose (mM)	4.9 ± 0.9	5.6 ± 3.2	5.1 ± 1.1	5.7 ± 1.6
HbA1c (%)	5.3 ± 0.3	5.5 ± 0.5	5.3 ± 0.6	5.9 ± 0.9*↓
Insulin (mU/L)	10.3 ± 7.0	11.7 ± 8.9	11.1 ± 8.0	18.0 ± 11.2*
C-peptide (nM)	0.9 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	1.3 ± 0.5*
Urea (mM)	4.7 ± 2.4	3.9 ± 0.7	4.4 ± 1.3	5.4 ± 2.2**
Creatine (µM)	67.0 ± 12.3	66.6 ± 12.7	73.6 ± 15.0	81.3 ± 34.8
eGFR (mL/min/1.73m ²)	86.7 ± 8.5	85.5 ± 8.8	87.0 ± 5.3	82.0 ± 15.8
Albumin (g/L)	37.7 ± 3.7	40.3 ± 8.7	40.0 ± 3.7	39.4 ± 4.7
Total Bilirubin (µM)	6.8 ± 3.8	8.9 ± 3.6	9.8 ± 6.5	14.6 ± 15.3*
ALT (U/L)	46.7 ± 75.2	41.3 ± 36.2	53.0 ± 38.0	56.8 ± 32.2
AST (U/L)	33.2 ± 30.7	31.3 ± 19.2	37.8 ± 19.8	41.6 ± 24.8
GGT (U/L)	27.1 ± 16.7	30.7 ± 17.7	32.6 ± 17.4	39.9 ± 24.9
ALP (U/L)	76.1 ± 19.8	70.7 ± 17.6	69.2 ± 19.7	86.9 ± 29.8↓
Iron (µM)	11.7 ± 4.0	13.1 ± 3.0	13.6 ± 4.7	12.0 ± 4.6
Ferritin (ng/mL)	98.6 ± 86.6	163.1 ± 197.0	170.7 ± 92.9	179.5 ± 101.8
Haemoglobin (g/L)	132.3 ± 15.3	138.2 ± 14.9	142.1 ± 9.0	140.6 ± 12.7
White Cell Count (*10 ⁹)	7.2 ± 2.0	6.5 ± 1.9	6.8 ± 2.1	7.7 ± 2.4
Platelets (*10 ⁹ /L)	257.6 ± 60.0	250.6 ± 60.78	280.2 ± 49.6	269.6 ± 68.4
MCV (fl)	89.2 ± 3.0	89.2 ± 3.7	88.7 ± 3.7	87.9 ± 5.0
MCH (pg)	28.7 ± 1.5	29.3 ± 1.6	28.9 ± 1.5	28.4 ± 2.4
MCHC (g/L)	321.8 ± 11.5	328.7 ± 13.5	324.9 ± 12.0	323.6 ± 13.2
TSH (mU/L)	5.3 ± 14	2.2 ± 0.9	1.5 ± 0.8	2.7 ± 2.4
Vitamin B12 (pg/mL)	366 ± 124.4	403.2 ± 266.6	416.8 ± 171.5	469.8 ± 217.5
Vitamin D (ng/mL)	59.9 ± 25.1	57.2 ± 17.9	57.2 ± 12.5	57.9 ± 29.2

ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; BMI, body mass index; eGFR, estimated glomerular filtration rate GORD, gastro-oesophageal reflux disease; GGT, gamma-glutamyl transferase; Hb, haemoglobin; HbA1c, glycosylated haemoglobin; HDL, High-density lipoprotein; LDL, low-density lipoprotein; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; OSA, obstructive sleep apnoea; TG, Triglycerides; TSH, Thyroid-stimulating hormone; WCC, white cell count. *Indicates significant difference ($p < 0.05$) compared to the no pathology group. **denotes significant difference compared to NAFLD F0. † denotes significant difference compared to NAFLD F1. Data displayed as mean $\pm$ SEM

3.2.6. NAFLD associated fibrosis does not drastically alter protein secretion

Our second analysis was to assess protein secretion from patients with NAFLD and significant fibrosis. Of the 3281 proteins assessed, ~10% were annotated by Human Protein Atlas as classically secreted in this stratification (Figure 3.4A). There were no proteins exclusively present or absent in any single grouping. We first sought to identify secreted proteins that were upregulated with NAFLD and significant fibrosis (NAFLD F2-3). There were surprisingly no differences between NAFLD F0 compared to those with no pathology or those with NAFLD F1 compared to NAFLD F0 (Figure 3.4B & C). Unexpectedly, we observed that individuals with NAFLD and significant fibrosis (NAFLD F2-3) exhibit no difference in liver secreted proteins compared to obese patients with no pathology (Figure 3.4D). However, when comparing NAFLD F2-3 with NAFLD F0 (Figure 3.4E), four proteins were increased, and one was decreased in NAFLD F2-3. The proteins enriched in the secreted medium were increased were elastin microfibril interfacer 1 (EMILIN1), amyloid P component (APCS), eukaryotic translation initiation factor 3 subunit K (EIF3K) and eukaryotic translation initiation factor 4 gamma 2 (EIF4G2). Protein tyrosine kinase 2 (PTK2) secretion was significantly reduced in NAFLD F2-3 (Figure 3.4E). Two of the 5 differentially regulated proteins, APCS and EMILIN1 are classically secreted, and the remainders were likely secreted unconventionally. No differences were observed when comparing NAFLD F2-3 to NAFLD F1 (Figure 3.4F) or NAFLD F1 to livers without pathology (data not shown). Bioinformatic assessment of the biological functions was performed but no enrichments were found.

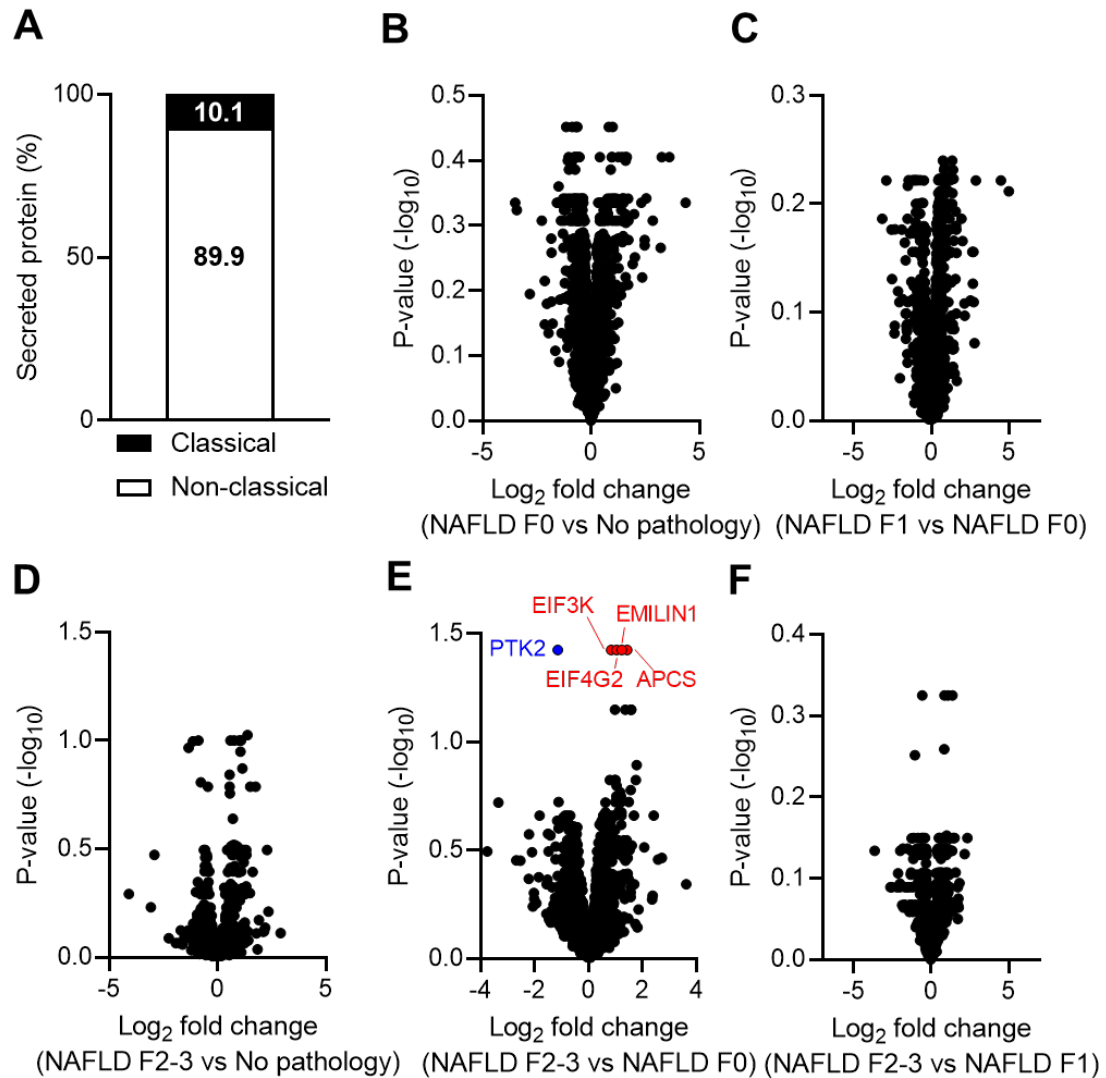


Figure 3.4: Effects of fibrosis of the liver-secreted proteins.(A) Percentage of classically secreted proteins secreted by human liver slices identified by The Human Protein Atlas. (B) Comparison of secreted proteins from livers with NAFLD F0 Vs No pathology, (C) NAFLD F1 Vs NAFLD F0 (D) Comparison of secreted proteins from livers with NAFLD F2-3 Vs No pathology, (E) NAFLD F2-3 Vs NAFLD F0. Significantly regulated proteins include amyloid P component (APCS), elastin microfibril interfacer 1 (EMILIN1), eukaryotic translation initiation factor 3 subunit K (EIF3K) and eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) and protein tyrosine kinase 2 (PTK2). (C) NAFLD F2-3 Vs NAFLD 1 (F) NAFLD F1 Vs NAFLD F0. Red and blue dots indicate significantly upregulated and downregulated proteins, respectively. Significance was determined by students t-test corrected by permutation-based false discovery rate of $q < 0.05$. No pathology, $n=26$; NAFLD F0, $n=21$; NAFLD F1; $n=23$; NAFLD F2-3, $n=26$. Proteins identified by gene name.

3.2.7. Liver secreted EMILIN1 is increased with NAFLD severity

The human proteomic analysis identified that EMILIN1 secretion was increased with NAFLD and significant fibrosis (Figure 3.4). EMILIN1 secretion was assessed when grouped based on fibrosis, NASH, and the Kleiner and brunt classifications and demonstrated that EMILIN1 secretion was increased by ~30% with NAFLD F2-3 compared to all other groups (Figure 3.5A). When assessed by NAFLD severity, we identified that EMILIN1 secretion increased with NASH and a NAS score of 4 or greater (Figure 3.5B & C). Further EMILIN1 secretion was increased with advanced steatosis, inflammation, and ballooning (Figure 3.5D-F).

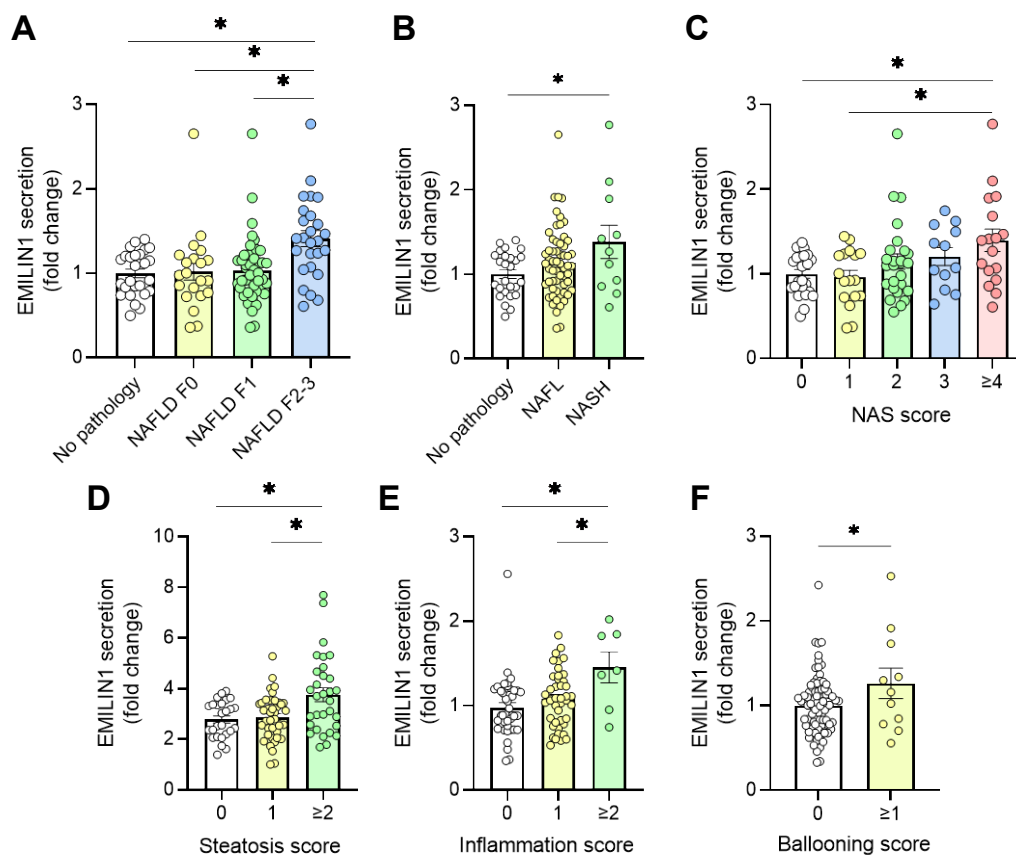


Figure 3.5: EMILIN1 is secreted from human livers with worsening NAFLD

pathology. Liver secreted EMILIN1 when grouped by (A) NAFLD associated fibrosis stage, (n=21-26/group) (B) NASH, (n=11-59/group) (C) NAFLD activity score, (n=11-26/group) (D) steatosis score, (n=26-39/group) (E) inflammation score (n=8-44/group) and (F) hepatocyte ballooning (n=21-85/group). Data were taken and analysed from the

human liver secreted proteome and stratified according to Kleiner/Brunt criteria [216]. Data displayed as mean $\pm$ SEM. Significance was assessed using one-way ANOVA followed by Bonferoni's multiple comparisons (Panels A-E) or unpaired t-test (panel F) and set at $p < 0.05$.

3.2.8. Liver *EMILIN1* mRNA is altered with NAFLD

We further wanted to identify if the mRNA levels of *EMILIN1* in the liver mirrored the secretion findings observed in Figure 3.5. Liver *EMILIN1* mRNA transcripts were increased in livers with NAFLD F2-3 and with NAFL or NASH compared to livers without adverse pathology (Figure 3.6A &B). *EMILIN1* expression was also increased in livers with a NAS score of 4 or greater, likely driven by steatosis (Figure 3.6C-D). *EMILIN1* expression was not altered by inflammation or hepatocyte ballooning (Figure 3.6E-F). Given the expression and secretion of EMILIN1 is increased in NAFLD and fibrosis, it remains plausible that EMILIN1 could be candidate biomarker for NAFLD-associated fibrosis.

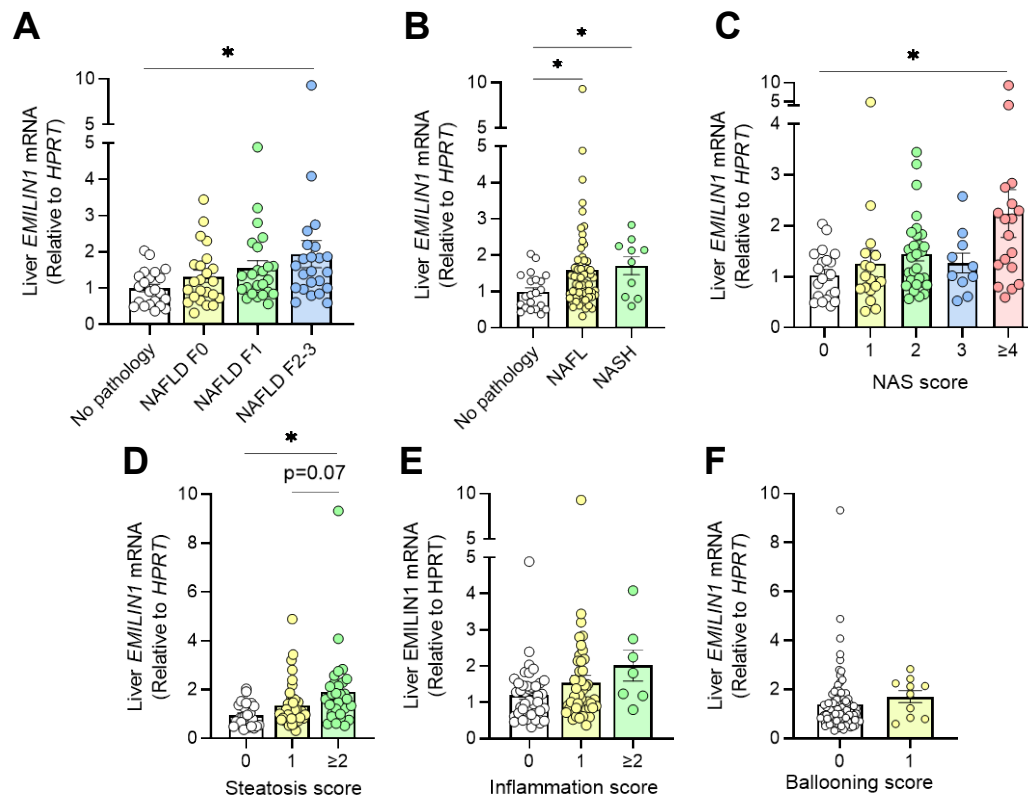


Figure 3.6: EMILIN1 is expressed in human livers with worsening NAFLD pathology. Hepatic *EMILIN1* expression when grouped by (A) NAFLD associated fibrosis stage, (n=22-24/group) (B) NASH, (n=10-61/group) (C) NAFLD activity score, (n=10-29/group) (D) steatosis score, (n=26-39/group) (E) inflammation score (n=7-47/group) and (F) hepatocyte ballooning (n=10-87/group). Data were taken and analysed from the human liver secreted proteome and stratified according to Kleiner /Brunt criteria [216]. Data displayed as mean ± SEM. Significance was assessed using one-way ANOVA followed by Bonferoni's multiple comparisons (panel A & E), Dunn's multiple comparisons (panel B, C, D) or unpaired t-test with significance set at $p < 0.05$.

3.2.9. Circulating EMILIN1 is increased with NASH but does not hold diagnostic value

To assess whether circulating EMILIN1 may be a potential biomarker for NAFLD F2-3, we assessed plasma levels of EMILIN1 by ELISA from our 96 patients, and an additional 219 obese patients with liver matched biopsies previously described elsewhere [270]. One patient in this combined cohort has hepatic cirrhosis (score of F4) however, the grouping remains identified as NAFLD F2-3 as it does not accurately reflect those with cirrhosis. Plasma EMILIN1 levels averaged 8.79 ± 0.05 ng/mL in our cohort and were not different with NAFLD F2-3 compared to any other group (Figure 3.7A). However, we did detect a significant increase in the plasma levels of EMILIN1 in patients with NASH compared to patients with NAFL or livers without adverse pathology, suggesting there may be clinical potential to aid in the diagnosis of NASH or NAFLD (Figure 3.9B). Circulating EMILIN1 was not altered by NAFLD severity as assessed by NAS score, or hepatic inflammation but increased with steatosis and hepatocyte ballooning (Figure 3.7C-F). The accuracy of non-invasive markers such as ALT, AST, and other scores (e.g., FIB-4) is to stratify patients with NASH, and hepatic fibrosis remains ineffective (Figure 3.1) [157]. Accordingly, we explored the potential use of plasma EMILIN1 as a biomarker of NAFL and NASH. The area under the receiver operating curve (AUROC) for EMILIN1 to diagnose NASH was 0.60 compared to the hepatic steatosis index that achieved an AUROC of 0.64 (Figure 3.7G). Further, when compared to the NAFLD fatty liver score (NLFS) with an AUROC of 0.75, EMILIN1 performed poorly with a score of 0.60 (Figure 3.7H). We then explored the potential of EMILIN1 to diagnose NAFLD (NASH and NAFL combined) and observed that EMILIN1 achieved an AUROC of 0.56, however, while NLFS achieved an AUROC of 0.69, and the hepatic steatosis index corresponded to 0.62 (Figure 3.7I & J).

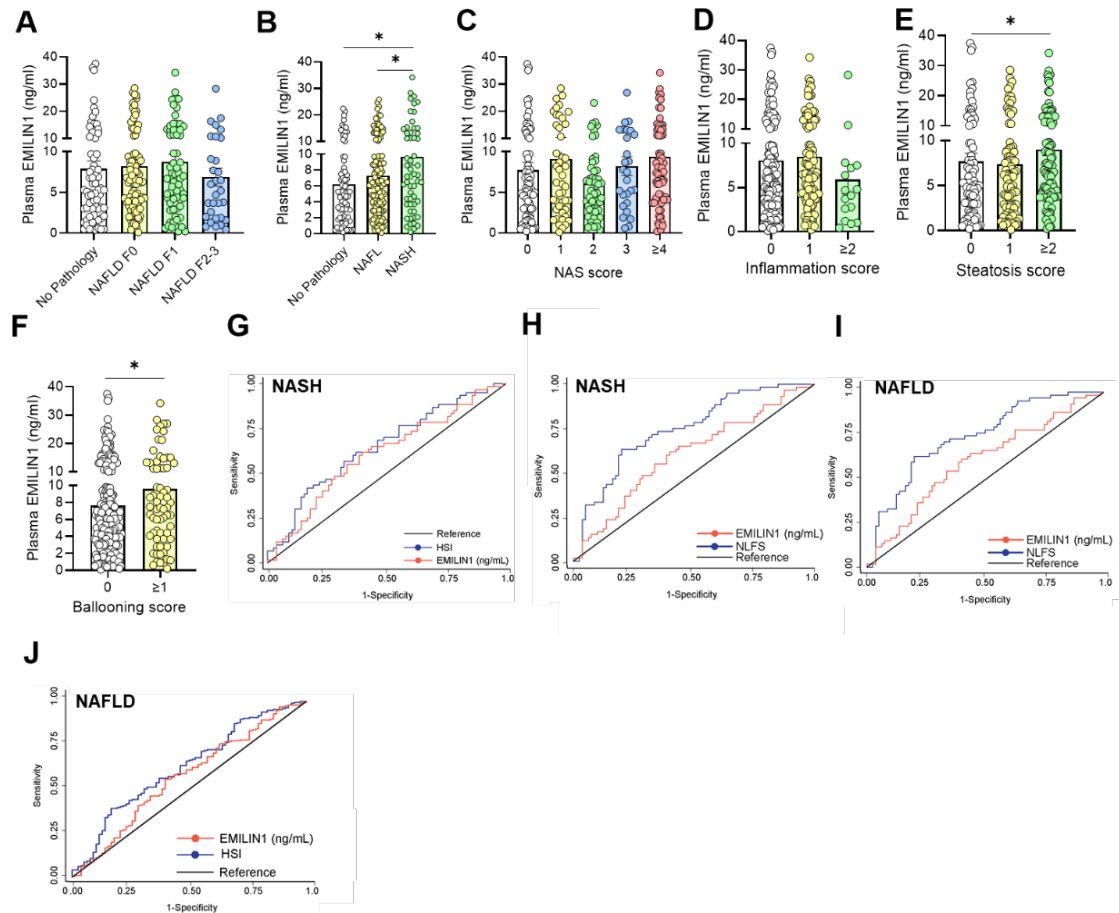


Figure 3.7: Circulating EMILIN1 is not an effective biomarker to diagnose NASH or NAFLD. (A) Plasma levels of EMILIN1 stratified by NAFLD associated fibrosis (n=56-130), (B) NASH, (C) NAFLD activity score (NAS) (n=81-132/group), (D) inflammation score (E) steatosis (n=82-102/group), (F) ballooning (n=68-200/group) and as specified by the Burnt/Kleiner criteria [147; 216] (n=16-106/group). (G) The diagnostic utility plasma EMILIN1 to diagnose NASH using a receiver operating characteristic curve and was compared against the hepatic steatosis index (HSI) and the (H) NAFLD liver fat score (NLFS). The diagnostic accuracy of plasma EMILIN1 was assessed to diagnose NAFLD and compared against the (I) NLFS and (J) HSI. Data displayed as mean $\pm$ SEM. Significance was assessed using one-way ANOVA followed by the Kruskal-Wallis test with Dunn's multiple comparisons (panel A-D, F) or Mann-Whitney U test with significance set at $p < 0.05$.

3.3. Discussion

Herein, we investigated the human liver secreted proteome as a resource to identify proteins whose secretion is altered in livers with NAFLD and NAFLD with significant fibrosis (NAFLD F2-3). We show that (1) current non-invasive measures do not adequately perform in our cohort, (2) that the liver secreted proteome is minimally altered with significant fibrosis, (3) that NASH remodels liver protein secretion, and these changes are enriched inflammatory pathways such as humoral immunity and acute inflammatory response, (4) EMILIN1 expression and secretion is altered in NAFLD F2-3 and NASH and circulating EMILIN1 levels does not represent an effective biomarker target to distinguish NASH or NAFLD.

There remains an unmet clinical need to develop a safe and tool to adequately detect and stage NAFLD, NASH, and hepatic fibrosis at large-scale. A suitable alternative to an invasive liver biopsy can be a blood test due to the reduced procedural risks and ease of large-scale use. However, few have assessed liver protein secretion, particularly in humans. Previous studies characterising the murine liver and plasma proteome have shown that the liver may be responsible for 25% of circulating proteins [271]. Further, the plasma proteome is altered in humans with NAFLD [272-274] and cirrhosis [274], suggesting that liver secreted proteins may be altered with NAFLD / NASH and hepatic fibrosis. Therefore, understanding what livers with NAFLD, NASH, or fibrotic livers are secreting into the blood and if it corresponds to a rise in circulating levels in the potential parameter exposes an exciting opportunity to address this unmet demand. To address this, we performed an intraoperative liver wedge biopsy in obese patients and generated precision-cut liver slices to assess liver protein secretion with or without various stages of NAFLD with the aim of identifying liver and pathology-specific

alterations in liver secreted proteins. A surprising finding was the reduction in secreted proteins detected in patients with NAFLD and NASH (Figure 3.2). Given that livers with NAFLD and NASH have 5- >66% of their area enriched with lipids, perhaps normalizing to the protein content would be more reflective of the liver physiology as it is benchmarked against the enzymatic machinery required to secrete these proteins.

Expanding on previous *in vitro* or *ex vivo* studies [5; 11; 275], we identified 3333 secreted proteins from human liver slices. NASH, but not hepatic fibrosis, was primarily responsible for the remodelling of protein secretion. Bioinformatic investigation revealed an enrichment of secreted proteins in integrin signalling, the regulation of ECM and ECM or 'matrisome' synthesis (Figure 3.4) with NASH, which is consistent with more progressive disease and may capture the liver crosstalk to explain why NASH livers progress to fibrosis to a greater extent than livers without adverse pathology [142]. Further computational assessment of biological processes from NASH livers predicts upregulation of humoral immunity, also termed antibody-mediated immunity and acute inflammatory responses, consistent with the inflammatory nature of NASH compared to 'normal' livers. When compared to NAFL livers, apoptosis, cell-substrate adhesion, and zymogen activation processes were upregulated with NASH, which is consistent with hepatocyte ballooning and enhanced interaction with the ECM in NASH livers [276]. Zymogen activation is commonplace in ECM proteins such as matrix metalloproteases (MMP) to control catalytic activity and digestion of the ECM [277; 278] and may be indicative of increased ECM synthesis. Collectively, we show that protein secretion is remodelled with NASH and speculate that hepatokine secretion may be responsible for the faster rate of fibrogenesis observed in these patients [32]. To

assess this, future studies could by apply conditioned medium containing NASH remodelled hepatokines to isolated HSCs and assess activation and proliferation.

A major cytokine that underpins the development and progression of NAFLD / NASH, fibrosis, and its associated co-morbidities is transforming growth factor- β 1 (TGF β 1) [279-281]. Thus, it was interesting that livers with more progressive disease (NAFLD F2-3, NASH, and NAS $\geq$ 4) expressed and secreted more EMILIN1 (Figure 3.5-3.7) given that EMILIN1 has previously been shown to bind to and prevent the cleavage of pro- TGF β 1 [282], which is suggestive of a counter-regulatory role in preventing uncontrolled TGF β signalling. Despite this, little is known about the physiological functions of EMILIN1 and its potential role in fibrosis, NASH, and metabolic disease and warrants further investigation. Given that expression and secretion of EMILIN1 was increased in NASH and NAFLD F2-3, and the circulating levels were unknown (Figure 3.6 & 3.7), we sought to determine plasma EMILIN1 levels in our cohort and an additional cohort previously characterised elsewhere [120; 270]. There was no difference in circulating levels of EMILIN1 in fibrosis or NAS score comparisons; however, we did observe a 30% increase in plasma levels in NASH patients, which corresponds to the ~30% increase in liver secretion with NASH (Figure 3.7B). Despite this, EMILIN1 was not effective as a potential biomarker to stratify NASH or NAFLD alone or in combination with various non-invasive scores (Figure 3.7). This may be suggestive of hepatokines perhaps having a greater autocrine, paracrine role as evidence by Fetuin B [11], SPARC Related Modular Calcium Binding 1 (SMOC1) [283], Arylsulfatase A (ARSA) [5] and Hexosaminidase A (HEXA) [284], however this is yet to be proven. Alternative, the variability in circulating levels could be attributed to differing histopathologists and their grading of NASH and or fibrosis, or more likely,

that circulating EMILIN1 alone does not represent liver function or damage. Another potential confounder regarding EMILIN1 is the cells or tissues that secrete EMILIN1. EMILIN1 protein is identified in every cell type within the liver [177]. However, little work has been described in tissue distribution at the protein level or secretion from other tissues that may be the primary source of circulating levels of EMILIN1.

Conceptually, identifying liver secreted proteins that are differentially regulated would correspond to a rise in the blood of said protein. However, it is not that simple. To be identified in the circulation, secreted factors need to be secreted, escape the extracellular matrix and digestive enzymes, avoid non-parenchymal cells in the sinusoid such as the HSC and Kupffer cells and then be shuttled through the liver sinusoidal endothelial cells [285] to be present in the circulation that is filled with circulating proteases. Indeed, our model is more representative of proteins likely to transverse to the plasma than isolated hepatocytes, however liver slices are devoid of infiltrating cells such as neutrophils that act to degrade ECM proteins like EMILIN1 [286; 287] and may explain its lack of clinical utility as a biomarker.

Perhaps one of the findings unique to our preparation or humans is that lipid accumulation alone does not remodel protein secretion (Figure 3.5), i.e., NAFL and NAFLD F0 compared to no pathology. This finding contrasts previous murine hepatocyte isolation studies with varying degrees of NAFL [11] or NASH [5], highlighting extensive changes in protein secretion. It is possible that species differences (human versus murine) or experimental preparations (intact liver versus isolated hepatocytes) may account for this discordance. Another likely explanation may lie in the clinical definition of steatosis. The brunt classification defines the threshold of

NAFL or steatosis at 5% in the field of view [147]. Our cohorts range from 1-3, representing 5% to >66% of the histological area (Table 1.1 – See Chapter 1). Given the vast variability of hepatic lipid accumulation within the NAFL group (from scores of 1-3), this may explain why protein secretion is not different. Perhaps a higher cut-off for the percentage of steatosis area may be required to elucidate these differences. This notion may explain the disconnect in the remodelling of protein secretion in isolated hepatocytes derived from low-fat and high-fat fed mice, as they do not exhibit the same variability in hepatic lipid accumulation. Moreover, we have performed global correlation analysis of liver secreted proteins normalised to the histopathologically diagnosed percentage of steatosis area and found that is significantly and positively correlated to secretion of 173 classically secreted proteins (data not shown), further supporting the notion that the amount of lipid storage may dictate protein secretion. Ongoing studies should regroup our liver proteome by steatosis score potentially by comparing a steatosis score of ≥ 2 vs 0, to identify differentially regulated proteins impacted by significant steatosis to resolve discrepancy.

Pioneers in our laboratory have characterized that murine livers have remodelled protein secretion from hepatocytes derived from NAFL and NASH mice which have led to the identification of several classically secreted proteins that regulate glycaemic control (e.g., Cathepsin S, Fetuin B, SMOC1, ARSA) [5; 11; 201; 288] and dyslipidaemia (HEXA) [284]. While the focus of this chapter is to identify liver-specific biomarkers, it is interesting that none of these proteins were significantly altered in the secretome of NASH patients and may reinforce the discrepancy in isolated hepatocyte and precision-cut liver slices. Despite this, we provide a novel resource characterising hepatokine

secretion in human NASH and NAFLD F2-3 and that some of which are yet to be functionally characterized in the liver and NAFLD.

Our study is not without limitations. We cannot exclude self-selection bias that is present in all translational studies that are not randomized. However, recruited patients were unaware of their liver pathology and grouping prior to diagnosis, with exceptions for few patients $n = <10 / 265$. Further, the prevalence of NAFLD and NASH is aligned with previous observations in the same obese populations [145; 270] and in our discovery cohort we have few patients with NASH. Notably, we have collapsed two cohorts sampled forming one discovery cohort to identify plasma biomarkers and lack a validation cohort. The use of liver biopsies to diagnosis NAFLD and fibrosis, coupled with histological interpretation are subject to limitations. Sampling variability is evident in liver biopsies [289] and without performing multiple biopsies, we may have incorrect diagnosis of NAFLD and fibrosis severity. Further, biopsies were interpreted and scored using a defined scoring system by an independent pathologist, two pathologists when including the additional 176 patients and have not assessed the kappa value between pathologists for assessing the diagnosis of NAFLD / NASH and the severity of fibrosis. Given liver biopsies are considered the gold standard to which all non-invasive algorithms are benchmarked against the use of an imperfect reference may lower accuracy of non-invasive scores.

In conclusion, we have developed a novel resource characterising the proteomic changes in secreted proteins from human livers with NAFLD F2-3 and NASH. These studies identified altered hepatokine secretion, particularly in NASH. Investigation into liver-secreted protein EMILIN1 did not provide significant clinical benefit. Assessment

of secretion does not necessarily correspond with changes in circulating levels. Future studies should directly assess plasma proteins and match to liver secrete proteins to identify NASH and NAFLD F2-3 pathophysiology specific biomarkers.

Chapter 4: Plasma proteomics discovers biomarkers that stratify NASH and hepatic fibrosis

4.1. Introduction

NAFLD is the most prevalent liver disease worldwide [8-10]. It comprises a spectrum of histological conditions, including NAFL and its more progressive form NASH. NAFL is characterised by steatosis that comprises $\geq 5\%$ in the field of view assessed by light microscopy and progresses to NASH when accompanied by lobular inflammation and hepatocyte ballooning [216]. Fibrosis can be present at any stage of NAFLD, and it is associated with treatment complications, liver-related and overall mortality [33; 140; 141]. NAFLD diagnosis is typically incidental [290], and progression to more severe disease is typically asymptomatic, with patients often presenting later at end-stage liver disease with limited treatment options [291].

Despite the high prevalence of NAFLD, there remains a lack of practical, effective, and non-invasive screening options to identify early-stage disease before disease progression. The current gold standard is the liver biopsy to diagnose NAFLD, NASH and liver fibrosis [96]. However, widescale use of the liver biopsy is not practical given the time, risks of adverse events and high volume of patients at risk of NAFLD [8-10]. On the other hand, there are several unvalidated simple diagnostic tools that do not require special tests include APRI, Fib-4 and other commercial biomarker panels such as the enhanced liver fibrosis test [292]. Not only do these tests perform poorly in obese individuals [157] that are at high risk of NASH and hepatic fibrosis [98; 293; 294], these tests are currently not approved by Australian or USA regulatory agencies and do not effectively reflect the NASH or fibrosis pathology [157]. Significant advancements

in imaging modalities to diagnose NAFLD, NASH and advanced fibrosis. Magnetic resonance imaging (MRI) has strong predictability to diagnose hepatic fibrosis [295] and has a modest ability to differentiate NAFL from NASH [162; 296]. Similarly, transient elastography (TE) effectively stratifies obese patients with severe fat accumulation but not effective at differentiating NASH from NAFL [297]. Both imaging approaches, MRI, and TE, require visitation to specialised centres that not practical for population level screening. Therefore, novel practical approaches to develop biomarker panels or complex scores that can identify NASH and hepatic fibrosis remain unmet for patients with and at risk of NAFLD.

Mass spectrometry-based proteomics provides a global and unbiased assessment of differentially regulated proteins and provides insight into their biological functions within a sample [298]. Plasma is an easily accessible fluid that can provide insight into various clinical phenotypes such as NAFLD and is effective for population-level screening [274; 299; 300]. Given that ~25% of circulating proteins are likely derived from the liver [177], and liver secreted proteins are altered in NASH and mildly with NAFLD and significant fibrosis (NAFLD F2-3, Chapter 3), this suggests a high likelihood that NAFLD and NASH will affect the plasma proteome. Plasma proteomics can facilitate biomarker discovery [273; 274; 301; 302] by generating up to thousands of candidate proteins to develop a panel of proteins that effectively stratify patients with NASH or NAFLD-associated fibrosis.

We previously characterised protein secretion from livers with patients with NASH and NAFLD F2-3 (Chapter 3). EMILIN1 was identified as a highly expressed and secreted protein from livers of patients with NASH, and despite higher circulating levels, was

ineffective at stratifying patients with NASH. Therefore, we speculated that liver secretion might not correspond to circulating levels, and a more appropriate approach would be to assess plasma proteins. An inherent problem in plasma proteomics is mitigating the over 13 orders of magnitude [303] limiting protein detection. We elected to take a more nuanced approach targeting high abundant proteins given 61.6% of proteins detected in the top200-1000 proteins [227] were also detected in our mass spectrometry proteomic analysis of human liver secreted proteins (Chapter 3). Identifying plasma proteins that can effectively reflect the underlying NASH and NAFLD F2-3 pathophysiology would provide a safe and routine procedure that is effective at a population level. Therefore, we aimed to characterise the plasma proteome to identify novel biomarkers for NASH and NAFLD F2-3. We hypothesised that the plasma proteome will differ between patients with NASH or NAFLD F2-3 compared to patients with no pathology. We further hypothesise that the identification of NASH and fibrosis-induced plasma proteins can be used as novel non-invasive biomarkers for these diseases.

4.2. Results

4.2.1. Plasma proteome is altered with NASH and NAFLD F2-3

Given that our liver secreted proteome did not lead to the identification of an effective biomarker for NASH and NAFLD F2-3, we chose to assess the plasma proteome of our 96 patients described in Chapter 3 and an additional 219 obese patients previously described [270], totalling 315 patients. Significant haemolysis was evident in the plasma of 33 patients, who were subsequently excluded from further analysis. Protein identification was defined by the detection of $x > 2$ unique peptides. Patients with other liver diseases and patients with fibrosis without NAFLD were excluded ($n=17$ removed), giving a total of 265 patients for plasma proteomic analysis. We identified a total of 240 proteins and a highly reproducible average of 233 proteins (median = 235) per patient. Our plasma proteome spanned five orders of magnitude (Figure 4.1B) and 41.3% of the proteins identified in the plasma were considered “liver-specific” when compared to current annotation data from the Human Protein Atlas [304] (Figure 4.1C). Correlational analysis of matched plasma and liver secreted proteins from our discovery cohort (Chapter 3, $n=96$) identified 148 proteins detected in the liver and plasma proteomes identifies a positive but weak relationship (Figure 4.1E), indicating that changes in secretion does not necessarily correspond to alterations in circulating levels.

Surprisingly, there was no difference in the proteome of obese patients with NAFL compared to No pathology (Figure 4.1F), and no proteins were only detected in one group. Patients with NASH had 11 proteins markedly altered in the plasma, of which eight were increased, and three were decreased compared to those with No pathology (Figure 4.1G). When NASH was compared to NAFL, we identified nine altered proteins, including a reduction in apolipoprotein F (APOF), pregnancy zone protein

(PZP), CD5 molecule like (CD5L), and an increase in Ficolin 3 (FCN3), transforming growth factor beta induced protein (TGFB1) and immunoglobulin kappa variable 2-28 (IGKV2-28; Figure 4.1H). APOF and PZP were decreased in patients with NASH compared to NAFL and No pathology, and ACTA2, FCN3 and TGFB1 were increased with NASH compared to NAFL and No pathology and may be effective potential as biomarkers for NASH.

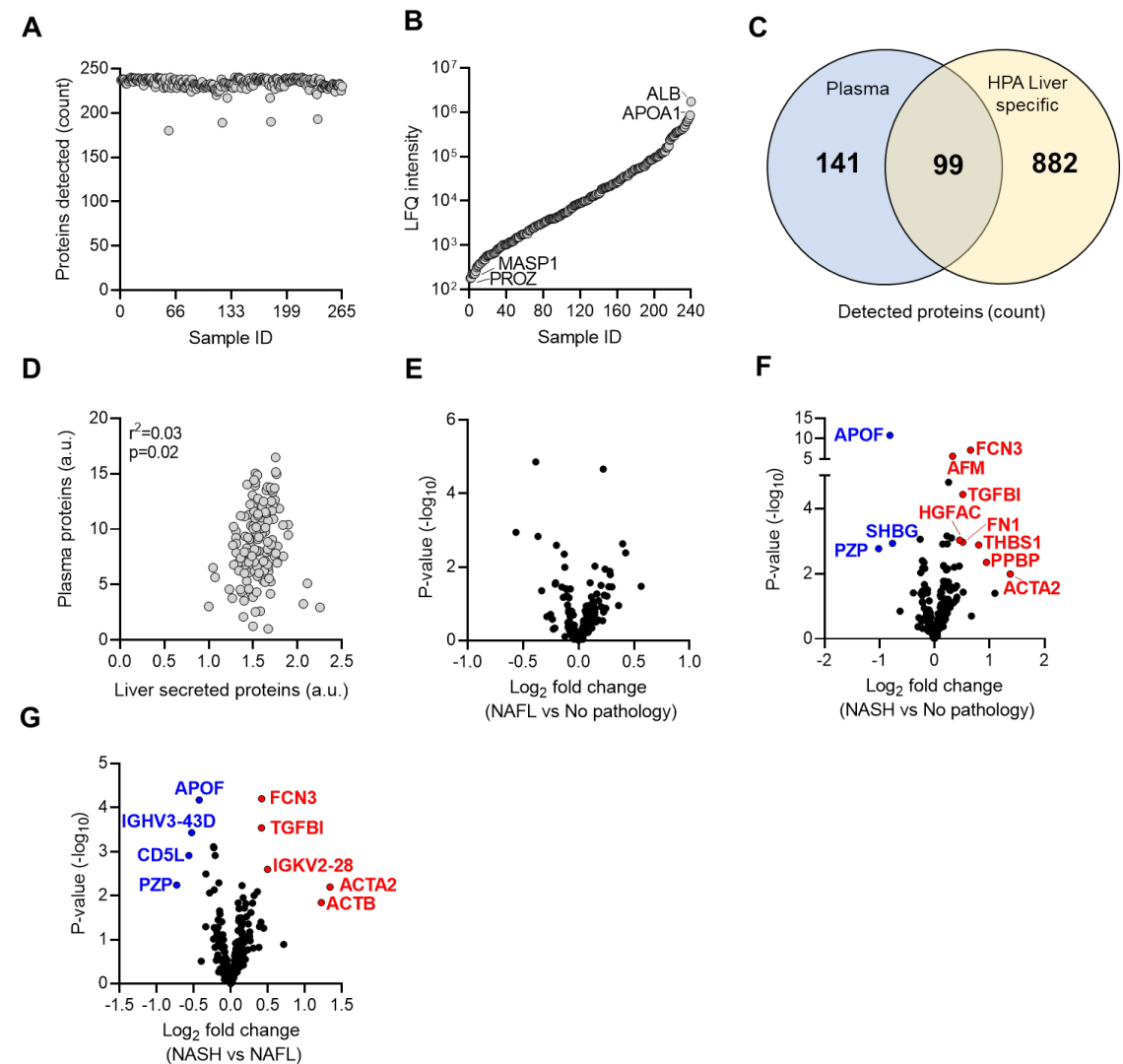


Figure 4.1: Identification of differentially abundant plasma proteins in patients with NASH.(A) The number of proteins detected in the plasma of each patient. (B) The dynamic range of quantified proteins (LFQ, label-free intensity, ALB Albumin, APOA1, Apolipoprotein A1; MASP 1, Mannan-binding lectin serine protease 1; PROZ, Protein Z, Vitamin K Dependent Plasma Glycoprotein). (C) The number of “liver-specific” proteins in our plasma proteome according to the Human Protein Atlas (HPA)

(D) Number of proteins detected in the liver and plasma secretome (n=96). **(E)** Pearson's correlation coefficient of plasma protein abundance in patients with NAFL vs No pathology, **(F)** NASH vs No pathology, **(G)** NASH vs NAFL. Red and blue dots indicate significantly upregulated and downregulated proteins, respectively. Significance was determined by student's unpaired t-test corrected by permutation-based false discovery rate of $q < 0.05$. No pathology, n=72; NAFL, n= 136; NASH, n= 57, unless specified otherwise. Proteins are identified by gene name.

4.2.2. Identification and assessment of potential NASH biomarkers

To assess whether the plasma proteins differentially regulated in NASH could serve as practical biomarkers, we assessed the relative levels and performed receiver operating characteristic curves of APOF, FCN3, IGKV2-28, and AFM in NASH. These candidates were pursued based on significance, significance across multiple screens including liver secretion (Chapter 3), and not regulated by sex (e.g., PZP) [305] were examined further.

APOF was significantly reduced in the plasma of NASH patients compared to those with no liver pathology (Figure 4.2A) and obtained an excellent AUROC of 0.82 (95% CI of 0.75 – 0.89) (Figure 4.2B). A specified cut-off of < -3.548 achieved a sensitivity of 75.0% and a specificity of 78.1%. We then assessed whether circulating APOF could discriminate NASH from patients without NASH (i.e., NAFL and No pathology = No NASH). Patients with NASH had lower circulating APOF levels than those without NASH and achieved an acceptable AUROC of 0.73 (95% CI 0.66-0.80). A threshold of < -3.552 achieves a sensitivity of 75.0% with a specificity of 63.6%.

FCN3 was increased in the plasma of patients with NASH compared to those with No pathology and achieved an AUROC of 0.77 (95% CI 0.69 - 0.86, Figure 4.2E & F). When a threshold of > -1.820 was applied, FCN3 achieved a sensitivity of 80.7% and specificity of 70.1%. FCN3 levels were higher in NASH patients than those without

NASH (Figure 4.2G). ROC curve analysis demonstrated an AUROC of 0.73 (Figure 4.2H) with a sensitivity of 72.0% and specificity of 71.4% at a cut-off of >-1.545 .

IGKV2-28 was increased in the plasma of patients with NASH compared to those with No pathology (Figure 4.2I), and IGKV2-28 obtained an AUROC of 0.63 ($p<0.0073$, 95% CI 0.54 – 0.73, Figure 4.2J). A threshold of >0.04 achieved a sensitivity of 70.1% and specificity of 50.0%. Similarly, there was an increase in IGKV2-28 levels in patients with NASH compared to those without NASH, and poorly discriminates NASH with an AUROC of 0.63 ($p<0.0023$, 95% CI 0.55 – 0.71, Figure 4.2K & L). The sensitivity and specificity were 70.1% and 51.8%, respectively, when a cut off of >0.068 was applied.

Liver Afamin (AFM) secretion was increased with NASH compared to No pathology (Chapter 3). AFM was increased with NASH compared to those with No pathology (Figure 4.2M) and obtained an acceptable AUROC of 0.74 ($p<0.0001$, 95% CI 0.66 – 0.83, Figure 4.2N). When a cut-off of >1.130 was applied, a sensitivity of 70% and specificity of 66.0% was achieved. Similarly, there was an increase in AFM levels in patients with NASH compared to those without NASH (Figure 4.2O) and the AUROC was 0.66 ($p=0.0014$, 95% CI 0.55 – 0.72, Figure 4.2P). Application of a threshold of >1.130 obtains a sensitivity of 70.2% and specificity of 52.2%.

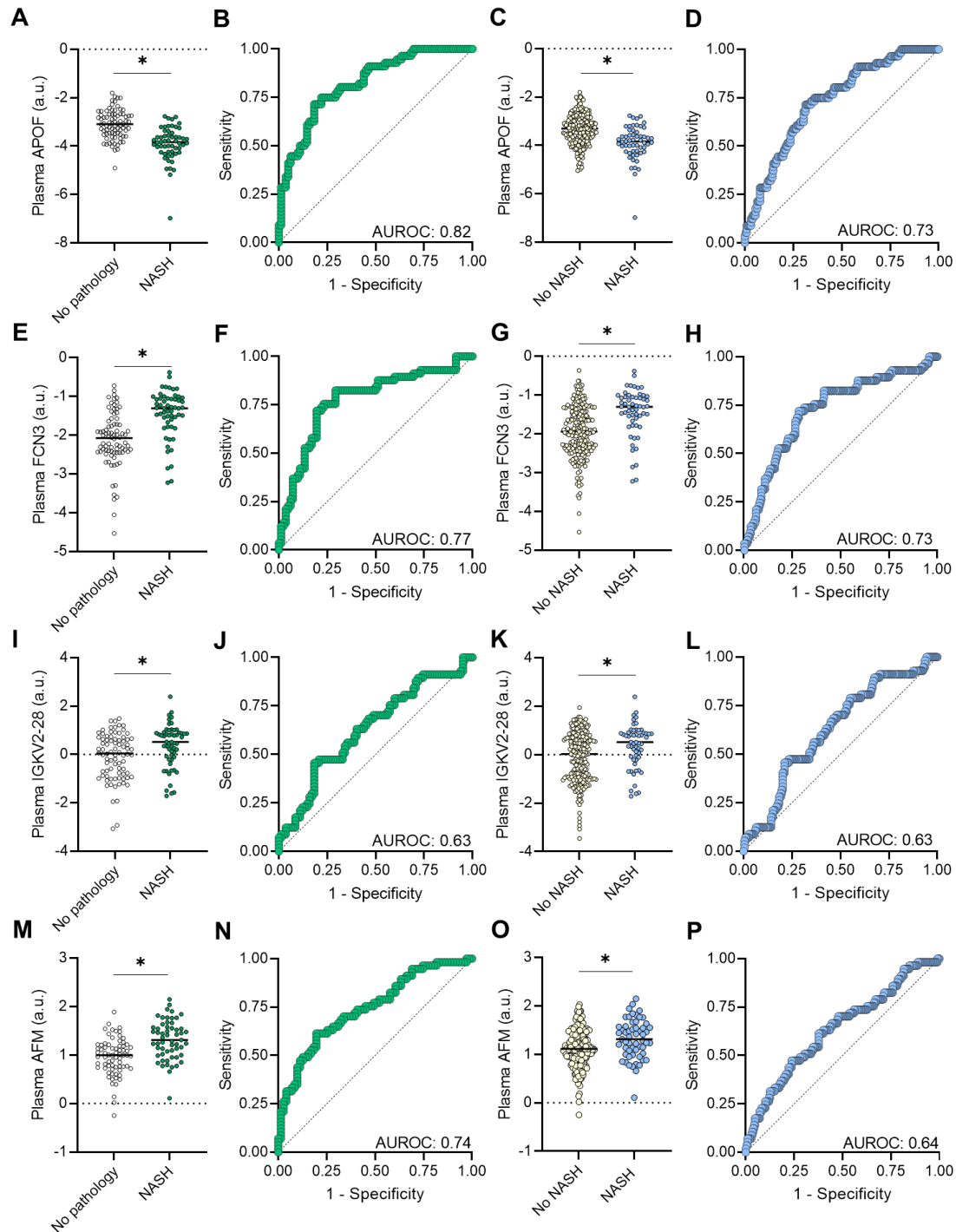


Figure 4.2: Diagnostic utility of plasma proteomics for NASH. (A) Plasma APOF levels were stratified for patients with No pathology and with NASH. (B) AUROC curves of APOF4 show diagnostic utility to identify obese patients with NASH compared to obese individuals without adverse liver pathology. (C) Plasma APOF levels were grouped for patients with and without NASH. (D) AUROC curves of APOF4 show diagnostic utility to stratify patients with NASH against those without NASH. (E) Plasma FCN3 levels were stratified for patients with No pathology and with NASH. (F) AUROC curves of FCN3 show diagnostic utility to stratify patients with NASH against obese patients without liver pathology. (G) Plasma FCN3 levels were stratified for patients without and with NASH. (H) AUROC curves of FCN3 show diagnostic utility to stratify patients with NASH against those without NASH. (I)

Plasma IGKV2-28 levels were stratified for patients with No pathology and with NASH. **(J)** AUROC curves of IGKV2-28 show diagnostic utility to stratify patients with NASH against obese patients without liver pathology. **(K)** Plasma IGKV2-28 levels were grouped for patients with and without NASH. **(L)** AUROC curves of IGKV2-28 show diagnostic utility to stratify patients with NASH against those without NASH. Data were analysed using student's unpaired t-test of the Log₂ transformed and median subtracted values. Line denotes median value (Panel A, C, E, G, I, K, M & O). Significance was set at * P<0.05. No pathology n=72; NASH, n=57; No NASH, n=217; NASH, n=57.

4.2.3. Identification of potential biomarkers for NAFLD F2-3

We then sought to assess whether NAFLD-associated fibrosis was associated with alterations in circulating proteins. APOF was decreased and FCN3 and fibronectin 1 (FN1) were increased in patients with NAFLD F0 compared to those with No pathology (Figure 4.3A). In patients with NAFLD F2-3, seven proteins were altered in the plasma compared to those with No pathology (Figure 4.3B), with decreased levels of IGKV2-28, PZP, sex hormone binding globulin (SHBG), and insulin like growth factor binding protein acid labile subunit (IGFALS), and increased levels of retinol binding protein 4 (RBP4) [306; 307], CD5L, and mannan-binding lectin serine protease 1 (MASP1) in NAFLD F2-3 (Figure 4.3B). When comparing NAFLD F2-3 to NAFLD F0, there were ten altered plasma proteins, including reduced levels of IGKV2-28, TGFBI, and ACTA2, and increased levels of RBP4 and CD5L (Figure 4.3C). CD5L, IGKV2-28, RBP4, and PZP were significantly altered in NAFLD F2-3 compared to both No pathology and NAFLD F0 and there were no differences in the plasma proteome when comparing patients with NAFLD F1 to NAFLD F0 or NAFLD F2-3 to NAFLD F1 (Figure 4.3D & E).

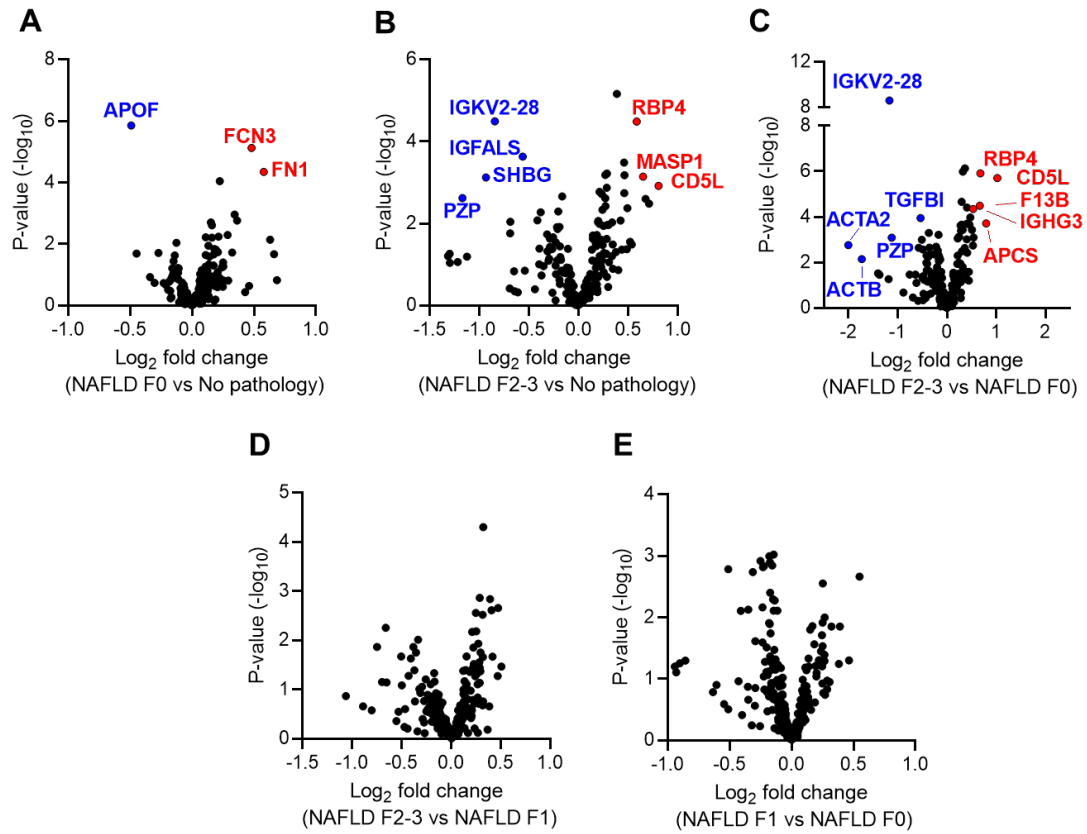


Figure 4.3: Identification of differentially abundant plasma proteins in patients with NAFLD F2-3. Identification of plasma proteins grouped by (A) NAFLD F0 vs No pathology, (B) NAFLD F2-3 vs No pathology, (C) NAFLD F2-3 vs NAFLD F0, (D) NAFLD F2-3 vs NAFLD F1, (E) NAFLD F1 vs NAFLD F0. Red and blue dots indicate significantly upregulated and downregulated proteins, respectively. Significance was determined by students t-test corrected by permutation-based false discovery rate of $q < 0.05$. NAFLD F2-3 $n=32$; NAFLD F1, $n=58$; NAFLD F0, $n=103$; No pathology, $n=72$. Proteins were identified by gene name.

4.2.4. Assessment of potential NAFLD F2-3 biomarkers

We next asked if the differentially regulated proteins identified in NAFLD F2-3 (Figure 4.3) could be used as biomarkers for disease stratification. Patients with No pathology and NAFLD F0 were grouped to form F0 (n=175). Patients with NAFLD F2-3 had significantly lower circulating levels of IGKV2-28 than those with F0 (Figure 4.4A), and this achieved an acceptable AUROC of 0.79 ($p<0.0001$, 95% CI 0.7 – 0.87, Figure 4.4B). When a cut-off of <-0.1873 was applied, IGKV2-28 obtained a sensitivity of 78% and a specificity of 70%. Circulating CD5L was increased with NAFLD F2-3 when compared with F0 (Figure 4.4C), and it achieved an acceptable AUROC of 0.74 ($p<0.0001$, 95% CI 0.65 – 0.83, Figure 4.4D) with a sensitivity of 71.7%, specificity of 62.9% when a cut off >1.156 was applied. The known hepatokine RBP4 was increased in NAFLD F2-3 compared with F0 (Figure 4.4E), and AUROC analysis obtained an acceptable value of 0.77 ($p<0.0001$, 95% CI 0.689 – 0.843, Figure 4.4F) with a sensitivity and specificity of 71.8% and 69.1% (cut off >1.135), respectively. Lastly, plasma APCS was increased in NAFLD F2-3 compared with F0 (Figure 4.4G) and obtained an acceptable AUROC of 0.71 ($p=0.0002$, 95% CI 0.614 – 0.800, Figure 4.4H), a cut-off of >1.046 achieved a sensitivity of 71.9% and specificity of 67.4%.

We next assessed the ability of current non-invasive scores to discriminate NAFLD F2-3 in our cohort. ROC analysis of the NAFLD fibrosis score and BARD score was not significantly different than random chance (defined as AUROC= 0.5) with an AUROC of 0.52 and 0.55, respectively (Figure 4.4I). APRI achieved a poor discrimination with an AUROC of 0.65 (Figure 4.4I) and was greater than random chance.

Other non-invasive measures such as the Fib-4 score, and ALT/AST ratio were not different than random chance (Figure 4.4J). In comparison, IGKV2-28 achieved an

AUROC of 0.79 (Figure 4.4B & I-J) and outperformed all non-invasive scores (Figure 4.4I & J) for discrimination of NAFLD F2-3.

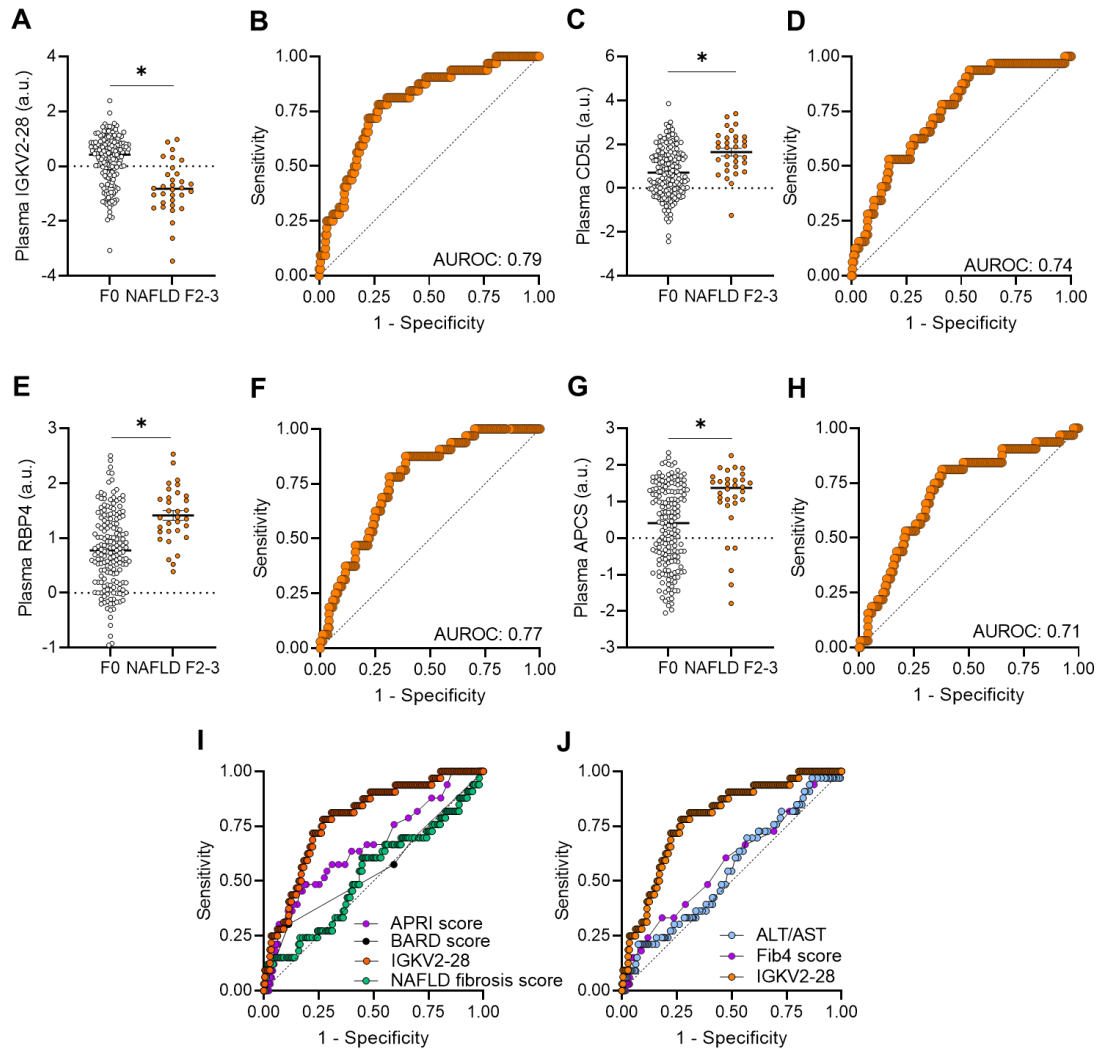


Figure 4.4: Diagnostic utility of plasma proteins for stratification of NAFLD F2-3 in obese patients. (A) Plasma IGKV2-28 levels were stratified for patients with F0 (No pathology and NAFLD F0 combined) and those with NAFLD F2-3. (B) Receiver operative characteristic (ROC) curve for the prediction of NAFLD F2-3 by IGKV2-28 plasma levels. (C) Plasma CD5L levels stratified for patients with F0 and NAFLD F2-3. (D) ROC curve for the prediction of NAFLD F2-3 by plasma CD5L levels. (E) Plasma RBP4 levels stratified for patients with F0 and NAFLD F2-3. (F) ROC curve for the prediction of NAFLD F2-3 by plasma RBP4 levels. (G) Plasma APCS levels stratified for patients with F0 and NAFLD F2-3. (H) ROC curve for the prediction of NAFLD F2-3 by plasma APCS levels. (I) ROC curve for the prediction of NAFLD F2-3 by plasma IGKV2-28, the APRI, BARD and NAFLD Fibrosis score. (J) ROC curve for the prediction of NAFLD F2-3 by plasma IGKV2-28, ALT/AST ratio and the Fib-4 score. Line denotes median value (Panel A, C, E & G). Each dot represents a single patient. Significance was assessed by unpaired t-test or Wilcoxon rank sign test. Significance set at * $P < 0.05$. F0, $N = 175$; NAFLD F2-3, $n = 32$.

4.3. Discussion

Obese patients are more likely to develop NAFLD and NASH and progress to more severe disease such as cirrhosis and HCC [308-310]. There remains to be no safe, effective, and practical screening tool to effectively stratify obese patients with NASH and NAFLD F2-3. Identifying patients at earlier stages of disease before end stage is of paramount importance. In this study, we performed plasma proteomics to identify high abundant plasma proteins that can effectively stratify obese patients with NASH and NAFLD F2-3. We show that (1) assessment of the high abundance proteins in the plasma proteome contains high similarity to the liver secreted proteome, (2) NASH and NAFLD F2-3 results in modest changes in circulating proteins when compared to those without pathology and NAFL, and (3) APOF and FCN3 can effectively identify NASH and APCS and IGKV2-28 can stratify patients with NAFLD F2-3, thereby meeting the criteria as candidate biomarkers.

Plasma proteomics is often performed for biomarker analysis in various liver pathologies [273]. A caveat of plasma proteomics is often plagued by the impaired detection of low abundance proteins as plasma proteins span over 13 orders of magnitude [303]. It is common place to remove these high abundant proteins to reduce the orders of magnitude and increase detection of low abundance plasma proteins [311]. However, our liver secreted proteome exhibited strong overlap with previous analyses of the top 200-1000 proteins [227]. Further, others show similar findings in humans with alcoholic liver disease where 77.6% of the plasma proteins identified, were also detected in liver tissue [301]. Because a large proportion of highly abundant plasma proteins are derived from the liver [312], we reasoned that assessment of the top 200-1000 plasma proteins may identify biomarkers that reflect NASH and NAFLD F2-3

liver pathophysiology. We identified modest alterations in the plasma proteome of obese patients with NASH and NAFLD F2-3, some of which aligned with previous studies examining the human plasma proteome using mass spectrometry proteomics, mirroring the changes observed in AFM, TGFBI, and SHBG with NASH [272; 274; 313].

We initially focused on NASH given it is the more severe form of NAFLD, patients with NASH have accelerated progression of hepatic fibrosis and development of HCC [294] and is difficult to detect with other non-biopsy techniques. The best biomarker to stratify NASH was the *bona fide* hepatokine [304] APOF. APOF was markedly reduced in the plasma of NASH patients and with an AUROC of 0.83 and performed excellently to stratify patients with NASH and appears to perform better than AFM (AUROC of 0.74). Others have also shown that APOF is reduced in the plasma of patients with NASH [314; 315] and it is currently part of a panel of proteins patented for mass spectrometry mediated diagnosis of NAFLD (WO2018/037229 A1).

Liver fibrosis regardless of steatosis or inflammation, is associated with overall and liver-related mortality [141; 153]. Despite this, there are no non-invasive approaches to effectively stratify those with advanced fibrosis. The best biomarker to stratify NAFLD F2-3 in this analysis was IGKV2-28 (Figure 4.4) that was reduced in the plasma of NAFLD F2-3 patients and performed near excellently (AUROC: 0.79) to stratify these patients. Little is known about IGKV2-28, other than hepatic expression of IGKV2-28 in NAFLD patients was associated with decompensated cirrhosis and HCC [316]. We also identified RBP4 and APCS whose plasma levels were increased with NAFLD F2-3 and achieved acceptable AUROCs to stratify NAFLD F2-3. Both RBP4 and APCS have

previously been identified as biomarkers for NAFLD [272; 317] and are yet to be effectively incorporated into a scoring panel to stratify patients with NAFLD F2-3. We further re-emphasise (Chapter 3) that non-invasive scores including NAFLD fibrosis score, and the BARD score do not perform well in our obese cohort (Figure 4.4) and that IGKV2-28 outperform these scores. This discovery cohort provides justification to validate our biomarkers in an additional cohort of NAFLD patients. More detailed statistical comparisons are required in ours and additional cohorts to statistically compare the IGKV2-28 ROC curves to APCS, or APRI and BARD ROC curves to quantify improvement in NAFLD F2-3 stratification. This is an ongoing assessment however, the AUROC alone is remarkably different for IGKV2-28 (0.79) vs APRI vs (0.66) and it is highly likely that IGKV2-28 remains a more worthwhile candidate to diagnose NAFLD F2-3 than the APRI score.

Closer examination of the individuals with NAFLD F2-3 reveals the same six patients whose plasma levels of IGKV2-28, APCS, and CD5L are similar to those in the F0 group. Interestingly five of these six patients are males with poorly controlled fasting blood glucose and elevated HbA1c levels, and four have been previously diagnosed with T2D. This observation highlights two critical points. Firstly, this warrants re-analysis of sex-specific effects, noting our lack of power in male individuals, and second and potentially sex-specific biomarkers may be a worthwhile pursuit. Second, we could enhance our biomarkers sensitivity and specificity by potentially incorporating measures of glycaemic control. One protein, IGKV2-28, was quite surprising, being lower in the plasma of NASH patients yet higher in those with significant fibrosis. This may seem counter-intuitive; however, NASH and fibrosis are associated, not present hand in hand [120]. In our study, we have 8 / 57 NASH patients with have significant

fibrosis (F2-3). Perhaps IGKV2-28 could have dual uses in identifying those with NASH and NAFLD F2-3 in separate scores. It is unlikely that a single marker alone can stratify NASH and is likely required to be part of an algorithm that incorporates routine biochemical measures such as circulating fasting blood glucose and HbA1c may enhance the sensitivity and specificity of NASH diagnosis. The multivariate analysis would be required as previously performed by our group [4; 120] and is the focus of ongoing research in our laboratory.

Similar to our liver secreted findings (Chapter 3) and contrasting previous studies [272], we observed no difference in the plasma proteome in those with NAFL vs No pathology (Figure 4.1F). However, our approach assessed higher abundant proteins [227] and not a comprehensive approach to quantify the total plasma proteome [272], and therefore may be limited by low abundance proteins that would be primarily impacted. Alternate approaches could include global correlational analysis comparing the relative abundance of plasma proteins compared to the percentage of liver fat scored by pathologists in their assessment of steatosis [216], or liver triglyceride content in the snap-frozen human liver tissue to more accurately reflect changes in circulating proteins steatosis.

Combination of hepatokine secretion (Chapter 3), and plasma proteomics (Figure 4.1) analyses identified only two proteins, Afamin (AFM) and APCS, that were increased in the liver secreted and plasma proteome with NASH and NAFLD F2-3, respectively. Increased plasma APCS has been identified previously in murine plasma proteome studies comparing long (>6 months) and short term (<2 months) high fat feeding [274]. However, the authors hypothesised that this increase was likely due to an increased APCS expression in visceral adipose, not an increase in liver secretion with NAFLD

severity [274]. On the other hand, plasma AFM levels were increased in patients with NAFLD [272], in NAFLD with T2D compared to those with T2D without NAFLD [274], and circulating concentrations are associated with waist circumference and fasting blood glucose [318]. However, when combined with plasma proteomics, we identified liver-specific [304] and NAFLD F2-3 reflective biomarker APCS and NASH liver reflective AFM and these two proteins warrant use to reflect NAFLD liver pathologies.

A limitation of our study is the size of our discovery cohort and that we have not prospectively validated the efficacy of the identified biomarkers in a validation cohort comprised of individuals at risk of NAFLD or NASH. Our assessment of the top200-1000 proteins lacks coverage of low abundant proteins. Future analysis will utilise the small protein enrichment assay (SPEA) [319] to quantify low abundant proteins in the plasma for further identification of NAFLD-related fibrosis biomarkers. Finally, similar to previously discussed limitations in Chapter 3, our cohort comprises women primarily, and we have not assessed the differences in circulating proteins in males compared to females. For example, sex-specific differences in PZP [305] and SHBG [320] and other under studied proteins may hinder the interpretation of the current findings.

In conclusion, plasma proteomics analysis of 265 obese individuals identified IGKV2-28 and APOF as biomarkers to effectively stratify patients with NAFLD F2-3 and NASH, respectively. In addition, APCS secretion from livers with NAFLD F2-3 and circulating levels are increased, positing it as a pathophysiology-specific biomarker. Future studies validating these biomarkers in an additional cohort and development of antibody-mediated approaches to quantify the concentration of IGKV2-28 and APOF

are warranted to develop high-throughput use to stratify patients with NAFLD F2-3 and NASH effectively.

Chapter 5: Characterisation of a novel hepatokine

EMILIN1 in NASH and NAFLD-associated fibrosis

5.1 Introduction

NAFLD is the most common liver disease worldwide [8-10]. It comprises a spectrum of histological conditions, NAFL and its more progressive form, NASH. Histologically, NAFL is characterised by steatosis that comprises $\geq 5\%$ in the field of view assessed by light microscopy and progresses to NASH when accompanied by lobular inflammation and hepatocyte ballooning [216]. Fibrosis can be present at any stage of the NAFLD spectrum of disease, and at any stage, it is associated with treatment complications, liver-related and overall mortality [33; 140; 141]. Further patients with NASH display fibrosis progression at twice the rate of a patient with NAFL [32], indicative of distinct cross talk between multiple cell types within the NASH liver. While the global prevalence of NAFLD is approximately 25% [19], and 37% of all NAFL and NASH patients will develop progressive fibrosis [32], there is an unmet demand for safe and effective pharmacological therapies for the treatment of NAFLD or fibrosis [96].

Side effects of currently available pharmaceuticals present a barrier to the effective treatment for NAFLD and NASH. Currently available drugs to treat NASH, but not fibrosis, include pioglitazone and vitamin E [96; 321]. However, caution has been excised in wide-scale use of both drugs for the treatment of NAFLD/NASH given that pioglitazone increases the risk of fractures in women [322] and weight gain even after discontinuation of treatment [323], while high dose vitamin E is associated with increased prostate cancer risk and all-cause mortality [324; 325]. Notably, there are no approved drugs to treat hepatic fibrosis. Overall, this demonstrates the need to identify

novel targets and develop new therapies that inhibit fibrosis or promote resolution of NAFLD/NASH and hepatic fibrosis, and its associated co-morbidities such as insulin resistance.

TGF β 1 is the primary cytokine that governs the initiation and progression of fibrosis [279-281]. TGF β 1 is secreted bound to its latency-associated peptide (LAP) forming a small latent complex (SLC). Subsequently, the SLC binds to the latent TGF β binding protein forming the large latent complex (LLC) that is then secreted into the ECM, crosslinked to fibrillin and or fibronectin microfibrils that can sequester the LLC [326-330]. Upon structural deformation or proteolytic cleavage of LAP, this releases the mature TGF β dimer that can bind to TGF β receptor II (TGF β RII), which is highly expressed on the hepatic stellate cell (HSC) compared to other cell types in the liver. Activation of the TGF β receptor leads to activation and differentiation of HSCs into *Acta2* expressing myofibroblast-like cells that produce and secrete extracellular matrix proteins in the liver. These matrix proteins include the interstitial collagens collagen type one or three α 1 chain (COL1A1 and COL3A1) along with tissue inhibitor of metalloproteases -1 and -3 (TIMP1 and -3) [331; 332]. Reducing TGF β 1 signalling can resolve fibrosis, reduce hepatic lipid accumulation, attenuate dyslipidaemia, and improve blood glucose control, which are common to NAFLD disease pathogenesis [280; 333; 334]. Further, liver-specific TGF β 1 knock-out mice were protected from diet-induced obesity, hyperglycaemia, and insulin resistance [333]. In line with these findings, hepatocyte specific TGF β RII deletion reduces hepatic steatosis, inflammation, ballooning, and hepatic fibrosis in mice [334], while administration of anti-TGF β 1 antibodies reduces weight gain, improves glucose tolerance and insulin sensitivity, and resolves hepatic lipid accumulation in high-fat fed and Lep^{ob/ob} mice [280]. Despite this

extensive body of evidence to support the use of anti-TGF β therapeutics, few have been successful in clinical trials owing to their off-target side effects [335]. Therefore, altering how we target TGF β signalling may be yield clinical benefit without unwanted side effects and provide an effective treatment for NAFLD, fibrosis, and its associated metabolic co-morbidities.

EMILIN1 is a 110 kDa protein (intact form) that is secreted to a greater extent from the liver of patients with NASH or NAFLD and significant fibrosis compared to livers without adverse pathology (Chapter 3). Further, we previously speculated that hepatokines may exert a more profound autocrine and paracrine effect given a weak correlation to of liver secreted proteins to their corresponding circulating levels (Chapter 4). EMILIN1 is expressed in all cell types of the liver [175] and previous findings report EMILIN1 as a binding partner of pro-TGF β 1, which prevents TGF β 1 release from the LLC and effectively reduces TGF β 1 signalling in *vitro*, *Xenopus* embryos and in mice. [282]. As mentioned above, TGF β 1 coordinates the production and secretion of extracellular matrix proteins in the liver and contributes to fibrosis and the development of metabolic co-morbidities in NAFLD. Accordingly, this study aimed to determine whether EMILIN1 can resolve NASH and hepatic fibrosis and resolve NASH-associated metabolic co-morbidities in mice.

5.2 Results

5.2.1. Human liver secreted Emilin1 correlates with measures of glycaemic control

We have previously shown that EMILIN1 expression and secretion is increased in liver derived from patients with NASH and NAFLD F2-3, and that plasma EMILIN1 levels are also increased in NASH patients (Chapter 3). Given the close links of NAFLD/NASH to impaired glycaemic control, we first assessed the relationship between liver EMILIN1 secretion (Chapter 3), and markers of glycaemic control assessed in patient-matched blood samples. Liver EMILIN1 secretion was weakly correlated to fasting blood glucose, glycated haemoglobin (HbA1c), and C-peptide levels (Figure 5.1A-D). Further, EMILIN1 secretion was significantly increased in patients with NAFLD and T2D compared to those without T2D and / or NAFLD (Figure 5.1E). We next assessed whether circulating levels of EMILIN1 were correlated with measures of glycaemic control. Plasma EMILIN1 was positively correlated with fasting blood glucose and HbA1c (Figure 5.1F& G) but were not associated with insulin and C-peptide levels (Figure 5.1H & I).

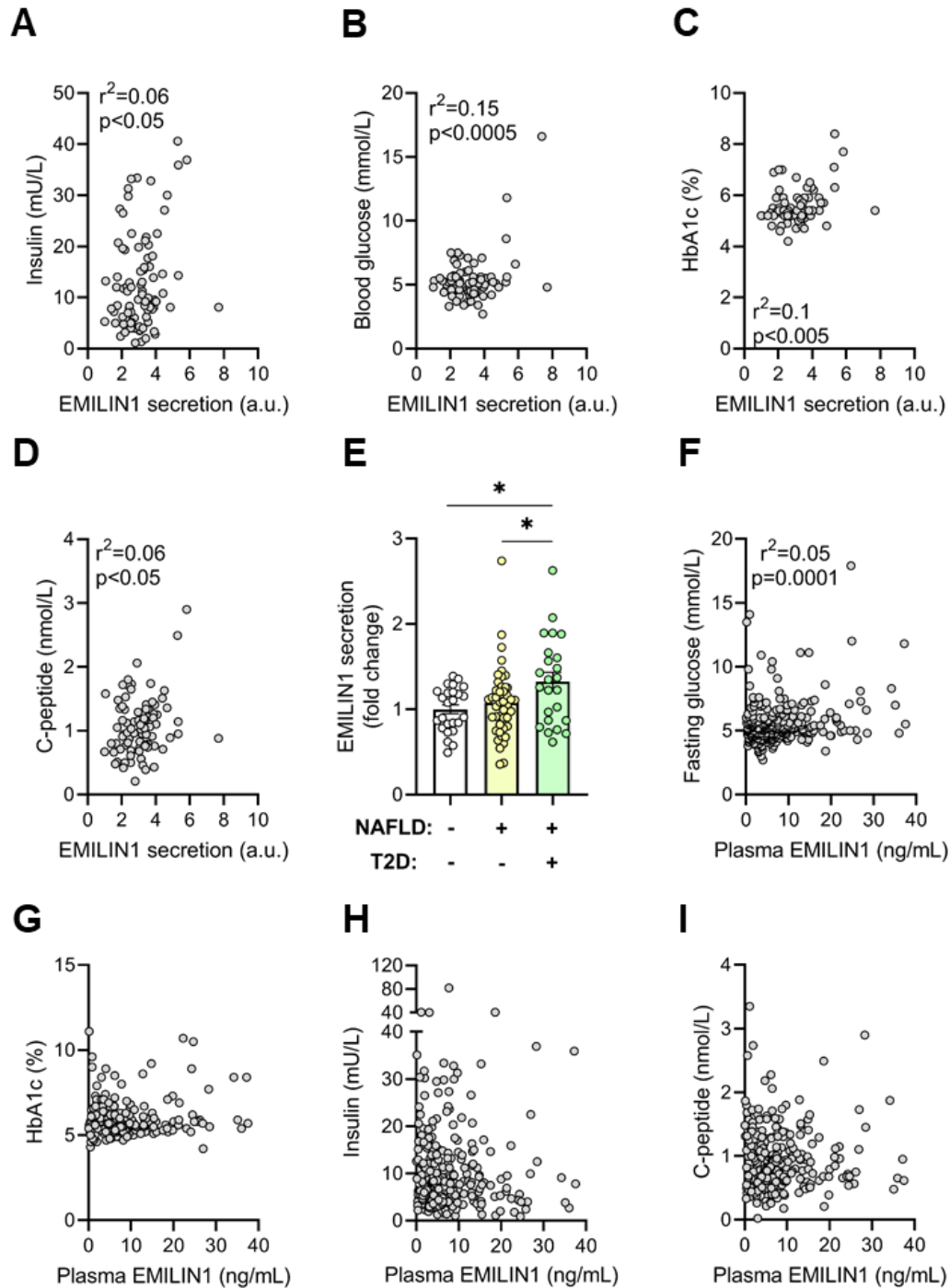


Figure 5.1: Liver secreted EMILIN1 correlates with measures of glycaemic control. Liver secreted EMILIN1 correlated with (A) fasting blood glucose, (B) glycosylated haemoglobin (HbA1c), (C) plasma insulin (D) plasma C-peptide in matched patients. (E) Liver secreted EMILIN1 with or without NAFLD and type 2 diabetes (T2D). Plasma EMILIN1 correlated to (F) Fasting blood glucose, (G) HbA1c, (H) insulin, and (I) C-peptide levels. For correlation analyses, N=83-90. For NAFLD/T2D, N= 25, 46 and 24, respectively. Data is displayed as mean $\pm$ SEM. * P <0.05. Significance was determined by Pearson's correlation co-efficient (panel A-D, F-I) or one-way ANOVA with Bonferoni's post hoc test (panel E).

5.2.2. Liver-specific Emilin1 overexpression in mice with NASH

EMILIN1 inhibits TGF β 1 signalling in HEK293T, mouse embryonic fibroblasts and in mice [282]. Hence, we surmised that overexpression of *Emilin1* in the liver would inhibit TGF β 1 signalling and reduce hepatic steatosis and fibrosis in murine NAFLD. Mice were fed a CHOL diet for 20 weeks and were segregated into experimental groups after matching for body weight and glucose tolerance (data not shown). Mice aged 20 weeks and were injected i.v with an AAV8 vector containing *Emilin1* cDNA (*Emilin1*-AAV) or scrambled DNA (Control-AAV) and were then fed a CHOL diet for further 12 weeks totalling 32 weeks of feeding. Liver mRNA expression of *Emilin1* was increased ~12 fold following *Emilin1*-AAV administration with no differences in heart, kidney, white adipose tissue and skeletal muscle (Figure 5.2A). We attempted to validate overexpression of EMILIN1 protein in the liver by immunoblot analysis; however, we could not detect EMILIN1 overexpression via immunoblot (Figure 5.2B) and is likely due to degradation of EMILIN1 into several effector proteins by proteases such as neutrophil elastase (NE) as previously described (Figure 5C taken from Pivetta et al, 2014) [287] and agrees with previous studies showing degradation in various human cancers [287]. *Emilin1*-AAV administration did not alter body weight, individual tissue weights, adiposity, or liver damage markers ALT and AST compared with Control-AAV (5.2 B-E).

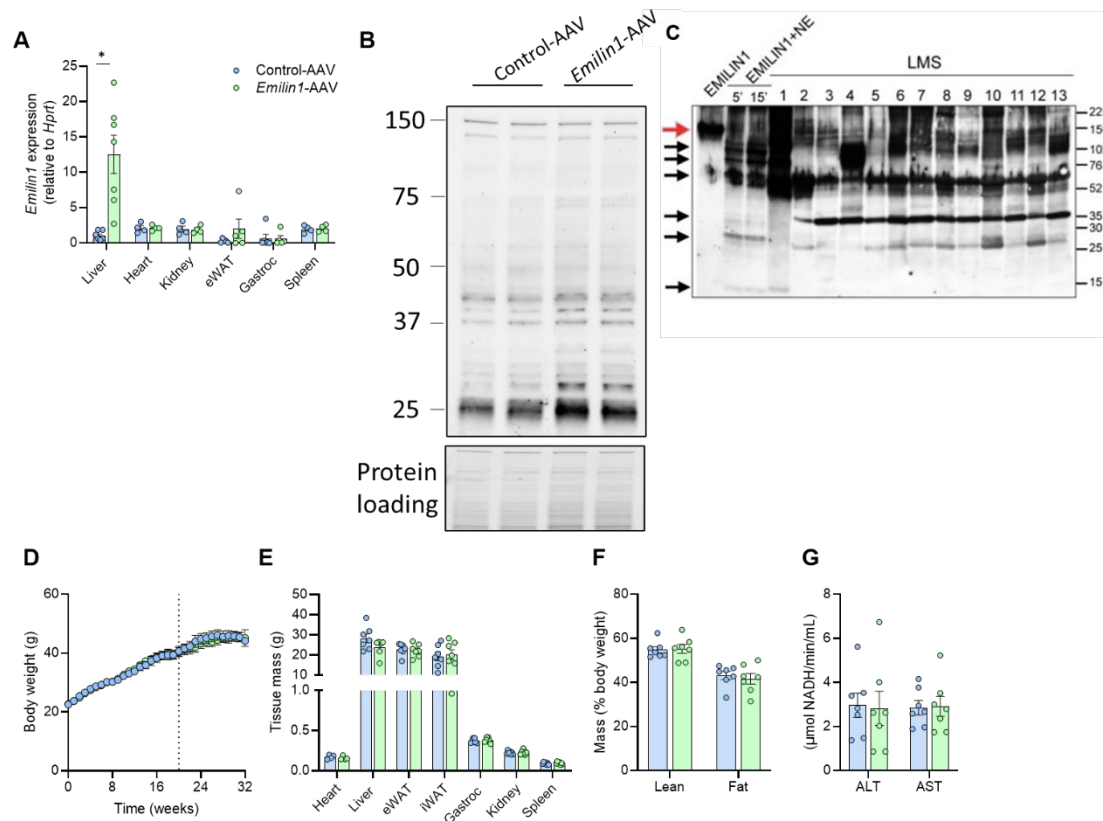


Figure 5.2: Effects of hepatic *Emilin1* overexpression on metabolic parameters following 32 weeks of CHOL feeding. (A) *Emilin1* expression was assessed in the liver and other tissues. eWAT, epididymal adipose tissue; Sk.Muscle, skeletal muscle. Immunoblot of EMILIN1 in Control-AAV or EMILIN1-AAV CHOL fed mice. (C) Figure taken from Pivetta et al 2014 [287]. Immunoblot staining for EMILIN1. Lanes include of recombinant EMILIN1 incubated with or without neutrophil elastase (NE) for 5 or 15 min and 13 patients with leiomyosarcomas (LMS). Red arrow depicts intact EMILIN1, and black arrows highlight NE dependent EMILIN1 fragmentation. (D) Assessment of body weight (dotted line indicates *Emilin1*- or Control-AAV injection). (E) Tissue mass at cull. (F) Lean and fat mass assessed by EchoMRI, and (G) plasma alanine transferase (ALT) and aspartate transferase (AST) levels. N=7 in each group. Data is displayed as mean $\pm$ SEM. * $P < 0.05$ vs Control-AAV. Significance was assessed by unpaired two-tailed t-test (panels A, E-G) or two-way ANOVA followed by Bonferoni post hoc test (panel D).

5.2.3. Emilin1 overexpression in the liver does not impact hepatic glucose and lipid metabolism

Histological assessment of steatosis on H&E sections revealed no difference between treatment groups. Biochemical evaluation showed no effect of *Emilin1*-AAV administration on liver triglyceride content (Figure 5.3A & B). Consistent with these findings, radiometric determination assays of liver glucose and fatty acid oxidation revealed no effects of *Emilin1*-AAV administration on glucose oxidation, *de novo* lipogenesis, fatty acid uptake, oxidation, and incorporation into various lipid species when compared with *Emilin1*-AAV administration (Figure 5.3F - H).

NAFLD is often accompanied by inflammation and hepatic fibrosis, and TGF β 1 signalling is implicated in these processes. There was no significant effect of *Emilin1*-AAV on inflammation, hepatocellular damage or fibrosis as assessed by histological assessment of Masson trichrome stained liver sections. Macrophage marker, *F4/80* was not different following *Emilin1*-AAV administration (Figure 5.3H), however there tended to be a reduction in C–C motif chemokine ligand 2 (*Ccl2*) expression in livers with Emilin1 overexpression (Figure 5.3H). The expression of tumour necrosis factor α (*Tnfa*) and *Il1b* was not altered with Emilin1-AAV treatment (Figure 5.3H).

Furthermore, there were no differences in the expression of hepatic *Acta2*, *Tgfb1*, *Colla1* with *Emilin1*-AAV compared to Control-AAV (Figure 5.3I). Liver *Col3a1* and *Timp1* tended to be reduced with *Emilin1*-AAV administration ($p=0.1$); however, this did not reach statistical significance (Figure 5.3I). *Timp3* expression was not different between groups. All together, these results suggest that *Emilin1* overexpression is unlikely to confer therapeutic benefits in early-stage NASH.

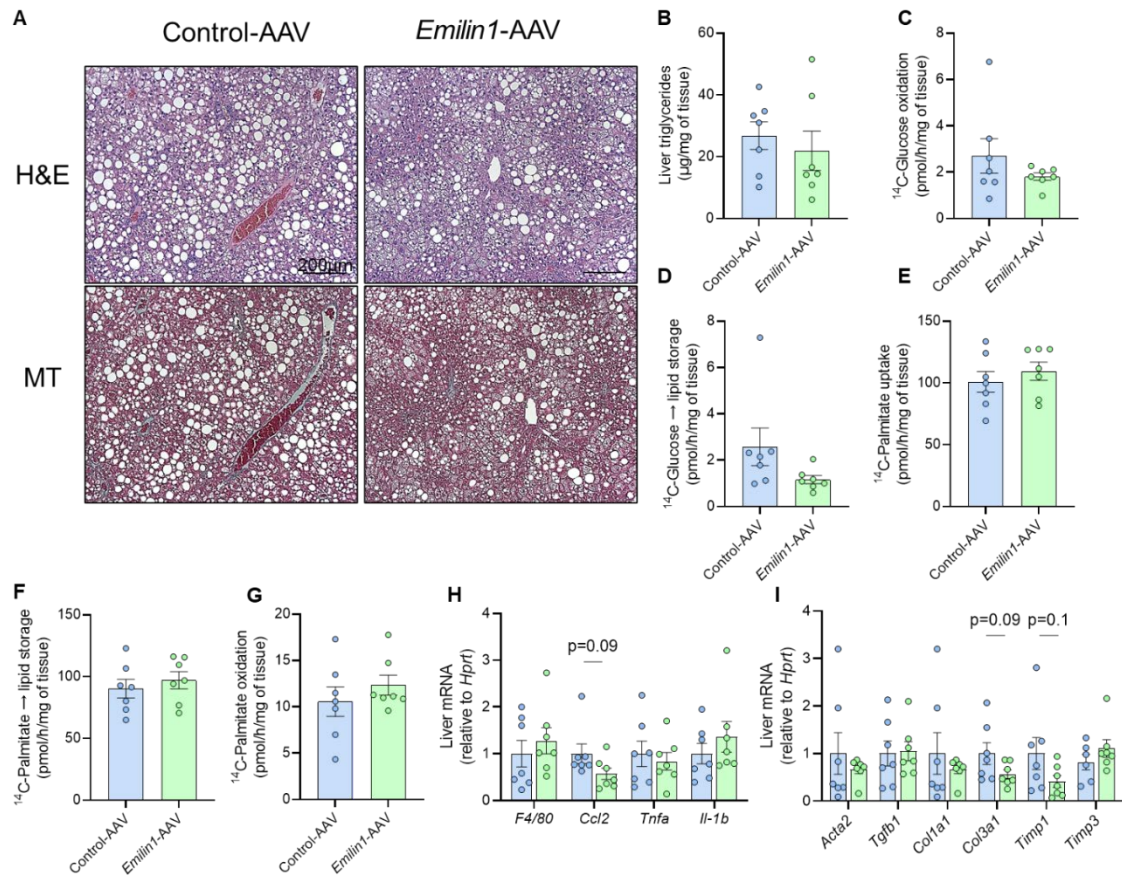


Figure 5.3: Liver-specific overexpression *Emilin1* does not alter hepatic lipid metabolism or hepatic fibrosis in CHOL mice. (A) Representative liver histology stained with hematoxylin and eosin and Masson's trichrome at 10x magnification. Scale bar denotes 200 μm. (B) Hepatic triglyceride content (C) radiometric tracing of glucose oxidation and (D) *de novo* lipogenesis. (E) Radiometric tracing of the rate of fatty uptake, (F) lipid storage, and (G) oxidation. (H) Liver mRNA expression of proinflammatory genes. (I) Liver mRNA expression of profibrotic genes N=7 in each group unless specified otherwise. Data is displayed as mean ± SEM. * P < 0.05 vs Control-AAV. Significance was assessed by unpaired two-tailed t-test (Panel B, E-I) or Mann-Whitney test (Panel C & D).

5.2.4. Hepatic Emilin1 reduces insulin secretion in mice with NASH and metabolic disease

To assess whether Emilin1 could alter common metabolic comorbidities of NAFLD, we assessed whole-body measurements of glycaemic control and dyslipidaemia that are also augmented with reductions in TGF β 1 signalling [280; 333]. We observed no difference in fasting blood glucose levels or glucose tolerance between treatment groups (Figure 5.4A & B). Fasting plasma insulin levels ($p=0.08$) and plasma insulin levels in response to an oral glucose load were lower with *Emilin1*-AAV (Figure 5.4C). Whole body insulin sensitivity measured by an insulin tolerance test was not different between groups (Figure 5.4D). Circulating levels of triglycerides, free fatty acids, and cholesterol were not different between groups (Figure 5.4E - G).

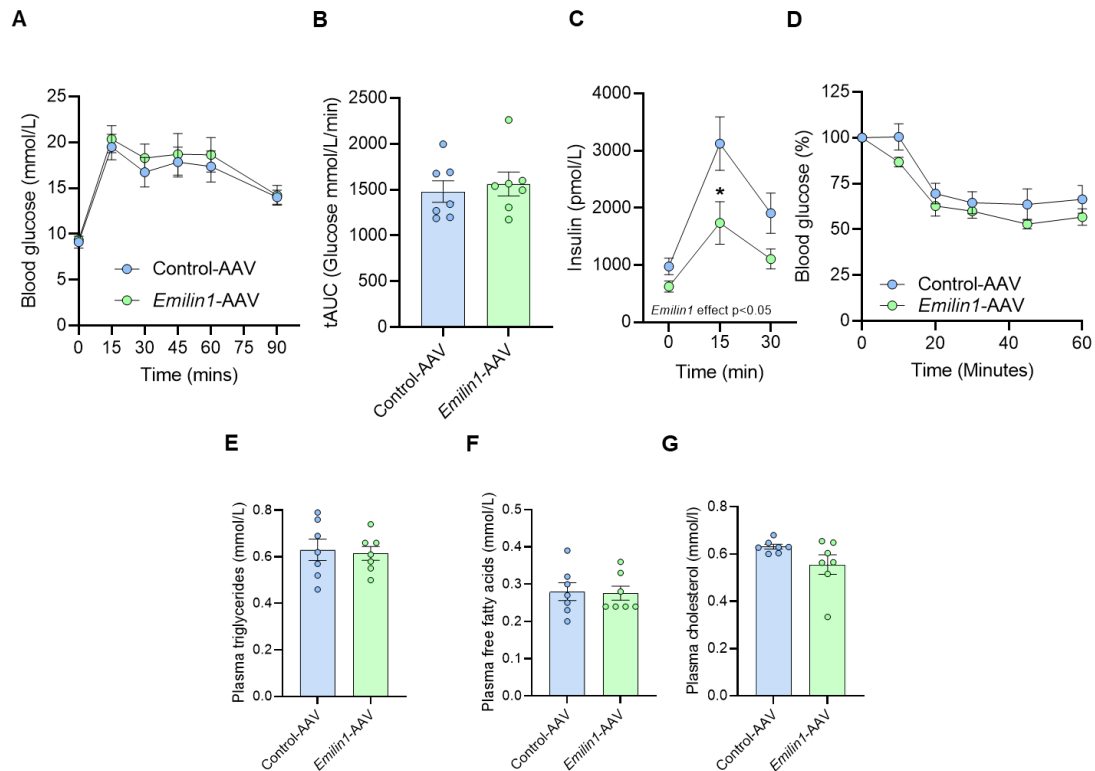


Figure 5.4: Effect of liver-specific overexpression of Emilin1 on NAFLD-related metabolic comorbidities. (A) Assessment of glucose tolerance (2 g/kg, oral) and (B) total glucose area under the curve. (C) Plasma insulin levels assessed before and during the glucose tolerance test (n=6 Control-AAV and n=7 *Emilin1*-AAV) (D) Assessment of insulin sensitivity using an insulin tolerance test (1 u/kg). Fasting plasma (E) triglycerides, (F) free fatty acids, and (G) cholesterol. N=7 in each group unless specified otherwise. Data is displayed as mean $\pm$ SEM. * P < 0.05 vs Control-AAV. Significance was assessed by two-way ANOVA followed by Bonferoni post hoc test (panel A, C and D) or unpaired two-tailed t-test (panels B, E-G).

5.2.5. Hepatic Emilin1 does not alter body weight or glycaemic control in mice with severe NASH

While CHOL feeding adequately recapitulates human NAFL and NAFLD-associated metabolic comorbidities, it does not induce severe NASH or significant hepatic fibrosis [336]. To overcome this limitation, we assessed whether *Emilin1* overexpression could reduce NAFLD severity and hepatic fibrosis in mice fed an MCD, a dietary model that induces severe NASH and fibrosis. Following four weeks of MCD feeding, mice were injected intravenously with *Emilin1*-AAV or Control-AAV and NAFLD and its

associated co-morbidities were subsequently assessed. After four weeks of AAV administration, we observed a two-fold increase in liver *Emilin1* expression with *Emilin1*-AAV compared with Control-AAV (Figure 5.5A). We attempted to assess protein levels by immunoblot but as previously shown [287] (Figure 5.2B & C), we detected EMILIN1 degradation (data not shown). Emilin1 overexpression did not change body or tissue weights (Figure 5.5D & E), nor circulating liver damage markers AST or ALT levels over time (Figure 5.5D & E). .

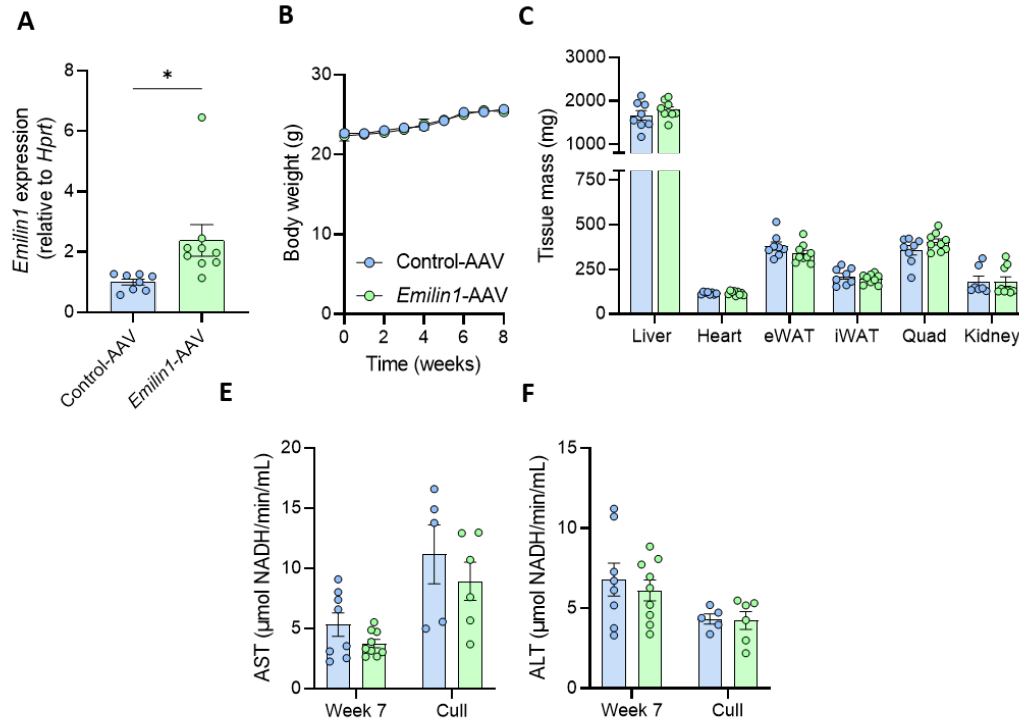


Figure 5.5: Metabolic characterisation of hepatic *Emilin1* in short-term MCD feeding. (A) Hepatic mRNA levels of *Emilin1* in MCD mice following four weeks of *Emilin1*-AAV or Control-AAV overexpression. (B) assessment of body weight over the 8 weeks of MCD feeding, (C) tissue mass and (D) plasma aspartate transferase (AST), at week 7 Control-AAV n=9, *Emilin1*-AAV=8; at cull Control-AAV n=5, *Emilin1*-AAV n=6 and (E) alanine transferase (ALT) at week 7 (Control-AAV n=9, *Emilin1*-AAV n=8); at cull (Control-AAV, n=5; *Emilin1*-AAV n=6). Data is displayed as mean $\pm$ SEM, N=8 Control-AAV and n=9 *Emilin1*-AAV unless specified otherwise, * P <0.05 vs Control-AAV. Significance was assessed by unpaired two-tailed t-test (panels A, E, F, and G) or two-way ANOVA coupled with Bonferroni's post hoc test (panel D).

5.2.6. Short term overexpression of Emilin1 reduces expression of hepatic fibrosis

Histological assessment of the liver in *Emilin1*-AAV mice revealed no differences in hepatic steatosis (Figure 5.6A); or liver triglyceride content (Figure 5.3B) however, there was a marked reduction in hepatic fibrosis on the Masson's trichrome stain (Figure 5.6A & C). Further, *Emilin1*-AAV administration did not alter the expression of macrophage marker F4/80 but we did observe a 50% decrease in the expression of the CCL2 (Figure 5.6D). We did not detect a reduction in the expression of pro-inflammatory cytokines (*Tnfa*) and *Il-1b* (Figure 5.6D). Consistent with these histological findings, *Emilin1*-AAV administration reduced the expression of pro-fibrotic Tgf β 1-responsive genes, including *Colla1*, *Col3a1*, *Timp1*, and *Timp3* (p=0.06). *Col3a1*, *Acta2* and *Tgfb1* expression was not different between treatment groups (Figure 5.6E). *Timp3* was not detected in three Control and three *Emilin1*-AAV mice in two independent runs and thus not included in the analysis.

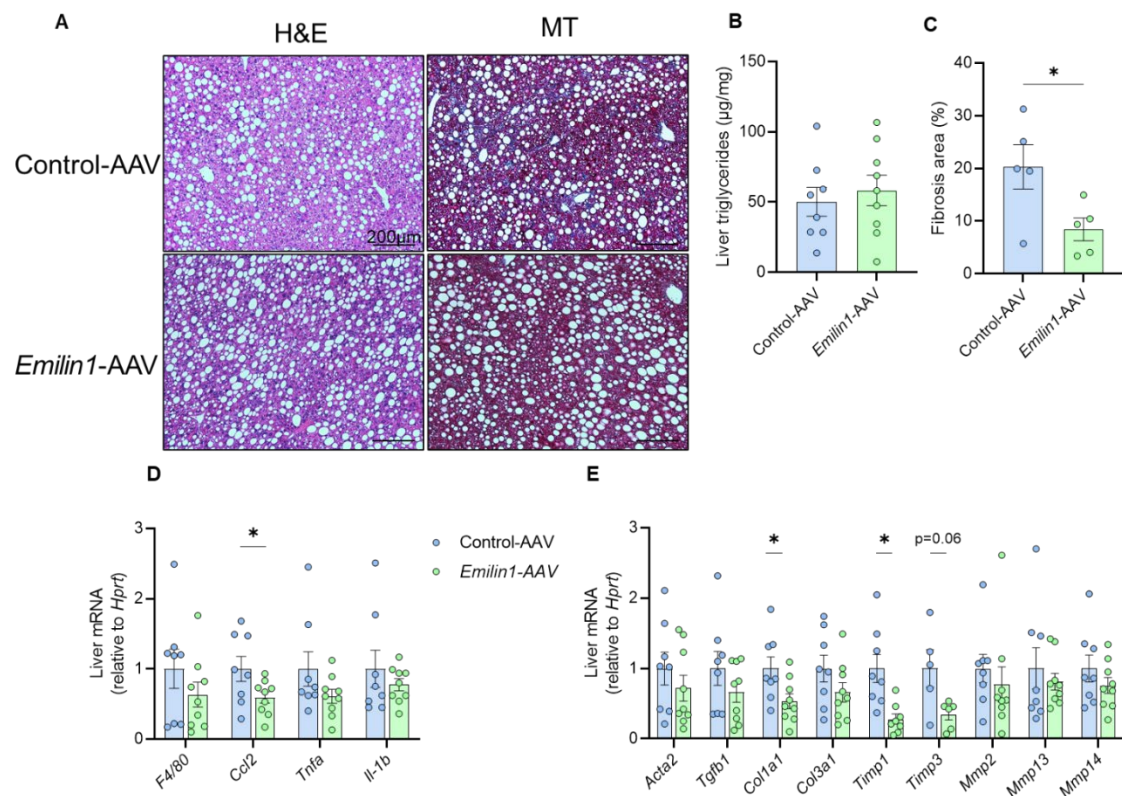


Figure 5.6: *Emilin1*-AAV hepatic fibrosis but not steatosis in 8 weeks of MCD feeding. (A) Representative images of the liver architecture stained with hematoxylin and eosin stain or Masson's trichrome stain. Scale bar denotes 200µm. (B) Assessment of liver triglyceride content (C) Orbit analysis of the average area on the Masson's trichrome image taken up by collagen deposition (n=5 per group). Quantification was performed on five images per section per mouse. (D) Liver mRNA expression of proinflammatory genes. (E) Liver mRNA expression of profibrotic genes. *Timp3* expression, Control -AAV, N=5; *Emilin1*-AAV, n=6. Data is displayed as mean ± SEM, N=8 Control-AAV, n=*Emilin1*-AAV n=9 unless specific otherwise, * P < 0.05 vs Control-AAV. Significance was assessed by unpaired two-tailed t-test (panels A, C, D, E, and G) or two-way ANOVA coupled with Bonferroni's post hoc test (panels B and F).

5.2.7. Characterisation of hepatic *Emilin1* with long term MCD feeding

Given the improvements in hepatic fibrosis with short-term *Emilin1* overexpression in mice fed the MCD diet, we conducted a second study extending feeding of the MCD diet for a further four weeks. An additional control group fed a dietary matched control diet methionine choline supplemented (MCS) diet was added to highlight the effects of the MCD on liver pathology. *Emilin1* expression was significantly increased ~3-fold with MCD feeding compared to the MCS diet (Figure 5.7A). As expected, *Emilin1*-AAV administration resulted in a further increase in *Emilin1* compared with MCD-fed mice injected with Control-AAV. Given that *Emilin1* was increased with MCD feeding (Figure 5.7A), we posited that perhaps lipids accumulation within the hepatocyte may be driving *Emilin1* expression. Consistent with this notion, the expression of *Emilin1* was dramatically increased in isolated hepatocytes cultured in the presence of lipids compared to all other metabolic substrates (Figure 5.7B).

Mice fed the MCS diet gained more weight than the MCD fed mice, with or without *Emilin1* overexpression, and this was due to differences in fat mass but not lean mass (Figure 5.7C-D). Liver weight was increased in mice fed the MCD compared with the control MCS diet, and there was no difference in liver mass between Control-AAV and *Emilin1*-AAV injected mice (Figure 5.7D). Plasma ALT and AST were elevated in mice fed the MCD vs. MCS diet but were not different between Control-AAV and *Emilin1*-AAV mice fed an MCD diet (Figure 5.7E & F).

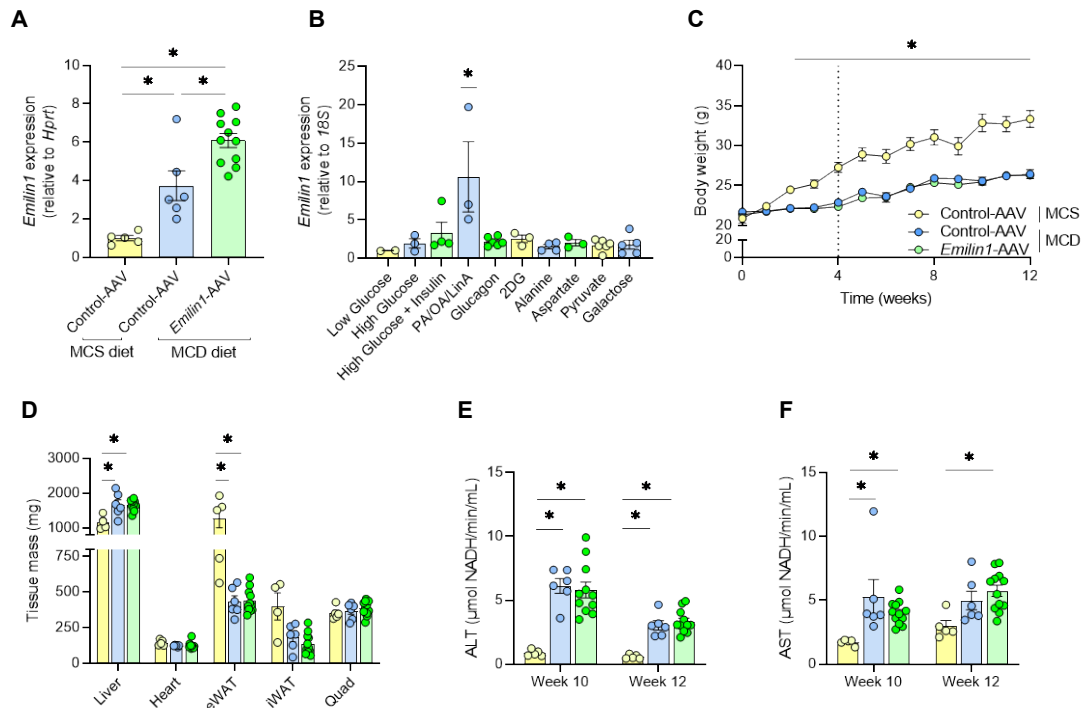


Figure 5.7: Metabolic characterisation of liver specific *Emilin1* overexpression following 12 weeks of MCD feeding. (A) Liver mRNA levels of *Emilin1*. (B) *Emilin1* expression in primary mouse hepatocytes after 8-hour stimulation with insulin (10 nM), high glucose (25 mM) high glucose and insulin, glucagon (25 pM), 2-deoxy-glucose (2-DG) (25 mM), alanine, aspartate, pyruvate (each 25mM), a fatty acid mixture conjugated to 2% bovine serum albumin (palmitic acid, oleic acid, linoleic acid (PA/OA/LinA), 2:2:1, 500 μM), galactose (25 mM), $n = 3$ (hepatocytes isolated from four mice; experiments carried out in duplicate). (C) Assessment of body weight (dotted line indicative of Control-AAV or *Emilin1*-AAV injection) (D) tissue mass at cull, (E) plasma alanine transferase (ALT), and (F) aspartate transferase (AST). Data is displayed as mean $\pm$ SEM, $N=7-10/\text{group}$, Significance was assessed by one-way ANOVA (panels A, B, D- F) or two-way ANOVA with Bonferroni's post hoc test (panels C). * $P < 0.05$.

5.2.8. Eight-week overexpression of *Emilin1* reduces markers of liver fibrosis

Assessment of liver histology revealed the expected absence of steatosis and fibrosis in mice fed the MCS diet. MCD feeding induced significant steatosis and fibrosis regardless of group (Figure 5.8A & B). Histological examinations of liver sections show more lipid droplets present on the H&E stain (Figure 5.8A) with *Emilin1* overexpression compared to MCD Control-AAV mice. However, *Emilin1* overexpression reduced fibrosis on the Masson's trichrome stain ($p=0.09$; Figure 5.8A & B). Consistent with these findings, biochemical determination of liver triglyceride content showed that MCD feeding increased hepatic triglycerides compared to MCS, with no difference in triglyceride content in *Emilin1*-AAV compared to MCD Control-AAV mice ($p=0.3$, Figure 5.8B). Expectedly, there was a significant increase in *Acta2* and *Tgfb1* expression in Control-AAV mice fed a MCD diet compared to the control MCS diet (Figure 5.8C & D). *Emilin1* overexpression reduced *Acta2* and *Tgfb1* when compared to MCD Control-AAV mice, and there was no difference between *Emilin1*-AAV and the MCS Control-AAV mice (Figure 5.8C & D). *Col1a1* expression was increased in livers of mice fed the MCD diet compared to the MCS diet; however, there was no difference between the MCD fed Control-AAV and *Emilin1*-AAV mice (Figure 5.8E). There was a striking ~30-fold increase in *Col3a1* in the Control-AAV mice fed an MCD diet compared to the Control-AAV MCS, and *Emilin1*-AAV partially suppressed this increase in *Col3a1* (Figure 5.8F). In accordance with previous from the short-term intervention (Figure 5.6E), *Timp1* and *Timp3* expression was significantly reduced in the livers of *Emilin1*-AAV mice compared to MCD Control-AAV mice such that levels were indistinguishable from mice fed the control MCS diet (Figure 5.8G & H).

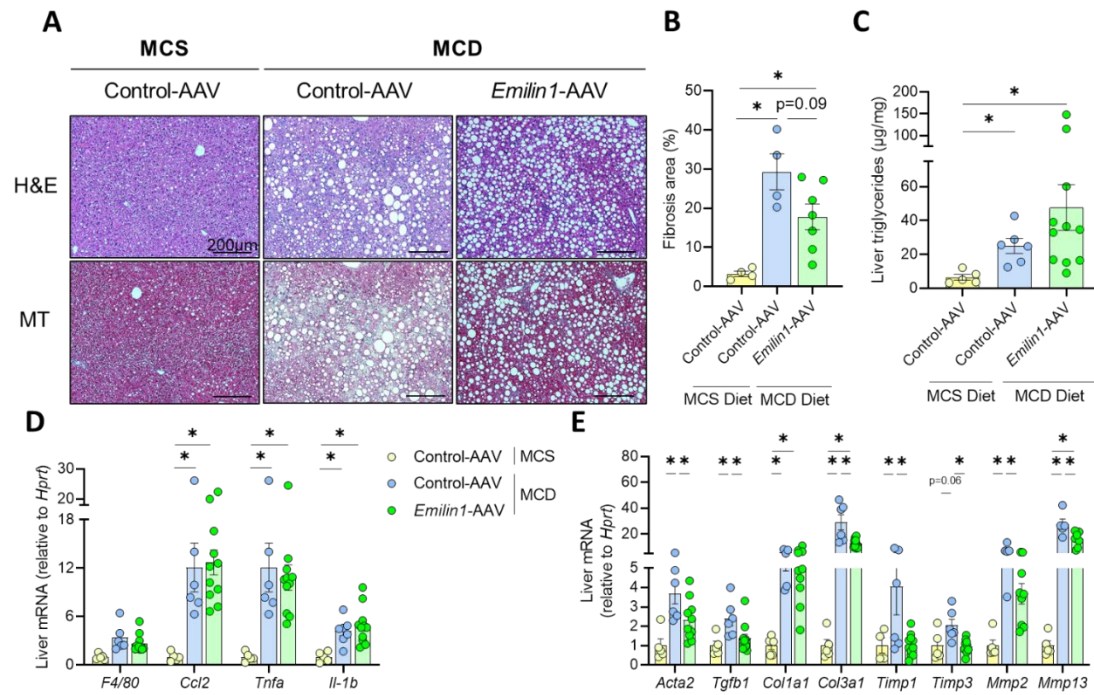


Figure 5.8: Emilin1 overexpression reduces hepatic fibrosis in 12 weeks of MCD feeding. (A) Representative images of the liver architecture stained with hematoxylin and eosin stain or Masson's trichrome stain from mice fed the methionine choline supplemented (MCS) or methionine choline deficient (MCD) diet. Scale bar denotes 200µm. (B) Orbit analysis of the average area on the Masson's trichrome image taken up by collagen deposition (n=3-5 per group), MCS, n=4; MCD-Control AAV, n=5; and MCD *Emilin1*-AAV, n=7. (C) Assessment of liver triglyceride content. (D) liver mRNA expression of pro-inflammatory and (E) pro-fibrotic genes. MCS, n=5; MCD-Control AAV, n=6; and MCD *Emilin1*-AAV, n=11 unless specified otherwise in each group unless specified otherwise. Data is displayed as mean ± SEM. * P < 0.05 vs Control-AAV. Significance was assessed by one-way ANOVA followed by Bonferroni's post hoc test (panels B-E).

5.3. Discussion

The prevalence of NAFLD is rising alongside its associated morbidities, including insulin resistance and hepatic fibrosis [8-10]. With ~25% of the world estimated to have NAFLD [9], the number of individuals at risk of disease progression to more severe liver disease, such as cirrhosis and hepatocellular carcinoma, and metabolic diseases such as type 2 diabetes and dyslipidaemia are immense. Therefore, there is an urgent need to find new pharmacotherapies to adequately treat NAFLD and hepatic fibrosis, both at the onset of disease, and in the advanced fibrotic state. In this study, we used adeno-associated viruses to overexpress the hepatokine Emilin1 in the livers of mice and identify Emilin1 as an effective target to reduce fibrosis in severe NASH, but not steatosis in NAFLD.

Liver secreted proteins represent a large proportion of the body's circulating proteins [2; 271], and these hepatokines exert a myriad of biological actions [337-340]. We previously identified EMILIN1 as a hepatokine whose secretion was markedly increased from livers with significant fibrosis (Chapter 3). TGF β 1 signalling induces NAFLD, NASH and hepatic fibrosis, and can lead to impairments in whole-body glucose homeostasis in mice and humans [280; 281; 333; 334]. Inhibition of TGF β 1 can reduce / prevent the activation of hepatic stellate cells and consequently reduce the deposition of extracellular matrix proteins and thereby fibrosis in the liver [341-344]. There has been immense interest in targeting TGF β signalling to treat hepatic fibrosis [281; 344; 345] and various human cancers [346]. Current approaches focus on targeting TGF β 1-3 proteins or the TGF β receptor [281; 344; 345], however few are exploiting the innate TGF β regulatory network that governs its activity.

Herein, we show that increasing hepatic *Emilin1* levels reduced fibrosis in advanced NASH (i.e., induced by feeding mice a MCD diet) likely by preventing TGF β release from the ECM. Further, *Emilin1*-AAV treatment does not lead to adverse pathologies such as neoplasms in mice fed a western-style NASH-inducing diet. Hepatic fibrosis is a dynamic process characterised by the net accumulation of ECM and understanding the mechanisms that regulate this process is critical for developing antifibrotic strategies [347; 348]. Our results are consistent with previous observations that EMILIN1 inhibits TGF β and collagen deposition in smooth muscle fibres by binding to pro-TGF β 1 and preventing release of the mature TGF β peptide [282; 343]. Secreted, not intracellular EMILIN1 has the capacity to bind to pro-TGF β 1 and prevent TGF β 1 signalling [282; 349]. Consistent with *in vitro* findings where EMILIN1 cloned with KDEL motif to prevent secretion demonstrated that intracellular EMILIN1 could not inhibit TGF β 1 signalling [282]. Further, patients with a missense mutation of c.64G>A (p.A22T) mapped to the signal peptide had reduced EMILIN1 in the ECM and displayed defects in ECM integrity [349]. Future studies are required to confirm accumulation of EMILIN1 in the ECM following *Emilin1* overexpression via immunohistochemistry as previously shown [282; 349]. We hypothesise that *Emilin1*-AAV induced EMILIN1 accumulation in the ECM given we observed consistent reductions in the expression of TGF β -regulated genes (*Timp1*, 3, *Acta2*, *Colla1*, *Col3a1*) [281] and reduced fibrosis induced by administration of *Emilin1*-AAV.

Fibrosis is characterised by the net accumulation of ECM proteins. The factors regulating the major ECM constituent, collagen deposition, and subsequent fibrosis in the liver are multifaceted and not fully understood, [281] but at a basic level, this process requires translation of collagen mRNA and complex regulatory processes to

secrete and stabilise in the ECM alongside equal degradation to prevent ECM accumulation and thus fibrosis [281]. Collagen turnover or degradation is thought to be mediated by MMP which are inhibited by TIMP proteins in a 1:1 stoichiometric ratio [350]. Further, in the HSC cell line LX2, TGF β 1 signalling not only increases collagen synthesis and the expression of TIMPs but also reduces the expression of MMPs ensuring collagen deposition and net accumulation [351]. Following 4 weeks of *Emilin1* overexpression we observed no difference in ECM degrading proteins in MMPs but observed reductions in the expression of *Timp1* and *-3* indicative of decreased TGF β 1 signalling and increased ECM degradation as MMP expression was not reduced. On the other hand, 8 weeks of *Emilin1* overexpression led to reductions in hepatic fibrosis and was associated with a marked decrease in *Timp1*, *-3*, *Mmp2* and *-13* and markers of HSC activation (*Acta2*, *Tgfb1*), suggesting that long term EMILIN1 expression mitigates ECM production compared to MCD fed Control-AAV. The strength of 4- and 8-week time points demonstrated a temporal effect of Emilin1-AAV, with a sustained reduction in liver fibrosis. However, given that there was no difference in inflammatory gene expression, ALT, AST, triglyceride content, and lipid droplets appeared more prevalent on H&E staining, these findings suggests that hepatocyte damage, steatosis and inflammation occur prior to the induction of fibrosis. Further and increasing EMILIN1 content can hinder the pro-fibrotic capability of these lipotoxic and inflammatory factors to prevent the progression of hepatic fibrosis. Complete resolution of NAFLD and significant fibrosis will likely require combination treatment of potentially EMILIN1-AAV with drugs that reduce lipid uptake.

A noteworthy finding was the lipid-regulated expression of EMILIN1. Parallel lines of evidence in isolated hepatocytes and MCD Control-AAV livers demonstrate marked

increases in *Emilin1* expression with lipid accumulation. This is unexpected given that differences in EMILIN1 secretion from human liver slices were only noted in patients with significant fibrosis (Chapter 3), and that EMILIN1 does not seem to have a role in regulating lipid metabolism (Figure 5.4). Indeed, assessment of *Emilin1* expression in isolated hepatocytes was characterised by differing metabolites should be repeated with pro-fibrotic and pro-inflammatory modulators, like TGF β . However, the mechanism of how lipid overload/lipid droplet accumulation increases *Emilin1* expression warrants further investigation. Previous work in cells and mice have eloquently described that *EMILIN1* harbours an specificity protein 1 (Sp1) promoter and activator protein (AP1) binding site and that there is likely co-operation between multiple promoter and enhancer regions perhaps distant from the coding region to regulate *EMILIN1* expression [352]. While other transcription factors that may drive *Emilin1* expression are not yet identified, the role of Sp1 and AP1 in NAFLD-mediated fibrosis is becoming increasingly clear. Sp1 transcription factor activity is increased in response to TGF β 1 signalling [353; 354] and blocking Sp1 protein from binding to its promoter region that is present on pro-fibrotic genes TGF β 1, platelet-derived growth factor- BB and cell cycle genes including cyclin D1 reduces the mRNA expression of *Tgfb1*, *Acta2* and *Colla1* in HSCs [355]. On the other hand, the AP1 protein complex that is comprised of the proteins c-JUN, c-FOS, and ATF [356] is upregulated in the livers of humans and mice with NAFLD or NASH [357; 358] and is required for TGF β 1 to direct its regulate its own synthesis [359] and secretion, and is thereby linked to the development of hepatic fibrosis. Given that EMILIN1 contains the AP1 DNA binding site, this suggests that EMILIN1 may be under the same promotion control as TGF β 1. Taken together, these parallel lines of evidence suggest that EMILIN1 may be under the control of

TGFβ1 responsive transcription factors to form a negative feedback loop to regulate TGFβ1 signalling and block uncontrolled fibrosis in NAFLD.

The CHOL dietary model exhibits mild fibrosis synonymous with human F1, however, the activity and signalling of TGFβ1 may not be sufficient to be effectively inhibited by *Emilin1* overexpression. Further, previous work comparing a CHOL and MCD demonstrates that the MCD diet exhibits greater oxidative, endoplasmic reticulum and autophagocytic stress that can activate TGFβ1, remove it from its LLC sequestration and bind to transforming growth factor beta 1 receptor 2 (TGFBRII) to induce signalling [360]. Therefore, we surmise that the livers in mice fed a CHOL diet did not exhibit enough cellular stresses to induce sufficient TGFβ1 signalling for EMILIN1 to induce an anti-fibrotic effect [360].

Glycaemic control is often impaired in patients with NAFLD and is thought to contribute to fibrosis severity [361]. Given that multi-faceted therapies are highly desirable in therapeutic development, determining whether EMILIN1 can efficiently treat hepatic fibrosis and other metabolic co-morbidities is of clinical importance. Previous approaches that have diminished TGFβ1 signalling, such as neutralising antibody treatment [280] or *Tgfb1* or *Tgfbrii* liver-specific gene deletion [333; 334] have reported improvements on glycaemic control. Contrasting associative human evidence (Figure 5.1), *Emilin1* overexpression did not impact blood glucose control or insulin sensitivity. However, we identified reductions in fasting and glucose-stimulated plasma insulin (Figure 5.3) [280], suggestive of a liver-pancreas axis mediated by EMILIN1. The effect of EMILIN1 on the pancreas remains unknown, although

EMILIN1 is enriched in pancreatic islets, suggestive of a possible role in regulating insulin secretion [362; 363]. Although beyond the scope of this study, future examination of EMILIN1 effects on glucose-stimulated insulin secretion are warranted.

On the other hand, hyperinsulinemia, which is often present in patients with NAFLD, can induce chronic over-expression of hepatic transcription factors that increase DNL and consequently liver lipid content [12; 62; 84; 85; 87]. Reciprocally, NAFLD can induce hyperinsulinemia and impair insulin clearance [92]. It is surprising that Emilin1-AAV in mice fed a NASH-inducing diet tended to reduce fasting and did reduce glucose-stimulated insulin levels without impacting fasting glucose, hepatic DNL, liver or plasma lipid levels. Given insulin sensitivity was also unchanged, perhaps a reduction in insulin clearance is present with Emilin1-AAV and this occurs independent of liver fat content [92]. Alternatively, ECM density [364] and composition [365] can regulate insulin action via various integrin receptors. Increasing hepatic EMILIN1, an ECM protein, may alter the composition of other ECM proteins and or impact integrin signalling [366] however, this remains functionally to be proven. Future work assessing insulin clearance is warranted.

There are several limitations to this study. While we observed the predicted reductions in markers of TGF β 1 signalling, in this study, we did not recapitulate that Emilin1 binds to TGF β 1 [282]. Indeed, this has been proven previously [282], and is required to demonstrate our phenotype is due to reductions in TGF β signalling. Our assessment of hepatic fibrosis relied largely on staining and gene expression and provides strong preliminary evidence for EMILIN1 as a potential therapeutic that requires further

exploration. Functional assessment such as assessing collagen synthesis rates [348] with or without EMILIN1 overexpression is needed to better understand the mechanistic underpinnings of EMILIN1's anti-fibrotic actions. Further, confirmation of EMILIN1 overexpression and reductions in markers of fibrosis at the protein level are of paramount importance and required to support our assertions. While we are unaware of a previous study overexpressing EMILIN1, it is abundantly clear that immunofluorescence seems to be the mainstay when assessing the relative protein abundance of EMILIN1 and is likely used to avoid the observation of the EMILIN1 degraded products [287]. Perhaps targeted mass spectrometry would curb this issue and is a necessary approach to validate EMILIN1 protein overexpression. Neutrophil elastase degrades EMILIN1, and this hinders confirmation of liver-specific overexpression. One could argue that its degradation would render it an ineffective therapy [287]. However, *in vitro* assessment of the EMILIN1 degraded components revealed that EMILIN1 could no longer suppress proliferation [287]. These findings pose the question: What is the biological activity of each of these 'degraded' products and their consequent effects on hepatic fibrosis and insulin secretion? Do these products work in synergy to govern inflammatory or pro-fibrotic pathways, and do they still inhibit markers of TGF β 1 signalling observed in *Emilin1*-AAV MCD mice? Or more simply, could application of neutrophil elastase inhibitors reduce hepatic fibrosis in mice fed an MCD diet? Investigations to answer these unresolved questions fundamental to EMILIN1 biology and as a potential therapeutic are warranted.

Taken together, these data provide murine *in vivo* evidence that *Emilin1* has the potential to treat hepatic fibrosis, particularly in advanced cases. Future studies are

warranted to recapitulate these findings in other models of liver fibrosis and to elucidate the molecular mechanisms of the anti-fibrotic actions of EMILIN1.

Chapter 6: Proteomic analysis reveals exercise training induced remodelling of hepatokine secretion and uncovers syndecan-4 as a regulator of hepatic lipid metabolism

6.1. Introduction

NAFLD is the most prevalent chronic liver disease, affecting an estimated 25% of the world's population [19]. It is defined as excessive accumulation of lipids in the liver and encompasses a range of pathologies ranging from hepatic steatosis to the more severe form of the disease, NASH, which is accompanied by inflammation and hepatocyte damage, with or without fibrosis [7; 96; 138]. Given the dramatic rise in NAFLD prevalence [8; 9], there is an urgent requirement for the development of effective and safe interventions to reduce disease severity and progression to worsening pathologies, such as hepatic cirrhosis and HCC [8].

Exercise training is effective for the treatment of NAFLD [367], and in the absence of approved pharmacotherapies, remains the current frontline treatment option for patients [96]. Exercise training reduces the severity of hepatic steatosis [368] and causes regression of NASH, with or without accompanying weight loss [369; 370]. This is thought to occur by the actions of inter-connected metabolic processes, including increased hepatic fatty acid oxidation, reduced *de novo* lipogenesis, and enhanced insulin sensitivity [371]. However, adherence to exercise training programs is not consistently achievable, particularly in obesity where NAFLD incidence is highest [372], necessitating the development of pharmacotherapies for NAFLD. We posit that understanding the processes by which exercise reduces NAFLD severity may inform new therapeutic opportunities for the treatment of this disease.

It is well documented that tissues actively secrete hormones (notably proteins), cytokines, metabolites, and nucleic acids, which can induce autocrine, paracrine and endocrine actions to regulate nutrient metabolism and systemic energy homeostasis [338]. Liver secreted proteins, also termed ‘hepatokines’, are important regulators of inter-tissue communication [2]. It is estimated that up to 25% of circulating proteins are liver-derived and a growing body evidence has shown that hepatokines act on numerous organs to induce diverse signalling events and to regulate many biological processes [271]. Notably, many hepatokines are important regulators of lipid and glucose metabolism, insulin action, energy expenditure and food intake, and dysregulated hepatokine secretion is a recognised feature of NAFLD [2]. Studies in mice indicate that the liver is highly susceptible to exercise-induced cellular stress [340], resulting in changes in the mRNAs encoding secreted proteins [373], thereby suggesting the likelihood of exercise-induced adaptations in liver protein secretion. However, very few *bona fide* hepatokines have been identified as exercise responsive and it is unknown whether chronic exercise training alters hepatokine secretion [337; 338], and whether exercise-regulated hepatokines impact metabolism in the liver or other tissues. We tested the hypothesis that chronic exercise training induces stable alterations in hepatokine secretion and that such changes can alter processes to alleviate the metabolic burden of NAFLD.

Herein, we demonstrate that exercise training induces marked remodelling of the hepatocyte proteome and secretome, and that hepatocyte secreted factors from exercise-trained mice improves fatty acid metabolism and insulin action in glucoregulatory tissues. We further identify syndecan-4 (SDC4) as an exercise-responsive hepatokine, which regulates hepatic lipid metabolism.

6.2. Results

6.2.1. Validation of the endurance exercise training program

NAFLD was established by feeding mice a HFD for six weeks. Mice were matched for body weight and glucose tolerance (Supplementary figure 8.1A-B) and allocated to either the sedentary group (Sedentary) or the endurance exercise training group (Exercise). Exercise training was performed for a further six weeks and all mice were maintained on the HFD (Figure 6.1A). Sedentary mice gained ~5.3 g body weight during this intervention period while exercise trained mice were refractory to HFD-induced body weight gain (Figure 6.1B). The difference in body mass gain was associated with less adipose tissue mass in exercise-trained compared with sedentary mice (Figure 6.1C). Skeletal muscle mass (mixed quadriceps) was not different between groups (Figure 6.1C). Exercise training reduced liver triglyceride content by ~52% compared with Sedentary (Figure 6.1D). The efficacy of the exercise program was demonstrated at the whole-body level by marked improvements in maximal running capacity (Figure 6.1E), and at the molecular level by increased skeletal muscle citrate synthase activity (Figure 6.1F), increased protein content of PGC1 α , a master regulator of mitochondrial biogenesis (Figure 6.1G-H), as well as increased protein expression of various subunits of the electron transport chain in skeletal muscle (Figure 6.1I-J). Furthermore, exercise training reduced fasting blood glucose and mildly improved glucose tolerance in mice (Supplementary figure 8.1C-D).

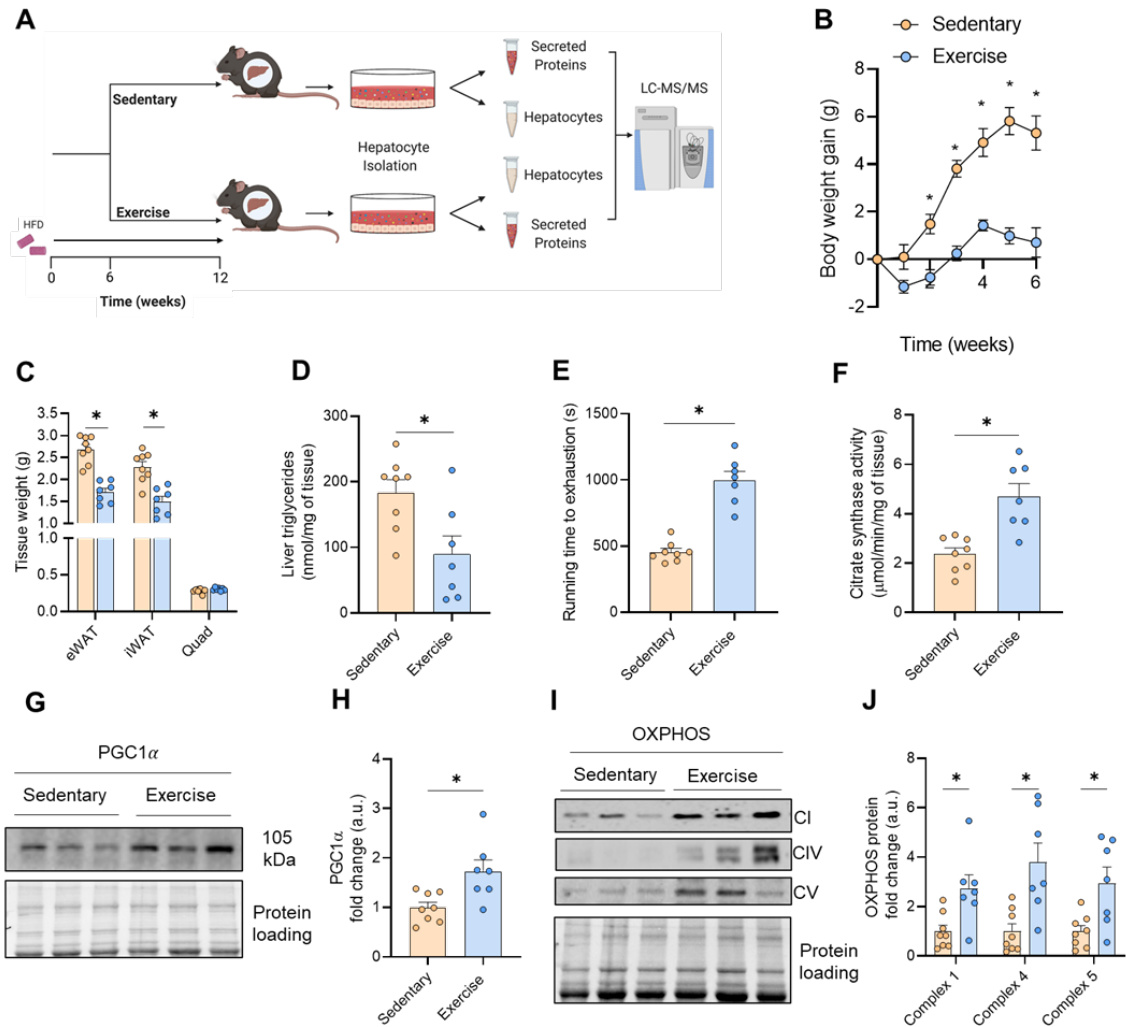


Figure 6.1: Validation of exercise adaptations in exercise trained mice. (A) Schematic of the study design. C57Bl/6 mice were fed a high fat diet for 6 weeks and were endurance exercise trained (Exercise) or remained sedentary (Sedentary) for a further 6 weeks, after which their hepatocytes were isolated and cultured for mass spectrometry proteomic evaluation of the intracellular and secreted proteins. (B) Body weight, (C) epididymal, inguinal adipose tissue and mixed quadriceps mass, (D) maximal running velocity during an incremental test to exhaustion, (E) citrate synthase activity in quadriceps muscle (F) hepatic triglyceride content, (G-H) representative immunoblot of PGC1α in quadriceps muscle and quantification, and (I-J) representative immunoblot of complex I, IV and V of the electron transport chain in quadriceps muscle and quantification. Data are presented as mean ± SEM, with n=7-8 per group. *P<0.05 vs. Sedentary. Significance was assessed by two-way ANOVA followed by Bonferroni's post hoc test (panel B) or students two tailed unpaired t-test (panels C-I). Figure 1A was created with BioRender.com.

6.2.2. Exercise training remodels the hepatocyte proteome and hepatokine secretion

Hepatocytes were isolated from mice 72 h after the last exercise bout to assess adaptive changes to exercise training rather than the effects of an acute exercise bout. The hepatocyte intracellular proteome was assessed using liquid chromatography coupled with tandem mass spectrometry and label-free quantification revealed pronounced differences between the sedentary and exercise trained mice. The analysis identified a total of 2657 individual proteins within hepatocytes, of which 57 proteins were significantly increased, and 80 proteins were significantly decreased with exercise training compared with sedentary conditions (Figure 6.2A). Protein LFQ intensity spanned five orders of magnitude and proteins previously shown to be highly enriched in liver [304] were abundant in this analysis, including Carbamoyl-phosphate synthase 1 (CPS), Acetyl-CoA transferase 2 (ACAA2) and Aldehyde dehydrogenase 2 (ALHD2) (Figure 6.2B). Proteins detected at lower intensities included Coiled-coil domain containing 51 (CCDC51) and N-alpha-acetyltransferase 15 (NAA15). Principle component analysis of the hepatocyte proteomes highlighted that, in general, the Exercise group clustered tightly whereas there was more variability in the Sedentary group (Figure 6.2C). Qiagen Ingenuity Pathway Analysis (IPA) of canonical pathways highlighted exercise-induced activation of the tricarboxylic acid (TCA) cycle, mTOR and sirtuin signalling, which is indicative of improved mitochondrial function, and suppression of oxidative stress-related pathways (e.g., NRF response, EIF2 signalling) (Figure 6.2D). Further analysis of potential upstream regulators highlighted enrichment of several tumour suppressive proteins (e.g., PTEN) and proteins involved in lipid metabolism (e.g., RICTOR, PPAR α), and suppression of oncogenes (e.g., MYC, MYCN) and regulators of oxidative stress (e.g., NFE2L2) (Figure 6.2E).

We next assessed exercise-induced adaptations in proteins secreted from hepatocytes and identified a total of 1593 proteins across both treatment groups (Figure 6.2F) spanning six orders of magnitude (Figure 6.2G). Proteins detected in the secreted medium at higher intensities included Annexin 4 (ANXA4), CPS1, Fatty acid binding protein 1 (FABP1) and Albumin. Hepatocytes secreted other plasma proteins known to be of hepatic origin including Hemopexin [304], Mannose binding lectin [304], Fibrinogen alpha chain [304], Hydroxyacid oxidase 1 (HAO1) [304], Thrombin [304], Complement C9 [304], Apolipoprotein H [304], DPP4 [374; 375] and HEXA [284]. In contrast to the intracellular proteome, there was clear clustering of hepatocyte secreted proteins by group as determined by PCA (Figure 6.2H). Of all secreted proteins, 20.5% were denoted as classically secreted by the presence of a N-terminal signal sequence, while 79.5% were predicted to be secreted via unconventional processes (*i.e.*, in extracellular vesicles) (Figure 6.2I), which is consistent with previous reports [275; 376]. Of the classically secreted proteins, 26 were increased and 9 were decreased following exercise training (Figure 6.2J). IPA identified overrepresentation of secreted proteins involved in lipid oxidation and transport and downregulation of proteins associated with hepatic steatosis, oxidative stress and liver cancer (Figure 6.2K). Furthermore, canonical pathways related to heparan sulphate synthesis and degradation of serotonin were overrepresented while cholesterol synthesis was downregulated with exercise training (Figure 6.2L). Bioinformatic prediction of upstream regulators identified an increase in Acyl-CoA oxidase 1 (ACOX1) and Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), and reductions in Nuclear factor-erythroid factor 2-related factor 2 (NRF2) and Peroxisome proliferator activated receptor α (PPAR α) (Figure 6.2M). Together, the results indicate that exercise training

regulates the intracellular proteome and protein secretion from hepatocytes and that these adaptive changes are predicted to modulate various aspects of lipid metabolism.

The mRNA expression or intracellular protein abundance is often used as a proxy to predict protein secretion from cells, despite the requirement for extensive posttranslational glycosylation and organellar shuttling to enable protein secretion [1]. We identified a significant negative correlation ($r^2=0.22$, $p<0.0001$) between the abundance of intracellular proteins and the corresponding secreted proteins (Figure 6.2N). This data suggests that the change in the intracellular abundance of a protein with an exercise intervention is a poor predictor of the likely change in protein secretion from hepatocytes.

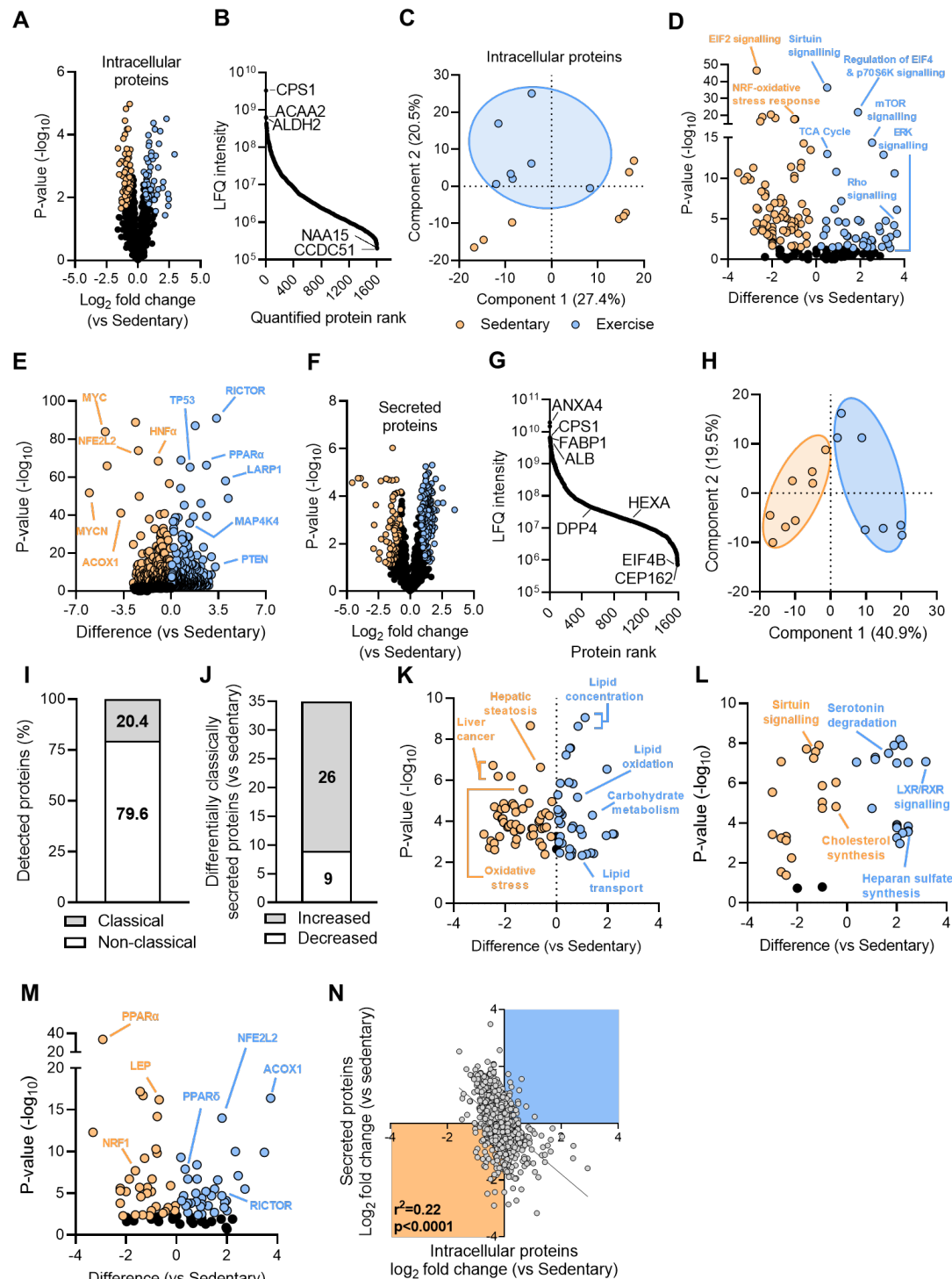


Figure 6.2: Identification of the hepatocyte intracellular proteome and hepatocyte secreted proteins.(A) Volcano plot showing hepatocyte intracellular proteins with those increased by exercise training denoted in blue and those decreased denoted in orange. (B) The dynamic range of quantified intracellular proteins. (C) Principal component analysis of the intracellular proteins. (D) Ingenuity Pathway Analysis showing enrichment of upstream factors in exercise-trained mice. (E) Canonical pathways within the hepatocyte. (F) Volcano plot showing hepatocyte secreted proteins with those increased by exercise training denoted in blue and those decreased denoted in orange. (G) The dynamic range of the quantified secreted proteins. (H) Principal component analysis of the hepatocyte secreted proteins. (I) Percentage of proteins that

are classically or non-classically secreted. **(J)** Number of classically secreted proteins that are significantly increased or decreased with exercise training. **(K-M)** Ingenuity Pathway Analysis showing enrichment of exercise mediated changes in **(K)** diseases and biological functions, **(L)** canonical pathways and **(M)** upstream factors. **(N)** Correlation between exercise-training induced changes in intracellular proteome and corresponding secreted protein. Shown are means $\pm$ SEM, N=7-8 per group. *P<0.05 vs. Sedentary. Significance was assessed by two-way ANOVA followed by Bonferroni's post hoc test (panel B) or students two tailed unpaired t-test (panels C-I). Proteins identified by corresponding gene name.

6.2.3. Hepatocyte secreted factors of exercise trained mice improve lipid metabolism and insulin action in isolated tissues

Hepatocyte secreted factors derived from mice with NAFL cause inflammation and insulin resistance [11], and numerous hepatokines contribute to dysregulated glucose and lipid metabolism in NAFLD [1; 2]. We tested whether hepatocyte-derived factors from exercise trained mice would improve metabolic processes that are often dysfunctional in NAFLD. To do this, we bathed isolated epididymal adipose tissue, intact soleus muscles and precision-cut liver slices from healthy donor mice in the medium containing hepatocyte secreted factors from sedentary or exercise trained mice and assessed metabolism.

Exercise-secreted factors did not alter lipolysis or insulin-stimulated 2-deoxyglucose uptake in epididymal adipose tissue (Figure 6.3A-B). In contrast, hepatocyte secreted factors from exercise trained mice significantly increased insulin-stimulated 2-deoxyglucose uptake in skeletal muscle (Figure 6.3C) when compared to effects induced by hepatocyte secreted factors from sedentary mice. Liver glucose output was also reduced by hepatocyte secreted factors from exercised compared with sedentary mice (Figure 6.3D). Fatty acid uptake (Figure 6.3E), triglyceride synthesis (Figure 6.3F), phospholipid synthesis (Figure 6.3G), and total lipid synthesis in the liver (Figure 6.3H) were not significantly different between treatment conditions, whereas hepatocyte

secreted factors from exercise trained mice increased fatty acid oxidation by 39% when compared to hepatocyte factors secreted from sedentary mice (Figure 6.3I). Collectively, these data provide evidence that regular endurance exercise training alters the complement of secreted factors from hepatocytes, which in turn improves metabolic functions that are often dysregulated in NAFLD.

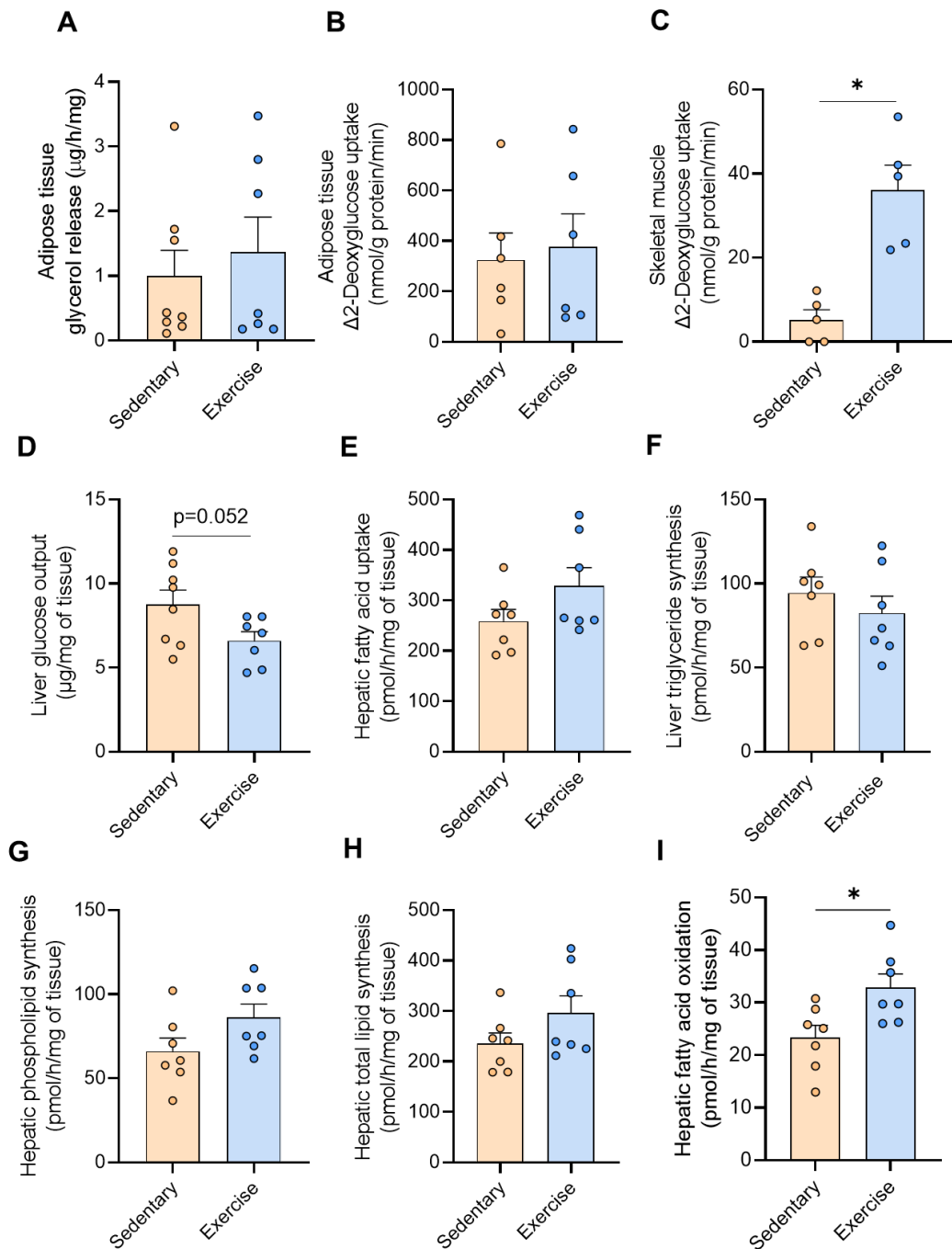


Figure 6.3: Impact of secreted factors derived from hepatocytes of sedentary and exercise-trained mice on metabolism. Hepatocyte secreted factors from Sedentary or

Exercise trained mice were applied to tissues followed by metabolic assessment. **(A)** Lipolysis and **(B)** insulin stimulated 2-deoxyglucose uptake in epididymal adipose tissue. **(C)** Insulin stimulated glucose uptake in soleus muscle. **(D)** Glucose output from precision cut liver slices. **(E-I)** Radiometric tracing of lipid metabolism in liver slices showing **(E)** fatty acid uptake, **(F)** triglyceride synthesis, **(G)** phospholipid synthesis, **(H)** total lipid storage, and **(I)** fatty acid oxidation. Data are presented as mean $\pm$ SEM, with n=5-8 per group. *P<0.05 vs. Sedentary. Significance was assessed by students two tailed unpaired t-test.

6.2.4. Identification of syndecan-4 as an exercise-responsive hepatokine

We next sought to identify the secreted proteins that may be mediating these metabolic effects and detected 55 proteins that were exclusively secreted from hepatocytes of exercise trained mice and 51 proteins secreted only from hepatocytes of sedentary mice (Figure 6.4A). A small proportion of these proteins were denoted as classically secreted (Figure 6.4B) and these are shown in Figure 6.4C. Classically secreted proteins exclusively secreted by hepatocytes in Sedentary mice included Coagulation factor XIII a chain (F13A1), Lactotransferrin (LTF), S100 calcium-binding protein A9 (S100A9) and Perlecan (HSPG2), which is associated with glucose intolerance [377] and adiposity [378]. Classically secreted proteins only secreted from hepatocytes of exercise trained mice (Figure 6.4C) included Insulin like growth factor binding protein, which was previously shown to protect against cardiovascular pathologies [379], and Retinoic acid receptor responder 2 (RARRES2), also known as Chemerin, which was shown to improve glycaemic control [380].

One protein that sparked our interest was Syndecan-4 (SDC4) (Figure 6.4C). SDC4 is a transmembrane heparan sulfate proteoglycan localized to the plasma membrane, which is cleaved by a range of extracellular proteases [381; 382] to release the ectodomain for secretion. We detected three peptides of SDC4 in our mass spectrometry analysis and all are contained in the ectodomain, which spans amino acids 28-41, 106-116, and 100-116

(peptides: 28-ETEVIDPQDLLEGR-41, 106-ELEENEVIPK-116, 100-VPSEPKELEENEVIPK-116). We did not detect peptides of the transmembrane or cytosolic domains, indicating detection of the secreted form of SDC4. Circulating SDC4 levels have been reported to be ~10-20 ng/mL in humans [383]. Plasma SDC4 is increased during exercise in humans [383] and polymorphisms in *SDC4* are associated with abdominal fat [384]. Moreover, ablation of the SDC4 orthologue in *Drosophila* (known as *Sdc*) reduced *Spargel* (i.e., PGC1 α) expression and decreased metabolic rate [385], whereas, somewhat paradoxically, plasma SDC4 levels are higher in men with dysregulated glycemic control compared to normoglycemic men [383]. Notably, *Sdc4* expression was significantly increased in the livers of exercise trained compared with sedentary mice (Figure 6.4D).

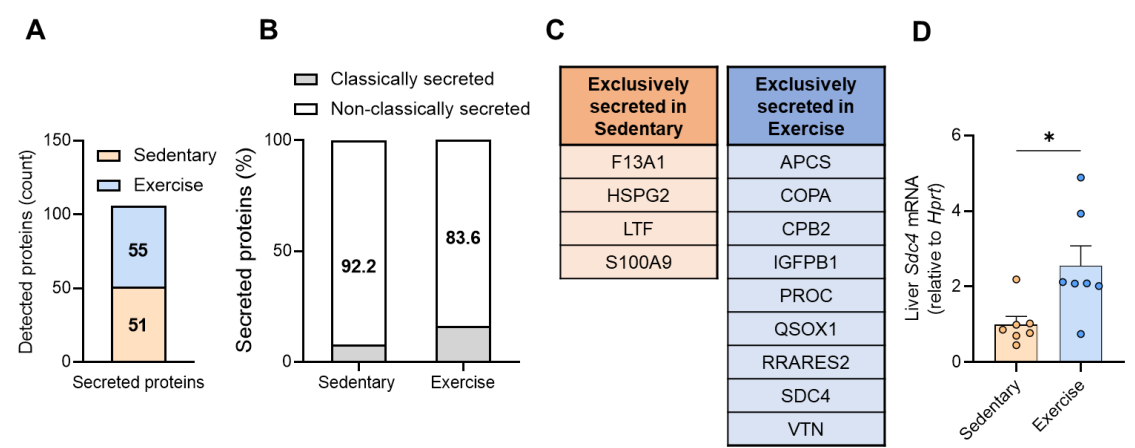


Figure 6.4: Identification of SDC4 as an exercise training-responsive hepatokine.(A) Identification of proteins only secreted from hepatocytes derived from Sedentary and Exercise-trained mice. (B) The percentage of classically and non-classically proteins secreted from hepatocytes derived from Sedentary and Exercise-trained mice. (C) Classically secreted hepatokines derived from hepatocytes of Sedentary and Exercise-trained mice. (D) *Sdc4* mRNA in the livers of Sedentary and Exercise-trained mice. Data are presented as mean $\pm$ SEM, with n=7-8 per group. *P<0.05 vs. Sedentary. Significance was assessed by students two tailed unpaired t-test (panel D).

6.2.5. Hepatocyte-specific *Sdc4* overexpression in mice with NAFLD

To investigate the metabolic roles of hepatic SDC4 and its potential to reduce the burden of NAFLD and its associated co-morbidities, mice were fed a high-fat diet for six weeks to induce mild obesity and NAFLD (Figure 6.5A). Mice were segregated into experimental groups after matching for body weight, fat mass and glucose tolerance (Supplementary figure 8.2A-C). Mice were then injected i.v with an AAV8 vector containing a hepatocyte-specific promotor (TBG) and *Sdc4* (*Sdc4*-AAV) or the same AAV8 vector containing scrambled DNA (Control-AAV) (Figure 5A). *Sdc4* expression was increased 2-fold in the liver following *Sdc4*-AAV administration, in the absence of changes in *Sdc4* expression in other tissues (Figure 6.5B), and this occurred independent of changes in the expression of other SDC family members, including *Sdc1*, *Sdc2* and *Sdc3* (Figure 6.5C). SDC4 secretion from precision-cut liver slices was increased by 80% in *Sdc4*-AAV compared with Control-AAV (Figure 6.5D), noting that the same three ectodomain spanning peptides in the exercise secretome were detected in this preparation. We were unable to detect SDC4 in the plasma of mice when using a commercially available ELISA or targeted mass spectrometry, which has been reported previously [386] and is thought to be due to excessive glycosylation [387].

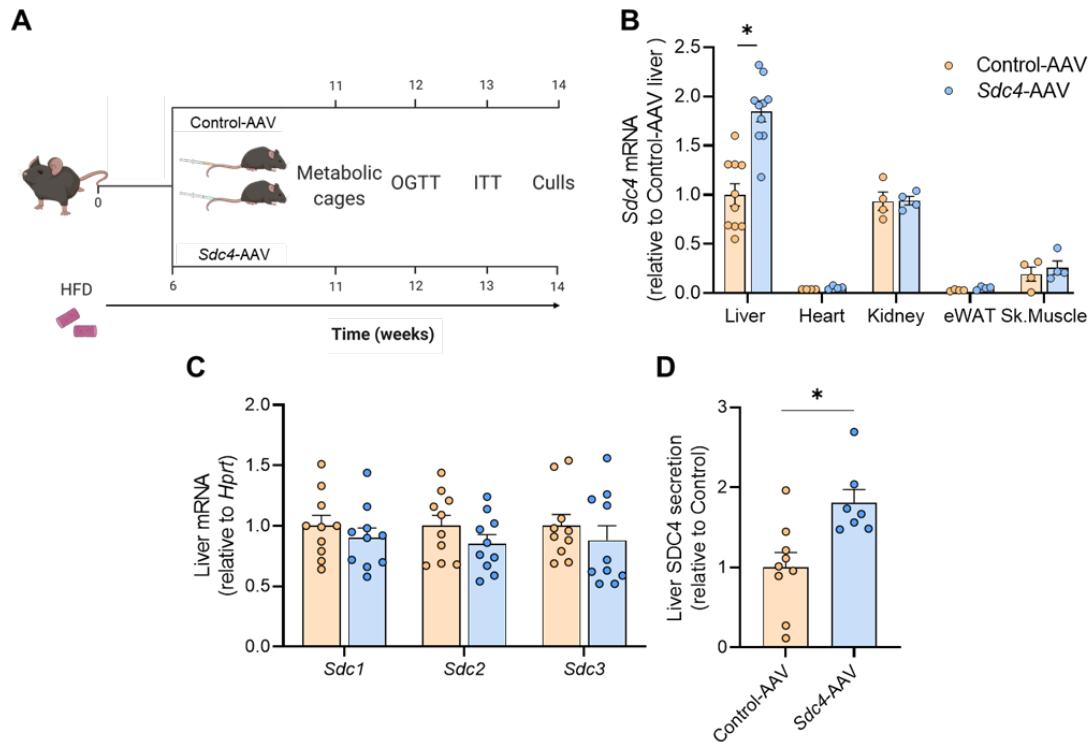


Figure 6.5: Validation of *Sdc4* overexpression in mice. (A) Schematic representation of the experimental design. C57BL/6 mice were fed a high fat diet (HFD) for 6 weeks then injected with *Sdc4*-AAV or Control-AAV. Following AAV administration, mice were maintained on a high fat diet for a further 6 weeks. (B) *Sdc4* expression in liver, heart, kidney, skeletal muscle and epididymal adipose tissue. (C) *Sdc1*, *Sdc2* and *Sdc3* mRNA expression in the liver. (D) Targeted mass spectrometry analysis of SDC4 secretion from precision cut liver slices. Data are presented as mean $\pm$ SEM, with n=4-10 per group. *P<0.05 vs. Control-AAV. Significance was assessed by students two tailed unpaired t-test. Figure 5A was created with Biorender.com.

6.2.6. Hepatic overexpression of *Sdc4* does not alter energy homeostasis or glycaemic control

Given the known links between SDC4, metabolism and energy expenditure in other species [384; 388; 389], we first assessed the impact of *Sdc4* overexpression on body mass regulation and energy homeostasis. Body weight, individual tissue weights, adiposity, food intake, daily energy expenditure and substrate partitioning were not different between *Sdc4*-AAV and Control-AAV (Figure 6.6A-F). Similarly, increasing *Sdc4* expression in the liver did not alter glucose tolerance (Figure 6.6G), fasting or glucose-stimulated insulin secretion (Figure 6.6H), or whole-body insulin action (Figure 6.6I). Plasma lipids including free fatty acids, total cholesterol and triglycerides were not different between groups (Figure 6.6J-L).

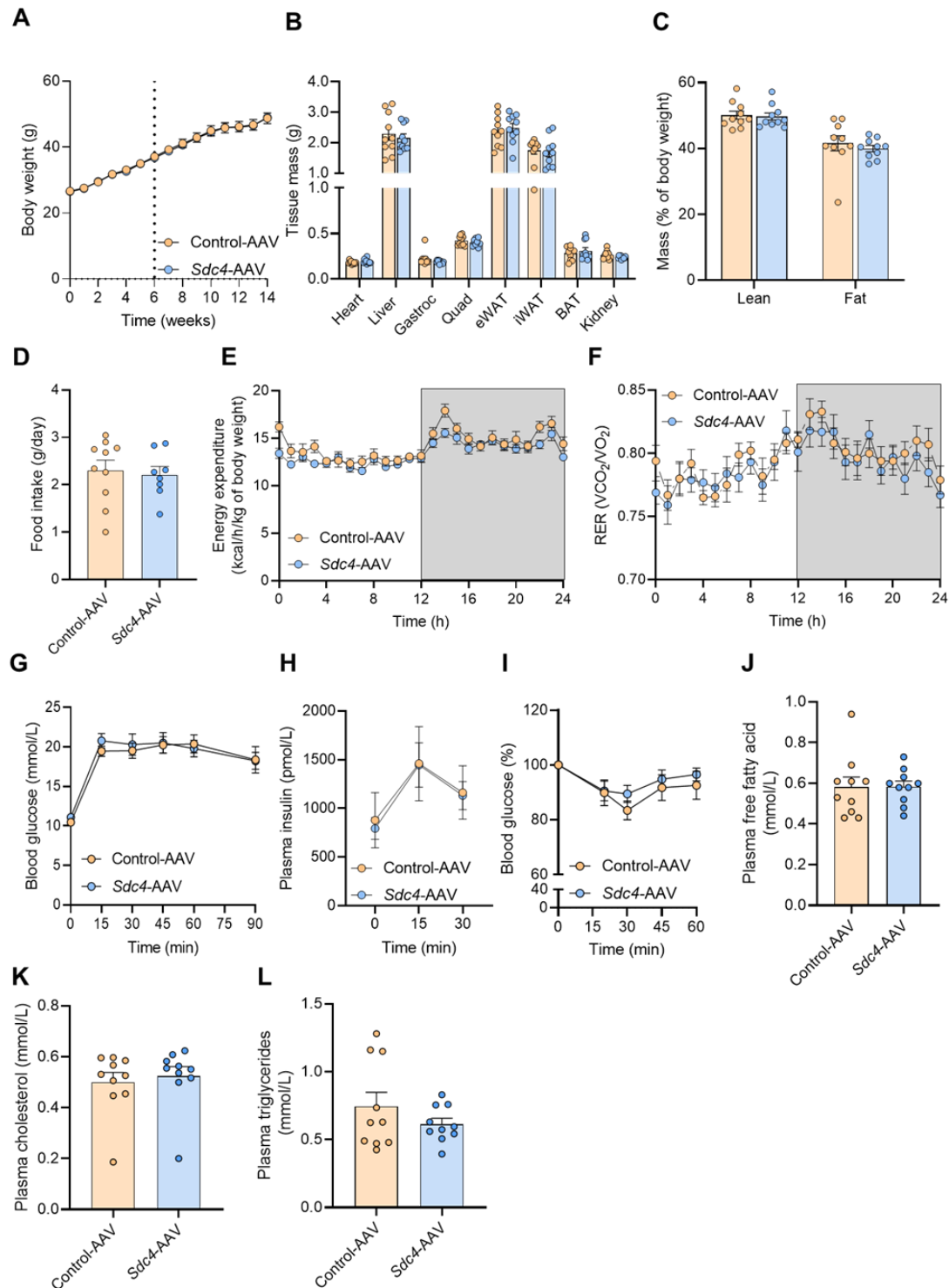


Figure 6.6: Hepatic *Sdc4* overexpression does not alter whole body energy homeostasis or glycaemic control.(A) Body weight (dotted line indicates *Sdc4*-AAV or Control-AAV injection), (B) tissue weights, (C) lean and fat mass, (D) daily food intake, (E) energy expenditure, (F) respiratory exchange ratio (RER), (G) glucose tolerance following oral glucose administration (2 g/kg), (H) fasting and glucose-stimulated plasma insulin levels, and (I) blood glucose in response to i.p. insulin administration (1 U/kg). (J) Plasma free fatty acids, (K) cholesterol and (L) triglycerides. Data are presented as mean $\pm$ SEM, with $n=7-10$ per group. * $P<0.05$ vs. Control-AAV. Significance was assessed by students two tailed unpaired t-test (panels

B, C, D, J, K, L) or two-way ANOVA followed by Bonferroni's post hoc test (panels A, E, F, G, H, I).

6.2.7. SDC4 reduces fatty acid uptake and markers of inflammation and fibrosis in the liver

Histological assessment of H&E-stained liver sections revealed a reduction in macrosteatosis in *Sdc4*-AAV compared with Control-AAV livers (Figure 6.7A). Unbiased assessment of lipid droplet number and size demonstrated an overall reduction of total lipid droplet area with *Sdc4*-AAV (Figure 6.7B), which was associated with a change in the size distribution of lipid droplets. SDC4 overexpression increased the number of 'smaller' lipid droplets ($<10\ \mu\text{m}^2$) without altering the number of larger lipid droplets ($10\text{-}50\ \mu\text{m}^2$) (Figure 6.7C). However, relative to the total number of lipid droplets, there was a 40% reduction in lipid droplets with an area $>50\ \mu\text{m}^2$ in mice overexpressing *Sdc4* in the liver and, importantly, the average area of 'larger' lipid droplets ($>50\ \mu\text{m}^2$) was reduced in *Sdc4*-AAV compared with Control-AAV (Figure 6.7E). These findings are consistent with the histological finding of reduced macrosteatosis in *Sdc4*-AAV mice (Figure 6.7A). Despite the significant lipid droplet remodelling, we did not observe any differences in total liver triglyceride levels as determined by biochemical assessment (Figure 6.7F). Taken together, this remodelling of lipid droplet size and number indicated a plausible role for SDC4 in regulating hepatic lipid metabolism.

In light of these alterations in liver lipid droplets, and given that SDC4 is localized to the plasma membrane [390] and is linked to triglyceride storage in *Drosophila* [391], we surmised that SDC4 might induce autocrine / paracrine regulation of lipid metabolism. Accordingly, we investigated the effect of SDC4 overexpression on lipid

metabolism in precision-cut liver slices. *De novo* lipogenesis was assessed by tracing ^{14}C -glucose into cellular lipid pools and was not different between groups (Figure 6.7G). In contrast, fatty acid uptake was significantly reduced in *Sdc4*-AAV compared with Control-AAV (Figure 6.7H), and this coincided with a 25% reduction in fatty acid incorporation into cellular lipids (Figure 6.7I). Fatty acid oxidation was also reduced ($P=0.06$) in livers overexpressing *Sdc4* (Figure 6.7J). Given the marked reduction in fatty acid uptake, we assessed the expression of genes encoding proteins involved in lipid transport and intracellular trafficking. There was no difference in the expression of fatty acid transporters including fatty acid translocase (Cd36), fatty acid transport protein -1 (Fatp1), -2 (Fatp2) and -5 (Fatp5). Diacylglycerol O-acyltransferase 2 (Dgat2), a key enzyme involved in triglyceride synthesis, was also similar between groups. In contrast, caveolin-1 (*Cav-1*) expression was increased with SDC4 overexpression compared with control (Figure 6.7K). This latter finding is of interest because Cav-1 is required to create membrane invaginations termed caveolae and form a complex with CD36 and other proteins to regulate fatty acid uptake and intracellular trafficking [392; 393].

Inflammation and fibrosis are common features of NAFLD. While this short-term HFD dietary intervention does not induce significant adverse histopathology such as lobular inflammation, hepatocellular ballooning, or fibrosis (data not shown), *Sdc4*-AAV reduced the expression of pro-inflammatory genes including *Tnfa* and *Il-1b* and several pro-fibrotic genes including transforming growth factor β 1 (*Tgfb1*) and interstitial collagen type I α 2 chain (*Colla2*) (Figure 6.7L). Thus, increasing SDC4 in the liver may reduce the rate of fatty acid uptake, and may prevent the eventual induction of inflammation and fibrosis in NAFLD.

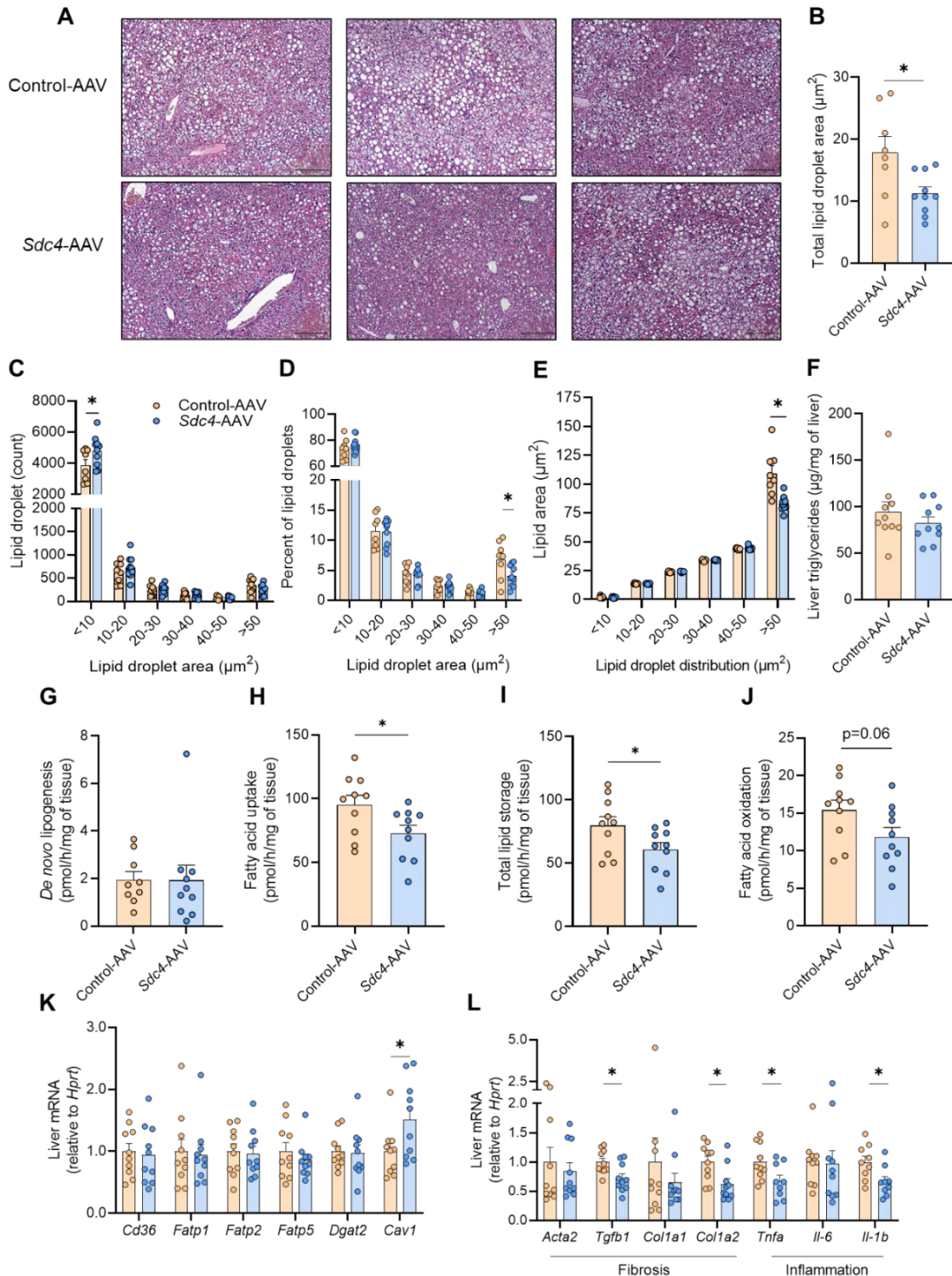


Figure 6.7: SDC4 regulates hepatic lipid metabolism in mice. (A) Representative liver histology stained with hematoxylin and eosin. Scale bar denotes 200 μ m. Sections from n=3 individual mice per group. (B) Quantification of the average lipid droplet area in liver sections. (C) Lipid droplet number in livers presented as a function of lipid droplet size. (D) Proportion of different sized lipid droplets in livers of mice. (E) Average area of lipid droplets highlighting a reduction in the area of larger (>50 μ m²) lipid droplets in Sdc4-AAV vs. Control-AAV mice. (F) Liver triglyceride content. (G) De novo lipogenesis, (H) fatty acid uptake, (I) total lipid storage and (J) fatty acid oxidation in liver. (K) mRNA expression of lipid transporter and esterification enzymes.

(L) mRNA expression pro-fibrotic and pro-inflammatory genes in the liver. Data are presented as mean $\pm$ SEM, with n=8-10 per group. *P<0.05 vs. Control-AAV. Significance was assessed by students two tailed unpaired t-test.

6.2.8. SDC4 ectodomain mediates changes in fatty acid metabolism and lipid droplet remodelling in hepatocytes

While *Sdc4* overexpression in mice induced changes in hepatocyte fatty acid metabolism and lipid droplet morphology, we were unable to definitively ascribe these effects to SDC4's function as a hepatokine. Accordingly, we next assessed the role of the SDC4 ectodomain in regulating these processes. To do this, we cultured intact soleus muscle and isolated hepatocytes with recombinant SDC4 ectodomain (rSDC4, 1 μ g/mL) and assessed insulin action and fatty acid metabolism. rSDC4 did not affect insulin-stimulated glucose uptake in intact soleus muscle (Figure 6.8A) whereas rSDC4 reduced fatty acid uptake (p=0.07) (Figure 6.8B) and lipid storage (p=0.06) (Figure 6.8C) in hepatocytes without significant changes in fatty acid oxidation (Figure 6.8D).

To ascertain whether the secreted form of SDC4 was responsible for altering lipid droplet morphology, primary hepatocytes were isolated and cultured with or without rSDC4 for 48 h. Visual inspection revealed marked remodelling of lipid droplet size with rSDC4 treatment (Figure 6.8E). While there was no difference in the number of lipid droplets between conditions (Figure 6.8F), rSDC4 administration reduced the average cellular lipid droplet area by ~20% (Figure 6.8C), and the total area occupied by lipid droplets within the cell by 25% (Figure 6.8D). Further, these changes were associated with changes in the size distribution of lipid droplets, with reductions in the number of lipid droplets with an area of 2-3, 3-4 and $>5 \mu\text{m}^2$ with rSDC4 treatment (Figure 6.8E). Thus, the secreted ectodomain of SDC4 impacts fatty acid metabolism and reduces lipid droplet size in hepatocytes.

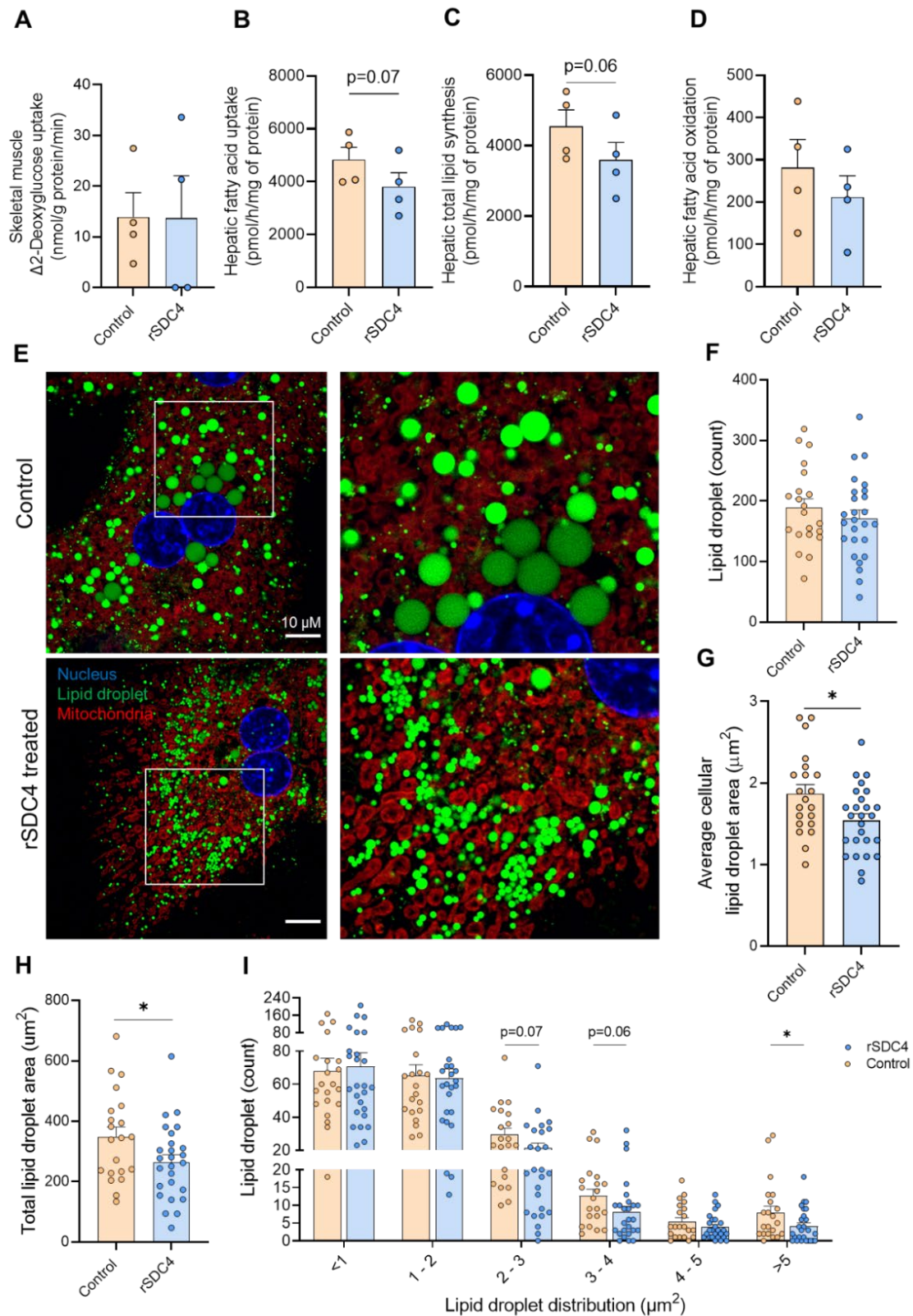


Figure 6.8: SDC4 ectodomain regulates fatty acid metabolism and lipid droplet distribution. (A) Representative image of lipid droplet morphology stained with Bodipy, Mitotracker and DAPI. Scale bar denotes 10 μm . Hepatocytes were isolated from $n=3$ mice, 5-8 hepatocytes were imaged across two technical replicates and quantified per mouse totalling $n=21$ Control and $n=26$ rSDC4 treated hepatocytes. (B) Quantification of the lipid droplet number within the cell, (C) the total area that lipid

droplets occupy in each hepatocyte, and **(D)** the average lipid droplets area within the hepatocyte. **(E)** Proportion of different sized lipid droplets in isolated hepatocytes. **(F)** Insulin stimulated glucose uptake with or without recombinant SDC4 in isolated soleus muscle. **(G)** Radiometric tracing of lipid metabolism in isolated hepatocytes with or without acute recombinant SDC4 treatment showing fatty acid uptake, **(H)** total lipid storage and, **(I)** fatty acid oxidation. Data are presented as mean $\pm$ SEM, * $P < 0.05$ vs. Control-AAV, with $n=4$ per group for paired analyses. Significance was assessed by students two tailed unpaired t-test (panel B-E) or paired t-test (panel G-I). rSDC4, recombinant SDC4.

6.3. Discussion

Exercise training induces a myriad of ‘health promoting’ effects in many tissues, including the liver [171]. However, the role of exercise in regulating the liver proteome and secretome is not well described, particularly in the context of NAFLD, which is the world’s most common liver disease. In this study, we employed unbiased mass spectrometry-based proteomics analyses to identify substantial remodelling of the intracellular proteome and secreted proteins from hepatocytes and offer insights into changes that may contribute to the metabolic benefits associated with regular exercise training. First, we identified exercise training regulation of the liver proteome. Second, we described the effect of exercise training on liver secreted proteins. Third, we show that the complement of liver secreted factors is altered with exercise training, which in turn alters glucose and lipid metabolism in liver and skeletal muscle. And lastly, we identified SDC4 as a novel hepatokine that is increased with exercise training and its secreted ectodomain regulates hepatic lipid metabolism and lipid droplet morphology in NAFLD.

The effects of exercise on liver metabolism are becoming increasingly well described [394] and include increased mitochondrial content and glycolysis, and reduced expression of lipid synthesis proteins [395; 396], which is associated with partial resolution of hepatic steatosis [367]. Furthermore, transcriptomic analysis conducted in

the liver of mice immediately after an acute bout of exercise revealed marked increases in the expression of genes involved in mitogen-activated protein kinase (MAPK) and PPAR signalling, and fatty acid metabolism [373]. However, whether such acute changes in gene expression can lead to stable adaptations at the protein level is not well defined [396]. To address this knowledge gap, we performed unbiased assessment of the hepatocyte proteome following chronic exercise training. Aligned with previous transcriptomic analysis [373] and the known protective effects of exercise training on NAFLD and HCC [397; 398], our bioinformatic investigation of the hepatocyte proteome highlighted upregulation of metabolic processes including the TCA cycle, mTOR signalling and PPAR α signalling, downregulation of pathways involved in cancer and inflammation (*e.g.*, Nrf2-oxidative stress response, Myc), and upregulation of tumour suppressor pathways (*e.g.*, PTEN and TP53). Collectively, these findings support the notion that the hepatocyte proteome is remodelled in response to regular exercise training in the context of NAFLD, and that these changes are associated with improved metabolic health and anti-tumorigenic effects.

The liver is an active endocrine organ that produces and secretes a large proportion of the body's circulating proteins [2; 271] and these hepatokines influence a myriad of biological functions [337-340]. Expanding this knowledge base, we identified 1593 hepatocyte secreted proteins, of which 20.5% are classically secreted. This analysis has the advantage of providing cell-specific knowledge of the hepatocyte secretome. However, hepatocyte isolation and prolonged *ex vivo* incubation/culture of cells may influence protein secretion and future studies are required to compare the hepatocyte secretome with the secretion pattern of liver tissue. Bioinformatic investigation revealed an enrichment of proteins regulating carbohydrate metabolism, lipid transport and lipid

oxidation following chronic exercise training. This prediction is supported by our finding that exercise-responsive factors secreted from hepatocytes improved skeletal muscle insulin action, reduced liver glucose output and increased liver fatty acid oxidation, adaptations that are often reported with exercise training [371]. It should be noted that secreted factors other than proteins, including metabolites, lipids and extracellular vesicles (containing lipids, non-coding RNA, proteins) [2; 399] are likely to contribute to these metabolic changes. We also identified significant alterations in specific hepatokines with chronic exercise training, including reductions in known mediators of insulin resistance (*e.g.*, dipeptidyl peptidase) [375] pathological angiogenesis (*e.g.*, pantetheinase) [400], adipose tissue lipolysis (*e.g.*, cathepsin B) [401] and fatty acid uptake (*e.g.*, fatty acid binding protein 4). On the other hand we identified increased secretion of proteins involved in the preservation of insulin sensitivity (*e.g.*, insulin degrading enzyme) [402], alleviation of oxidative stress (*e.g.*, n-myc downstream regulated gene 2, heme oxygenase-1) [403; 404] and the liver X receptor / retinoid X receptor activation that dampens the inflammatory response (α -1-microglobulin, transthyretin, Serpina1, α -1-antitrypsin) [405]. Notably, of the 26 hepatokines upregulated with exercise-training, there remains a paucity of evidence describing their role in glucose and lipid metabolism. An intriguing observation from these studies was the clear negative correlation between secreted and intracellular proteins in exercise-trained hepatocytes. It is possible that secreted proteins induce negative feedback loops, whereby increased protein degradation and turnover, and proteolytic ectodomain shedding of proteins caused the negative correlation in secreted versus intracellular proteins. It is also possible that there is a 'lag' in protein translation behind transcription of highly secreted proteins. Indeed, others have reported that a group of proteins in the secretome of activated macrophages did not show a concomitant

change in transcript or intracellular protein levels [406], and further studies combining isotopic labelling and mass spectrometry to measure the rate of protein synthesis, cellular distribution and secretion are required to test these possibilities in hepatocytes. Together, the present study shows that remodelling of hepatokine secretion is an adaptation to regular exercise training, and that these changes in hepatokine secretion are associated with changes in metabolism in the liver (autocrine/paracrine effects) and in distant tissues that would be predicted to resolve several pathogenic features of NAFLD.

The proteomic analysis identified SDC4 as an exercise-responsive hepatokine. SDC4 is a transmembrane proteoglycan that comprises an extracellular ectodomain, a transmembrane domain, and a cytoplasmic tail that binds to the actin cytoskeleton and intracellular binding partners. Extracellular proteases can cleave the ectodomain of SDC4 [407] and subsequently regulate its secretion. SDC4 has been implicated in controlling triglyceride storage [388] and dyslipidaemia [389], while SNPs in *Sdc4* are associated with energy expenditure, fasting blood glucose, insulin sensitivity, and abdominal fat [408], processes known to be impacted in NAFLD and by exercise training. However, previous studies by our group have not detected SDC4 secretion from hepatocytes isolated from mice fed a chow or a high-fat diet [5; 204], indicating that SDC4 secretion is unlikely to be regulated by an obesogenic diet *per se* and potentially related to cortisol and adrenaline. In this study, the ectodomain of SDC4 was reproducibly detected in the secretome of hepatocytes derived from exercise trained mice, indicating that SDC4 is an exercise-responsive hepatokine.

Our studies using recombinant protein of the SDC4 ectodomain and hepatocyte-specific AAV-mediated SDC4 overexpression in mice identified a role for SDC4 in regulating

hepatic lipid metabolism. Histological assessment revealed a reduction in macrosteatosis and concomitant accumulation of smaller lipid droplets, without altering total triglyceride content using AAV-mediated overexpression and with chronic rSDC4 exposure in primary hepatocytes. Such lipid droplet remodelling is reminiscent of the changes seen in liver and muscle after exercise training [368; 409] and aligns with the view that smaller lipid droplets differ from larger lipid droplets with respect to their protein composition, inter-organelle contacts and functions [410]. Smaller lipid droplets are considered ‘healthier’ than larger lipid droplets [409; 411], a notion supported by parallel lines of evidence demonstrating that exercise training reduces intramyocellular lipid droplet size without altering lipid droplet quantity or intramyocellular lipid content in patients with type 2 diabetes [409], and that athletes, when compared to individuals with obesity and type 2 diabetes, have a reduction in lipid droplet area and size of ‘larger’ lipid droplets, independent of changes in the total number of lipid droplets and intramyocellular lipid content [411]. Whether this association holds true in the human liver remains to be determined.

SDC4 reduced fatty acid uptake in hepatocytes, which was associated with less incorporation of fatty acids into cellular lipids, factors that might contribute to the reduced prevalence of larger lipid droplets in *Sdc4*-AAV mice and hepatocytes chronically exposed to the SDC4 ectodomain. Changes in the expression of fatty acid transporters do not appear to be mediating SDC4 effects on fatty acid uptake and storage. Rather, SDC4 overexpression was associated with increased *Cav1* expression in the liver. While the role of CAV-1 in mediating fatty acid uptake remains contentious [412], previous studies show that *Cav1* overexpression decreases fatty acid uptake in multiple cell types and deletion of *Cav1* in the liver reduces hepatic lipid accumulation

[413; 414]. Further, others report binding of SDC4 to CAV-1 [415], which triggers caveolar dependent endocytosis [386], a process that influences fatty acid transport and lipid droplet growth [416]. Together, these findings provide a possible mechanism to explain the reduction in SDC4-mediated fatty acid uptake and lipid droplet storage reported in the present study.

Loss of SDC4 results in an aggravated inflammatory response in the lung and impairments in wound healing in the skin and kidney [386; 417-419]. Here, we showed that increasing *Sdc4* content and secretion in the liver reduces the expression of pro-inflammatory and pro-fibrotic genes in NAFLD. Given that reductions in hepatic inflammation and fibrosis are associated with improved health outcomes [420] and progression to NASH [421], further studies examining the anti-inflammatory and anti-fibrotic effects of SDC4 in NASH are warranted.

In summary, we have developed a novel resource detailing the proteomic changes that occur in hepatocytes with regular exercise training in mice with NAFLD. These studies identify marked remodelling of the liver intracellular proteome and altered hepatokine secretion with exercise training, effects that impacted metabolism in the liver and skeletal muscle. SDC4 was identified as a novel exercise-responsive hepatokine that decreases fatty acid uptake, reduces macrosteatosis and dampens the expression of inflammatory and pro-fibrotic genes in the liver. By understanding the proteomic changes in hepatocytes with exercise, we believe that these findings have strong potential for the discovery of new therapeutic targets for NAFLD, which remains an unmet clinical need.

Chapter 7: General Discussion

Inter-tissue communication comprises a complex, multifaceted network that tightly regulates energy homeostasis and metabolism, and perturbations in this process frequently leads to defects in lipid metabolism, NASH, and hepatic fibrosis [422]. In recent years, hepatokines have been ascribed various roles as biomarkers of NAFLD [423-425] or regulators of glucose and lipid metabolism [187; 202; 288]; however, little is known about the broad changes in hepatokine secretion that occur with advanced NAFLD / NASH with or without significant fibrosis or how hepatokine secretion is remodelled with exercise, a frontline treatment for NAFLD. Therefore, this thesis focused on using mass spectrometry proteomic to characterise the changes in liver-secreted and plasma proteins in humans and mice to investigate their clinical utility to diagnose and treat NAFLD and its associated comorbidities

Adequate clinical management of patients with NAFLD remains ineffective, which is partially due to ineffective or impractical screening and stratification. The liver biopsy is an invasive, costly, and impractical approach that is currently required to adequately diagnose NASH and hepatic fibrosis in obese individuals [145; 157]. Non-invasive algorithms using plasma borne proteins and metabolites are being increasingly used to diagnose NASH and hepatic fibrosis [256; 426; 427]; however, there remains a lack of clinical efficacy and validation, particularly in obese cohorts or against other fibrotic diseases [145; 157]. For example, the Forns index developed in non-obese patients with hepatitis C with significant fibrosis [255] resulted in poor sensitivity and specificity in obese individuals (Chapter 4) [157] and required lowering of the threshold to improve diagnostic accuracy [157], highlighting the need for validation of existing non-invasive algorithms in obese cohorts. Another issue relates to specificity. In this regard, a novel

algorithm, ADAPT [426], utilises the Pro-C3 fragment formed from type III collagen synthesis [428], and demonstrated high sensitivity for stratifying those with significant fibrosis [426]. However, obesity promotes fibrosis in extra-hepatic organs such as the kidney [429; 430] of which the PRO-C3 marker is currently investigated as a potential biomarker in kidney fibrosis [431]. Thus, it is possible that the PRO-C3 marker and ADAPT algorithm are indicative of generalised fibrosis in the body rather than liver fibrosis specifically. Therefore, non-invasive tests that enter the clinic should adequately inform on the presence of fibrosis and the tissue location. Collectively, the lack of sensitive and specific tests have led the American Association for the Study of Liver Diseases to advise against screening patients in high-risk groups (*e.g.*, obese and or diabetic) due to the unreliability of these diagnostic tests and the lack of treatment options for these patients [6; 96].

In pursuit of a novel biomarker, we interrogated EMILIN1 whose circulating concentration was unknown. Plasma EMILIN1 levels assessed by ELISA were shown not to be an adequate biomarker to stratify patients with NASH or hepatic fibrosis despite increases in expression and secretion from livers of individuals with biopsy confirmed NASH and fibrosis. This finding aligned with a poor correlation of liver-secreted and plasma proteins shown more broadly among high abundant plasma proteins and highlights the complexity of protein secretion into the blood. Classically secreted proteins undergo extensive modification from protein synthesis, folding, quality control, and trafficking in a tissue-specific manner [432]. In addition, little is known about the process regulating plasma protein levels after their secretion from the cell. With respect to hepatokines, it is still unknown how proteins avoid extracellular degrading enzymes, highly phagocytic resident and infiltrating immune cells, and

bypass the extensive collagen network to be transported through the sinusoidal epithelial cells to enter the systemic circulation [285; 433; 434]. These are areas that need further investigation.

To overcome the lack of diagnostic capabilities in patients with NAFLD and NAFLD-associated fibrosis, we profiled liver-secreted, and plasma proteins to identify novel biomarkers that could distinguish disease progression. Our findings are arguably reconciled with the current difficulty in developing sensitive and specific biomarkers that reflect NAFLD pathophysiology, given that only two significantly altered plasma proteins, AFM and APCS, also exhibited increased hepatic secretion. Further, the hepatokines APOF and IGKV2-28 appeared to outcompete AFM and APCS as biomarkers to stratify patients with NASH and NAFLD-associated fibrosis. Little is known about IGKV2-28, particularly in obesity and NAFLD. However, circulating APOF was previously shown to be reduced with hypertriglyceridemia but increased with hypercholesterolemia [435]; both conditions are common in patients with NAFLD. Therefore, non-invasive tests that utilise APOF as a non-invasive biomarker for NAFLD may require accounting for circulating lipid levels for accurate stratification. Together, these findings provide novel insights into the hepatokine and plasma proteome remodelling with NASH and NAFLD-associated fibrosis and identify biomarkers that could be exploited to stratify patients with, or are at risk of NASH and NAFLD-associated fibrosis. Specifically, we have identified several proteins that alone perform better than current non-invasive tests for the detection of NAFLD F2-3, and NASH. Future analysis that combines these markers with other biochemical and clinical measures may prove to be an effective and safe approach to diagnose patients with NASH and NAFLD F2-3 non-invasively.

Our second and third aims converged to identify potential treatments for NAFLD and hepatic fibrosis based on the prior knowledge that other hepatokines have pleiotropic roles and impact whole body energy homeostasis and metabolism [2]. Our two ‘omics’ screens identified two ECM proteins, EMILIN1 and SDC4, that were under appreciated in the context of NAFLD and hepatic fibrosis. Both SDC4 and EMILIN1 were previously reported to influence cell functions by direct interaction with cell-surface receptors [436] or indirectly by regulating growth factor availability [282]. While the understanding of ECM-mediated control on metabolism is becoming better appreciated [437; 438], there remains a paucity of information in this field, particularly in NAFLD. Here we examined the autocrine, paracrine, and endocrine role of SDC4 and EMILIN1 in NAFLD.

To date, few studies have assessed the role of SDC4 in the liver, let alone in NAFLD [439]. Serum SDC4 is an excellent predictor of NAFLD in humans [440], and the druggable stimulation of SDC4 transmembrane protein has shown therapeutic potential in treating patients with HCC [436]. In the context of obesity, high-fat feeding female SDC4^{-/-} mice resulted in increased liver TG and adipose tissue inflammation [439] consistent with our observed reductions in hepatic steatosis with SDC4 overexpression. Further, our approaches utilising recombinant protein and liver-specific AAV that overexpressed SDC4 demonstrate that the ectodomain of SDC4 is likely responsible for reducing fatty acid uptake, lipid droplet size and inflammation. Although other SDC family members are considered to have overlapping functions with SDC4 and SDC1-3 levels are increased in SDC4^{-/-} cells and mice [439; 441; 442], the expression of SDC1-

3 were not different our study, hence it is unlikely that compensation from other SDC proteins were mediating the observed reductions in hepatic fatty acid metabolism and inflammation. SDC4s role in inflammation and fibrosis has been increasingly interrogated [386; 417; 419; 442-444]. Previous assessment of SDC4 knockdown in primary human umbilical vein endothelial cells revealed ~3-fold increases in the secretion of chemokines, expression of pro-inflammatory genes, and impairments in wound healing [442]. Impaired wound healing has been recapitulated in the skin and kidney *in vivo* [417; 444]. Further, ours and previous findings in SDC4^{-/-} females [439] are consistent with other reports demonstrating that the secreted ectodomain of cardiac SDC4 is responsible for immune cell recruitment and promotion of ECM degradation, rather than the transmembrane protein [443]. As such, improvements in NAFLD severity are likely driven by the secreted form of SDC4. We have not assessed the mechanism of how the ectodomain of SDC4 mediates reduces fatty acid uptake and remodels lipid droplet morphology. Application of SDC4 ectodomain onto isolated hepatocytes and performing phosphoproteomics may define potential pathways mediating this response.

EMILIN1 is not unique in its capacity to regulate TGF β signalling. Extracellular matrix protein 1 (ECM1) is produced by hepatocytes and inhibits pro-TGF β cleavage, reduces fibrogenesis, and is patented for the treatment of liver fibrosis [445] (Patent; CN-111135311-A, WO-2020088070-A1). Similarly, microfibril-associated glycoprotein 1 (MAGP1, encoded by the gene *Mfap2*) binds to the mature TGF β ligand, and when MAGP1 is deficient, this predisposes mice to NAFLD, hyperlipidaemia, hyperglycaemia, and impairments in glucose handling and reduced energy expenditure [446]. EMILIN1 is an abundant ECM protein in the healthy human liver [447; 448],

with approximately 8-fold greater levels of EMILIN1 compared to ECM1 [447; 448]. Despite this, the functional assessment of EMILIN1 in the liver, let alone under pathological conditions including NAFLD, hepatic fibrosis, and HCC, remains to be described. Further, given its higher abundance, it is feasible that a more pronounced phenotype of hepatic fibrosis will occur with hepatic EMILIN1 knockdown. We showed that injection with *Emilin1*-AAV reduced hepatic fibrosis and glucose-stimulated insulin secretion in two dietary models of NASH (Chapter 5). This thesis did not identify the molecular mechanisms mediating these EMILIN1 effects; however, it may be attributed to its EMI and gC1q domain. Previous reports show that the EMI domain prevents the release of pro-TGF β release [282] while the gC1q domain that is shared with adiponectin and TNF superfamily of proteins [449] was shown to inhibit proliferation *in vitro* [286; 287; 450]. Together the EMI and gC1q domain may exert the same inhibition on HSC activation and proliferation to prevent the development of fibrosis. Lastly, overexpression of hepatocyte EMILIN1 reduced glucose-stimulated insulin levels, while it is plausible that it could be due to a reduction in TGF β activity in the pancreas [451] however, this assertion requires further investigation.

EMILIN1 turnover within tissues or in the plasma is not fully understood. Neutrophil elastase can degrade EMILIN1 *in vitro* [287] and may be, in part, responsible for EMILIN1 turnover in the tissue or the circulation. In the tissues, following NE mediated EMILIN1 degradation the components may exert biological functions [286] or can targeted for endocytosis and digestion [452]. On the other hand, circulating EMILIN1 would be likely be translocated from the plasma to tissues for degradation. However, the main tissues and cells within the tissues that are responsible for EMILIN1 endocytosis and degradation are not yet known. Whether key disposal sites are similar to other

plasma proteins like albumin that are largely degraded by the muscle, liver, and kidney [453] are plausible. A novel approach combining isotopic tracers and mass spectrometry could be utilised to determine the rate of EMILIN1 synthesis and turnover [348]. Or potentially performing a modified ‘bleach-chase’ and measure EMILIN1 disappearance [454]. Further, whether plasma EMILIN1 is the full intact protein, the degraded components, or a combination of the two and if this ratio of intact vs degraded is modified in NAFLD and or fibrosis are unknown. These fundamental questions regarding fundamental EMILIN1 biology warrant further exploration.

NAFLD is a multifaceted metabolic disease, and successful treatment requires a reduction in inflammation, fibrosis, alterations in lipid metabolism and may require systemic changes to promote whole-body metabolic improvements. There is currently no approved drug to treat NAFLD and hepatic fibrosis and identifying proteins that can reduce NAFLD severity is of paramount importance [6; 96]. Our hepatokine screens and *in vivo* studies shows that SDC4 is a novel inhibitor of fatty acid uptake and dampens inflammation, while EMILIN1 is an inhibitor of hepatic fibrosis. Moreover, both proteins independently reduce NAFLD severity by different approaches, but neither completely resolves the disease. In the case of EMILIN1, the CHOL dietary model, that is a model of NASH but not fibrosis, demonstrated that EMILIN1 did not impact lipid accumulation, hepatic lipid, or glucose metabolism, markers of inflammation or hepatic fibrosis. Indeed, this model does induce hyperinsulinemia and dyslipidaemia that was not altered with *Emilin1*-AAV. While in mice fed the MCD diet exhibited *Emilin1*-AAV reduced hepatic fibrosis without altering inflammatory gene expression in the MCD model. Collectively, these findings suggest that EMILIN1 is working downstream of inflammatory signalling and reduces the fibrosis that develops as a consequence to

NAFLD-associated inflammation. In a similar manner, overexpression of hepatic Syndecan-4 did not alter dyslipidaemia or insulinemia but did reduce macrosteatosis, which was associated with reduced inflammatory and fibrotic gene expression. Given that Sdc4-AAV was administered after NAFLD was already induced, it suggests when coupled with the reduction in hepatic fatty acid uptake findings, that it, like EMILIN1, acts following induction of NAFLD to reduce NAFLD severity but is not sufficient to alleviate the disease without pharmacological intervention. Identifying one target that underpins the NAFLD and fibrosis network without extensive side effects may be naïve and has led to the view that combination therapies to reduce hepatic lipid accumulation, inflammation, and fibrosis are required for effective NAFLD treatment [455; 456]. While one could suggest a double AAV approach to overexpress SDC4 and EMILIN1 to synergise NAFLD therapies to reduce hepatic macrosteatosis, inflammation, and fibrosis with potential improvements in insulin secretion (Chapter 4 & 5), we may be inhibited by lack of readily available measures to detect these secreted proteins in the circulation, mechanisms and the extensive clinical translation required to develop novel pharmaceuticals. Perhaps a more readily translational approach would be the combination treatment of EMILIN1 and firsocostat, also termed GS-0976. Firsocostat is an extensively studied liver-specific acetyl-CoA carboxylase inhibitor that reduces DNL and increases β -oxidation to reduce hepatic steatosis effectively [457-462] and is currently being investigated as a combination therapy with several small molecules [463; 464] to overcome the effect of hypertriglyceridemia associated with Firsocostat monotherapy. While improvements in human hepatic fibrosis were not assessed in the most recent clinical trial [464], co-administration of firsocostat and DGAT2 inhibitor reduced hepatic fibrosis, *Acta2* and *Colla1* expression in rats fed a choline-deficient, amino acid-defined, high-fat diet [464]. Perhaps a combination of Firsocostat and

Emilin1 overexpression could successfully resolve end stage NAFLD with advanced fibrosis. On the other hand, combination of FirscoStat and SDC4 may resolve hepatic steatosis by reducing fatty acid uptake and storage, DNL, and increasing fatty acid oxidation. Such possibilities warrant further investigation.

The third primary aim of this thesis was to assess whether hepatokine secretion was altered following exercise in obese mice with NAFLD. This work was informed by previous studies assessing the secreted proteome from hepatocytes isolated from high fat-fed mice and mice with NASH [5; 11] that demonstrated marked remodelling of classically secreted proteins and led to the discovery of Fetuin B that impairs glucose tolerance [11], and SMOC1 and ARSA that improve glycaemic control and insulin sensitivity in mice [5; 288]. We reasoned that because exercise is an effective treatment for NAFLD and NAFLD comorbidities, that exercise training might remodel the hepatocyte intracellular and secreted proteome and such changes might positively regulate lipid and glucose metabolism. Reports on how exercise impacts liver metabolism are well described [373; 395; 396]. However, whether exercise training alters the hepatocyte intracellular and secreted proteome remained to be elucidated [337-340; 396]. We identified marked remodelling of the hepatocyte intracellular and secreted proteins with exercise training, and the metabolic effect of the exercise-trained secreted medium on isolated tissues *ex vivo* revealed marked improvements in insulin sensitivity in skeletal muscle, reduction in basal hepatic glucose output and increased fatty acid oxidation, effects that are consistent with the known beneficial effects of exercise training [371; 465-467]. Congruent with our findings, studies using the inverse approach, that is assessing the effects of the hepatocyte secreted medium isolated from obese untrained mice, showed markedly reduced insulin-stimulated glucose uptake in

cultured myotubes [11], suggesting that exercise training remodelling liver secreted factors to prevent the reduction in insulin-stimulated glucose uptake. A central unanswered question of this body of work is how is the liver informed of exercise? It may be communicated in combination by cortisol and adrenaline, exercise responsive myokines [468], muscle-derived extracellular vesicles [172] and or by the central nervous system [469] to orchestrate transcription factor remodelling and subsequent changes in protein expression and secretion. Do the lack of these extra-hepatic signals contribute the mismatch in protein synthesis and secretion (Chapter 6) or are there negative feedback mechanisms mediated by extracellular proteins that regulate their intracellular synthesis? However, despite these unanswered questions, we reinforce the notion that the liver adapts to chronic exercise training and understanding of the exercise-induced adaptations can be harnessed to develop novel therapies.

In conclusion, the findings in this thesis provide insight into the divergent functions of human and exercise-induced hepatokines and how they can be used to understand biology and for clinical gain. The field currently lacks a non-invasive method to diagnose or stratify patients with NAFLD and NASH, and there are no approved treatment options to resolve the disease. To address this, we used two ‘omics’ approaches to identify NASH and NAFLD F2-3 biomarkers, demonstrating remodelling of the liver-secreted and plasma proteome. The liver secreted proteome identified EMILIN1 which was not a biomarker for NASH but may be an effective anti-fibrotic for patients with NASH. Plasma proteomic analysis identified a panel of biomarkers that perform better than current non-invasive scores for the stratification of NAFLD F2-3. Future investigations should combine these values with clinical parameters to develop more sensitive and reliable non-invasive scores to diagnose NASH and NAFLD-

associated fibrosis. Lastly, exercise-training remodels hepatocyte protein secretion, and from this, we identified SDC4, a novel regulator of hepatic fatty acid uptake and inflammation. Further study is required to define the molecular mechanisms of how SDC4 and EMILIN1 proteins exert their phenotypic functions. Together, these findings highlight the therapeutic potential of exploiting hepatokines for clinical gain. This body of work supports further interrogation of novel hepatokines and their impact on NAFLD and metabolic co-morbidities.

Chapter 8: Supplementary information

8.1. Supplementary figures

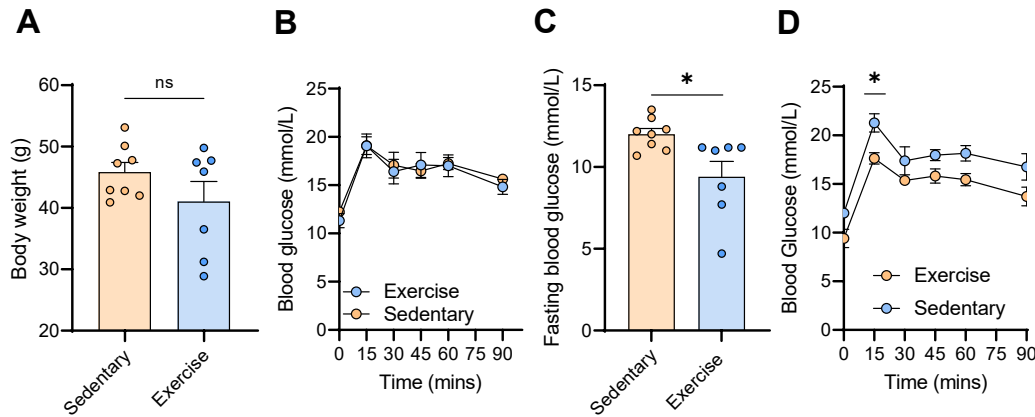


Figure 8.1: Grouping of exercise and sedentary mice.(A) Prior to exercise or sedentary allocation, mice were matched for body weight and (B) glucose tolerance following oral glucose administration (2 g/kg). (C) Fasting blood glucose following exercise training. (D) glucose tolerance following oral glucose administration (2 g/kg) and the calculated. Data are presented as mean $\pm$ SEM, with $n=7-8$ per group. * $P<0.05$ vs. Sedentary. Significance was assessed by two-tailed unpaired t-test (panel A), or two-way ANOVA followed by Bonferroni's post hoc test (panel B). Ns, not significant.

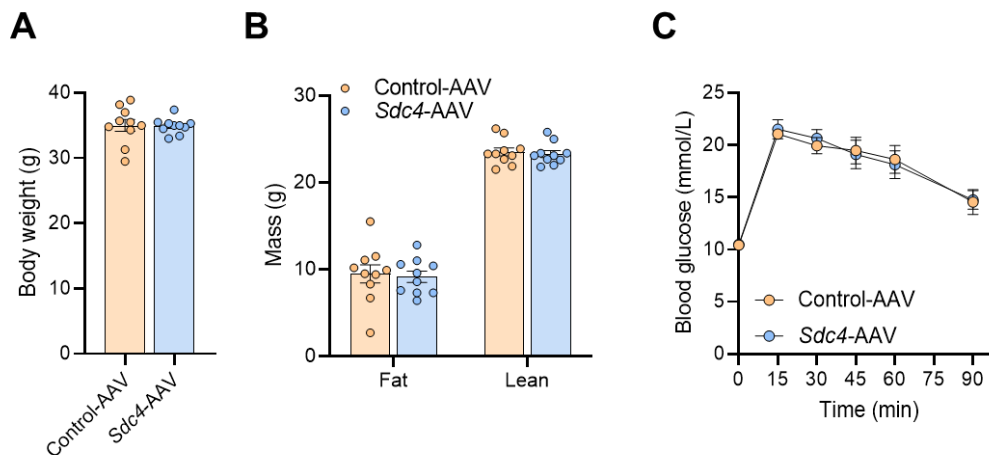


Figure 8.2: Grouping of Sdc4-AAV and Control-AAV mice.(A) Prior to *Sdc4*-AAV or Control-AAV allocation, mice were grouped based on body weight (B) lean and fat mass and (C) glucose tolerance. Data are presented as mean $\pm$ SEM, with $n=10$ per group. * $P<0.05$ vs. Control-AAV. Significance was assessed by students two tailed unpaired t-test (panels A, B) or two-way ANOVA followed by Bonferroni's post hoc test (panel C).

8.2. Abbreviations

Table 8.1. Abbreviations

Abbreviation	Definition
2DG	2-deoxyglucose
AAV	Adeno-associated virus
ACAA2	Acetyl-CoA transferase
ACC	Acetyl-CoA transferase
ACN	Acetonitrile
ACOX1	Acyl-CoA oxidase 1
ACTA2	Actin alpha 2
AFM	Afamin
AGC	Automatic gain control
ALB	Albumin
ALDH2	Aldehyde dehydrogenase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ANXA4	Annexin 4
AP1	Activator protein
APCS	Amyloid P component
APOF	Apolipoprotein F
APRI	Aspartate aminotransferase to platelet ratio index
ARSA	Arylsulfatase A
ASM	Acid-soluble metabolites
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUROC	Area under the receiver operating curve
BARD	BMI, AST/ALT ratio, T2D
BCA	Bicinchoninic acid assay
BMI	Body mass index
BSA	Bovine serum albumin
CAV1	Caveolin-1
CCDC51	Coiled-coil domain containing 51
CCL2	C–C motif chemokine ligand 2
CD36	Fatty acid translocase
CD5L	CD5 molecule like
CHOL	High-fat, high-fructose, high-cholesterol diet
ChREBP	Carbohydrate response element-binding protein
CID	collision induced dissociation
Col1a1	collagens collagen type one alpha one chain

Col1a2	collagens collagen type one alpha two chain
Col3a1	collagens collagen type three alpha one chain
CPS	Carbamoyl-phosphate synthase 1
CPT1	Carnitine O-palmitoyltransferase 1
CRN	Clinical research network
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DGAT2	Diacylglycerol O-acyltransferase 2
DIA	Data-independent acquisition mode
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNL	De novo lipogenesis
DPP4	Dipeptidyl peptidase-4
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIF3K	Eukaryotic translation initiation factor 3 subunit K
EIF4G2	Eukaryotic translation initiation factor 4 gamma 2
ELF	Enhanced liver fibrosis
EMILIN1	Elastin microfibril interfacer 1
ER	Endoplasmic reticulum
ESI	Electrospray Ionization
eWat	Epididymal adipose tissue
F13A1	Coagulation factor XIII alpha chain
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FABP4	Fatty acid binding protein
FAS	Fatty acid synthase
FATP	Fatty acid transporter protein
FBS	Foetal bovine serum
FCN3	Ficolin 3
FFA	Free fatty acid
FGF21	Fibroblast growth factor 21
FIB4	Fibrosis-4
GGT	Gamma-glutamyl transferase
GORD	Gastro-oesophageal reflux disease
H&E	Hematoxylin and eosin
HAO1	Hydroxyacid oxidase 1
HbA1c	Glycosylated haemoglobin
HBSS	Hank's Buffered Salt Solution
HCC	Hepatocellular carcinoma
HCD	Higher-energy C-trap dissociation
HDL	High-density lipoprotein

HEXA	Hexosaminidase A
HFD	High fat diet
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine Guanine Phosphoribosyltransferase
HSC	Hepatic stellate cell
HSPG2	Perlecan
IGKV2-28	Immunoglobulin kappa variable 2-28
IHTG	Intrahepatic triglyceride content
IL	Interleukin
Il1b	Interleukin one beta
IL-1r	Interleukin one receptor
IPA	Ingenuity pathway analysis
IR	Insulin resistance
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LFQ	Label free quantitation
LLC	Large latent complex
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTF	Lactotransferrin
M199	Medium 199
MAPK	Mitogen-activated protein kinase
MASP1	Mannan-binding lectin serine protease
MCD	Methionine and choline deficient
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCS	Methionine choline supplemented
MCV	Mean corpuscular volume
MMP	Matrix metalloprotease
mtDNA	Mitochondrial DNA
NAA15	N-alpha-acetyltransferase 15
NADH	Nicotinamide adenine
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NCE	Normalised collision energy
NFkB	Nuclear factor kappa- B
NFS	NAFLD fibrosis score
NK	Natural killer
NRF2	Nuclear factor-erythroid factor 2-related factor 2
OSA	Obstructive sleep apnoea

OXPHOS	Oxidative phosphorylation complex
P/S	Penicillin-streptomycin
PA/OA/LinA	Palmitic acid, oleic acid, linoleic acid
PBS	Phosphate buffered saline
PGC1a	Proliferator-activated receptor- γ coactivator-1 α
PKC	Protein kinase C
PPAR	Peroxisome proliferator activated receptor
PTK2	Protein tyrosine kinase 2
PUMA	p53 upregulated modulator of apoptosis
PZP	Pregnancy zone protein
RARRES2	Chimerin
RBP4	Retinol-binding protein 4
RER	Respiratory exchange ratio
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
RIPA	Radioimmunoprecipitation assay
ROC	Receiver operating curve
ROS	Reactive oxygen species
rSDC4	Recombinant syndecan-4
SD	Standard deviation
SDC	Syndecan
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
Sk. Muscle	Skeletal muscle
SLC	Small latent complex
SMOC1	SPARC Related Modular Calcium Binding 1
SP1	Specificity protein 1
SREBP-1c	Sterol response element-binding protein 1c
T2D	Type 2 diabetes
TBG	Thyroxine binding globulin
TCA	Tricarboxylic acid cycle
TCEP	Tris(2-carboxyethyl) phosphine
TFA	Trifluoroacetic acid
TG	Triglyceride
Tgfb1	Transforming growth factor beta 1
TGFBI	Transforming growth factor beta induced protein
TGFBRII	Transforming growth factor beta 1 receptor 2
TIMP	Tissue inhibitor of metalloprotease
TLC	Thin layer chromatography
TLR	Toll like receptor
TNFa	Tumour necrosis factor a
TSH	Thyroid-stimulating hormone

UHPLC	Ultra-high performance liquid chromatography
USA	United states of America
VCO2	Volume of carbon dioxide
VO2	Volume of oxygen
WCC	White cell count

Chapter 9: References:

- [1] Montgomery, M.K., De Nardo, W., Watt, M.J., 2019. Impact of Lipotoxicity on Tissue “Cross-Talk” and Metabolic Regulation. *Physiology* 34(2):134-149.
- [2] Watt, M.J., Miotto, P.M., De Nardo, W., Montgomery, M.K., 2019. The Liver as an Endocrine Organ—Linking NAFLD and Insulin Resistance. *Endocrine Reviews* 40(5):1367-1393.
- [3] Keenan, S.N., De Nardo, W., Lou, J., Schittenhelm, R.B., Montgomery, M.K., Granneman, J.G., et al., 2021. Perilipin 5 S155 phosphorylation by PKA is required for the control of hepatic lipid metabolism and glycemic control. *Journal of Lipid Research* 62:100016-100016.
- [4] Watt, M., Bayliss, J., Ooi, G., De Nardo, W., Shah, Y., Montgomery, M., et al., 2021. Ectodysplasin A is increased in nonalcoholic fatty liver disease but is not associated with type 2 diabetes. *Frontiers in Endocrinology*
- [5] Montgomery, M.K., Bayliss, J., Nie, S., De Nardo, W., Keenan, S.N., Miotto, P.M., et al., 2022. Deep proteomic profiling unveils arylsulfatase A as a non-alcoholic steatohepatitis inducible hepatokine and regulator of glycemic control. *Nature Communications* 13(1):1259.
- [6] Chalasani, N., Younossi, Z., Lavine, J.E., Diehl, A.M., Brunt, E.M., Cusi, K., et al., 2012. The Diagnosis and Management of Non-alcoholic Fatty Liver Disease: Practice Guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 142(7):1592-1609.
- [7] Loomba, R., Sanyal, A.J., 2013. The global NAFLD epidemic. *Nature Reviews Gastroenterology & Hepatology* 10:686.
- [8] Adams, L.A., Roberts, S.K., Strasser, S.I., Mahady, S.E., Powell, E., Estes, C., et al., 2020. Nonalcoholic fatty liver disease burden: Australia, 2019-2030. *J Gastroenterol Hepatol* 35(9):1628-1635.
- [9] Estes, C., Razavi, H., Loomba, R., Younossi, Z., Sanyal Arun, J., 2017. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. *Hepatology* 67(1):123-133.
- [10] Estes, C., Anstee, Q.M., Arias-Loste, M.T., Bantel, H., Bellentani, S., Caballeria, J., et al., 2018. Modeling NAFLD disease burden in China, France, Germany, Italy, Japan, Spain, United Kingdom, and United States for the period 2016–2030. *Journal of Hepatology* 69(4):896-904.
- [11] Meex, Ruth C., Hoy, Andrew J., Morris, A., Brown, Russell D., Lo, Jennifer C.Y., Burke, M., et al., 2015. Fetuin B Is a Secreted Hepatocyte Factor Linking Steatosis to Impaired Glucose Metabolism. *Cell metabolism* 22(6):1078-1089.
- [12] Seppälä-Lindroos, A., Vehkavaara, S., Häkkinen, A.-M., Goto, T., Westerbacka, J., Sovijärvi, A., et al., 2002. Fat Accumulation in the Liver Is Associated with Defects in Insulin Suppression of Glucose Production and Serum Free Fatty Acids Independent of Obesity in Normal Men. *The Journal of Clinical Endocrinology & Metabolism* 87(7):3023-3028.
- [13] Caldwell, S., Ikura, Y., Dias, D., Isomoto, K., Yabu, A., Moskaluk, C., et al., 2010. Hepatocellular ballooning in NASH. *Journal of Hepatology* 53(4):719-723.
- [14] Rinella, M.E., 2015. Nonalcoholic fatty liver disease: A systematic review. *JAMA* 313(22):2263-2273.
- [15] Hardy, T., Oakley, F., Anstee, Q.M., Day, C.P., 2016. Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum. *Annual Review of Pathology: Mechanisms of Disease* 11(1):451-496.

- [16] Global Burden of Disease Cancer, C., 2017. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: A systematic analysis for the global burden of disease study. *JAMA Oncology* 3(4):524-548.
- [17] Ludwig J, V.T., McGill DB, Oh BJ., 1980. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 55(7):434-438.
- [18] Leung, C., Rivera, L., Furness, J.B., Angus, P.W., 2016. The role of the gut microbiota in NAFLD. *Nature Reviews Gastroenterology & Hepatology* 13:412.
- [19] Younossi, Z.M., Koenig, A.B., Abdelatif, D., Fazel, Y., Henry, L., Wymer, M., 2016. Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64(1):73-84.
- [20] Economics, D.A., 2013. The economic cost and health burden of liver diseases in Australia.
- [21] Abarca-Gómez, L., Abdeen, Z.A., Hamid, Z.A., Abu-Rmeileh, N.M., Acosta-Cazares, B., Acuin, C., et al., 2017. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *The Lancet* 390(10113):2627-2642.
- [22] Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., et al., 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet* 384(9945):766-781.
- [23] Oddy, W.H., Herbison, C.E., Jacoby, P., Ambrosini, G.L., O'Sullivan, T.A., Ayonrinde, O.T., et al., 2013. The Western Dietary Pattern Is Prospectively Associated With Nonalcoholic Fatty Liver Disease in Adolescence. *The American Journal Of Gastroenterology* 108:778.
- [24] Ooi, G.J., Burton, P.R., Earnest, A., Laurie, C., Kemp, W.W., Nottle, P.D., et al., 2018. Visual Liver Score to Stratify Non-Alcoholic Steatohepatitis Risk and Determine Selective Intraoperative Liver Biopsy in Obesity. *Obes Surg* 28(2):427-436.
- [25] Ooi, G.J., Burton, P.R., Bayliss, J., Raajendiran, A., Earnest, A., Laurie, C., et al., 2019. Effect of Body Mass Index, Metabolic Health and Adipose Tissue Inflammation on the Severity of Non-alcoholic Fatty Liver Disease in Bariatric Surgical Patients: a Prospective Study. *Obes Surg* 29(1):99-108.
- [26] Farrell, G.C., Wong, V.W.-S., Chitturi, S., 2013. NAFLD in Asia—as common and important as in the West. *Nature Reviews Gastroenterology & Hepatology* 10:307.
- [27] Das, K., Das, K., Mukherjee Partha, S., Ghosh, A., Ghosh, S., Mridha Asit, R., et al., 2010. Nonobese population in a developing country has a high prevalence of nonalcoholic fatty liver and significant liver disease. *Hepatology* 51(5):1593-1602.
- [28] Lazo, M., Clark, J., 2008. The Epidemiology of Nonalcoholic Fatty Liver Disease: A Global Perspective. *Seminars in Liver Disease* 28 (4):339-350.
- [29] Welsh, J.A., Karpen, S., Vos, M.B., 2013. Increasing Prevalence of Nonalcoholic Fatty Liver Disease Among United States Adolescents, 1988–1994 to 2007–2010. *The Journal of pediatrics* 162(3):496-500.e491.
- [30] ABS, 2015. 4364.0.55.001—National Health Survey: First Results, 2014-2015.
- [31] Mercedes de Onis, Monika Blössner, Borghi, E., 2010. Global prevalence and trends of overweight and obesity among preschool children. *American Journal of Clinical Nutrition* 92:1257-1264.
- [32] Singh, S., Allen, A.M., Wang, Z., Prokop, L.J., Murad, M.H., Loomba, R., 2015. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a

- systematic review and meta-analysis of paired-biopsy studies. *Clin Gastroenterol Hepatol* 13(4):643-e640.
- [33] Hagstrom, H., Nasr, P., Ekstedt, M., Hammar, U., Stal, P., Hultcrantz, R., et al., 2017. Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J Hepatol* 67(6):1265-1273.
- [34] Lazo, M., Hernaez, R., Eberhardt, M.S., Bonekamp, S., Kamel, I., Guallar, E., et al., 2013. Prevalence of Nonalcoholic Fatty Liver Disease in the United States: The Third National Health and Nutrition Examination Survey, 1988–1994. *American Journal of Epidemiology* 178(1):38-45.
- [35] Ratzliff, V., Voiculescu, M., Poynard, T., Touching some firm ground in the epidemiology of NASH. *Journal of Hepatology* 56(1):23-25.
- [36] Musso, G., Gambino, R., Cassader, M., Pagano, G., 2011. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 43(8):617-649.
- [37] Portillo-Sanchez, P., Bril, F., Maximos, M., Lomonaco, R., Biernacki, D., Orsak, B., et al., 2015. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. *J Clin Endocrinol Metab* 100(6):2231-2238.
- [38] Ortiz-Lopez, C., Lomonaco, R., Orsak, B., Finch, J., Chang, Z., Kochunov, V.G., et al., 2012. Prevalence of Prediabetes and Diabetes and Metabolic Profile of Patients With Nonalcoholic Fatty Liver Disease (NAFLD). *Diabetes Care* 35(4):873.
- [39] Masarone, M., Rosato, V., Aglitti, A., Bucci, T., Caruso, R., Salvatore, T., et al., 2017. Liver biopsy in type 2 diabetes mellitus: Steatohepatitis represents the sole feature of liver damage. *PLoS ONE* 12(6):e0178473-e0178473.
- [40] Bugianesi, E., Gastaldelli, A., Vanni, E., Gambino, R., Cassader, M., Baldi, S., et al., 2005. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* 48(4):634-642.
- [41] Korenblat, K.M., Fabbrini, E., Mohammed, B.S., Klein, S., 2008. Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* 134(5):1369-1375.
- [42] Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., et al., 2001. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 50(8):1844-1850.
- [43] Kraegen, E.W., Clark, P.W., Jenkins, A.B., Daley, E.A., Chisholm, D.J., Storlien, L.H., 1991. Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40(11):1397-1403.
- [44] Turner, N., Kowalski, G.M., Leslie, S.J., Risis, S., Yang, C., Lee-Young, R.S., et al., 2013. Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. *Diabetologia* 56(7):1638-1648.
- [45] van Herpen, N.A., Schrauwen-Hinderling, V.B., Schaart, G., Mensink, R.P., Schrauwen, P., 2011. Three Weeks on a High-Fat Diet Increases Intrahepatic Lipid Accumulation and Decreases Metabolic Flexibility in Healthy Overweight Men. *The Journal of Clinical Endocrinology & Metabolism* 96(4):E691-E695.
- [46] María del Carmen Manzano-Robleda, Beatriz Barranco-Fragoso, Misael Uribe, Méndez-Sánchez, N., 2015. Portal vein thrombosis: What is new? *Annals of Hepatology* 14(1):20-27.
- [47] Madry, K., Lut, W., Lepert, R., Borkowska, M. & Papiernik, E, 1976. Lipid composition of plasma obtained from various parts of the vascular system of the rabbit. *Acta Physiol. Pol* 27:485-492.

- [48] Lindeboom, L., de Graaf, R.A., Nabuurs, C.I., van Ewijk, P.A., Hesselink, M.K.C., Wildberger, J.E., et al., 2016. Quantum coherence spectroscopy to measure dietary fat retention in the liver. *JCI Insight* 1(13):e84671.
- [49] Del Prato, S., Enzi, G., Vigili de Kreutzenberg, S., Lisato, G., Riccio, A., Maifreni, L., et al., 1990. Insulin regulation of glucose and lipid metabolism in massive obesity. *Diabetologia* 33(4):228-236.
- [50] Lindeboom, L., Nabuurs, C.I., Hesselink, M.K., Wildberger, J.E., Schrauwen, P., Schrauwen-Hinderling, V.B., 2015. Proton magnetic resonance spectroscopy reveals increased hepatic lipid content after a single high-fat meal with no additional modulation by added protein. *Am J Clin Nutr* 101(1):65-71.
- [51] Silverstein, R.L., Febbraio, M., 2009. CD36, a Scavenger Receptor Involved in Immunity, Metabolism, Angiogenesis, and Behavior. *Science signaling* 2(72):re3-re3.
- [52] Petersen, K.F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R.E., Shulman, G.I., 2005. Reversal of Nonalcoholic Hepatic Steatosis, Hepatic Insulin Resistance, and Hyperglycemia by Moderate Weight Reduction in Patients With Type 2 Diabetes. *Diabetes* 54(3):603-608.
- [53] Marra, F., Lotersztajn, S., 2013. Pathophysiology of NASH: perspectives for a targeted treatment. *Current Pharmaceutical Design* 19(29):5250-5269.
- [54] Raajendiran, A., Tsiloulis, T., Watt, M.J., 2016. Adipose tissue development and the molecular regulation of lipid metabolism. *Essays Biochem* 60(5):437-450.
- [55] Laurencikiene, J., Skurk, T., Kulyte, A., Heden, P., Astrom, G., Sjolín, E., et al., 2011. Regulation of lipolysis in small and large fat cells of the same subject. *J Clin Endocrinol Metab* 96(12):E2045-2049.
- [56] Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., et al., 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112(12):1821-1830.
- [57] Seki, E., Brenner, D.A., Karin, M., 2012. A Liver Full of JNK: Signaling in Regulation of Cell Function and Disease Pathogenesis, and Clinical Approaches. *Gastroenterology* 143(2):307-320.
- [58] Zeyda, M., Stulnig, T.M., 2009. Obesity, Inflammation, and Insulin Resistance – A Mini-Review. *Gerontology* 55(4):379-386.
- [59] Meex, R.C.R., Watt, M.J., 2017. Hepatokines: linking nonalcoholic fatty liver disease and insulin resistance. *Nature Reviews Endocrinology* 13:509.
- [60] Arner, P., Langin, D., Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance. *Trends in Endocrinology & Metabolism* 25(5):255-262.
- [61] Donnelly, K.L., Smith, C.I., Schwarzenberg, S.J., Jessurun, J., Boldt, M.D., Parks, E.J., 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *Journal of Clinical Investigation* 115(5):1343-1351.
- [62] Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., et al., Relationship Between Hepatic/Visceral Fat and Hepatic Insulin Resistance in Nondiabetic and Type 2 Diabetic Subjects. *Gastroenterology* 133(2):496-506.
- [63] McQuaid, S.E., Hodson, L., Neville, M.J., Dennis, A.L., Cheeseman, J., Humphreys, S.M., et al., 2011. Downregulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition? *Diabetes* 60(1):47-55.
- [64] Fabbrini, E., Mohammed, B.S., Magkos, F., Korenblat, K.M., Patterson, B.W., Klein, S., 2008. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology* 134(2):424-431.

- [65] Fabbrini, E., Tiemann Luecking, C., Love-Gregory, L., Okunade, A.L., Yoshino, M., Fraterrigo, G., et al., 2016. Physiological Mechanisms of Weight Gain-Induced Steatosis in People With Obesity. *Gastroenterology* 150(1):79-81.e72.
- [66] Chan, D.C., Watts, G.F., Gan, S., Wong, A.T., Ooi, E.M., Barrett, P.H., 2010. Nonalcoholic fatty liver disease as the transducer of hepatic oversecretion of very-low-density lipoprotein-apolipoprotein B-100 in obesity. *Arterioscler Thromb Vasc Biol* 30(5):1043-1050.
- [67] Enooku, K., Tsutsumi, T., Kondo, M., Fujiwara, N., Sasako, T., Shibahara, J., et al., 2020. Hepatic FATP5 expression is associated with histological progression and loss of hepatic fat in NAFLD patients. *J Gastroenterol* 55(2):227-243.
- [68] Kawano, Y., Cohen, D.E., 2013. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *Journal of gastroenterology* 48(4):434-441.
- [69] Tolwani, R.J., Hamm, D.A., Tian, L., Sharer, J.D., Vockley, J., Rinaldo, P., et al., 2005. Medium-Chain Acyl-CoA Dehydrogenase Deficiency in Gene-Targeted Mice. *PLoS Genetics* 1(2):e23.
- [70] Ibdah, J.A., Perlegas, P., Zhao, Y., Angdisen, J., Borgerink, H., Shadoan, M.K., et al., Mice Heterozygous for a Defect in Mitochondrial Trifunctional Protein Develop Hepatic Steatosis and Insulin Resistance. *Gastroenterology* 128(5):1381-1390.
- [71] Hodson, L., McQuaid, S.E., Humphreys, S.M., Milne, R., Fielding, B.A., Frayn, K.N., et al., 2010. Greater dietary fat oxidation in obese compared with lean men: an adaptive mechanism to prevent liver fat accumulation? *Am J Physiol Endocrinol Metab* 299(4):E584-592.
- [72] Schrauwen, P., Schrauwen-Hinderling, V., Hoeks, J., Hesselink, M.K.C., 2010. Mitochondrial dysfunction and lipotoxicity. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1801(3):266-271.
- [73] Rosca, M.G., Vazquez, E.J., Chen, Q., Kerner, J., Kern, T.S., Hoppel, C.L., 2012. Oxidation of fatty acids is the source of increased mitochondrial reactive oxygen species production in kidney cortical tubules in early diabetes. *Diabetes* 61(8):2074-2083.
- [74] Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., et al., 2015. Adaptation of Hepatic Mitochondrial Function in Humans with Non-Alcoholic Fatty Liver Is Lost in Steatohepatitis. *Cell metabolism* 21(5):739-746.
- [75] Sunny, N.E., Parks, E.J., Browning, J.D., Burgess, S.C., 2011. Excessive Hepatic Mitochondrial TCA Cycle and Gluconeogenesis in Humans with Nonalcoholic Fatty Liver Disease. *Cell metabolism* 14(6):804-810.
- [76] Moore, M.P., Cunningham, R.P., Meers, G.M., Johnson, S.A., Wheeler, A.A., Ganga, R.R., et al., 2022. Compromised hepatic mitochondrial fatty acid oxidation and reduced markers of mitochondrial turnover in human NAFLD. *Hepatology* n/a(n/a).
- [77] Timlin MT, P., Elizabeth J., 2005. Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am J Clin Nutr.* 81(1):35-42.
- [78] Lambert, J.E., Ramos-Roman, M.A., Browning, J.D., Parks, E.J., 2014. Increased De Novo Lipogenesis Is a Distinct Characteristic of Individuals With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 146(3):726-735.
- [79] Ameer, F., Scandiuzzi, L., Hasnain, S., Kalbacher, H., Zaidi, N., De novo lipogenesis in health and disease. *Metabolism - Clinical and Experimental* 63(7):895-902.
- [80] Iizuka, K., Bruick, R.K., Liang, G., Horton, J.D., Uyeda, K., 2004. Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well

as glycolysis. *Proceedings of the National Academy of Sciences of the United States of America* 101(19):7281.

[81] Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of Clinical Investigation* 109(9):1125-1131.

[82] Eissing, L., Scherer, T., Tödter, K., Knippschild, U., Greve, J.W., Buurman, W.A., et al., 2013. De novo lipogenesis in human fat and liver is linked to ChREBP- β and metabolic health. *Nat Commun* 4:1528.

[83] Uyeda, K., Yamashita, H., Kawaguchi, T., 2002. Carbohydrate responsive element-binding protein (ChREBP): a key regulator of glucose metabolism and fat storage. *Biochemical Pharmacology* 63(12):2075-2080.

[84] Oehler G, B.H., Matthes KJ., 1982. Hyperinsulinemia in hepatic steatosis. *International Journal of Obesity* 6(1):137-144.

[85] Ameer, F., Scandiuizzi, L., Hasnain, S., Kalbacher, H., Zaidi, N., 2014. De novo lipogenesis in health and disease. *Metabolism - Clinical and Experimental* 63(7):895-902.

[86] Schwarz JM, L.P., Dare D, Aghajanian K., 2003. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr.* 77(1):43-50.

[87] Haas, J.T., Miao, J., Chanda, D., Wang, Y., Zhao, E., Haas, M.E., et al., 2012. Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression. *Cell metabolism* 15(6):873-884.

[88] Softic, S., Cohen, D.E., Kahn, C.R., 2016. Role of Dietary Fructose and Hepatic de novo Lipogenesis in Fatty Liver Disease. *Digestive Diseases and Sciences* 61(5):1282-1293.

[89] Rhee, E.J., Lee, W.Y., Cho, Y.K., Kim, B.I., Sung, K.C., 2011. Hyperinsulinemia and the development of nonalcoholic Fatty liver disease in nondiabetic adults. *Am J Med* 124(1):69-76.

[90] Steneberg, P., Sykaras, A.G., Backlund, F., Straseviciene, J., Söderström, I., Edlund, H., 2015. Hyperinsulinemia Enhances Hepatic Expression of the Fatty Acid Transporter Cd36 and Provokes Hepatosteatosis and Hepatic Insulin Resistance. *J Biol Chem* 290(31):19034-19043.

[91] Peiris, A.N., Mueller, R.A., Smith, G.A., Struve, M.F., Kissebah, A.H., 1986. Splanchnic insulin metabolism in obesity. Influence of body fat distribution. *J Clin Invest* 78(6):1648-1657.

[92] Bril, F., Lomonaco, R., Orsak, B., Ortiz-Lopez, C., Webb, A., Tio, F., et al., 2014. Relationship between disease severity, hyperinsulinemia, and impaired insulin clearance in patients with nonalcoholic steatohepatitis. *Hepatology* 59(6):2178-2187.

[93] Marques-Lopes I, Ansorena D, Astiasaran I, Forga L, JA., M., 2001. Postprandial de novo lipogenesis and metabolic changes induced by a high-carbohydrate, low-fat meal in lean and overweight men. *Am J Clin Nutr.* 73(2):253-261.

[94] Wilke, M.S., French, M.A., Goh, Y.K., Ryan, E.A., Jones, P.J., Clandinin, M.T., 2009. Synthesis of specific fatty acids contributes to VLDL-triacylglycerol composition in humans with and without type 2 diabetes. *Diabetologia* 52(8):1628-1637.

[95] Vedala, A., Wang, W., Neese, R.A., Christiansen, M.P., Hellerstein, M.K., 2006. Delayed secretory pathway contributions to VLDL-triglycerides from plasma NEFA, diet, and de novo lipogenesis in humans. *Journal of Lipid Research* 47(11):2562-2574.

[96] Chalasani, N., Younossi, Z., Lavine, J.E., Charlton, M., Cusi, K., Rinella, M., et al., 2017. The diagnosis and management of nonalcoholic fatty liver disease: Practice

- guidance from the American Association for the Study of Liver Diseases. *Hepatology* 67(1):328-357.
- [97] Harrison, S.A., Oliver, D., Arnold, H.L., Gogia, S., Neuschwander-Tetri, B.A., 2008. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. *Gut* 57(10):1441.
- [98] Dixon JB, Bhathal PS, PE., O.B., 2001. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* 121(1):91-100.
- [99] Toshikuni, N., Tsuchishima, M., Fukumura, A., Arisawa, T., Tsutsumi, M., 2017. Associations of Fatty Liver Disease with Hypertension, Diabetes, and Dyslipidemia: Comparison between Alcoholic and Nonalcoholic Steatohepatitis. *Gastroenterology Research and Practice* 2017:9127847.
- [100] Eckardt, K., Taube, A., Eckel, J., 2011. Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity. *Rev Endocr Metab Disord* 12(3):163-172.
- [101] Morigny, P., Houssier, M., Mouisel, E., Langin, D., 2016. Adipocyte lipolysis and insulin resistance. *Biochimie* 125:259-266.
- [102] Unger, R.H., Orci, L., 2002. Lipoapoptosis: its mechanism and its diseases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1585(2):202-212.
- [103] Lee, J.Y., Sohn, K.H., Rhee, S.H., Hwang, D., 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J Biol Chem* 276(20):16683-16689.
- [104] Weber, K., Schilling, J.D., 2014. Lysosomes integrate metabolic-inflammatory cross-talk in primary macrophage inflammasome activation. *J Biol Chem* 289(13):9158-9171.
- [105] Harte, A.L., da Silva, N.F., Creely, S.J., McGee, K.C., Billyard, T., Youssef-Elabd, E.M., et al., 2010. Elevated endotoxin levels in non-alcoholic fatty liver disease. *Journal of Inflammation* 7(1):15.
- [106] Kakisaka, K., Cazanave, S.C., Fingas, C.D., Guicciardi, M.E., Bronk, S.F., Werneburg, N.W., et al., 2012. Mechanisms of lysophosphatidylcholine-induced hepatocyte lipoapoptosis. *Am J Physiol Gastrointest Liver Physiol* 302(1):G77-84.
- [107] Han, M.S., Park, S.Y., Shinzawa, K., Kim, S., Chung, K.W., Lee, J.H., et al., 2008. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. *J Lipid Res* 49(1):84-97.
- [108] Fricker, M., O'Prey, J., Tolkovsky, A.M., Ryan, K.M., 2010. Phosphorylation of Puma modulates its apoptotic function by regulating protein stability. *Cell death & disease* 1(7):e59-e59.
- [109] Tavares De Almeida, I., Cortez-Pinto, H., Fidalgo, G., Rodrigues, D., Camilo, M.E., 2002. Plasma total and free fatty acids composition in human non-alcoholic steatohepatitis. *Clinical Nutrition* 21(3):219-223.
- [110] Lehmann, R., Franken, H., Dammeier, S., Rosenbaum, L., Kantartzis, K., Peter, A., et al., 2013. Circulating lysophosphatidylcholines are markers of a metabolically benign nonalcoholic fatty liver. *Diabetes Care* 36(8):2331-2338.
- [111] ter Horst, K.W., Gilijamse, P.W., Versteeg, R.I., Ackermans, M.T., Nederveen, A.J., la Fleur, S.E., et al., 2017. Hepatic Diacylglycerol-Associated Protein Kinase Cε Translocation Links Hepatic Steatosis to Hepatic Insulin Resistance in Humans. *Cell reports* 19(10):1997-2004.

- [112] Jornayvaz, F.R., Birkenfeld, A.L., Jureczak, M.J., Kanda, S., Guigni, B.A., Jiang, D.C., et al., 2011. Hepatic insulin resistance in mice with hepatic overexpression of diacylglycerol acyltransferase 2. *Proc Natl Acad Sci U S A* 108(14):5748-5752.
- [113] Petersen, M.C., Madiraju, A.K., Gassaway, B.M., Marcel, M., Nasiri, A.R., Butrico, G., et al., 2016. Insulin receptor Thr1160 phosphorylation mediates lipid-induced hepatic insulin resistance. *J Clin Invest* 126(11):4361-4371.
- [114] Samuel, V.T., Liu, Z.X., Wang, A., Beddow, S.A., Geisler, J.G., Kahn, M., et al., 2007. Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *J Clin Invest* 117(3):739-745.
- [115] Turpin, S.M., Nicholls, H.T., Willmes, D.M., Mourier, A., Brodesser, S., Wunderlich, C.M., et al., 2014. Obesity-induced CerS6-dependent C16:0 ceramide production promotes weight gain and glucose intolerance. *Cell Metab* 20(4):678-686.
- [116] Montgomery, M.K., Brown, S.H., Lim, X.Y., Fiveash, C.E., Osborne, B., Bentley, N.L., et al., 2016. Regulation of glucose homeostasis and insulin action by ceramide acyl-chain length: A beneficial role for very long-chain sphingolipid species. *Biochim Biophys Acta* 1861(11):1828-1839.
- [117] Ashraf, N.U., Sheikh, T.A., 2015. Endoplasmic reticulum stress and Oxidative stress in the pathogenesis of Non-alcoholic fatty liver disease. *Free Radic Res* 49(12):1405-1418.
- [118] Holland, W.L., Brozinick, J.T., Wang, L.P., Hawkins, E.D., Sargent, K.M., Liu, Y., et al., 2007. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab* 5(3):167-179.
- [119] Martinez, L., Torres, S., Baulies, A., Alarcon-Vila, C., Elena, M., Fabrias, G., et al., 2015. Myristic acid potentiates palmitic acid-induced lipotoxicity and steatohepatitis associated with lipodystrophy by sustaining de novo ceramide synthesis. *Oncotarget* 6(39):41479-41496.
- [120] Ooi, G.J., Meikle, P.J., Huynh, K., Earnest, A., Roberts, S.K., Kemp, W., et al., 2021. Hepatic lipidomic remodeling in severe obesity manifests with steatosis and does not evolve with non-alcoholic steatohepatitis. *Journal of Hepatology* 75(3):524-535.
- [121] Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al., 2009. A core gut microbiome in obese and lean twins. *Nature* 457(7228):480-484.
- [122] Lau, E., Carvalho, D., Freitas, P., 2015. Gut Microbiota: Association with NAFLD and Metabolic Disturbances. *BioMed research international* 2015:979515-979515.
- [123] Zhu, L., Baker, S.S., Gill, C., Liu, W., Alkhouri, R., Baker, R.D., et al., 2013. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology* 57(2):601-609.
- [124] Jiang, W., Wu, N., Wang, X., Chi, Y., Zhang, Y., Qiu, X., et al., 2015. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Scientific Reports* 5:8096.
- [125] Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., Gusovsky, F., 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274(16):10689-10692.
- [126] Latz, E., Xiao, T.S., Stutz, A., 2013. Activation and regulation of the inflammasomes. *Nature reviews. Immunology* 13(6):397-411.
- [127] Stienstra, R., Saudale, F., Duval, C., Keshtkar, S., Groener, J.E., van Rooijen, N., et al., 2010. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. *Hepatology* 51(2):511-522.

- [128] Miura, K., Kodama, Y., Inokuchi, S., Schnabl, B., Aoyama, T., Ohnishi, H., et al., 2010. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1 β in mice. *Gastroenterology* 139(1):323-334.e327.
- [129] Yoshiji, H., Kuriyama, S., Miyamoto, Y., Thorgeirsson, U.P., Gomez, D.E., Kawata, M., et al., 2000. Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. *Hepatology* 32(6):1248-1254.
- [130] Garcia-Martinez, I., Santoro, N., Chen, Y., Hoque, R., Ouyang, X., Caprio, S., et al., 2016. Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of TLR9. *The Journal of Clinical Investigation* 126(3):859-864.
- [131] Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F., Lanzavecchia, A., 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6(8):769-776.
- [132] Sanyal, A.J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W.B., Contos, M.J., Sterling, R.K., et al., 2001. Nonalcoholic steatohepatitis: Association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 120(5):1183-1192.
- [133] Perez-Carreras, M., Del Hoyo, P., Martin, M.A., Rubio, J.C., Martin, A., Castellano, G., et al., 2003. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 38(4):999-1007.
- [134] Cortez-Pinto, H., Chatham, J., Chacko, V.P., Arnold, C., Rashid, A., Diehl, A.M., 1999. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* 282(17):1659-1664.
- [135] Kendrick, A.A., Choudhury, M., Rahman, S.M., McCurdy, C.E., Friederich, M., Van Hove, J.L., et al., 2011. Fatty liver is associated with reduced SIRT3 activity and mitochondrial protein hyperacetylation. *Biochem J* 433(3):505-514.
- [136] Shimada, K., Crother, T.R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., et al., 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36(3):401-414.
- [137] Chalasani, N., Younossi, Z., Lavine, J.E., Diehl, A.M., Brunt, E.M., Cusi, K., et al., 2012. The diagnosis and management of non-alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 55(6):2005-2023.
- [138] Kleiner, D.E., Berk, P.D., Hsu, J.Y., Courcoulas, A.P., Flum, D., Khandelwal, S., et al., 2014. Hepatic Pathology Among Patients Without Known Liver Disease Undergoing Bariatric Surgery: Observations and a Perspective from the Longitudinal Assessment of Bariatric Surgery (LABS) Study. *Seminars in Liver Disease* 34(1):98-107.
- [139] Vernon, G., Baranova, A., Younossi, Z.M., 2011. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 34(3):274-285.
- [140] Angulo, P., Kleiner, D.E., Dam-Larsen, S., Adams, L.A., Bjornsson, E.S., Charatcharoenwitthaya, P., et al., 2015. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 149(2):389-397.e310.
- [141] Ekstedt, M., Hagström, H., Nasr, P., Fredrikson, M., Stål, P., Kechagias, S., et al., 2015. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 61(5):1547-1554.
- [142] Farrell, G.C., van Rooyen, D., Gan, L., Chitturi, S., 2012. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. *Gut Liver* 6(2):149-171.

- [143] Buzzetti, E., Pinzani, M., Tsochatzis, E.A., The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism - Clinical and Experimental* 65(8):1038-1048.
- [144] Brunt, E.M., Kleiner, D.E., Wilson, L.A., Belt, P., Neuschwander-Tetri, B.A., 2011. The NAS and The Histopathologic Diagnosis in NAFLD: Distinct Clinicopathologic Meanings. *Hepatology (Baltimore, Md.)* 53(3):810-820.
- [145] Ooi, G.J., Mgaith, S., Eslick, G.D., Burton, P.R., Kemp, W.W., Roberts, S.K., et al., 2018. Systematic review and meta-analysis: non-invasive detection of non-alcoholic fatty liver disease related fibrosis in the obese. *Obesity Reviews* 19(2):281-294.
- [146] Rockey DC, C.S., Goodman ZD, Nelson RC, Smith, AD., 2009. Liver biopsy – AASLD position paper. *Hepatology*(49):1017–1044.
- [147] Brunt, E.M., Kleiner, D.E., Wilson, L.A., Belt, P., Neuschwander-Tetri, B.A., 2011. Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology* 53(3):810-820.
- [148] Dulai, P.S., Singh, S., Patel, J., Soni, M., Prokop, L.J., Younossi, Z., et al., 2017. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology* 65(5):1557-1565.
- [149] Vilar-Gomez, E., Calzadilla-Bertot, L., Wai-Sun Wong, V., Castellanos, M., Aller-de la Fuente, R., Metwally, M., et al., 2018. Fibrosis Severity as a Determinant of Cause-Specific Mortality in Patients With Advanced Nonalcoholic Fatty Liver Disease: A Multi-National Cohort Study. *Gastroenterology* 155(2):443-457.e417.
- [150] Wong, V.W., Adams, L.A., de Ledinghen, V., Wong, G.L., Sookoian, S., 2018. Noninvasive biomarkers in NAFLD and NASH - current progress and future promise. *Nat Rev Gastroenterol Hepatol* 15(8):461-478.
- [151] Mofrad, P., Contos, M.J., Haque, M., Sargeant, C., Fisher, R.A., Luketic, V.A., et al., 2003. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 37(6):1286-1292.
- [152] Taitano, A.A., Markow, M., Fiana, J.E., Wheeler, D.E., Gonzalvo, J.P., Murr, M.M., 2014. Bariatric surgery improves histological features of nonalcoholic fatty liver disease and liver fibrosis. *J Gastrointest Surg* 19(3):429-436.
- [153] Angulo, P., Kleiner, D.E., Dam-Larsen, S., Adams, L., Bjornsson, E.S., Charatcharoenwitthaya, P., et al., 2015. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology* 149:389-397.
- [154] Merriman, R.B., Ferrell, L.D., Patti, M.G., Weston, S.R., Pabst, M.S., Aouizerat, B.E., et al., 2006. Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. *Hepatology* 44(4):874-880.
- [155] Patel, V., Sanyal, A.J., Sterling, R., 2016. Clinical Presentation and Patient Evaluation in Nonalcoholic Fatty Liver Disease. *Clin Liver Dis* 20(2):277-292.
- [156] Rockey, D.C., Caldwell, S.H., Goodman, Z.D., Nelson, R.C., Smith, A.D., 2009. Liver biopsy. *Hepatology* 49(3):1017-1044.
- [157] Ooi, G.J., Burton, P.R., Doyle, L., Wentworth, J.M., Bhathal, P.S., Sikaris, K., et al., 2017. Modified thresholds for fibrosis risk scores in nonalcoholic fatty liver disease are necessary in the obese. *Obes Surg* 27(1):115-125.
- [158] Petta, S., Maida, M., Macaluso, F.S., Di Marco, V., Camma, C., Cabibi, D., et al., 2015. The severity of steatosis influences liver stiffness measurement in patients with nonalcoholic fatty liver disease. *Hepatology* 62(4):1101-1110.
- [159] Wilder, J., Patel, K., 2014. The clinical utility of FibroScan® as a noninvasive diagnostic test for liver disease. *Medical Devices (Auckland, N.Z.)* 7:107-114.

- [160] Imajo, K., Kessoku, T., Honda, Y., Tomeno, W., Ogawa, Y., Mawatari, H., et al., Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. *Gastroenterology* 150(3):626-637.e627.
- [161] Singh, S., Venkatesh, S.K., Loomba, R., Wang, Z., Sirlin, C., Chen, J., et al., 2016. Magnetic Resonance Elastography for Staging Liver Fibrosis in Non-alcoholic Fatty Liver Disease: A Diagnostic Accuracy Systematic Review and Individual Participant Data Pooled Analysis. *European radiology* 26(5):1431-1440.
- [162] Loomba, R., Wolfson, T., Ang, B., Hooker, J., Behling, C., Peterson, M., et al., 2014. Magnetic Resonance Elastography Predicts Advanced Fibrosis in Patients With Nonalcoholic Fatty Liver Disease: A Prospective Study. *Hepatology (Baltimore, Md.)* 60(6):1920-1928.
- [163] Naveau, S., Lamouri, K., Pourcher, G., Njiké-Nakseu, M., Ferretti, S., Courie, R., et al., 2014. The Diagnostic Accuracy of Transient Elastography for the Diagnosis of Liver Fibrosis in Bariatric Surgery Candidates with Suspected NAFLD. *Obesity Surgery* 24(10):1693-1701.
- [164] Conn, V.S., Koopman, R.J., Ruppar, T.M., Phillips, L.J., Mehr, D.R., Hafdahl, A.R., 2014. Insulin Sensitivity Following Exercise Interventions: Systematic Review and Meta-Analysis of Outcomes Among Healthy Adults. *Journal of primary care & community health* 5(3):211-222.
- [165] Orzi, L.A., Gariani, K., Oldani, G., Delaune, V., Morel, P., Toso, C., 2016. Exercise-based Interventions for Nonalcoholic Fatty Liver Disease: A Meta-analysis and Meta-regression. *Clin Gastroenterol Hepatol* 14(10):1398-1411.
- [166] Vilar-Gomez, E., Martinez-Perez, Y., Calzadilla-Bertot, L., Torres-Gonzalez, A., Gra-Oramas, B., Gonzalez-Fabian, L., et al., 2015. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. *Gastroenterology* 149(2):367-378.e365; quiz e314-365.
- [167] Cuthbertson, D.J., Shojaee-Moradie, F., Sprung, V.S., Jones, H., Pugh, C.J., Richardson, P., et al., 2016. Dissociation between exercise-induced reduction in liver fat and changes in hepatic and peripheral glucose homeostasis in obese patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)* 130(2):93-104.
- [168] Musso, G., Cassader, M., Rosina, F., Gambino, R., 2012. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. *Diabetologia* 55(4):885-904.
- [169] Oh, S., Tsujimoto, T., Kim, B., Uchida, F., Suzuki, H., Iizumi, S., et al., 2021. Weight-loss-independent benefits of exercise on liver steatosis and stiffness in Japanese men with NAFLD. *JHEP Rep* 3(3):100253.
- [170] Hallsworth, K., Fattakhova, G., Hollingsworth, K.G., Thoma, C., Moore, S., Taylor, R., et al., 2011. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut* 60(9):1278-1283.
- [171] Hawley, John A., Hargreaves, M., Joyner, Michael J., Zierath, Juleen R., 2014. Integrative Biology of Exercise. *Cell* 159(4):738-749.
- [172] Whitham, M., Parker, B.L., Friedrichsen, M., Hingst, J.R., Hjorth, M., Hughes, W.E., et al., 2018. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell metabolism* 27(1):237-251.e234.
- [173] Chen, L., Yang, Y., Wang, Y., Qiu, L., Xia, H., Wang, A., et al., 2018. Proteomic response of the rat liver in differential swimming modes. *Clin Exp Pharmacol Physiol* 45(6):581-590.

- [174] Kim, M.S., Pinto, S.M., Getnet, D., Nirujogi, R.S., Manda, S.S., Chaerkady, R., et al., 2014. A draft map of the human proteome. *Nature* 509(7502):575-581.
- [175] Azimifar, S.B., Nagaraj, N., Cox, J., Mann, M., 2014. Cell-type-resolved quantitative proteomics of murine liver. *Cell Metab* 20(6):1076-1087.
- [176] Michael, D.J., Cai, H., Xiong, W., Ouyang, J., Chow, R.H., 2006. Mechanisms of peptide hormone secretion. *Trends in endocrinology and metabolism: TEM* 17(10):408-415.
- [177] Lai, K.K., Kolippakkam, D., Beretta, L., 2008. Comprehensive and quantitative proteome profiling of the mouse liver and plasma. *Hepatology* 47(3):1043-1051.
- [178] Yoshida, M., Satoh, A., Lin, J.B., Mills, K.F., Sasaki, Y., Rensing, N., et al., 2019. Extracellular Vesicle-Contained eNAMPT Delays Aging and Extends Lifespan in Mice. *Cell metabolism* 30(2):329-342.e325.
- [179] Wang, J., Li, L., Zhang, Z., Zhang, X., Zhu, Y., Zhang, C., et al., 2022. Extracellular vesicles mediate the communication of adipose tissue with brain and promote cognitive impairment associated with insulin resistance. *Cell metabolism* 34(9):1264-1279.e1268.
- [180] Gao, H., Jin, Z., Bandyopadhyay, G., Wang, G., Zhang, D., Rocha, K.C.e., et al., 2022. Aberrant iron distribution via hepatocyte-stellate cell axis drives liver lipogenesis and fibrosis. *Cell metabolism* 34(8):1201-1213.e1205.
- [181] Stefan, N., Häring, H.-U., 2013. The role of hepatokines in metabolism. *Nature Reviews Endocrinology* 9:144.
- [182] Iroz, A., Couty, J.-P., Postic, C., 2015. Hepatokines: unlocking the multi-organ network in metabolic diseases. *Diabetologia* 58(8):1699-1703.
- [183] Mraz, M., Bartlova, M., Lacinova, Z., Michalsky, D., Kasalicky, M., Haluzikova, D., et al., 2009. Serum concentrations and tissue expression of a novel endocrine regulator fibroblast growth factor-21 in patients with type 2 diabetes and obesity. *Clin Endocrinol (Oxf)* 71(3):369-375.
- [184] Chavez, A.O., Molina-Carrion, M., Abdul-Ghani, M.A., Folli, F., Defronzo, R.A., Tripathy, D., 2009. Circulating fibroblast growth factor-21 is elevated in impaired glucose tolerance and type 2 diabetes and correlates with muscle and hepatic insulin resistance. *Diabetes Care* 32(8):1542-1546.
- [185] Arner, P., Pettersson, A., Mitchell, P.J., Dunbar, J.D., Kharitonov, A., Ryden, M., 2008. FGF21 attenuates lipolysis in human adipocytes - a possible link to improved insulin sensitivity. *FEBS Lett* 582(12):1725-1730.
- [186] Xu, J., Lloyd, D.J., Hale, C., Stanislaus, S., Chen, M., Sivits, G., et al., 2009. Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* 58(1):250-259.
- [187] Badman, M.K., Pissios, P., Kennedy, A.R., Koukos, G., Flier, J.S., Maratos-Flier, E., 2007. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab* 5(6):426-437.
- [188] Gaich, G., Chien, J.Y., Fu, H., Glass, L.C., Deeg, M.A., Holland, W.L., et al., 2013. The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes. *Cell Metab* 18(3):333-340.
- [189] Zhu, J., Wan, X., Wang, Y., Zhu, K., Li, C., Yu, C., et al., 2017. Serum fetuin B level increased in subjects of nonalcoholic fatty liver disease: a case-control study. *Endocrine* 56(1):208-211.
- [190] Li, Z., Lin, M., Liu, C., Wang, D., Shi, X., Chen, Z., et al., 2018. Fetuin-B links nonalcoholic fatty liver disease to type 2 diabetes via inducing insulin resistance: Association and path analyses. *Cytokine* 108:145-150.

- [191] Gerst, F., Wagner, R., Kaiser, G., Panse, M., Heni, M., Machann, J., et al., 2017. Metabolic crosstalk between fatty pancreas and fatty liver: effects on local inflammation and insulin secretion. *Diabetologia* 60(11):2240-2251.
- [192] Drucker, D.J., Nauck, M.A., 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 368(9548):1696-1705.
- [193] Baumeier, C., Schlüter, L., Saussenthaler, S., Laeger, T., Rödiger, M., Alaze, S.A., et al., 2017. Elevated hepatic DPP4 activity promotes insulin resistance and non-alcoholic fatty liver disease. *Mol Metab* 6(10):1254-1263.
- [194] Scheen, A.J., 2018. The safety of gliptins : updated data in 2018. *Expert Opin Drug Saf* 17(4):387-405.
- [195] Zhang, Y., Cai, T., Zhao, J., Guo, C., Yao, J., Gao, P., et al., 2020. Effects and Safety of Sitagliptin in Non-Alcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis. *Horm Metab Res* 52(7):517-526.
- [196] Leite Nathalie, C., Villela-Nogueira Cristiane, A., Pannain Vera, L.N., Bottino Adriana, C., Rezende Guilherme, F.M., Cardoso Claudia, R.L., et al., 2011. Histopathological stages of nonalcoholic fatty liver disease in type 2 diabetes: prevalences and correlated factors. *Liver International* 31(5):700-706.
- [197] Schauer, P.R., Kashyap, S.R., Wolski, K., Brethauer, S.A., Kirwan, J.P., Pothier, C.E., et al., 2012. Bariatric Surgery versus Intensive Medical Therapy in Obese Patients with Diabetes. *New England Journal of Medicine* 366(17):1567-1576.
- [198] Ungerleider, N.A., Bonomi, L.M., Brown, M.L., Schneyer, A.L., 2013. Increased activin bioavailability enhances hepatic insulin sensitivity while inducing hepatic steatosis in male mice. *Endocrinology* 154(6):2025-2033.
- [199] Hashimoto, O., Funaba, M., Sekiyama, K., Doi, S., Shindo, D., Satoh, R., et al., 2018. Activin E Controls Energy Homeostasis in Both Brown and White Adipose Tissues as a Hepatokine. *Cell Rep* 25(5):1193-1203.
- [200] Kumar, K.G., Trevaskis, J.L., Lam, D.D., Sutton, G.M., Koza, R.A., Chouljenko, V.N., et al., 2008. Identification of adropin as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism. *Cell Metab* 8(6):468-481.
- [201] Karimkhanloo, H., Keenan, S.N., Sun, E.W., Wattchow, D.A., Keating, D.J., Montgomery, M.K., et al., 2020. Circulating cathepsin S improves glycemic control in mice. *J Endocrinol*.
- [202] Awazawa, M., Gabel, P., Tsaousidou, E., Nolte, H., Krüger, M., Schmitz, J., et al., 2017. A microRNA screen reveals that elevated hepatic ectodysplasin A expression contributes to obesity-induced insulin resistance in skeletal muscle. *Nature medicine* 23:1466.
- [203] Ou, H.Y., Wu, H.T., Hung, H.C., Yang, Y.C., Wu, J.S., Chang, C.J., 2012. Endoplasmic reticulum stress induces the expression of fetuin-A to develop insulin resistance. *Endocrinology* 153(7):2974-2984.
- [204] Meex, R.C., Hoy, A.J., Morris, A., Brown, R.D., Lo, J.C., Burke, M., et al., 2015. Fetuin B is a secreted hepatocyte factor linking steatosis to impaired glucose metabolism. *Cell metabolism* 22(6):1078-1089.
- [205] Wu, H.T., Ou, H.Y., Hung, H.C., Su, Y.C., Lu, F.H., Wu, J.S., et al., 2016. A novel hepatokine, HFREP1, plays a crucial role in the development of insulin resistance and type 2 diabetes. *Diabetologia* 59(8):1732-1742.
- [206] Wu, H.T., Lu, F.H., Ou, H.Y., Su, Y.C., Hung, H.C., Wu, J.S., et al., 2013. The role of hepassocin in the development of non-alcoholic fatty liver disease. *J Hepatol* 59(5):1065-1072.

- [207] Lan, F., Misu, H., Chikamoto, K., Takayama, H., Kikuchi, A., Mohri, K., et al., 2014. LECT2 functions as a hepatokine that links obesity to skeletal muscle insulin resistance. *Diabetes* 63(5):1649-1664.
- [208] Borg, M.L., Andrews, Z.B., Duh, E.J., Zechner, R., Meikle, P.J., Watt, M.J., 2011. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. *Diabetes* 60(5):1458-1466.
- [209] Crowe, S., Wu, L.E., Economou, C., Turpin, S.M., Matzaris, M., Hoehn, K.L., et al., 2009. Pigment epithelium-derived factor contributes to insulin resistance in obesity. *Cell Metab* 10(1):40-47.
- [210] Misu, H., Takamura, T., Takayama, H., Hayashi, H., Matsuzawa-Nagata, N., Kurita, S., et al., 2010. A liver-derived secretory protein, selenoprotein P, causes insulin resistance. *Cell Metab* 12(5):483-495.
- [211] Sáez-López, C., Salcedo-Allende, M.T., Hernandez, C., Simó-Servat, O., Simó, R., Selva, D.M., 2019. Sex Hormone-Binding Globulin Expression Correlates With Acetyl-Coenzyme A Carboxylase and Triglyceride Content in Human Liver. *J Clin Endocrinol Metab* 104(5):1500-1507.
- [212] Mouchiroud, M., Camiré, É., Aldow, M., Caron, A., Jubinville, É., Turcotte, L., et al., 2019. The hepatokine Tsukushi is released in response to NAFLD and impacts cholesterol homeostasis. *JCI Insight* 4(15):e129492.
- [213] Xiong, X., Wang, Q., Wang, S., Zhang, J., Liu, T., Guo, L., et al., 2019. Mapping the molecular signatures of diet-induced NASH and its regulation by the hepatokine Tsukushi. *Mol Metab* 20:128-137.
- [214] Mouchiroud, M., Camiré, É., Aldow, M., Caron, A., Jubinville, É., Turcotte, L., et al., 2019. The Hepatokine TSK does not affect brown fat thermogenic capacity, body weight gain, and glucose homeostasis. *Molecular Metabolism* 30:184-191.
- [215] Saunders, J.B., Aasland, O.G., Babor, T.F., de la Fuente, J.R., Grant, M., 1993. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* 88(6):791-804.
- [216] Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., et al., 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 41(6):1313-1321.
- [217] Kruger, F.C., Daniels, C.R., Kidd, M., Swart, G., Brundyn, K., van Rensburg, C., et al., 2011. APRI: a simple bedside marker for advanced fibrosis that can avoid liver biopsy in patients with NAFLD/NASH. *S Afr Med J* 101(7):477-480.
- [218] Subasi, C.F., Aykut, U.E., Yilmaz, Y., 2015. Comparison of noninvasive scores for the detection of advanced fibrosis in patients with nonalcoholic fatty liver disease. *Eur J Gastroenterol Hepatol* 27(2):137-141.
- [219] Wu, S.-D., Wang, J.-Y., Li, L., 2010. Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: a comparative study. *World Journal of Gastroenterology* 16(4):501-507.
- [220] Lee, J.H., Kim, D., Kim, H.J., Lee, C.H., Yang, J.I., Kim, W., et al., 2010. Hepatic steatosis index: a simple screening tool reflecting nonalcoholic fatty liver disease. *Dig Liver Dis* 42(7):503-508.
- [221] Angulo, P., Hui, J.M., Marchesini, G., Bugianesi, E., George, J., Farrell, G.C., et al., 2007. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 45(4):846-854.
- [222] Kotronen, A., Peltonen, M., Hakkarainen, A., Sevastianova, K., Bergholm, R., Johansson, L.M., et al., 2009. Prediction of Non-Alcoholic Fatty Liver Disease and Liver Fat Using Metabolic and Genetic Factors. *Gastroenterology* 137(3):865-872.

- [223] Li, M., Parker, B.L., Pearson, E., Hunter, B., Cao, J., Koay, Y.C., et al., 2020. Core functional nodes and sex-specific pathways in human ischaemic and dilated cardiomyopathy. *Nature Communications* 11(1):2843.
- [224] Bruderer, R., Bernhardt, O.M., Gandhi, T., Xuan, Y., Sondermann, J., Schmidt, M., et al., 2017. Optimization of Experimental Parameters in Data-Independent Mass Spectrometry Significantly Increases Depth and Reproducibility of Results *. *Molecular & Cellular Proteomics* 16(12):2296-2309.
- [225] Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., et al., 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13:731.
- [226] Willforss, J., Chawade, A., Levander, F., 2019. NormalyzerDE: Online Tool for Improved Normalization of Omics Expression Data and High-Sensitivity Differential Expression Analysis. *J Proteome Res* 18(2):732-740.
- [227] Harney, D.J., Hutchison, A.T., Hatchwell, L., Humphrey, S.J., James, D.E., Hocking, S., et al., 2019. Proteomic Analysis of Human Plasma during Intermittent Fasting. *J Proteome Res* 18(5):2228-2240.
- [228] Messner, C.B., Demichev, V., Bloomfield, N., Yu, J.S.L., White, M., Kreidl, M., et al., 2021. Ultra-fast proteomics with Scanning SWATH. *Nature Biotechnology* 39(7):846-854.
- [229] Demichev, V., Messner, C.B., Vernardis, S.I., Lilley, K.S., Ralser, M., 2020. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nature Methods* 17(1):41-44.
- [230] Cox, J., Hein, M.Y., Lubner, C.A., Paron, I., Nagaraj, N., Mann, M., 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & cellular proteomics : MCP* 13(9):2513-2526.
- [231] Mohktar, R.A., Montgomery, M.K., Murphy, R.M., Watt, M.J., 2016. Perilipin 5 is dispensable for normal substrate metabolism and in the adaptation of skeletal muscle to exercise training. *Am J Physiol Endocrinol Metab* 311(1):E128-137.
- [232] Borg, M.L., Omran, S.F., Weir, J., Meikle, P.J., Watt, M.J., 2012. Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice. *The Journal of Physiology* 590(17):4377-4389.
- [233] Thakker, G.D., Frangogiannis, N.G., Bujak, M., Zymek, P., Gaubatz, J.W., Reddy, A.K., et al., 2006. Effects of diet-induced obesity on inflammation and remodeling after myocardial infarction. *American Journal of Physiology - Heart and Circulatory Physiology* 291(5):H2504.
- [234] Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* 26:1367.
- [235] Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., et al., 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods* 13:731.
- [236] Krämer, A., Green, J., Pollard, J., Jr., Tugendreich, S., 2014. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics (Oxford, England)* 30(4):523-530.
- [237] MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen, B., et al., 2010. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics (Oxford, England)* 26(7):966-968.

- [238] Deutsch, E.W., 2010. The PeptideAtlas Project. *Methods in molecular biology* (Clifton, N.J.) 604:285-296.
- [239] Lydic, T.A., Busik, J.V., Reid, G.E., 2014. A monophasic extraction strategy for the simultaneous lipidome analysis of polar and nonpolar retina lipids [S]. *Journal of Lipid Research* 55(8):1797-1809.
- [240] Liebisch, G., Vizcaíno, J.A., Köfeler, H., Trötz Müller, M., Griffiths, W.J., Schmitz, G., et al., 2013. Shorthand notation for lipid structures derived from mass spectrometry. *Journal of Lipid Research* 54(6):1523-1530.
- [241] Seger, S., Stritt, M., Vezzali, E., Nayler, O., Hess, P., Groenen, P.M.A., et al., 2018. A fully automated image analysis method to quantify lung fibrosis in the bleomycin-induced rat model. *PLoS ONE* 13(3):e0193057.
- [242] Stritt, M., Bär, R., Freyss, J., Marrie, J., Vezzali, E., Weber, E., et al., 2011. Supervised Machine Learning Methods for Quantification of Pulmonary Fibrosis, MDA.
- [243] Berg, S., Kutra, D., Kroeger, T., Straehle, C.N., Kausler, B.X., Haubold, C., et al., 2019. ilastik: interactive machine learning for (bio)image analysis. *Nature Methods* 16(12):1226-1232.
- [244] Younossi, Z.M., Otgonsuren, M., Henry, L., Venkatesan, C., Mishra, A., Erario, M., et al., 2015. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. *Hepatology* 62(6):1723-1730.
- [245] Sanyal, A., Poklepovic, A., Moyneur, E., Barghout, V., 2010. Population-based risk factors and resource utilization for HCC: US perspective. *Curr Med Res Opin* 26(9):2183-2191.
- [246] Younossi, Z., Anstee, Q.M., Marietti, M., Hardy, T., Henry, L., Eslam, M., et al., 2018. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 15(1):11-20.
- [247] Statistics, A.B.o., 2018. National Health Survey: First results. 2017-2018.
- [248] Ward, Z.J., Bleich, S.N., Craddock, A.L., Barrett, J.L., Giles, C.M., Flax, C., et al., 2019. Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity. *New England Journal of Medicine* 381(25):2440-2450.
- [249] Janssen, F., Bardoutsos, A., Vidra, N., 2020. Obesity Prevalence in the Long-Term Future in 18 European Countries and in the USA. *Obesity Facts* 13(5):514-527.
- [250] Pelusi, S., Cespiati, A., Rametta, R., Pennisi, G., Mannisto, V., Rosso, C., et al., 2019. Prevalence and Risk Factors of Significant Fibrosis in Patients With Nonalcoholic Fatty Liver Without Steatohepatitis. *Clin Gastroenterol Hepatol* 17(11):2310-2319.e2316.
- [251] Rockey, D.C., Caldwell, S.H., Goodman, Z.D., Nelson, R.C., Smith, A.D., American Association for the Study of Liver, D., 2009. Liver biopsy. *Hepatology* 49(3):1017-1044.
- [252] Asrani, S.K., 2015. Incorporation of Noninvasive Measures of Liver Fibrosis Into Clinical Practice: Diagnosis and Prognosis. *Clin Gastroenterol Hepatol* 13(12):2190-2204.
- [253] Hashemi, S.A., Alavian, S.M., Gholami-Fesharaki, M., 2016. Assessment of transient elastography (FibroScan) for diagnosis of fibrosis in non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Caspian J Intern Med* 7(4):242-252.
- [254] Ratzl, V., Giral, P., Charlotte, F., Bruckert, E., Thibault, V., Theodorou, I., et al., 2000. Liver fibrosis in overweight patients. *Gastroenterology* 118(6):1117-1123.

- [255] Wai, C.T., Greenson, J.K., Fontana, R.J., Kalbfleisch, J.D., Marrero, J.A., Conjeevaram, H.S., et al., 2003. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 38(2):518-526.
- [256] Wong, V.W.-S., Adams, L.A., de Lédinghen, V., Wong, G.L.-H., Sookoian, S., 2018. Noninvasive biomarkers in NAFLD and NASH — current progress and future promise. *Nature Reviews Gastroenterology & Hepatology* 15(8):461-478.
- [257] Armstrong, M.J., Hazlehurst, J.M., Parker, R., Koushiappi, E., Mann, J., Khan, S., et al., 2014. Severe asymptomatic non-alcoholic fatty liver disease in routine diabetes care; a multi-disciplinary team approach to diagnosis and management. *QJM : monthly journal of the Association of Physicians* 107(1):33-41.
- [258] Aron-Wisnewsky, J., Vigliotti, C., Witjes, J., Le, P., Holleboom, A.G., Verheij, J., et al., 2020. Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders. *Nature Reviews Gastroenterology & Hepatology* 17(5):279-297.
- [259] Sutti, S., Albano, E., 2020. Adaptive immunity: an emerging player in the progression of NAFLD. *Nature Reviews Gastroenterology & Hepatology* 17(2):81-92.
- [260] Piccinin, E., Villani, G., Moschetta, A., 2019. Metabolic aspects in NAFLD, NASH and hepatocellular carcinoma: the role of PGC1 coactivators. *Nature Reviews Gastroenterology & Hepatology* 16(3):160-174.
- [261] Parthasarathy, G., Revelo, X., Malhi, H., 2020. Pathogenesis of Nonalcoholic Steatohepatitis: An Overview. *Hepatology communications* 4(4):478-492.
- [262] Iredale, J.P., 2007. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 117(3):539-548.
- [263] Kazankov, K., Jørgensen, S.M.D., Thomsen, K.L., Møller, H.J., Vilstrup, H., George, J., et al., 2019. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nature Reviews Gastroenterology & Hepatology* 16(3):145-159.
- [264] Campana, L., Iredale, J.P., 2017. Regression of Liver Fibrosis. *Semin Liver Dis* 37(1):1-10.
- [265] Ramachandran, P., Pellicoro, A., Vernon, M.A., Boulter, L., Aucott, R.L., Ali, A., et al., 2012. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* 109(46):E3186-3195.
- [266] Younossi, Z.M., Golabi, P., de Avila, L., Paik, J.M., Srishord, M., Fukui, N., et al., 2019. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of Hepatology* 71(4):793-801.
- [267] Zhang, Q.-Q., Lu, L.-G., 2015. Nonalcoholic Fatty Liver Disease: Dyslipidemia, Risk for Cardiovascular Complications, and Treatment Strategy. *Journal of clinical and translational hepatology* 3(1):78-84.
- [268] Ryoo, J.H., Suh, Y.J., Shin, H.C., Cho, Y.K., Choi, J.M., Park, S.K., 2014. Clinical association between non-alcoholic fatty liver disease and the development of hypertension. *J Gastroenterol Hepatol* 29(11):1926-1931.
- [269] Kwak, M.-S., Kim, D., Chung, G.E., Kang, S.J., Park, M.J., Kim, Y.J., et al., 2012. Serum bilirubin levels are inversely associated with nonalcoholic fatty liver disease. *Clinical and molecular hepatology* 18(4):383-390.
- [270] Ooi, G.J., Burton, P.R., Bayliss, J., Raajendiran, A., Earnest, A., Laurie, C., et al., 2018. Effect of Body Mass Index, Metabolic Health and Adipose Tissue Inflammation on the Severity of Non-alcoholic Fatty Liver Disease in Bariatric Surgical Patients: a Prospective Study. *Obes Surg*.

- [271] Lai, K.K.Y., Kolippakkam, D., Beretta, L., 2008. Comprehensive and quantitative proteome profiling of the mouse liver and plasma. *Hepatology* 47(3):1043-1051.
- [272] Bell, L.N., Theodorakis, J.L., Vuppalandhi, R., Saxena, R., Bemis, K.G., Wang, M., et al., 2010. Serum Proteomics and Biomarker Discovery Across the Spectrum of Nonalcoholic Fatty Liver Disease. *Hepatology (Baltimore, Md.)* 51(1):111-120.
- [273] Luo, Y., Wadhawan, S., Greenfield, A., Decato, B.E., Oseini, A.M., Collen, R., et al., 2021. SOMAscan Proteomics Identifies Serum Biomarkers Associated With Liver Fibrosis in Patients With NASH. *Hepatology communications* 5(5):760-773.
- [274] Niu, L., Geyer, P.E., Wewer Albrechtsen, N.J., Gluud, L.L., Santos, A., Doll, S., et al., 2019. Plasma proteome profiling discovers novel proteins associated with non-alcoholic fatty liver disease. *Mol Syst Biol* 15(3):e8793.
- [275] Franko, A., Hartwig, S., Kotzka, J., Ruoff, M., Nüssler, A.K., Königsrainer, A., et al., 2019. Identification of the Secreted Proteins Originated from Primary Human Hepatocytes and HepG2 Cells. *Nutrients* 11(8):1795.
- [276] McQuitty, C.E., Williams, R., Chokshi, S., Urbani, L., 2020. Immunomodulatory Role of the Extracellular Matrix Within the Liver Disease Microenvironment. *Frontiers in Immunology* 11(2903).
- [277] Rosenblum, G., Meroueh, S., Toth, M., Fisher, J.F., Fridman, R., Mobashery, S., et al., 2007. Molecular Structures and Dynamics of the Stepwise Activation Mechanism of a Matrix Metalloproteinase Zymogen: Challenging the Cysteine Switch Dogma. *Journal of the American Chemical Society* 129(44):13566-13574.
- [278] Ra, H.-J., Parks, W.C., 2007. Control of matrix metalloproteinase catalytic activity. *Matrix biology : journal of the International Society for Matrix Biology* 26(8):587-596.
- [279] Zeybel, M., Hardy, T., Wong, Y.K., Mathers, J.C., Fox, C.R., Gackowska, A., et al., 2012. Multigenerational epigenetic adaptation of the hepatic wound-healing response. *Nat Med* 18(9):1369-1377.
- [280] Yadav, H., Quijano, C., Kamaraju, A.K., Gavrilova, O., Malek, R., Chen, W., et al., 2011. Protection from obesity and diabetes by blockade of TGF- β /Smad3 signaling. *Cell metabolism* 14(1):67-79.
- [281] Kim, K.K., Sheppard, D., Chapman, H.A., 2018. TGF- β 1 Signaling and Tissue Fibrosis. *Cold Spring Harbor Perspectives in Biology* 10(4):a022293.
- [282] Zacchigna, L., Vecchione, C., Notte, A., Cordenonsi, M., Dupont, S., Maretto, S., et al., 2006. Emilin1 links TGF-beta maturation to blood pressure homeostasis. *Cell* 124(5):929-942.
- [283] Montgomery, M., Bayliss, J., Devereux, C., Bezawork-Geleta, A., Roberts, D., Huang, C., et al., 2020. SMOC1 is a glucose-responsive hepatokine and therapeutic target for glycaemic control. *Science Translational Medicine* 12(559):eaaz8048.
- [284] Montgomery, M.K., Taddese, A.Z., Bayliss, J., Nie, S., Williamson, N.A., Watt, M.J., 2021. Hexosaminidase A (HEXA) regulates hepatic sphingolipid and lipoprotein metabolism in mice. *The FASEB Journal* 35(12):e22046.
- [285] Shetty, S., Lalor, P.F., Adams, D.H., 2018. Liver sinusoidal endothelial cells — gatekeepers of hepatic immunity. *Nature Reviews Gastroenterology & Hepatology* 15(9):555-567.
- [286] Maiorani, O., Pivetta, E., Capuano, A., Modica, T.M.E., Wassermann, B., Bucciotti, F., et al., 2017. Neutrophil elastase cleavage of the gC1q domain impairs the EMILIN1- α 4 β 1 integrin interaction, cell adhesion and anti-proliferative activity. *Scientific Reports* 7(1):39974.

- [287] Pivetta, E., Danussi, C., Wassermann, B., Modica, T.M., Del Bel Belluz, L., Canzonieri, V., et al., 2014. Neutrophil elastase-dependent cleavage compromises the tumor suppressor role of EMILIN1. *Matrix Biol* 34:22-32.
- [288] Montgomery, M.K., Bayliss, J., Devereux, C., Bezawork-Geleta, A., Roberts, D., Huang, C., et al., 2020. SMOC1 is a glucose-responsive hepatokine and therapeutic target for glycemic control. *Sci Transl Med* 12(559).
- [289] Ratziu, V., Charlotte, F., Heurtier, A., Gombert, S., Giral, P., Bruckert, E., et al., 2005. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 128(7):1898-1906.
- [290] Williams, C.D., Stengel, J., Asike, M.I., Torres, D.M., Shaw, J., Contreras, M., et al., 2011. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 140(1):124-131.
- [291] Friedman, S.L., Neuschwander-Tetri, B.A., Rinella, M., Sanyal, A.J., 2018. Mechanisms of NAFLD development and therapeutic strategies. *Nature medicine* 24(7):908-922.
- [292] Vilar-Gomez, E., Chalasan, N., 2018. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *J Hepatol* 68(2):305-315.
- [293] Li, L., Liu, D.W., Yan, H.Y., Wang, Z.Y., Zhao, S.H., Wang, B., 2016. Obesity is an independent risk factor for non-alcoholic fatty liver disease: evidence from a meta-analysis of 21 cohort studies. *Obes Rev* 17(6):510-519.
- [294] Wang, L., Guo, J., Lu, J., 2016. Risk factor compositions of nonalcoholic fatty liver disease change with body mass index in males and females. *Oncotarget* 7(24):35632-35642.
- [295] Dulai, P.S., Sirlin, C.B., Loomba, R., 2016. MRI and MRE for non-invasive quantitative assessment of hepatic steatosis and fibrosis in NAFLD and NASH: Clinical trials to clinical practice. *Journal of Hepatology* 65(5):1006-1016.
- [296] Loomba, R., Cui, J., Wolfson, T., Haufe, W., Hooker, J., Szeverenyi, N., et al., 2016. Novel 3D Magnetic Resonance Elastography for the Noninvasive Diagnosis of Advanced Fibrosis in NAFLD: A Prospective Study. *Official journal of the American College of Gastroenterology | ACG* 111(7).
- [297] Garg, H., Aggarwal, S., Shalimar, Yadav, R., Datta Gupta, S., Agarwal, L., et al., 2018. Utility of transient elastography (fibroscan) and impact of bariatric surgery on nonalcoholic fatty liver disease (NAFLD) in morbidly obese patients. *Surg Obes Relat Dis* 14(1):81-91.
- [298] Larance, M., Lamond, A.I., 2015. Multidimensional proteomics for cell biology. *Nat Rev Mol Cell Biol* 16(5):269-280.
- [299] Gold, L., Ayers, D., Bertino, J., Bock, C., Bock, A., Brody, E.N., et al., 2010. Aptamer-based multiplexed proteomic technology for biomarker discovery. *PLoS ONE* 5(12):e15004.
- [300] Sun, B.B., Maranville, J.C., Peters, J.E., Stacey, D., Staley, J.R., Blackshaw, J., et al., 2018. Genomic atlas of the human plasma proteome. *Nature* 558(7708):73-79.
- [301] Niu, L., Thiele, M., Geyer, P., Rasmussen, D.N., Webel, H.E., Santos, A., et al., 2020. A paired liver biopsy and plasma proteomics study reveals circulating biomarkers for alcohol-related liver disease. *bioRxiv*.
- [302] Geyer, P.E., Holdt, L.M., Teupser, D., Mann, M., 2017. Revisiting biomarker discovery by plasma proteomics. *Mol Syst Biol* 13(9):942-942.
- [303] Schwenk, J.M., Omenn, G.S., Sun, Z., Campbell, D.S., Baker, M.S., Overall, C.M., et al., 2017. The Human Plasma Proteome Draft of 2017: Building on the Human

Plasma PeptideAtlas from Mass Spectrometry and Complementary Assays. *J Proteome Res* 16(12):4299-4310.

- [304] Kampf, C., Mardinoglu, A., Fagerberg, L., Hallström, B.M., Edlund, K., Lundberg, E., et al., 2014. The human liver-specific proteome defined by transcriptomics and antibody-based profiling. *Faseb j* 28(7):2901-2914.
- [305] Folkersen, J., Teisner, B., Grunnet, N., Grudzinskas, J.G., Westergaard, J.G., Hindersson, P., 1981. Circulating levels of pregnancy zone protein: normal range and the influence of age and gender. *Clin Chim Acta* 110(2-3):139-145.
- [306] Moraes-Vieira, P.M., Yore, M.M., Dwyer, P.M., Syed, I., Aryal, P., Kahn, B.B., 2014. RBP4 activates antigen-presenting cells, leading to adipose tissue inflammation and systemic insulin resistance. *Cell Metab* 19(3):512-526.
- [307] Fedders, R., Muenzner, M., Weber, P., Sommerfeld, M., Knauer, M., Kedziora, S., et al., 2018. Liver-secreted RBP4 does not impair glucose homeostasis in mice. *J Biol Chem* 293(39):15269-15276.
- [308] Huang, D.Q., El-Serag, H.B., Loomba, R., 2021. Global epidemiology of NAFLD-related HCC: trends, predictions, risk factors and prevention. *Nature Reviews Gastroenterology & Hepatology* 18(4):223-238.
- [309] Borena, W., Strohmaier, S., Lukanova, A., Bjørge, T., Lindkvist, B., Hallmans, G., et al., 2012. Metabolic risk factors and primary liver cancer in a prospective study of 578,700 adults. *Int J Cancer* 131(1):193-200.
- [310] Welzel, T.M., Graubard, B.I., Zeuzem, S., El-Serag, H.B., Davila, J.A., McGlynn, K.A., 2011. Metabolic syndrome increases the risk of primary liver cancer in the United States: A study in the SEER-medicare database. *Hepatology* 54(2):463-471.
- [311] Keshishian, H., Burgess, M.W., Specht, H., Wallace, L., Clauser, K.R., Gillette, M.A., et al., 2017. Quantitative, multiplexed workflow for deep analysis of human blood plasma and biomarker discovery by mass spectrometry. *Nature Protocols* 12(8):1683-1701.
- [312] Malmström, E., Kilsgård, O., Hauri, S., Smeds, E., Herwald, H., Malmström, L., et al., 2016. Large-scale inference of protein tissue origin in gram-positive sepsis plasma using quantitative targeted proteomics. *Nature Communications* 7:10261-10261.
- [313] Wood, G.C., Chu, X., Argyropoulos, G., Benotti, P., Rolston, D., Mirshahi, T., et al., 2017. A multi-component classifier for nonalcoholic fatty liver disease (NAFLD) based on genomic, proteomic, and phenomic data domains. *Sci Rep* 7:43238.
- [314] Kumar, A., Gangadharan, B., Cobbold, J., Thursz, M., Zitzmann, N., 2017. Absolute quantitation of disease protein biomarkers in a single LC-MS acquisition using apolipoprotein F as an example. *Sci Rep* 7(1):12072.
- [315] Gangadharan, B., Bapat, M., Rossa, J., Antrobus, R., Chittenden, D., Kampa, B., et al., 2012. Discovery of novel biomarker candidates for liver fibrosis in hepatitis C patients: a preliminary study. *PLoS ONE* 7(6):e39603.
- [316] Wegermann, K., Henao, R., Diehl, A.M., Murphy, S.K., Abdelmalek, M.F., Moylan, C.A., 2018. Branched chain amino acid transaminase 1 (BCAT1) is overexpressed and hypomethylated in patients with non-alcoholic fatty liver disease who experience adverse clinical events: A pilot study. *PLoS ONE* 13(9):e0204308-e0204308.
- [317] Veyel, D., Wenger, K., Broermann, A., Bretschneider, T., Luippold, A.H., Krawczyk, B., et al., 2020. Biomarker discovery for chronic liver diseases by multi-omics - a preclinical case study. *Sci Rep* 10(1):1314.
- [318] Kronenberg, F., Kollerits, B., Kiechl, S., Lamina, C., Kedenko, L., Meisinger, C., et al., 2014. Plasma Concentrations of Afamin Are Associated With the Prevalence

- and Development of Metabolic Syndrome. *Circulation: Cardiovascular Genetics* 7(6):822-829.
- [319] Harney, D.J., Hutchison, A.T., Su, Z., Hatchwell, L., Heilbronn, L.K., Hocking, S., et al., 2019. Small-protein Enrichment Assay Enables the Rapid, Unbiased Analysis of Over 100 Low Abundance Factors from Human Plasma. *Molecular & cellular proteomics : MCP* 18(9):1899-1915.
- [320] Zhao, J.V., Schooling, C.M., 2021. Sex-specific Associations of Sex Hormone Binding Globulin with CKD and Kidney Function: A Univariable and Multivariable Mendelian Randomization Study in the UK Biobank. *J Am Soc Nephrol* 32(3):686-694.
- [321] Sanyal, A.J., Chalasani, N., Kowdley, K.V., McCullough, A., Diehl, A.M., Bass, N.M., et al., 2010. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 362(18):1675-1685.
- [322] Hampton, T., 2007. Diabetes Drugs Tied to Fractures in Women. *JAMA* 297(15):1645-1645.
- [323] Lutchman, G., Modi, A., Kleiner, D.E., Promrat, K., Heller, T., Ghany, M., et al., 2007. The effects of discontinuing pioglitazone in patients with nonalcoholic steatohepatitis. *Hepatology* 46(2):424-429.
- [324] Klein, E.A., Thompson, I.M., Jr., Tangen, C.M., Crowley, J.J., Lucia, M.S., Goodman, P.J., et al., 2011. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* 306(14):1549-1556.
- [325] Miller, E.R., 3rd, Pastor-Barriuso, R., Dalal, D., Riemersma, R.A., Appel, L.J., Guallar, E., 2005. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 142(1):37-46.
- [326] Isogai, Z., Ono, R.N., Ushiro, S., Keene, D.R., Chen, Y., Mazzei, R., et al., 2003. Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. *J Biol Chem* 278(4):2750-2757.
- [327] Zilberberg, L., Todorovic, V., Dabovic, B., Horiguchi, M., Couroussé, T., Sakai, L.Y., et al., 2012. Specificity of latent TGF- β binding protein (LTBP) incorporation into matrix: role of fibrillins and fibronectin. *J Cell Physiol* 227(12):3828-3836.
- [328] Hildebrand, A., Romarís, M., Rasmussen, L.M., Heinegård, D., Twardzik, D.R., Border, W.A., et al., 1994. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *The Biochemical journal* 302 (Pt 2)(Pt 2):527-534.
- [329] Hyytiäinen, M., Penttinen, C., Keski-Oja, J., 2004. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 41(3):233-264.
- [330] Hinck, A.P., Mueller, T.D., Springer, T.A., 2016. Structural Biology and Evolution of the TGF- β Family. *Cold Spring Harb Perspect Biol* 8(12).
- [331] Leof, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorleto, P.E., Moses, H.L., 1986. Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. *Proceedings of the National Academy of Sciences* 83(8):2453.
- [332] Seki, E., Brenner, D.A., 2015. Recent advancement of molecular mechanisms of liver fibrosis. *J Hepatobiliary Pancreat Sci* 22(7):512-518.
- [333] Pan, Q., Chen, Y., Yan, H.U.I., Yang, W., Shen, Z., Dahanayaka, S.A., et al., 2018. Regulation of Hepatic Glucose Production by Transforming Growth Factor Beta 1 via Protein Kinase A. *Diabetes* 67(Supplement 1):2441-PUB.
- [334] Yang, L., Roh, Y.S., Song, J., Zhang, B., Liu, C., Loomba, R., et al., 2014. Transforming growth factor beta signaling in hepatocytes participates in steatohepatitis

through regulation of cell death and lipid metabolism in mice. *Hepatology* (Baltimore, Md.) 59(2):483-495.

[335] Teixeira, A.F., ten Dijke, P., Zhu, H.-J., 2020. On-Target Anti-TGF- β Therapies Are Not Succeeding in Clinical Cancer Treatments: What Are Remaining Challenges? *Frontiers in Cell and Developmental Biology* 8.

[336] Febbraio, M.A., Reibe, S., Shalapour, S., Ooi, G.J., Watt, M.J., Karin, M., 2019. Preclinical Models for Studying NASH-Driven HCC: How Useful Are They? *Cell metabolism* 29(1):18-26.

[337] Ennequin, G., Sirvent, P., Whitham, M., 2019. Role of exercise-induced hepatokines in metabolic disorders. *Am J Physiol Endocrinol Metab* 317(1):E11-e24.

[338] Murphy, R.M., Watt, M.J., Febbraio, M.A., 2020. Metabolic communication during exercise. *Nature Metabolism* 2(9):805-816.

[339] Seo, D.Y., Park, S.H., Marquez, J., Kwak, H.-B., Kim, T.N., Bae, J.H., et al., 2021. Hepatokines as a Molecular Transducer of Exercise. *Journal of Clinical Medicine* 10(3).

[340] Hoene, M., Weigert, C., 2010. The stress response of the liver to physical exercise. *Exerc Immunol Rev* 16:163-183.

[341] Fan, X., Zhang, Q., Li, S., Lv, Y., Su, H., Jiang, H., et al., 2013. Attenuation of CCl₄-induced hepatic fibrosis in mice by vaccinating against TGF- β 1. *PLoS ONE* 8(12):e82190.

[342] Ling, H., Roux, E., Hempel, D., Tao, J., Smith, M., Lonning, S., et al., 2013. Transforming growth factor β neutralization ameliorates pre-existing hepatic fibrosis and reduces cholangiocarcinoma in thioacetamide-treated rats. *PLoS ONE* 8(1):e54499.

[343] Akhurst, R.J., Hata, A., 2012. Targeting the TGF β signalling pathway in disease. *Nat Rev Drug Discov* 11(10):790-811.

[344] Kim, J.-Y., An, H.-J., Kim, W.-H., Gwon, M.-G., Gu, H., Park, Y.-Y., et al., 2017. Anti-fibrotic Effects of Synthetic Oligodeoxynucleotide for TGF- β 1 and Smad in an Animal Model of Liver Cirrhosis. *Molecular Therapy - Nucleic Acids* 8:250-263.

[345] Beilfuss, A., Sowa, J.-P., Sydor, S., Beste, M., Bechmann, L.P., Schlattjan, M., et al., 2015. Vitamin D counteracts fibrogenic TGF- β signalling in human hepatic stellate cells both receptor-dependently and independently. *Gut* 64(5):791-799.

[346] Liu, S., Ren, J., ten Dijke, P., 2021. Targeting TGF β signal transduction for cancer therapy. *Signal Transduction and Targeted Therapy* 6(1):8.

[347] Lee, U.E., Friedman, S.L., 2011. Mechanisms of hepatic fibrogenesis. *Best Pract Res Clin Gastroenterol* 25(2):195-206.

[348] Decaris, M.L., Li, K.W., Emson, C.L., Gatmaitan, M., Liu, S., Wang, Y., et al., 2017. Identifying nonalcoholic fatty liver disease patients with active fibrosis by measuring extracellular matrix remodeling rates in tissue and blood. *Hepatology* 65(1):78-88.

[349] Capuano, A., Bucciotti, F., Farwell, K.D., Tippin Davis, B., Mroske, C., Hulick, P.J., et al., 2016. Diagnostic Exome Sequencing Identifies a Novel Gene, EMILIN1, Associated with Autosomal-Dominant Hereditary Connective Tissue Disease. *Human Mutation* 37(1):84-97.

[350] Duarte, S., Baber, J., Fujii, T., Coito, A.J., 2015. Matrix metalloproteinases in liver injury, repair and fibrosis. *Matrix biology : journal of the International Society for Matrix Biology* 44-46:147-156.

[351] Robert, S., Gicquel, T., Bodin, A., Lagente, V., Boichot, E., 2016. Characterization of the MMP/TIMP Imbalance and Collagen Production Induced by IL-1 β or TNF- α Release from Human Hepatic Stellate Cells. *PLoS ONE* 11(4):e0153118.

- [352] Fabbro, C., de Gemmis, P., Braghetta, P., Colombatti, A., Volpin, D., Bonaldo, P., et al., 2005. Analysis of Regulatory Regions of Emilin1 Gene and Their Combinatorial Contribution to Tissue-specific Transcription*. *Journal of Biological Chemistry* 280(16):15749-15760.
- [353] D'Souza, R.C.J., Knittle, A.M., Nagaraj, N., Dinther, M.v., Choudhary, C., Dijke, P.t., et al., 2014. Time-resolved dissection of early phosphoproteome and ensuing proteome changes in response to TGF- β . *Science signaling* 7(335):rs5-rs5.
- [354] Feng, X.-H., Lin, X., Derynck, R., 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15Ink4B transcription in response to TGF- β . *The EMBO Journal* 19(19):5178-5193.
- [355] Chen, H., Zhou, Y., Chen, K.Q., An, G., Ji, S.Y., Chen, Q.K., 2012. Anti-fibrotic effects via regulation of transcription factor Sp1 on hepatic stellate cells. *Cell Physiol Biochem* 29(1-2):51-60.
- [356] Garces de Los Fayos Alonso, I., Liang, H.-C., Turner, S.D., Lagger, S., Merkel, O., Kenner, L., 2018. The Role of Activator Protein-1 (AP-1) Family Members in CD30-Positive Lymphomas. *Cancers* 10(4):93.
- [357] Schattenberg, J.M., Singh, R., Wang, Y., Lefkowitz, J.H., Rigoli, R.M., Scherer, P.E., et al., 2006. Jnk1 but not jnk2 promotes the development of steatohepatitis in mice. *Hepatology* 43(1):163-172.
- [358] Schulien, I., Hockenjos, B., Schmitt-Graeff, A., Perdekamp, M.G., Follo, M., Thimme, R., et al., 2019. The transcription factor c-Jun/AP-1 promotes liver fibrosis during non-alcoholic steatohepatitis by regulating Osteopontin expression. *Cell Death & Differentiation* 26(9):1688-1699.
- [359] Kim, S.J., Angel, P., Lafyatis, R., Hattori, K., Kim, K.Y., Sporn, M.B., et al., 1990. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. *Molecular and cellular biology* 10(4):1492-1497.
- [360] Machado, M.V., Michelotti, G.A., Xie, G., Almeida Pereira, T., Boursier, J., Bohnic, B., et al., 2015. Mouse models of diet-induced nonalcoholic steatohepatitis reproduce the heterogeneity of the human disease. *PLoS ONE* 10(5):e0127991-e0127991.
- [361] Nakahara, T., Hyogo, H., Yoneda, M., Sumida, Y., Eguchi, Y., Fujii, H., et al., 2014. Type 2 diabetes mellitus is associated with the fibrosis severity in patients with nonalcoholic fatty liver disease in a large retrospective cohort of Japanese patients. *J Gastroenterol* 49(11):1477-1484.
- [362] Sackett, S.D., Tremmel, D.M., Ma, F., Feeney, A.K., Maguire, R.M., Brown, M.E., et al., 2018. Extracellular matrix scaffold and hydrogel derived from decellularized and delipidized human pancreas. *Sci Rep* 8(1):10452.
- [363] Naba, A., Clauser, K.R., Mani, D.R., Carr, S.A., Hynes, R.O., 2017. Quantitative proteomic profiling of the extracellular matrix of pancreatic islets during the angiogenic switch and insulinoma progression. *Sci Rep* 7:40495.
- [364] Li, Q., Hata, A., Kosugi, C., Kataoka, N., Funaki, M., 2010. The density of extracellular matrix proteins regulates inflammation and insulin signaling in adipocytes. *FEBS Lett* 584(19):4145-4150.
- [365] Williams, A.S., Kang, L., Wasserman, D.H., 2015. The extracellular matrix and insulin resistance. *Trends in Endocrinology & Metabolism* 26(7):357-366.
- [366] Capuano, A., Pivetta, E., Sartori, G., Bosisio, G., Favero, A., Cover, E., et al., 2019. Abrogation of EMILIN1- β 1 integrin interaction promotes experimental colitis and colon carcinogenesis. *Matrix Biol* 83:97-115.

- [367] Hashida, R., Kawaguchi, T., Bekki, M., Omoto, M., Matsuse, H., Nago, T., et al., 2017. Aerobic vs. resistance exercise in non-alcoholic fatty liver disease: A systematic review. *J Hepatol* 66(1):142-152.
- [368] la Fuente, F.P., Quezada, L., Sepúlveda, C., Monsalves-Alvarez, M., Rodríguez, J.M., Sacristán, C., et al., 2019. Exercise regulates lipid droplet dynamics in normal and fatty liver. *Biochim Biophys Acta Mol Cell Biol Lipids* 1864(12):158519.
- [369] Promrat, K., Kleiner, D.E., Niemeier, H.M., Jackvony, E., Kearns, M., Wands, J.R., et al., 2010. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* 51(1):121-129.
- [370] Eckard, C., Cole, R., Lockwood, J., Torres, D.M., Williams, C.D., Shaw, J.C., et al., 2013. Prospective histopathologic evaluation of lifestyle modification in nonalcoholic fatty liver disease: a randomized trial. *Therap Adv Gastroenterol* 6(4):249-259.
- [371] Rector, R.S., Thyfault, J.P., Morris, R.T., Laye, M.J., Borengasser, S.J., Booth, F.W., et al., 2008. Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol* 294(3):G619-626.
- [372] Inelmen, E.M., Toffanello, E.D., Enzi, G., Gasparini, G., Miotto, F., Sergi, G., et al., 2005. Predictors of drop-out in overweight and obese outpatients. *Int J Obes (Lond)* 29(1):122-128.
- [373] Hoene, M., Franken, H., Fritsche, L., Lehmann, R., Pohl, A.K., Häring, H.U., et al., 2010. Activation of the mitogen-activated protein kinase (MAPK) signalling pathway in the liver of mice is related to plasma glucose levels after acute exercise. *Diabetologia* 53(6):1131-1141.
- [374] Baumeier, C., Schluter, L., Saussenthaler, S., Laeger, T., Rodiger, M., Alaze, S.A., et al., 2017. Elevated hepatic DPP4 activity promotes insulin resistance and non-alcoholic fatty liver disease. *Mol Metab* 6(10):1254-1263.
- [375] Ghorpade, D.S., Ozcan, L., Zheng, Z., Nicoloro, S.M., Shen, Y., Chen, E., et al., 2018. Hepatocyte-secreted DPP4 in obesity promotes adipose inflammation and insulin resistance. *Nature* 555:673.
- [376] Meex, Ruth C., Hoy, Andrew J., Morris, A., Brown, Russell D., Lo, Jennifer C.Y., Burke, M., et al., 2016. Fetuin B Is a Secreted Hepatocyte Factor Linking Steatosis to Impaired Glucose Metabolism. *Cell metabolism* 22(6):1078-1089.
- [377] Templin, A.T., Mellati, M., Soininen, R., Hogan, M.F., Esser, N., Castillo, J.J., et al., 2019. Loss of perlecan heparan sulfate glycosaminoglycans lowers body weight and decreases islet amyloid deposition in human islet amyloid polypeptide transgenic mice. *Protein Eng Des Sel* 32(2):95-102.
- [378] Yamashita, Y., Nakada, S., Yoshihara, T., Nara, T., Furuya, N., Miida, T., et al., 2018. Perlecan, a heparan sulfate proteoglycan, regulates systemic metabolism with dynamic changes in adipose tissue and skeletal muscle. *Scientific Reports* 8(1):7766.
- [379] Rajwani, A., Ezzat, V., Smith, J., Yuldasheva, N.Y., Duncan, E.R., Gage, M., et al., 2012. Increasing circulating IGFBP1 levels improves insulin sensitivity, promotes nitric oxide production, lowers blood pressure, and protects against atherosclerosis. *Diabetes* 61(4):915-924.
- [380] Takahashi, M., Takahashi, Y., Takahashi, K., Zolotaryov, F.N., Hong, K.S., Kitazawa, R., et al., 2008. Chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. *FEBS Lett* 582(5):573-578.
- [381] Rodríguez-Manzaneque, J.C., Carpizo, D., Plaza-Calonge, M.d.C., Torres-Collado, A.X., Thai, S.N.M., Simons, M., et al., 2009. Cleavage of syndecan-4 by

- ADAMTS1 provokes defects in adhesion. *The international journal of biochemistry & cell biology* 41(4):800-810.
- [382] Ramnath, R., Foster, R.R., Qiu, Y., Cope, G., Butler, M.J., Salmon, A.H., et al., 2014. Matrix metalloproteinase 9-mediated shedding of syndecan 4 in response to tumor necrosis factor α : a contributor to endothelial cell glycocalyx dysfunction. *The FASEB Journal* 28(11):4686-4699.
- [383] Lee, S., Kolset, S.O., Birkeland, K.I., Drevon, C.A., Reine, T.M., 2019. Acute exercise increases syndecan-1 and -4 serum concentrations. *Glycoconjugate Journal* 36(2):113-125.
- [384] De Luca, M., Klimentidis, Y.C., Casazza, K., Chambers, M.M., Cho, R., Harbison, S.T., et al., 2010. A conserved role for syndecan family members in the regulation of whole-body energy metabolism. *PLoS ONE* 5(6):e11286.
- [385] Davenport, L., Johari, Y., Klejn, A., Laurie, C., Smith, A., Ooi, G.J., et al., 2019. Improving Compliance with Very Low Energy Diets (VLEDs) Prior to Bariatric Surgery-a Randomised Controlled Trial of Two Formulations. *Obes Surg* 29(9):2750-2757.
- [386] Bass, M.D., Williamson, R.C., Nunan, R.D., Humphries, J.D., Byron, A., Morgan, M.R., et al., 2011. A syndecan-4 hair trigger initiates wound healing through caveolin- and RhoG-regulated integrin endocytosis. *Developmental cell* 21(4):681-693.
- [387] Karamanos, N.K., Piperigkou, Z., Theocharis, A.D., Watanabe, H., Franchi, M., Baud, S., et al., 2018. Proteoglycan Chemical Diversity Drives Multifunctional Cell Regulation and Therapeutics. *Chem Rev* 118(18):9152-9232.
- [388] De Luca, M., Yi, N., Allison, D.B., Leips, J., Ruden, D.M., 2005. Mapping quantitative trait loci affecting variation in *Drosophila* triacylglycerol storage. *Obes Res* 13(9):1596-1605.
- [389] Rose, G., Crocco, P., De Rango, F., Corsonello, A., Lattanzio, F., De Luca, M., et al., 2015. Metabolism and successful aging: Polymorphic variation of syndecan-4 (SDC4) gene associate with longevity and lipid profile in healthy elderly Italian subjects. *Mech Ageing Dev* 150:27-33.
- [390] Agere, S.A., Kim, E.Y., Akhtar, N., Ahmed, S., 2018. Syndecans in chronic inflammatory and autoimmune diseases: Pathological insights and therapeutic opportunities. *J Cell Physiol* 233(9):6346-6358.
- [391] Eveland, M., Brokamp, G.A., Lue, C.-H., Harbison, S.T., Leips, J., De Luca, M., 2016. Knockdown expression of Syndecan in the fat body impacts nutrient metabolism and the organismal response to environmental stresses in *Drosophila melanogaster*. *Biochem Biophys Res Commun* 477(1):103-108.
- [392] Pohl, J., Ring, A., Stremmel, W., 2002. Uptake of long-chain fatty acids in HepG2 cells involves caveolae: analysis of a novel pathway. *J Lipid Res* 43(9):1390-1399.
- [393] Stremmel, W., Staffer, S., Wannhoff, A., Pathil, A., Chamulitrat, W., 2014. Plasma membrane phospholipase A2 controls hepatocellular fatty acid uptake and is responsive to pharmacological modulation: implications for nonalcoholic steatohepatitis. *Faseb j* 28(7):3159-3170.
- [394] Thyfault, J.P., Rector, R.S., 2020. Exercise Combats Hepatic Steatosis: Potential Mechanisms and Clinical Implications. *Diabetes* 69(4):517.
- [395] Trefts, E., Williams, A.S., Wasserman, D.H., 2015. Exercise and the Regulation of Hepatic Metabolism. *Progress in molecular biology and translational science* 135:203-225.

- [396] Li, F., Li, T., Liu, Y., 2016. Proteomics-Based Identification of the Molecular Signatures of Liver Tissues from Aged Rats following Eight Weeks of Medium-Intensity Exercise. *Oxidative medicine and cellular longevity* 2016:3269405-3269405.
- [397] Arfianti, A., Pok, S., Barn, V., Haigh, W.G., Yeh, M.M., Ioannou, G.N., et al., 2020. Exercise retards hepatocarcinogenesis in obese mice independently of weight control. *Journal of Hepatology* 73(1):140-148.
- [398] Saran, U., Guarino, M., Rodríguez, S., Simillion, C., Montani, M., Foti, M., et al., 2018. Anti-tumoral effects of exercise on hepatocellular carcinoma growth. *Hepatology communications* 2(5):607-620.
- [399] Ji, Y., Luo, Z., Gao, H., Dos Reis, F.C.G., Bandyopadhyay, G., Jin, Z., et al., 2021. Hepatocyte-derived exosomes from early onset obese mice promote insulin sensitivity through miR-3075. *Nature Metabolism* 3(9):1163-1174.
- [400] Povero, D., Eguchi, A., Niesman, I.R., Andronikou, N., de Mollerat du Jeu, X., Mulya, A., et al., 2013. Lipid-induced toxicity stimulates hepatocytes to release angiogenic microparticles that require Vanin-1 for uptake by endothelial cells. *Science signaling* 6(296):ra88-ra88.
- [401] Mizunoe, Y., Kobayashi, M., Hoshino, S., Tagawa, R., Itagawa, R., Hoshino, A., et al., 2020. Cathepsin B overexpression induces degradation of perilipin 1 to cause lipid metabolism dysfunction in adipocytes. *Scientific Reports* 10(1):634.
- [402] Villa-Pérez, P., Merino, B., Fernández-Díaz, C.M., Ciudad, P., Lobatón, C.D., Moreno, A., et al., 2018. Liver-specific ablation of insulin-degrading enzyme causes hepatic insulin resistance and glucose intolerance, without affecting insulin clearance in mice. *Metabolism: clinical and experimental* 88:1-11.
- [403] Anderson, K.J., Russell, A.P., Foletta, V.C., 2015. NDRG2 promotes myoblast proliferation and caspase 3/7 activities during differentiation, and attenuates hydrogen peroxide - But not palmitate-induced toxicity. *FEBS Open Bio* 5:668-681.
- [404] Malaguarnera, L., Madeddu, R., Palio, E., Arena, N., Malaguarnera, M., 2005. Heme oxygenase-1 levels and oxidative stress-related parameters in non-alcoholic fatty liver disease patients. *J Hepatol* 42(4):585-591.
- [405] Zelcer, N., Tontonoz, P., 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. *The Journal of Clinical Investigation* 116(3):607-614.
- [406] Eichelbaum, K., Krijgsveld, J., 2014. Rapid temporal dynamics of transcription, protein synthesis, and secretion during macrophage activation. *Molecular & cellular proteomics : MCP* 13(3):792-810.
- [407] Rodríguez-Manzanque, J.C., Carpizo, D., Plaza-Calonge Mdel, C., Torres-Collado, A.X., Thai, S.N., Simons, M., et al., 2009. Cleavage of syndecan-4 by ADAMTS1 provokes defects in adhesion. *Int J Biochem Cell Biol* 41(4):800-810.
- [408] De Luca, M., Klimentidis, Y.C., Casazza, K., Moses Chambers, M., Cho, R., Harbison, S.T., et al., 2010. A Conserved Role for Syndecan Family Members in the Regulation of Whole-Body Energy Metabolism. *PLoS ONE* 5(6):e11286.
- [409] Daemen, S., Gemmink, A., Brouwers, B., Meex, R.C.R., Huntjens, P.R., Schaart, G., et al., 2018. Distinct lipid droplet characteristics and distribution unmask the apparent contradiction of the athlete's paradox. *Molecular Metabolism* 17:71-81.
- [410] Zhang, S., Wang, Y., Cui, L., Deng, Y., Xu, S., Yu, J., et al., 2016. Morphologically and Functionally Distinct Lipid Droplet Subpopulations. *Sci Rep* 6:29539.
- [411] Gemmink, A., Daemen, S., Brouwers, B., Hoeks, J., Schaart, G., Knoop, K., et al., 2021. Decoration of myocellular lipid droplets with perilipins as a marker for in vivo lipid droplet dynamics: A super-resolution microscopy study in trained athletes and

- insulin resistant individuals. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1866(2):158852.
- [412] Fernandez-Rojo, M.A., Ramm, G.A., 2016. Caveolin-1 Function in Liver Physiology and Disease. *Trends in Molecular Medicine* 22(10):889-904.
- [413] Mattern, H.M., Raikar, L.S., Hardin, C.D., 2009. The effect of caveolin-1 (Cav-1) on fatty acid uptake and CD36 localization and lipotoxicity in vascular smooth muscle (VSM) cells. *International journal of physiology, pathophysiology and pharmacology* 1(1):1-14.
- [414] Li, M., Chen, D., Huang, H., Wang, J., Wan, X., Xu, C., et al., 2017. Caveolin1 protects against diet induced hepatic lipid accumulation in mice. *PLoS ONE* 12(6):e0178748-e0178748.
- [415] Mathiesen, S.B., Lunde, M., Aronsen, J.M., Romaine, A., Kaupang, A., Martinsen, M., et al., 2019. The cardiac syndecan-4 interactome reveals a role for syndecan-4 in nuclear translocation of muscle LIM protein (MLP). *Journal of Biological Chemistry* 294(22):8717-8731.
- [416] Hao, J.-W., Wang, J., Guo, H., Zhao, Y.-Y., Sun, H.-H., Li, Y.-F., et al., 2020. CD36 facilitates fatty acid uptake by dynamic palmitoylation-regulated endocytosis. *Nature Communications* 11(1):4765.
- [417] Scarpellini, A., Huang, L., Burhan, I., Schroeder, N., Funck, M., Johnson, T.S., et al., 2014. Syndecan-4 Knockout Leads to Reduced Extracellular Transglutaminase-2 and Protects against Tubulointerstitial Fibrosis. *Journal of the American Society of Nephrology* 25(5):1013.
- [418] Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhez, F., Detmar, M., et al., 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J Clin Invest* 107(2):R9-r14.
- [419] Tanino, Y., Chang, M.Y., Wang, X., Gill, S.E., Skerrett, S., McGuire, J.K., et al., 2012. Syndecan-4 regulates early neutrophil migration and pulmonary inflammation in response to lipopolysaccharide. *Am J Respir Cell Mol Biol* 47(2):196-202.
- [420] Dong, J., Viswanathan, S., Adami, E., Singh, B.K., Chothani, S.P., Ng, B., et al., 2021. Hepatocyte-specific IL11 cis-signaling drives lipotoxicity and underlies the transition from NAFLD to NASH. *Nature Communications* 12(1):66.
- [421] Kamari, Y., Shaish, A., Vax, E., Shemesh, S., Kandel-Kfir, M., Arbel, Y., et al., 2011. Lack of interleukin-1 α or interleukin-1 β inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. *Journal of Hepatology* 55(5):1086-1094.
- [422] Kim, K., Kim, K.H., 2020. Targeting of Secretory Proteins as a Therapeutic Strategy for Treatment of Nonalcoholic Steatohepatitis (NASH). *International Journal of Molecular Sciences* 21(7):2296.
- [423] Flisiak-Jackiewicz, M., Bobrus-Chociej, A., Wasilewska, N., Tarasow, E., Wojtkowska, M., Lebensztejn, D.M., 2019. Can hepatokines be regarded as novel non-invasive serum biomarkers of intrahepatic lipid content in obese children? *Adv Med Sci* 64(2):280-284.
- [424] Zhang, X., Yeung, D.C., Karpisek, M., Stejskal, D., Zhou, Z.G., Liu, F., et al., 2008. Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes* 57(5):1246-1253.
- [425] Stastna, M., Van Eyk, J.E., 2012. Secreted proteins as a fundamental source for biomarker discovery. *Proteomics* 12(4-5):722-735.
- [426] Daniels, S.J., Leeming, D.J., Eslam, M., Hashem, A.M., Nielsen, M.J., Krag, A., et al., 2019. ADAPT: An Algorithm Incorporating PRO-C3 Accurately Identifies Patients With NAFLD and Advanced Fibrosis. *Hepatology* 69(3):1075-1086.

- [427] Vilar-Gomez, E., Chalasani, N., 2018. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *Journal of Hepatology* 68(2):305-315.
- [428] Nielsen, M.J., Veidal, S.S., Karsdal, M.A., Ørsnes-Leeming, D.J., Vainer, B., Gardner, S.D., et al., 2015. Plasma Pro-C3 (N-terminal type III collagen propeptide) predicts fibrosis progression in patients with chronic hepatitis C. *Liver Int* 35(2):429-437.
- [429] Kovesdy, C.P., Furth, S.L., Zoccali, C., World Kidney Day Steering, C., 2017. Obesity and Kidney Disease: Hidden Consequences of the Epidemic. *Canadian journal of kidney health and disease* 4:2054358117698669-2054358117698669.
- [430] Foster, M.C., Hwang, S.J., Larson, M.G., Lichtman, J.H., Parikh, N.I., Vasan, R.S., et al., 2008. Overweight, obesity, and the development of stage 3 CKD: the Framingham Heart Study. *Am J Kidney Dis* 52(1):39-48.
- [431] Genovese, F., Akhgar, A., Lim, S.S., Farris, A.B., Battle, M., Cobb, J., et al., 2021. Collagen Type III and VI Remodeling Biomarkers Are Associated with Kidney Fibrosis in Lupus Nephritis. *Kidney360* 2(9):1473.
- [432] Feizi, A., Gatto, F., Uhlen, M., Nielsen, J., 2017. Human protein secretory pathway genes are expressed in a tissue-specific pattern to match processing demands of the secretome. *npj Systems Biology and Applications* 3(1):22.
- [433] Sanz-García, C., Fernández-Iglesias, A., Gracia-Sancho, J., Arráez-Aybar, L.A., Nevzorova, Y.A., Cubero, F.J., 2021. The Space of Disse: The Liver Hub in Health and Disease. *Livers* 1(1).
- [434] Schulze, R.J., Schott, M.B., Casey, C.A., Tuma, P.L., McNiven, M.A., 2019. The cell biology of the hepatocyte: A membrane trafficking machine. *Journal of Cell Biology* 218(7):2096-2112.
- [435] Morton, R.E., Mihna, D., 2022. Apolipoprotein F concentration, activity, and the properties of LDL controlling ApoF activation in hyperlipidemic plasma. *Journal of Lipid Research* 63(2):100166.
- [436] Yang, H., Liu, Y., Zhao, M.-M., Guo, Q., Zheng, X.-K., Liu, D., et al., 2021. Therapeutic potential of targeting membrane-spanning proteoglycan SDC4 in hepatocellular carcinoma. *Cell death & disease* 12(5):492.
- [437] Romani, P., Brian, I., Santinon, G., Pocaterra, A., Audano, M., Pedretti, S., et al., 2019. Extracellular matrix mechanical cues regulate lipid metabolism through Lipin-1 and SREBP. *Nat Cell Biol* 21(3):338-347.
- [438] Bays, J.L., Campbell, H.K., Heidema, C., Sebbagh, M., DeMali, K.A., 2017. Linking E-cadherin mechanotransduction to cell metabolism through force-mediated activation of AMPK. *Nat Cell Biol* 19(6):724-731.
- [439] De Luca, M., Vecchie, D., Athmanathan, B., Gopalkrishna, S., Valcin, J.A., Swain, T.M., et al., 2019. Genetic Deletion of Syndecan-4 Alters Body Composition, Metabolic Phenotypes, and the Function of Metabolic Tissues in Female Mice Fed A High-Fat Diet. *Nutrients* 11(11):2810.
- [440] Sato, Y., Tanino, Y., Wang, X., Nikaido, T., Sato, S., Misa, K., et al., 2017. Baseline serum syndecan-4 predicts prognosis after the onset of acute exacerbation of idiopathic interstitial pneumonia. *PLoS ONE* 12(5):e0176789-e0176789.
- [441] Vanhoutte, D., Schellings, M.W., Götte, M., Swinnen, M., Herias, V., Wild, M.K., et al., 2007. Increased expression of syndecan-1 protects against cardiac dilatation and dysfunction after myocardial infarction. *Circulation* 115(4):475-482.
- [442] Vuong, T.T., Reine, T.M., Sudworth, A., Jenssen, T.G., Kolset, S.O., 2015. Syndecan-4 is a major syndecan in primary human endothelial cells in vitro, modulated

by inflammatory stimuli and involved in wound healing. *J Histochem Cytochem* 63(4):280-292.

[443] Strand, M.E., Aronsen, J.M., Braathen, B., Sjaastad, I., Kvaløy, H., Tønnessen, T., et al., 2015. Shedding of syndecan-4 promotes immune cell recruitment and mitigates cardiac dysfunction after lipopolysaccharide challenge in mice. *J Mol Cell Cardiol* 88:133-144.

[444] Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhez, F., Detmar, M., et al., 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *The Journal of Clinical Investigation* 107(2):R9-R14.

[445] Fan, W., Liu, T., Chen, W., Hammad, S., Longerich, T., Hausser, I., et al., 2019. ECM1 Prevents Activation of Transforming Growth Factor β , Hepatic Stellate Cells, and Fibrogenesis in Mice. *Gastroenterology* 157(5):1352-1367.e1313.

[446] Craft, C.S., Pietka, T.A., Schappe, T., Coleman, T., Combs, M.D., Klein, S., et al., 2014. The extracellular matrix protein MAGP1 supports thermogenesis and protects against obesity and diabetes through regulation of TGF- β . *Diabetes* 63(6):1920-1932.

[447] Naba, A., Clauser, K.R., Whittaker, C.A., Carr, S.A., Tanabe, K.K., Hynes, R.O., 2014. Extracellular matrix signatures of human primary metastatic colon cancers and their metastases to liver. *BMC Cancer* 14(1).

[448] Arteel, G.E., Naba, A., 2020. The liver matrisome – looking beyond collagens. *JHEP Reports* 2(4):100115.

[449] Kishore, U., Ghai, R., Greenhough, T.J., Shrive, A.K., Bonifati, D.M., Gadjeva, M.G., et al., 2004. Structural and functional anatomy of the globular domain of complement protein C1q. *Immunology letters* 95(2):113-128.

[450] Danussi, C., Petrucco, A., Wassermann, B., Pivetta, E., Modica, T.M., Del Bel Belluz, L., et al., 2011. EMILIN1- α 4/ α 9 integrin interaction inhibits dermal fibroblast and keratinocyte proliferation. *J Cell Biol* 195(1):131-145.

[451] Sekine, N., Yamashita, N., Kojima, I., Miyazaki, J., Ogata, E., 1994. Bimodal effect of transforming growth factor-beta on insulin secretion in MIN6 cells. *Diabetes Res Clin Pract* 26(1):7-14.

[452] Hershko, A., Ciechanover, A., 1998. The ubiquitin system. *Annu Rev Biochem* 67:425-479.

[453] Yedgar, S., Carew, T.E., Pittman, R.C., Beltz, W.F., Steinberg, D., 1983. Tissue sites of catabolism of albumin in rabbits. *American Journal of Physiology-Endocrinology and Metabolism* 244(1):E101-E107.

[454] Eden, E., Geva-Zatorsky, N., Issaeva, I., Cohen, A., Dekel, E., Danon, T., et al., 2011. Proteome Half-Life Dynamics in Living Human Cells. *Science* 331(6018):764-768.

[455] Dufour, J.-F., Caussy, C., Loomba, R., 2020. Combination therapy for non-alcoholic steatohepatitis: rationale, opportunities and challenges. *Gut* 69(10):1877.

[456] Corey, K.E., Chalasani, N., 2011. Should combination therapy be the paradigm for future nonalcoholic steatohepatitis clinical trials? *Hepatology (Baltimore, Md.)* 54(5):1503-1505.

[457] Alkhouri, N., Lawitz, E., Noureddin, M., DeFronzo, R., Shulman, G.I., 2020. GS-0976 (Firsocostat): an investigational liver-directed acetyl-CoA carboxylase (ACC) inhibitor for the treatment of non-alcoholic steatohepatitis (NASH). *Expert opinion on investigational drugs* 29(2):135-141.

[458] Savage, D.B., Choi, C.S., Samuel, V.T., Liu, Z.-X., Zhang, D., Wang, A., et al., 2006. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *The Journal of Clinical Investigation* 116(3):817-824.

- [459] Ross, T.T., Crowley, C., Kelly, K.L., Rinaldi, A., Beebe, D.A., Lech, M.P., et al., 2020. Acetyl-CoA Carboxylase Inhibition Improves Multiple Dimensions of NASH Pathogenesis in Model Systems. *Cell Mol Gastroenterol Hepatol* 10(4):829-851.
- [460] Stiede, K., Miao, W., Blanchette, H.S., Beysen, C., Harriman, G., Harwood, H.J., Jr., et al., 2017. Acetyl-coenzyme A carboxylase inhibition reduces de novo lipogenesis in overweight male subjects: A randomized, double-blind, crossover study. *Hepatology* 66(2):324-334.
- [461] Loomba, R., Kayali, Z., Nouredin, M., Ruane, P., Lawitz, E.J., Bennett, M., et al., 2018. GS-0976 Reduces Hepatic Steatosis and Fibrosis Markers in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 155(5):1463-1473.e1466.
- [462] Kim, C.W., Addy, C., Kusunoki, J., Anderson, N.N., Deja, S., Fu, X., et al., 2017. Acetyl CoA Carboxylase Inhibition Reduces Hepatic Steatosis but Elevates Plasma Triglycerides in Mice and Humans: A Bedside to Bench Investigation. *Cell Metab* 26(2):394-406.e396.
- [463] Loomba, R., Nouredin, M., Kowdley, K.V., Kohli, A., Sheikh, A., Neff, G., et al., 2021. Combination Therapies Including Cilofexor and Firsocostat for Bridging Fibrosis and Cirrhosis Attributable to NASH. *Hepatology* 73(2):625-643.
- [464] Calle, R.A., Amin, N.B., Carvajal-Gonzalez, S., Ross, T.T., Bergman, A., Aggarwal, S., et al., 2021. ACC inhibitor alone or co-administered with a DGAT2 inhibitor in patients with non-alcoholic fatty liver disease: two parallel, placebo-controlled, randomized phase 2a trials. *Nat Med* 27(10):1836-1848.
- [465] Henriksson, J., 1995. Influence of exercise on insulin sensitivity. *J Cardiovasc Risk* 2(4):303-309.
- [466] Gregory, J.M., Muldowney, J.A., Engelhardt, B.G., Tyree, R., Marks-Shulman, P., Silver, H.J., et al., 2019. Aerobic exercise training improves hepatic and muscle insulin sensitivity, but reduces splanchnic glucose uptake in obese humans with type 2 diabetes. *Nutrition & diabetes* 9(1):25.
- [467] McGee, S.L., Hargreaves, M., 2020. Exercise adaptations: molecular mechanisms and potential targets for therapeutic benefit. *Nature Reviews Endocrinology* 16(9):495-505.
- [468] Berzigotti, A., Saran, U., Dufour, J.-F., 2016. Physical activity and liver diseases. *Hepatology* 63(3):1026-1040.
- [469] Jakovljevic, D.G., Hallsworth, K., Zalewski, P., Thoma, C., Klawe, J.J., Day, C.P., et al., 2013. Resistance exercise improves autonomic regulation at rest and haemodynamic response to exercise in non-alcoholic fatty liver disease. *Clin Sci (Lond)* 125(3):143-149.