Lean NAFLD: A distinct entity shaped by differential metabolic adaptation

Dr. Fei Wen Chen

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Medicine

University of Sydney

2020

DECLARATION

Unless otherwise acknowledged, the work described within this thesis was carried out personally by the author, at the Westmead Institute of Medical Research and University of Sydney, between March 2017 and December 2019.

None of this work has been submitted previously for the purpose of obtaining any other degree.

Fei Wen Chen

ACKNOWLEDGEMENTS

Undertaking this PhD has been a truly life-changing experience that would not have been possible without the support from key people and organisations.

Firstly, I would like to express my sincere gratitude to my supervisors for this project, Professor Jacob George and A/Prof Mohammed Eslam, without whom this project would not have been possible. Thank you very much for your guidance, patience, feedback and motivation and for always believing in the potential that this project has despite the many setbacks and doubts casted by many.

I would also like to thank the Storr liver unit, all the scientists, nurses, patients, collaborators as well as the funding bodies, the Research Training Program scholarship and the Westmead Institute for Medical Research Top-up grant, for their time, intellectual and financial contribution towards this PhD.

A very heartfelt thank you to my family and their support during my PhD journey. My parents, Thiam Wong Chen and Meh Nio Kwan, who are both scientists themselves, have been my constant rock of support during every ups and downs of this journey. My mum, especially, who has spent as much time travelling between Kuala Lumpur, China and Sydney, as I have preparing this thesis, juggling between work, caring for my father, my other siblings, her other grandchildren and me, in order to lend me a hand so I could finish this PhD on time.

To my wonderful husband, Joko Boentarya, father of our beautiful daughter Josie Boentarya and our newborn son Jordan, who has put up with me through all these years of medical studies and training, and my PhD journey. Thank you for always being by my side through all this, keeping our family together and helping me cope with motherhood, being a student and a doctor at the same time.

And to all my friends and colleagues, and all those I have not mentioned, thank you for sparing your time and ears, and being a part of this memorable journey, without your support this project would not have been possible.

TABLE OF CONTENTS

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) prevalence is growing dramatically with epidemic of obesity. A subset of patients with NAFLD is lean, but the pathophysiology of this sub-group is still not well known. This project aims to investigate the roles of metabolic health and metabolic adaptation in the pathogenesis of lean NAFLD, using well-characterised Caucasian subjects with lean and non-lean NAFLD, and comparing them with the lean and non-lean healthy controls, and murine models. We investigated in detail their demographics, genetic background, bile acid profile, gut microbiota and their bile acid regulatory activity to further understand the underlying pathophysiology governing the development and progression of lean NAFLD. We then compared our findings in humans with that of mice models of lean and non-lean NAFLD. Finally, we performed an untargeted metabolomics analysis on lean and non-lean NAFLD patients to determine other metabolic pathways and biomarkers, which may be relevant to lean NAFLD.

PUBLICATIONS

Chen, F., et al.; *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation;* Hepatology (2020 Apr), 71(4), 1213-1227 DOI: 10.1002/hep.30908

Chen, F; George, J; Eslam, M; *Lean NAFLD in Asians*; accepted for publication in Clinical liver disease on 28 December 2019; DOI: 10.1002/cld.930

Chen, F, et al.; *The role of metabolic health and metabolic adaptation in lean NAFLD;* invited review and submitted for publication in Nature Reviews Gastroenterology and Hepatology 28 January 2020; manuscript no: NRGH-19-237V1

AWARDS AND GRANTS

- The University of Sydney and Commonwealth Government Research Training Program Scholarship
- Westmead Institute of Medical Research Top-Up Grant
- CPC Westmead Research Forum – winner of Rapid Research 3-minute Oral Presentation Competition August 2018
- Westmead Hospital Week Research Symposium – winner for Best Oral Presentation August 2019
- Westmead clinical school postgraduate seminar – winner September 2019
- Jerry Koutts Young Investigator Award 2019 – Runner-up

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ASBT – Apical sodium bile acid transporter

BA – Bile acids

BMI – Body mass index

BSL – Blood sugar levels

ChR – Choledsterol rich

CRN – Clinical research network

DCA – Deoxycholic acid

FC – Fold change

FGF-19/fgf-15 – Fibroblast growth factor 19/15

FXR – Farsenoid-X-Receptor

GLP-1 – Glucagon-like Peptide -1

HCA – Hierarchical cluster analysis

HS – High sucrose

LCA – Lithocholic acid

NAFLD – Non-alcoholic fatty liver disease

NAS – NAFLD activity scores

NASH – Non-alcoholic steatohepatitis

SAT – Subcutaneous adipose tissue

TGR-5 – Takeda-G receptor 5

UDCA – Ursodeoxycholic acid

VAT – Visceral adipose tissue

WHR – Waist hip ratio

CHAPTER ONE

INTRODUCTION

The majority of this chapter has been submitted for publication in *Nature Reviews Gastroenterology and Hepatology*, and is currently in revision:

Chen, F, et al.; *The role of metabolic health and metabolic adaptation in lean NAFLD;* invited review and submitted for publication in Nature Reviews Gastroenterology and Hepatology, 28 January 2020; manuscript no: NRGH-19-237V1

1 INTRODUCTION

1.1 BURDEN OF LIVER DISEASE

Chronic liver disease affects a major proportion of the global population and accounts for about 2 million deaths worldwide, which is roughly 3.5% of all deaths, an increase in rate from the previous reported rate of 3% in the year 2000 (Asrani et al. 2019). At least half of the mortalities caused by chronic liver disease is contributed by liver cirrhosis, where it ranks within the top 20 causes of deaths globally (Asrani et al. 2019). The aetiology and distribution of chronic liver disease varies geographically, where non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease account for the majority of causes of cirrhosis in Western countries and viral hepatitis still remains the predominant cause of cirrhosis in Asian countries (Lozano et al. 2012). In addition, NAFLD represents a significant economic burden to society that reduces quality of life including through increased symptoms of fatigue and decreased mental well-being. This affects how well a person is able to function in their daily activities (Sayiner et al. 2016). In the United States alone, patients with NAFLD are reported to have higher annual health care expenditure ($19,390 versus $5,567) with higher rates of unemployment (55% versus 30%) and disability related unemployment (30.5% versus 6.6%) compared to those without chronic liver disease. In Europe, NAFLD is estimated to have an annual cost of about €35billion (from €354 to €1,163 per patient; highest in patients aged 45-65) (Stepanova et al. 2017; Z. M. Younossi et al. 2016b).

1.2 EPIDEMIOLOGY AND DEFINITION OF NAFLD

Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause for liver disorder in Western industrialized nations, with a prevalence ranging between 6 – 35% (median 20%) worldwide (Bellentani 2017). NAFLD can be defined as the presence of more than 5% hepatic steatosis without evidence of hepatocellular injury in the form of hepatocyte ballooning, in the absence of other causes for secondary hepatic fat accumulation such as excessive alcohol consumption or use of steatogenic medications (Chalasani et al. 2012; Le et al. 2017). The majority of NAFLD cases are associated with presence of metabolic risk factors such as obesity, diabetes, hypertension and dysipidaemia (Le et al. 2017). This is reflected with the parallel increase of metabolic syndrome (the Adult Treatment Panel III diagnosis of metabolic syndrome requires the presence of at least three of: waist circumference >102cm in men and >88cm in women, triglyceride level of 150 mg/dL (or 1.7 mmol/L) or greater, high density lipoprotein (HDL) level of less than 40 mg/dL (or 1.0 mmol/L) in men and less than 50 mg/dL (or 1.3 mmol/L) in women or use of lipid medications, systolic blood pressure greater than 130mmHg or diastolic blood pressure greater than 85mmHg or use of anti-hypertensive medications, and fasting plasma glucose level of 110 mg/dL (or 5.6 mmol/L) or greater or use of diabetic medications (Chalasani et al. 2012)) with NAFLD in Western countries (Le et al. 2017).

Although the majority of NAFLD is associated with obesity, a small but significant proportion of patients with NAFLD do not have obesity. This sub-group, also known as "lean NAFLD" has been understudied in the literature and will be the focus of this thesis.

1.3 FACTORS AFFECTING NAFLD DEVELOPMENT

1.3.1 Modifiable risk factors

Lifestyle factors

Studies have shown that lifestyle habits may partially explain the heterogeneity of metabolic health and non-alcoholic fatty liver disease. Even overweight and obese individuals may have the same overall mortality risk as normal weight people. Population cross-sectional studies have shown that metabolically healthy obesity is more prevalent in younger and female adults, and that these individuals are more likely to exercise and less likely to smoke or drink heavily (Goday et al. 2016; Matheson et al. 2012). A recent study estimated the prevalence of insufficient physical activity at around 23.3% in 2010 (Hallal et al. 2012). Alarmingly, a more recent report on worldwide trends in physical inactivity between 2001-2016 suggested that the prevalence of physical inactivity has not altered since 2001, with the rate being twice as high in high income countries, and rising over time (Guthold et al. 2018).

The possible underlying mechanism governing this may lie in how individuals modulate whole body energy metabolism, as evidenced by the fact that concurrent physical activity increases fatty acid oxidation during high calorie intake period (S. R. Smith et al. 2000). In addition, lower fasting respiratory quotient has been shown to be positively associated with the ability to extract energy from fat (Pujia et al. 2016). Other lifestyle factors which have been shown to contribute to cardiometabolic health risk include sleep duration and sleep quality factors (Koren and Taveras 2018). A study in China has

found that patients with NAFLD have shorter duration of sleep compared to healthy controls (C. Li et al. 2019). The most commonly used definition of healthy lifestyle include the adaptation of four healthy habits which include moderate alcohol intake, not smoking, 30 minutes of exercise daily and eating five or more servings of vegetables and fruits daily (Matheson et al. 2012).

Role of diet and microbiome

About 10-100 trillion micro-organisms composed of bacteria, fungi, archae, and viruses live inside or on the human body. The majority of these microbial symbionts (collectively known as the microbiota) reside within the digestive tract (Turnbaugh et al. 2007). Four main phyla of bacteria make up the human microbiome: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. The gut microbiome plays a role in the bile acid pathway in the conversion of primary to secondary bile acids (bile acids will be discussed in more detail in later section). The conversion of primary bile acids to secondary bile acids require the initial deconjugation by Bile Salt Hydrolase (BSH) before downstream modifications by 7-alpha dehydroxylase to produce deoxycholic acid (DCA) and lithocholic acid (LCA) or by 7-alpha hydroxysteroid dehydrogenase to produce ursodeoxycholic acid (UDCA) (Jiao et al. 2017; Ridlon et al. 2006). BSH activity is present in all major gut bacterial species, however the conversion of primary to secondary bile acid by 7-dehydroxylation are carried out only by bacteria with bile acid inducible genes. These include those belonging to the genera *Clostridium* (clusters XIVa and XI), *Eubacterium, Blautia, Ruminococcaceae* and *Lachnospiraceae*, all of which belong to the *Firmicutes* phylum (Wahlstrom et al. 2016a; Yokota et al. 2012).

Given the anatomical link between the intestine and the liver, the microbiota has been proposed to play a role in the pathogenesis of various hepatic pathologies. A number of studies have demonstrated the role of intestinal dysbiosis in a variety of human diseases, including NAFLD (Wieland et al. 2015). These studies have been performed using both murine models as well as in humans. Unfortunately, due to the large variation in the study design, population, sample sizes, and clinical endpoints, the results remain controversial and difficult to interpret. Common to all published studies in the literature on the role of microbiome in NAFLD, however, is that these studies have demonstrated a measurable difference in the microbiome between different stages of NAFLD and NASH, compared to their healthy controls. However, causality has not been proven and further studies are required to delineate mechanistic links.

Multiple factors influence microbiome composition. These include age, BMI, genetics and diet (Wu et al. 2011). Diet has been shown to exert predominant effect on microbiome composition, irrespective of the host's genotypes (Carmody 2015). Studies have shown that long-term dietary habits are strongly associated with specific enterotype clustering of microbiota, where *Bacteroides* enterotype was associated with subjects who have diet rich in animal fat and protein and the *Prevotella* enterotype with individuals with carbohydrate rich diet (De Filippo et al. 2010; Wu et al. 2011). Of interest, it has been suggested that cholesterol intake is higher in lean compared to obese NAFLD (Enjoji et al. 2012; Musso et al. 2003; Yasutake et al. 2009b). Furthermore, although alterations in diet have been shown to change the microbiota composition within 24 hours, an individual's enterotype identity is only affected by their long-term dietary habit (Wu et al. 2011). Few studies on the microbiota profile of patients in lean and non-lean NAFLD have demonstrated evidence of microbial dysbiosis in lean

NAFLD compared with non-lean NAFLD and healthy controls, but the specific changes, as well as the role of these changes in the pathophysiology of lean NAFLD remain controversial (B. Wang et al. 2016; Wieland et al. 2015).

Role of bile acids and its regulators

The pathogenesis of NAFLD and the progression of NAFLD from simple steatosis have not been fully understood. It has been thought that certain factors such as genetic predisposition, insulin resistance, inflammatory events involving mediators such as endotoxins, adiponectin, oxidative stress as well as hepatotoxic bile acid played a role in the disease progression (Arab et al. 2017; Perez and Briz 2009). Bile acids are steroid molecules synthesized in the liver from cholesterol. The primary bile acids chenodeoxycholate (CDCA) and cholate (CA) are synthesized from the cholesterol in the liver, conjugated into their taurine or glycine conjugates and excreted into the bile, where they assist in fat emulsification and absorption. Primary bile acids also undergo conversion into the secondary bile acids deoxycholate (DCA), lithocolic acid (LCA) and ursodeoxycholic acid (UDCA) by the intestinal bacteria and mostly reabsorbed in the distal ileum via the enterohepatic circulation (Arab et al. 2017; Khalid et al. 2015). Besides their role in the digestion and absorption of fat and fat soluble vitamins, bile acids have also been recognised as signalling molecules involved in the regulation of lipid and glucose metabolism, as well as inflammatory modulators in the liver and several other tissues (Arab et al. 2017; Chavez-Talavera et al. 2017; Khalid et al. 2015). This is mediated through their actions on specific bile acid receptors, including members of the farsenoid X receptor (FXR), pregnane X receptor (PXR), Vitamin D receptor and Takeda G protein coupled receptor 5 (TGR5) (Arab et al. 2017). The

binding of bile acids to the FXR in the ileocytes trigger the transcription and production of Fibroblast Growth Factor 19 (FGF-19), which is then transported to the liver where it binds to the tyrosine kinase FGF receptor 4 (FGF4R4) (Khalid et al. 2015). This then activates the c-Jun N terminal-kinases 1/2 signalling pathways which subsequently down regulates the CYP7A1, a key cytochrome P450 enzyme in the bile acid synthesis pathway (Khalid et al. 2015). FXR can be stimulated by most bile acids, although at varying potency, with CDCA displaying the highest potency, followed by LCA and DCA then CA (Khalid et al. 2015). In addition to regulating bile acid synthesis, the FGF-19 have also been shown to play a significant role in the glucose and cholesterol homeostasis, by promoting hepatic glycogen storage, fatty acid beta oxidation and decreasing hepatic lipogenesis (Arab et al. 2017; Khalid et al. 2015). The enzymes involved in bile acid synthesis are controlled tightly in response to the changing metabolic conditions, and dysregulation of bile acid synthesis and metabolism is often an indication of liver dysfunction. Bile acid levels, therefore, have been recognized as sensitive indicators of hepatobiliary diseases and have been implicated to play a role in several diseases including alcoholic and non-alcoholic fatty liver diseases (Xie et al. 2015).

Given the limited resources in the drug treatment of NAFLD other than promotion of lifestyle changes in diet and exercise habits as well as control of comorbidities (type 2 diabetes, hypertension and dyslipidaemia), bile acid (BA) derivatives and compounds that influence BA-related signalling pathways are emerging as potentially useful therapeutic agents for NAFLD and NASH.

For metabolic homeostasis, in addition to the neuroendocrine axis, caloric intake and physical activity, the enterohepatic circulation, including bile acids (BA) and their metabolites, and gut microbiota are intimately involved. Bile acids are the principal route for cholesterol catabolism, and recent evidence demonstrates that a high intake of dietary cholesterol (Ioannou et al. 2009), elevated levels of hepatic cholesterol (Min et al. 2012; Puri et al. 2007; Simonen et al. 2013; Van Rooyen et al. 2011) and disrupted hepatic cholesterol homeostasis are pivotal drivers of the pathogenesis of NAFLD (Arab et al. 2017; Puri et al. 2007; Simonen et al. 2013). Despite the increasing number of studies, our knowledge into the role of bile acids in NAFLD pathogenesis and progression is still incomplete. A number of studies looking at the metabolomics profile of patients with NAFLD/NASH have shown that the progression from NAFLD to NASH is characterised by increase in total serum bile acid concentration as well as variations in the level of primary and secondary bile acid compositions (Ferslew et al. 2015; Jiao et al. 2017; Kalhan et al. 2011; Puri et al. 2017). Interestingly, total bile acid concentration was also found to be higher in patients who have achieved weight loss through previous gastric bypass as compared to those without previous gastric bypass with similar preoperative or current BMI, possibly through improved insulin sensitivity (Dutia et al. 2015; Kohli et al. 2013; Legry et al. 2017; Patti et al. 2009; Sachdev et al. 2016; Werling et al. 2013). There is strong evidence that activation of bile acid signalling induces improvements in metabolic (glucose and lipid) phenotype in murine models (Pierre et al. 2016). Furthermore, in human and murine models, elevated bile acids play a role in the metabolic improvements after bariatric surgery, including in type 2 diabetes, dyslipidaemia and NASH resolution, even before significant weight loss (A. P. Chambers et al. 2011; Kohli et al. 2015; Patti et al. 2009; Pournaras et al. 2012). The role and regulation of bile acid, through its specific

nuclear receptors such as the Farsenoid X receptor (FXR), in the pathogenesis and treatment of NAFLD has been described in multiple studies (Khalid et al. 2015; Mudaliar et al. 2013; Puri et al. 2017). Activation of FXR receptors by binding of bile acids or with FXR agonists has been shown to result in improvement of lipid and glucose metabolism through the release of Fibroblast Growth Factor 19 (FGF-19), which not only results in down regulation of CYP7A1, the rate-limiting enzyme in bile acid synthesis but also in promotion of fatty acid beta oxidation and inhibition of glycogen synthesis (Arab et al. 2017; Khalid et al. 2015). Activation of FXR activity on the hepatic stellate cells has also been shown to provide protection against liver fibrosis in murine models (Schumacher et al. 2020).A number of drugs targeting the bile acid pathways such as the FXR agonists Obeticholic acid and FGF-19 analogue NGM282, are currently in phase 3 and 2 clinical trials respectively for the treatment of NAFLD, and have been shown to improve liver histology in patients with NAFLD (Harrison et al. 2018; Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015).

Interestingly, insulin resistance improved only with FGF-19 analogue treatment, but not with FXR agonist treatment (Harrison et al. 2018; Neuschwander-Tetri et al. 2015). This suggests that there may be another receptor which, together with the FXR receptors, play a role in regulating metabolic pathways involved in mediating glucose homeostasis and insulin resistance. Indeed, recent studies looking at the role of bile acid in murine models undergoing bile acid diversion surgery to mimic Roux-en-Y gastric bypass described the importance of FXR receptors and Takeda G-protein coupled receptors 5 (TGR 5) in metabolic improvements post bariatric surgery, through the down-stream production of Glucagon-like-peptide 1 (GLP-1) (Albaugh et al. 2019; Pierre et al. 2019). Studies regarding the effect of weight loss after bariatric surgery on

the intestinal FXR and TGR5 activities have been controversial. Some mice studies have shown that intestinal FXR and TGR5 are inversely related, however studies on the effect of intestinal FXR agonist Fexaramine have shown increased GLP-1 secretion and improved glucose tolerance in mice (Browning et al. 2019; Pathak et al. 2018; Trabelsi et al. 2015). Due to inter-species differences in BA physiology, these findings have not been directly extended into human studies, and findings regarding BA physiology changes post bariatric surgery in humans also remain conflicting. Previous studies have shown that the gene expression of the major BA target, the FXR, is increased in the liver but decreased in the small intestine, whereas the intestinal TGR5 receptor is increased after Roux-en-Y gastric bypass in obese patients (Browning et al. 2019). However, along with the increased BA levels, both FGF-19 and GLP-1 levels have also been shown to increase post bariatric surgery, which are presumed to support the metabolic benefits post bariatric surgery (Browning et al. 2019; Cole et al. 2015).

FXR and TGR5 receptors are also differentially expressed in adipocytes, FXR in white, and TGR5 in brown adipocytes, respectively, as well as in certain immune-inflammatory cells in adipocytes (E. P. Broeders 2015). In adipocytes, FXR regulates the differentiation and functions of adipocytes and promotes peroxisome proliferator-activated receptor-γ (PPARγ) activity which interferes with the Wnt/β-catenin pathway, while TGR5 activates the thyroid hormone receptor to uncouple mitochondrial function and increase thermogenesis in brown adipose tissue, which further contributes to their anti-inflammatory and insulin-sensitizing effects (Abdelkarim et al. 2010; Watanabe et al. 2006). The enzymes involved in bile acid synthesis are controlled tightly in response to changing metabolic conditions and metabolic alterations, along with chronic low-

grade inflammation, which are characteristics of meta-inflammatory disorders such as obesity, type 2 diabetes and NAFLD (Chavez-Talavera et al. 2017; Xie et al. 2015).

Therefore, the interplay between an individual's lifestyle factors, combined with their microbiota and bile acid profile, shaped in part by their dietary composition and genetic as well as epigenetic backgrounds, has a significant impact on an individual's overall metabolic health. This in turn governs the risk for metabolic disorders, including NAFLD.

1.3.2 **Non-modifiable risk factors**

Genetic factors

Genetic factors in NAFLD

NAFLD is a complex disease phenotype. Multiple twins and familial studies have shown that first degree relatives of NAFLD patients are at increased risk of the disease than the general population and that about 50% of hepatic fat are inherited. The hepatic fat content subsequently affects the risk of metabolic disease and liver fibrosis (Eslam et al. 2018a; Schwimmer et al. 2009).

In addition, NAFLD also demonstrates interethnic variability as shown in multiple epidemiological studies. Individuals from South America, Asia, Hispanic descents in the United States are at increased risk of NAFLD, whereas those of European and

African descent showed lower NAFLD prevalence, irrespective of the socioeconomic status, insulin resistance and adiposity (Guerrero et al. 2009; Z. M. Younossi et al. 2016a).

Various genome-wide association studies (GWAS) in the last decade have been the focus of extensive research to elucidate the role of genetic influence in many disease processes, including NAFLD. The discovery of the single nucleotide polymorphism (SNP) rs738409 C/G variant in *PNPLA3* (patatin-like phospholipase domain containing 3, also known as adiponutrin or calcium-independent phospholipase A2-epsilon), which is now regarded as a major genetic component of NAFLD/NASH in the first GWAS of NAFLD has significantly contributed to our understanding of the genetic component of the disease. This SNP encodes for the amino acid substitution I148M and is significantly associated with the accumulation of fat in the liver as well as histological severity and progression of NAFLD (Romeo et al. 2008; Sookoian and Pirola 2011). This substitution induces loss of function of the enzymatic hydrolase activity, resulting in entrapment of triglycerides and retinyl esters in lipid droplets of hepatocytes and hepatic stellate cells, and subsequently leading to liver damage and accumulation of extracellular protein, with the end result being liver fibrosis development (Eslam et al. 2018a). The expression of rs738409 C>G in PNPLA3 allele was found to be higher in Asian lean NAFLD compared to obese NAFLD, although in another study involving Western lean NAFLD, there was no significant difference found in the frequency of the allele expression between lean and non-lean group(Fracanzani et al. 2017; Wei et al. 2015). Nevertheless, studies have demonstrated independent association of this allele with NASH development and higher degree of fibrosis (grade 2 or more) in lean NAFLD (Fracanzani et al. 2017; Wei et al. 2015). In addition, the rs738409 variant also

explains some sexual dysmorphism in NAFLD, with higher effect seen in women compared with men (Sookoian and Pirola 2011).

Aside from the discovery of highly replicated variant such as rs738409, other variants in multiple loci with diverse functions in NAFLD have also been uncovered through GWAS. These include variants in multi-gene locus called NCAN/TM6SF2/CILP2/PBX4 located in the TM6SF2 (transmembrane 6 superfamily member 2) gene, which is non-synonymous for the rs58542926 variant, which encodes the amino acid substitution p.Glu167Lys (E167K) involved in the enrichment of triglycerides to apolipoprotein B100 in the pathway of very low density lipoprotein secretion from hepatocytes (Eslam et al. 2016b). Carriers of this mutation has been shown to have higher liver triglyceride content and lower circulating lipoproteins, resulting in greater risk of NAFLD progression but interestingly lower risk of cardiovascular diseases (Eslam et al. 2018a). Additionally, in a recent study, carriers of this variant were significantly associated with increased endotoxemia and elevated alanine transaminase level as well as increased hepatic triglyceride content independent of obesity, insulin resistance and alcohol intake (Kozlitina et al. 2014; Pang et al. 2017). Compared to obese NAFLD, lean NAFLD has been shown to carry higher prevalence of rs58542926 C>T in TM6SF2 allele (Fracanzani et al. 2017).

Another variant discovered through NAFLD-GWAS was the variant rs780094 in the *GCKR* (glucokinase regulatory gene), whose missense is associated with a modest risk of having a fatty liver, with pooled odds ratio of 1.25 (Speliotes et al. 2011; Zain et al. 2015). A number of other variants have been found to be associated with NAFLD

disease severity and progression, but all these variants have quite a diverse effect on NAFLD susceptibility, conferring only a small to moderate increment in risk, and explaining only a minor proportion of familial clustering (Manolio et al. 2009).

Recently, the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) rs641738 C>T variant was found to be associated with the risk of NAFLD, inflammation and fibrosis, as well as risk of NAFLD progression to hepatocellular carcinoma (HCC). This protein is involved in the remodelling of phosphatidylinositol with arachidonic acid as part of the Land's cycle. The rs641738 C>T variant results in downregulation of MBOAT7 at an mRNA and protein level, which subsequently reduces the level of phosphatidyl-inositol containing arachidonic acid in hepatocytes and in the circulation (Mancina et al. 2016).

While gene polymorphisms undoubtedly play a role in development of liver fibrosis in NAFLD, it does not fully explain the inter-individual variability in the rate of fibrosis development (Zeybel et al. 2015). In the recent study looking into the role of genetic polymorphism on the pathogenesis of NAFLD in non-obese patients, for example, no significant association has been found between the presence of the alleles *PNPLA3 and TM6SF2* with histological severity (J. C. Leung et al. 2017a). Furthermore, there is increasing understanding that in most complex disease and phenotype, the predictive value of these genetic variants towards clinical practice outcome is only limited (Hardy and Mann 2016). It has become more apparent that studies looking at the gene-gene or gene-environment factors may improve our understanding of the inter-patient variability on the disease progression (Eslam and George 2016; Hardy et al. 2016).

Genetic contribution of obesity and fat distribution

Considerable inter-individual variation exists with regards to metabolic risk for a given BMI. The evidence for the role of genetics in determining how an individual respond to excess energy dates back to more than 25 years ago where a study involving 12 pairs of identical twins showed variations in weight gain and fat distribution among the pairs in response to overfeeding(Bouchard et al. 1990). The waist hip ratio, which has been used as surrogate measure of regional fat distribution is estimated to be heritable in up to 60%, independent of the risk for overall obesity (Schleinitz et al. 2014). With the era of genome wide association studies (GWAS), several genetic loci have been identified to be involved in regulating obesity and controlling body extra fat distribution as well as the metabolic profile of excess adiposity (i.e. metabolically healthy obesity vs metabolically unhealthy obesity)(Iacobini et al. 2019). The single nucleotide polymorphism (SNP) near *MC4R* gene has been involved in obesity and remained one of the major loci associated with waist circumference (J. C. Chambers et al. 2008). A meta-analysis of GWAS in 2010 have uncovered 13 loci associated with WHR adjusted for BMI (*RSPO3, VEGFA, NISCH-STAB1*, *TBX15-WARS2, NFE2L3, GRB14-COBLL1, DNM3-PIGC, ITPR2-SSPN, LY86, HOXC13, ADAMTS9, ZNRF3-KREMEN1, CPEB4*) and the known association signal at *LYPLAL1* involved in lipase activity was also confirmed, with effect sizes reaching 0.059 per risk allele in women (Heid et al. 2010). Many of these loci have also been showed to be associated with metabolic traits such as fasting glucose, insulin, adiponectin levels and BMI, as well as with metabolic conditions such as type 2 diabetes, hypertension and coronary artery disease (Kilpelainen et al. 2011; Schleinitz et al. 2014). Several genetic variants have also been associated with lower risk of metabolic abnormalities despite BMI in the obese range (Yaghootkar et al. 2016). Interestingly, the same genetic variants have also been shown

to share similar pattern of metabolic trait association with the monogenic lipodystrophy phenotype, including lower BMI, higher VAT to SAT ratio, impaired insulin sensitivity and increased risk of type 2 diabetes (Iacobini et al. 2019; Yaghootkar et al. 2014). This suggests that unlike in specific altered fat distribution condition like lipodystrophy where there is a clear genetic mutation involved, there seems to be a polygenic nature influencing fat distribution (visceral vs subcutaneous) and metabolic trait, with further influence from other factors such as epigenetic, environmental and biologic factors (Schleinitz et al. 2014).

Epigenetic factors

Despite the advances of genetic analyses to identify polymorphisms associated with WHR and fat distribution, these can only explain a small proportion of phenotypic variance and genetic heritability. Therefore, other factors linking genetic to environmental factors such as epigenetics need to be considered. The study of epigenetics encompasses the study of how non-genetic factors act on the gene and affect its expression and phenotype (Hardy and Mann 2016). The epigenetic mechanisms act as interphase between an individual's genetic background and his environmental cues and are dynamically regulated throughout the individual's lifetime (Hardy and Mann 2016). These mechanisms comprise of DNA methylation, histone modifications and chromatin remodeling, and non-coding RNAs (Hardy and Mann 2016).

DNA methylation refers to the addition of a methyl group to the fifth carbon position on the cytosine base in the cytosine-phosphoguanine (CpG) dinucleotide region to form 5-

methylcytosine. This process occurs throughout the genome, but when it occurs in the gene promoter region, in the region rich in CpG dinucleotides (CpG islands), this then causes gene repression by affecting its ability to affect transcription factor binding and chromatin structure (Bergman and Cedar 2013; Bian et al. 2013). DNA methylation profile is not inherited from the gametes, but rather re-established at the time of implantation at the very early embryo stage. This profile is then maintained through every cell division and plays an important role in various important processes including genomic imprinting, embryonic development, cellular differentiation and chromosomal stability (Bergman and Cedar 2013; Zeybel et al. 2015).

Two distinct classes of enzymes, namely the DNA methyltransferase (DNMT1, DNMT3a and DNMT 3b) and the Ten-Eleven Translocation (TET1-3) enzymes, regulate the process of DNA methylation (Hardy and Mann 2016). DNA methyltransferase 1 (DNMT1) is responsible for maintaining DNA methylation profile in daughter cells during mitosis, whereas DNMT3a and DNMT3b are responsible for regulating de novo DNA methylation in the absence of cell division. TET enzymes, on the other hand, are responsible for restoring unmodified cytosine residue by catalysing oxidation of methyl groups on DNA (Hardy and Mann 2016; Mann 2014).

Histone modifications include methylation (mono-, di- or tri-), acetylation and citrullination of one or more amino acids in the N-terminal tails of core histones. Non-coding RNAs (ncRNA), including short micro RNA (miRNA), Piwi-interacting RNA (piRNA) and large intervening non-coding RNA (lincRNA), can self-propagate and

transmit regulatory information independent of the underlying DNA (Bernstein et al. 2007; Zaratiegui et al. 2007).

DNA methylation is the most common and best-studied epigenetic mark. One of the earliest study of the role of DNA methylation in human disease involved the work by Feinberg et al in 1983 where they discovered global methylation changes of DNA in human tumours (Feinberg and Vogelstein 1983). Subsequently, a number of studies have followed demonstrating the roles of DNA methylation in tumorigenesis, such as hypermethylation of tumour-suppressor genes as well as the role of DNA methylation in the inactivation of microRNA (miRNA) (Esteller 2008). Studies into the roles of DNA methylation in many other non-malignant human diseases have also emerged over the next decade, including the role of DNA methylation in aging process, as well as in a number of complex diseases such as Type 1 Diabetes, liver fibrosis, many autoimmune conditions.

The role of DNA methylation in NAFLD patients has also been the focus of a number of studies in the past decade. Methylation in the promoter region of anti-fibrogenic gene such as peroxisome proliferator-activated receptor α (*PPARα*) has been correlated with peripheral insulin resistance, fasting insulin level and homeostasis model assessment of insulin resistance (HOMA-IR) in patients with NAFLD (Sookoian et al. 2010; Zeybel et al. 2015). In a study comparing DNA methylation level within several fibrosis related genes, Zeybel et al has shown that DNA methylation level at specific CpGs within genes known to affect fibrosis differ between patients with mild versus severe NAFLD. In this study, there was more methylation seen in anti-fibrogenic genes such as PPARα

and PPARδ, and less methylation in profibrogenic genes such as transforming growth factor β1 (*TGFβ1*), *Collagen 1A1* and platelet derived growth factor α (*PDGFα*) in patients with severe NAFLD compared to those with mild NAFLD (Zeybel et al. 2015).

The strong adipose tissue-specific expression patterns of genes playing important role in early development have been found to be preserved from one pre-adipocyte to the next over several generations, suggesting the existence of yet unknown mechanism to maintain expression profiles over time (Schleinitz et al. 2014). Genome-wide methylation analysis using methylated DNA immunoprecipitation sequencing of eight different adipose depots in three pig breeds displaying different fat levels despite living in comparable environments demonstrated functionally relevant methylation differences between different adipose depots. These differences were reflected in the visceral adipose tissue, which carries the metabolic risk factors associated with impaired inflammatory and immune responses (M. Li et al. 2012).

Several human studies have also supported the role of epigenetics in the regulation of fat distribution. DNA methylation levels at the *LEP* promoter encoding for the protein leptin, which is the main player in regulation of energy homeostasis, were shown to be related to its tissue distribution. Furthermore, dynamic changes in adipose tissue leptin expression as a result of weight loss are not associated with alterations in leptin promoter methylation patterns (Marchi et al. 2011). In addition, in another recent study, altered DNA methylation at the *IGF2/H19* locus as a result of adverse in-utero environments have been associated with changes in subcutaneous fat measures, but not visceral or central adiposity (Huang et al. 2012).

It has been widely shown in multiple studies involving several species that oscillations of intrauterine and early postnatal nutritional, metabolic and hormonal environments may increase susceptibilities to the development of metabolic disorders and diseases in later life (Plagemann 2004). Furthermore, maternal nutrition during pregnancy contributes to the perinatal programming of the genome which has influence on fetal body composition and adverse fat distribution, and ultimately risk of obesity and metabolic diseases later in life (Blumfield et al. 2012). These 'embryonic or fetal programming' suggests that metabolic health and adiposopathy is a transgenerational disease (H. Bays and Scinta 2015).

1.4 FACTORS AFFECTING NAFLD PROGRESSION

NAFLD covers a spectrum of liver condition ranging from simple steatosis (non-alcoholic fatty liver) to non-alcoholic steatohepatitis (NASH) involving hepatocyte injury and inflammation with or without fibrosis (Bedossa 2016). The histologic diagnosis of NASH requires the presence of more than 5% hepatic steatosis and inflammation with hepatocyte injury (eg ballooning), with or without any fibrosis (Kleiner et al. 2005a). Although steatosis is the hallmark of NAFLD, it can be absent in some cases, including advanced stages of the disease (van der Poorten et al. 2013). This phenomenon of "burnt out" NASH was thought to be due to increased adiponectin levels in individuals with advanced NASH fibrosis. Adiponectin is the most abundant human adipocytokine, which acts directly on hepatocytes to upregulate fatty acid oxidation, inhibit fatty acid synthesis and improve insulin sensitivity, and hence plays a key role in hepatic steatosis (van der Poorten et al. 2013; Xu et al. 2003).

In multiple previous studies including a study by Angulo et al, it was demonstrated that presence and degree of fibrosis independently predict the overall and liver-related mortality/ liver transplantation or liver-related events, regardless of the presence of other histologic features (Angulo et al. 2015). Several studies have shown that compared with non-lean NAFLD, patients with lean NAFLD tend to show less severe histological features (Sookoian and Pirola 2018). However, despite the more favourable histological features at baseline, several long-term studies on lean NAFLD patients have demonstrated worse prognosis with respect to development of severe liver disease, independent of other confounders (A. C. e. a. Dela Cruz 2014; Hagstrom et al. 2018).

1.5 METABOLIC HEALTH

1.5.1 Definition

Although there has been no universally accepted definition of metabolic health in the literature, the most widely accepted and perhaps one of the first definition of metabolic health is the absence of insulin resistance, no evidence of subclinical inflammation as determined by high sensitivity C-reactive protein (CRP), together with only one component of the metabolic syndrome according to the Adult Treatment Panel III criteria **(Table 1)** (Lorenzo et al. 2007; Wildman et al. 2008).

Table 1. Clinical parameters used for the diagnosis of metabolic health

Systemic inflammation	hs-CRP level < 0.1mg/L
Insulin resistance	HOMA-IR < 5.13
Plus only **one** (or none) of the following components:	
Clinical parameter	**Criteria for metabolic abnormality**
Blood pressure	Systolic/diastolic blood pressure ≥ 130/85 mmHg or anti-hypertensive drug use
Triglyceride level	Fasting triglyceride level ≥ 150mg/dL (or ≥ 1.7 mmol/L)
HDL-C level	HDL-C level < 40mg/dL (or < 1.0 mmol/L) in men or < 50mg/dL (or < 1.3 mmol/L) in women or use of lipid lowering medication
Glucose level	Fasting glucose level ≥ 100mg/dL (or ≥ 5.6 mmol/L) or use of anti-diabetic medication

Metabolic health is defined as absence of systemic inflammation with only one (or none) other component of metabolic syndrome. Abbreviations: hs-CRP, high sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model of insulin resistance.

More recently, a more rigorous definition for metabolic health, especially metabolically healthy obesity, was suggested so as to determine the true prevalence and outcome of this group of people. This new definition is based on the absence of cardiometabolic diseases, a healthy cardiometabolic blood profile, normal blood pressure and intrahepatic triglyceride content and normal insulin sensitivity (G. I. Smith et al. 2019).

1.5.2 **Adiposopathy**

While it is not entirely clear as to the underlying mechanisms explaining individual variation in metabolic health, the term "adiposopathy" or defective/sick adipose tissue has been introduced. Adiposopathy governs an individual's cardiometabolic risk, above and beyond BMI alone (H. E. Bays 2011) and refers to the pathogenic enlargement of fat cells and fat tissue, resulting in anatomic and functional disturbances leading to altered lipid metabolism, adipose inflammation and adverse clinical outcomes (H. E. Bays et al. 2008).

However, given that adipose tissue is not a single, functionally uniform organ, it is not only how fat is stored (adipocyte proliferation versus adipocyte hypertrophy) that matters, but where the fat is stored (visceral versus subcutaneous, upper body versus lower body) and the type of fat (brown versus white). The 'where' and 'type' of adiposity has a greater impact on an individual's metabolic health than total fat mass (Iacobini et al. 2019). Thus, visceral and subcutaneous adipose tissues differ with regards to their contribution for metabolic risk. Visceral adipose tissue (VAT) as well as ectopic fat in or around the liver, heart and skeletal muscle lipid content (intramyocellular) have been linked to impaired glucose homeostasis, insulin resistance and cardiovascular disease (Lim and Meigs 2013). On the other hand, subcutaneous adipose tissue (SAT), especially lower body SAT (gluteofemoral body fat) which is a characteristic of metabolically healthy individuals is associated with lower risk for metabolic diseases (Goodpaster et al. 2005; Manolopoulos et al. 2010). The only exception is upper body subcutaneous fat which has been shown to be the primary source of circulating free fatty acids and hence plays an important role in determining insulin resistance and metabolic impairment. This has been demonstrated in several

disease states associated with accumulation of upper body fat, including Cushing's syndrome, lipodystrophy and human immunodeficiency virus associated lipodystrophy (Ebbert and Jensen 2013; J. J. Lee et al. 2017).

The adipocyte responds to positive energy balance through adipocyte hypertrophy as well as adipocyte hyperplasia (i.e. recruitment and proliferation of adipocyte precursors). Adipose tissue expandability and the increase in fat mass, especially SAT expansion, has been linked in previous studies to metabolic improvement and protection from type 2 diabetes (J. Y. Kim et al. 2007; McLaughlin et al. 2011). Whereas SAT expansion protects from metabolic risk, expansion of VAT or limited expansion of SAT is strongly associated with insulin resistance due to its hyperlipolytic state that is resistant to the anabolic actions of insulin, thereby producing larger amounts of circulating free fatty acids (Despres and Lemieux 2006; O'Connell et al. 2010). Although both SAT and VAT sizes correlate with the degree of fatty liver, only VAT size is related to metabolic health and progression from hepatic steatosis to fibrosis (O'Connell et al. 2010). Previous studies have shown that surgical removal of abdominal SAT through liposuction does not improve insulin resistance in obese individuals, whereas transplantation of SAT into the abdominal cavity results in improved insulin sensitivity and glucose metabolism. This supports the notion that differences in metabolic health appear to be reflected by the "fitness" of SAT, while dysfunctional SAT (adiposopathy) is characteristic of the metabolically unhealthy state (Iacobini et al. 2019; Klein et al. 2004; Tran et al. 2008). **Figure 1** shows a schematic representation of the different adiposity phenotype based on metabolic health status and body weight.

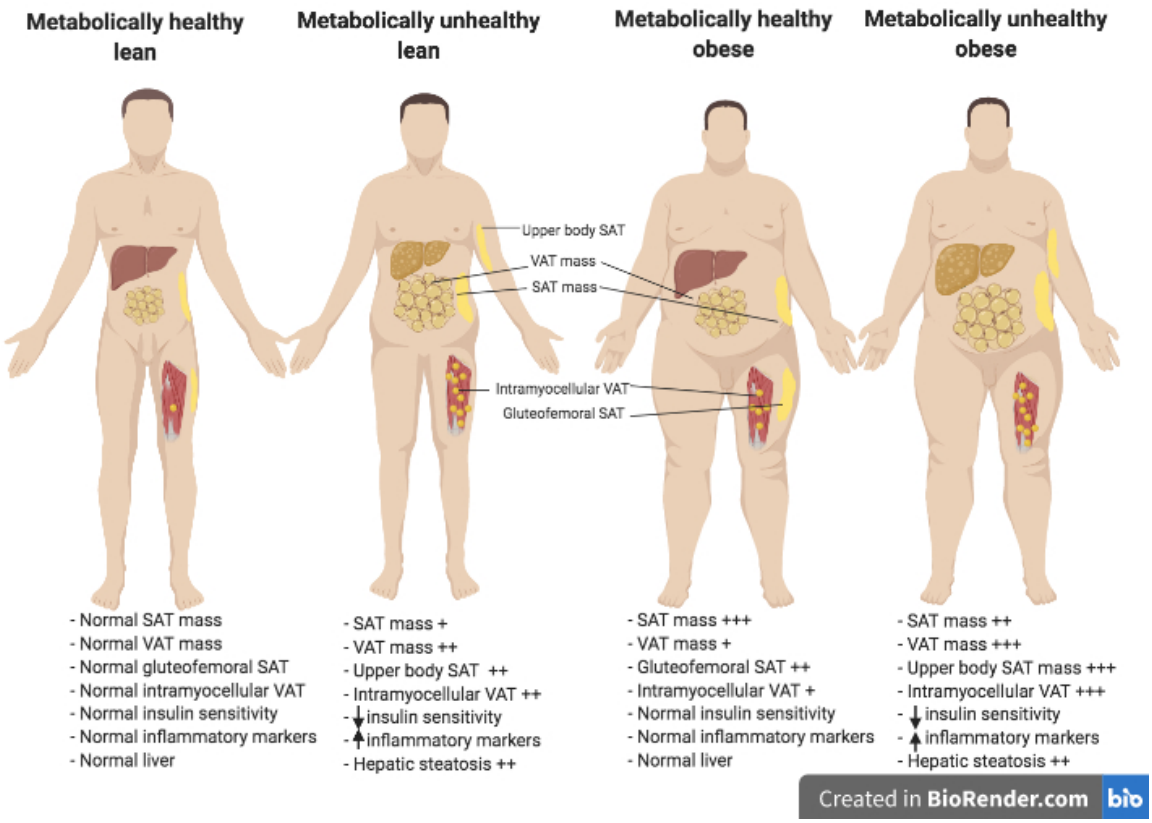


Figure 1. Adiposity phenotype based on metabolic health status and body weight

The difference in fat depots, insulin sensitivity, inflammatory marker and hepatic fat content in individuals with metabolically healthy lean, metabolically unhealthy lean (lean NAFLD), metabolically healthy obese and metabolically unhealthy obese phenotypes. Abbreviations: SAT – Subcutaneous adipose tissue; VAT – Visceral adipose tissue; NAFLD – Non-alcoholic fatty liver disease.

1.5.3 **Effect of metabolic health on NAFLD**

Given that metabolic health status (defined as per Table 1) is an integral aspect of NAFLD pathophysiology, several studies have investigated the relationship between metabolic health and NAFLD. In these, the risk of developing steatohepatitis and significant fibrosis increases progressively as the number of metabolic risk factors increases (Ampuero et al. 2018). Consistently, a cross-sectional study of more than 1,000 patients with biopsy proven NAFLD demonstrated that metabolic health has a greater impact on the risk of NASH development, significant fibrosis, atherogenic dyslipidaemia and kidney dysfunction than obesity or BMI alone(Ampuero et al. 2018). That study also found a similar risk for steatohepatitis and fibrosis in a metabolically unhealthy group, regardless of their body weight, suggesting that metabolic health has a greater impact on the severity of liver disease than BMI, possibly through unfavourable body fat distribution (and/or as yet unknown factors) with a long but important period of subclinical systemic inflammation (Ampuero et al. 2018). Similar findings have been demonstrated in Asian and Mexican populations (Gutierrez-Grobe et al. 2017; M. K. Lee et al. 2015; Sung et al. 2014).

Despite this data, metabolically healthy obesity cannot be considered entirely benign as it carries almost double the risk of steatohepatitis compared to individuals who are metabolically healthy and normal weight(Sung et al. 2014). This implies that healthy obesity (acting through subclinical or as yet be discovered impacts on metabolic health) perhaps represents a “honeymoon phase” that in some individuals eventually progresses to a metabolically unhealthy obese state (Kramer et al. 2013). A decline in insulin sensitivity and increased fasting blood glucose levels, especially in those with higher

BMI, older age, presence of more severe metabolic dysfunction and poor lifestyle index, have been identified to be major factors associated with the conversion from metabolically healthy obesity to metabolically unhealthy obesity (G. I. Smith et al. 2019). In addition, the presence of NAFLD can promote (or at least be associated with) the conversion of an individual's metabolic health from metabolically healthy to metabolically unhealthy, independent of age, sex, BMI, lifestyle factors, individual components of metabolic syndrome and insulin resistance. The effect is greater in those with a lower BMI and body fat mass compared to those with high BMI and body fat mass (Hwang et al. 2019).

1.6 METABOLIC ADAPTATION

The human body has great capacity to maintain body weight homeostasis through effects on food intake and energy expenditure. The ability of the body to increase or decrease energy expenditure beyond the obligatory energy costs of depositing and maintaining new tissues, digesting food, moving and maintaining body mass, without any change in body mass is defined as metabolic adaptation (Johannsen et al. 2019). Adaptation is achieved through a fine balance of regulatory systems through the interaction of hormones, chemokine signals and the neuroendocrine axis (Johannsen et al. 2019). In response to certain nutrition and/or physical activity conditions, several cytokines or peptides secreted from muscles (myokines), adipose tissue (adipokines) and liver (hepatokines) engage in cross-talk to maintain energy homeostasis by governing lipid and glucose metabolism as well as mediating local and systemic inflammation. Any perturbations in the systems involved results in loss of metabolic

adaptation, resulting in abnormal expansion of adipose tissue and obesity, hepatic fat accumulation, and insulin resistance (Oh et al. 2016).

In addition, the enterohepatic circulation including bile acids (BA) and their metabolites, as well as gut microbiota play important roles in metabolic adaptation which occurs in part due to genetic and developmental influences (Wahlstrom et al. 2016a). A number of early experiments involving protein overfeeding have shown large variations in weight gain among nonrelated subjects but high correlation within twin pairs (Bouchard et al. 1990). Further, studies have shown that the change in energy expenditure was due to a change in non-exercise activity thermogenesis (Diaz et al. 1992; Leibel et al. 1995). This concept of metabolic adaptation may explain why some individuals appear to be obesity resistant while others gain weight easily when challenged with caloric abundance.

Given the complex and multifactorial pathogenesis of NAFLD (Buzzetti et al. 2016) and knowing that not all obese people have NAFLD and not all NAFLD patients are obese(Younes and Bugianesi 2019), how an individual **adapts** to an unfavorable set of metabolic circumstances will govern when he/she will manifest fatty liver disease. This adaptive ability is the capability of the body to increase or decrease energy expenditure beyond obligatory energy requirements without any change in body mass.

1.7 LEAN NAFLD

1.7.1 Definition and epidemiology

NAFLD classically presents in close association with metabolic syndrome or one of its components, including obesity, hypertension, type 2 diabetes mellitus and dyslipidaemia (Chalasani et al. 2012; Z. M. Younossi et al. 2016a). The prevalence of NAFLD has risen in parallel with obesity recently, with nearly a third of adults in the USA having BMI more than 30kg/m^2 (Ng et al. 2014). A meta-analysis of 21 cohort studies in 2016 found obesity to be a 3.5-fold increased risk of developing NAFLD, with a dose-dependent relationship between BMI and NAFLD risk (L. Li et al. 2016). However, not all obese patients suffer from the metabolic disturbances related to obesity, including NAFLD. This concept of "metabolically healthy obesity", present in up to 30% of all obese individuals, refers to obese individuals with no evidence of metabolic or cardiovascular complications (Wildman et al. 2008). Similarly, a fair proportion of patients (10-30%) develop NAFLD despite having normal body mass index (BMI < 25 kg/m^2) (Das and Chowdhury 2013; Z. M. Younossi et al. 2012). This subset of individuals is known as lean NAFLD, which is most commonly defined as NAFLD in the population with BMI less than 25 kg/m^2 in Western studies and less than 23 kg/m^2 in Eastern studies (D. Kim and Kim 2017). Lean NAFLD was first reported in Asian countries and may represent the other end of the spectrum known as the "metabolically unhealthy normal weight" NAFLD (D. Kim and Kim 2017; J. C. Leung et al. 2017a; Sookoian and Pirola 2017; Stefan et al. 2017).

The first population study describing lean NAFLD was conducted in Korea in 2004, where lean NAFLD was present in 23.4% of the non-obese population with associated metabolic disorders (H. J. Kim et al. 2004). Since then, lean NAFLD has been described in several Asian and Caucasian reports. **Figure 2** shows geographical data on available lean NAFLD prevalence worldwide. These data indicate that there are patients with fatty liver who are lean by BMI criteria and secondly that disease prevalence is between 5 - 26% in Asian and 7 - 20% in Western populations (Younes and Bugianesi 2019). In one study, up to 75% of patients with NAFLD and significant liver disease prevalence was shown to have normal BMI in a non-obese Asian population (Das et al. 2010). However, owing to the lack of a widely accepted definition of "lean" across studies, as well as the heterogeneity in NAFLD diagnostic criteria, the current data suffers from many limitations.

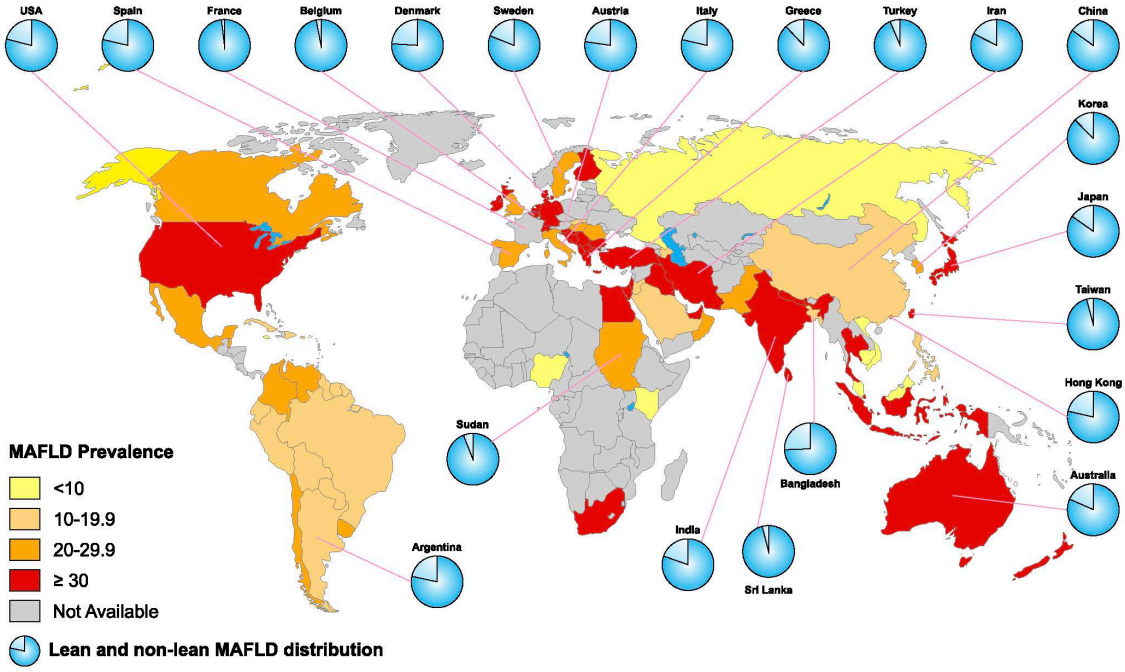


Figure 2. Worldwide prevalence of NAFLD and of lean NAFLD as a proportion of total NAFLD

Worldwide distribution of NAFLD with data on the prevalence of lean NAFLD (light blue; where available). Abbreviation: NAFLD – Non-alcoholic fatty liver disease

1.7.2 Histological characteristics

The histological characteristics of NAFLD vary between ethnic groups including for the subgroup with lean disease. A recent systematic review from cross sectional studies shows that liver fibrosis stage is significantly lower in lean compared to overweight/obese NAFLD (Sookoian and Pirola 2018). Similarly, the NAFLD activity scores and presence of steatohepatitis are lower compared to overweight/obese patients, although there was substantial heterogeneity in the results (Sookoian and Pirola 2018). Some studies however have reported a more severe histological picture in lean patients with higher rates of advanced fibrosis, ballooning and lobular inflammation, as well as

greater steatohepatitis compared to their non-lean counterparts (Denkmayr et al. 2018; Q. Wang et al. 2019).

1.7.3 **Pathogenesis**

Over the past decade, there has been a surge in the number of studies describing lean NAFLD and its characteristics. However, studies looking into the pathogenesis of lean NAFLD are lacking. **Table 2** lists selected published studies on lean NAFLD, including the number of patients included, definition of lean NAFLD and main findings. Although lean NAFLD has been shown to share metabolic features and hepatic pathology as the classical obese NAFLD, patients with lean NAFLD lack any linear association with adiposity. This suggests that the distribution of adipose tissue in the body has more relevance in the pathogenesis of lean NAFLD (Das and Chowdhury 2013). In a recent cross-sectional study of lean and overweight individuals with and without NAFLD, insulin resistance was positively and significantly associated with hepatic triglyceride content, which has been shown to be closely associated with NAFLD, above and beyond their BMI and waist circumference measurements (Gonzalez-Cantero et al. 2018). These findings indicate that it is likely that factors other than adiposity may come into play in the pathogenesis of NAFLD (F. Chen et al. 2019).

1.7.4 **Prognosis**

In contrast to studies that examine the prevalence and presentation of lean NAFLD, data on its long-term prognosis have been scarce and conflicting. Some reports suggest that

clinical events and prognosis are worse in the obese compared to the lean NAFLD population, with higher cardiovascular events and death (Fracanzani et al. 2011; J. C. Leung et al. 2017a). One recent study with a median follow up of 49 months reported a clinical event rate of 11.9% in obese compared to 8.3% in the lean NAFLD population (J. C. Leung et al. 2017a). However a study in 2014 by Delacruz et al. looking at the long-term prognosis of lean patients with NAFLD and a median follow up of 11 years has challenged this finding (A. C. e. a. Dela Cruz 2014). This international cohort study included 483 patients with biopsy-proven disease and suggested that the median survival free of liver transplantation was in fact lower in those who were lean compared to obese. This occurred despite having a better metabolic profile and less advanced liver fibrosis (A. C. e. a. Dela Cruz 2014). This result was supported by another report of 646 patients with biopsy proven NAFLD and a median of 19.9 years follow up. The study showed that although patients with lean disease did not have increased mortality, they had an increased risk for the development and progression to severe liver disease compared to obese patients (hazard ratio 2.69, $p = 0.007$) (Hagstrom et al. 2018).

While lean NAFLD reflects the hepatic manifestation of a metabolically unhealthy normal weight, studies involving other organ systems also indicate that individuals with a metabolically unhealthy phenotype may suffer a worse prognosis despite a normal BMI. Studies of diabetes mellitus in underweight or normal weight people suggest a distinct, albeit less characterized pathophysiology to disease in the overweight/obese population, with higher mortality rates(George et al. 2015). Similarly, metabolic health (as measured by the number of components of metabolic syndrome) has been shown to be a stronger predictor for myocardial dysfunction than simply BMI or fat mass alone (Dobson et al. 2016).

Table 2. Summary of a selection of published studies on lean NAFLD

Author, year, country	Definition of lean NAFLD	Sample size	Main findings
Kim, HJ, 2004, Korea (H. J. Kim et al. 2004)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	74 lean and 106 non-lean NAFLD; 386 lean healthy and 202 non-lean healthy controls	Metabolic disorders are present in NAFLD subjects with normal weight
Chen, CH, 2006, Taiwan (C. H. Chen et al. 2006)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	61 lean and 291 non-lean NAFLD; 1383 lean healthy and 654 non-lean healthy controls	Hypertriglyceridaemia was related to NAFLD in non-obese subjects
Das, K, 2010, India (Das et al. 2010)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound, confirmed on CT	123 lean and 41 non-lean NAFLD; 1660 lean healthy and 87 non-lean healthy controls	Lean NAFLD is present in 75% of this predominantly non-obese population, with potentially significant liver disease
Younossi, 2012, USA (Z. M. Younossi et al. 2012)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	431 lean and 2061 non-lean NAFLD; 4026 lean healthy and 5095 non-lean healthy controls	Lean NAFLD patients (20.9%) are younger, have lower metabolic syndrome and is more common in females
Margariti, 2012, Greece (Margariti et al. 2012)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	19 lean and 143 non-lean NAFLD	Lean NAFLD patients (12%) have lower metabolic syndrome and higher ALT/AST than non-lean NAFLD
Bhat, 2013, India (Bhat et al. 2013)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	30 lean and 120 non-lean NAFLD	Lean NAFLD present in 20% of patients. Insulin resistance is common amongst patients with NAFLD, including lean NAFLD (80%)
Kumar, 2013, India (Kumar et al. 2013)	Lean BMI < 23 kg/m^2, biopsy proven NAFLD	27 lean and 141 non-lean NAFLD	Lean NAFLD patients (13.2%) have less severe histology and lower insulin resistance than non-lean NAFLD
Delacruz, 2014, Australia (A. C. e. a. Dela Cruz 2014)	Lean BMI < 25 kg/m^2, biopsy proven NAFLD	125 lean and 965 non-lean NAFLD	Lean NAFLD patients (11.5%) have higher mortality than patients with non-lean NAFLD despite presenting with healthier metabolic profile
Alam, 2014, India (Alam et al. 2014)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound, biopsy in some (220/465)	119 lean and 346 non-lean NAFLD	Lean NAFLD patients (25.6%) were metabolically and histologically similar to non-lean NAFLD patients, with similar rates

			of NASH and fibrosis
Feng, 2014, China (Feng et al. 2014)	Lean BMI < 24 kg/m^2, hepatic steatosis on liver ultrasound	134 lean and 764 non-lean NAFLD; 597 lean healthy and 284 non-lean healthy controls	Lean NAFLD patients (14.9%) had higher visceral adiposity index and comparable metabolic risk profile to non-lean NAFLD
Vendhan, 2014, India (Vendhan et al. 2014)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	48 lean and 125 non-lean NAFLD	Lean NAFLD patients (27.7%) had better metabolic profile but similar association to coronary artery disease as non-lean NAFLD
Wei, 2015, Hong Kong (Wei et al. 2015)	Lean BMI < 25 kg/m^2, liver fat assessed by proton-magnetic resonance spectroscopy	135 lean and 127 non-lean NAFLD	Lean NAFLD patients (19.3%) had similar intrahepatic triglyceride content, but lower cytokeratin-18 fragments and liver fibrosis. PNPLA3 G allele was more common in lean NAFLD.
Nishioji, 2015, Japan (Nishioji et al. 2015)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	411 lean and 394 non-lean NAFLD; 2285 lean healthy and 181 non-lean healthy controls	Lifestyle and metabolic factors (higher triglycerides and waist circumference) increases the risk of NAFLD, even in lean patients (15.2%)
Cho, 2016, Korea (Cho 2016)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	213 lean and 347 non-lean NAFLD; 1498 lean healthy controls	Lean NAFLD patients (12.4%) had higher proportion of females, lower insulin resistance and fewer metabolic risk factors than non-lean NAFLD
Feldman, 2017, Austria (Feldman et al. 2017b)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	55 lean and 61 non-lean NAFLD; 71 lean healthy controls	Lean NAFLD patients (29.4%) had impaired glucose tolerance, low adiponectin concentrations and a distinct metabolic profile with increased PNPLA3 risk allele carriage
Leung, 2017, Hong Kong (J. C. Leung et al. 2017a)	Lean BMI < 25 kg/m^2, biopsy-proven NAFLD	72 lean and 235 non-lean NAFLD	Lean NAFLD patients (23.5%) had less severe disease and better prognosis than non-lean NAFLD. Hypertriglyceridaemia and high creatinine were associated with advanced liver disease in lean NAFLD
Fracanzani, 2017, Italy (Fracanzani et al. 2017)	Lean BMI < 25 kg/m^2, biopsy-proven NAFLD	143 lean and 526 non-lean NAFLD	Lean NAFLD patients (21.4%) had higher TM6SF2 risk allele carriage and lower metabolic syndrome, less NASH and lower

			fibrosis but thinner carotid intima compared to non-lean NAFLD
Sookoian, 2017, Argentina (Sookoian and Pirola 2018)	Systematic review, lean BMI ≤ 25 kg/m^2	493 lean and 2209 non-lean NAFLD	Lean patients tended to have milder histological features compared to non-lean NAFLD
Sookoian, 2017, Argentina (Sookoian and Pirola 2017)	Systematic review with meta-analysis, lean BMI ≤ 25 kg/m^2, hepatic steatosis on liver ultrasound	1966 lean and 5938 non-lean NAFLD; 9946 lean healthy and 6027 obese healthy controls	Lean NAFLD shared common altered metabolic and cardiovascular profile compared to non-lean NAFLD, although the effect is less severe in lean NAFLD
Hagstorm, 2017, Sweden (Hagstrom et al. 2018)	Lean BMI < 25 kg/m^2, biopsy proven NAFLD	123 lean, 335 overweight and 188 obese NAFLD	Lean NAFLD patients (19%) had lower fibrosis at better metabolic profile at baseline but increased risk of development of severe liver disease
Denkmayr, 2018, Austria (Denkmayr et al. 2018)	Lean BMI ≤ 25 kg/m^2, biopsy proven NAFLD	72 lean, 242 overweight and 150 obese NAFLD	Lean NAFLD patients (15.9%) had severe histological features similar to obese but more progressed than overweight NAFLD
Tobari, 2018, Japan (Tobari et al. 2018)	Lean BMI < 25 kg/m^2, biopsy proven NAFLD	116 lean, 173 overweight and 115 obese NAFLD	Advanced fibrosis was not associated with BMI but histological steatosis was more common in lean NAFLD
Li, 2019, China (C. Li et al. 2019)	Lean BMI < 24 kg/m^2, hepatic steatosis on liver ultrasound	84 lean and 85 non-lean NAFLD; 90 lean healthy and 92 non-lean healthy controls	Lean NAFLD patients had comparable total caloric, calorigenic nutrition, iron, sleep duration and overtime work as obese NAFLD
Niriella, 2019, Srilanka (Niriella et al. 2019)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	120 lean and 816 non-lean NAFLD; 1206 healthy controls	Lean NAFLD patients (4%) had similar risk of developing metabolic comorbidities compared to non-lean NAFLD, with higher NAFLD associated with PNPLA3 incidence
Yilmaz, 2019, Turkey (Yilmaz et al. 2019)	Lean BMI < 25 kg/m^2, biopsy proven NAFLD	30 lean and 428 non-lean NAFLD	Lean NAFLD was present in 6.4% of the study sample, with metabolic syndrome present in 63% of the sample population
Wang, 2019, China (Q. Wang et al. 2019)	Lean BMI < 25 kg/m^2, biopsy confirmed NAFLD	36 lean and 48 non-lean NAFLD	Lean NAFLD patients (42.9%) have a female predominance and more advanced fibrosis compared to non-lean NAFLD patients

1.8 HYPOTHESIS AND AIMS

We hypothesize that there is a distinct underlying metabolic adaptation governing the pathophysiology of lean NAFLD, which may explain its unique baseline characteristics and long-term prognosis.

The aims of this project are to:

1. Compare the characteristics of lean NAFLD patients to lean and non-lean healthy controls
2. Compare the characteristics of lean NAFLD compared to non-lean NAFLD patients in terms of their bile acid profile and regulation, gut microbiota profile and metabolic adaptation capacity
3. Explore murine models of lean and non-lean NAFLD to investigate the replicability of our hypothesis from the human results
4. Explore the metabolomic characterisation of patients with lean NAFLD compared to patients with non-lean NAFLD to investigate if there are other metabolic pathways other than that of bile acid pathways which are significantly different

CHAPTER TWO

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Polymerase chain reaction (PCR) primers

The real-time polymerase chain reaction (PCR) primers were ordered from GeneWorks. The primers were designed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and reaction conditions were optimised for a standard curve of pooled cDNA. **Table 3** shows the sequence of primers used for qPCR in this thesis.

Table 3 List of mouse primers used for qPCR analysis

Gene	Primer sequence
GAPDH	GAAGGTGAAGGTCGGAGTC (forward) GAAGATGGTGATGGGATTTC (reverse)
ASBT	TGGGTTTCTTCCTGGCTAGACT (forward) TGTTCTGCATTCCAGTTTCCAA (reverse)
BSEP	CAGACACCATGTCTGACTCAGTGA (forward) GGCCACACTCAGACCTATGACGGC (reverse)
CYP7A1	AGCAACTAAACAACCTGCCAGTACTA (forward) GTCCGGATATTCAAGGATGCA (reverse)
CYP8B1	TGAATTCTTGAAGGGGATGC (forward) CCTTGCTCCCTCAGAAACTG (reverse)
CYP27A1	TTCTCAGACACGATCTATGGCTGT (forward) CTACTGTCTCTGCAGAAAGCGTA (reverse)

FGF-15	ACGGGCTGATTCGCTACTC (forward) TGTAGCCTAAACAGTCCATTTCCT (reverse)
FGFR4	CGAGGCATGCAGTATCTGG (forward) CAAAGTCAGCGATCTTCATCACA (reverse)
FXR	CGGAACAGAAACCTTGTTTCG (forward) TTGCCACATAAATATTCATTGAGATT (reverse)
HNF4A	CCAAGAGGTCCATGGTGTTTAAG (forward) GTGCCGAGGGACGATGTAGT (reverse)
Mrp2	TCCAGGACCAAGAGATTTGC (forward) TCTGTGAGTGCAAGAGACAGGT (reverse)
NTCP	GGGTCGGAGGATGGAGGCGCACAA (forward) GGACGTTTTGGAATCCTGTTTCCA (reverse)
OST beta	GTATTTTCGTGCAGAAGATGCG (forward) TTTCTGTTTGCCAGGATGCTC (reverse)
SHP	CAGCGCTGCCTGGAGTCT (forward) AGGATCGTGCCCTTCAGGTA (reverse)

2.1.2 **Sources of Clinical Information and Human Biological Tissue**

2.1.2.1 **Clinical and laboratory assessments**

Physical examination was performed on all patients on the day of the liver biopsy including measurement of body mass index. Weight (in kilograms) and height (in centimetres) were measured by staff at the time of biopsy and used to calculate BMI, expressed as kg/m^2. Following WHO criteria for Western populations, patients with BMI of less than $25kg/m^2$ were defined as lean, and $\geq 25\ kg/m^2$ as non-lean (Z. M.

Younossi et al. 2012). Hypertension was defined as a registered diagnosis in patient medical records, a resting blood pressure of ≥140/90 mm Hg, or having any antihypertensive medication prescribed. Type 2 diabetes mellitus (T2DM) was defined as a registered diagnosis in patient medical records, a fasting plasma glucose value ≥ 7 mmol/L (or 126 mg/dL) or having any antidiabetic medication prescribed.

Venous bloods were collected on the morning of liver biopsy after an overnight 12-hour fast for serum transaminases, bilirubin, albumin, lipid profile, glucose and insulin. Serum insulin was determined by a radioimmunoassay technique (Phadaseph Insulin RIA; Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). All other biochemical tests were performed using conventional automated analyzers within each local department. Insulin resistance was calculated using the homeostasis model (HOMA-IR) using the formula: HOMA-IR = fasting insulin (mU/L) x plasma glucose (mmol/L)/22.5 (Eslam et al. 2011; Matthews et al. 1985).

2.1.2.2 **NAFLD cohort**

Patients were recruited from hepatology clinics at four centres: Australia (Storr Liver Centre, Westmead Hospital, Sydney and Sir Charles Gairdner Hospital Unit, University of Western Australia) and Italy (Unit of Metabolic Diseases and Clinical Dietetics, University of Bologna; Gastroenterology unit, University of Palermo, and Division of Gastroenterology and Hepatology, University of Turin).

Inclusion criteria were patients who had liver biopsy for suspected NAFLD with available serum samples and clinical data. Individuals with alternative diagnoses were excluded including excess alcohol intake (>20 g per day for women; and >30 g per day for men), chronic viral hepatitis (hepatitis B and hepatitis C), autoimmune liver diseases, hereditary hemochromatosis, α1-antitrypsin deficiency, Wilson's disease and drug-induced liver injury. In addition, a phosphatidylethanol assay was performed in all NAFLD patients to avoid misclassification of alcoholic liver disease in this population. Patients with any secondary cause of steatohepatitis including previous gastrointestinal surgery or ingestion of medications known to impact hepatic steatosis or bile acid metabolism or with decompensated liver disease were excluded as previously described in another study (van der Poorten et al. 2013).

2.1.2.3 **Healthy controls**

Healthy Caucasians controls were recruited from volunteers if the following applied: age between 40-65 years, BMI < 25 kg/m^2 for lean healthy controls or BMI > 25 kg/m^2 for non-lean healthy controls, alcohol intake ≤ 20g per day for women and ≤ 30g per day for men, metabolically healthy (defined as absence or having only one component of the metabolic syndrome according to the Adult Treatment Panel III criteria, including triglyceride level of 150 mg/dL (1.7 mmol/L) or greater, high density lipoprotein (HDL) level of less than 40 mg/dL (1.0 mmol/L) in men and less than 50mg/dL (1.3 mmol/L) in women, systolic blood pressure greater than 130 mmHg or diastolic blood pressure greater than 85mmHg and fasting plasma glucose level of 100 mg/dL (5.6 mmol/L) or greater (Lorenzo et al. 2007; Wildman et al. 2008)). In addition, they also needed to have normal liver tests (normal transaminases

(less than 30 U/L for ALT and less than 35 U/L for AST), with normal levels of serum bilirubin (less than 15 umol/L) and albumin (38-50 g/L) and metabolic parameters (fasting blood glucose and blood cholesterol levels), as well as absence of liver steatosis on ultrasonography. Written informed consent was obtained from all participants.

2.1.3 **Sources of mice tissue**

Male C57BL/6 mice obtained from Animal Resources Centre (Perth, Australia) were used for diet studies commencing at week 8 and were exposed to a 12-hr light/dark cycle with free access to food and water. Mice were fed either a 33% sucrose diet (SF09-079, Specialty Feed Service, Glen Forest, Australia, see **Table 4A**) or a diet containing 33% Sucrose, 2% cholesterol and 0.5% cholate (SF09-080, Specialty Feed Service, Glen Forest, Australia, **see Table 4B**) starting at 8 weeks of age for 16 weeks.

In addition, a separate group of mice were fed the cholesterol rich diet containing 33% sucrose, 2% cholesterol and 0.5% cholate, along with a sodium dependant bile acid transporter (ASBT) inhibitor (ASBTi, SC-435, Lumena/Shire Pharmaceuticals) for 8 weeks. At the time of harvest, mice were anesthetized with i.p. ketamine (100 mg/kg)/xylazine (10 mg/kg) injection after a 4-hr fasting period. Blood was collected by cardiac puncture. Liver and ileum samples were harvested, rapidly snap frozen in liquid nitrogen and stored at -80 ºC. A thin slice of liver tissue was formalin fixed for histology. All procedures were approved by the Western Sydney Local Health District Animal Ethics Committee and conducted in accordance with Animal Experimentation

guidelines of the National Health and Medical Research Council (NHMRC) of Australia. Mice tissues were obtained through collaboration with Dr Saeed Esmaili, Storr Liver Centre and University of Sydney.

Table 4. A) Diet composition and nutritional parameters for mice fed the high sucrose diet. B) Diet composition and nutritional parameters for mice fed an atherogenic diet

A.

Ingredients	
Casein (Acid)	193 g/Kg
Sucrose	337 g/Kg
Cocoa Butter	19 g/Kg
Soya Oil	24 g/Kg
Cellulose	48 g/Kg
Wheat Starch	303 g/Kg
Dextrinised Starch	34 g/Kg
DL Methionine	2.9 g/Kg
Calcium Carbonate	12.6 g/Kg
Sodium Chloride	2.5 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.4 g/Kg
Potassium Dihydrogen Phosphate	6.6 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.4 g/Kg
AIN93 Vitamins	9.6 g/Kg

Calculated Nutritional Parameters	
Protein	18.60%
Total Fat	4.35%
Total Carbohydrate	62.01%
Crude Fibre	4.50%
AD Fibre	4.50%
Digestible Energy	15.7 MJ/Kg
% Total calculated digestible energy from lipids	15.30%
% Total calculated digestible energy from protein	20.90%

B.

Ingredients	
Casein (Acid)	188 g/Kg
Sucrose	329 g/Kg
Cocoa Butter	19 g/Kg
Soya Oil	23 g/Kg
Cellulose	47 g/Kg
Wheat Starch	296 g/Kg
Dextrinised Starch	33 g/Kg
DL Methionine	2.8 g/Kg
Calcium Carbonate	12.3 g/Kg
Sodium Chloride	2.4 g/Kg
AIN93 Trace Minerals	1.3 g/Kg
Potassium Citrate	2.3 g/Kg
Potassium Dihydrogen Phosphate	6.4 g/Kg
Potassium Sulphate	1.5 g/Kg
Choline Chloride (75%)	2.4 g/Kg
AIN93 Vitamins	9.4 g/Kg
Sodium Cholate	5 g/Kg
Cholesterol	20 g/Kg

Calculated Nutritional Parameters	
Protein	18.20%
Total Fat	4.20%
Crude Fibre	4.40%
AD Fibre	4.40%
Digestible Energy	16.2 MJ/Kg
% Total calculated digestible energy from lipids	9.50%
% Total calculated digestible energy from protein	19.70%

2.2 METHODS

2.2.1 Histopathology

A single expert liver pathologist at each centre who was blinded to patient clinical characteristics and serum measurements interpreted the liver biopsies. All biopsies had a minimum of 11 portal tracts, and inadequate biopsies were excluded. Disease activity was assessed according to the NAFLD Activity Score; fibrosis was staged according to the NAFLD clinical research network (CRN) (Kleiner et al. 2005b). Some of these patients have been the subjects of previous publications (Eslam et al. 2015a; Eslam et al. 2016a; Eslam et al. 2016c). The concordance between pathologists within this cohort was very good for steatosis and fibrosis, with coefficients for inter-observer agreement for fibrosis stage and steatosis grade of 0.78 and 0.85, respectively (Kazankov et al. 2016).

2.2.2 Phosphatidylethanol measurement

The serum phosphatidylethanol level was measure using the Human Peth (phosphatidylethanol) ELISA kit (Elabscience) on all lean NAFLD subjects according to the manufacturer's instructions. Briefly, a standard working solution stock is prepared and 100uL is added to each well in the first two columns, followed by 100uL of serum samples. The plate is covered and incubated for 90 minutes at 37^0C. After this, the liquid is removed from the wells and 100uL of biotinylated detection antibody working solution is added to each well, followed by a 1-hour incubation period at 37^{o}C after gentle mixing. The solution is then discarded and the wells are washed

three times with the wash solution and patted dry. Following this, 100uL of HRP conjugated working solution is added to each well and the plate is covered and incubated at 37ºC for 30 minutes. This is followed by another wash done five times as per previously. Next, 90uL of substrate reagent is added to each well and the plate is covered and incubated for 15 minutes at 37ºC, protected from the light. 50uL stop solution is then added to each well and the optical density is determined with a micro-plate reader at 450nm. Values above 253 ng/mL are regarded as significant alcohol consumption in the past few weeks (Kechagias et al. 2015).

2.2.3 **Methods for bile acid quantification**

2.2.3.1 **Bile acid extraction**

Bile acids were extracted from serum samples as previously described (van der Poorten et al. 2013; Xie et al. 2015). Briefly, 80µL of acetonitrile containing internal standard (cholic2,2,4,4-d4acid, Quebec, Canada) was added to 20µL of the serum sample. After centrifugation, the supernatant was evaporated to dryness and stored at -20^0 C until time of analysis.

2.2.3.2 **Bile acid measurement**

The dried bile acid residue was reconstituted in mobile phase containing 50:50 water and acetonitrile and analysed on a Ultra Performance Liquid Chromatography (UPLC, Shimadzu, Kyoto, Japan) system using an ACQUITY (WATERS, Milford, MA) column in combination with a Q-TRAP 5500 Mass Spectrometer (AB SCIEX,

Toronto, Canada) to quantify concentrations of 19 bile acids. The mass spectrometer was operated in negative ion mode. The calibration solution containing all 19 analytes was prepared at a series of concentrations in pooled naïve plasma depleted of bile acids using activated charcoal to generate the calibration curve. The detection limit for individual bile acids was 0.01 – 0.05 umol/L.

2.2.4 **Method of FGF-19 measurement**

FGF19 level was measured using the Human FGF19 Elisa kit (EHFGF19, Thermo Scientific) on the serum of subjects according to the manufacturer's instructions. Briefly, a series of concentrations of standard solution is prepared. 100uL of standard or diluted serum samples (1 in 2 using assay diluent C) or blank is added to each well, and the plate is covered and incubated for 2.5 hours at room temperature after gentle shaking. After this, the plate is washed 4-5 times with the wash buffer and patted dry using an absorbent towel. To each well, 100uL of diluted biotinylated antibody solution (1 in 80 dilution) is added and the plate covered and incubated at room temperature for 1 hour. The plate is then washed 3-4 times again with the wash buffer. After this, 20uL of diluted streptavidin solution (1 in 500 dilution) is added to each well and the plate is covered and incubated for 45 minutes at room temperature. This is followed by another 3-4 washes with the wash buffer. Next, 100uL of TMB substrate solution is added to each well and the plate incubated at room temperature for 30 minutes away from the light. After this, 50uL stop solution is added to each well and the plate is read within 30 minutes using a micro-plate reader at 450nm.

2.2.5 **Method of C4 measurement**

7-alpha-hydroxy-4-cholesten-3-one (C4) was purchased from Toronto Research Chemicals (Toronto, Canada). Cortisol-1,2-d_2 was purchased from CDN isotopes (Hornsby, NSW, Australia) and charcoal stripped serum (CSS) was purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Additional reagents and solvents were of HPLC grade. To 50µL of serum, 200µL of ice-cold acetonitrile containing 2% formic acid and 16 ηg of the assay internal standard, cortisol-1,2-d_2 was added. The mixture was vortexed for 1 minute and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and evaporated under vacuum at room temperature. The samples were then reconstituted in the assay mobile phase and transferred to a 96 well plate for analysis. Stock solution of C4 (1µM) was prepared in CSS and diluted to give final concentrations of 0.01-1µM. The standards were treated in the same way as the samples. A Nextera UPLC (SHIMADZU, Kyoto, Japan) system was used in combination with a Q-TRAP 5500 Mass Spectrometer (AB SCIEX, Toronto, Canada) with Analyst software 1.6.2. Chromatographic separations were performed with an ACQUITY (WATERS, Milford, MA, USA) UPLC BEH C18 column (1.7microns 2.1x100mm). The temperature of the column and auto sampler was 65ºC and 12ºC, respectively. Sample injection was 1µL. The mobile phase consisted of 10% acetonitrile and 10% methanol in water containing 0.1% formic acid (mobile phase A) and 10% methanol in acetonitrile 0.1% formic acid (mobile phase B) delivered as a gradient: 0-3-min mobile phase B 20%; 3-3.5-min mobile phase B 80%, 7-9min mobile phase B with a constant 80% flow rate of 0.5ml/min. The mass spectrometer was operated in positive electro-spray mode working in the multiple reaction mode (MRM). Transition MRMs for C4 and the internal standard

cortisol-1,2-d_2 were 401.2→177.2 and 365.2→122.2 respectively. Operating parameters were: curtain gas 30psi; ion spray voltage 4500 V; temperature 55°C; ion source gas 1 60psi; ion source gas 2 65psi. Declustering potential, entrance potentials and collision cell exit potentials were optimised using the Analyst software.

2.2.6 **Genotyping**

Genotyping for *TM6SF2* rs58542926 and *PNPLA3* rs738409 was performed on all available DNA samples (n = 471 (88%) using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA). All genotyping was blinded to clinical variables and some was extracted from previous reports (Eslam et al. 2016a).

2.2.7 **Method of RNA extraction from animal tissues**

RNA was extracted from animal tissues as per protocol. Briefly, a piece of animal tissue (about 30mg each) is cut, and 350uL FARB buffer and 3.5uL β-mercaptoethanol are added to the tissue, along with 1 microbead (Qiagen). The mixture is then spun using the rotor-stator tissue homogenizer at 3000rpm for 3 minutes and incubated for 5 minutes at room temperature. The mixture is then passed through a filter column to a collection tube and centrifuged for 2 minutes at 18,000rpm. After this, the supernatant is collected in a new microcentrifuge tube and an equal volume of 70% RNA-ase free ethanol solution is added. Next, the mixture is vortexed and passed through a FARB mini column to a collection tube and centrifuged for 1 minute at 18,000rpm. The flow

through is discarded and the FARB mini column is returned to the collection tube. 500uL of wash buffer 1 is then added to the FARB column and centrifuged for 1 minute at 18,000rpm. Then, 750uL of wash buffer 2 is added to the FARB column after discarding the flow through and the mixture is centrifuged for 1 minute at 18,000 rpm. This step is repeated again once after which the FARB mini column is centrifuged for an additional 3 minutes at 18,000rpm to dry the column. The FARB mini column is then placed to an elution tube and 45uL RNA-ase free water is added to the membrane centre of the FARB mini column and left for one minute before centrifuging at 18,000rpm for 1 minute to elute the RNA. The RNA concentration is then measured using Nano-drop and stored at -80ºC.

2.2.8 **Method of cDNA synthesis**

cDNA was reverse transcribed from total RNA using qScript® cDNA SuperMix cat# 95048 (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer‘s instructions. In brief, 1-10 µg RNA and 4µl qScript cDNA SuperMix were added to a sterile RNase-free microcentrifuge tube and the volume was completed to 20 µl by RNase/DNase-free water. The mixture was incubated for 5 minutes at 25°C, then 30 minutes at 42°C, 5 minutes at 85°C and finally held at 4°C using Mastercycler gradient 5331 (Eppendorf AG, Hamburg, Germany). The synthesized cDNA was stored at -20 °C for further experiments.

2.2.9 **Method of qPCR**

Real-time PCR was performed in duplicate on Applied Biosystems, Foster City, CA, USA. Using TaqMan™ Fast Advanced Master Mix Catalog number: 4444556. In each PCR tube, 10 µl of Master Mix was added to 1 µl of the probe, 6 µl of DNAse/RNAse free water and 3 µl of the diluted cDNA template (dilution of cDNA was 1:50 of dH2O). The mRNA levels of the murine liver tissue were normalised to the expression of housekeeping gene 36B4, using TaqMan Fam labelled gene expression 36B4 probe (Mm99999915_g1), catalogue number: 4331182. Expression was measured using CT values, normalized to that of 36B4 (ΔCT = CT (36B4) - CT (target) and then expressed as $2^{-\Delta CT}$.

2.2.10 **Method of mice ileal *fgf-15* measurement**

Mice ileal *fgf15* was assessed using the Mouse Fgf15 ELISA kit (Competitive EIA, LifeSpan BioSciences, Inc.) as per the manufacturer's protocol. Briefly, a series of concentrations of standard solution is prepared with sample diluent solution. To each well, 50uL of working standard solution, sample or blank is added, followed by 50uL of detection reagent A working solution. The plate is then covered and incubated at 37ºC for 1 hour. The solution is aspirated and washed with the wash buffer 3 times before drying by gently tapping against clean absorbent paper. Then, 100uL detection reagent B working solution is added to each well and the plate is covered, mixed and incubated for 30 minutes at 37ºC. The liquid is then aspirated and washed 5 times. After this, 90uL of TMB substrate solution is added to each well and the plate is

covered and incubated for 10-20 minutes at 37ºC, protected from light. This is periodically monitored until optimal colour development has been achieved, after which 50uL stop solution is added to each well and the optical density value for each well is immediately determined using a microplate reader set to 450nm.

2.2.11 Microbiota analysis

A single stool sample was collected from patients with biopsy-proven lean and non-lean NAFLD, as well as lean healthy controls. Genomic DNA isolation from these materials were performed using the QIAGEN DNeasy Powerlyzer Powersoil kit (QIAGEN, Germany) according to the manufacturer's instructions. The DNA extracts were used for sequencing of the V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene as previously described(Choo et al. 2015). Briefly, amplicons were generated and indexed using the Illumina Miseq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html) with modifications. PCR amplification was performed using the following program: 95°C for 3 min, followed by 25 cycles of 95°C for 30s, 50°C for 30s and 72°C for 30s, and a final extension step of 72°C for 30s. Dual-indexing of the amplicons was performed using 8 cycles of the same program.

Paired-end 16S rRNA sequencing (2 x 300 bp sequence reads) was performed on an Illumina Miseq platform at the David R Gunn Genomics Facility (South Australian Health and Medical Research Institute). Bioinformatics processing of the 16S rRNA

sequence reads were performed using the Quantitative Insights into Microbial Ecology (QIIME) software (version 2-2018.2). Denoising was performed using the DADA2 pipeline, and chimera filtering and operational taxonomic unit (OTU) assignment was performed against the SILVA 16S rRNA reference database (release v132) clustered at 97% similarity. A minimum subsampling depth of 8,335 reads and 10,698 reads was selected for microbiota composition analysis of the human stool and mice caecum samples, respectively. Taxa present in ≥3 samples and in > 5 sequence reads were used to analyse genera that are differentially abundant between groups. The Benjamini-Hochberg method was used to control the false discovery rate for multiple testing correction. Stool processing and sequencing were performed at the South Australian Healthy and Medical Research Institute, Australia, in collaboration with Dr Geraint Rogers and Dr Jocelyn Choo.

2.2.12 Inflammatory cytokines measurement

Inflammatory cytokines were measured on human serum samples using a human routine 16-plex cytokine panel kit, performed by Crux Biolab, Victoria, Australia.

2.2.13 Statistical analysis

Data was analysed using SPSS version 24.0 (IBM, Armonk, NY). Values are expressed as mean ± standard deviation, median and interquartile range or frequency (percentage) as appropriate. P-values for comparisons of distributions between groups were assessed using Fisher's exact test. The Mann-Whitney non-parametric test was

used to obtain significance between two means of continuous variables. The strength of associations between continuous variables was reported using Spearman's rank correlations. Univariable analysis of variance (ANOVA) was used to examine factors associated with increasing total secondary bile acid levels as continuous variables. Multiple regression analysis was then undertaken to determine which factors significant on ANOVA remained independent predictors for total secondary bile acid levels when adjusted for other clinically relevant variables including age, gender, BMI, hypertension, diabetes, dyslipidaemia, total cholesterol, HOMA and histological profile (fibrosis, steatosis, ballooning, portal inflammation, lobular inflammation and NAS).

Hepatic steatosis was graded from 0 to 3 and was dichotomized into mild steatosis (NASH CRN grades 0-2) and more severe steatosis (grade 3) for the purposes of statistical analysis. Hepatocyte ballooning was dichotomized into no ballooning and any ballooning for analysis purposes. Lobular and portal inflammation was dichotomized to mild (grade 0-1) and severe (grade 2 or more) (Brunt et al. 2011). Fibrosis stage was dichotomized to mild fibrosis (F0-1) and significant fibrosis (F2-4). This was based on a recent systematic review, which showed that the risk of liver-related mortality increases exponentially from stage 2 fibrosis onwards (Eslam et al. 2016c). Statistical significance was considered as $p<0.05$ throughout.

CHAPTER THREE

COMPARISON OF LEAN NAFLD WITH LEAN AND NON-LEAN HEALTHY CONTROLS

Parts of this chapter has been published in:

Chen, F., et al.; *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation;* accepted in Hepatology August 2019; DOI: 10.1002/hep.30908

3 COMPARISON OF LEAN NAFLD WITH LEAN AND NON-LEAN HEALTHY CONTROLS

3.1 INTRODUCTION

The diagnosis of NAFLD and its sequelae NASH require the presence of hepatic steatosis as well as hepatocyte damage evidenced by inflammation and/or fibrosis. Excess cholesterol intake seen in some patients with NAFLD results in free cholesterol accumulation in the hepatocyte mitochondrial membrane, which leads to increased susceptibility to hepatocyte death in response to other noxious stimuli by promoting glutathione loss from mitochondrial and making the hepatocytes sensitive to TNF-induced cytotoxicity (Duwaerts and Maher 2014). In addition, factors such as the genetics and the gut microbiota add to the complexity of NAFLD and NASH pathogenesis. Changes in gut microbiota profile in response to high energy feeding has been linked to enhanced endotoxin absorption from the gut, or "metabolic endotoxemia", as well as promoting the production of pro-inflammatory cytokines and ethanol which eventually leads to hepatotoxicity (Cani et al. 2008; Duwaerts and Maher 2014).

While many studies have shown that increasing body weight is associated with increased mortality, recent evidence has suggested that not all obese or overweight people carry the same metabolic risk and mortality (Kramer et al. 2013; Stefan et al. 2017). Studies in the literature have started to delineate between obese people who are at risk of cardio-metabolic disease (or the "metabolically unhealthy obesity") and obese people who are healthy, the so-called "metabolically healthy obesity". Similarly,

metabolically unhealthy normal weight individuals carry risk for cardiovascular events and/or increased mortality (Eckel et al. 2015; Stefan et al. 2017). A large meta-analysis recently showed that the risk for cardiovascular events and/or all-cause mortality was higher among metabolically unhealthy normal weight individuals compared to the metabolically healthy normal weight people (RR 3.14, 95% CI 2.36 – 3.93) (Kramer et al. 2013).

A recent large study involving 981 subjects with BMI in the normal range, overweight and obese, classified as metabolically healthy or unhealthy (metabolic health defined as having less than 2 parameters of metabolic syndrome) showed that within normal weight individuals, metabolically unhealthy individuals had significantly higher liver fat content and prevalence of NAFLD, significantly higher visceral fat mass and carotid-intima media thickness and a significantly lower percentage of subcutaneous leg fat mass and lower insulin sensitivity and secretion (Stefan et al. 2017). Factors that have been correlated with a metabolically unhealthy phenotype in normal weight individuals include older age and lower physical activity, after adjusting for male sex and waist circumference in a large population study (Wildman et al. 2008).

Therefore, we aimed to test this finding in our cohort of patients using a cohort of patients with lean NAFLD (n=99) and comparing them to lean healthy controls (n=30) as well as non-lean healthy controls (n=46).

3.2 METHODS

We compared a cohort of lean healthy controls (n=30) and non-lean healthy controls (n=46) with biopsy-proven lean NAFLD (n=99). Inclusion and exclusion criteria for these patients were outlined in section 2.1.2.2 and 2.1.2.3. Clinical and laboratory assessments of the participants were described in section 2.1.2.1. All lean NAFLD patients had a liver biopsy performed and histopathology was assessed as per section 2.2.1.

Methods of bile acid extraction and quantification, as well as serum FGF-19 and C4 measurements were also described in the methods sections 2.2.3 to 2.2.5. Stools of a subset of patients were sent for microbiota analysis at the South Australian Health and Medical Research Institute (SAHMRI) as per section 2.2.11. In addition, serum inflammatory cytokine levels were measured at the Crux Biolab, Victoria as per section 2.2.12.

Data was analysed using SPSS version 24.0 (IBM, Armonk, NY). Further details of statistical analysis were provided in section 2.2.13.

3.3 RESULTS

3.3.1 Patient characteristics

The baseline characteristics of lean and non-lean healthy controls as well as lean NAFLD patients are outlined in **Table 5.** All the patients in the lean and non-lean

healthy controls as well as lean NAFLD groups were matched by age and gender. There was no significant difference in the levels of serum fasting blood sugar level, total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides between the three groups.

Metabolic health was defined as the absence of insulin resistance with no evidence of subclinical inflammation as determined by high sensitivity C-reactive protein (CRP), together with none or only one component of the metabolic syndrome according to the Adult Treatment Panel III criteria (hypertension, elevated fasting BSL, dyslipidaemia, elevated plasma triglyceridaemia or low plasma HDL cholesterol) (Lorenzo et al. 2007; Wildman et al. 2008). With this definition, metabolically healthy patients had a significantly lower prevalence of hypertension compared to metabolically unhealthy normal weight patient, regardless of their BMI.

Table 5. Characteristics of lean and non-lean healthy controls and lean NAFLD patients

	Lean control (MHNW) (n = 30)	Non-lean control (MHO) (n=46)	Lean NAFLD (MUNW) (n = 99)	p-value#
Age (years)	46.7 ± 12	48.6 ± 11.2	46 ± 11.7	0.530
Male (%)	22 (73.3)	35 (76.1)	69 (69.7)	0.502
BMI (kg/m^2)	22.8 ± 1.9	28.5 ± 3.2	23.2 ± 1.5	**<0.001**
ALT (IU/ml)	27.1 ± 10.3	26.3 ± 8.7	57.9 ± 35.6	**<0.001**
Fasting BSL (mmol/L)	4.9 ± 0.5	5.1 ± 0.3	5.3 ± 1.8	0.244
Total Cholesterol (mmol/L)	5.0 ± 1.1	5.3 ± 1.1	5.1 ± 1.2	0.375
HDL Cholesterol (mmol/L)	1.4 ± 0.4	1.5 ± 0.4	1.5 ± 0.6	0.943
LDL Cholesterol (mmol/L)	3.0 ± 0.8	3.2 ± 0.9	3.6 ± 1.6	0.084
Triglycerides (mmol/L)	1.3 ± 1.0	1.5 ± 0.8	1.6 ± 1.4	0.353
Diabetes (%)	0	0	11 (11.1)	
Hypertension (%)	1 (3.3)	7 (15.2)	25 (25.3)	
Dyslipidaemia (%)	9 (30.0)	25 (54.3)	43 (43.4)	

#p-value was calculated using one-way Anova test. Abbreviations: MHNW: Metabolically healthy normal weight; MHO: Metabolically healthy obese; MUNW: Metabolically unhealthy normal weight. Metabolic health was defined as the absence of insulin resistance/diabetes with none or only one of the metabolic syndrome components.

3.3.2 Metabolic health status has more impact on bile acid levels than BMI alone

To investigate the effect of metabolic health on bile acid metabolism, we first compared the total bile acid levels between lean and non-lean healthy controls as well as lean NAFLD patients. Interestingly, total bile acid level was not significantly different between the lean and non-lean healthy controls, however, the total bile acid level was significantly higher in patients with lean NAFLD suggesting the greater impact of metabolic health status on the bile acid level, beyond their BMI alone (**Figure 3**).

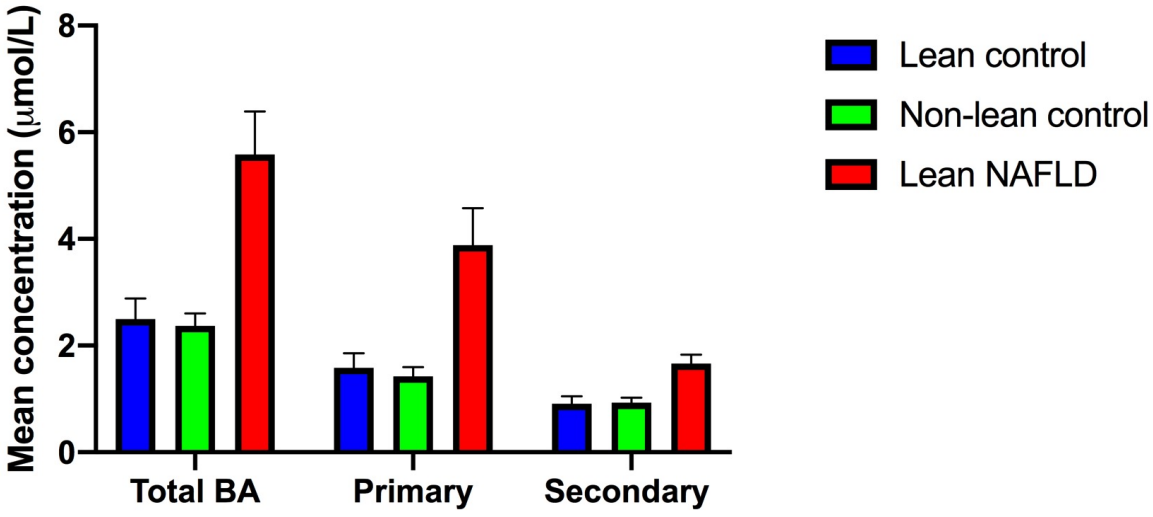


Figure 3. Total bile acids, total primary bile acids and total secondary bile acid levels between lean and non-lean healthy controls as well as lean NAFLD patients.

The x-axis showed lean healthy controls (n=30), non-lean healthy controls (n=46), and lean NAFLD (n=99) patients and the y-axis showed mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

3.3.3 Lean NAFLD patients had distinct bile acid profile

The composition of the BA pool also differed between the groups. Lithocolic acid (LC) is abundant in the lean control group but not in the non-lean control and lean NAFLD groups (**Figure 4**). In addition, the proportion of conjugated primary bile acids, for example, glycocholic acid (GCA) (1.2275 ± 3.53182 umol/L in lean NAFLD vs 0.3403 ± 0.47052 umol/L in lean healthy controls and 0.2627 ± 0.28027 umol/L in non-lean healthy controls, $p = 0.092$), taurochenodeoxycholic acid (TCDCA) (0.2538

± 0.70753 umol/L vs 0.0746 ± 0.09019 umol/L and 0.0625 ± 0.06001 umol/L, p = 0.004) and taurocholic acid (TCA) (0.2919 ± 1.04185 umol/L vs 0.0533 ± 0.8936 umol/L and 0.0327 ± 0.03954 umol/L, p = 0.069) were higher in the lean NAFLD group compared to the two healthy control groups.

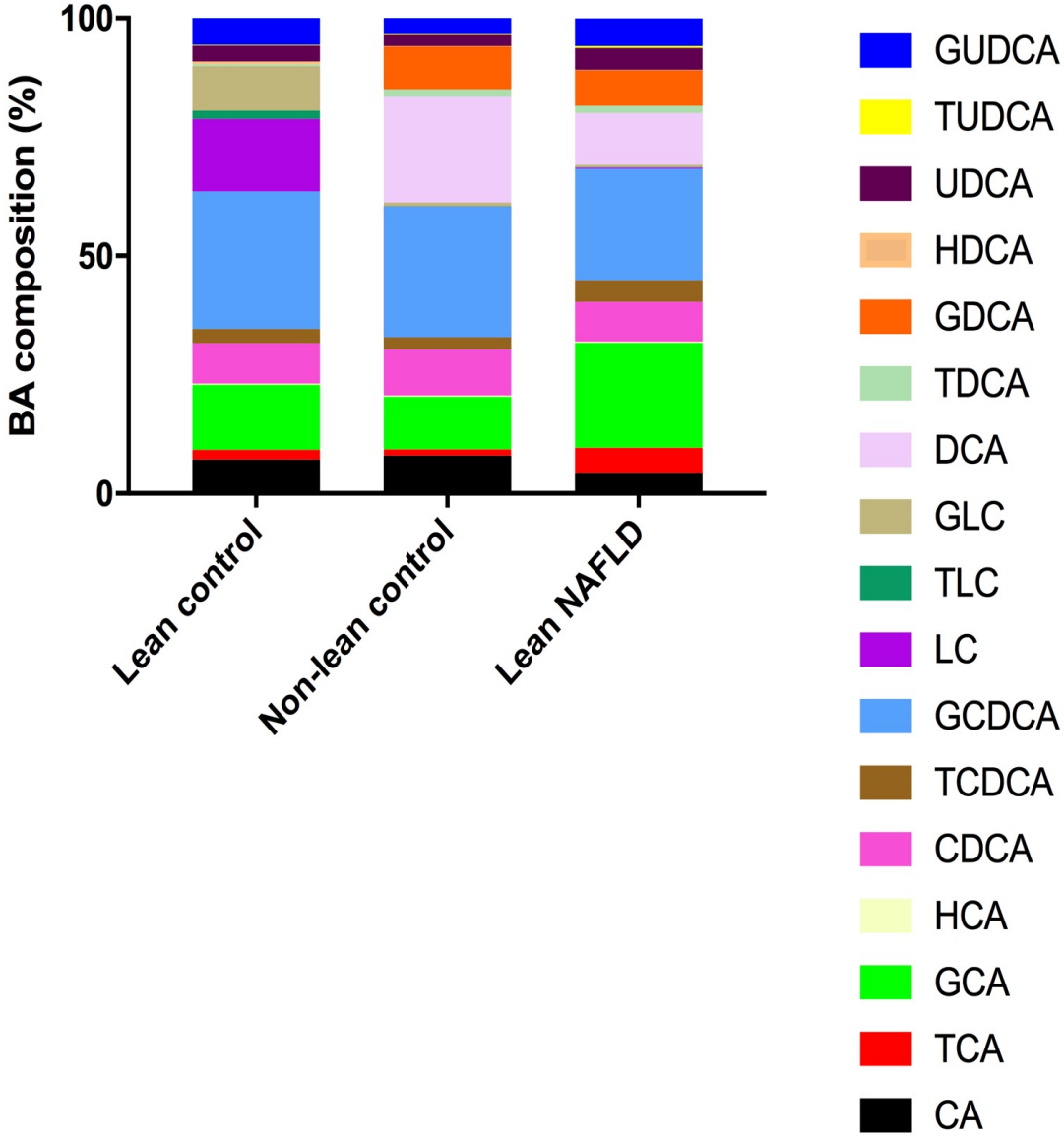


Figure 4. Bile acid distribution in lean healthy controls and lean NAFLD patients.

Bile acid composition as a percentage according to hepatic fibrosis. The x-axis shows lean healthy controls (n = 30) and lean NAFLD patients (n = 99), and the y-axis shows the percentage composition of each individual bile acid in %.

3.3.4 Lean NAFLD patients had elevated individual serum bile acid levels

Having demonstrated the importance of metabolic health on bile acid levels above and beyond the BMI, we next compared the bile acid profile and their regulation between lean NAFLD and lean healthy controls. The levels of total bile acids (5.56 ± 7.47 uM vs 2.50 ± 2.11 uM, p=0.002) as well as total primary BA (3.79 ± 6.42 uM vs 1.58 ± 1.49 uM, p=0.018) and total secondary BA (1.73 ± 1.76 uM vs 0.91 ± 0.76 uM, p=0.003) were significantly higher in lean NAFLD patients compared to lean healthy controls (**Figure 5A**).

In terms of primary bile acids, the concentration of both the cholic acid (CA) and chenodeoxycholic acid (CDCA) were higher in lean NAFLD compared to lean healthy controls although it was only significant for CDCA levels (0.47 ± 0.63 uM vs 0.21 ± 0.27 uM, p=0.003, **Figure 5B-C**).

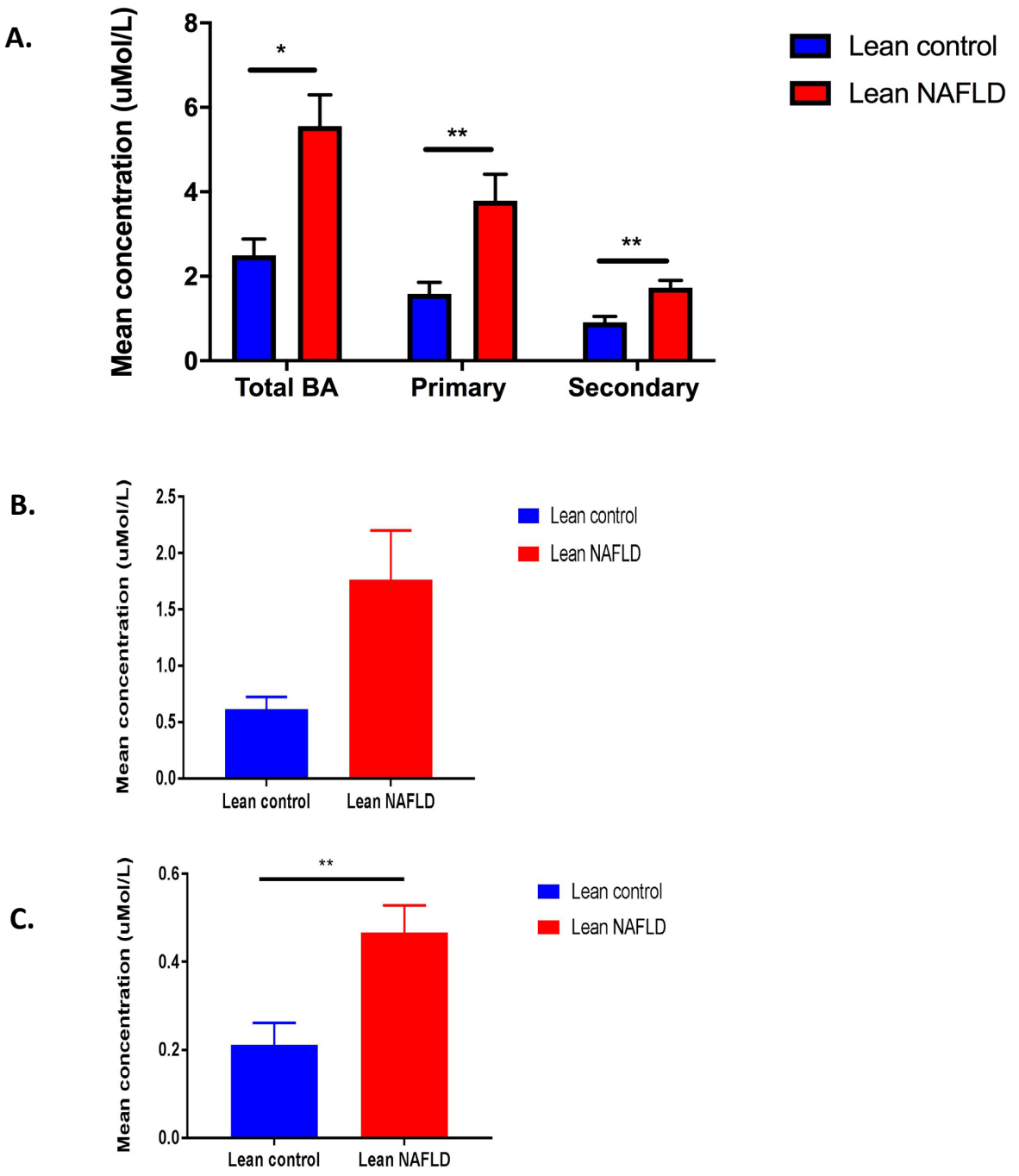


Figure 5. A) Bile acid levels in lean healthy controls and lean NAFLD B) Cholic acid (CA) and C) Chenodeoxycholic acid (CDCA) levels between lean healthy controls and lean NAFLD patients.

The x axis shows lean healthy controls (n = 30, blue bar) and lean NAFLD patients (n = 99, red bar) and the y axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

Similarly, the secondary bile acid deoxycholic acid was significantly higher in lean NAFLD patients compared to lean healthy controls (0.60 ± 0.57 uM vs 0.38 ± 0.39 uM, p=0.019, **Figure 6A**) with higher secondary to primary bile acid ratio in the lean healthy controls (**Figure 6B**).

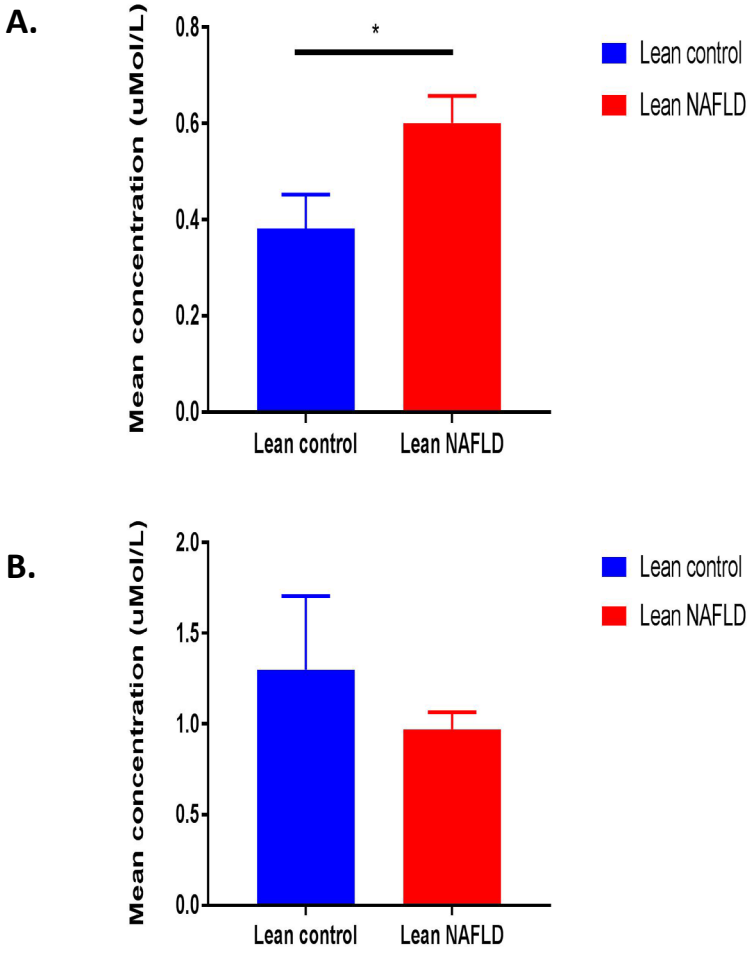


Figure 6. A) Deoxycholic acid (DCA) levels and B) secondary/primary bile acid ratio between lean healthy controls and lean NAFLD patients.

The x axis shows lean healthy controls (n = 30, blue bar) and lean NAFLD patients (n = 99, red bar) and the y axis shows the mean concentration of bile acid levels in µmol/L in A, and secondary to primary bile acid ratio in B. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. P < 0.05, ** P<0.01, *** P<0.001.

3.3.5 Lean NAFLD patients had comparable FGF-19 levels to lean healthy controls in the early, but not in later stages of the disease

There was no significant difference in levels of FGF-19 between lean healthy controls and lean NAFLD patients with none/mild fibrosis (F0-1). However, in lean NAFLD patients with moderate/severe fibrosis (F2-4), the level of FGF-19 was significantly lower compared to that in lean healthy controls (**Figure 7**).

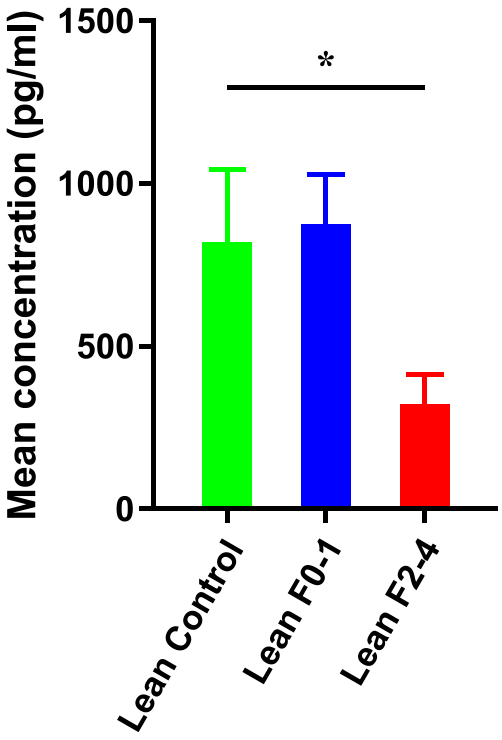


Figure 7. FGF-19 levels in lean healthy controls and lean NAFLD patients stratified by fibrosis stage.

Mean concentration of FGF19 levels according to BMI and hepatic fibrosis. The x axis shows lean healthy controls (n = 30, green bar) and lean NAFLD patients with absent/mild (F0–F1, blue bar, n = 75) and moderate/severe (F2-4, red bar, n = 24) hepatic fibrosis; the y axis shows the mean concentration of FGF19 in pg/mL. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

3.3.6 C4 levels in lean NAFLD compared to lean healthy controls

When C4 level (a serum marker for BA synthesis) was measured, lean NAFLD patients had significantly higher levels of C4 compared to lean healthy controls. This was more so for lean NAFLD patients with more advanced fibrosis compared to early fibrosis (**Figure 8**).

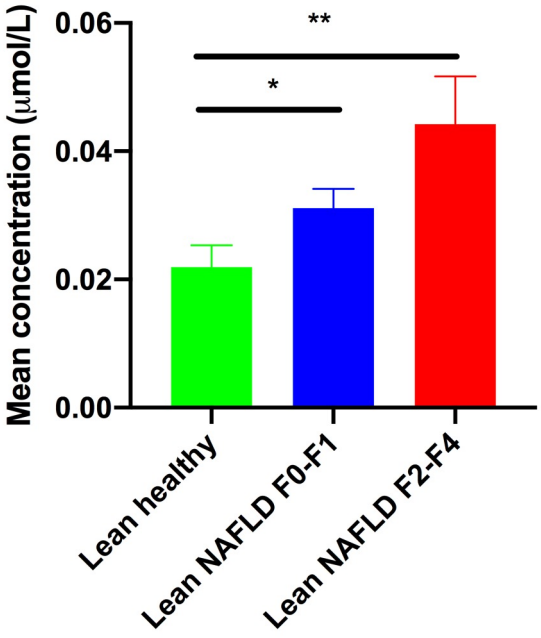


Figure 8. C4 levels between lean healthy controls and lean NAFLD patients

Mean concentration of C4 levels according to BMI and hepatic fibrosis. The x axis shows lean healthy controls (n = 30, green bar) and lean NAFLD patients with absent/mild (F0–F1, blue bar, n = 75) and moderate/severe (F2-4, red bar, n = 24) hepatic fibrosis; the y axis shows the mean concentration of C4 in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

3.3.7 Microbiota profile in lean NAFLD patient is distinct from lean healthy controls

A selection of patients from the lean healthy controls and lean NAFLD groups were used for the comparison of their microbiota profile. The patient characteristics are shown **Table** 6. Analysis of microbiota demonstrated a distinct separation in profiles between lean healthy controls and lean NAFLD (PERMANOVA P = 0.069, Pseudo-F = 2.019) (**Figures 9A-C**). More specifically, in the lean NAFLD group there was an increased abundance of the species *Dorea* and a reduction in the relative abundance of a number species, including *Marvinbryantia* and the *Christensellenaceae R7* group.

Table 6. Characteristics of lean healthy controls and lean NAFLD patients used in microbiota analysis

	Lean control (n = 9)	Lean NAFLD (n = 5)	p-value
Age (years)	56.1 ± 8.5	49 ± 8.5	0.259
Male (%)	3 (33.3)	2 (40)	1.0
BMI (kg/m^2)	22.7 ± 1.9	24.0 ± 1.5	0.274
ALT (IU/ml)	25.3 ± 9.6	97.5 ± 64.1	0.0051
Total Cholesterol (mmol/L)	4.9 ± 1.3	5.2 ± 0.6	0.218
Fasting BSL (mmol/L)	4.7 ± 0.3	7.1 ± 4.8	0.645

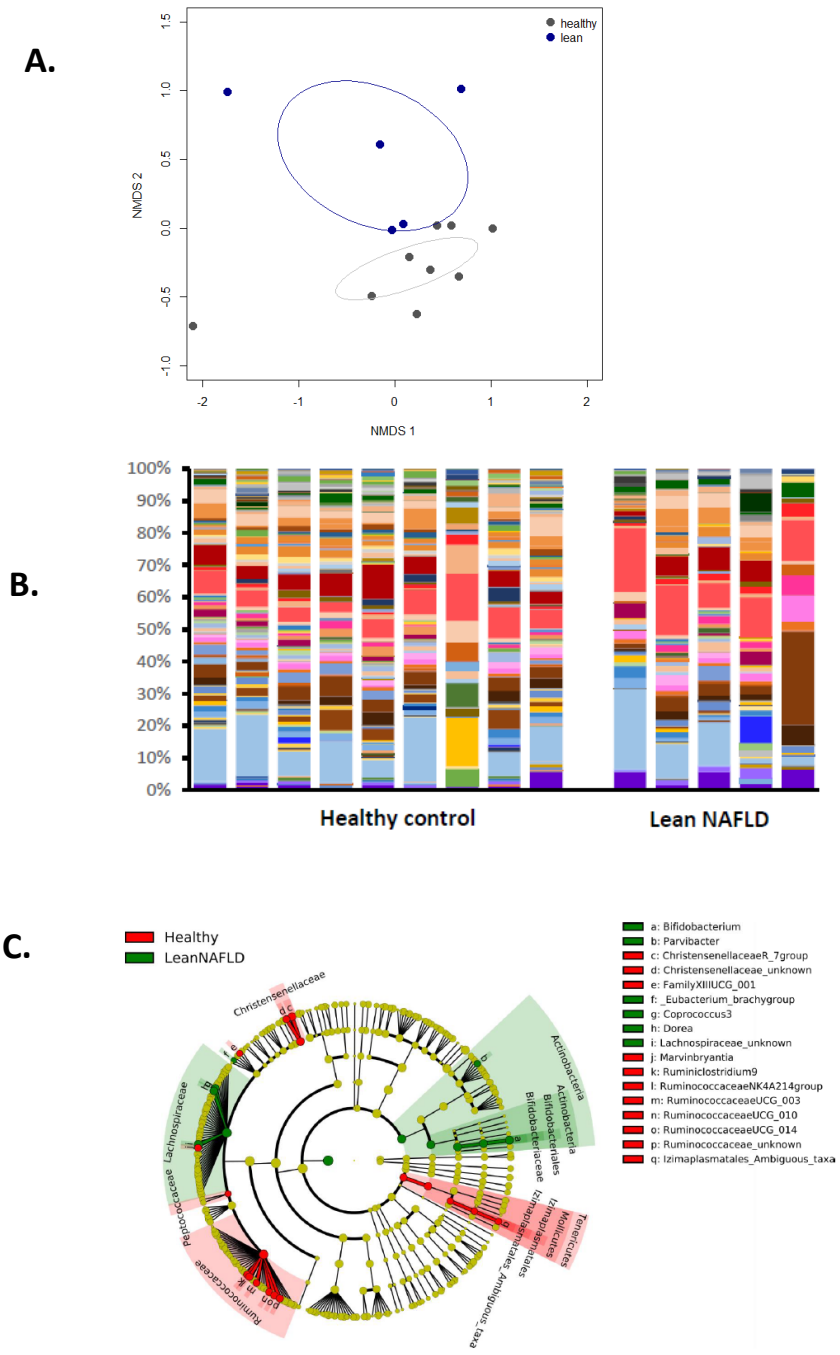


Figure 9. Microbiota profile of lean healthy controls and lean NAFLD patients.

A) Non-metric Multi-dimensional scaling (NMDS) plot of distribution of gut microbiota in lean healthy controls (n = 9) and lean NAFLD patients (n = 5) showing distinct separation between the two groups. Circles represent 95% confidence limit of the standard error of samples in each group. B) Operational taxonomic unit (OUT) table between lean healthy controls (n = 9) and lean NAFLD patients (n = 5). C) Taxa differences between lean healthy controls (n = 9) and lean NAFLD patients (n = 5).

3.3.8 Lean NAFLD patients had higher inflammatory cytokine profile

We next compared the inflammatory cytokine profiles from serum of lean healthy controls and lean NAFLD patients. Interestingly, patients with lean NAFLD had higher levels, although insignificant, of several inflammatory cytokines, including IL-1 beta ($p = 0.6905$), IL-4 ($p = 0.1646$) and TNF-alpha ($p = 0.1096$) (**Figures 10A-C**).

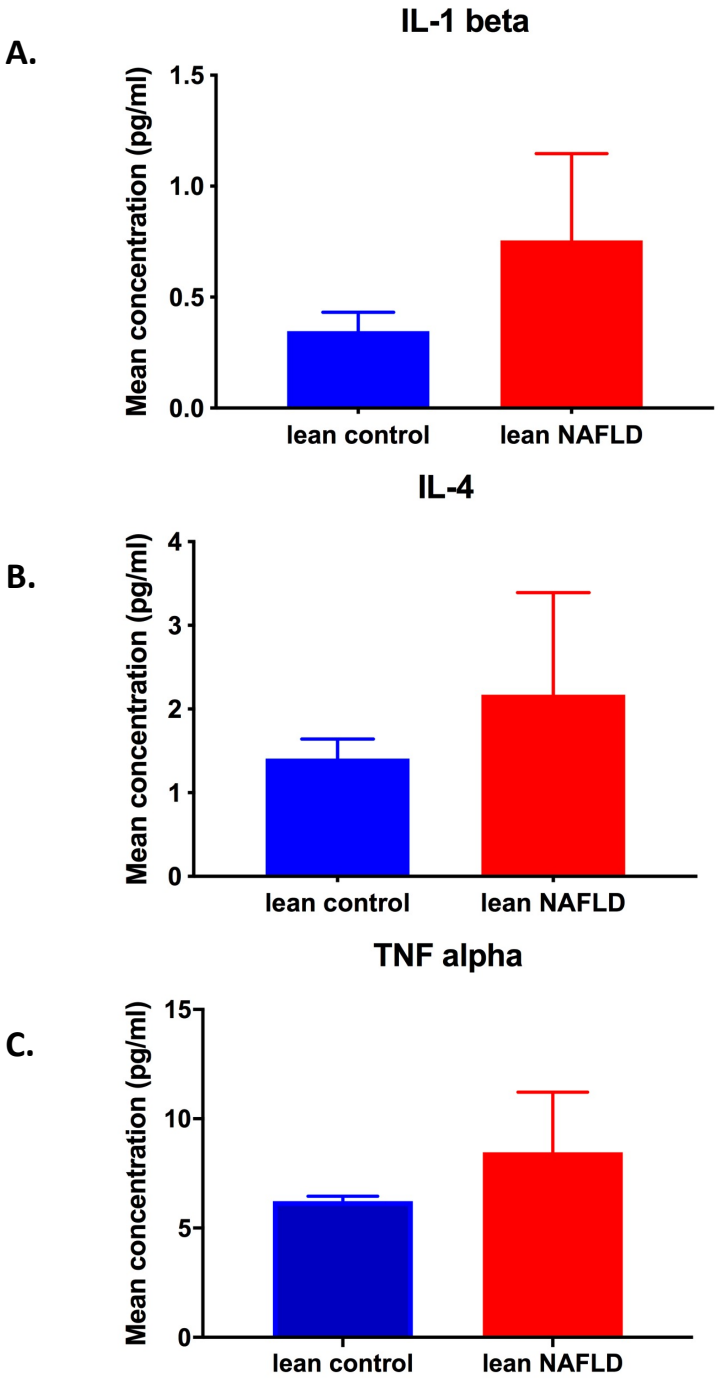


Figure 10. Inflammatory cytokines level between lean healthy control and lean NAFLD

The x axis shows lean healthy controls (n = 30) and lean NAFLD (n = 99); the y axis shows the mean concentration of inflammatory cytokines in pg/mL. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

3.4 DISCUSSION

In this chapter, we first compared the characteristics of patients with lean NAFLD with groups of lean healthy controls as well as non-lean healthy controls. We were first interested to see the effect of metabolic health on the baseline metabolic profile of patients as well as on the bile acid profile. Lean NAFLD patients and metabolically unhealthy patients had a higher prevalence of hypertension compared to metabolically healthy patients regardless of their BMI. When we compared their bile acid profiles (including comparing it to the non-lean NAFLD patients), we found that patients who were metabolically unhealthy had significantly higher levels of bile acid levels compared to metabolically healthy patients, regardless of their BMI.

We then focused on the comparison of lean healthy controls (to represent metabolically healthy patients) with lean NAFLD (to represent metabolically unhealthy patients). Here we found that patients with lean NAFLD had significantly higher total BA, total primary BA and total secondary BA compared to lean healthy controls with levels higher for certain individual BA like chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA), both of which are potent FXR agonists.

Next we compared the metabolic adaptation capacity between the two groups by measuring FGF-19 levels in the serum as markers of FXR activity. Although overall there was no significant difference in FGF-19 levels between lean healthy and lean NAFLD patients, when we stratified the lean NAFLD patients according to their fibrosis severity we found a striking difference in metabolic adaptation. Patients with

early stages of lean NAFLD (F0-F1) had similar levels of FGF-19 compared to lean healthy controls but this level significantly dropped in the later stages of the disease (F2-F4) suggesting a loss of metabolic adaptation as the disease progresses in the lean NAFLD patients. Exactly what triggers the switch or loss of metabolic adaptation is not clear and is beyond the scope of our study. It would be interesting for future studies to explore this further through longitudinal follow up of patients.

The serum C4 level, which is a marker for bile acid synthesis (Arab et al. 2017) also reflected the FGF-19 levels with the level being lowest in the lean healthy controls, and highest in lean NAFLD with advanced fibrosis. Interestingly, however, unlike the FGF-19 levels, which were not significantly different between the lean healthy controls and lean NAFLD with early fibrosis, we saw significantly higher C4 levels in lean NAFLD with early fibrosis compared to lean healthy controls. This may reflect the metabolic adaptation that lean NAFLD possesses early on in the disease course, where the increased dietary intake of cholesterol is compensated by increased bile acid production to maintain serum cholesterol level and body weight. This may also explain the lower secondary to primary bile acid ratios seen in lean NAFLD patients compared to lean healthy controls.

Lean NAFLD had a distinct separation in microbiota profile compared to the healthy controls with an increased abundance of *Dorea* that has been implicated in the pathogenesis and progression of NASH (Del Chierico et al. 2017b; Del Chierico et al. 2017a), and a decrease in several species protective for NAFLD such as *Marvinbryantia* and *Christensellenaceae R7* group. Both are known to play a role in

the modulation and production of beneficial short chain fatty acids and in providing a desirable intestinal environment for the growth of probiotic bacteria (Ma et al. 2017; Zhou et al. 2017).

Certain gut microbiota, especially those belonging to the Firmicutes phyla such as the *Ruminococcus, Marvinbryantia* and *Christensellenaceae* are known butyrate producers. Butyrate is a beneficial short chain fatty acids (SCFA) and are products of dietary fibre fermentation in the gut (Zhou and Fan 2019). Apart from providing energy for the intestinal epithelium, SCFAs also play significant roles in regulation of immunity, lipid and glucose metabolism as well as maintenance of gut microbiota homeostasis (Z. H. Zhao et al. 2019). Studies have shown that supplementation with butyrate-producing probiotics corrected high fat diet (HFD) induced steatohepatitis in mice through the production of butyrate, as well as improving the gastrointestinal barrier, thereby inhibiting the delivery of gut derived endotoxin to the liver (Zhou et al. 2017). The trend towards higher inflammatory cytokines seen in lean NAFLD compared to lean healthy controls further supports a gut-derived pathogenesis of lean NAFLD.

3.5 CONCLUSION

In this chapter we have demonstrated the importance of metabolic health on baseline metabolic risk profiles as well as bile acid levels. In addition, we have shown that lean NAFLD patients had altered gut microbiota, which increases their risk for NAFLD development and progression, as well as increases in their pro-inflammatory milieu

compared to lean healthy controls. Lean NAFLD however, demonstrated good metabolic adaptation, especially early in the disease process, with FGF19 levels comparable to those seen in the lean healthy controls.

CHAPTER FOUR

COMPARISON OF METABOLIC ADAPTATION IN LEAN NAFLD WITH NON-LEAN NAFLD PATIENTS

Parts of this chapter has been published in:

Chen, F., et al.; *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation;* accepted in Hepatology August 2019; DOI: 10.1002/hep.30908

4 COMPARISON OF METABOLIC ADAPTATION IN LEAN NAFLD WITH NON-LEAN NAFLD PATIENTS

4.1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects about 20-30% of the world's population and is a leading cause for end-stage liver disease, cancer and transplantation (Z. Younossi et al. 2018a). Despite this, the existence and clinical course of the entity known as "lean NAFLD" or "NAFLD in lean patients" has been the subject of intense debate and controversy. To many, lean NAFLD refers to individuals manifesting the disease in the context of a normal body mass index (BMI), but having excess visceral adiposity and insulin resistance, as well as metabolic dysfunction that is typically observed in people with obesity (Ruderman et al. 1998), the so called metabolically obese normal-weight (MONW) individual. The prevalence of lean NAFLD varies widely according to the criteria used for its definition but ranges from 5 to 45% (Ding et al. 2016). By this interpretation, lean NAFLD is similar if not identical to NAFLD associated with overweight and obesity, with insulin resistance at its core.

Accumulating evidence however suggests that lean NAFLD might be a distinct patho-physiological entity with about half (47-65%) having NASH (Z. M. Younossi et al. 2016a). While "lean NAFLD" was first described in Asia, it has since been recognised globally (Z. Younossi et al. 2018a). Most aspects of lean NAFLD including its operational classification have not been systematically characterised. The most frequently used definition is that of hepatic steatosis with a BMI < 25 kg/m^2 (or less

than 23 kg/m^2 in Asians) in the absence of significant alcohol intake (Das and Chowdhury 2013). The natural history of lean NAFLD is even less well characterised; some data suggests that they have worse mortality and accelerated disease progression, despite a more favourable metabolic risk profile (A. C. Dela Cruz et al. 2014; Hagstrom et al. 2018). Lastly, the pathogenesis and mechanisms for their favourable metabolic profile compared to obese NAFLD is puzzling and poorly understood, while therapeutic options for lean NAFLD remain undefined.

We hypothesized that the pathogenesis of lean and obese NAFLD and their distinct metabolic and histological profiles is caused by more than just differences in body weight and body mass index. We considered that the clinical phenotype of lean NAFLD might reflect differences in the integration of signals from the diet and the systemic metabolic milieu, as also the enterohepatic axis comprising both bile acids and gut microbiota. We tested this hypothesis in a large well-phenotyped biopsy proven cohort of 538 Caucasian patients with NAFLD.

4.2 METHODS

The cohort comprised five hundred and thirty-eight consecutive Caucasian patients with histologically characterized NAFLD. The inclusion criteria and clinical and laboratory assessments and histopathology are described in detail in methods chapter. Ethics approval was obtained from the Human Ethics committee of the Western Sydney Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees.

Methods of genotyping, high throughput bile acid profiling, quantification of C4, FGF19, and microbiome analysis are provided in the methods chapter. In addition, serum phosphatidylethanol level was measured in all patients to rule out significant alcohol intake in the past few weeks prior to recruitment. Details on the method of serum phosphatiylethanol level measurement is also described in chapter 2, methods.

Data were analysed using SPSS version 24.0 (IBM, Armonk, NY). Values are expressed as mean ± standard deviation, median and interquartile range or frequency (percentage) as appropriate. Statistical significance was considered as $p<0.05$ throughout; details are provided in chapter 2, methods.

4.3 RESULTS

4.3.1 Clinical, histological and genetic characteristics of patients with lean NAFLD

A total of 538 patients with biopsy proven NAFLD were recruited for the study. Ninety-nine patients (18%) were lean. The clinical and biochemical characteristics of lean NAFLD compared to their counterpart non-lean patients are presented in **Table 7**. Both groups had similar non-significant phosphatidylethanol level in the blood therefore ruling out significant alcohol intake (values above 253 ng/mL are regarded as significant alcohol consumption in the past weeks (Kechagias et al. 2015)). In

addition to lower BMI, lean patients had lower waist hip ratio (WHR) and a better metabolic profile, including a significantly lower frequency of diabetes, a higher serum HDL, and lower serum triglycerides, fasting blood glucose and HOMA-IR values, compared to their non-lean counterparts. Histologically, lean patients had higher prevalence of none or mild fibrosis and lower NAS scores ($p<0.001$ for both), as well as lower serum ALT **(Figure 11)**. In total, lean patients have favourable metabolic and histological features compared to non-lean NAFLD.

Lean NAFLD patients had a significantly higher prevalence of carriage of the *TM6SF2* rs58542926 (T) allele compared to non-lean patients, but a similar prevalence of the *PNPLA3* GG polymorphism (**Table 7**). To adjust for the effect of confounding factors, the *TM6SF2* rs58542926 (T) allele still associated with lean NAFLD in a multivariable model adjusting for age, gender, ALT, diabetes, total cholesterol level, fibrosis, steatosis and *PNPLA3* genotype (OR 2.567, 95% confidence interval 1.426-4.619, p = 0.002).

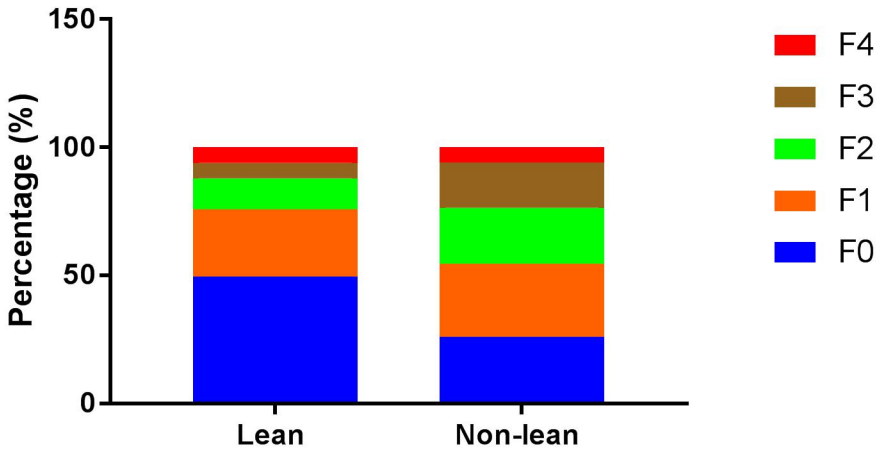


Figure 11. Fibrosis grade distribution

The distribution of fibrosis grades amongst patients with lean NAFLD (n=99) and non-lean NAFLD (n=439)

Table 7. Clinical and histological characteristics of lean and non-lean NAFLD patients

	Lean NAFLD (n = 99)	Non-lean NAFLD (n = 439)	p-value
Age (years)	46 ± 11.7	47 ± 13.0	0.445
Male (%)	69 (69.7)	290 (64.9)	0.413
BMI (kg/m^2)	23.2 ± 1.5	30.8 ± 4.7	**<0.001**
PNPLA3 I148M (no, %) (CC/CG/GG)	32(32.3)/39(39.4)/16(16.2)	145(32.4)/172(38.5)/67(14.9)	**0.973**
TM6SF2 E167K (no, %) (CC/CT/TT)	59(59.6)/22(22.2)/3(3.0)	321(71.8)/50(11.8)/6(1.3)	**0.005**
ALT (IU/ml)	57.9 ± 35.6	72.3 ± 46.8	**<0.001**
Waist/ Hip ratio (WHR)*	0.919 ± 0.062	0.971 ± 0.079	**<0.001**
Normal WHR	25 (29.2)	46 (10.3)	
Elevated WHR	29 (29.3)	189 (42.3)	
Phosphatidylethanol level (ng/mL)	66.35 ± 48.59	66.45 ± 52.72	0.8829
Diabetes (%)	11 (11.1)	128 (28.6)	**<0.001**
Hypertension (%)	25 (25.3)	158 (35.3)	0.060
Dyslipidaemia (%)	43 (43.4)	242 (54.1)	0.059
Total Cholesterol (mmol/L)	5.1 ± 1.2	5.2 ± 1.2	0.472
HDL-C (mmol/L)	1.5 ± 0.6	1.2 ± 0.3	**<0.001**
LDL-C (mmol/L)	3.6 ± 1.6	3.5 ± 1.6	0.667
Triglyceride (mmol/L)	1.6 ± 1.4	1.9 ± 1.2	0.083
Fasting BSL (mmol/L)	5.3 ± 1.8	5.9 ± 1.8	**0.006**
HOMA-IR	2.8 ± 1.9	5.4 ± 5.9	**<0.001**
Fibrosis (%)			
F0-1 (%)	75 (75.8)	239 (54.6)	**<0.001**
F2-4 (%)	24 (24.2)	200 (45.4)	
Ballooning (%)			
No ballooning (%)	37 (37.4)	131 (30.4)	0.1510
Any ballooning (%)	62 (62.6)	308 (69.6)	
Steatosis (%)			
Grade 1-2 (%)	85 (85.9)	351 (81.2)	0.2530
Grade 3 (%)	14 (14.1)	88 (18.8)	
Lobular inflammation			
Grade 0-1 (%)	88 (88.9)	352 (80.5)	0.0782
Grade 2 or more (%)	11 (11.1)	87 (19.5)	
NAS score	3 ± 2	4 ± 2	**0.001**

*Values are mean±SD, or number (%), p-value was calculated using Fisher's exact test and student's t-test. *WHR based on 54 lean patients and 235 non-lean patients. Normal WHR defined as less than 0.90 for males and less than 0.85 for females. DNA was available for 471 patients (86%).*

4.3.2 Serum bile acid profile is associated with NAFLD severity, but not steatosis

Although there is increasing evidence to suggest a critical role for BAs in metabolic diseases including NAFLD, their correlation with disease severity is conflicting, likely due to the limited sample sizes and various methodologies adapted in previous studies. We explored the association between the BA profile and liver histology.

No differences in the total BA, total primary or total secondary BA levels were noted between patients with mild steatosis (S1-S2) compared to those with severe steatosis (S3) (**Figure 12**). Next, the association with steatohepatitis activity including the severity of inflammation and hepatocyte ballooning was tested. In this analysis, significantly higher total BAs ($p = 0.006$), primary BAs ($p = 0.031$) and secondary BAs ($p < 0.001$) were found in patients with hepatocyte ballooning, compared to those without ballooning (**Figure 13**).

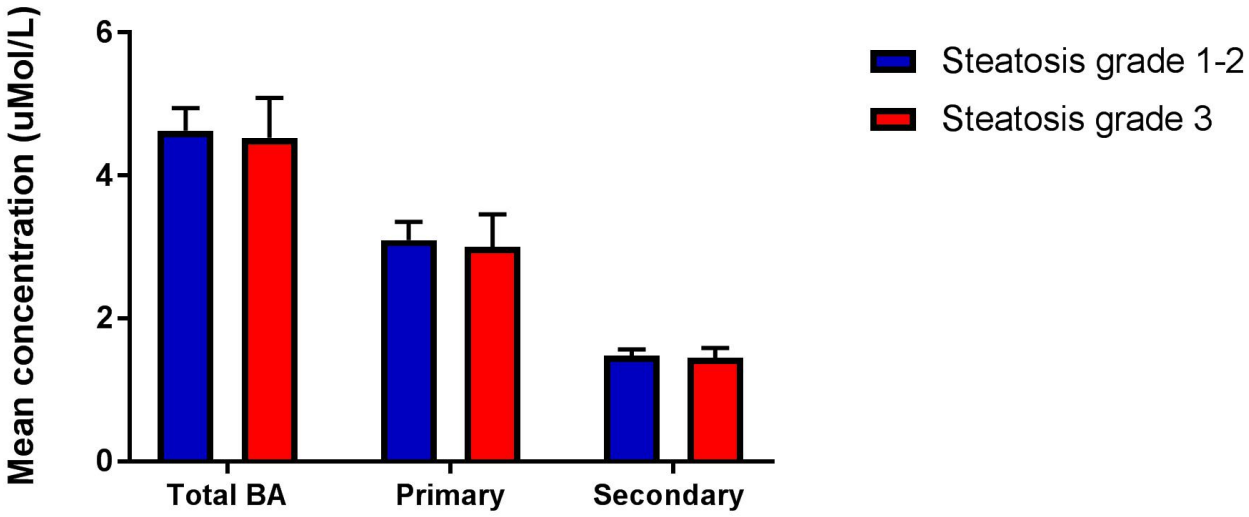


Figure 12. Steatosis grade and bile acid concentration

Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to hepatic steatosis. The x axis shows hepatic steatosis dichotomized as mild (grade 1-2, n = 436) or moderate/severe (grade 3, n = 102), and the y axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

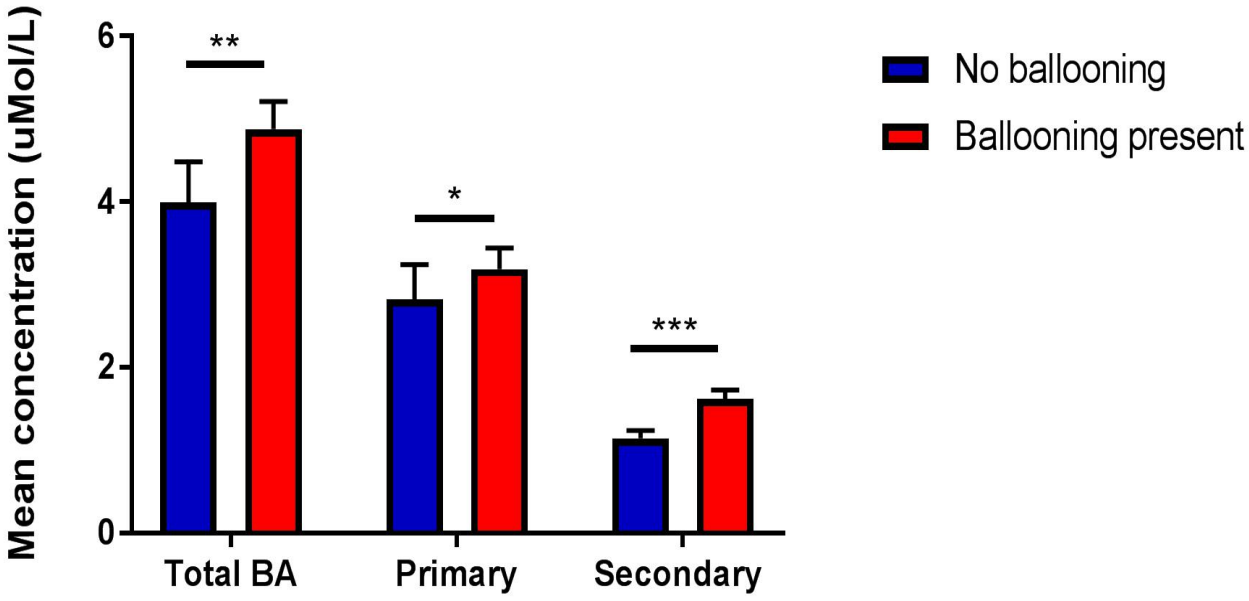
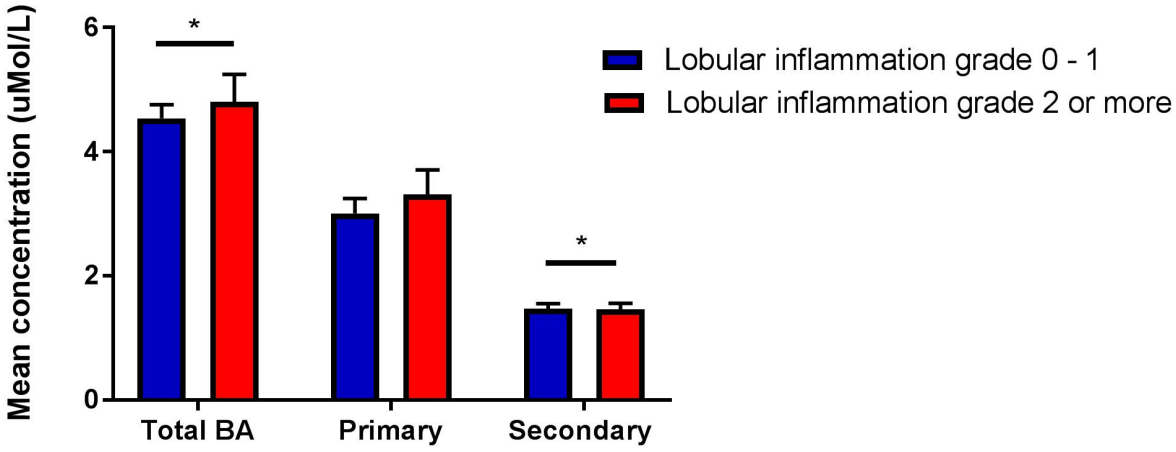


Figure 13. Ballooning grade and bile acid concentration

Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to hepatocyte ballooning. The x axis shows hepatic ballooning dichotomized as no ballooning (n = 168) or any ballooning (n = 370), and the y axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

Next, we investigated the relationship between BA and lobular and portal inflammation. When comparing degrees of inflammation, higher grades of lobular inflammation were associated with higher total (p = 0.027) and secondary BAs (1.4680 ± 1.8109 vs 1.4563 ± 1.0038, p = 0.021), while there was no significant difference in primary BA levels (p = 0.073). Similarly, there was no significant difference in the total BA, primary and secondary BA levels with different grades of portal inflammation (**Figures 14A and B**).

A.



B.

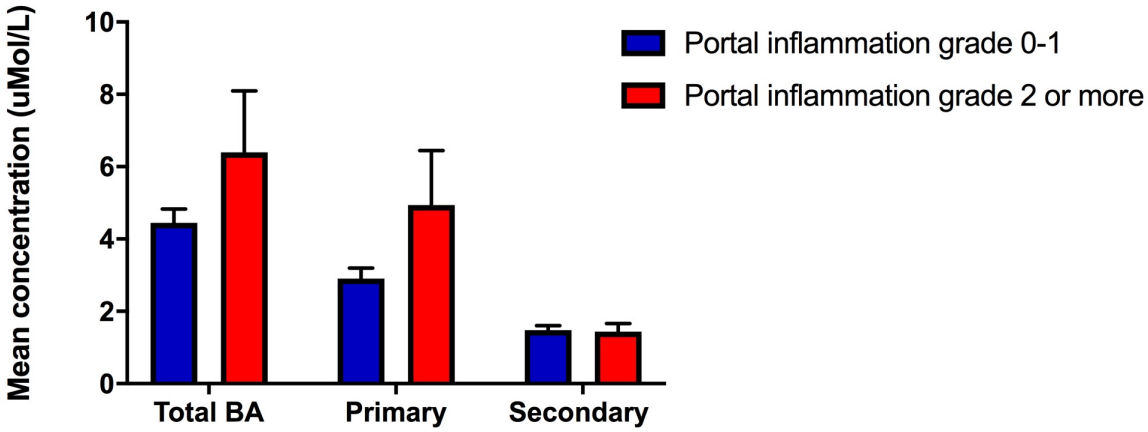


Figure 14. Bile acid levels and their associations with inflammation.

A. Mean serum bile acid levels in different lobular inflammation grades, dichotomized as lobular inflammation grade 0-1 and grade 2 or more. The x axis shows lobular inflammation dichotomized as absent/mild (grade 0-1, n = 440) or moderate/severe (grade 2 or more, n = 98), and the y axis shows the mean concentration of bile acid levels in µmol/L. B. Mean serum bile acid levels in different portal inflammation grades dichotomized as grade 0-1 and grade 2 or more. The x axis shows portal inflammation dichotomized as absent/mild (grade 0-1, n = 440) or moderate/severe (grade 2 or more, n = 98), and the y axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

Higher levels of total BAs ($p = 0.001$), primary BAs ($p = 0.001$) and secondary BAs ($p = 0.002$) were seen in patients with higher NAS score, defined as > 3 compared to patients with lower scores (**Figure 15**). Similarly, patients with NASH had higher levels of total, primary and secondary BAs, compared to patients with steatosis, but only the secondary BA levels were significantly different between the two groups ($p = 0.047$).

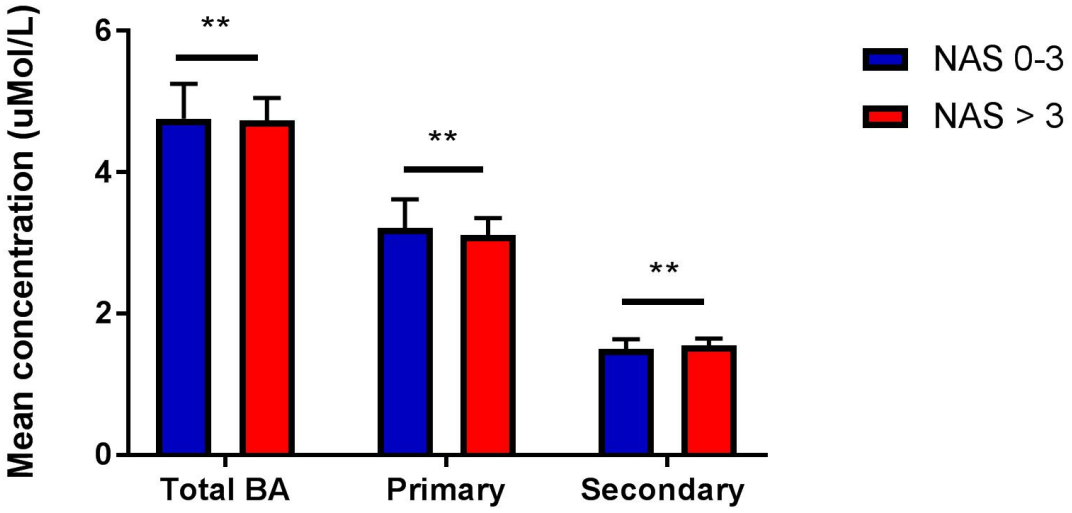


Figure 15. Bile acid distribution with respect to NAFLD activity scores (NAS).

Mean concentration of total bile acids, total primary bile acids, and total secondary bile acids according to NAS. The x-axis shows NAS dichotomized as absent/mild (score 0-3, n = 251) or moderate/severe (grade 3 or more, n = 287), and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

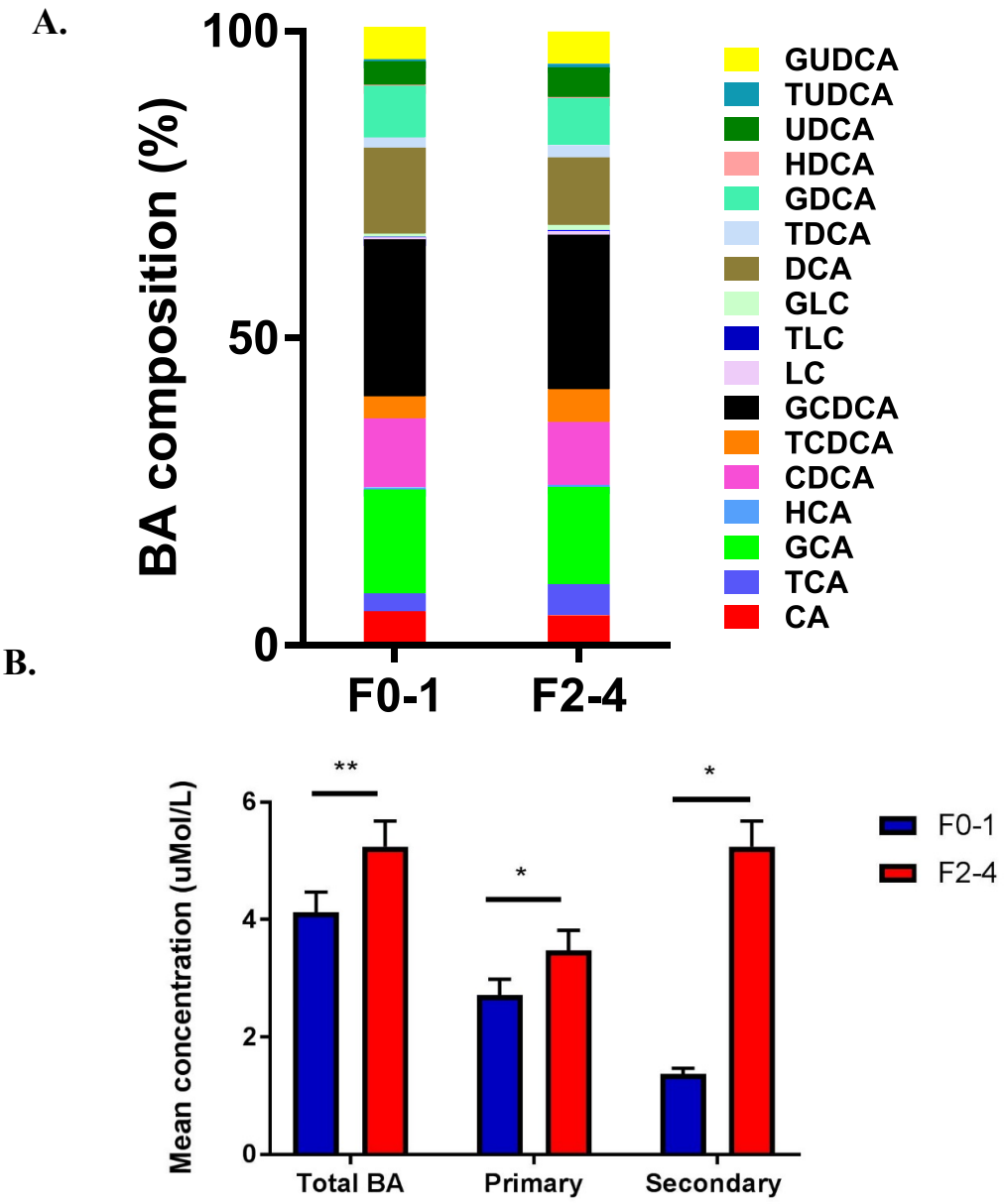


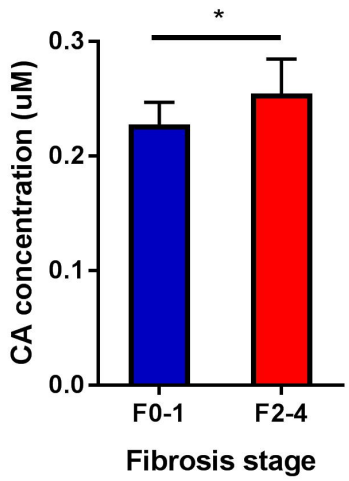
Figure 16. Bile acid distribution with respect to fibrosis grade

A) Bile acid composition as a percentage according to hepatic fibrosis. The x-axis shows patients with absent/mild (F0-F1, left, n = 314) and moderate/severe fibrosis (F2-F4, right, n = 224), and the y-axis shows the percentage composition of each individual bile acid in %. B) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224) and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

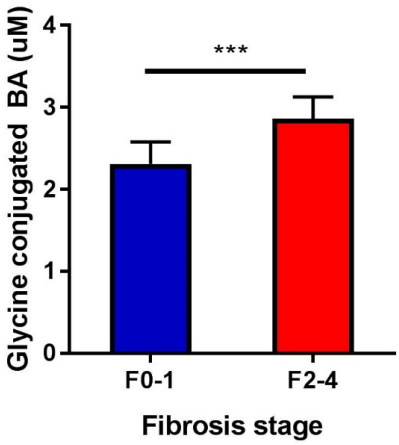
Lastly, the association of BAs with NAFLD fibrosis stage was tested. The distribution of all individual BAs between patients with none/mild fibrosis and those with significant fibrosis is depicted in **Figure 16A**. Patients with significant fibrosis (≥F2) had higher total ($p = 0.017$), primary ($p = 0.018$) and secondary BA levels ($p = 0.045$) compared to those with none/mild fibrosis (F0-1) (**Figure 16B**). This is consistent with previous studies (Puri et al. 2017).

At the level of individual BAs, the level of cholic acid was significantly higher in patients with significant fibrosis compared to those with none/mild fibrosis ($p = 0.026$) **(Figure 15A)**. Similarly, the levels of glycine and taurine conjugated BAs were significantly higher in patients with significant fibrosis compared to those with none/mild fibrosis ($p = 0.002$ and $p < 0.001$ respectively) **(Figures 17B-C)**.

A.



B.



C.

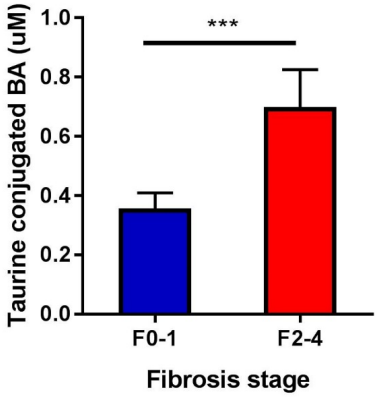


Figure 17. Individual bile acid between fibrosis grades

A) Mean concentration of cholic acid (CA) according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224), and the y-axis shows the mean concentration of bile acid levels in µmol/L. B) Mean concentration of glycine conjugated bile acids according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224), and the y-axis shows the mean concentration of bile acid levels in µmol/L. C) Mean concentration of taurine conjugated bile acids according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224), and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

4.3.3 **Lean NAFLD patients have higher serum bile acid levels**

Next we explored the bile acid profile between patients with lean and non-lean NAFLD. Interestingly, patients with lean NAFLD had higher total, primary and secondary BA levels compared to those with non-lean NAFLD, though this was only significant for the secondary bile acids (p=0.010) (**Figure 18B**). The composition of individual BAs also differed between lean and non-lean NAFLD patients, wherein lean patients had lower Deoxycholic acid (DCA), Glycochenodeoxycholic acid (GCDCA) and chenodeoxycholic acid (CDCA), but more glycocholic acid (GCA) compared to the non-lean patients (**Figure 18A).** Lean NAFLD patients also had non-significantly higher total conjugated and total unconjugated BAs compared to non-lean NAFLD patients.

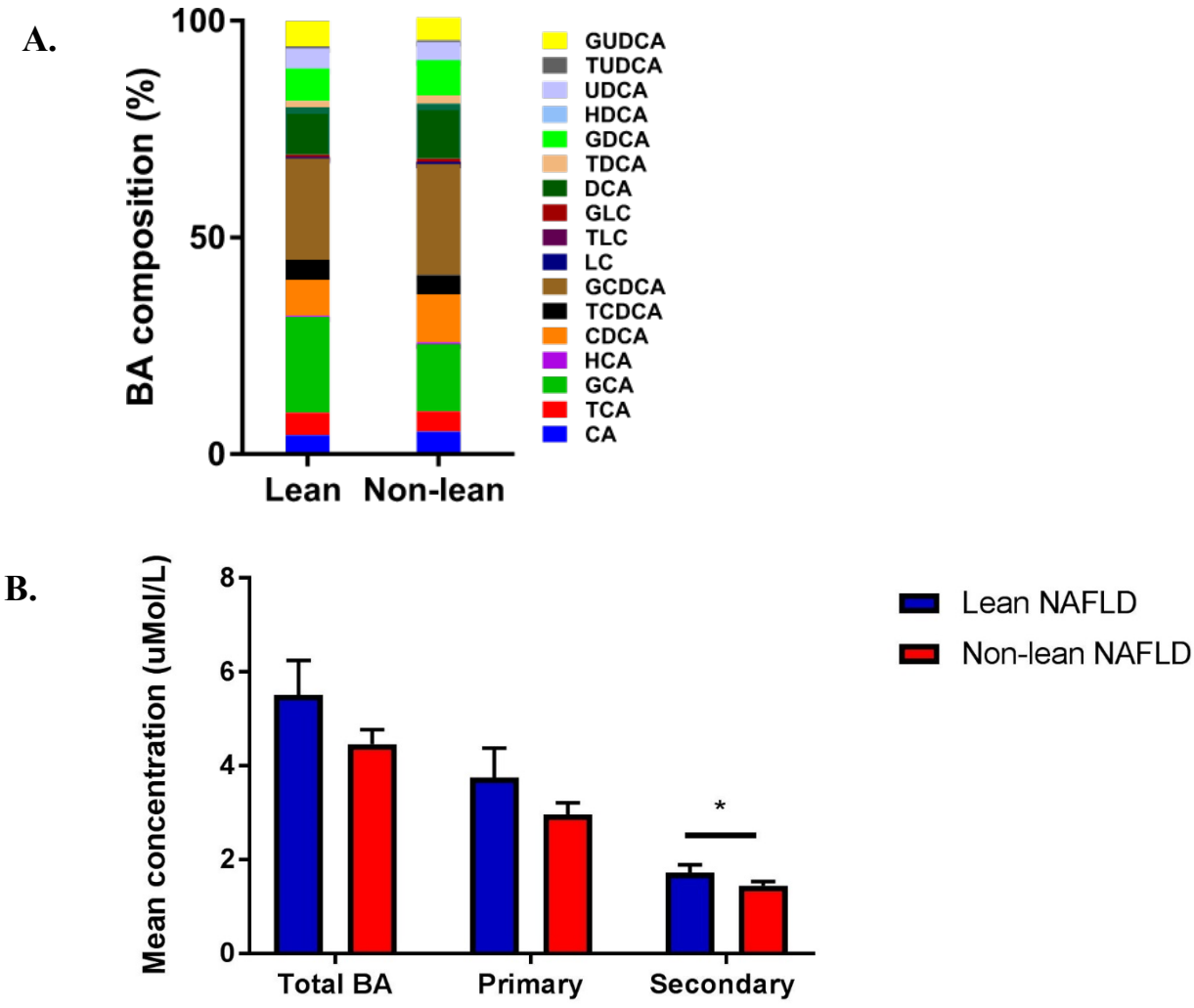
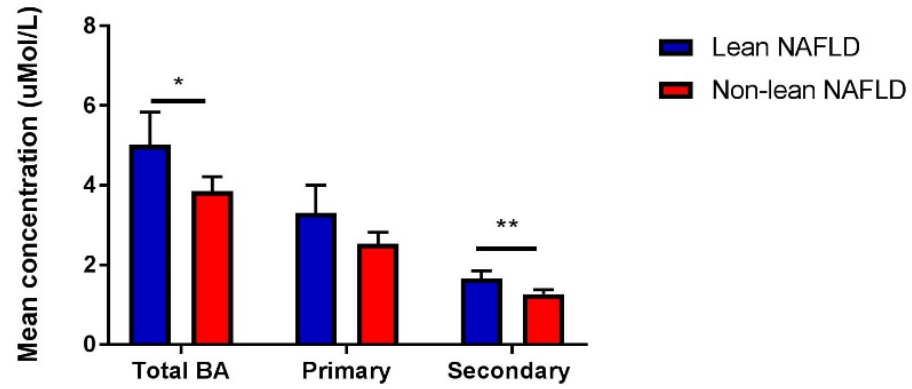


Figure 18. Bile acid distribution in lean and non-lean NAFLD patients.

A) Bile acid composition in percentage between lean and non-lean NAFLD patients. The x-axis shows lean (n = 99) and non-lean NAFLD patients (n = 439), and the y-axis shows the percentage composition of each individual bile acid in %. B) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids in lean and non-lean NAFLD patients. The x-axis shows lean (n = 99) and non-lean NAFLD patients (n = 439) and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

Given the strong correlation between BA profiles and fibrosis, we examined the relationship between bile acids and lean NAFLD stratified by fibrosis stage. When stratified in this way, in those with mild fibrosis (F0-1), higher total secondary BA levels were observed in lean compared to non-lean NAFLD patients ($p = 0.004$). No significant difference between lean and non-lean patients was observed in those with more severe fibrosis **(Figures 19A and B).** The predominant secondary BAs contributing to this difference were deoxycholate (DCA) and ursodeoxycholic acid (UDCA) ($p<0.05$ for both) **(Figures 20A and B)**. Glycocholic acid (GCA) was also higher in lean NAFLD patients; however, this difference was not significant (**Figure 21A**). The secondary to primary BA ratio was significantly higher in patients with lean compared to non-lean NAFLD ($p = 0.018$) **(Figure 21B)**.

A.



B.

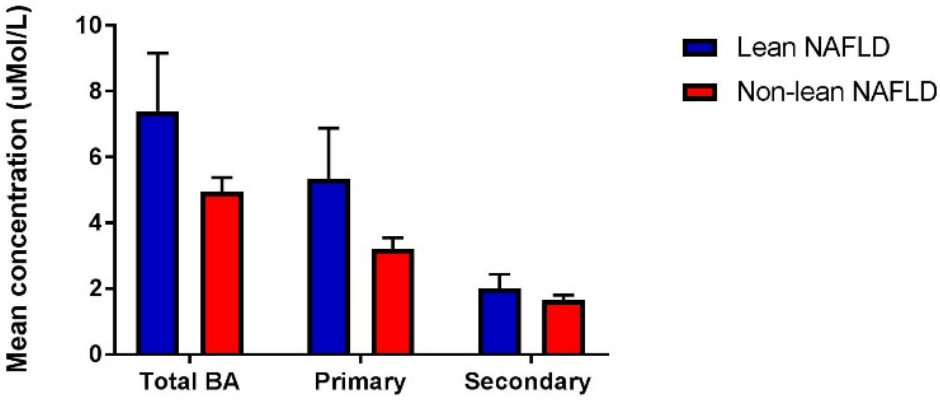
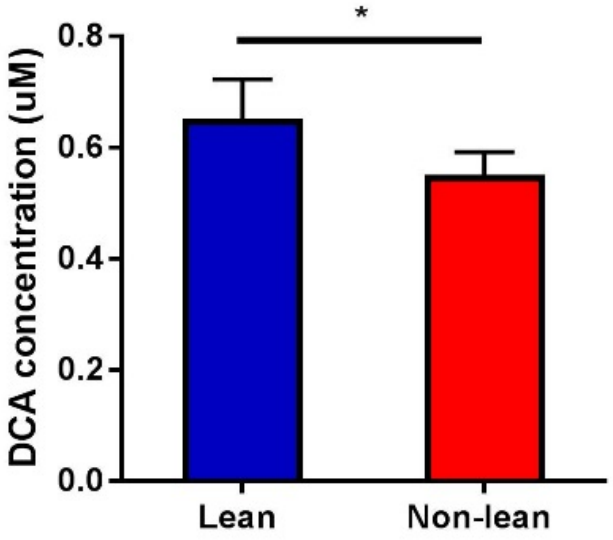


Figure 19. Bile acid levels between lean and non-lean stratified by fibrosis degrees

A) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids in lean and non-lean patients with absent/mild fibrosis. The x-axis shows lean (n = 75) and non-lean (n = 239) patients with absent/mild (F0–F1) hepatic fibrosis and the y-axis shows the mean concentration of bile acid levels in µmol/L. B) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids in lean and non-lean patients with moderate/severe fibrosis. The-x axis shows lean (n = 24) and non-lean (n = 200) patients with moderate/severe (F2–F4) hepatic fibrosis and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

A.



B.

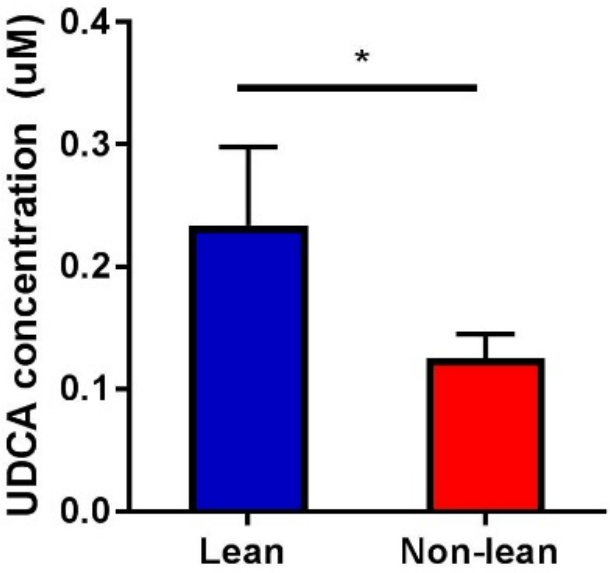
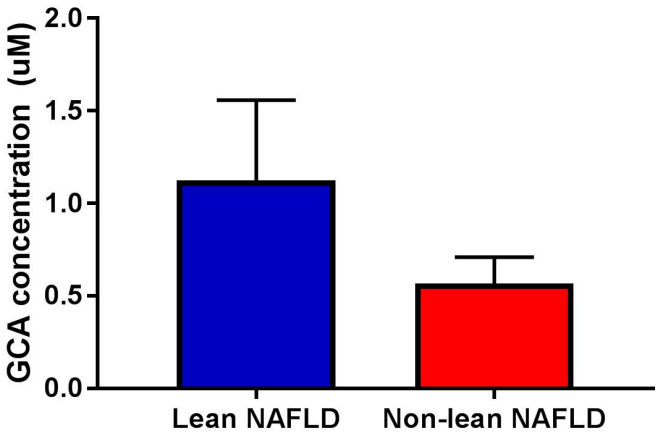


Figure 20. Individual bile acids between lean and non-lean

A) Mean concentration of deoxycholic acid (DCA). The x-axis shows lean (n = 99) and non-lean patients (n = 439), and the y-axis shows the mean concentration of bile acid levels in µmol/L. B) Mean concentration of Ursodeoxycholic acid (UDCA). The x-axis shows lean (n = 99) and non-lean NAFLD patients (n = 439), and the y-axis shows the mean concentration of bile acid levels in µmol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

A.



B.

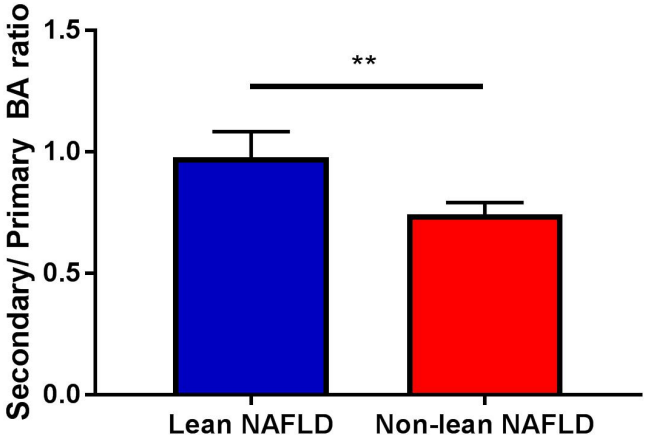


Figure 21. GCA levels and secondary/primary BA ratio in lean and non-lean NAFLD

A) Mean concentration of glycocholic acid (GCA). The x-axis shows lean (n = 99) and non-lean patients (n = 439), and the y-axis shows the mean concentration of bile acid levels in µmol/L. B) Secondary to primary BA ratio in lean and non-lean NAFLD patients. The x-axis shows lean (n = 99) and non-lean NAFLD patients (n = 439), and the y-axis shos secondary to primary BA ratio. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

In a subsequent analysis we determined the relevant clinical factors associated with secondary BA levels. Consistently, on univariable analysis, BMI, fibrosis and ballooning were associated with secondary bile acid levels. On multivariable analysis, only BMI and fibrosis stage remained independently, negatively and positively associated with secondary BA levels, respectively (**Table 8**).

Table 8. Univariable and multivariable analysis of total secondary bile acids with relevant clinical factors

Clinical factors	Univariable analysis				Multivariable analysis			
	β	95% CI		p-value	β	95% CI		p-value
Age	0.072	-0.001	0.022	0.082				
Gender	-0.021	-0.385	0.222	0.599				
BMI	-0.09	-0.059	-0.003	**0.029**	-0.125	-0.077	-0.014	**0.005**
Diabetes	-0.046	-0.536	0.148	0.266				
Hypertension	0.016	-0.248	0.369	0.7				
Dyslipidaemia	-0.036	-0.413	0.16	0.386				
Total cholesterol	-0.046	-0.191	0.053	0.266				
HOMA-IR	0.027	-0.025	0.042	0.625				
Fibrosis	0.141	0.082	0.333	**0.001**	0.128	0.052	0.326	**0.007**
Steatosis	-0.055	-0.331	0.074	0.214				
Ballooning	0.135	0.114	0.513	**0.002**	0.094	0.009	0.429	0.051
Lobular inflammation	-0.037	-0.36	0.142	0.396				
Portal Inflammation	-0.03	-0.442	0.248	0.58				
NAS	0.007	-0.09	0.107	0.869				

4.3.4 **Lean NAFLD patients have higher serum FGF19 levels**

FXR dysregulation has been implicated in the pathogenesis of NAFLD so we were interested to determine if differential effects are observed in lean versus non-lean NAFLD (Jiao et al. 2017; Puri et al. 2017). To examine for this, serum FGF19 a surrogate marker of FXR activity was measured. In this analysis, reduced levels of FGF19 were observed with the advancement of fibrosis stage ($p = 0.030$) (**Figure 22**). Patients with lean NAFLD had significantly higher FGF19 levels compared to non-lean NAFLD patients ($p = 0.028$) (**Figure 23A**). Interestingly, when stratified according to fibrosis severity, the differences were more profound in those with mild fibrosis (F0-F1) ($p = 0.005$), with the reverse being true as fibrosis severity increased; this was however not significant **(Figure 23B-C)**.

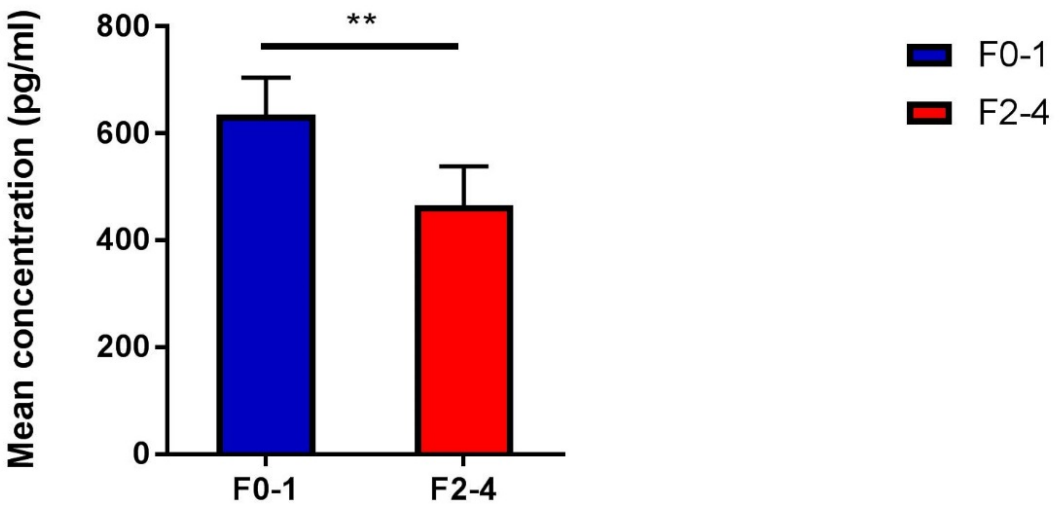


Figure 22. FGF-19 in early and late fibrosis

Mean concentration of FGF19 according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224), and the y-axis shows the mean concentration of FGF19 in pg/mL. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

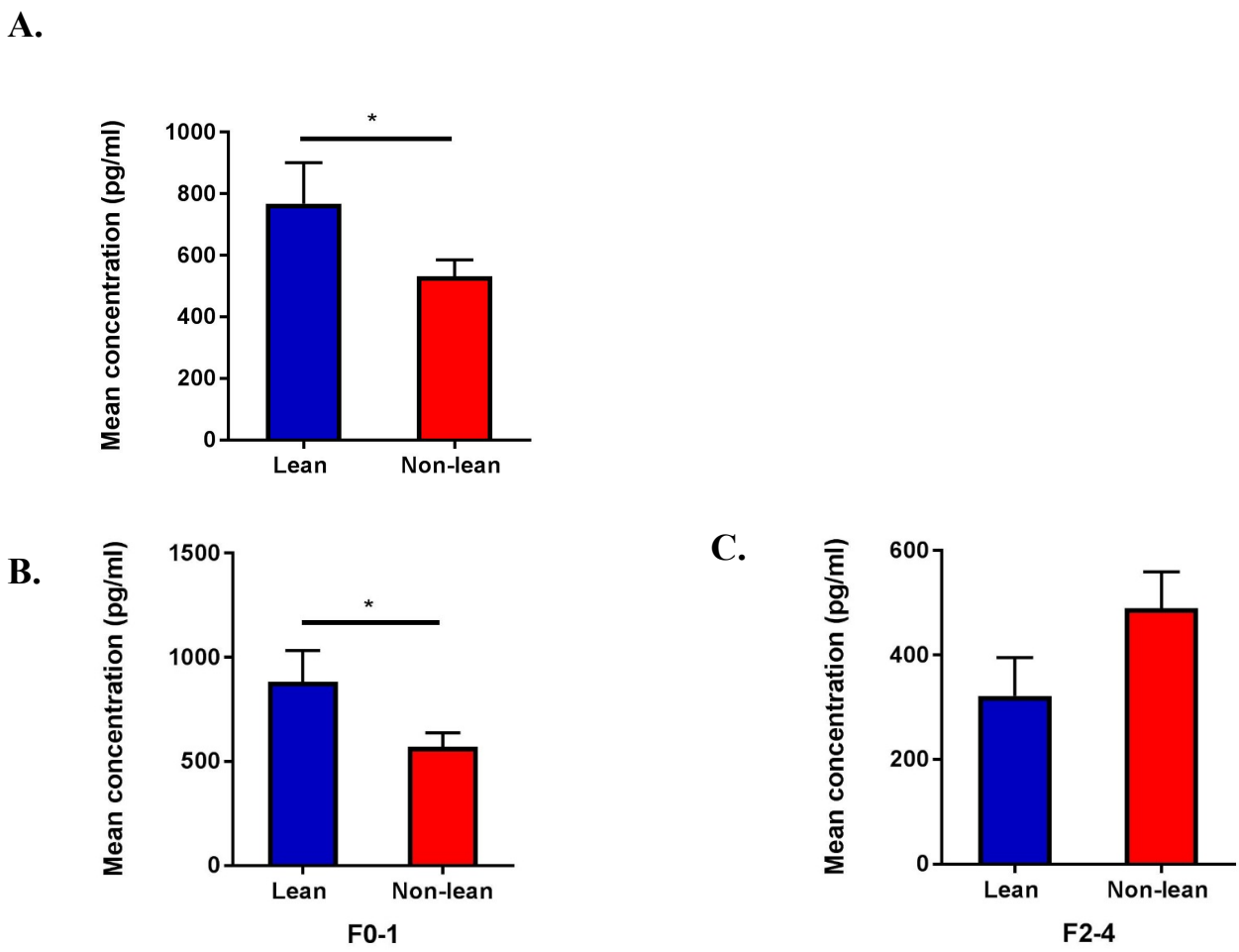


Figure 23. FGF-19 in lean and non-lean, and stratified by fibrosis degree

A) Mean concentration of FGF19 in lean and non-lean NAFLD patients. The x-axis shows lean (n = 99) and non-lean (n = 439) NAFLD patients, and the y-axis shows the mean concentrations of FGF-19 levels in pg/mL. B) Mean concentration of FGF19 levels in lean and non-lean patients according to BMI and hepatic fibrosis. The x-axis shows lean and non-lean NAFLD patients with absent/mild fibrosis (F0–F1, n = 75 for lean and n = 239 for non-lean NAFLD) and and the y-axis shows the mean concentrations of FGF-19 levels in pg/mL. C) Mean concentration of FGF19 levels in lean and non-lean patients according to BMI and hepatic fibrosis. The x-axis shows lean and non-lean NAFLD patients with moderate/severe fibrosis (F2-4, n = 24 for lean and n = 200 for non-lean); and the y-axis shows the mean concentrations of FGF-19 levels in pg/mL. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

4.3.5 **Lean NAFLD patients have lower C4 levels**

To investigate differences in bile acid metabolism between lean and non-lean NAFLD, C4 levels, a bile acid synthesis intermediate was measured as a marker of *de novo* BA synthesis. In this analysis, no difference in levels of C4 was observed according to fibrosis stage (**Figure 24A**). However, as expected, patients with lean NAFLD had significantly lower C4 levels compared to their non-lean counterparts ($p = 0.016$) **(Figure 24B)**. This difference was more predominant in those with mild fibrosis (F0-F1) ($p = 0.010$), but not in those with moderate/severe fibrosis (F2-F4) **(Figure 24C-D)**.

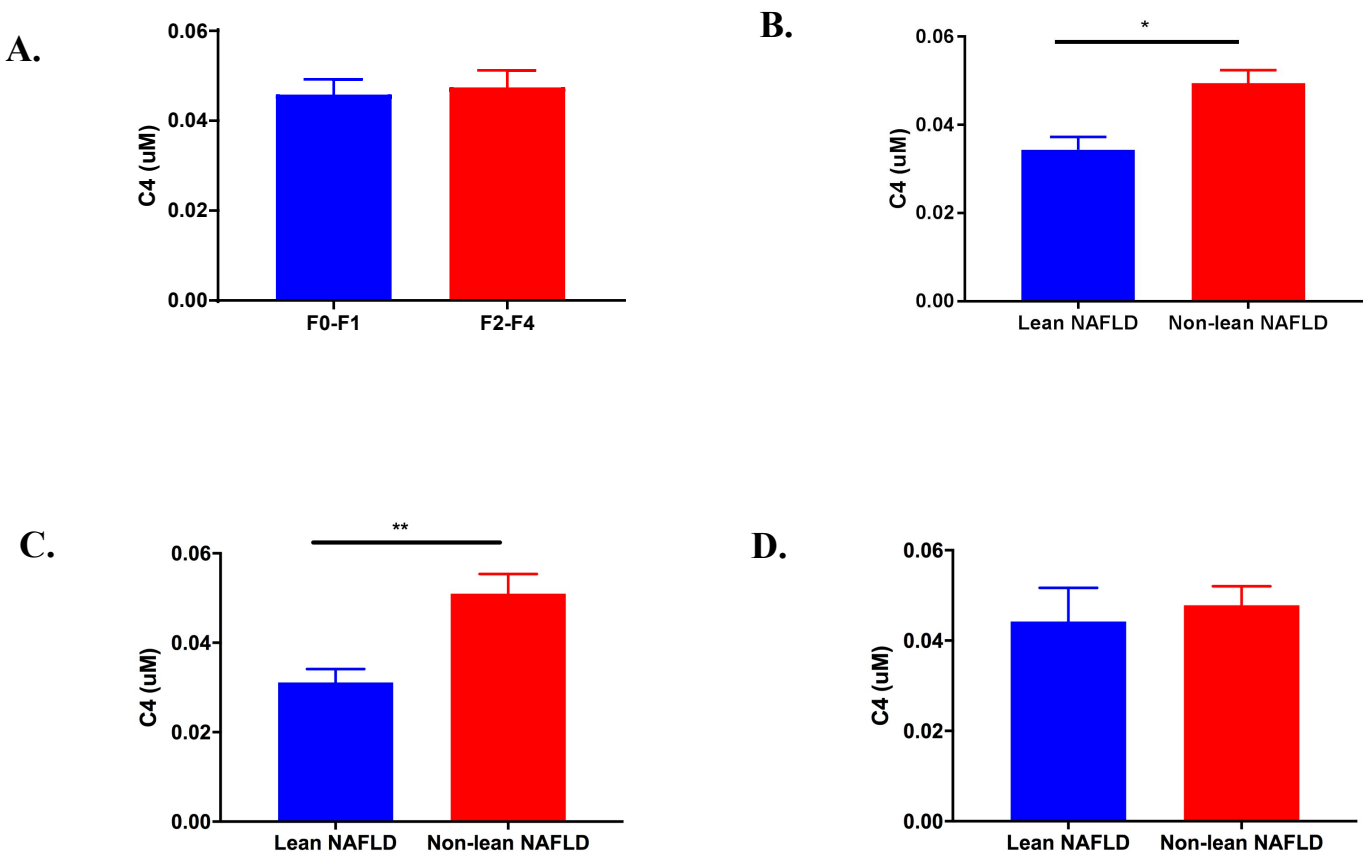


Figure 24. C4 levels between lean and non-lean

A) Mean concentration of C4 according to hepatic fibrosis. The x-axis shows hepatic fibrosis dichotomized as absent/mild (F0–F1, n = 314) or moderate/severe (F2–F4, n = 224), and the y-axis shows the mean concentration of C4 in umol/mL. B) Mean concentration of C4 in lean and non-lean NAFLD patients. The x-axis shows lean (n = 99) and non-lean (n = 439) NAFLD patients, and the y-axis shows the mean concentrations of C4 in umol/mL. C) Mean concentration of C4 levels according to BMI and hepatic fibrosis. The x-axis shows lean and non-lean NAFLD patients with absent/mild fibrosis (F0–F1, n = 75 for lean and n = 239 for non-lean NAFLD) and the y-axis shows the mean concentration of C4 in umol/mL D) Mean concentration of C4 levels according to BMI and hepatic fibrosis. The x-axis shows lean and non-lean NAFLD patients with moderate/severe fibrosis (F2-4, right panel, n = 24 for lean and n = 200 for non-lean); and the y-axis shows the mean concentration of C4 in umol/mL. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

4.3.6 **Lean NAFLD patients have a distinct microbiota profile**

The composition of the gut microbiome and their interaction with BAs affects FXR-mediated signalling in both the liver and intestine and is implicated in NAFLD pathogenesis (Jiao et al. 2017; Ramirez-Perez et al. 2017). Hence, we determined gut microbiome composition in a small exploratory subset of patients with available stool samples by 16S rRNA amplicon sequencing. At the phylum level, no differences in taxonomic composition of the gut microbiome were observed according to lean versus obese BMI status. At the genus level, Erysipelotrichaceae UCG-003, as well as several bacterial genera within the Clostridiales order including *Ruminococcus, Clostridium sensu stricto 1, Romboutsia* and Ruminococcaceae UCG-008 were enriched in lean patients, while *Ruminiclostridium* and *Streptococcus* were enriched in obese NAFLD patients (Mann-Whitney test, $p<0.05$) (**Figures 25 and 26A-F**). These changes remained significant for Ruminococcaceae UCG-008 when corrected for multiple comparison testing (FDR $p= 0.010$).

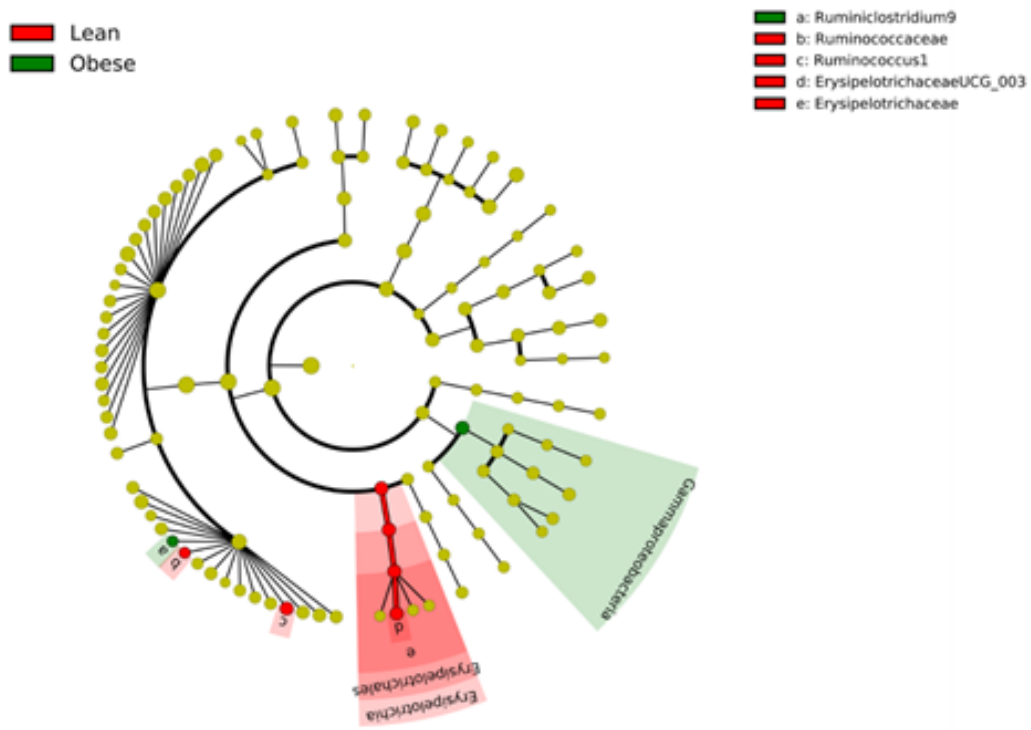


Figure 25. Microbiota profile between lean and non-lean

Microbiota abundance differences between the lean and non-lean NAFLD patients. The colour denoting each taxa represents the group in which the taxa was identified to be significantly increased compared to the comparison group ($p < 0.05$).

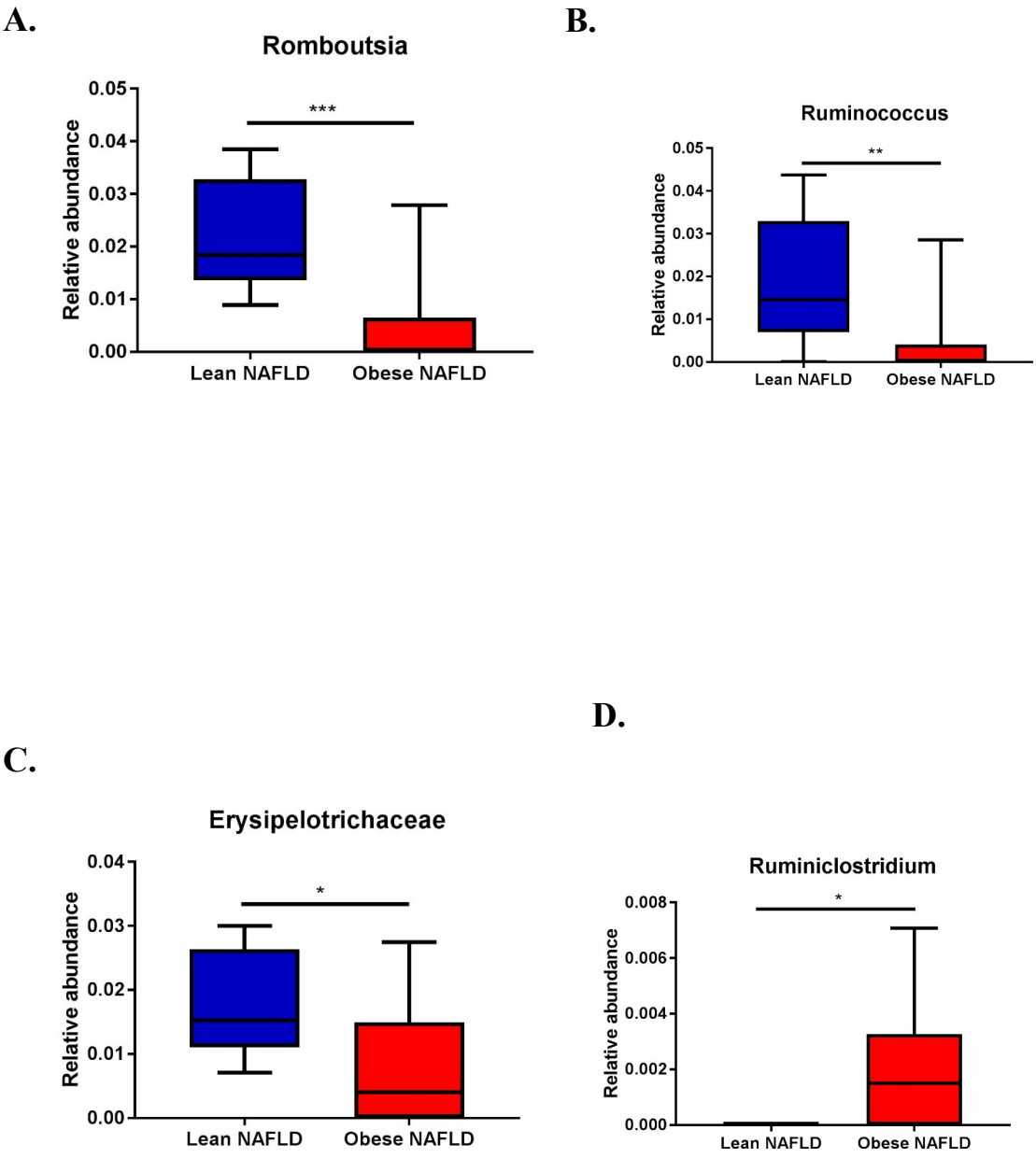


Figure 26. Individual taxa differences between lean and non-lean

Abundance of bacterial genera and species that differ between patients with lean (n=5) and obese NAFLD (n=24). A) Romboutsia, B) Ruminococcus, C) Erysipelotrichaceae, D) Ruminoclostridium. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

4.4 DISCUSSION

Lean NAFLD constitutes a significant proportion of NAFLD patients though its pathogenesis is not well understood. Herein we provide a testable hypothesis for the pathophysiological distinction between lean and non-lean NAFLD that can be examined in other cohorts. Using biopsy proven Caucasian patients in whom the lean NAFLD entity is less frequent than in cohorts from Asia, we demonstrate that lean patients have distinct metabolic, genetic, histologic and bile acid profiles, C4 levels, as well as differences in FXR activity and gut microbiota compared to their non-lean counterparts.

Consistent with other reports (Z. Younossi et al. 2018a), around 1 in 5 Caucasian patients with NAFLD are lean and have a favourable metabolic and pathological profile, with less insulin resistance and dyslipidaemia, and milder liver histology. A reciprocal and intimate interaction between bile acids and gut microbiota is associated with, and thought to regulate, metabolic and hepatic traits (Arab et al. 2017; Schnabl and Brenner 2014). Although myriad factors could explain the differences we observed, our results *in toto* suggest that the balance and interaction between the systemic metabolic milieu and changes in the intestinal microbiome and bile acid physiology govern the expression of hepatic disease and the onset and progression of NAFLD in patients with a normal BMI.

To elaborate, increased bile acid levels as we observed in lean NAFLD, are reported to mediate resistance to diet-induced obesity, a phenomenon called “obesity-

resistance" (Watanabe et al. 2006; Watanabe et al. 2011). Obesity-resistant rodents can burn more dietary fat by increasing energy expenditure. Of relevance, bile acids (including major bile acid species such as CA, TCA, DCA and CDCA) increase energy expenditure (Watanabe et al. 2006) and CDCA increases human brown adipose tissue activity (E. P. M. Broeders et al. 2015). FGF19 which was also increased in lean NAFLD, is reported to be a key regulator of energy expenditure and improves glucose and lipid homeostasis (Fu et al. 2004), while gut-restricted FXR agonism promotes metabolic improvements and enhances thermogenesis and browning of white adipose tissue (WAT) in mice (Fang et al. 2015). At microbiota level, patients with lean NAFLD had distinct gut microbiota compared to those who were non-lean. Lean NAFLD had an increased abundance of members belonging to the *Clostridium* genus, and as well *Ruminococcaceae* that are involved in the formation of bile acids (Kakiyama et al. 2013; Wahlstrom et al. 2016b). Thus, we surmise that patients with lean NAFLD have an obesity-resistant phenotype in part mediated by greater levels of bile acids, FGF19 and microbiota changes.

The milder disease and favourable metabolic profile of patients with lean NAFLD could be explained by the current findings. There is strong evidence that activation of bile acid signalling induces improvements in metabolic (glucose and lipid) phenotype in murine models, mediated through the actions of FXR activity and Takeda G protein-coupled receptor 5 (TGR5) (Pathak et al. 2018; Pierre et al. 2016). Activation of the FXR in hepatic stellate cells has also been shown in murine models to protect against liver fibrosis formation (Schumacher et al. 2020). Furthermore, in both humans and murine models, elevated bile acids play a role in the metabolic improvements after bariatric surgery, including in type 2 diabetes,

dyslipidemia and NASH, even before significant weight loss (A. P. Chambers et al. 2011; Kohli et al. 2015; Patti et al. 2009). Thus, we suggest that lean patients can adapt metabolically and excrete greater amounts of bile acids while their obese counterparts are those less able to excrete adequate amounts of bile acids to rid themselves of excess cholesterol, even if they are able to maintain a plasma cholesterol level comparable to that of lean patients. Consistently, in humans, lean and obese patients have differential defence mechanisms to maintain stable serum cholesterol levels, wherein dietary cholesterol appears to preferentially induce bile acid synthesis in lean compared to obese patients (Klass et al. 2006).

Notably, we did not observe any association between bile acid levels and hepatic steatosis, indicating a potential lack of a protective effect of bile acids on the development of steatosis, as opposed to changes in peripheral tissues. Alternatively, changes in microbiota might explain the development of steatosis (Chu et al. 2018). Similarly, compared to non-lean NAFLD they had an increased relative abundance of several phylotypes within the Erysipelotrichaceae family in both patient and murine models that have been repeatedly linked to host lipid and cholesterol phenotypes in different species (humans, mice, and hamsters) and positively associates with changes in liver fat in humans (Martinez et al. 2013). Use of plant sterol esters (PSE) to reduce cholesterol in hamsters likewise reduced Erysipelotrichaceae abundance (Martinez et al. 2013). Ruminococcaceae UCG-008, *Clostridium* sensu stricto 1 and *Romboutsia*, which were also enriched in lean NAFLD are reported to be strongly correlate with hepatic triglycerides (L. Zhao et al. 2018).

At a genetic level, we demonstrated that while there was no significant difference in the proportions of patients with *PNPLA3* rs738409 GG genotype, a significantly greater proportion of patients with lean NAFLD carried the *TM6SF2* rs58542926 (T) allele than non-lean NAFLD patients. Interestingly, TM6SF2 is implicated in cholestrol synthesis (Fan et al. 2016) and *TM6SF2*, but not *PNPLA3* genotypes correlate with endotoxemia (Pang et al. 2017). Hence, the lean NAFLD phenotype might be consistent with "obesity-resistance", where individuals are still prone to develop steatosis in response to an obesogenic environment (and perhaps a diet enriched in cholesterol), likely by genetic and gut-driven mechanisms.

We observed that differences between lean and non-lean patients were more profound in those with early stages of liver fibrosis. This suggests that with disease progression, homeostatic responses might possibly no longer be able to limit inflammation and fibrosis, leading ultimately to long-term adverse outcomes, despite a favourable baseline metabolic and histological profile (A. C. Dela Cruz et al. 2014; Hagstrom et al. 2018). This hypothesis is supported by the higher serum bile acid levels and lower FGF19 levels in patients with significant fibrosis. Longitudinal studies would be needed to confirm the findings.

The strengths of our report include the study of a large, well-defined, biopsy-proven Caucasian cohort, and as detailed an investigation as is possible, from cross-sectional data. However, our study also has limitations. First, patients were seen in tertiary referral centres, and may suffer from selection bias. In addition, dietary histories were not available given the accumulation of cohorts over several years,

while the cross-sectional design did not allow for interventions or longitudinal outcomes and thus, a causal relationship cannot be demonstrated. Lastly, our study is limited by the small sample size with regards to microbiome analysis. It would also be interesting in future studies to measure differences in faecal bile acids.

4.5 CONCLUSION

In conclusion, in contrast to non-lean NAFLD, lean patients are likely to have a distinct pathophysiology. We suggest that the onset of disease occurs at a lower BMI set point (with lower measures of insulin resistance and dyslipidaemia) and is shaped by the genetics background and early alterations in bile acid and gut microbiota profile. These changes might reflect altered dietary composition (perhaps with an excess of dietary cholesterol, as previously reported in patients with lean NAFLD (Enjoji et al. 2012; Musso et al. 2003; Yasutake et al. 2009b)), altered cholesterol metabolism, limitations in adipocyte numbers in childhood, or differences in mucosal immunology. Secondary or concomitant alterations in gut microbiota composition also drives the phenotype to a greater extent than in patients with non-lean NAFLD. This hypothesis does not negate the possibility that there are overweight/obese NAFLD patients with a similar pattern of compensatory mechanisms but suggests that lean patients have a preponderance of a gut-mediated phenotype. Further studies are needed to investigate the contribution of early-stage adaptive mechanisms on the long-term hepatic and extrahepatic outcomes of this disease. Our hypothesis would suggest that these individuals will have more severe and progressive liver disease as it has been suggested before (A. C. Dela Cruz et al. 2014; Hagstrom et al. 2018), but this hypothesis needs further confirmation.

CHAPTER FIVE

CHARACTERISATION OF METABOLIC ADAPTATION IN MICE MODELS OF LEAN AND NON-LEAN NAFLD

Parts of this chapter has been published in:

Chen, F., et al.; *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation;* accepted in Hepatology August 2019; DOI: 10.1002/hep.3090

5 CHARACTERISATION OF METABOLIC ADAPTATION IN MICE MODELS OF LEAN AND NON-LEAN NAFLD

5.1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a heterogeneous disease with a spectrum ranging from simple steatosis to the more severe form, non-alcoholic steatohepatitis (NASH) with its associated morbidity and mortality. Various modifiable factors such as diet, microbiota and lifestyle, as well as non-modifiable factors such as genetics and epigenetics influence NAFLD pathogenesis.

Given the complexity of NAFLD pathogenesis, animal models have become an integral part of elucidating the pathophysiology and the effects of treatment for NAFLD. Generally, the C57BL/6 strain in mice and Wistar and Sprague Dawley rat strains are preferred for NAFLD models due to the intrinsic predilection to develop obesity, type 2 diabetes mellitus and NAFLD (Van Herck et al. 2017).

NAFLD and its metabolic syndrome components can be induced in animal models using nutritional, chemical or genetic models, or a combination of these. The ideal animal model of NAFLD should reflect the pathophysiology of human NAFLD/NASH with the hepatic as well as metabolic manifestations. Although many of the genetic models of NASH induce steatohepatitis, they do not occur very quickly as in the dietary models, in addition to them being more costly. Furthermore, many genetic models of NASH require a secondary stimulus such as dietary or endotoxin to

promote progression from steatosis to steatohepatitis. Lastly, the implicated mutations used in the genetic models of NASH are often not prevalent in human patients, making the models less applicable (Jacobs et al. 2016). Chemically induced animal models like the genetic models also often require a combination of dietary interventions to achieve the desired outcomes. Hence these models are more useful for investigation of a more advanced NAFLD phenotype such as advanced fibrosis, cirrhosis and HCC (Jacobs et al. 2016). Therefore, dietary animal models of NAFLD are more commonly utilised.

One of the diets commonly used in NAFLD animal models is the atherogenic (Ath) diet. This diet contains a relatively high dose of cholesterol and cholic acid. The atherogenic diet induces steatosis and inflammation typically after 6 weeks, with hepatocellular ballooning and fibrosis. In addition, these animals display increased levels of ALT and total cholesterol, with minimal weight gain. The addition of a high-fat component to the Ath diet can increase hepatic insulin resistance and further accelerate disease progression (Van Herck et al. 2017).

The excessive intake of fructose, a monosaccharide primarily metabolized in the liver has been associated with the development and progression of NAFLD by promoting fat deposition, inflammation, oxidative stress, insulin resistance and fibrosis (Stephenson et al. 2018). Fructose-supplemented drinking water in both rats and mice induces simple steatosis after 8 weeks with significant increases in body weight, plasma triglycerides and glucose levels. In addition, there is intestinal bacterial

overgrowth observed, followed by increased endotoxin levels in the portal blood and activation of Kupffer cells (Van Herck et al. 2017).

In our study, we were interested to see if the findings found in lean and non-lean NAFLD patients could be replicated in mice models. For this, we used C57BL/6 mice fed either an atherogenic (Ath) diet to replicate lean NAFLD given its ability to induce steatosis and inflammation without inducing weight gain, or a high sucrose (HS) diet to replicate our non-lean NAFLD models given its ability to promote NAFLD development and progression with insulin resistance and the associated significant weight gain.

5.2 METHODS

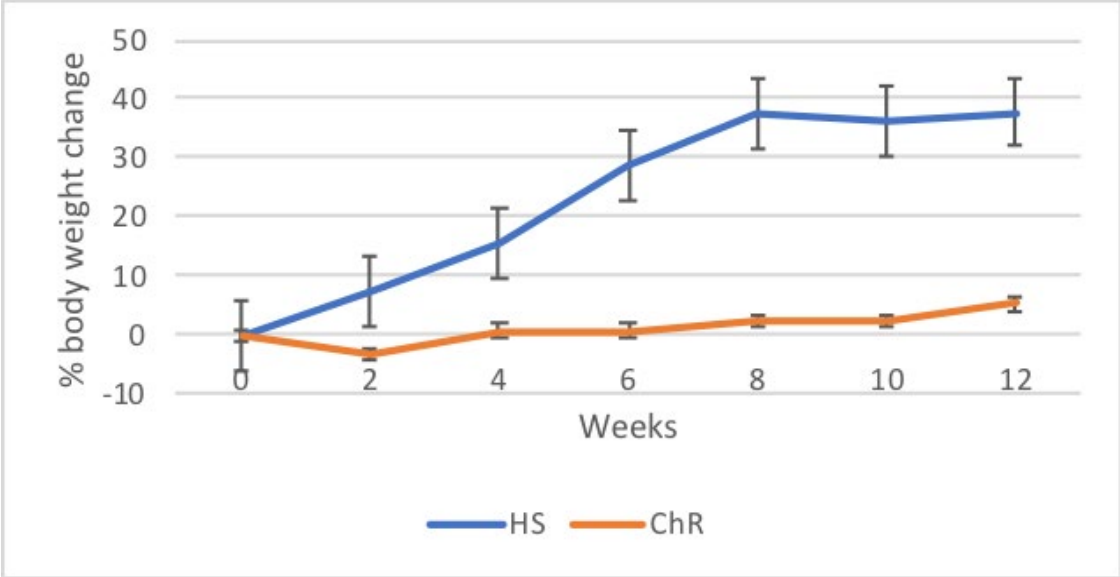
Five to six male C57BL/6 mice were fed either a high sucrose diet (HS) diet or cholesterol rich diet (ChR) to recapitulate human non-lean and lean NAFLD features, respectively. Details of the source of mice, dietary composition for the two groups as well as methods for RNA extraction from mice tissues, cDNA synthesis and qPCR are described in chapter 2. Bile acid profile measurement, serum fgf15 level and gut microbiota profile methods are described in chapter 2.

5.3 RESULTS

5.3.1 High sucrose, but not the cholesterol rich diet results in weight gain and increased steatosis

Mice fed the high sucrose (HS) diet gained an average 30-40% of weight compared to their baseline weight. This is in contrast to mice fed the ChR diet where they remained lean (**Figure 27A and B**)

A.



B.

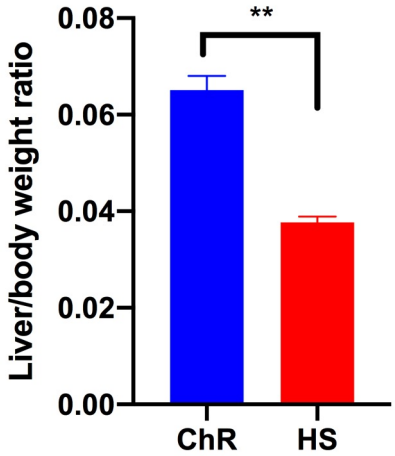
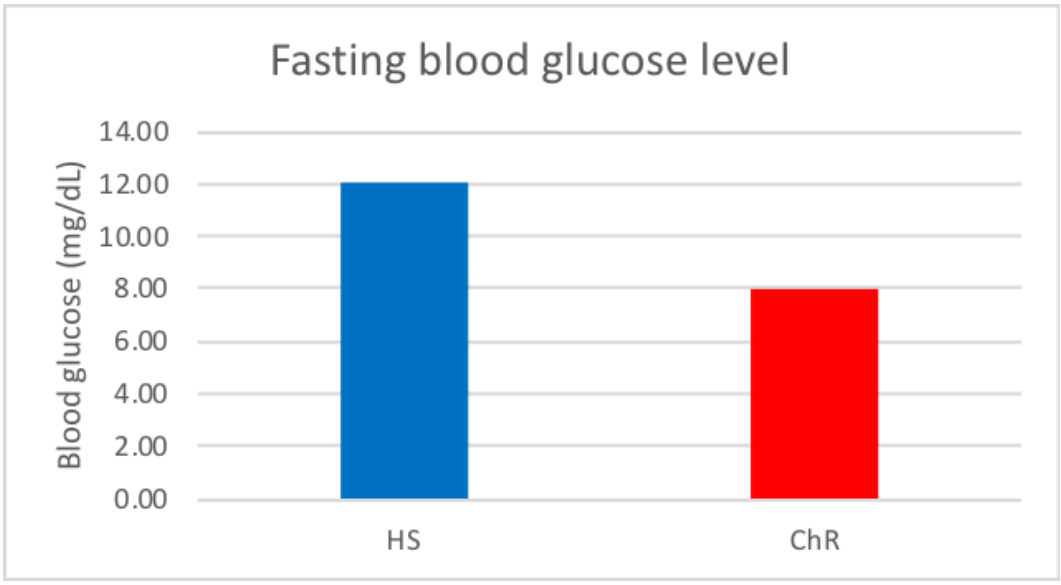


Figure 27. Body weight and liver/body weight ratio in mice fed cholesterol rich (ChR) and high sucrose (HS) diet

A. Changes in body weight over time. The x-axis shows the number of weeks and the y-axis shows the % change in body weight from baseline. B. Liver/body weight ratio in ChR diet mice and HS diet mice. The x-axis shows the ChR and HS fed mice and the y-axis shows the ratio of the liver to total body weight. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

Additionally, mice fed the HS diet had higher fasting serum glucose level with worse glucose tolerance test compared to mice fed the ChR diet (**Figure 28A and B**). In both groups, a glucose loading dose was administered and blood sugar level was measured every 20 minutes. There was a peak of blood glucose concentration seen in both groups after a glucose loading, followed by more rapid resolution of the glucose peak in the ChR mice group, indicating better glucose tolerance.

A.



B.

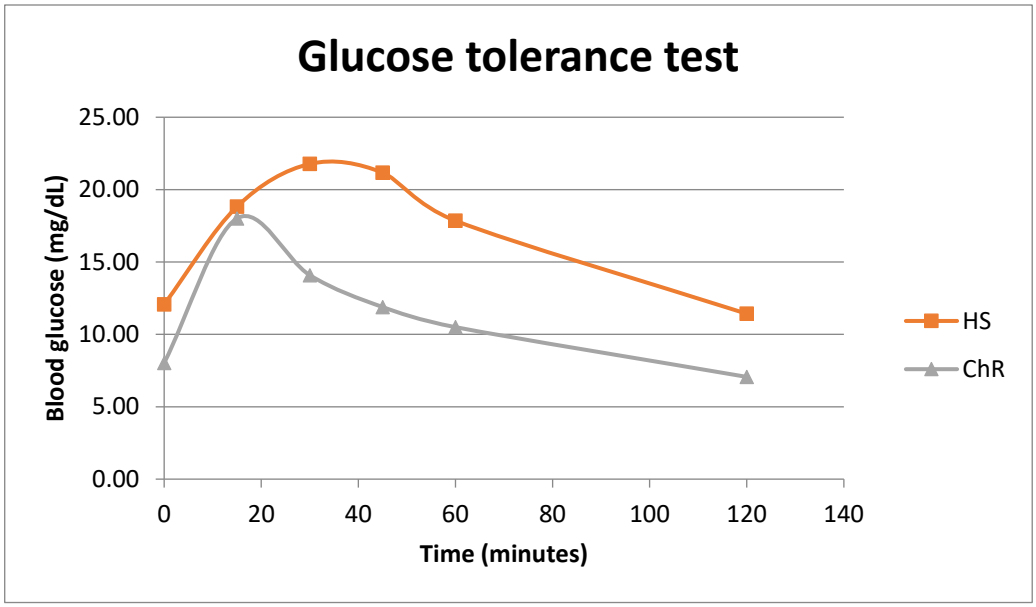


Figure 28. A. Fasting blood glucose levels and B. glucose tolerance test between mice fed cholesterol rich (ChR) and high sucrose (HS) diet.

The x-axis shows the ChR (n=5) and HS (n=6) groups in A, and time after blood glucose loading in minutes in B and the y-axis shows blood glucose levels in mg/dL.

All mice in both groups developed NAFLD at the end of the study. As shown in the representative histology images in **Figure 29**, mice fed the HS diet developed increased steatosis; mice fed the ChR diet developed steatosis with increased inflammatory infiltrates.

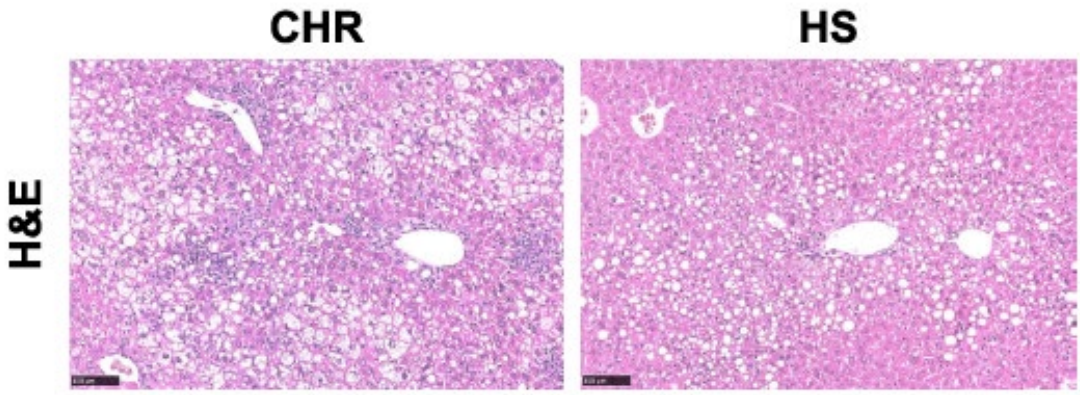


Figure 29. Histology images of mice fed cholesterol rich (ChR) and high sucrose (HS) diet.

Hematoxylin and Eosin (H&E) images of liver biopsies taken from ChR (n=5) and HS (n=6) mice

5.3.2 Lean NAFLD mice models have higher bile acid levels with distinct profile

Like our human results, our lean NAFLD mice models had higher total (p = 0.01), primary (p = 0.02) and secondary bile acids (p = 0.06) compared to the non-lean NAFLD mice models, although this was only significant for total and primary BA levels (**Figure 30**).

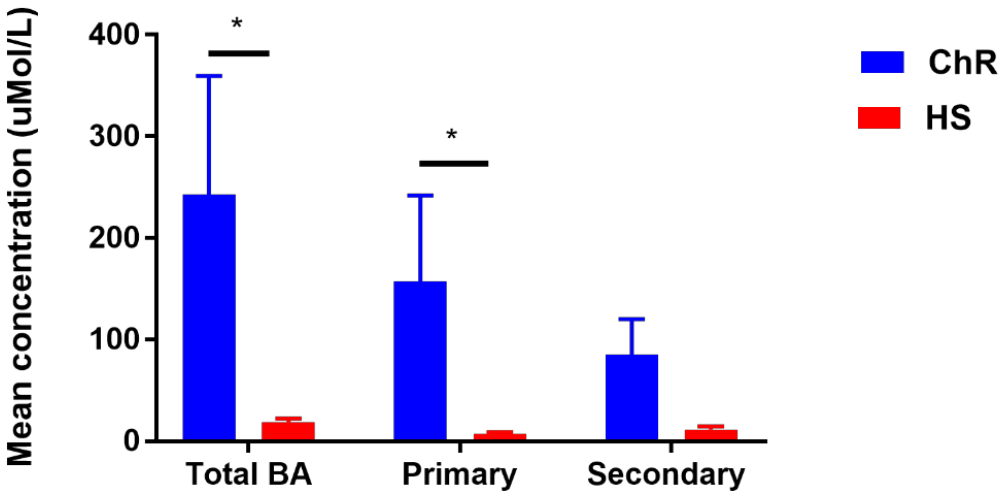


Figure 30. Total bile acids, total primary bile acids and total secondary bile acids level in mice fed cholesterol rich (ChR) and high sucrose (HS) diet.

The x-axis shows total BA, total primary BA and total secondary BA in ChR (blue, n=5) and HS (red, n=6) diet fed mice. The y-axis shows mean BA concentration in uMol/L. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. *P < 0.05, ** P<0.01, *** P<0.001.

In terms of BA distribution profile, the primary BAs cholic acid (CA) and its conjugate taurocholic acid (TCA), as well as taurodeoxycholic acid (TDCA) were present in higher proportion in the ChR group compared to the HS group (**Figure 31**). On the other hand, muricholic acid (MCA), deoxycholic acid (DCA) and lithocholic acid (LC) were present in a higher proportion in the HS compared to the ChR group (**Figure 31**).

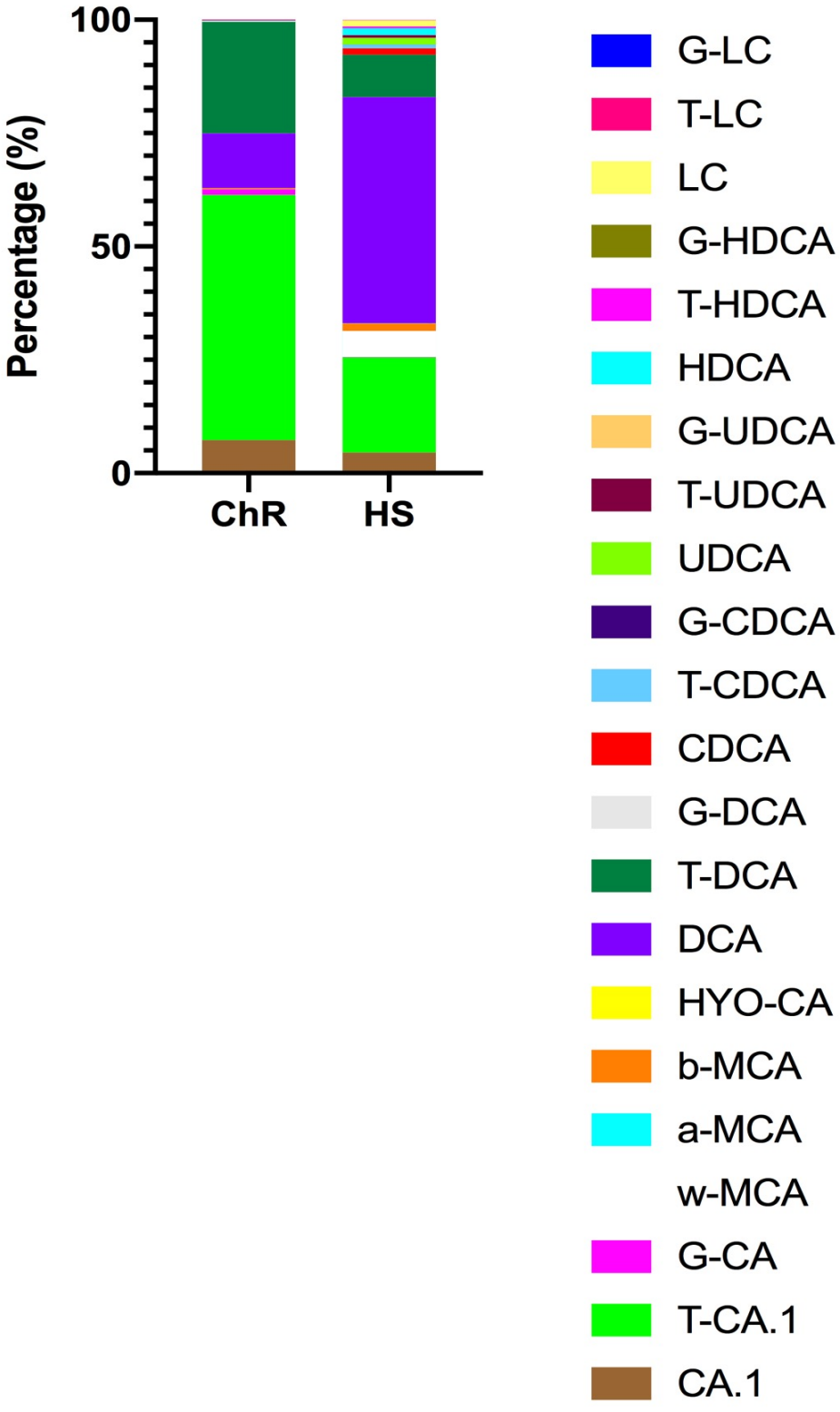


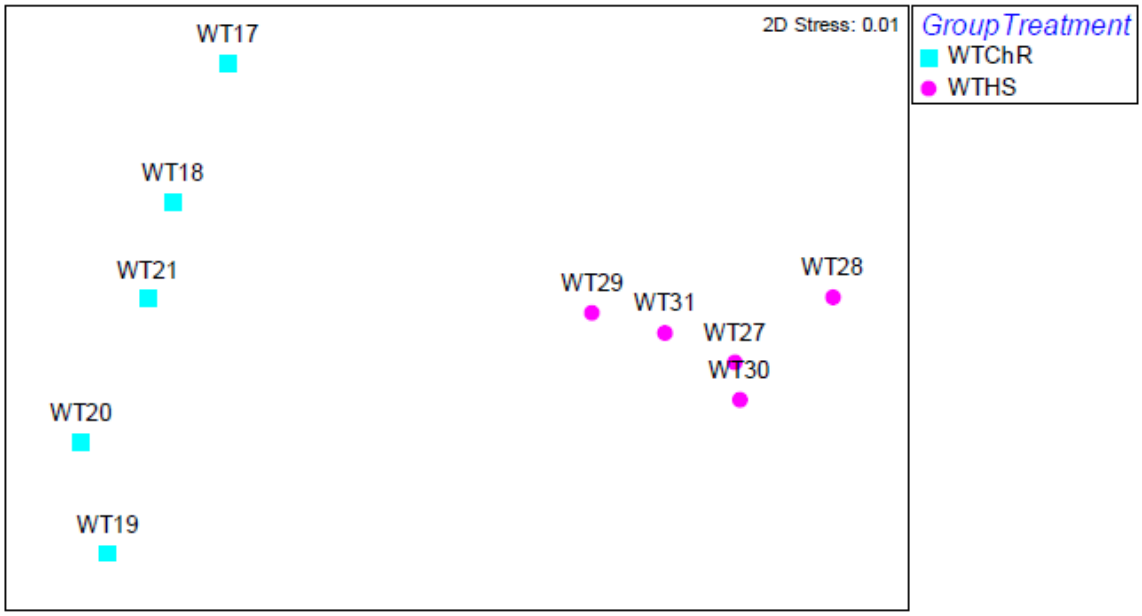
Figure 31. Bile acid distribution between mice fed cholesterol rich (ChR) and high sucrose (HS) diet.

The x-axis shows the cholesterol rich (ChR, n=5) and high sucrose (HS, n=6) fed mice groups. The y-axis shows proportion of each BA in %.

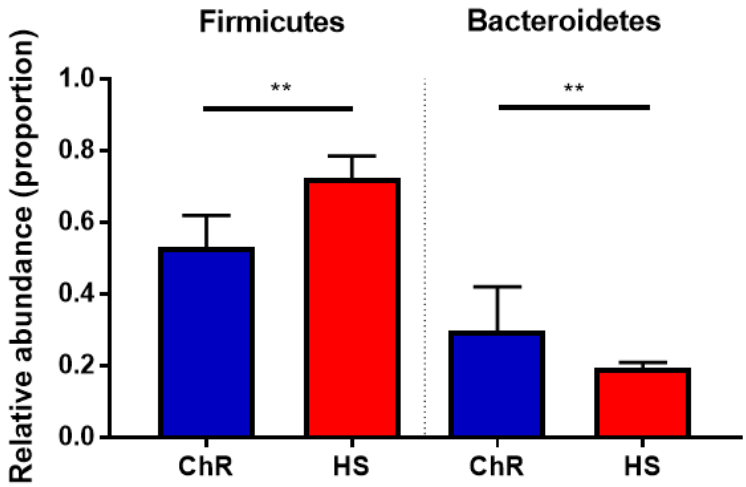
5.3.3 Lean NAFLD mice model has a distinct gut microbiota profile

Analysis of the microbiota demonstrated a distinct and separate microbiota profile between lean NAFLD and non-lean NAFLD mice models, with changes in composition (PERMANOVA P= 0.009, pseudo-F= 18.58, 126 permutations, **Figure 32A**) as reflected broadly by significant changes in the relative abundances of the two major phyla, Firmicutes and Bacteroidetes, as well as in Actinobacteria, Cyanobacteria and Proteobacteria. Mice fed the ChR diet were observed to have an increased relative abundance of Bacteroidetes and a decrease in Firmicutes compared to those fed high sucrose **(Figure 32B).** As we observed in humans, similar trends were noted for the abundance of members of the Ruminococcaceae bacterial family in the high cholesterol diet fed (lean NAFLD) mice. These changes were also observed for several phylotypes within the Erysipelotrichaceae (**Figures 32C and D**).

A.


2D Stress: 0.01
Group Treatment
WTChR
WTHS
WT17
WT18
WT21
WT20
WT19
WT29
WT31
WT28
WT27
WT30

B.


Firmicutes
Bacteroidetes
Relative abundance (proportion)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
ChR
HS
ChR
HS

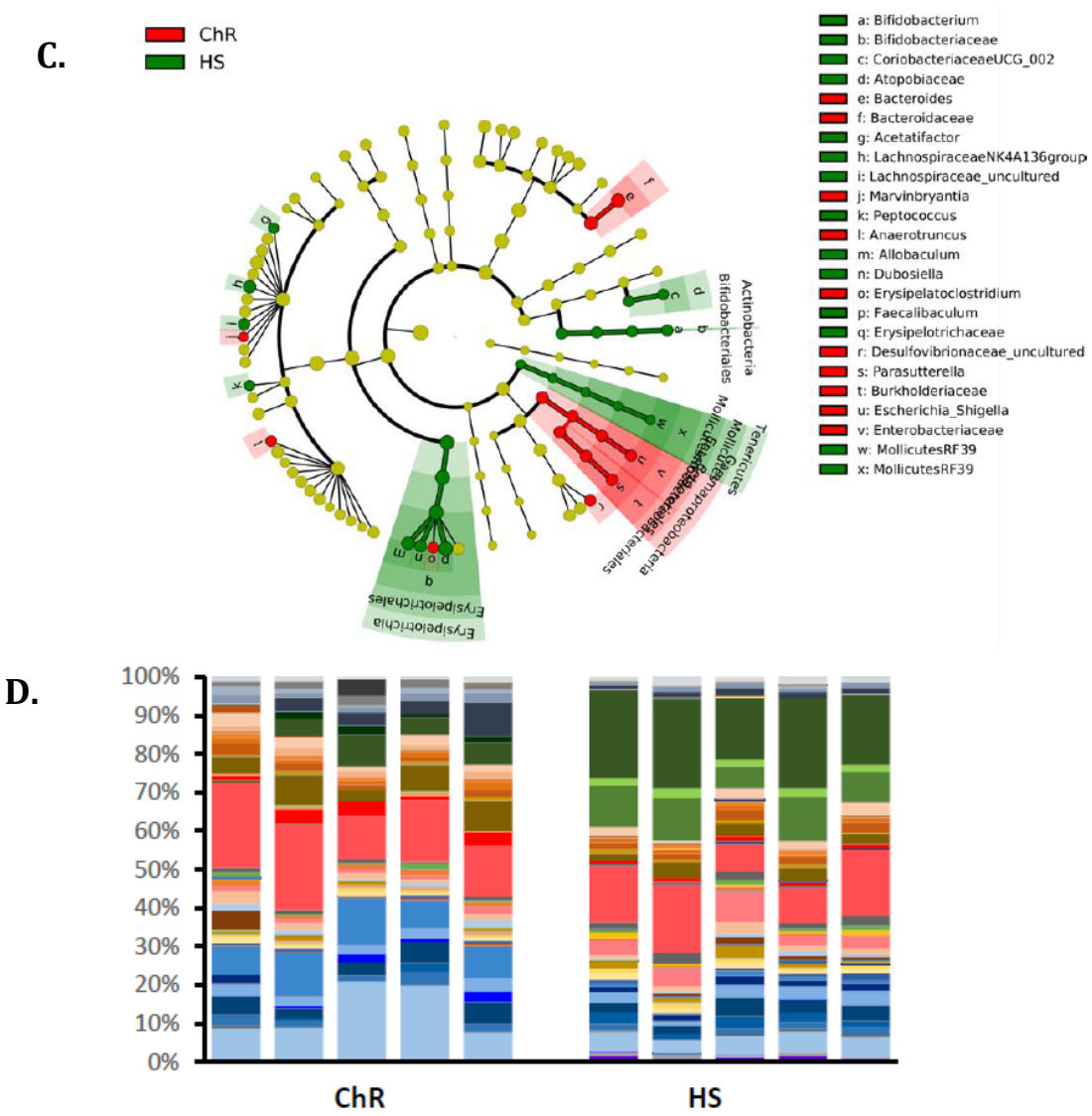


Figure 32. Gut microbiota profile in mice fed cholesterol rich (ChR) diet and mice fed high sucrose (HS) diet.

A. Microbiota composition between the two groups showing distinct separation of profiles. B. Comparison of the abundance of the phyla Firmicutes and Bacteroidetes between the two groups. C. Microbiota abundance differences between the two groups. The colour denoting each taxa represents the group in which the taxa was identified to be significantly increased compared to the comparison group ($p < 0.05$). D. Microbiota composition between the two groups. Each colour denotes each taxa and the y-axis represents the % abundance.

5.3.4 The increased bile acids in lean NAFLD mice model is due to increased bile acid synthesis

To investigate if the increased levels of BA seen in the lean NAFLD mice model is due to increased BA production or increased BA reuptake from the enterohepatic circulation, we performed qPCR on mice liver and ileal tissues to quantify the mRNA levels of several BA synthetic enzymes as well as BA transporters.

Our results showed that in the lean NAFLD model, BA synthetic enzyme mRNAs in the liver were significantly higher compared to those in the non-lean NAFLD mice model. This was true for both the BA synthetic enzymes involved in the classical pathway ($p = 0.0016$ for CYP7A1 (**Figure 33A and C)** and $p = 0.001$ for CYP8B1) as well as alternative pathway ($p = 0.0031$ for CYP27A1) (**Figure 33B**).

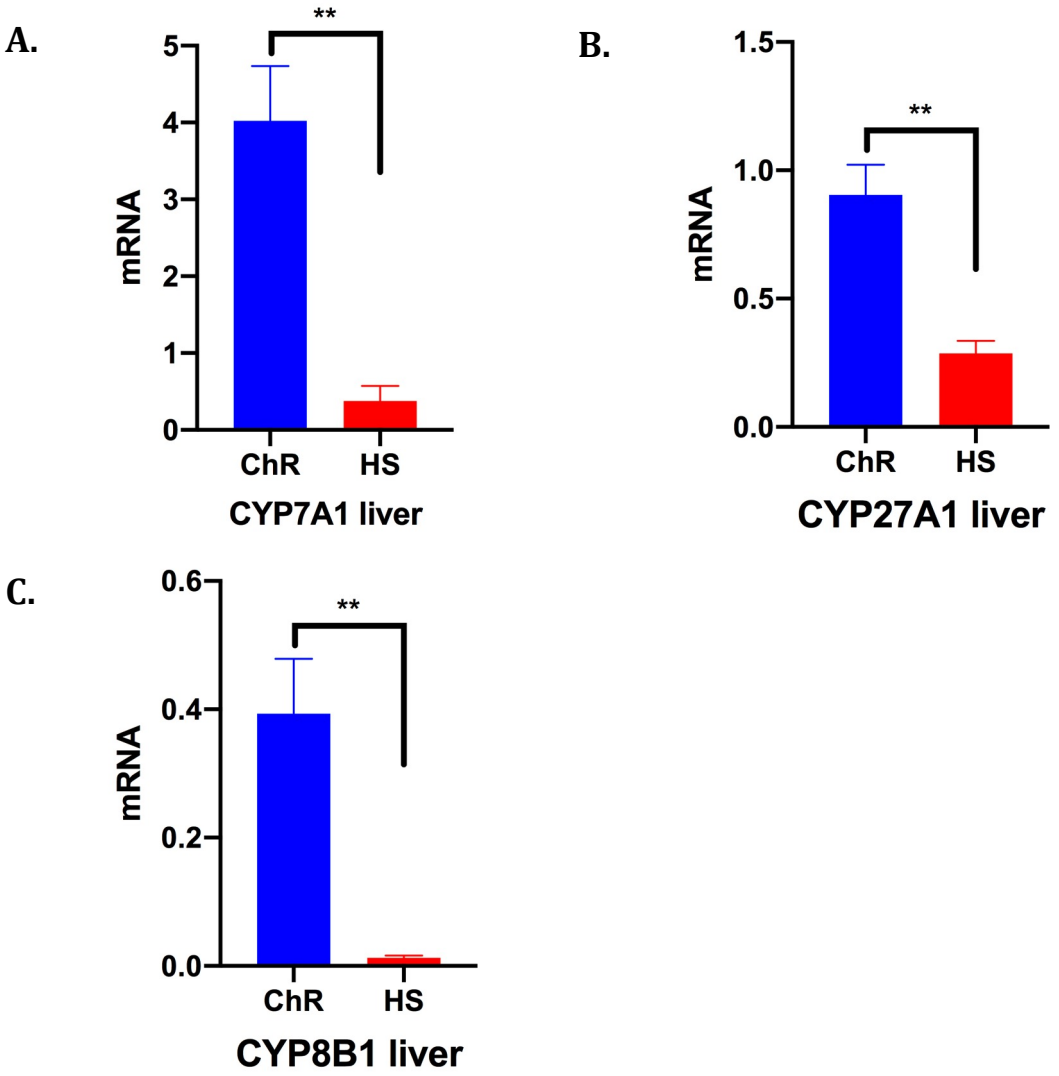
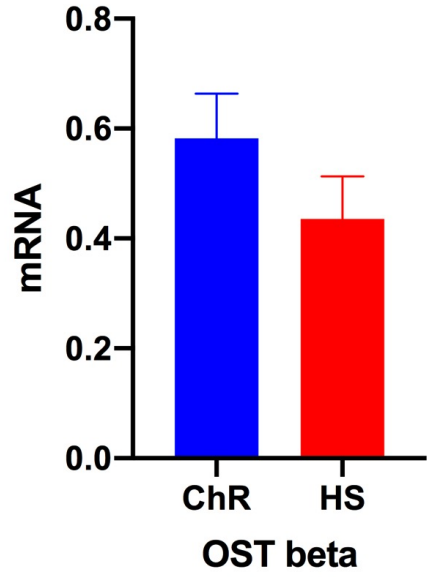


Figure 33. Liver bile acid synthetic enzyme mRNA levels in mice fed a cholesterol rich (ChR) or a high sucrose (HS) diets.

A. Cytochrome P450 7A1 (CYP7A1, classical pathway). B. Cytochrome P450 27A1 (CYP27A1, alternative pathway) and C. Cytochrome P450 8B1 (CYP8B1, classical pathway). The x-axis shows the two groups, cholesterol rich (ChR, n=5) versus high sucrose (HS, n=6) fed mice. The y-axis shows mRNA levels in folds, using 36B4 as housekeeping gene. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

When we compared the BA transporter mRNA levels between lean and non-lean NAFLD models, although it appeared that there was increased expression of BA transporters OST beta and ASBT in the ileum of lean NAFLD models, this was not significant ($p = 0.1049$ for OST beta and $p = 0.3450$ for ASBT) (**Figure 34A and B**).

A.



B.

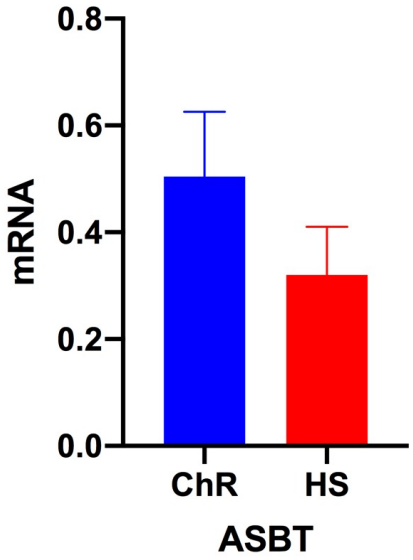


Figure 34. Ileal bile acid transporters organic solute transporter beta (OST beta) and apical sodium bile acid transporter (ASBT) in mice fed a cholesterol rich (ChR) or a high sucrose (HS) diet

A. OST beta bile acid transporter and B. ASBT bile acid transporter. The x-axis shows the two groups, cholesterol rich (ChR, n=5) versus high sucrose (HS, n=6) fed mice. The y-axis showed mRNA levels in folds, using 36B4 as housekeeping gene. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

5.3.5 **Lean NAFLD mice model have elevated FXR activity**

Analysis of ileal fibroblast growth factor 15 (*fgf15*), the mouse equivalent of human FGF-19 showed significantly higher levels in our lean NAFLD, compared to the non-lean NAFLD mice (**Figure 35A**). The increased *fgf15* levels activates the FGF receptor in the liver to stimulate hepatic FXR activity (**Figure 35B**).

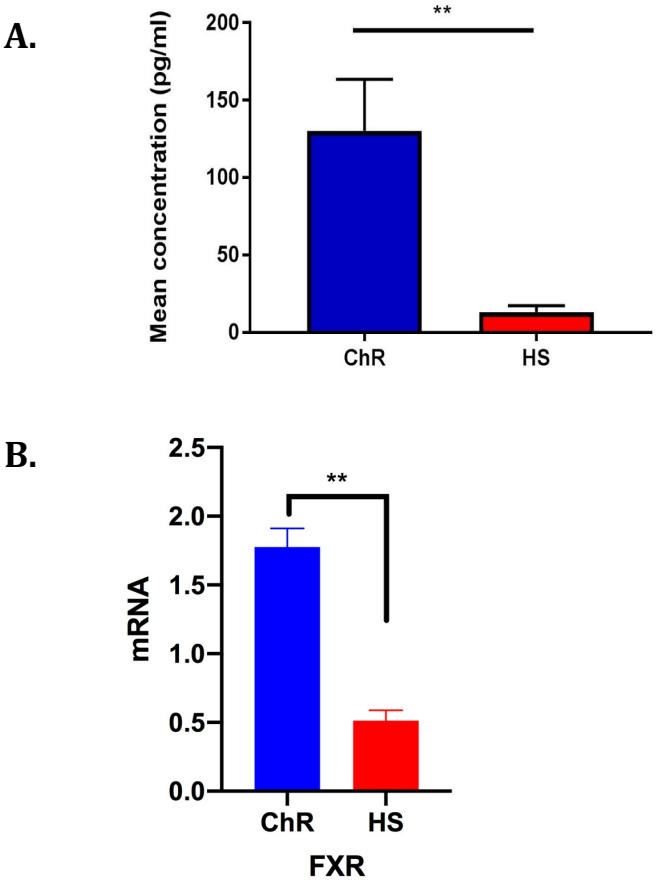


Figure 35. Serum fgf15 levels (A) and FXR mRNA levels (B) in mice fed a cholesterol rich (ChR) or a high sucrose (HS) diet.

The x-axis shows the cholesterol rich (ChR, blue, n=5) and high sucrose (HS, red, n=6) diet fed mice. The y-axis showed the mean serum fgf15 levels in pg/ml in A, and mRNA levels in folds in B, using 36B4 as housekeeping gene. Results are expressed as mean ± SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P<0.01$, *** $P<0.001$.

5.4 DISCUSSION

We used mice fed a cholesterol rich diet (ChR) to replicate human lean NAFLD and mice fed a high sucrose (HS) diet to replicate non-lean NAFLD patients. Mice fed the ChR diet like in human lean NAFLD maintained their weight throughout the period, as opposed to mice fed a HS diet. The latter gained significant amounts of weight during the experiment. Furthermore, mice in the ChR diet group had significantly better fasting glucose levels as well as a better response to the glucose tolerance test suggesting better insulin sensitivity. Histologically, both mice groups developed steatosis, however, the lean NAFLD mice demonstrated increased inflammatory infiltrates.

Like in human NAFLD, the gut microbiota profile in the mice models showed similar patterns between the groups. There was increased Bacteroidetes and decreased Firmicutes in the lean NAFLD compared to the non-lean NAFLD mice. Previous studies have demonstrated possible roles of certain families of microbiota including in Bacteroidaceae and Erysipelotrichaceae with BA metabolism. Increased BA levels are associated with increased levels of these families (Zietak 2016). This may explain our finding of increased BA production through up-regulation of BA synthetic enzyme mRNA. Furthermore, certain taxa belonging to the family Erysipelotrichaceae and Coriobacteriaceae have been shown to be decreased in non-obese humans or those who have lost weight after gastric bypass compared to obese humans (Zhang et al. 2009). This is similar to our mice where the levels of the taxa belonging to Erysipelotrichaceae was lower in the lean compared to the non-lean NAFLD mice.

We were able to demonstrate increased levels of bile acids in lean NAFLD mice which is similar to what was seen in human NAFLD patients. Further testing also suggested that the increased level of bile acid is due to increased bile acid production, as evidenced by the up regulation of BA synthetic enzymes involved in both the classical and alternative pathways.

The increased levels of ileal bile acid transporter mRNAs for ASBT and OSTβ (which accounts for more than 95% of intestinal BA reabsorption from the distal ileum enterocytes (Chavez-Talavera et al. 2017)), despite not being significant, may reflect the better metabolic adaptation seen in lean NAFLD mice, as in our human lean patients. Bile acids stimulate secretion of several hormones including *fgf15* (mouse equivalent of human FGF-19) from the enteroendocrine cells which has positive metabolic effects, including improved glucose tolerance. In addition, *fgf15* is involved in metabolic adaptation through mediating bile acid synthesis in the liver, acting via the FXR pathway.

Despite the many similarities and benefits of using mice models to study human disease in terms of their homogeneity and more standardized nutritional manipulation, there are also differences between mice and humans. Thus , results from mice models must be interpreted with caution. Mice for example have distinct bile acid homeostasis compared to humans. Whereas in humans the primary bile acid pool is made up of cholic acid (CA) and chenodeoxycholic acid (CDCA) and their conjugates, in mice the majority of the primary BA pool is comprised of T-βmuricholic acid (T-βMCA) and

T-αmuricholic acid (T-αMCA) which are formed through hydroxylation of CDCA and UDCA respectively, via the enzyme CYP2c70 (McGlone et al. 2019). This difference in BA pool composition also affects the physiology seen in the two species, as MCA are FXR antagonists, whereas CDCA is a potent FXR agonist (McGlone et al. 2019). In addition, there are also differences in the homeostasis of BA in humans and mice. In mice, bile acids are almost exclusively conjugated with taurine which has greater solubility, whereas in humans, the majority of bile acids are conjugated with glycine, with only a small proportion being conjugated to taurine (McGlone et al. 2019).

5.5 CONCLUSION

Lean NAFLD is a unique sub-group of NAFLD with unclear pathophysiology. In this study we demonstrated using mice models fed a cholesterol rich or a high sucrose diet, certain similarities to human NAFLD patients, namely in their histological and metabolic profile, as well as in bile acid homeostasis. We also demonstrated a distinct and improved metabolic adaptation in the lean mice similar to human patients with respect to increased FXR activity. Although the results suggests the potential to undertake interventional experiments on NAFLD pathophysiology, including lean NAFLD, mice models have key differences with respect to their bile acid physiology and results must therefore be interpreted with caution.

CHAPTER SIX

METABOLOMIC ANALYSIS OF LEAN NAFLD COMPARED TO NON-LEAN NAFLD

6 METABOLOMIC ANALYSIS OF LEAN NAFLD COMPARED TO NON-LEAN NAFLD AND LEAN HEALTHY CONTROLS

6.1 INTRODUCTION

The search for non-invasive biomarkers for the diagnosis and staging of NAFLD is an unmet need. Liver biopsy remains the gold standard for diagnosing and staging NAFLD, but it is invasive and subject to sampling error and inter-observer variability (Brunt et al. 2011). Although multiple non-invasive methods have been studied for use in quantification of hepatic steatosis and fibrosis including magnetic resonance imaging proton density fat fraction (MRI-PDFF) and magnetic resonance elastography (MRE), these modalities lack the ability to quantify hepatic inflammation, ballooning and injury, all essential for the diagnosis of NASH (Mayo et al. 2018).

Current therapeutic options for NAFLD and NASH are limited to lifestyle intervention but the optimal dietary nutrient composition and exercise requirement are still debatable to achieve the greatest histologic benefit. Although several drugs have been considered useful for use in NAFLD, their benefit and long-term efficacy as well as safety are uncertain. Hence, metabolomics might provide an interesting tool to achieve early and better diagnosis, understand disease pathogenesis and suggest targets for development of new treatments (Gitto et al. 2018).

Previous studies on metabolomic profiling in patients with NAFLD and NASH have shown modifications in the metabolites including lipids, amino acids, glucose and bile

acid pathways. The results however remain conflicting due to the vast range of sample sizes, differences in populations as well as methods of metabolomic profiling (Gitto et al. 2018).

We identified the bile acid pathway to be significantly altered between different stages of NAFLD as well as between lean and non-lean NAFLD. Hence, in this chapter we aim to further explore this from a metabolomics perspective to determine if other metabolic pathways are different between the lean and non-lean NAFLD groups.

6.2 METHODS

We selected a sub-group of patients from our large cohort for metabolomics analysis keeping the ratio between the lean and non-lean NAFLD groups similar to that of our previous comparisons. A total of 181 patients were analysed (19 lean and 162 non-lean NAFLD). We performed untargeted metabolomics analysis using two different platforms, the AMIDE (negative ion mode) and the HILIC (positive ion mode) methods, which look into different metabolic pathways as described below.

6.2.1 AMIDE METHOD

The AMIDE method measures polar compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) in the negative ion mode. Positive mode can also be used. Analytes include amino acids, nucleotides, nucleosides, nucleotide triphosphates, high-energy intermediates, organic acids, TCA cycle intermediates, bile acids and vitamins.

Briefly, 80µL of amide IS-IS is added to 20µL of each serum sample and the mixture vortexed to promote protein precipitation. The sample mixtures are centrifuged at 14000rpm for 20 minutes at 4ºC. After that, 75µL of the supernatant is transferred into glass vials with inserts, taking care to avoid transferring protein pellet particles. The vial is capped and stored at -30ºC until time of analysis. Further details on reagent preparation, mass spectrometer settings and list of metabolites are in the Appendix section.

6.2.2 **HILIC METHOD**

The HILIC method measures polar compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) in the positive ion mode. Analytes include amino acids, nucleotides, neurotransmitters and selected medications and vitamins.

Briefly, to 10µL of serum, 90 µL of HILIC IS-IS is added and the mixture vortexed to promote protein precipitation. The sample mixtures are then centrifuged at 14000 rpm for 20 minutes at 4ºC. After that, 75µL of supernatant is transferred into glass vials with inserts, taking care to avoid transferring protein pellet particles. The vial is capped tightly and stored at -30ºC until time of analysis. Further details on reagent preparation, mass spectrometer settings and list of metabolites are in the Appendix section.

6.2.3 **STATISTICAL ANALYSIS**

Ions from both ESI- and ESI+ are merged and imported into the SIMCA-P program (version 14.1) for multivariate analysis. A principal components analysis (PCA) is first used as an unsupervised method for data visualization and outlier identification. Supervised regression modelling is then performed on the data set by use of partial least squares discriminant analysis (PLS-DA) or orthogonal partial least squares discriminant analysis (OPLS-DA) to identify the potential biomarkers. The biomarkers are filtered and confirmed by combining the results of the VIP values (VIP > 1.5) and t-test ($p < 0.05$). The quality of the fitting model can be explained by R^2 and Q^2 values. R^2 displays the variance explained in the model and indicates the quality of the fit. Q^2 displays the variance in the data indicating the model's predictability. **Figure 36** shows a flowchart of how metabolomics analysis was performed.

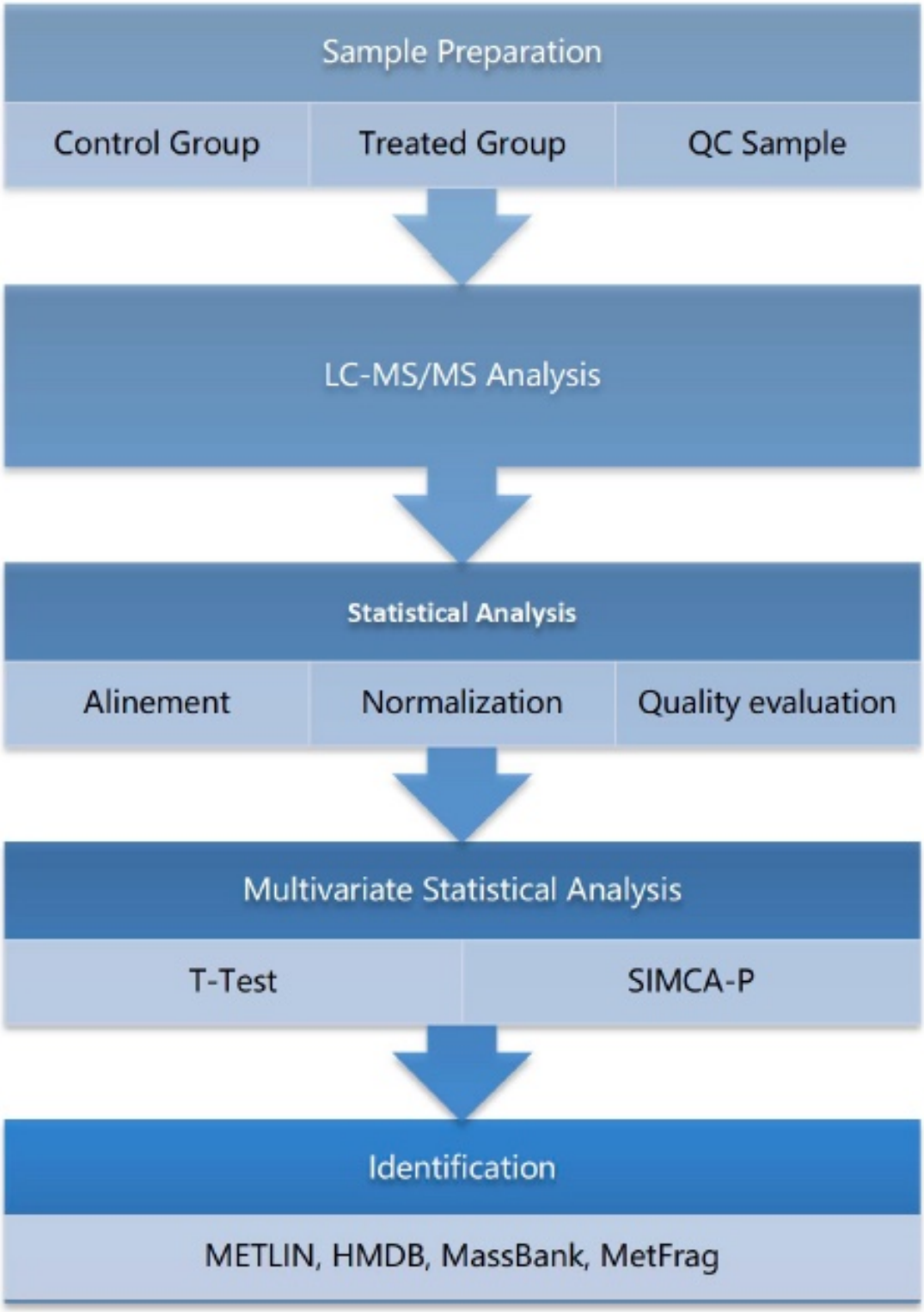


Figure 36. Flowchart of metabolomics analysis from sample preparation to identification

6.3 RESULTS

6.3.1 Patient demographics

We analysed a total of 181 well-characterised, biopsied NAFLD patients (19 lean and 162 non-lean). The baseline patient demographics are shown in **Table 9**.

Patients in both lean and non-lean NAFLD groups were selected to match in terms of their age and gender. Apart from patients in the non-lean NAFLD patients having significantly more diabetes, there was no significant difference in the total cholesterol level as well as their histological profile between the two groups.

Table 9. Baseline characteristics of lean and non-lean NAFLD patients for metabolomics analysis

	Lean NAFLD (n = 19)	Non-lean NAFLD (n = 162)	p-value
Age (years)	50 ± 8.01	51 ± 13.6	0.7541
Male (%)	10 (52.6)	81 (50)	1.000
BMI (kg/m^2)	23.0 ± 1.3	32.4 ± 5.8	**<0.001**
ALT (IU/ml)	65.2 ± 36.0	74.0 ± 52.8	0.4808
Diabetes (%)	2 (10.5)	39 (24.1)	**<0.001**
Hypertension (%)	7 (36.8)	58 (35.8)	1.000
Dyslipidaemia (%)	9 (47.4)	90 (55.6)	0.6274
Total Cholesterol (mmol/L)	5.3 ± 1.2	5.1 ± 1.2	0.4928
Fibrosis (%) **F 0-1 (%)** **F 2-4 (%)**	 14 (73.7) 5 (26.3)	 100 (61.7) 62 (38.3)	 0.4519
Ballooning (%) **No ballooning (%)** **Any ballooning (%)**	 14 (73.7) 5 (26.3)	 105 (64.8) 57 (35.2)	 0.6105
Steatosis (%)** **Grade 1-2 (%)** **Grade 3 (%)**	 15 (78.9) 4 (11.1)	 133 (82.1) 29 (17.9)	 0.7548
Portal inflammation **No inflammation (%)** **Inflammation (%)**	 10 (52.6) 9 (47.4)	 78 (48.1) 84 (51.9)	 0.8181
Lobular inflammation **No inflammation (%)** **Inflammation (%)**	 8 (42.1) 11 (57.9)	 77 (47.5) 85 (52.5)	 0.8089
NAS score	2 ± 2	3 ± 2	**0.0407**

Values are mean±SD, or number (%), p-value was calculated using Fisher's exact test and student's t-test.

6.3.2 Principal component analysis demonstrates unclear groupings between the two groups

After normalization is carried out, a line plot was used and all the peaks are merged and imported into the SIMCA-P software for multivariate statistical analysis. As shown in **Figure 37**, the line plot demonstrated relative system instability during sample analysis

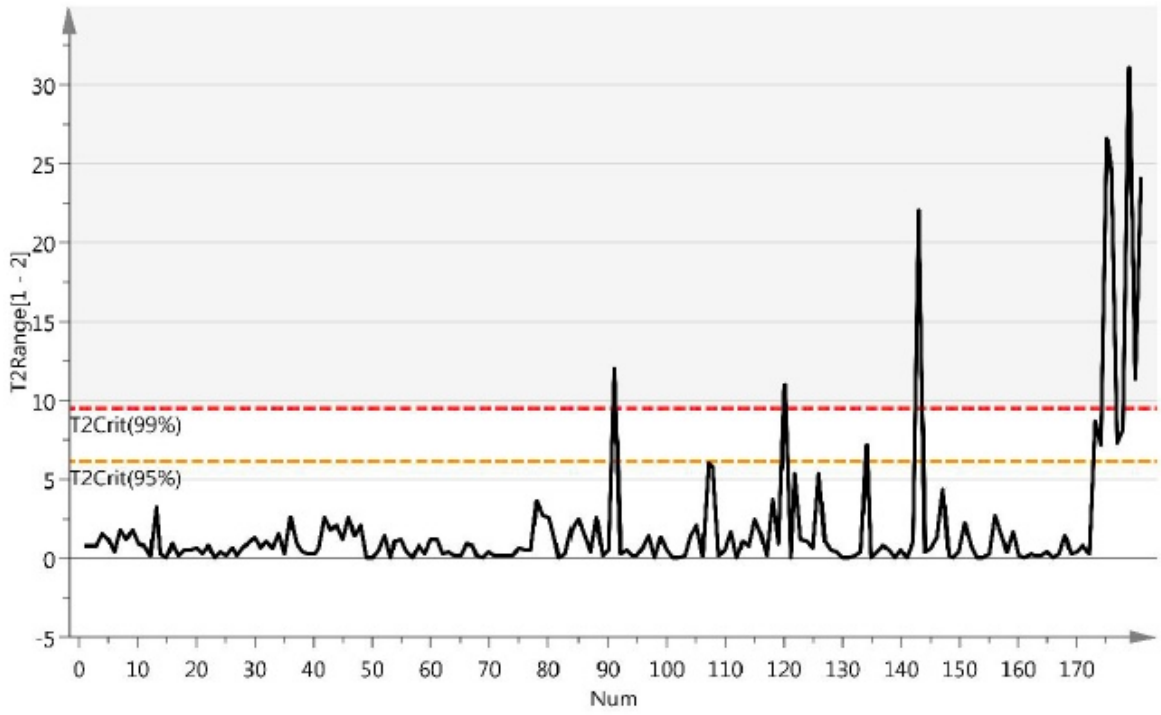


Figure 37. The line plot of samples

Line plot of samples to evaluate the methodology. The x-axis indicates the number of samples and the y-axis indicates the 95% confidence interval.

To investigate global metabolism variations, we first use PCA to analyse all observations acquired in both ion modes. PCA is a technique used to emphasize

variation and bring out strong patterns in a dataset. It is often used to make data easy to explore and visualize. It also acts as an unsupervised pattern recognition method for handling metabolomics data and can classify the metabolic phenotypes based on all imported samples. Due to the unsupervised pattern, the result can be unsatisfactory sometimes. As shown in the PCA plot (**Figure 38**), an overview of all samples in the data can be observed and exhibit an unclear grouping trend between the two groups.

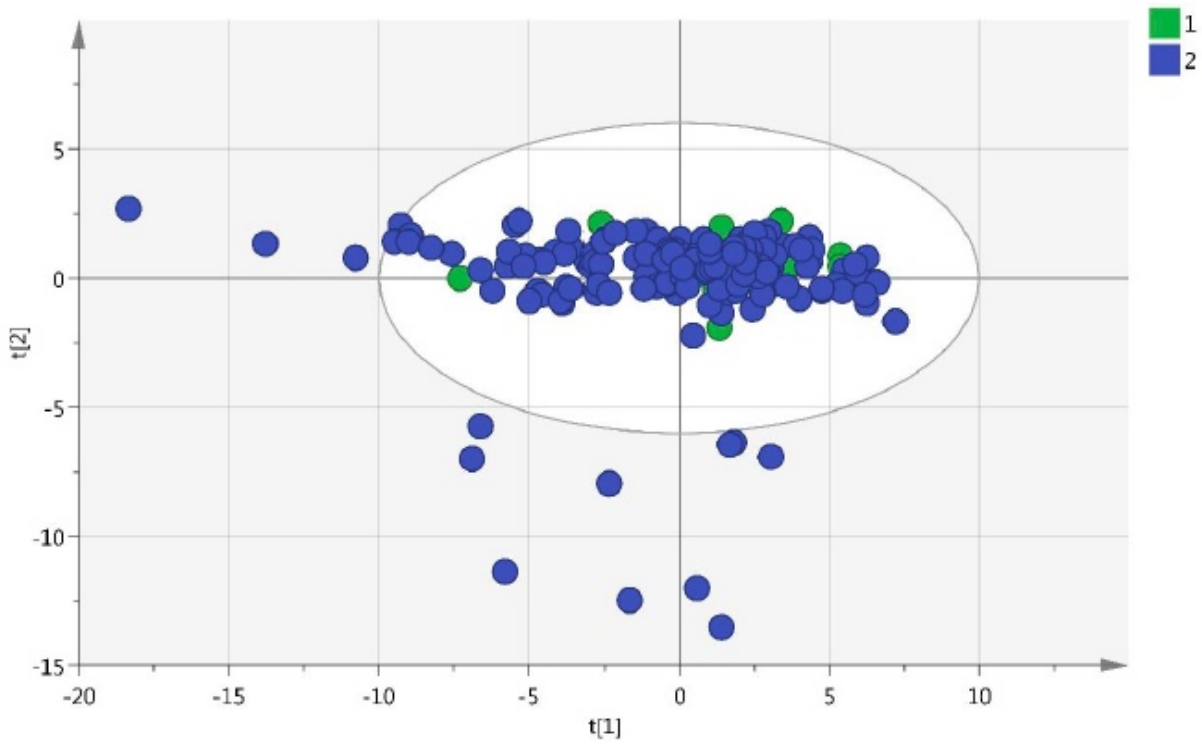


Figure 38. The scores scatter plot of the PCA model.

The x-axis represents the first component and the y-axis represents the second component. The two groups are denoted as 1 (lean NAFLD) and 2 (non-lean NAFLD).

6.3.3 Metabolic changes in the two groups

In order to eliminate any non-specific effects of the operative technique and confirm the biomarkers, partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-DA) was performed to compare metabolic changes in the two groups, respectively.

In PLS-DA score plot, as well as the OPLS-DA score plot, an unclear separation of the two groups is observed (**Figures 39 and 40**).

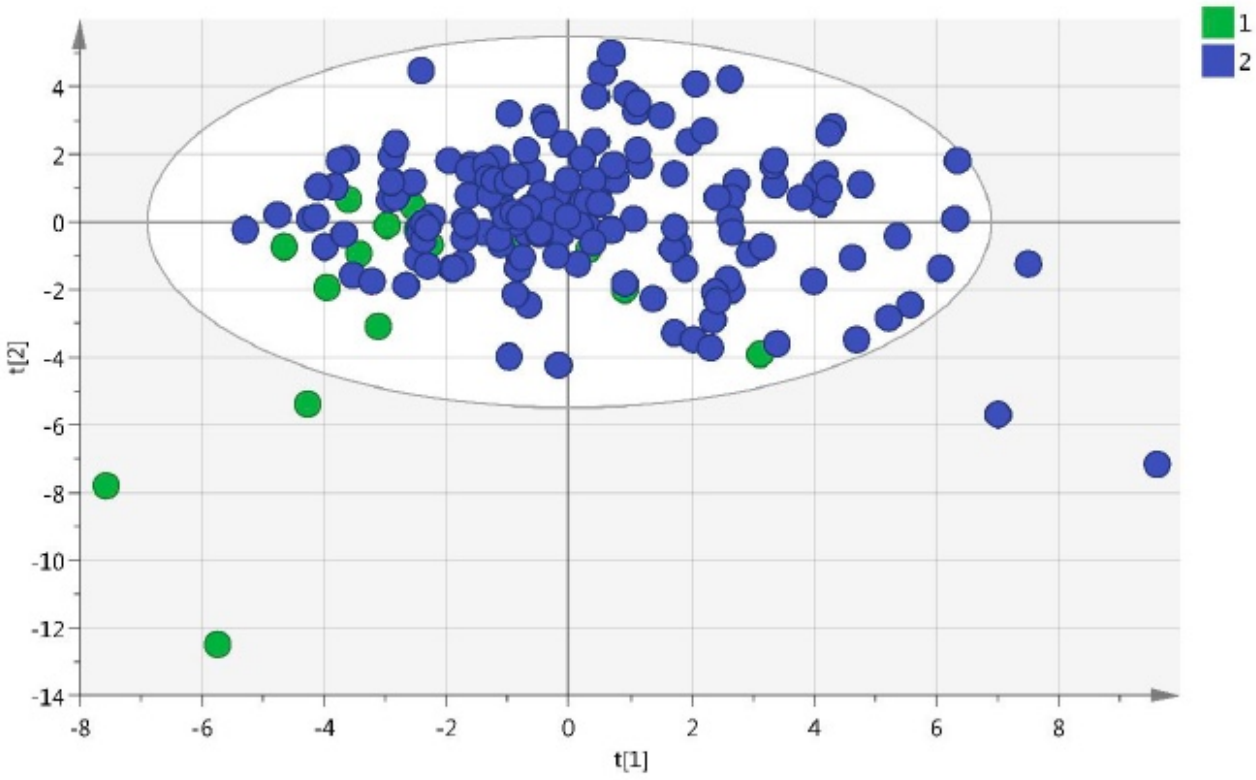


Figure 39. The scores scatter plot of partial least squares discriminant analysis (PLS-DA) model between lean and non-lean NAFLD.

The groups are marked as 1 (lean NAFLD) and 2 (non-lean NAFLD)

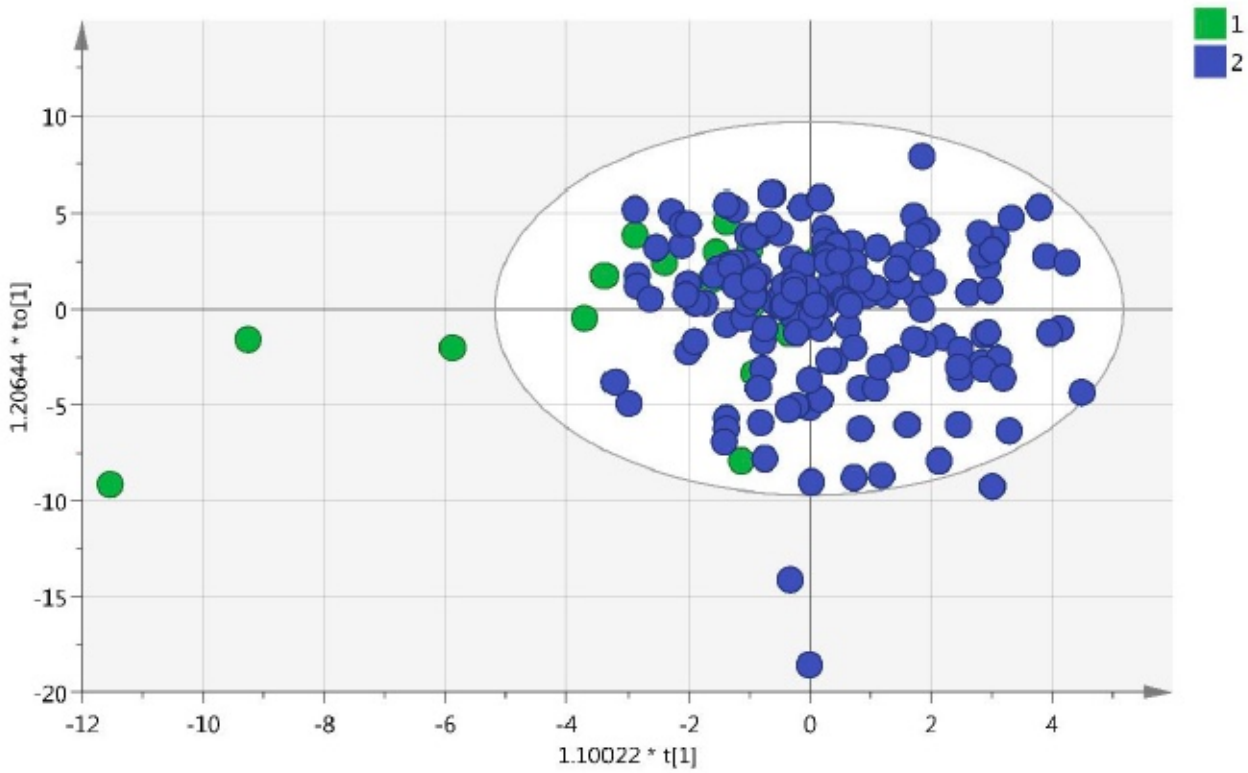


Figure 40. The scores scatter plot of the orthogonal partial least squares discriminant analysis (OPLS-DA) model between lean and non-lean NAFLD

The groups are marked as 1 (lean NAFLD) and 2 (non-lean NAFLD).

Interestingly, while there was no clear separation between the two groups when analysed based on lean and non-lean NAFLD groups overall, there was distinct separation in the OPLS-DA model between the extremes of lean NAFLD patients with advanced fibrosis and non-lean NAFLD patients with none/mild fibrosis (**Figure 41**). This could reflect differences in metabolism between advanced and none/mild fibrosis.

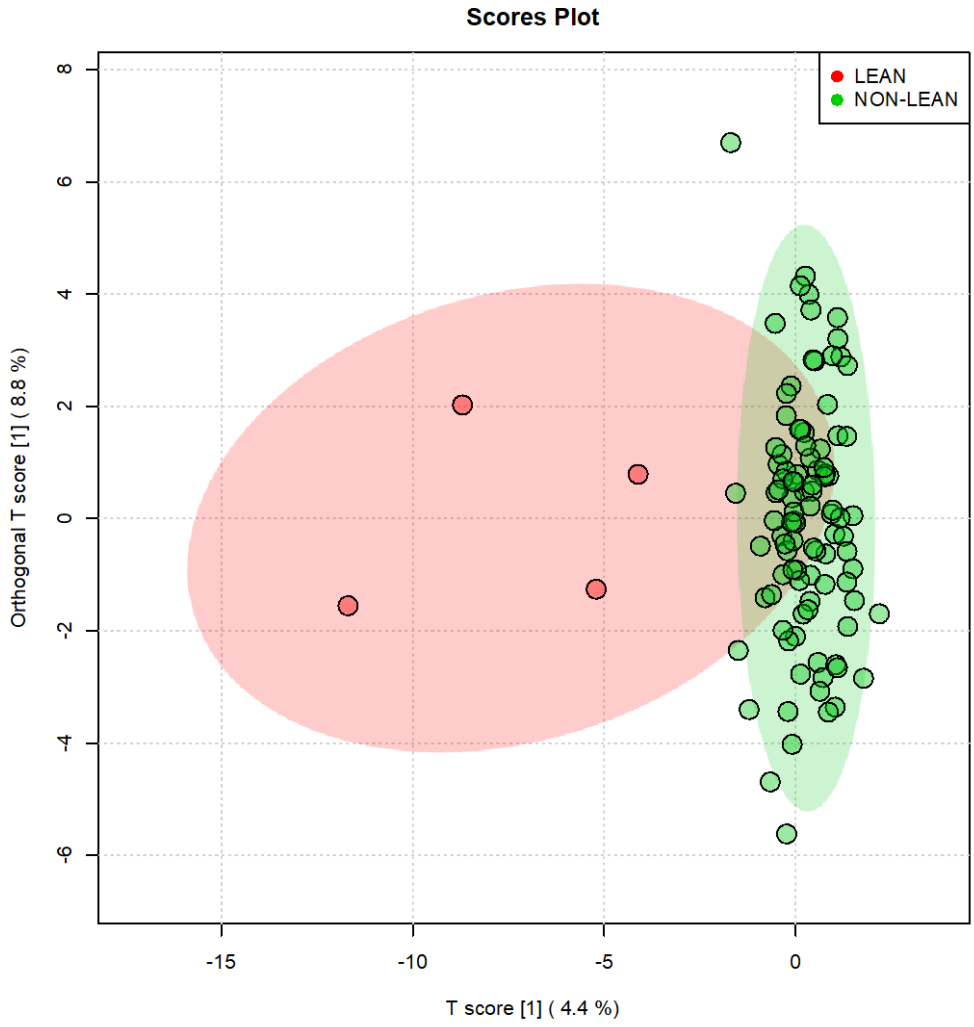


Figure 41. Scores scatter plot of the orthogonal partial least squares discriminant analysis (OPLS-DA) between lean NAFLD with advanced fibrosis and non-lean NAFLD with none/mild fibrosis

6.3.4 Single variable analysis based on significant variable importance of projection (VIP) values

Significantly changed metabolites between the groups were filtered out based on the variable importance of projection (VIP) values. Significant VIP values are considered to be VIP > 1.5. The PLS-DA loading plot is shown in **Figure 42** with metabolites in red labelled as significant compounds (VIP > 1.5).

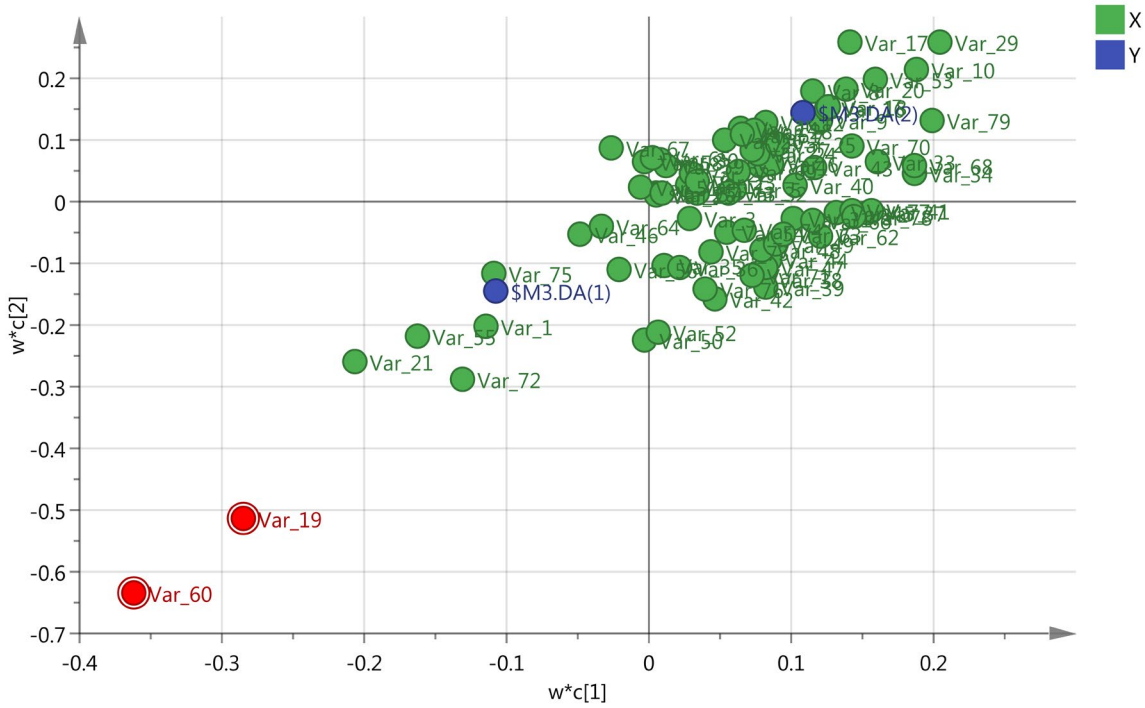


Figure 42. The loading plot of the PLS-DA model, with metabolites in red labelled as significant compounds (VIP>1.5).

Var_60 is Cystamine and Var_19 is Pyruvate.

Univariate analysis was then performed on a volcano plot, shown in **Figure 43**, including fold change analysis and t-test. As shown, the result from the univariate

analysis did not show any significance, likely due to the small number of samples and the instability of sample analysis.

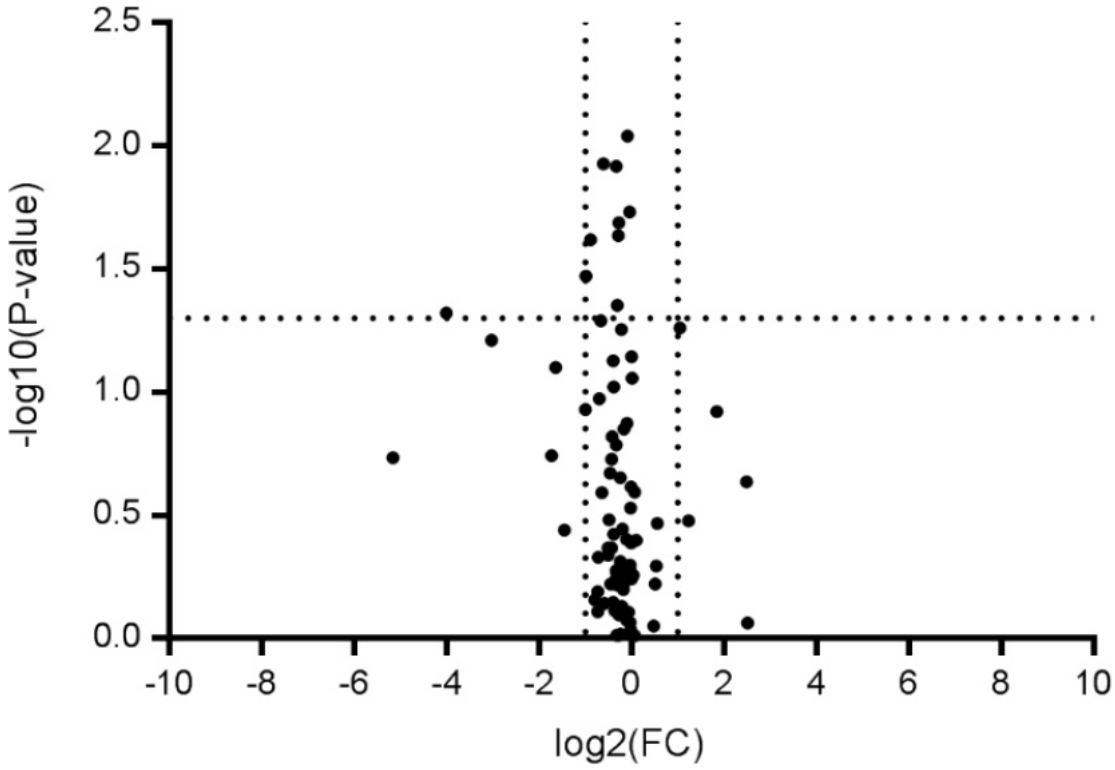


Figure 43. Volcano plot of data.

Univariate analysis of metabolites with values of Y > 1.30 and X > 1 considered to be a significant increase; values of Y > 1.30 and X < 1 considered to be a significant decrease.

6.3.5 Identification of potential biomarkers

The chemical structures of important metabolites were then identified according to online databases such as the Human Metabolome Database (www.hmdb.ca), Metlin (www.metlin.scripps.edu) and massbank (www.massbank.jp) using the data of accurate masses and MS/MS fragments. When necessary, further confirmation was acquired through comparisons with authentic standards including retention times and MS/MS fragmentation patterns.

6.3.6 Cluster analysis

Mean values of metabolite contents are used to calculate the metabolite ratio. After log transformation of the data, median centred ratios are then normalized. Hierarchical clustering analysis (HCA) is performed using the complete linkage algorithm of the program Cluster 3.0 (Stanford University) and the results are visualized using Treeview (Stanford University). Metabolite ratios from two independent experiments of every significant metabolite are used for the HCA. Colour intensity correlates with degree of increase (red) and decrease (blue) relative to the mean metabolite ratio. The HCA of metabolomics data from the lean and non-lean NAFLD groups is shown in **Figure 44**.

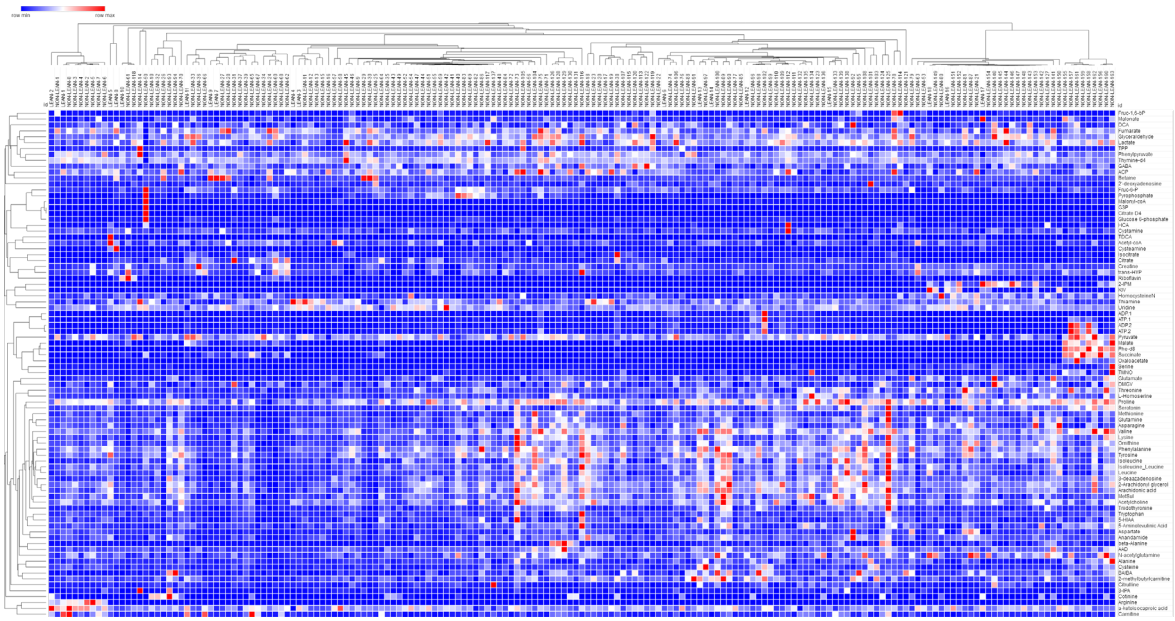


Figure 44. Hierarchical cluster analysis of metabolomics data from significant metabolites

Hierarchical cluster analysis (HCA) from metabolite ratios of every significant metabolite. Red denotes degree of increase and blue denotes degree of decrease relative to the mean metabolite ratio.

After performing the HCA, the top 3 metabolites with the most significant fold change were identified. **Table 10** shows the fold change for the top 3 metabolites and the t-test analysis between the two groups.

Table 10. T-test analysis of the top 3 metabolites

Metabolite	Mean metabolite ratio	Fold change (FC)	Log_2FC	p-value
ATP	11.6739	0.655834	-0.6086	0.011797
Fructose-6-phosphate	1.013142	0.795882	-0.32943	0.01206
Cysteine	1.331316	0.944543	-0.08231	0.009122

6.3.7. Correlation network of metabolites

To investigate the latent relationships of the metabolites, a correlation network diagram was constructed based on the KEGG databases and MBRole. All significant metabolites are imported to obtain the categorical annotations, including pathways, enzyme interactions and other biological annotations, using the limiting condition of $p < 0.05$ in the MBRole.

A metabolic pathway map is then constructed based on relevant literature and the KEGG database. The correlation map is shown in **Figure 45**.

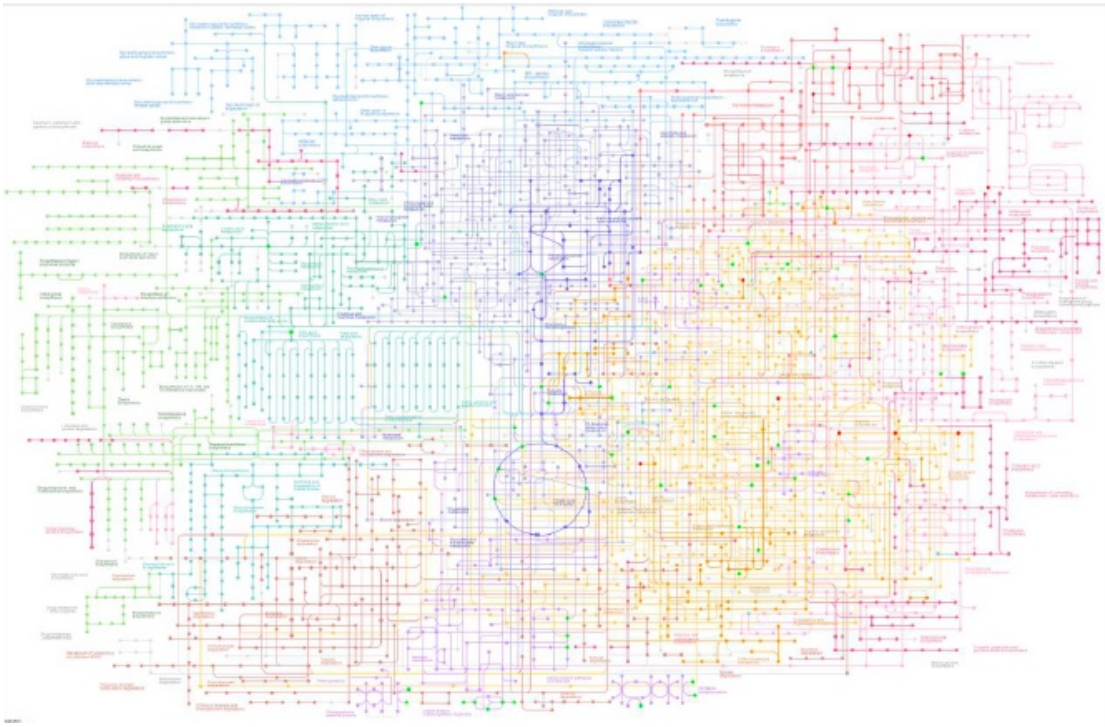


Figure 45. Metabolic network of the significantly changed metabolites.

Red represents increase and green represents decrease.

6.4 DISCUSSION

In this chapter, we performed non-targeted metabolomics analysis comparing metabolites in lean and non-lean NAFLD patients. As previously outlined by us and other studies, lean NAFLD represents a distinct subgroup characterised by better metabolic adaptation mediated by the interplay of gut microbiota, bile acid metabolism, genetic background and lifestyle factors. We aimed to perform untargeted metabolomics analysis to determine if we were able to find signature metabolites which are characteristic of lean NAFLD. The metabolomics analysis demonstrated a few salient findings.

Firstly, one of the top 3 metabolites that were identified to be different between the two groups was fructose-6-phosphate. Fructose-6-phosphate is a metabolite from glucose and fructose metabolism which has been implicated in human and animal studies to be involved in the pathogenesis of NAFLD and NASH through promotion of fat accumulation in the liver due to both increased lipogenesis and decreased fat oxidation (Jensen et al. 2018). This is partly mediated through the metabolism of fructose by fructokinase C with associated ATP consumption, which results in nucleotide turnover and uric acid generation. This promotes fat accumulation (Jensen et al. 2018). Interestingly, ATP was also identified as one of the significantly different metabolites between lean and non-lean NAFLD groups, further supporting this hypothesis.

Cysteine, a trans-sulphuration product from a sulphur containing amino acid homocysteine, was also identified to be significantly different between lean and non-lean NAFLD patients. Disturbances in the metabolism of homocysteine and cysteine has been implicated in a number of conditions such as cardiovascular disease and type-2- diabetes, as well as in obese NAFLD through promotion of hepatic oxidative stress (Francque et al. 2016; Kalhan et al. 2011). Furthermore, a study on metabolomics profile in paediatric patients with NAFLD also found a positive correlation between plasma cysteine level and presence of hepatic fibrosis suggesting its potential role in disease progression (Pastore et al. 2014)

The significantly altered ABC (ATP-binding cassette) transporters pathway between lean and non-lean NAFLD is also interesting. ABC transporters are a group of membrane transporters, which consist of a wide variety of proteins that hydrolyze ATP to actively transport xenobiotics, endobiotics and their conjugates across cellular membranes. These efflux transporters reside on the sinusoidal and canalicular membranes of hepatocytes and transport substrates into the blood and bile, respectively (Hardwick et al. 2011). Disruptions of these transporter functions have been implicated in multiple diseases including NAFLD. In one study of ABC transporter functions during progression of NAFLD to NASH, increased expression of multiple efflux transporters and altered cellular localization of ABC transporters, especially ABCC2 was found in NASH (Hardwick et al. 2011).

Bile acid metabolism although not considered to be the top significantly altered pathway in our study remained significantly different between lean and non-lean

NAFLD groups (p = 0.0101). This is consistent with results from our previous chapters as well as multiple other studies in NAFLD.

Metabolomic profiling between lean and non-lean NAFLD has previously been undertaken. In one study, the serum metabolome of 187 subjects from lean healthy, lean NAFLD and obese NAFLD was compared; this showed differences in phosphatidylcholine and lysine, and branched chain amino acids lysine, tyrosine and valine to be significantly different on multiple logistic regression analysis (Feldman et al. 2017b). Unfortunately, due to the instability of the sample analysis (owing to technical reasons which resulted in the run being interrupted several times) as well as the underpowered sample size, most of the results were insignificant. However, when samples were analysed based on the extremes of BMI in the two groups, the scatter plot demonstrated a much clearer separation between two groups, although this could be due to differences in metabolism between patients with advanced versus mild fibrosis and not necessarily BMI driven. Unfortunately, there were only a small number of patients with advanced fibrosis in the lean group making this analysis limited.

Future directions would involve increasing the number of samples, especially those at extremes of BMI and fibrosis to hopefully distinguish the two groups better and potentially identify metabolites which are able to be used as potential biomarkers for diagnosis or prognostication purposes in patients with lean NAFLD.

6.5 CONCLUSION

In conclusion, metabolomics analysis remains a useful tool for identification of biomarkers for many diseases including lean NAFLD. Our analysis, although mostly insignificant, suggested a few differences in metabolism between lean and non-lean patients, especially for those involved in fructose/glucose metabolism as well as in active transporters. Further studies are required to better characterise this important subgroup of NAFLD.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

7 SUMMARY AND CONCLUSIONS

7.1 SUMMARY OF FINDINGS

This series of chapters are part of a project to investigate the underlying pathogenesis of lean NAFLD, a subgroup which is under-recognized with unclear mechanisms.

We have introduced the concept of metabolic health and how it governs an individual's risk for development of metabolic disease, including NAFLD. This is mediated through the regulation of adiposity, insulin resistance, inflammation and bile acid metabolism. In the first result chapter we investigated the role of metabolic health in bile acid metabolism in lean NAFLD compared to lean and non-lean healthy controls. We found that metabolic health status played a greater role in determining their bile acid levels. Subsequently, we investigated the difference in bile acid metabolism and regulatory pathways between lean NAFLD and lean healthy controls in more detail and found that in addition to lean patients demonstrating higher levels of bile acids, they also had a distinct gut microbiota profile. Taken together, this appears to have increased their inflammatory profile as shown by the trend to an increase in inflammatory cytokines compared to their lean healthy counterparts.

In the next chapter we compared the characteristics of lean NAFLD patients with those who were non-lean. We discovered that bile acid levels tended to increase as fibrosis degree increased. Interestingly, the bile acid levels, especially secondary bile acid levels, were significantly higher in lean NAFLD patients compared to their non-

lean counterparts. Lean patients also demonstrated a higher proportion of *TM6SF2* risk allele carriage compared to the non-lean NAFLD patients, as well as a distinct microbiota profile. The enriched species have been described to be involved in bile acid synthesis and hepatic steatosis formation. However, on further investigation we discovered that although the bile acid levels were higher in lean NAFLD, these patients tended to have milder histology and fibrosis at baseline. This suggests a compensatory mechanism to counteract the increased bile acid levels. We looked into the bile acid regulatory mechanisms and discovered that there is increased level of FXR activity (measured through serum FGF-19 levels) in the lean NAFLD patients, especially in the early stages of the disease. This increase in FXR activity may act as a compensatory metabolic adaptation mechanism by decreasing bile acid formation (as shown by the reduced level of serum C4) as well as non-bile acid mediated pathway through its action on glucose and insulin sensitivity and reduction in liver fibrosis (Pathak et al. 2018; Schumacher et al. 2020). At least in the early stages of the disease this may explain the milder histology. The increased bile acid levels along with increased FXR activity and altered gut microbiota profile may also support the metabolic adaptation capacity that lean NAFLD patients possess in order to rid themselves of excess cholesterol and remain obesity-resistant. However, this adaptation capacity tends to be lost with disease progression as shown by the marked decrease in FXR activity, increased serum C4 levels and subsequently increased bile acid levels, resulting in rapid progression of liver disease. The proposed hypothesis is summarised in **Figure 46**.

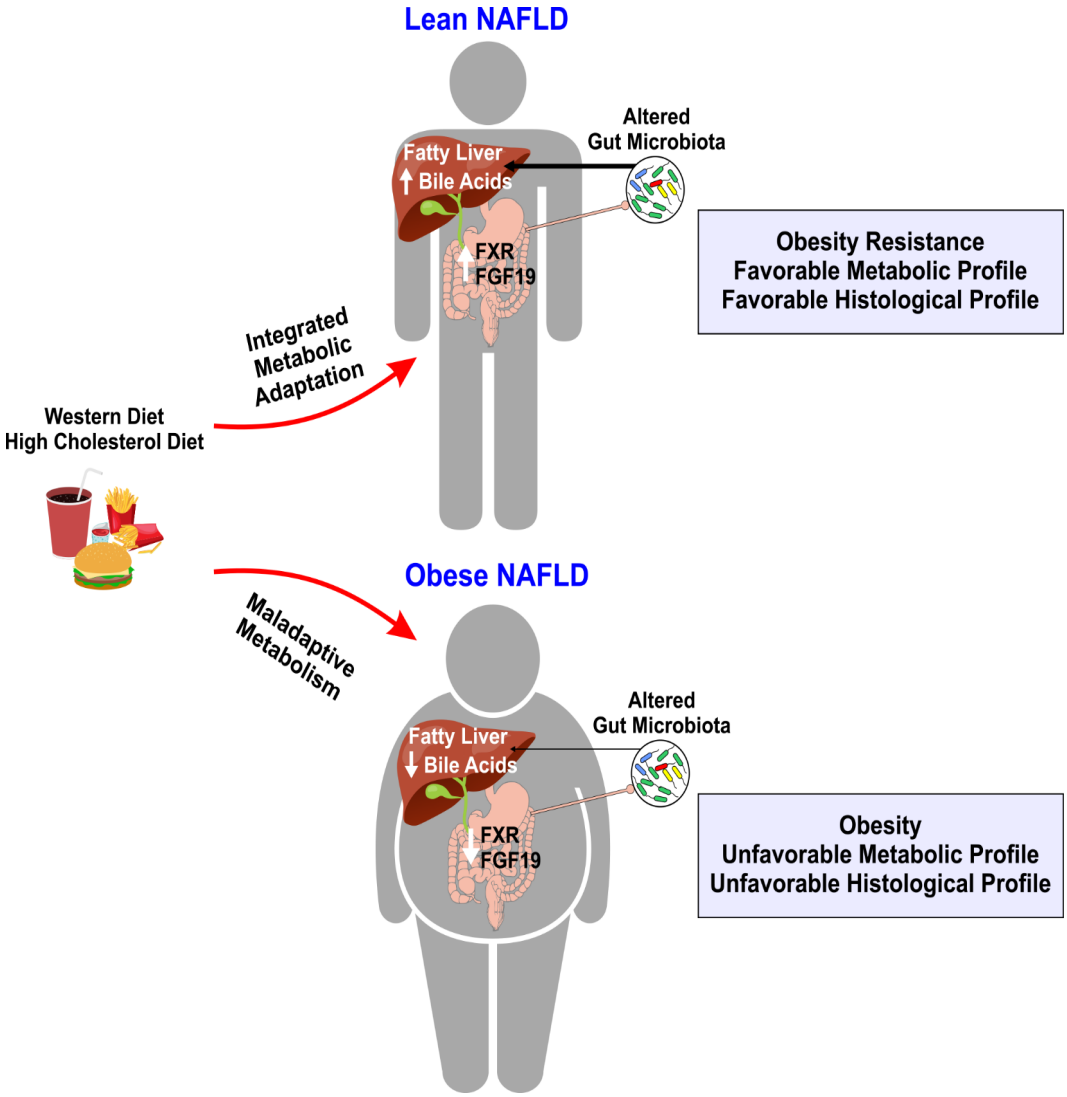


Figure 46. Proposed model for the differential pathophysiology between lean and obese patients with NAFLD.

Lean patients have better metabolic and liver histology profiles. Consistent with the notion that lean patients have appropriate metabolic adaptation to an obesogenic environment, they are obesity resistant. The compensatory mechanisms include increases in bile acids and FXR activity and distinct gut microbiota profiles despite steatosis development. Similar features were observed in murine models of lean and obese NAFLD. We suggest that the relative contribution of the systemic milieu versus that of the gut governs the lean versus non-lean phenotype.

Having demonstrated the metabolic adaptive capacity of lean NAFLD in humans, we proceeded to replicate the findings using mice models. Using mice fed a cholesterol rich diet to simulate lean NAFLD and mice fed a high sucrose diet to simulate non-lean NAFLD, their bile acid profile, gut microbiota and bile acid regulatory components were investigated. We found that like in humans, lean NAFLD mice had significantly higher serum bile acid levels. In addition, further interrogation uncovered increased bile acid production and increased bile acid reabsorption through the ileum as possible underlying reasons for the increased bile acid levels. Interestingly, like in human cohorts, lean mice models also demonstrated better metabolic adaptive capacity as shown by increased serum *fgf15* levels, the orthologue of human FGF-19. We also demonstrated a distinct gut microbiota profile further supporting a predominant gut-driven pathogenesis for lean NAFLD.

Having explored the difference in the bile acid pathway between lean and non-lean NAFLD, we were interested to explore other potential pathways and markers, which may be useful for understanding pathogenesis. Although our experiment was underpowered with respect to the number of samples and the quality of the analysis, it did reveal a couple of salient findings. Firstly, bile acid metabolism remained significantly different between lean and non-lean NAFLD confirming our earlier findings. Secondly, a number of metabolites, namely fructose-6-phosphate, ATP and cysteine, which have been implicated in previous studies were significantly different between the two groups.

7.2 SIGNIFICANCE OF FINDINGS

Our series of experiments have shown an evolution in our understanding of lean NAFLD pathogenesis through the integration of bile acid metabolism, gut microbiota, genetic background and lifestyle factors. Studies on lean NAFLD in the literature have previously focussed on descriptive reports comparing them to healthy controls or obese NAFLD. Hence, this is the first study to examine the pathogenesis of lean NAFLD in detail using large, well-characterised samples.

This project is also the first to introduce the concepts of metabolic health and metabolic adaptation and describe their roles in the pathogenesis of lean NAFLD development and progression. While metabolic health governs an individual's risk for developing metabolic diseases including NAFLD, a person's metabolic adaptive capacity dictates when the person develops the metabolic disease. Given that metabolic health and metabolic adaptive capacity have an impact on the risk for and progression of NAFLD, BMI itself may be a less robust predictor of NAFLD outcomes. Instead, BMI should perhaps be better considered a marker of maladaptation.

Moreover, an individual may respond to increased dietary cholesterol or caloric intake with appropriate metabolic adaptation to maintain body weight, or they may have complete loss of metabolic adaptation resulting in weight gain, increased adiposity and hepatic steatosis. In some individuals however, increased caloric intake may only result in partial loss of metabolic adaptation where the outcome is lean NAFLD. In this scenario, as outlined in **Figure 47**, increased dietary cholesterol intake in lean

NAFLD (as suggested by earlier studies) in the context of perturbed metabolic adaptive capacity associates with some metabolic adaptation through increased production of bile acids, especially secondary bile acids and increased FXR activity to maintain body weight and serum cholesterol levels, as well as maintain favourable liver histology (F. Chen et al. 2019; Enjoji et al. 2012; Schumacher et al. 2020).

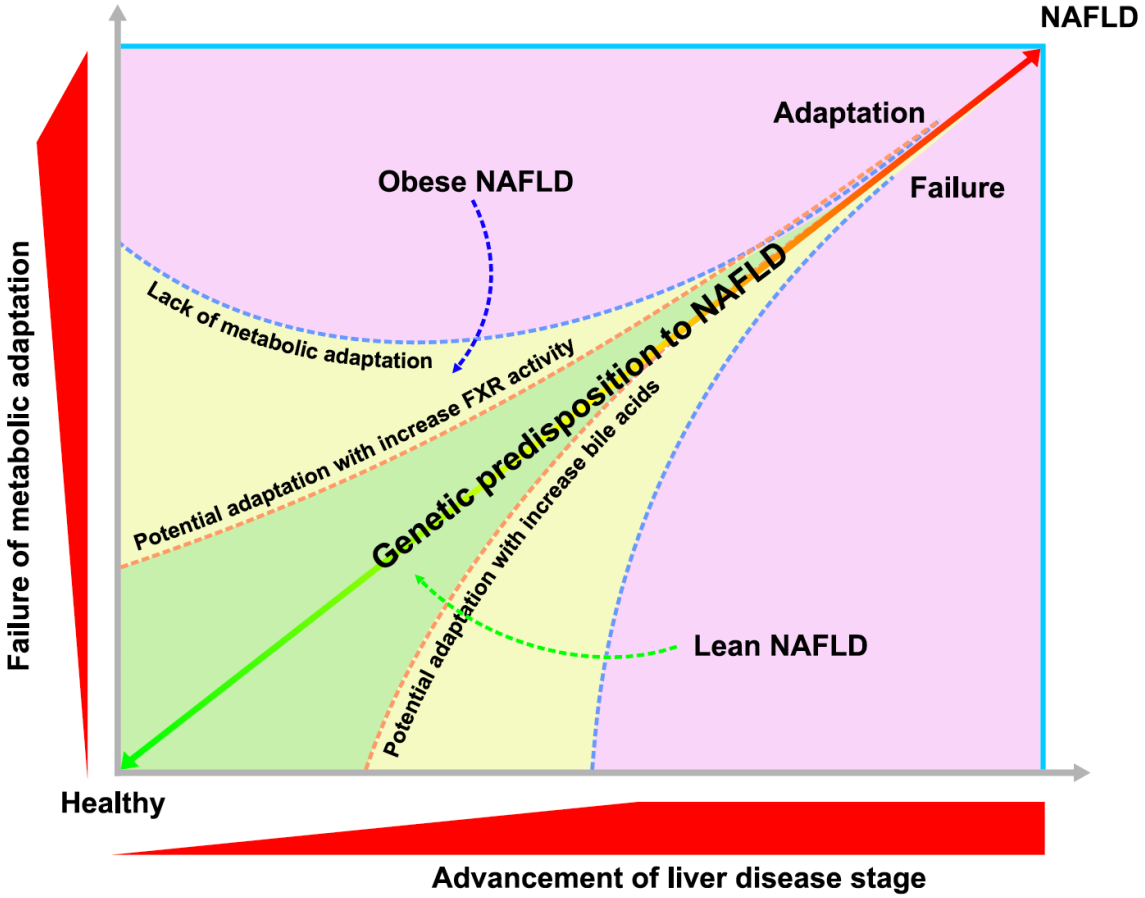


Figure 47. The role of metabolic adaptation in lean NAFLD

Schematic representation of differences in metabolic adaptation between individuals with lean and obese NAFLD. In obese patients there is relatively poor metabolic adaptation resulting in adiposity and the development of liver disease. In contrast, among lean NAFLD patients there is partial metabolic adaptation at least in the early stages of the disease. In the example shown, this is through increased bile acid production and FXR activity (other mechanisms may also be operative). This results in an "obesity resistant" phenotype" which appears to be lost as the disease progresses. Abbreviations: NAFLD – Non-alcoholic fatty liver disease; FXR – Farsenoid X receptor.

7.3 CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

In this thesis we presented data on the complex pathophysiology of lean NAFLD, a distinct subset of patients with poor metabolic health but good metabolic adaptation. In summary, lean and non-lean NAFLD represents one of the best examples of disease heterogeneity and the wide spectrum of fatty liver disease. An obvious implication is that future clinical trials should consider stratifying patients into lean and non-lean as the subgroups have a different underlying patho-biology and drivers, and likely differences in outcome. In addition, given the importance of metabolic health to NAFLD pathophysiology, classification of patients based on their metabolic health status warrants further attention.

Future studies on lean NAFLD should be directed at longitudinal follow-up of lean NAFLD patients to better understand its underlying pathogenesis and what triggers the loss of metabolic adaptation as the disease progresses into more advanced fibrosis. This should incorporate an understanding of lifestyle factors such as detailed dietary histories as well as information on physical activity. Measurements of other non-bile acid mediated pathway of the FXR activity, such as through the actions of other factors such as Takeda G-protein coupled receptor 5 (TGR5), Glucagon like peptide 1 (GLP1) or Peroxisome proliferator-activated receptor gamma (PPAR gamma) activity on liver histology and metabolism would also be of benefit to complete our understanding of the mechanism for which increased FXR acitivity protects against liver damage, especially in early stages of the lean NAFLD. Furthermore, measurement of faecal bile acids in addition to serum bile acids would benefit to further understand bile acid metabolism in these patients.

Another potential source for improvement in our understanding would be to increase the number of samples analysed for the gut microbiota profile and metabolomics analysis in lean and non-lean patients as well as in lean healthy controls in order to increase the power of the analysis.

Lastly, a potential application of our results would be to implement an FXR based treatment in the mouse models to determine if there is a role for this in the management of lean patients. Another potential application would be to use the bile acid metabolic signatures for the development of an algorithm to prognosticate in lean NAFLD patients to identify those with poorer metabolic adaptation who are at risk of rapid liver disease progression.

7.4 CONCLUSIONS

In conclusion, lean NAFLD presents as a unique sub-phenotype of patients with fatty liver disease. Metabolic health status plays a major role in the development of NAFLD and among lean individuals with the disease, their genetic, epigenetic, gut microbiota and bile acid profiles, enterohepatic circulation and lifestyle factors explain their phenotype despite a normal BMI. The distinct and better adaptation of lean patients allows them to respond to adverse metabolic inputs to maintain body weight despite an increase in cardiometabolic risk. Whether or not this partial metabolic adaptation is preserved in the long run and what triggers the switch to maladaptation with disease progression remains to be elucidated.

CHAPTER EIGHT

REFERENCES

8 REFERENCES

1. Asrani, S.K., et al., *Burden of liver diseases in the world.* J Hepatol, 2019. **70**(1): p. 151-171.
2. Lozano, R., et al., *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2095-128.
3. Sayiner, M., et al., *Assessment of health utilities and quality of life in patients with non-alcoholic fatty liver disease.* BMJ Open Gastroenterol, 2016. **3**(1): p. e000106.
4. Stepanova, M., et al., *Direct and Indirect Economic Burden of Chronic Liver Disease in the United States.* Clin Gastroenterol Hepatol, 2017. **15**(5): p. 759-766.e5.
5. Younossi, Z.M., et al., *The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe.* Hepatology, 2016. **64**(5): p. 1577-1586.
6. Bellentani, S., *The epidemiology of non-alcoholic fatty liver disease.* Liver Int, 2017. **37 Suppl 1**: p. 81-84.
7. Le, M.H., et al., *Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States.* PLoS One, 2017. **12**(3): p. e0173499.
8. Chalasani, N., et al., *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, 2012. **142**(7): p. 1592-609.
9. Matheson, E.M., D.E. King, and C.J. Everett, *Healthy lifestyle habits and mortality in overweight and obese individuals.* J Am Board Fam Med, 2012. **25**(1): p. 9-15.
10. Goday, A., et al., *Prevalence and clinical characteristics of metabolically healthy obese individuals and other obese/non-obese metabolic phenotypes in a working population: results from the Icaria study.* BMC Public Health, 2016. **16**: p. 248.
11. Hallal, P.C., et al., *Global physical activity levels: surveillance progress, pitfalls, and prospects.* The Lancet, 2012. **380**(9838): p. 247-257.
12. Guthold, R., et al., *Worldwide trends in insufficient physical activity from 2001 to 2016: a pooled analysis of 358 population-based surveys with 1·9 million participants.* The Lancet Global Health, 2018. **6**(10): p. e1077-e1086.
13. Smith, S.R., et al., *Concurrent physical activity increases fat oxidation during the shift to a high-fat diet.* Am J Clin Nutr, 2000. **72**(1): p. 131-8.
14. Pujia, A., et al., *Individuals with Metabolically Healthy Overweight/Obesity Have Higher Fat Utilization than Metabolically Unhealthy Individuals.* Nutrients, 2016. **8**(1).
15. Koren, D. and E.M. Taveras, *Association of sleep disturbances with obesity, insulin resistance and the metabolic syndrome.* Metabolism, 2018. **84**: p. 67-75.
16. Li, C., et al., *Lean non-alcoholic fatty liver disease patients had comparable total caloric, carbohydrate, protein, fat, iron, sleep duration and overtime work as obese non-alcoholic fatty liver disease patients.* J Gastroenterol Hepatol, 2019. **34**(1): p. 256-262.
17. Turnbaugh, P.J., et al., *The human microbiome project.* Nature, 2007. **449**(7164): p. 804-10.
18. Ridlon, J.M., D.J. Kang, and P.B. Hylemon, *Bile salt biotransformations by human intestinal bacteria.* J Lipid Res, 2006. **47**(2): p. 241-59.
19. Jiao, N., et al., *Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD.* Gut, 2017.
20. Wahlstrom, A., et al., *Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism.* Cell Metab, 2016. **24**(1): p. 41-50.
21. Yokota, A., et al., *Is bile acid a determinant of the gut microbiota on a high-fat diet?* Gut Microbes, 2012. **3**(5): p. 455-9.
22. Wieland, A., et al., *Systematic review: microbial dysbiosis and nonalcoholic fatty liver disease.* Aliment Pharmacol Ther, 2015. **42**(9): p. 1051-63.

23. Wu, G.D., et al., *Linking long-term dietary patterns with gut microbial enterotypes.* Science, 2011. **334**(6052): p. 105-8.
24. Carmody, R.N., *Diet dominates host genotype in shaping the murine gut microbiota.* Cell Host Microbe, 2015. **17**: p. 72-84.
25. De Filippo, C., et al., *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14691-6.
26. Musso, G., et al., *Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis.* Hepatology, 2003. **37**(4): p. 909-16.
27. Yasutake, K., et al., *Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: The significance of dietary cholesterol.* Scandinavian Journal of Gastroenterology, 2009. **44**(4): p. 471-477.
28. Enjoji, M., et al., *Nutrition and nonalcoholic Fatty liver disease: the significance of cholesterol.* Int J Hepatol, 2012. **2012**: p. 925807.
29. Wang, B., et al., *Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver Disease.* Sci Rep, 2016. **6**: p. 32002.
30. Perez, M.J. and O. Briz, *Bile-acid-induced cell injury and protection.* World J Gastroenterol, 2009. **15**(14): p. 1677-89.
31. Arab, J.P., et al., *Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives.* Hepatology, 2017. **65**(1): p. 350-362.
32. Khalid, Q., I. Bailey, and V.B. Patel, *Non-Alcoholic Fatty Liver Disease: The Effect of Bile Acids and Farnesoid X Receptor Agonists on Pathophysiology and Treatment.* Liver Research - Open Journal, 2015. **1**(2): p. 32-40.
33. Chavez-Talavera, O., et al., *Bile Acid Control of Metabolism and Inflammation in Obesity, Type 2 Diabetes, Dyslipidemia, and Nonalcoholic Fatty Liver Disease.* Gastroenterology, 2017. **152**(7): p. 1679-1694 e3.
34. Xie, G., et al., *Profiling of serum bile acids in a healthy Chinese population using UPLC-MS/MS.* J Proteome Res, 2015. **14**(2): p. 850-9.
35. Ioannou, G.N., et al., *Association between dietary nutrient composition and the incidence of cirrhosis or liver cancer in the United States population.* Hepatology, 2009. **50**(1): p. 175-84.
36. Puri, P., et al., *A lipidomic analysis of nonalcoholic fatty liver disease.* Hepatology, 2007. **46**(4): p. 1081-90.
37. Simonen, M., et al., *Desmosterol in human nonalcoholic steatohepatitis.* Hepatology, 2013. **58**(3): p. 976-82.
38. Min, H.K., et al., *Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic Fatty liver disease.* Cell Metab, 2012. **15**(5): p. 665-74.
39. Van Rooyen, D.M., et al., *Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis.* Gastroenterology, 2011. **141**(4): p. 1393-403, 1403 e1-5.
40. Kalhan, S.C., et al., *Plasma metabolomic profile in nonalcoholic fatty liver disease.* Metabolism, 2011. **60**(3): p. 404-13.
41. Ferslew, B.C., et al., *Altered Bile Acid Metabolome in Patients with Nonalcoholic Steatohepatitis.* Dig Dis Sci, 2015. **60**(11): p. 3318-28.
42. Puri, P., et al., *The presence and severity of nonalcoholic steatohepatitis is associated with specific changes in circulating bile acids.* Hepatology, 2017.
43. Legry, V., et al., *Bile acid alterations are associated with insulin resistance, but not with NASH in obese subjects.* J Clin Endocrinol Metab, 2017.
44. Patti, M.E., et al., *Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism.* Obesity (Silver Spring), 2009. **17**(9): p. 1671-7.

45. Dutia, R., et al., *Temporal changes in bile acid levels and 12alpha-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes.* Int J Obes (Lond), 2015. **39**(5): p. 806-13.
46. Werling, M., et al., *Enhanced fasting and post-prandial plasma bile acid responses after Roux-en-Y gastric bypass surgery.* Scand J Gastroenterol, 2013. **48**(11): p. 1257-64.
47. Kohli, R., et al., *Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids.* J Clin Endocrinol Metab, 2013. **98**(4): p. E708-12.
48. Sachdev, S., et al., *FGF 19 and Bile Acids Increase Following Roux-en-Y Gastric Bypass but Not After Medical Management in Patients with Type 2 Diabetes.* Obes Surg, 2016. **26**(5): p. 957-65.
49. Pierre, J.F., et al., *Activation of bile acid signaling improves metabolic phenotypes in high-fat diet-induced obese mice.* Am J Physiol Gastrointest Liver Physiol, 2016. **311**(2): p. G286-304.
50. Chambers, A.P., et al., *Weight-independent changes in blood glucose homeostasis after gastric bypass or vertical sleeve gastrectomy in rats.* Gastroenterology, 2011. **141**(3): p. 950-8.
51. Kohli, R., et al., *Bile Acid Signaling: Mechanism for Bariatric Surgery, Cure for NASH?* Dig Dis, 2015. **33**(3): p. 440-6.
52. Pournaras, D.J., et al., *The Role of Bile After Roux-en-Y Gastric Bypass in Promoting Weight Loss and Improving Glycaemic Control.* Endocrinology, 2012. **153**(8): p. 3613-3619.
53. Mudaliar, S., et al., *Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease.* Gastroenterology, 2013. **145**(3): p. 574-82.e1.
54. Schumacher, J.D., et al., *Direct and Indirect Effects of Fibroblast Growth Factor (FGF) 15 and FGF19 on Liver Fibrosis Development.* Hepatology, 2020. **71**(2): p. 670-685.
55. Neuschwander-Tetri, B.A., et al., *Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial.* Lancet, 2015. **385**(9972): p. 956-65.
56. Harrison, S.A., et al., *NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial.* The Lancet, 2018. **391**(10126): p. 1174-1185.
57. Pierre, J.F., et al., *Bile Diversion Improves Metabolic Phenotype Dependent on Farnesoid X Receptor (FXR).* Obesity (Silver Spring), 2019. **27**(5): p. 803-812.
58. Albaugh, V.L., et al., *Role of Bile Acids and GLP-1 in Mediating the Metabolic Improvements of Bariatric Surgery.* Gastroenterology, 2019. **156**(4): p. 1041-1051 e4.
59. Pathak, P., et al., *Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism.* Hepatology, 2018. **68**(4): p. 1574-1588.
60. Trabelsi, M.S., et al., *Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells.* Nat Commun, 2015. **6**: p. 7629.
61. Browning, M.G., et al., *Changes in Bile Acid Metabolism, Transport, and Signaling as Central Drivers for Metabolic Improvements After Bariatric Surgery.* Curr Obes Rep, 2019.
62. Cole, A.J., et al., *The Influence of Bariatric Surgery on Serum Bile Acids in Humans and Potential Metabolic and Hormonal Implications: a Systematic Review.* Curr Obes Rep, 2015. **4**(4): p. 441-50.
63. Broeders, E.P., *The bile acid chenodeoxycholic acid increases human brown adipose tissue activity.* Cell Metab., 2015. **22**: p. 418-426.
64. Watanabe, M., et al., *Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation.* Nature, 2006. **439**(7075): p. 484-9.
65. Abdelkarim, M., et al., *The Farnesoid X Receptor Regulates Adipocyte Differentiation and Function by Promoting Peroxisome Proliferator-activated Receptor-γ and Interfering with the Wnt/β-Catenin Pathways.* Journal of Biological Chemistry, 2010. **285**(47): p. 36759-36767.

66. Schwimmer, J.B., et al., *Heritability of nonalcoholic fatty liver disease.* Gastroenterology, 2009. **136**(5): p. 1585-92.
67. Eslam, M., L. Valenti, and S. Romeo, *Genetics and epigenetics of NAFLD and NASH: Clinical impact.* J Hepatol, 2018. **68**(2): p. 268-279.
68. Younossi, Z.M., et al., *Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes.* Hepatology, 2016. **64**(1): p. 73-84.
69. Guerrero, R., et al., *Ethnic differences in hepatic steatosis: an insulin resistance paradox?* Hepatology, 2009. **49**(3): p. 791-801.
70. Sookoian, S. and C.J. Pirola, *Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease.* Hepatology, 2011. **53**(6): p. 1883-94.
71. Romeo, S., et al., *Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease.* Nat Genet, 2008. **40**(12): p. 1461-5.
72. Wei, J.L., et al., *Prevalence and Severity of Nonalcoholic Fatty Liver Disease in Non-Obese Patients: A Population Study Using Proton-Magnetic Resonance Spectroscopy.* Am J Gastroenterol, 2015. **110**(9): p. 1306-14; quiz 1315.
73. Fracanzani, A.L., et al., *Liver and Cardiovascular Damage in Patients With Lean Nonalcoholic Fatty Liver Disease, and Association With Visceral Obesity.* Clin Gastroenterol Hepatol, 2017. **15**(10): p. 1604-1611 e1.
74. Eslam, M., et al., *Diverse impacts of the rs58542926 E167K variant in TM6SF2 on viral and metabolic liver disease phenotypes.* Hepatology, 2016. **64**(1): p. 34-46.
75. Kozlitina, J., et al., *Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease.* Nat Genet, 2014. **46**(4): p. 352-6.
76. Pang, J., et al., *Significant positive association of endotoxemia with histological severity in 237 patients with non-alcoholic fatty liver disease.* Aliment Pharmacol Ther, 2017. **46**(2): p. 175-182.
77. Speliotes, E.K., et al., *Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits.* PLoS Genet, 2011. **7**(3): p. e1001324.
78. Zain, S.M., Z. Mohamed, and R. Mohamed, *Common variant in the glucokinase regulatory gene rs780094 and risk of nonalcoholic fatty liver disease: a meta-analysis.* J Gastroenterol Hepatol, 2015. **30**(1): p. 21-7.
79. Manolio, T.A., et al., *Finding the missing heritability of complex diseases.* Nature, 2009. **461**(7265): p. 747-53.
80. Mancina, R.M., et al., *The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent.* Gastroenterology, 2016. **150**(5): p. 1219-1230 e6.
81. Zeybel, M., et al., *Differential DNA methylation of genes involved in fibrosis progression in non-alcoholic fatty liver disease and alcoholic liver disease.* Clin Epigenetics, 2015. **7**: p. 25.
82. Leung, J.C., et al., *Histological severity and clinical outcomes of nonalcoholic fatty liver disease in nonobese patients.* Hepatology, 2017. **65**(1): p. 54-64.
83. Hardy, T. and D.A. Mann, *Epigenetics in liver disease: from biology to therapeutics.* Gut, 2016.
84. Hardy, T., et al., *Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease.* Gut, 2016.
85. Eslam, M. and J. George, *Genetic and epigenetic mechanisms of NASH.* Hepatol Int, 2016. **10**(3): p. 394-406.
86. Bouchard, C., et al., *The response to long-term overfeeding in identical twins.* N Engl J Med, 1990. **322**(21): p. 1477-82.
87. Schleinitz, D., et al., *The genetics of fat distribution.* Diabetologia, 2014. **57**(7): p. 1276-86.
88. Iacobini, C., et al., *Metabolically healthy versus metabolically unhealthy obesity*. Metabolism, 2019. **92**: p. 51-60.

89. Chambers, J.C., et al., *Common genetic variation near MC4R is associated with waist circumference and insulin resistance.* Nat Genet, 2008. **40**(6): p. 716-8.
90. Heid, I.M., et al., *Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution.* Nat Genet, 2010. **42**(11): p. 949-60.
91. Kilpelainen, T.O., et al., *Genetic variation near IRS1 associates with reduced adiposity and an impaired metabolic profile.* Nat Genet, 2011. **43**(8): p. 753-60.
92. Yaghootkar, H., et al., *Genetic Evidence for a Link Between Favorable Adiposity and Lower Risk of Type 2 Diabetes, Hypertension, and Heart Disease.* Diabetes, 2016. **65**(8): p. 2448-60.
93. Yaghootkar, H., et al., *Genetic evidence for a normal-weight "metabolically obese" phenotype linking insulin resistance, hypertension, coronary artery disease, and type 2 diabetes.* Diabetes, 2014. **63**(12): p. 4369-77.
94. Bergman, Y. and H. Cedar, *DNA methylation dynamics in health and disease.* Nat Struct Mol Biol, 2013. **20**(3): p. 274-81.
95. Bian, E.B., et al., *DNA methylation: new therapeutic implications for hepatic fibrosis.* Cell Signal, 2013. **25**(1): p. 355-8.
96. Mann, D.A., *Epigenetics in liver disease.* Hepatology, 2014. **60**(4): p. 1418-25.
97. Bernstein, B.E., A. Meissner, and E.S. Lander, *The mammalian epigenome.* Cell, 2007. **128**(4): p. 669-81.
98. Zaratiegui, M., D.V. Irvine, and R.A. Martienssen, *Noncoding RNAs and gene silencing.* Cell, 2007. **128**(4): p. 763-76.
99. Feinberg, A.P. and B. Vogelstein, *Hypomethylation distinguishes genes of some human cancers from their normal counterparts.* Nature, 1983. **301**(5895): p. 89-92.
100. Esteller, M., *Epigenetics in Cancer.* N Engl J Med, 2008. **358**: p. 1148 - 1159.
101. Sookoian, S., et al., *Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter.* Hepatology, 2010. **52**(6): p. 1992-2000.
102. Li, M., et al., *Co-methylated genes in different adipose depots of pig are associated with metabolic, inflammatory and immune processes.* Int J Biol Sci, 2012. **8**(6): p. 831-7.
103. Marchi, M., et al., *Human leptin tissue distribution, but not weight loss-dependent change in expression, is associated with methylation of its promoter.* Epigenetics, 2011. **6**(10): p. 1198-206.
104. Huang, R.C., et al., *DNA methylation of the IGF2/H19 imprinting control region and adiposity distribution in young adults.* Clin Epigenetics, 2012. **4**(1): p. 21.
105. Plagemann, A., *'Fetal programming' and 'functional teratogenesis': on epigenetic mechanisms and prevention of perinatally acquired lasting health risks.* J Perinat Med, 2004. **32**(4): p. 297-305.
106. Blumfield, M.L., et al., *Dietary balance during pregnancy is associated with fetal adiposity and fat distribution.* Am J Clin Nutr, 2012. **96**(5): p. 1032-41.
107. Bays, H. and W. Scinta, *Adiposopathy and epigenetics: an introduction to obesity as a transgenerational disease.* Curr Med Res Opin, 2015. **31**(11): p. 2059-69.
108. Bedossa, P., *Histological Assessment of NAFLD.* Dig Dis Sci, 2016. **61**(5): p. 1348-55.
109. Kleiner, D.E., et al., *Design and validation of a histological scoring system for nonalcoholic fatty liver disease.* Hepatology, 2005. **41**(6): p. 1313-21.
110. van der Poorten, D., et al., *Hepatic fat loss in advanced nonalcoholic steatohepatitis: are alterations in serum adiponectin the cause?* Hepatology, 2013. **57**(6): p. 2180-8.
111. Xu, A., et al., *The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice.* Journal of Clinical Investigation, 2003. **112**(1): p. 91-100.
112. Angulo, P., et al., *Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease.* Gastroenterology, 2015. **149**(2): p. 389-97 e10.

113. Sookoian, S. and C.J. Pirola, *Systematic review with meta-analysis: the significance of histological disease severity in lean patients with nonalcoholic fatty liver disease.* Aliment Pharmacol Ther, 2018. **47**(1): p. 16-25.
114. Hagstrom, H., et al., *Risk for development of severe liver disease in lean patients with nonalcoholic fatty liver disease: A long-term follow-up study.* Hepatol Commun, 2018. **2**(1): p. 48-57.
115. Dela Cruz, A.C.e.a., *Characteristics and long-term prognosis of lean patients with nonalcoholic fatty liver disease.* Gastroenterology, 2014. **146**(S-909).
116. Lorenzo, C., et al., *The National Cholesterol Education Program - Adult Treatment Panel III, International Diabetes Federation, and World Health Organization definitions of the metabolic syndrome as predictors of incident cardiovascular disease and diabetes.* Diabetes Care, 2007. **30**(1): p. 8-13.
117. Wildman, R.P., et al., *The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004).* Arch Intern Med, 2008. **168**(15): p. 1617-24.
118. Smith, G.I., B. Mittendorfer, and S. Klein, *Metabolically healthy obesity: facts and fantasies.* Journal of Clinical Investigation, 2019. **129**(10): p. 3978-3989.
119. Ampuero, J., et al., *The effects of metabolic status on non-alcoholic fatty liver disease-related outcomes, beyond the presence of obesity.* Aliment Pharmacol Ther, 2018. **48**(11-12): p. 1260-1270.
120. Gutierrez-Grobe, Y., et al., *Less liver fibrosis in metabolically healthy compared with metabolically unhealthy obese patients with non-alcoholic fatty liver disease.* Diabetes Metab, 2017. **43**(4): p. 332-337.
121. Sung, K.C., et al., *Metabolically healthy obese subjects are at risk of fatty liver but not of pre-clinical atherosclerosis.* Nutr Metab Cardiovasc Dis, 2014. **24**(3): p. 256-62.
122. Lee, M.K., et al., *Metabolic Health Is More Important than Obesity in the Development of Nonalcoholic Fatty Liver Disease: A 4-Year Retrospective Study.* Endocrinol Metab (Seoul), 2015. **30**(4): p. 522-30.
123. Kramer, C.K., B. Zinman, and R. Retnakaran, *Are metabolically healthy overweight and obesity benign conditions?: A systematic review and meta-analysis.* Ann Intern Med, 2013. **159**(11): p. 758-69.
124. Hwang, Y.C., H.Y. Ahn, and C.Y. Park, *Association Between Nonalcoholic Fatty Liver Disease and Future Deterioration of Metabolic Health: A Cohort Study.* Obesity (Silver Spring), 2019. **27**(8): p. 1360-1366.
125. Bays, H.E., *Adiposopathy is "sick fat" a cardiovascular disease?* J Am Coll Cardiol, 2011. **57**(25): p. 2461-73.
126. Bays, H.E., et al., *Is adiposopathy (sick fat) an endocrine disease?* Int J Clin Pract, 2008. **62**(10): p. 1474-83.
127. Lim, S. and J.B. Meigs, *Ectopic fat and cardiometabolic and vascular risk.* Int J Cardiol, 2013. **169**(3): p. 166-76.
128. Manolopoulos, K.N., F. Karpe, and K.N. Frayn, *Gluteofemoral body fat as a determinant of metabolic health.* Int J Obes (Lond), 2010. **34**(6): p. 949-59.
129. Goodpaster, B.H., et al., *Obesity, regional body fat distribution, and the metabolic syndrome in older men and women.* Arch Intern Med, 2005. **165**(7): p. 777-83.
130. Lee, J.J., et al., *Upper Body Subcutaneous Fat Is Associated with Cardiometabolic Risk Factors.* Am J Med, 2017. **130**(8): p. 958-966 e1.
131. Ebbert, J.O. and M.D. Jensen, *Fat depots, free fatty acids, and dyslipidemia.* Nutrients, 2013. **5**(2): p. 498-508.
132. Kim, J.Y., et al., *Obesity-associated improvements in metabolic profile through expansion of adipose tissue.* J Clin Invest, 2007. **117**(9): p. 2621-37.
133. McLaughlin, T., et al., *Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity.* J Clin Endocrinol Metab, 2011. **96**(11): p. E1756-60.

134. Despres, J.P. and I. Lemieux, *Abdominal obesity and metabolic syndrome.* Nature, 2006. **444**(7121): p. 881-7.
135. O'Connell, J., et al., *The relationship of omental and subcutaneous adipocyte size to metabolic disease in severe obesity.* PLoS One, 2010. **5**(4): p. e9997.
136. Klein, S., et al., *Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease.* N Engl J Med, 2004. **350**(25): p. 2549-57.
137. Tran, T.T., et al., *Beneficial effects of subcutaneous fat transplantation on metabolism.* Cell Metab, 2008. **7**(5): p. 410-20.
138. Johannsen, D.L., et al., *Metabolic adaptation is not observed after 8 weeks of overfeeding but energy expenditure variability is associated with weight recovery.* Am J Clin Nutr, 2019.
139. Oh, K.J., et al., *Metabolic Adaptation in Obesity and Type II Diabetes: Myokines, Adipokines and Hepatokines.* Int J Mol Sci, 2016. **18**(1).
140. Diaz, E.O., et al., *Metabolic response to experimental overfeeding in lean and overweight healthy volunteers.* Am J Clin Nutr, 1992. **56**(4): p. 641-55.
141. Leibel, R.L., M. Rosenbaum, and J. Hirsch, *Changes in energy expenditure resulting from altered body weight.* N Engl J Med, 1995. **332**(10): p. 621-8.
142. Buzzetti, E., M. Pinzani, and E.A. Tsochatzis, *The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD).* Metabolism, 2016. **65**(8): p. 1038-48.
143. Younes, R. and E. Bugianesi, *NASH in Lean Individuals.* Semin Liver Dis, 2019. **39**(1): p. 86-95.
144. Ng, M., et al., *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, 2014. **384**(9945): p. 766-781.
145. Li, L., et al., *Obesity is an independent risk factor for non-alcoholic fatty liver disease: evidence from a meta-analysis of 21 cohort studies.* Obes Rev, 2016. **17**(6): p. 510-9.
146. Das, K. and A. Chowdhury, *Lean NASH: distinctiveness and clinical implication.* Hepatol Int, 2013. **7 Suppl 2**: p. 806-13.
147. Younossi, Z.M., et al., *Nonalcoholic fatty liver disease in lean individuals in the United States.* Medicine (Baltimore), 2012. **91**(6): p. 319-27.
148. Kim, D. and W.R. Kim, *Nonobese Fatty Liver Disease.* Clin Gastroenterol Hepatol, 2017. **15**(4): p. 474-485.
149. Sookoian, S. and C.J. Pirola, *Systematic review with meta-analysis: risk factors for non-alcoholic fatty liver disease suggest a shared altered metabolic and cardiovascular profile between lean and obese patients.* Aliment Pharmacol Ther, 2017. **46**(2): p. 85-95.
150. Stefan, N., F. Schick, and H.U. Haring, *Causes, Characteristics, and Consequences of Metabolically Unhealthy Normal Weight in Humans.* Cell Metab, 2017. **26**(2): p. 292-300.
151. Kim, H.J., et al., *Metabolic significance of nonalcoholic fatty liver disease in nonobese, nondiabetic adults.* Arch Intern Med, 2004. **164**(19): p. 2169-75.
152. Das, K., et al., *Nonobese population in a developing country has a high prevalence of nonalcoholic fatty liver and significant liver disease.* Hepatology, 2010. **51**(5): p. 1593-602.
153. Denkmayr, L., et al., *Lean Patients with Non-Alcoholic Fatty Liver Disease Have a Severe Histological Phenotype Similar to Obese Patients.* J Clin Med, 2018. **7**(12).
154. Wang, Q., et al., *Non-obese histologically confirmed NASH patients with abnormal liver biochemistry have more advanced fibrosis.* Hepatology International, 2019. **13**(6): p. 766-776.
155. Gonzalez-Cantero, J., et al., *Insulin resistance in lean and overweight non-diabetic Caucasian adults: Study of its relationship with liver triglyceride content, waist circumference and BMI.* PLoS One, 2018. **13**(2): p. e0192663.
156. Chen, F., et al., *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation.* Hepatology, 2019.
157. Fracanzani, A.L., et al., *Risk of nonalcoholic steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease and low visceral adiposity.* J Hepatol, 2011. **54**(6): p. 1244-9.

158. George, A.M., A.G. Jacob, and L. Fogelfeld, *Lean diabetes mellitus: An emerging entity in the era of obesity.* World J Diabetes, 2015. **6**(4): p. 613-20.
159. Dobson, R., et al., *Metabolically healthy and unhealthy obesity: differential effects on myocardial function according to metabolic syndrome, rather than obesity.* Int J Obes (Lond), 2016. **40**(1): p. 153-61.
160. Chen, C.H., et al., *Prevalence and risk factors of nonalcoholic fatty liver disease in an adult population of taiwan: metabolic significance of nonalcoholic fatty liver disease in nonobese adults.* J Clin Gastroenterol, 2006. **40**(8): p. 745-52.
161. Margariti, E., et al., *Non-alcoholic fatty liver disease may develop in individuals with normal body mass index.* Ann Gastroenterol, 2012. **25**(1): p. 45-51.
162. Bhat, G., et al., *Insulin resistance and metabolic syndrome in nonobese Indian patients with non-alcoholic fatty liver disease.* Trop Gastroenterol, 2013. **34**(1): p. 18-24.
163. Kumar, R., et al., *Clinicopathological characteristics and metabolic profiles of non-alcoholic fatty liver disease in Indian patients with normal body mass index: Do they differ from obese or overweight non-alcoholic fatty liver disease?* Indian J Endocrinol Metab, 2013. **17**(4): p. 665-71.
164. Alam, S., et al., *Clinical, anthropometric, biochemical, and histological characteristics of nonobese nonalcoholic fatty liver disease patients of Bangladesh.* Indian J Gastroenterol, 2014. **33**(5): p. 452-7.
165. Feng, R.N., et al., *Lean-non-alcoholic fatty liver disease increases risk for metabolic disorders in a normal weight Chinese population.* World J Gastroenterol, 2014. **20**(47): p. 17932-40.
166. Vendhan, R., et al., *Comparison of characteristics between nonobese and overweight/obese subjects with nonalcoholic fatty liver disease in a South Indian population.* Diabetes Technol Ther, 2014. **16**(1): p. 48-55.
167. Nishioji, K., et al., *Prevalence of and risk factors for non-alcoholic fatty liver disease in a non-obese Japanese population, 2011-2012.* J Gastroenterol, 2015. **50**(1): p. 95-108.
168. Cho, H.C., *Prevalence and Factors Associated with Nonalcoholic Fatty Liver Disease in a Nonobese Korean Population.* Gut Liver, 2016. **10**(1): p. 117-25.
169. Feldman, A., et al., *Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver.* Am J Gastroenterol, 2017. **112**(1): p. 102-110.
170. Tobari, M., et al., *Characteristics of non-alcoholic steatohepatitis among lean patients in Japan: Not uncommon and not always benign.* J Gastroenterol Hepatol, 2018.
171. Niriella, M.A., et al., *Lean non-alcoholic fatty liver disease (lean NAFLD): characteristics, metabolic outcomes and risk factors from a 7-year prospective, community cohort study from Sri Lanka.* Hepatol Int, 2019. **13**(3): p. 314-322.
172. Yilmaz, Y., et al., *Growing burden of nonalcoholic fatty liver disease in Turkey: A single-center experience.* Turk J Gastroenterol, 2019. **30**(10): p. 892-898.
173. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-9.
174. Eslam, M., et al., *Use of HOMA-IR in hepatitis C.* J Viral Hepat, 2011. **18**(10): p. 675-84.
175. Kleiner, D.E., et al., *Design and validation of a histological scoring system for nonalcoholic fatty liver disease.* Hepatology, 2005. **41**(6): p. 1313-21.
176. Eslam, M., et al., *Interferon-lambda rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease.* Nature Communications, 2015. **6**.
177. Eslam, M., et al., *FibroGENE: A gene-based model for staging liver fibrosis.* Journal of Hepatology, 2016. **64**(2): p. 390-398.
178. Eslam, M., et al., *Diverse Impacts of the rs58542926 E167K Variant in TM6SF2 on Viral and Metabolic Liver Disease Phenotypes.* Hepatology, 2016. **64**(1): p. 34-46.
179. Kazankov, K., et al., *The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease.* Liver International, 2016. **36**(10): p. 1549-1557.

180. Kechagias, S., et al., *Phosphatidylethanol Compared with Other Blood Tests as a Biomarker of Moderate Alcohol Consumption in Healthy Volunteers: A Prospective Randomized Study.* Alcohol Alcohol, 2015. **50**(4): p. 399-406.
181. Choo, J.M., L.E.X. Leong, and G.B. Rogers, *Sample storage conditions significantly influence faecal microbiome profiles.* Scientific Reports, 2015. **5**.
182. Brunt, E.M., et al., *Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings.* Hepatology, 2011. **53**(3): p. 810-20.
183. Duwaerts, C.C. and J.J. Maher, *Mechanisms of Liver Injury in Non-Alcoholic Steatohepatitis.* Curr Hepatol Rep, 2014. **13**(2): p. 119-129.
184. Cani, P.D., et al., *Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet-Induced Obesity and Diabetes in Mice.* Diabetes, 2008. **57**(6): p. 1470-1481.
185. Eckel, N., et al., *Characterization of metabolically unhealthy normal-weight individuals: Risk factors and their associations with type 2 diabetes.* Metabolism, 2015. **64**(8): p. 862-71.
186. Del Chierico, F., et al., *Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach.* Hepatology, 2017. **65**(2): p. 451-464.
187. Del Chierico, F., et al., *Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach.* Hepatology, 2017. **65**(2): p. 451-464.
188. Zhou, D., et al., *Sodium butyrate attenuates high-fat diet-induced steatohepatitis in mice by improving gut microbiota and gastrointestinal barrier.* World J Gastroenterol, 2017. **23**(1): p. 60-75.
189. Ma, J., Q. Zhou, and H. Li, *Gut Microbiota and Nonalcoholic Fatty Liver Disease: Insights on Mechanisms and Therapy.* Nutrients, 2017. **9**(10).
190. Zhou, D. and J.G. Fan, *Microbial metabolites in non-alcoholic fatty liver disease.* World J Gastroenterol, 2019. **25**(17): p. 2019-2028.
191. Zhao, Z.H., et al., *Indole-3-propionic acid inhibits gut dysbiosis and endotoxin leakage to attenuate steatohepatitis in rats.* Exp Mol Med, 2019. **51**(9): p. 103.
192. Younossi, Z., et al., *Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention.* Nature Reviews Gastroenterology & Hepatology, 2018. **15**(1): p. 11-20.
193. Ruderman, N., et al., *The metabolically obese, normal-weight individual revisited.* Diabetes, 1998. **47**(5): p. 699-713.
194. Ding, C., Z.L. Chan, and F. Magkos, *Lean, but not healthy: the "metabolically obese, normal-weight' phenotype.* Current Opinion in Clinical Nutrition and Metabolic Care, 2016. **19**(6): p. 408-417.
195. Dela Cruz, A.C., et al., *Characteristics and Long-Term Prognosis of Lean Patients With Nonalcoholic Fatty Liver Disease.* Gastroenterology, 2014. **146**(5): p. S909-S909.
196. Ramirez-Perez, O., et al., *The Role of the Gut Microbiota in Bile Acid Metabolism.* Ann Hepatol, 2017. **16**(Suppl. 1: s3-105.): p. s15-s20.
197. Schnabl, B. and D.A. Brenner, *Interactions Between the Intestinal Microbiome and Liver Diseases.* Gastroenterology, 2014. **146**(6): p. 1513-1524.
198. Watanabe, M., et al., *Lowering Bile Acid Pool Size with a Synthetic Farnesoid X Receptor (FXR) Agonist Induces Obesity and Diabetes through Reduced Energy Expenditure.* Journal of Biological Chemistry, 2011. **286**(30): p. 26913-26920.
199. Broeders, E.P.M., et al., *The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity.* Cell Metabolism, 2015. **22**(3): p. 418-426.
200. Fu, L., et al., *Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes.* Endocrinology, 2004. **145**(6): p. 2594-603.
201. Fang, S., et al., *Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance.* Nature Medicine, 2015. **21**(2): p. 71-77.

202. Kakiyama, G., et al., *Modulation of the fecal bile acid profile by gut microbiota in cirrhosis.* Journal of Hepatology, 2013. **58**(5): p. 949-955.
203. Wahlstrom, A., et al., *Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism.* Cell Metabolism, 2016. **24**(1): p. 41-50.
204. Klass, D.M., et al., *Biliary lipids, cholesterol and bile synthesis: different adaptive mechanisms to dietary cholesterol in lean and obese subjects.* Aliment Pharmacol Ther, 2006. **23**(7): p. 895-905.
205. Chu, H., et al., *Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease.* Gut, 2018.
206. Martinez, I., et al., *Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters.* Appl Environ Microbiol, 2013. **79**(2): p. 516-24.
207. Zhao, L., et al., *A Glucagon-Like Peptide-1 Receptor Agonist Lowers Weight by Modulating the Structure of Gut Microbiota.* Front Endocrinol (Lausanne), 2018. **9**: p. 233.
208. Fan, Y., et al., *Hepatic Transmembrane 6 Superfamily Member 2 Regulates Cholesterol Metabolism in Mice.* Gastroenterology, 2016. **150**(5): p. 1208-1218.
209. Van Herck, M.A., L. Vonghia, and S.M. Francque, *Animal Models of Nonalcoholic Fatty Liver Disease-A Starter's Guide.* Nutrients, 2017. **9**(10).
210. Jacobs, A., et al., *An Overview of Mouse Models of Nonalcoholic Steatohepatitis: From Past to Present.* Curr Protoc Mouse Biol, 2016. **6**(2): p. 185-200.
211. Stephenson, K., et al., *Updates on Dietary Models of Nonalcoholic Fatty Liver Disease: Current Studies and Insights.* Gene Expr, 2018. **18**(1): p. 5-17.
212. Zietak, M., *Altered microbiota contributes to reduced diet-induced obesity upon cold exposure.* Cell Metab., 2016. **23**: p. 1216-1223.
213. Zhang, H., et al., *Human gut microbiota in obesity and after gastric bypass.* Proc Natl Acad Sci U S A, 2009. **106**(7): p. 2365-70.
214. McGlone, E.R., et al., *Bile acid mediated changes after bariatric surgery: what can we learn from mouse models?* Gastroenterology, 2019.
215. Mayo, R., et al., *Metabolomic-based noninvasive serum test to diagnose nonalcoholic steatohepatitis: Results from discovery and validation cohorts.* Hepatol Commun, 2018. **2**(7): p. 807-820.
216. Gitto, S., et al., *Study of the Serum Metabolomic Profile in Nonalcoholic Fatty Liver Disease: Research and Clinical Perspectives.* Metabolites, 2018. **8**(1).
217. Jensen, T., et al., *Fructose and sugar: A major mediator of non-alcoholic fatty liver disease.* J Hepatol, 2018. **68**(5): p. 1063-1075.
218. Francque, S.M., D. van der Graaff, and W.J. Kwanten, *Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanisms and implications.* J Hepatol, 2016. **65**(2): p. 425-43.
219. Pastore, A., et al., *Plasma levels of homocysteine and cysteine increased in pediatric NAFLD and strongly correlated with severity of liver damage.* Int J Mol Sci, 2014. **15**(11): p. 21202-14.
220. Hardwick, R.N., et al., *Variations in ATP-binding cassette transporter regulation during the progression of human nonalcoholic fatty liver disease.* Drug Metab Dispos, 2011. **39**(12): p. 2395-402.

CHAPTER NINE

APPENDIX

9 APPENDIX

9.1 PUBLICATIONS

9.1.1 Hepatology

Chen, F, Esmaili, S, Rogers, G, Bugianesi, E, Petta, S, Marchesini, G, Bayoumi, A, Metwally, M, Azardaryany, MK, Coulter, S, Choo, JM, Younes, R, Rosso, C, Liddle, C, Adams, LA, Craxi, A, George J, Eslam, M; *Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation;* accepted in Hepatology August 2019; DOI: 10.1002/hep.309

HEPATOLOGY

HEPATOLOGY, VOL. 0, NO. 0, 2020

Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation

Fei Chen,[1*] Saeed Esmaili ,[1,2*] Geraint B. Rogers,[3] Elisabetta Bugianesi,[4] Salvatore Petta,[5] Giulio Marchesini,[6] Ali Bayoumi,[1] Mayada Metwally,[1] Mahmoud Karimi Azardaryany,[1] Sally Coulter,[1] Jocelyn M. Choo,[3] Ramy Younes,[4] Chiara Rosso ,[4] Christopher Liddle,[1] Leon A. Adams,[7] Antonio Craxì,[5] Jacob George,[1] and Mohammed Eslam[1]

BACKGROUND AND AIMS: Nonalcoholic fatty liver disease (NAFLD) affects a quarter of the adult population. A significant subset of patients are lean, but their underlying pathophysiology is not well understood.

APPROACH AND RESULTS: We investigated the role of bile acids (BAs) and the gut microbiome in the pathogenesis of lean NAFLD. BA and fibroblast growth factor (FGF) 19 levels (a surrogate for intestinal farnesoid X receptor [FXR] activity), patatin-like phospholipase domain containing 3 (*PNPLA3*), and transmembrane 6 superfamily member 2 (*TM6SF2*) variants, and gut microbiota profiles in lean and nonlean NAFLD were investigated in a cohort of Caucasian patients with biopsy-proven NAFLD (n = 538), lean healthy controls (n = 30), and experimental murine models. Patients with lean NAFLD had a more favorable metabolic and histological profile compared with those with nonlean NAFLD (P < 0.05 for all). BA levels were significantly higher in NAFLD with advanced compared with earlier stages of liver fibrosis. Patients with lean NAFLD had higher serum secondary BA and FGF19 levels and reduced 7-alpha-hydroxy-4-cholesten-3-one (*C4*) levels (P < 0.05 for all). These differences were more profound in early compared with advanced stages of fibrosis (P < 0.05 for both). Lean patients demonstrated an altered gut microbiota profile. Similar findings were demonstrated in lean and nonlean murine models of NAFLD. Treating mice with an apical sodium-dependent BA transporter inhibitor (SC-435) resulted in marked increases in fgf15, a shift in the BA and microbiota profiles, and improved steatohepatitis in the lean model.

CONCLUSIONS: Differences in metabolic adaptation between patients with lean and nonlean NAFLD, at least in part, explain the pathophysiology and provide options for therapy. (Hepatology 2020;0:1-15).

Nonalcoholic fatty liver disease (NAFLD) affects approximately 20% to 30% of the world's population and is a leading cause for end-stage liver disease, cancer, and transplantation.[1] Despite this, the existence and clinical course of the entity known as "lean NAFLD" or "NAFLD in lean patients" has been the subject of intense debate and controversy. To many, "lean NAFLD" refers to individuals manifesting the disease in the context of a normal body mass index (BMI) but having excess visceral adiposity and insulin resistance as well as metabolic dysfunction that is typically observed in people with obesity,[2] the so-called metabolically obese normal-weight individual. The prevalence of lean NAFLD varies widely according to the criteria used for its definition but ranges from 5% to 45%.[3] By this interpretation, lean NAFLD is similar if not identical to NAFLD associated with obesity and being overweight, with insulin resistance at its core.

Accumulating evidence, however, suggests that lean NAFLD might be a distinct pathophysiological entity,

Abbreviations: ALT, alanine aminotransferase; BA, bile acid; BMI, body mass index; C4, 7-alpha-hydroxy-4-cholesten-3-one; CA, cholic acid; CDCA, chenodeoxycholic acid; ChR, cholesterol-rich; CI, confidence interval; DCA, deoxycholic acid; FGF, fibroblast growth factor; FXR, farnesoid X receptor; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; PERMANOVA, permutational multivariate analysis of variance; PNPLA3, patatin-like phospholipase domain containing 3; TM6SF2, transmembrane 6 superfamily member 2; UDCA, ursodeoxycholic acid.

Received June 11, 2019; accepted August 9, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30908/suppinfo.

**These authors contributed equally to this work.*

Supported by the Robert W. Storr Bequest to the Sydney Medical Foundation of the University of Sydney and National Health and Medical Research Council of Australia (NHMRC) program grants (APP1053206 and APP1149976) and project grants (APP1107178 and APP1108422) to M.E. and J.G; the Australian Government Research Training Program scholarship and the Westmead Institute of Medical Research Top-Up grant to F.C.; and a Matthew Flinders Research Fellowship and an NHMRC Senior Research Fellowship to G.R.

View this article online at wileyonlinelibrary.com.

with approximately half (47%-65%) having nonalcoholic steatohepatitis (NASH).[4] Although lean NAFLD was first described in Asia, it has since been recognized globally.[1] Most aspects of lean NAFLD, including its operational classification, have not been systematically characterized. The most frequently used definition is that of hepatic steatosis with a BMI < 25 kg/m^2 (or <23 kg/m^2 in Asians) in the absence of significant alcohol intake.[5] The natural history of lean NAFLD is even less well characterized; some data suggest that they have worse mortality and accelerated disease progression despite a more favorable metabolic risk profile.[6,7] Finally, the pathogenesis and mechanisms for their favorable metabolic profile compared with obese NAFLD are puzzling and poorly understood, and therapeutic options for lean NAFLD remain undefined.

For metabolic homeostasis, in addition to the neuroendocrine axis, caloric intake, and physical activity, the enterohepatic circulation, including bile acids (BAs) and their metabolites and gut microbiota, are intimately involved. BAs are the principal route for cholesterol catabolism, and recent evidence demonstrates that a high intake of dietary cholesterol,[8] elevated levels of hepatic cholesterol,[9-11] and disrupted hepatic cholesterol homeostasis are pivotal drivers of NAFLD.[9-11] However, individual responses to changes in dietary cholesterol vary widely, suggesting a modifying role for other environmental or genetic factors. It is of interest that it has been suggested that cholesterol intake is higher in lean compared with obese NAFLD.[12-14] BAs also regulate glucose and lipid metabolism and energy expenditure,[15] and in turn their production, transport, and metabolism are regulated by specific nuclear BA receptors, the farnesoid X receptor (FXR) and circulating fibroblast growth factor (FGF19), likely by means of dependent and independent mechanisms.[15,16] The gut microbiome is also intimately involved in the pathogenesis of several metabolic diseases, including body weight regulation, NAFLD, and liver cancer, in part through direct interactions with BAs.[17-19]

We hypothesized that the pathogenesis of lean and obese NAFLD and their distinct metabolic and histological profiles is caused by more than just differences in body weight and BMI. We considered that the clinical phenotype of lean NAFLD might reflect differences in the integration of signals from the diet and the systemic metabolic milieu as well as the enterohepatic axis comprising both BAs and gut microbiota. We tested this hypothesis in a large, well-phenotyped, biopsy-proven cohort of 538 Caucasian patients with NAFLD and 30 lean healthy controls.

DOI 10.1002/hep.30908

Potential conflict of interest: Dr. Marchesini consults for Pfizer and Eli Lilly. He advises AstraZeneca, Gilead, and Novo Nordisk.

ARTICLE INFORMATION:

From the [1]Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Westmead, NSW, Australia; [2]Liver and Pancreatobiliary Diseases Research Center, Digestive Disease Research Institute, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran; [3]SAHMRI Infection and Immunity Theme, School of Medicine, Flinders University, Adelaide, Australia; [4]Division of Gastroenterology and Hepatology, Department of Medical Science, University of Turin, Turin, Italy; [5]Section of Gastroenterology and Hepatology, Dipartimento Biomedico di Medicina Interna e Specialistica (Di.Bi.M.I.S.), University of Palermo, Palermo, Italy; [6]Unit of Metabolic Diseases & Clinical Dietetics, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy; [7]Medical School, Sir Charles Gairdner Hospital Unit, University of Western Australia, Nedlands, WA, Australia.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Jacob George, Ph.D.
Storr Liver Centre, Westmead Institute for Medical Research
Westmead Hospital and University of Sydney
176 Hawkesbury Rd.
2145 Westmead, NSW, Australia
E-mail: jacob.george@sydney.edu.au
Tel.: +1-612-88907705
or

Mohammed Eslam, Ph.D.
Storr Liver Centre, Westmead Institute for Medical Research
Westmead Hospital and University of Sydney
176 Hawkesbury Rd.
2145 Westmead, NSW, Australia
E-mail: mohammed.eslam@sydney.edu.au
Tel.: +1-612-88907705

Patients and Methods

PATIENT SELECTION

A total of 538 consecutive Caucasian patients with histologically characterized NAFLD and 30 lean healthy controls were included. The inclusion criteria and clinical and laboratory assessments and histopathology are described in detail in the Supporting Information. Ethics approval was obtained and conformed to the ethical guidelines of the 1975 Declaration of Helsinki, from the human ethics committee of the Western Sydney Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees. Written informed consent was obtained from all patients.

Methods of genotyping, high-throughput BA profiling, and quantification of 7-alpha-hydroxy-4-cholesten-3-one (C4), FGF19, fgf15, and microbiome analysis are provided in the Supporting Information.

NAFLD MICE MODELS

Male C57BL/6 mice obtained from the Animal Resources Centre (Perth, Australia) were used for diet studies commencing at week 8; details are provided in the Supporting Information. All procedures were approved by the Western Sydney Local Health District animal ethics committee and conducted in accordance with the animal experimentation guidelines of the National Health and Medical Research Council of Australia.

STATISTICAL ANALYSIS

Data were analyzed using SPSS version 24.0 (IBM, Armonk, NY). Values are expressed as mean ± standard deviation, median, and interquartile range or frequency (percentage) as appropriate. Statistical significance was considered as $P < 0.05$ throughout; details are provided in the Supporting Information.

Results

CLINICAL, HISTOLOGICAL, AND GENETIC CHARACTERISTICS OF PATIENTS WITH LEAN NAFLD

A total of 538 patients with biopsy-proven NAFLD were recruited for the study. Of the patients, 99 (18%) were lean. The clinical and biochemical characteristics of lean NAFLD compared with their counterpart nonlean patients are presented in Table 1 and representative images of histological images of liver biopsies are presented in Supporting Fig S1. In addition to lower BMI, lean patients had lower waist-to-hip ratios and better metabolic profiles, including significantly lower frequency of diabetes, higher serum high-density lipoprotein, and lower serum triglycerides, fasting blood glucose, and homeostasis model assessment of insulin resistance values compared with their nonlean counterparts. Histologically, lean patients had lower fibrosis scores and nonalcoholic fatty liver disease activity scores (NAS; $P < 0.001$ for both) as well as lower serum alanine aminotransferase (ALT). In total, lean patients have favorable metabolic and histological features compared with nonlean NAFLD.

Patients with lean NAFLD had a significantly higher prevalence of carriage of the *transmembrane 6 superfamily member 2* (*TM6SF2*) rs58542926 (T) allele compared with nonlean patients but a similar prevalence of the *patatin-like phospholipase domain containing 3* (*PNPLA3*) GG polymorphism (Table 1). To adjust for the effect of confounding factors, the *TM6SF2* rs58542926 (T) allele still associated with lean NAFLD in a multivariable model adjusting for age, sex, ALT, diabetes, total cholesterol level, fibrosis, steatosis, and *PNPLA3* genotype (odds ratio, 2.567; 95% confidence interval [CI], 1.426-4.619; $P = 0.002$).

SERUM BA PROFILE IS ASSOCIATED WITH NAFLD SEVERITY BUT NOT STEATOSIS

Although there is increasing evidence to suggest a critical role for BAs in metabolic diseases, including NAFLD, their correlation with disease severity is conflicting, which is likely because of the limited sample sizes of previous studies. We explored the association between the BA profile and liver histology.

No differences in total BA, total primary BA, or total secondary BA levels were noted between patients with mild steatosis (S1-S2) compared with those with severe steatosis (S3; Supporting Fig. S2C). Next, the association with steatohepatitis activity, including the severity of inflammation and hepatocyte ballooning, was tested. In this analysis, significantly higher total BAs ($P = 0.006$), primary BAs ($P = 0.031$), and secondary BAs ($P < 0.001$) were found in patients with

TABLE 1. Clinical and Histological Characteristics of Patients with Lean and Nonlean NAFLD

	Lean NAFLD (n = 99)	Nonlean NAFLD (n = 439)	*P* Value
Age (years)	46 ± 11.7	47 ± 13.0	0.445
Male (%)	69 (69.7)	290 (64.9)	0.413
BMI (kg/m^2)	23.2 ± 1.5	30.8 ± 4.7	**<0.001**
PNPLA3 I148M (CC/CG/GG)	32 (36.8)/39 (44.8)/16 (18.4)	145 (37.8)/172 (44.8)/67 (17.4)	**0.973**
TM6SF2 E167K (CC/CT/TT)	59 (70.2)/22 (26.2)/3 (3.6)	321 (85.1)/50 (13.3)/6 (1.6)	**0.005**
ALT (IU/mL)	57.9 ± 35.6	72.3 ± 46.8	**<0.001**
WHR*	0.919 ± 0.062	0.971 ± 0.079	**<0.001**
Normal WHR	25 (29.2)	46 (10.3)	
Elevated WHR	29 (29.3)	189 (42.3)	
Phosphatidylethanol level (ng/mL)	66.35 ± 48.59	66.45 ± 52.72	0.8829
Diabetes (%)	11 (11.1)	128 (28.6)	**<0.001**
Hypertension (%)	25 (25.3)	158 (35.3)	0.060
Dyslipidemia (%)	43 (43.4)	242 (54.1)	0.059
Total cholesterol (mmol/L)	5.1 ± 1.2	5.2 ± 1.2	0.472
HDL-C (mmol/L)	1.5 ± 0.6	1.2 ± 0.3	**<0.001**
LDL-C (mmol/L)	3.6 ± 1.6	3.5 ± 1.6	0.667
Triglyceride (mmol/L)	1.6 ± 1.4	1.9 ± 1.2	0.083
Fasting BSL (mmol/L)	5.3 ± 1.8	5.9 ± 1.8	**0.006**
HOMA-IR	2.8 ± 1.9	5.4 ± 5.9	**<0.001**
Fibrosis (%)			
F0-F1 (%)	75 (75.8)	239 (54.6)	**<0.001**
F2-F4 (%)	24 (24.2)	200 (45.4)	
Ballooning (%)			
No ballooning (%)	37 (37.4)	131 (30.4)	0.1510
Any ballooning (%)	62 (62.6)	308 (69.6)	
Steatosis (%)			
Grades 1-2 (%)	85 (85.9)	351 (81.2)	0.2530
Grade 3 (%)	14 (14.1)	88 (18.8)	
Lobular inflammation			
Grades 0-1 (%)	88 (88.9)	352 (80.5)	0.0782
Grade 2 or more (%)	11 (11.1)	87 (19.5)	
NAS	3 ± 2	4 ± 2	**0.001**

Values are mean ± SD or n (%). *P* value was calculated using Fisher's exact test and Student *t* test.
*WHR based on 54 lean patients and 235 nonlean patients. Normal WHR defined as <0.90 for males and <0.85 for females. DNA was available for 471 patients (86%). The bold values indicate statistically significant results. Abbreviation: HOMA-IR, homeostasis model assessment of insulin resistance.

hepatocyte ballooning compared with those without ballooning (Supporting Fig. S2A).

When comparing degrees of inflammation, higher grades of lobular inflammation were associated with higher total and secondary BAs (P = 0.027 and P = 0.026, respectively), whereas there was no significant difference in primary BA levels (P = 0.073; Supporting Fig. S2B). Finally, the association of BAs with NAFLD fibrosis stage was tested. The distribution of all individual BAs between patients with none/mild fibrosis and those with significant fibrosis is depicted in Fig. 1A. Patients with significant fibrosis (≥F2) had higher total (P = 0.017), primary (P = 0.018), and secondary (P = 0.045) BA levels compared with those with none/mild fibrosis (F0-F1; Fig. 1B). At the level of individual BAs, the level of cholic acid (CA) was significantly higher in patients with significant fibrosis compared with those with none/mild fibrosis (P = 0.026; Fig. 1C). Similarly, the levels of glycine and taurine-conjugated BAs were significantly higher in patients with significant fibrosis compared with those with none/mild fibrosis (P = 0.002 and P < 0.001, respectively; Fig. 1D,E). The association between BA levels and fibrosis remained significant

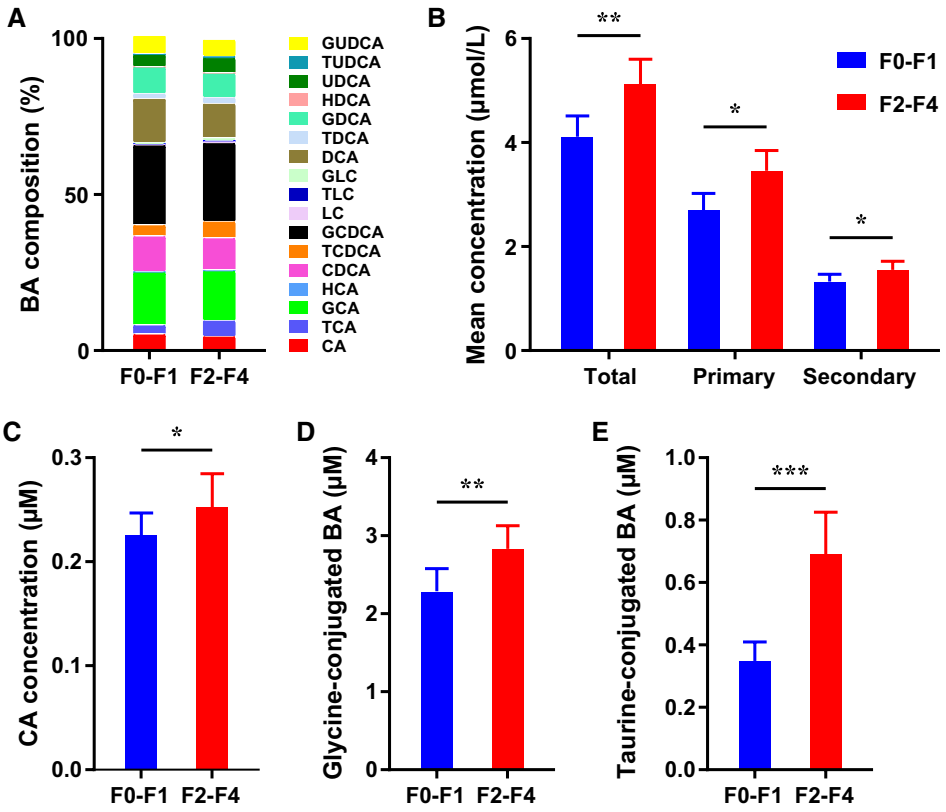


FIG. 1. Serum BA levels and liver fibrosis. (A) BA composition as a percentage according to hepatic fibrosis. The *x* axis shows patients with absent/mild (F0-F1, left, n = 314) and moderate/severe (F2-F4, right, n = 224) fibrosis, and the *y* axis shows the composition of each individual BA in percent. (B) Mean concentration of total BAs, total primary BAs, and total secondary BAs according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of BA levels in µmol/L. (C) Mean concentration of CA according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of BA levels in µmol/L. (D) Mean concentration of glycine-conjugated BAs according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of BA levels in µmol/L. (E) Mean concentration of taurine-conjugated BAs according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of BA levels in µmol/L. Results are expressed as mean ± SEM, and *P* value was calculated using the Mann-Whitney nonparametric *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLC, glycolithocholic acid; GUDCA, Glycoursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LC, lithocholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLC, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

after adjusting for age, BMI, sex, diabetes, ALT, and platelets in a multivariable linear regression analysis (β = 0.115; 95% CI, 0.061-0.168; $P < 0.01$ for total secondary BA levels, and β = 0.302; 95% CI, 0.145-0.458; $P < 0.01$ for ursodeoxycholic acid [UDCA]).

Higher levels of total BAs ($P = 0.001$), primary BAs ($P = 0.001$), and secondary BAs ($P = 0.002$) were seen in patients with higher NAS, defined as >3, compared with patients with lower scores (Supporting Fig. S3). Similarly, patients with NASH had higher levels of total, primary, and secondary BAs compared with patients with steatosis, but only the secondary BA levels were significantly different between the two groups ($P = 0.047$).

LEAN NAFLD PATIENTS HAVE HIGHER SERUM BA LEVELS

Next, we explored the differential BA profile between patients with lean and nonlean NAFLD.

Interestingly, patients with lean NAFLD had higher total, primary, and secondary BA levels compared with those with nonlean NAFLD, although this was only significant for the secondary BAs (P = 0.010; Fig. 2B). The composition of individual BAs also differed between patients with lean and nonlean NAFLD, wherein lean patients had lower deoxycholic acid (DCA), glycochenodeoxycholic acid, and chenodeoxycholic acid (CDCA) but more glycocholic acid (GCA) compared with the nonlean patients (Fig. 2A). Patients with lean NAFLD also had nonsignificantly higher total conjugated and total unconjugated BAs compared with patients with nonlean NAFLD.

Given the strong correlation between BA profiles and fibrosis, we examined the relationship between BAs and lean NAFLD stratified by fibrosis stage.

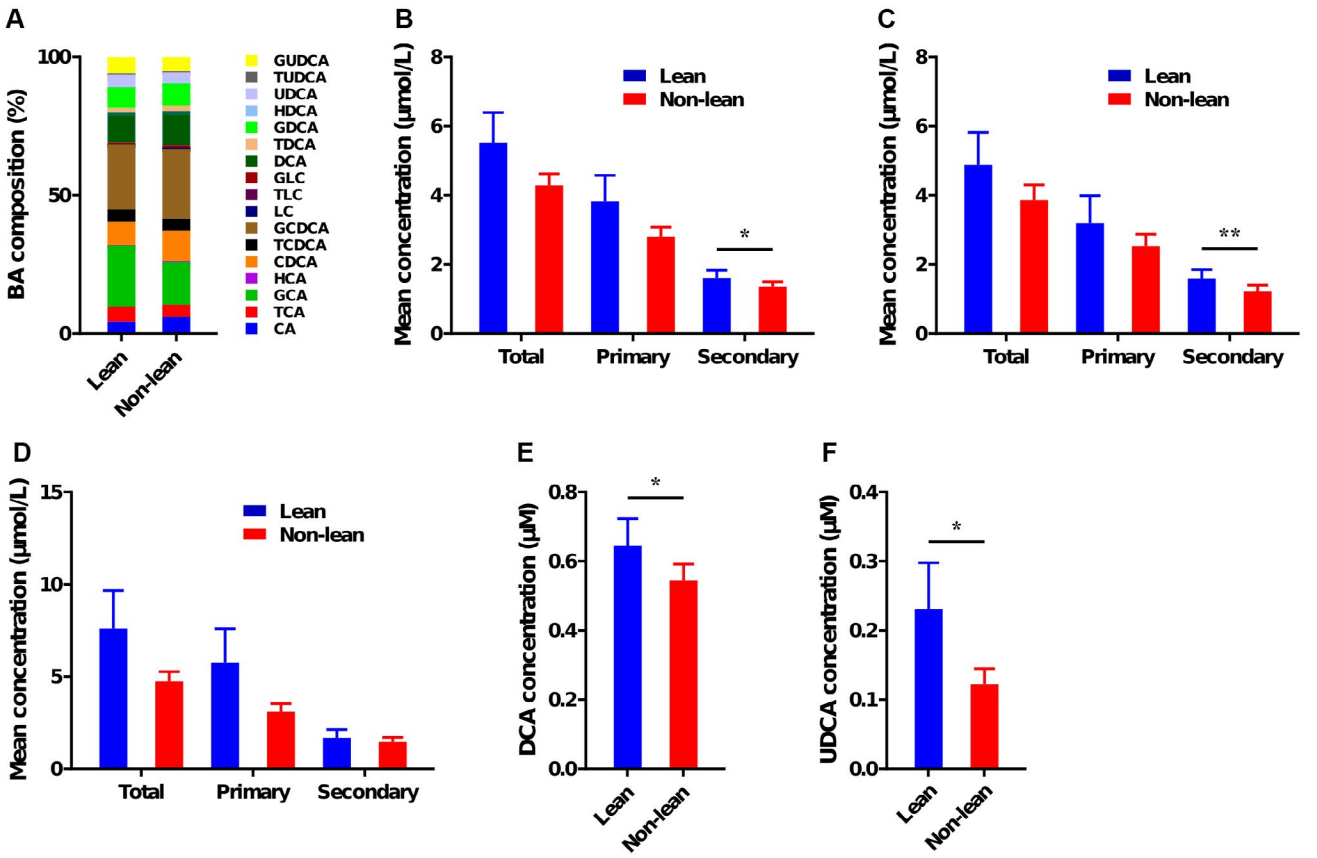


FIG. 2. Serum BA levels in patients with lean and nonlean NAFLD. (A) BA composition in percentage between patients with lean and nonlean NAFLD. The *x* axis shows patients with lean (n = 99) and nonlean (n = 439) NAFLD, and the *y* axis shows the composition of each individual BA in percent. (B) Mean concentration of total BAs, total primary BAs, and total secondary BAs in patients with lean and nonlean NAFLD. The *x* axis shows patients with lean (n = 99) and nonlean (n = 439) NAFLD, and the *y* axis shows the mean concentration of BA levels in µmol/L. (C) Mean concentration of total BAs, total primary BAs, and total secondary BAs in lean and nonlean patients with absent/mild fibrosis. The *x* axis shows lean (n = 75) and nonlean (n = 239) patients with absent/mild (F0-F1) hepatic fibrosis, and the *y* axis shows the mean concentration of BA levels in µmol/L. (D) Mean concentration of total BAs, total primary BAs, and total secondary BAs in lean and nonlean patients with moderate/severe fibrosis. The *x* axis shows lean (n = 24) and nonlean (n = 200) patients with moderate/severe (F2-F4) hepatic fibrosis, and the *y* axis shows the mean concentration of BA levels in µmol/L. (E) Mean concentration of DCA. The *x* axis shows lean (n = 99) and nonlean (n = 439) patients, and the *y* axis shows the mean concentration of BA levels in µmol/L. (F) Mean concentration of UDCA. The *x* axis shows patients with lean (n = 99) and nonlean (n = 439) NAFLD, and the *y* axis shows the mean concentration of BA levels in µmol/L. Results are expressed as mean ± SEM, and P value was calculated using the Mann-Whitney nonparametric t test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLC, glycolithocholic acid; GUDCA, Glycoursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LC, lithocholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLC, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

When stratified in this way, in those with mild fibrosis (F0-F1), higher total secondary BA levels were observed in patients with lean NAFLD compared with patients with nonlean NAFLD (P = 0.004). No significant difference between lean and nonlean patients was observed in those with more severe fibrosis (Fig. 2C,D). The predominant secondary BAs contributing to this difference were DCA and UDCA (P < 0.05 for both; Fig. 2E,F). GCA was also higher in patients with lean NAFLD; however, this difference was not significant (Supporting Fig. S4B). The secondary to primary BA ratio was significantly higher in patients with lean compared with nonlean NAFLD (P = 0.018; Supporting Fig. S4A).

In a subsequent analysis, we determined the relevant clinical factors associated with secondary BA levels. Consistently, on univariable analysis, BMI, fibrosis, and ballooning were associated with secondary BA levels. On multivariable analysis, only BMI and fibrosis stage remained independently, negatively, and positively associated with secondary BA levels, respectively (Supporting Table S1).

PATIENTS WITH LEAN NAFLD HAVE HIGHER SERUM FGF19 LEVELS

FXR dysregulation has been implicated in the pathogenesis of NAFLD; therefore, we were interested to determine if differential effects are observed in lean versus nonlean NAFLD.[20,21] To examine for this, serum FGF19, a surrogate marker of FXR activity, was measured. In this analysis, reduced levels of FGF19 were observed with the advancement of fibrosis stage (P = 0.030; Fig. 3A). Patients with lean NAFLD had significantly higher FGF19 levels compared with patients with nonlean NAFLD (P = 0.028; Fig. 3B). Interestingly, when stratified according to fibrosis severity, the differences were more profound in those with mild fibrosis (F0-F1; P = 0.005), with the reverse being true as fibrosis severity increased; this was, however, not significant (Fig. 3C).

PATIENTS WITH LEAN NAFLD HAVE LOWER C4 LEVELS

To investigate differences in BA metabolism between lean and nonlean NAFLD, levels of C4, a BA synthesis intermediate, were measured as a marker of *de novo* BA synthesis. In this analysis, no difference in levels of C4 was observed according to fibrosis stage (Fig. 3D). However, as expected, patients with lean NAFLD had significantly lower C4 levels compared with their nonlean counterparts (P = 0.016; Fig. 3E). This difference was more predominant in those with mild fibrosis (F0-F1; P = 0.010) but not in those with moderate/severe fibrosis (F2-F4; Fig. 3F).

PATIENTS WITH LEAN NAFLD HAVE A DISTINCT MICROBIOTA PROFILE

The composition of the gut microbiome and its interaction with BAs affects FXR-mediated signaling in both the liver and intestine and is implicated in NAFLD pathogenesis.[20,22] Hence, we determined gut microbiome composition in a small exploratory subset of patients with available stool samples by 16S ribosomal RNA amplicon sequencing. The clinical characteristics of these patients is found in Supporting Table S2. At the phylum level, no differences in taxonomic composition of the gut microbiome were observed according to lean versus obese BMI status. At the genus level, *Erysipelotrichaceae* UCG-003 as well as several bacterial genera within the *Clostridiales* order, including *Ruminococcus*, *Clostridium sensu stricto* 1, *Romboutsia*, and *Ruminococcaceae* UCG-008, were enriched in lean patients, and *Ruminiclostridium* and *Streptococcus* were enriched in patients with NAFLD who were obese (Mann-Whitney test; P < 0.05; Fig. 4A-F). These changes remained significant for Ruminococcaceae UCG-008 when corrected for multiple comparison testing (false discovery rate; P = 0.010).

PATIENTS WITH LEAN NAFLD HAVE DIFFERENTIAL BA AND MICROBIOTA PROFILES COMPARED WITH LEAN HEALTHY CONTROLS

We next compared our cohort of patients with lean NAFLD with lean healthy controls matched by age and sex. Their baseline demographics in comparison to patients with lean NAFLD are shown in Supporting Table S3. Patients with lean NAFLD had significantly higher total BAs, total primary BAs, and total secondary BAs compared with the lean healthy controls

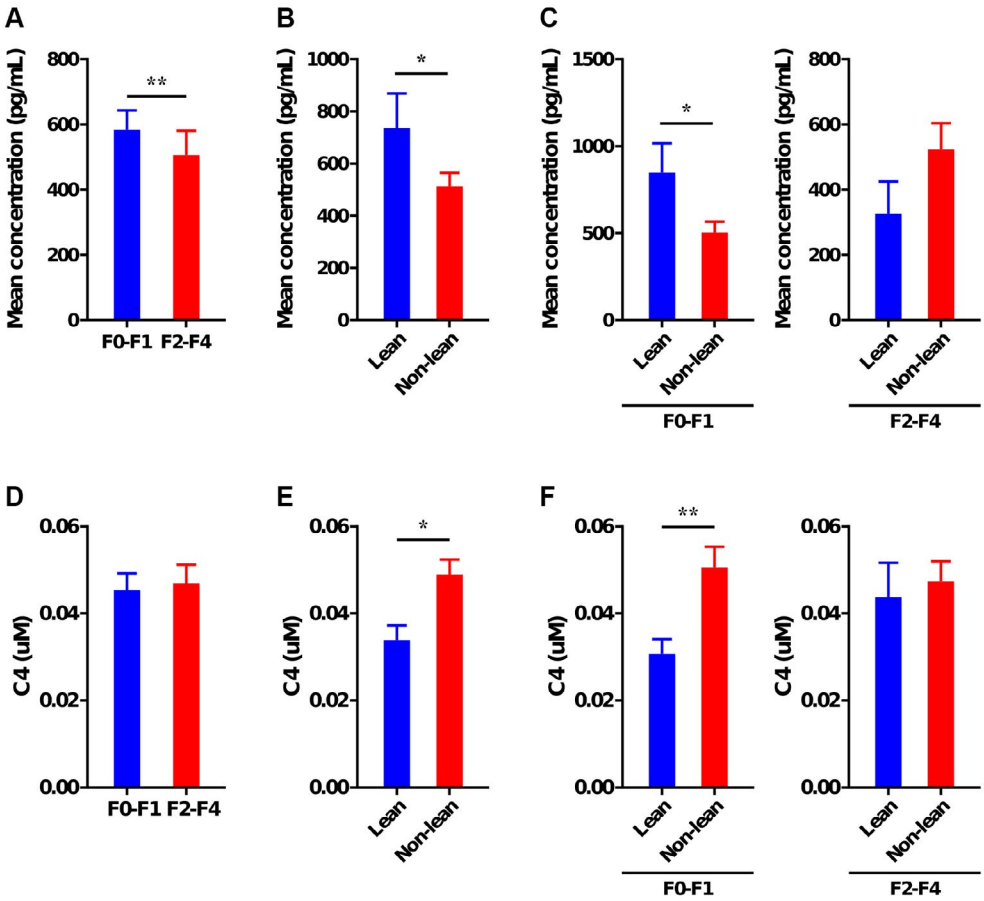


FIG. 3. Serum FGF19 levels and C4 levels. (A) Mean concentration of FGF19 according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of FGF19 in pg/mL. (B) Mean concentration of FGF19 in patients with lean and nonlean NAFLD. The *x* axis shows patients with lean (n = 99) and nonlean (n = 439) NAFLD, and the *y* axis shows the mean concentrations of FGF19 levels in pg/mL. (C) Mean concentration of FGF19 levels in lean and nonlean patients according to BMI and hepatic fibrosis. The *x* axis shows patients with lean and nonlean NAFLD with absent/mild fibrosis (F0-F1, left panel, n = 75 for lean and n = 239 for nonlean NAFLD) and moderate/severe fibrosis (F2-F4, right panel, n = 24 for lean and n = 200 for nonlean), and the *y* axis shows the mean concentrations of FGF19 levels in pg/mL. (D) Mean concentration of C4 according to hepatic fibrosis. The *x* axis shows hepatic fibrosis dichotomized as absent/mild (F0-F1, n = 314) or moderate/severe (F2-F4, n = 224), and the *y* axis shows the mean concentration of C4 in μmol/mL. (E) Mean concentration of C4 in patients with lean and nonlean NAFLD. The *x* axis shows patients with lean (n = 99) and nonlean (n = 439) NAFLD, and the *y* axis shows the mean concentrations of C4 in μmol/mL. (F) Mean concentration of C4 levels according to BMI and hepatic fibrosis. The *x* axis shows patients with lean and nonlean NAFLD with absent/mild fibrosis (F0-F1, left panel, n = 75 for lean and n = 239 for nonlean NAFLD) and moderate/severe fibrosis (F2-F4, right panel, n = 24 for lean and n = 200 for nonlean), and the *y* axis shows the mean concentration of C4 in μmol/mL. Results are expressed as mean ± SEM, and *P* value was calculated using the Mann-Whitney nonparametric *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Supporting Fig. S5A). There was no significant difference in levels of FGF19 between lean healthy controls and patients with lean NAFLD with none/mild fibrosis (F0-F1). However, in patients with lean NAFLD and moderate/severe fibrosis (F2-F4), the level of FGF19 was significantly lower compared with that in lean healthy controls (Supporting Fig. S5B). Analysis of microbiota demonstrated a distinct separation in profiles between lean healthy controls and lean NAFLD (permutational multivariate analysis of variance [PERMANOVA] $P = 0.069$, Pseudo-F = 2.019; Supporting Fig. S5C). More specifically, in the lean NAFLD group, there was an increased abundance of the species *Dorea* and a reduction in the relative abundance of a number of species, including *Marvinbryantia* and the *Christensellenaceae R7* group.

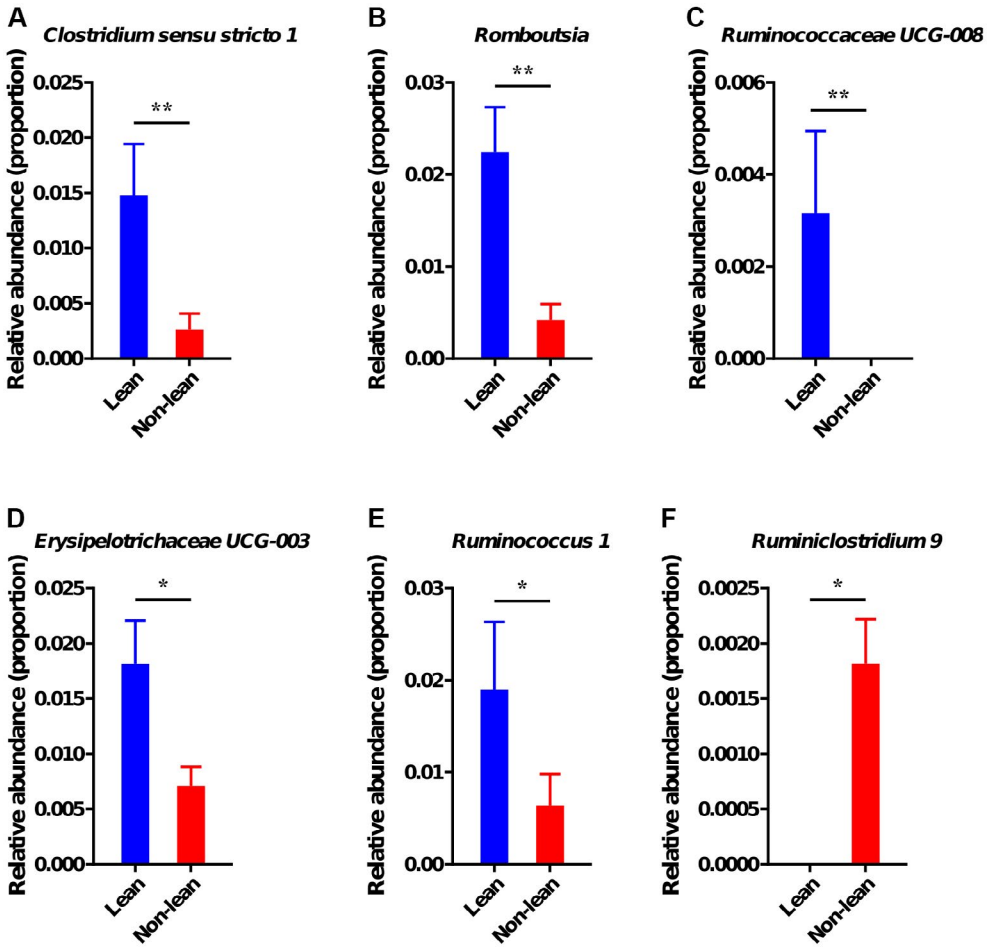


FIG. 4. Gut microbiota associated with lean NAFLD. Abundance of bacterial genera and species that differ between patients with lean (n = 5) and obese (n = 24) NAFLD. Results are expressed as mean ± SEM, and *P* value was calculated using the Mann-Whitney nonparametric *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

A MURINE LEAN NAFLD MODEL HAS INCREASED BAs AND ALTERED GUT MICROBIOTA

To further test our hypothesis, we investigated alterations in BA levels and the gut microbiome in a murine model of lean versus nonlean NAFLD. Mice were fed either a cholesterol-rich (ChR) or a high-sucrose diet for 16 weeks. Mice fed the ChR diet remained lean despite the development of NAFLD, which is consistent with other studies,[23,24] whereas those fed the high-sucrose diet demonstrated significant weight gain. Histology images are shown in Fig. 5A. Like our human results, mice fed the ChR diet had significantly higher total ($P = 0.01$), primary ($P = 0.02$), and secondary ($P = 0.06$) BAs (Fig. 5B). Analysis of ileal fgf15, the mouse equivalent of human FGF19, showed significantly higher levels in mice fed the ChR compared with the high-sucrose diet (Fig. 5C).

Analysis of the microbiota demonstrated a change in composition (PERMANOVA $P = 0.009$, pseudo-F = 18.58, 126 permutations) as reflected broadly by significant changes in the relative abundances of the two major phyla, Firmicutes and Bacteroidetes, as well as in Actinobacteria, Cyanobacteria, and Proteobacteria. Mice fed the ChR diet were observed to have an increased relative abundance of Bacteroidetes and a decrease in Firmicutes compared with those fed with high sucrose (Fig. 5D). As we observed in humans, similar trends were noted for the abundance of members of the Ruminococcaceae bacterial family in the mice fed the high-cholesterol diet

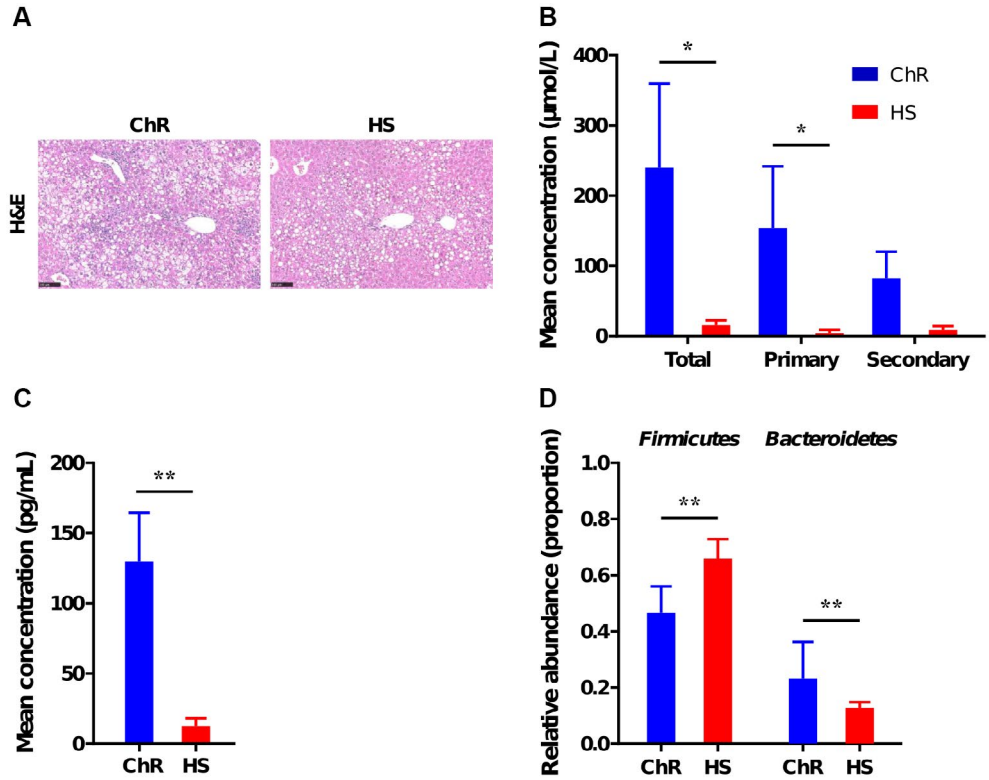


FIG. 5. Serum BA levels and microbiota profile in a murine experimental model of NAFLD. (A) Histology images from a mouse model of lean NAFLD. Hematoxylin and eosin (H&E) image of liver biopsy section from a mouse fed the ChR diet and a mouse fed the high-sucrose (HS) diet. (B) Mean concentrations of total BAs, total primary BAs, and total secondary BAs in a mouse model of lean NAFLD. The *x* axis shows mice fed a diet high in cholesterol (blue bar, n = 9) and mice fed a diet high in sucrose (red bar, n = 5), and the *y* axis shows the mean concentrations of BA levels in μmol/L. (C) Mean concentrations of ileal fgf15 in a mouse model of lean NAFLD. The *x* axis shows mice fed a diet high in cholesterol (blue bar, n = 9) and mice fed a diet high in sucrose (red bar, n = 5), and the *y* axis shows the mean concentrations of ileal fgf15 levels in pg/mL. (D) Relative abundance of the phyla *Firmicutes* and *Bacteroidetes* in ceca of mice fed the high-cholesterol or the high-sucrose diet, as determined by 16S ribosomal RNA sequencing. Results are expressed as mean ± SEM, and *P* value was calculated using the Mann-Whitney nonparametric *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(lean NAFLD). These changes were also observed for several phylotypes within the Erysipelotrichaceae. A correlation plot between different BAs and the bacterial taxa altered in both models is shown in Supporting Fig. S6F.

INHIBITING BA REABSORPTION RESULTED IN IMPROVED STEATOHEPATITIS IN A LEAN NAFLD MODEL

Finally, to investigate the therapeutic implications of our findings, we treated the ChR diet–fed mice (lean model) with or without apical sodium-dependent BA transporter inhibitor (SC-435), which mediates the active reabsorption of BAs into the enterohepatic circulation. Supplementing with SC-435 led to a reduction in liver/body weight ratio (Supporting Fig. S6A) and hepatic total and free cholesterol accumulation (Supporting Fig. S6B,C) but not body or epididymal weights. Histologically, SC-435 treatment resulted in reduced inflammation and fibrosis on hematoxylin and eosin and sirius red staining and reduced NAS (Fig. 6A) and serum ALT levels (Fig. 6B). As expected, this was accompanied by a reduction in the messenger RNA expression of inflammatory and fibrosis markers (Fig. 6C).

In additional analyses, treatment with SC-435 resulted in a shift in the BA profile to one with increased FXR agonistic BAs, such as DCA and

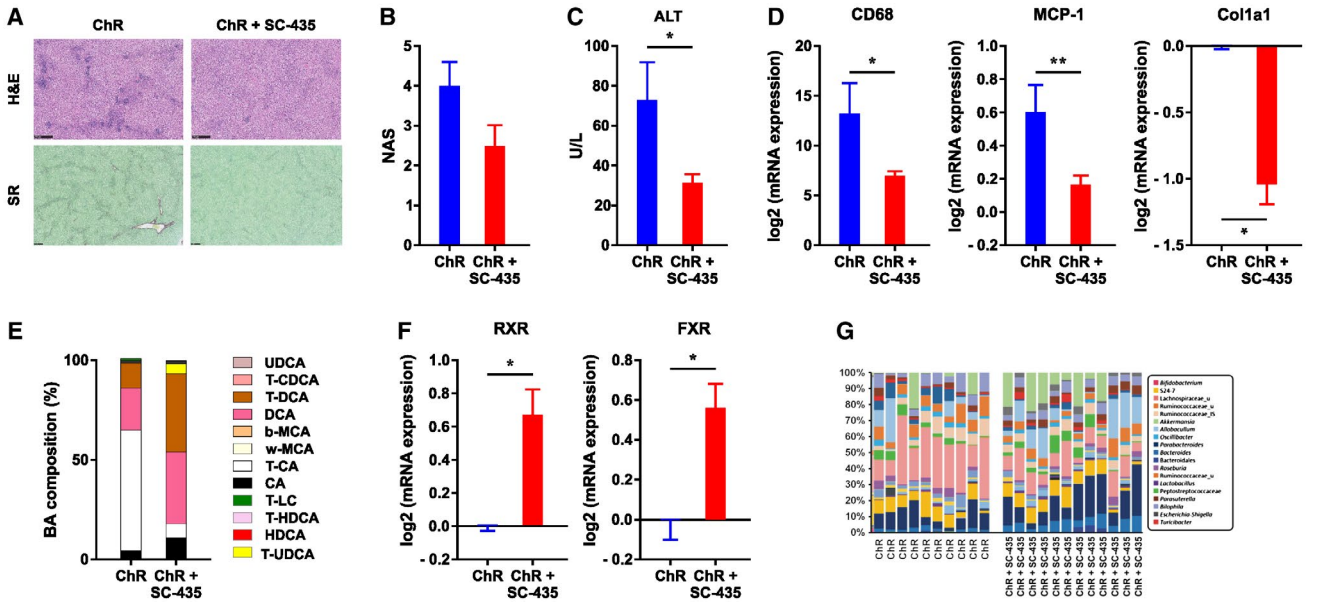


FIG. 6. Administration of SC-435 reduces NASH and alters BA and microbiota profiles in a lean NAFLD model. (A) H&E staining of liver sections indicates reductions in liver inflammation. Sirius red staining shows reduced liver fibrosis (scale bars, 250 μm). (B) NAS indicates a lower level of inflammation in mice treated with SC-435. (C) Serum ALT levels are decreased in mice treated with SC-435. (D) Reduction in mRNA expression of CD68, MCP-1 (macrophage and inflammatory markers), and Col1a1 (fibrotic marker). Data are presented as relative expression or log2 RNA expression. (E) SC-435 led to a shift in BA profiles. (F) SC-435 increased mRNA expression of FXR and retinoid X receptor, which are involved in BA regulation and FXR activity. (G) Analysis of taxa indicates significant differences between mice treated with and without SC-435 (PERMANOVA; pseudo-F = 8.64, p [perm] = 0.012). Data are presented as mean ± SEM, and *P* value was calculated using the Mann-Whitney nonparametric *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n = 5-6 mice per group. Abbreviations: b-MCA, beta muricholic acid; CA, cholic acid; ChR, cholesterol rich; DCA, deoxycholic acid; FXR, farsenoid X receptor; HDCA, hyodeoxycholic acid; NAS, NAFLD activity score; RXR, retinoid X receptor; T-CA, taurocholic acid; T-CDCA, taurochenodeoxycholic acid; T-DCA, taurodeoxycholic acid; T-HDCA, tauro hyodeoxycholic acid; T-LC, tauro lithocholic acid; T-UDCA, tauro ursodeoxycholid acid; UDCA, ursodeoxycholic acid; w-MCA, omega muricholic acid.

taurochenodeoxycholic acid (Fig. 6D), increased ileal fgf15 levels (Supporting Fig. S6E), and hepatic mRNA expression of genes involved in the regulation of BAs, such as retinoid X receptor and FXR (Fig. 6E). Interestingly, SC-435 increased the weight of ceca of mice that were fed the diet (Supporting Fig. S6D) and altered the gut microbiota, with an increase in the relative abundances of Bacteroides and a decreased relative abundance of members of the Lachnospiraceae family compared with control mice (Fig. 6F).

Discussion

Lean NAFLD constitutes a significant proportion of patients with NAFLD, although its pathogenesis is not well understood. Here, we provide a testable hypothesis for the pathophysiological distinction between lean and nonlean NAFLD that can be examined in other cohorts. Using biopsy-proven Caucasian patients in whom the lean NAFLD entity is less frequent than in cohorts from Asia, we demonstrate that lean patients have distinct metabolic, genetic, histologic, and BA profiles and C4 levels, as well as differences in FXR activity and gut microbiota, compared with their nonlean counterparts and lean healthy controls.

Consistent with other reports,[1] approximately 1 in 5 Caucasian patients with NAFLD are lean and have a favorable metabolic and pathological profile, with less insulin resistance and dyslipidemia and milder liver histology. A reciprocal and intimate interaction between BAs and gut microbiota is associated with, and thought to regulate, metabolic and hepatic traits.[15,18] Although myriad factors could explain

the differences we observed, our results *in toto* suggest that the balance and interaction between the systemic metabolic milieu and changes in the intestinal microbiome and BA physiology govern the expression of hepatic disease and the onset and progression of NAFLD in patients with a normal BMI.

To elaborate, increased BA levels, as we observed in lean NAFLD, are reported to mediate resistance to diet-induced obesity, a phenomenon called "obesity resistance."[25,26] Obesity-resistant rodents can burn more dietary fat by increasing energy expenditure. It is of relevance, BAs (including major BA species, such as CA, trichloroacetic acid, DCA, and CDCA) increase energy expenditure,[26] and CDCA increases human brown adipose tissue activity.[27] FGF19, which was also increased in lean NAFLD, is reported to be a key regulator of energy expenditure and improves glucose and lipid homeostasis,[28] and gut-restricted FXR agonism promotes metabolic improvements and enhances thermogenesis and browning of white adipose tissue in mice.[29] At the microbiota level, patients with lean NAFLD had distinct gut microbiota compared with those who were nonlean. Lean NAFLD had an increased abundance of members belonging to the *Clostridium* genus as well as *Ruminococcaceae,* which are involved in the formation of BAs.[30,31] Consistently, in an experimental model that involved feeding mice a ChR diet,[23,24] we recapitulated several features of the phenotype, including lean body weight, steatohepatitis, and less insulin resistance, compared with mice receiving a high-sucrose diet, with similar changes in BA profiles with higher total BAs and fgf15 levels and similar trends observed in gut microbiota. Thus, we surmise that patients with lean NAFLD have an obesity-resistant phenotype in part mediated by greater levels of BAs and FGF19 and microbiota changes. The decreased levels of C4 in lean NAFLD further support the concept of metabolic adaptation whereby the increased FXR activity (as represented by FGF-19 levels) results in negative feedback on BA synthesis.

The milder disease and favorable metabolic profile of patients with lean NAFLD could be explained by the current findings. There is strong evidence that activation of BA signaling induces improvements in metabolic (glucose and lipid) phenotypes in murine models.[32] Furthermore, in both humans and murine models, elevated BAs play a role in the metabolic improvements after bariatric surgery, including in type 2 diabetes, dyslipidemia, and NASH, even before significant weight loss.[33-35] Thus, we suggest that patients who are lean can adapt metabolically and excrete greater amounts of BAs, whereas their obese counterparts are those less able to excrete adequate amounts of BAs to rid themselves of excess cholesterol, even if they are able to maintain a plasma cholesterol level comparable to that of lean patients. Consistently, in humans, patients who are lean and patients who are obese have differential defense mechanisms to maintain stable serum cholesterol levels, wherein dietary cholesterol appears to preferentially induce BA synthesis in patients who are lean compared with patients who are obese.[36]

Notably, we did not observe any association between BA levels and hepatic steatosis, indicating a potential lack of a protective effect of BAs on the development of steatosis, as opposed to changes in peripheral tissues. Alternatively, changes in microbiota might explain the development of steatosis.[37] Lean NAFLD had a distinct separation in microbiota profile compared with the healthy controls, with an increased abundance of *Dorea* that has been implicated in the pathogenesis and progression of NASH[38] and a decrease of several species protective for NAFLD, such as *Marvinbryantia* and *Christensellenaceae R7* group.[39,40] Similarly, compared with nonlean NAFLD, they had an increased relative abundance of several phylotypes within the *Erysipelotrichaceae* family in both patient and murine models that have been repeatedly linked to host lipid and cholesterol phenotypes in different species (humans, mice, and hamsters) and positively associate with changes in liver fat in humans.[41] Use of plant sterol esters to reduce cholesterol in hamsters likewise reduced *Erysipelotrichaceae* abundance.[41] *Ruminococcaceae* UCG-008, *Clostridium sensu stricto* 1, and *Romboutsia*, which were also enriched in lean NAFLD, are reported to be strongly correlated with hepatic triglycerides.[42]

At a genetic level, we demonstrated that although there was no significant difference in the proportions of patients with *PNPLA3* rs738409 GG genotype, a significantly greater proportion of patients with lean NAFLD carried the *TM6SF2* rs58542926 (T) allele than patients with nonlean NAFLD. Interestingly, TM6SF2 has been implicated in cholesterol synthesis.[43] In addition, *TM6SF2*, but not *PNPLA3*, genotypes correlate with endotoxemia.[44] Hence, the lean NAFLD phenotype might be consistent with obesity resistance, in which individuals are still prone to

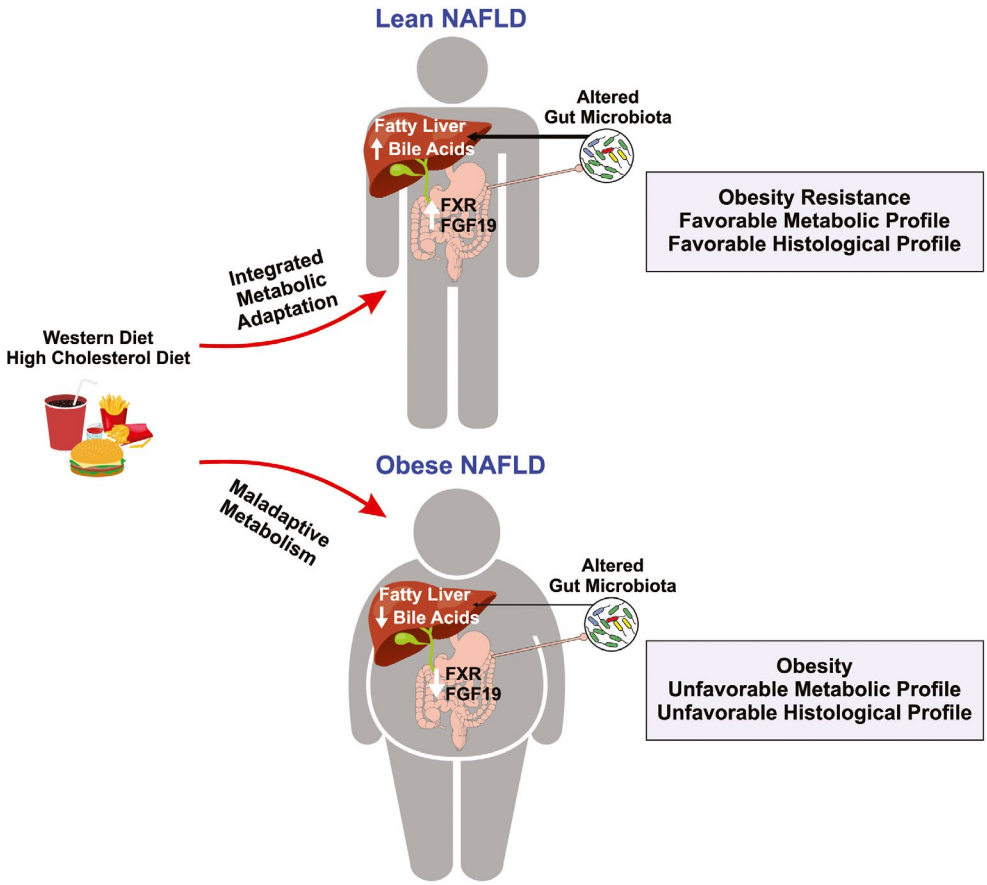


FIG. 7. Proposed model for the differential pathophysiology between patients who are lean and obese with NAFLD. Patients who were lean had better metabolic and liver histology profiles. Consistent with the notion that lean patients have appropriate metabolic adaptation to an obesogenic environment, they are obesity resistant. The compensatory mechanisms include increases in BAs and FXR activity and distinct gut microbiota profiles that explain the favorable profile despite steatosis development. Similar features were observed in murine models of lean and obese NAFLD.

develop steatosis in response to an obesogenic environment (and perhaps a diet enriched in cholesterol), likely by genetic and gut-driven mechanisms.

We observed that differences between lean and nonlean patients or lean controls were more profound in those with early stages of liver fibrosis. This suggests that with disease progression, homeostatic responses might possibly no longer be able to limit inflammation and fibrosis, leading ultimately to long-term adverse outcomes despite a favorable baseline metabolic and histological profile.[6,7] This hypothesis is supported by the higher serum BA levels and lower FGF19 levels in patients with significant fibrosis. Longitudinal studies would be needed to confirm the findings.

To investigate the therapeutic implications of our findings, we demonstrated that use of the apical sodium-dependent BA transporter inhibitor SC-435 results in marked increases in fgf15 and a shift in BA and microbiota profiles as well as improved steatohepatitis in the lean model.

The strengths of our report include the study of a large, well-defined, biopsy-proven Caucasian cohort and as detailed an investigation as possible from cross-sectional data and complementary mouse studies. However, our study also has limitations. First, patients were seen in tertiary referral centers and may suffer from selection bias. In addition, dietary histories were not available given the accumulation of cohorts over several years, and the cross-sectional design did not allow for interventions or longitudinal outcomes; thus, a causal relationship cannot be demonstrated. Finally, our study is limited by the small sample size

with regard to microbiome analysis. It would also be interesting in future studies to measure differences in fecal BAs.

In conclusion, in contrast to nonlean NAFLD, lean patients are likely to have a distinct pathophysiology. We suggest that the onset of disease occurs at a lower BMI set point (with lower measures of insulin resistance and dyslipidemia) and is shaped by the genetics background and early alterations in the BA and gut microbiota profile. These changes might reflect altered dietary composition (perhaps with an excess of dietary cholesterol, as reported in patients with lean NAFLD[12-14]), altered cholesterol metabolism, limitations in adipocyte numbers in childhood, or differences in mucosal immunology. Secondary or concomitant alterations in gut microbiota composition also drive the phenotype to a greater extent than in patients with nonlean NAFLD. This hypothesis does not negate the possibility that there are patients who are overweight/obese with NAFLD with a similar pattern of compensatory mechanisms but suggests that lean patients have a preponderance of a gut-mediated phenotype (Fig. 7). Further studies are needed to investigate the contribution of early-stage adaptive mechanisms on the long-term hepatic and extrahepatic outcomes of this disease. Our hypothesis would suggest that these individuals will have more severe and progressive liver disease, as has been suggested,[6,7] but this hypothesis needs further confirmation.

Acknowledgment: We thank all the patients for their participation in this study.

Author Contributions: F.C., J.G., and M.E. designed the study. F.C., S.E., S.C., M.K.A., A.B., M.M., and M.E. carried out the acquisition of data, analysis, and interpretation of data. F.C. and M.E. drafted the manuscript. E.B., S.P., R.Y., G.M., C.R., C.L., L.A.A., and A.C. were responsible for sample acquisition. G.R. and J.M.C. were responsible for microbiota analysis. All authors contributed to the writing of the manuscript and approved the submission of the final version of the manuscript.

REFERENCES

1) Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2018;15:11-20.
2) Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. Diabetes 1998;47:699-713.
3) Ding C, Chan Z, Magkos F. Lean, but not healthy: the 'metabolically obese, normal-weight' phenotype. Curr Opin Clin Nutr Metab Care 2016;19(6):408-417.
4) Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
5) Das K, Chowdhury A. Lean NASH: distinctiveness and clinical implication. Hepatol Int 2013;7:806-813.
6) Dela Cruz AC, Bugianesi E, George J, Day CP, Liaquat H, Charatcharoenwitthaya P, et al. 379 characteristics and long-term prognosis of lean patients with nonalcoholic fatty liver disease. Gastroenterology 2014;146:S-909.
7) Hagström H, Nasr P, Ekstedt M, Hammar U, Stål P, Hultcrantz R, et al. Risk for development of severe liver disease in lean patients with nonalcoholic fatty liver disease: a long-term follow-up study. Hepatol Commun 2017;2:48-57.
8) Ioannou GN, Morrow OB, Connole ML, Lee SP. Association between dietary nutrient composition and the incidence of cirrhosis or liver cancer in the United States population. Hepatology 2009;50:175-184.
9) Simonen M, Männistö V, Leppänen J, Kaminska D, Kärjä V, Venesmaa S, et al. Desmosterol in human nonalcoholic steatohepatitis. Hepatology 2013;58:976-982.
10) Min HK, Kapoor A, Fuchs M, Mirshahi F, Zhou H, Maher J, et al. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. Cell Metab 2012;15:665-674.
11) Van Rooyen DM, Larter CZ, Haigh WG, Yeh MM, Ioannou G, Kuver R, et al. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. Gastroenterology 2011;141:1393-1403.e5.
12) Musso G, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 2003;37:909-916.
13) Yasutake K, Nakamuta M, Shima Y, Ohyama A, Masuda K, Haruta N, et al. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol. Scand J Gastroenterol 2009;44:471-477.
14) Enjoji M, Yasutake K, Kohjima M, Nakamuta M. Nutrition and nonalcoholic fatty liver disease: the significance of cholesterol. Int J Hepatol 2012;2012:925807.
15) Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. Hepatology 2017;65:350-362.
16) Al-Khaifi A, Rudling M, Angelin B. An FXR agonist reduces bile acid synthesis independently of increases in FGF19 in healthy volunteers. Gastroenterology 2018;155:1012-1016.
17) Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. N Engl J Med 2016;375:2369-2379.
18) Schnabl B, Brenner DA. Interactions between the intestinal microbiome and liver diseases. Gastroenterology 2014;146:1513-1524.
19) Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature 2013;499:97-101. Erratum in: Nature 2014;506:396.
20) Jiao N, Baker SS, Chapa-Rodriguez A, Liu W, Nugent CA, Tsompana M, et al. Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. Gut 2018;67:1881-1891.

21) Puri P, Daita K, Joyce A, Mirshahi F, Santhekadur PK, Cazanave S, et al. The presence and severity of nonalcoholic steatohepatitis is associated with specific changes in circulating bile acids. Hepatology 2018;67:534-548.
22) Ramírez-Pérez O, Cruz-Ramón V, Chinchilla-López P, Méndez-Sánchez N. The role of the gut microbiota in bile acid metabolism. Ann Hepatol 2017;16:S21-S26.
23) Tu LN, Showalter MR, Cajka T, Fan S, Pillai VV, Fiehn O, et al. Metabolomic characteristics of cholesterol-induced non-obese nonalcoholic fatty liver disease in mice. Sci Rep 2017;7:6120.
24) Matsuzawa N, Takamura T, Kurita S, Misu H, Ota T, Ando H, et al. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology 2007;46:1392-1403.
25) Watanabe M, Horai Y, Houten SM, Morimoto K, Sugizaki T, Arita E, et al. Lowering bile acid pool size with a synthetic farnesoid x receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure. J Biol Chem 2011;286:26913-26920.
26) Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 2006;439:484-489.
27) **Broeders EP**, **Nascimento EB**, Havekes B, Brans B, Roumans KHM, Tailleux A, et al. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. Cell Metab 2015;22:418-426.
28) Fu L, John LM, Adams SH, Yu XX, Tomlinson E, Renz M, et al. Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. Endocrinology 2004;145:2594-2603.
29) Fang S, Suh JM, Reilly SM, Yu E, Osborn O, Lackey D, et al. Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance. Nat Med 2015;21:71-77.
30) Kakiyama G, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, et al. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. J Hepatol 2013;58:949-955.
31) Wahlström A, Sayin SI, Marschall HU, Bäckhed F. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. Cell Metabol 2016;24:41-50.
32) Pierre JF, Martinez KB, Ye H, Nadimpalli A, Morton TC, Yang J, et al. Activation of bile acid signaling improves metabolic phenotypes in high-fat diet-induced obese mice. Am J Physiol Gastrointest Liver Physiol 2016;311:G286-G304.
33) Chambers AP, Jessen L, Ryan KK, Sisley S, Wilson-Pérez HE, Stefater MA, et al. Weight-independent changes in blood glucose homeostasis after gastric bypass or vertical sleeve gastrectomy in rats. Gastroenterology 2011;141:950-958.
34) Kohli R, Myronovych A, Tan BK, Salazar-Gonzalez RM, Miles L, Zhang W, et al. Bile acid signaling: mechanism for bariatric surgery, cure for NASH? Dig Dis 2015;33:440-446.
35) Patti ME, Houten SM, Bianco AC, Bernier R, Larsen PR, Holst JJ, et al. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. Obesity (Silver Spring) 2009;17:1671-1677.
36) Klass DM, Bührmann K, Sauter G, Del Puppo M, Scheibner J, Fuchs M, et al. Biliary lipids, cholesterol and bile synthesis: different adaptive mechanisms to dietary cholesterol in lean and obese subjects. Aliment Pharmacol Ther 2006;23:895-905.
37) Chu H, Duan Y, Yang L, Schnabl B. Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease. Gut 2019;68:359-370.
38) Del Chierico F, Nobili V, Vernocchi P, Russo A, De Stefanis C, Gnani D, et al. Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. Hepatology 2017;65:451-464.
39) Zhou D, Pan Q, Xin FZ, Zhang RN, He CX, Chen GY, et al. Sodium butyrate attenuates high-fat diet-induced steatohepatitis in mice by improving gut microbiota and gastrointestinal barrier. World J Gastroenterol 2017;23:60-75.
40) Ma J, Zhou Q, Li H. Gut microbiota and nonalcoholic fatty liver disease: insights on mechanisms and therapy. Nutrients 2017;9:1124.
41) Martínez I, Perdicaro DJ, Brown AW, Hammons S, Carden TJ, Carr TP, et al. Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters. Appl Environ Microbiol 2013;79:516-524.
42) Zhao L, Chen Y, Xia F, Abudukerimu B, Zhang W, Guo Y, et al. A glucagon-like peptide-1 receptor agonist lowers weight by modulating the structure of gut microbiota. Front Endocrinol (Lausanne) 2018;9:233.
43) Fan Y, Lu H, Guo Y, Zhu T, Garcia-Barrio MT, Jiang Z, et al. Hepatic transmembrane 6 superfamily member 2 regulates cholesterol metabolism in mice. Gastroenterology 2016;150:1208-1218.
44) Pang J, Xu W, Zhang X, Wong GL, Chan AW, Chan HY, et al. Significant positive association of endotoxemia with histological severity in 237 patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2017;46:175-182.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30908/suppinfo.

9.1.2 Clinical Liver disease

Chen, F; George, J; Eslam, M; *Lean NAFLD in Asians*; accepted for publication in Clinical liver disease on 28 December 2019; DOI: 10.1002/cld.930

NAFLD in lean Asians

Mohammed Eslam, Fei Chen and Jacob George

Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, NSW, Australia

Author contributions: The authors contributed equally to all aspects of this article.

Corresponding Author:

Mohammed Eslam: Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Westmead 2145, NSW, Australia, Ph: 61-2-88907705. Email: mohammed.eslam@sydney.edu.au

Jacob George: Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Westmead 2145, NSW, Australia, Ph: 61-2-88907705; Fx 61-2-96357582. Email: jacob.george@sydney.edu.au

Acknowledgements: ME and JG are supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney; a National Health and Medical Research Council of Australia (NHMRC) Program Grant (APP1053206, APP1149976) and Project grants (APP1107178 and APP1108422).

Conflicts of interest: The authors declare no competing interests for this manuscript.

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects about 20-30% of the global population and increases the risk of hepatic and extra-hepatic complications including cardiovascular disease, diabetes and some types of cancer (Z. Younossi et al. 2018a). While NAFLD is strongly associated with obesity, not all obese subjects will develop disease; conversely, a significant proportion of patients will have a normal body mass index (BMI) and are commonly referred to as having 'lean NAFLD' or NAFLD in a lean person.

Definition

Lean NAFLD is defined as disease that develops in subjects with a normal BMI based on ethnic-specific cut-offs of 25 kg/m^2 in Caucasians and 23 kg/m^2 in Asian patients. A limitation of this definition is that it relies solely on BMI, an imperfect index of body fat topography and fails to identify body fatness in nearly half of adults. Notably, visceral adiposity is more strongly implicated in the predisposition to NAFLD development irrespective of BMI. Similarly, there is a lack of incorporation of concepts surrounding metabolic health in the current definition with nearly a third of lean individuals likely being metabolically unhealthy (**Figure 1**).

Prevalence

Lean NAFLD prevalence ranges from 5 to 26%; 5% to 45% in Asians and 5-20% in European populations (Z. Younossi et al. 2018a) (**Figure 2**). In China, of 6,905 subjects with a BMI <25 kg/m^2, 7.27% had ultrasonographic evidence of hepatic steatosis while in another study of 2,000 Chinese with BMI <24 kg/m^2, 18% had NAFLD. In Hong Kong the prevalence of NAFLD based on proton-magnetic resonance spectroscopy (^{1}HMRS)

spectroscopy was 19% in subjects with a BMI <25 kg/m^2. Other countries in Asia demonstrate a similar prevalence of BMI-based lean NAFLD (Japan: 15.2% in 3,271 non-obese subjects; India (urban West Bengal): 5% in those with BMI<25 kg/m^2 based on ultrasonography and subsequent CT validation; Korea: 12.6% in 29,994 health check non-obese participants). In western populations, the Dallas Heart Study revealed a prevalence of hepatic steatosis by ^{1}H-MRS that ranged from 11% in African Americans to 20% in Caucasians and 26% in Hispanics with a BMI <30 kg/m^2. Similarly, a large study including subjects from Australia and Italy suggested that the prevalence of NAFLD was 20% in those of Caucasian descent with BMI <25 kg/m^2(F. Chen et al. 2019). Data on the true population prevalence and ethnicity-based variations in lean NAFLD prevalence are still limited.

Clinical, histological characteristics and outcome of lean NAFLD

By definition, patients with lean NAFLD have a lower BMI but they also have a lower waist circumference and a more favourable metabolic profile with lower levels of dyslipidemia, diabetes, hypertension, glycemia and homeostasis model assessment insulin resistance index (HOMA-IRI) compared to their obese counterparts. In cross-sectional studies, lean patients also have less hepatic inflammation and fibrosis. Despite the favourable phenotype however (F. Chen et al. 2019; J. C. F. Leung et al. 2017b), lean patients with NAFLD may have a worse outcome and accelerated disease progression (A. C. Dela Cruz et al. 2014; Hagstrom et al. 2018), though one study in Chinese patients with shorter follow up (4 years) suggested that non-obese patients may have a better prognosis, though this was not significant (J. C. F. Leung et al. 2017b). As would be expected from the underlying metabolic abnormalities, lean NAFLD is associated with an increased risk

of incident diabetes and cardiovascular disease compared to those without NAFLD (Sinn et al. 2019).

Pathophysiology

The pathophysiological pathways underlying the development and progression of NAFLD in lean subjects are not entirely clear. However, emerging evidence indicates that lean NAFLD is a distinct entity shaped by the dynamic interaction of genetic predisposition, metabolic dysregulation, the gut microbiota and the enterohepatic circulation. Comparing lean and non-lean patients with NAFLD, the prevalence of the *PNPLA3* (G) allele was reported to be higher in lean individuals in some but not others reports (Eslam et al. 2018b; Feldman et al. 2017a). An increased prevalence of the *TM6SF2* (T) (F. Chen et al. 2019) and *IFNL3/IFNL4* (C) allele among lean patients has also been demonstrated (Eslam et al. 2015a; Petta et al. 2017a).

Lean NAFLD patients tend to have a distinct metabolic and gut microbiota profile with higher concentrations of lysine that is implicated in visceral fat accumulation (Feldman et al. 2017a). In another study, patients were reported to have increased bile acids and FXR activity (measured by FGF15/19), implying that they have better metabolic adaptation and are perhaps relatively obese resistant. Notably, this adaptation attenuates with progression of disease (F. Chen et al. 2019) (**Figure 3**). Intriguingly, pilot data suggest that patients with lean NAFLD may have a distinct gut microbiota profile with enrichment of species implicated in the generation of liver fat (F. Chen et al. 2019).

Approach to management

No specific guidelines exist for the management of lean, as opposed to non-lean NAFLD. The current recommendations of the American Association for the Study of Liver Disease and the European Association for the Study of the Liver recommends weight loss alone or accompanied by increased physical activity for all patients with NAFLD. Although weight loss might intuitively appear to be less beneficial in lean patients, there are demonstrable effects of lifestyle intervention even in this sub-group (V. W. S. Wong et al. 2018). High fructose and cholesterol intake has been reported in patients with lean NAFLD and it would seem appropriate to recommend reducing intake of these nutrients, while encouraging adoption of a Mediterranean-type diet. The latter also has beneficial effects on cardiovascular disease and visceral fat accumulation (Estruch et al. 2018). Similarly, emerging evidence indicates that exercise can reduce liver fat independent of weight loss (Johnson et al. 2009).

Lean NAFLD patients are underrepresented in ongoing clinical trials; thus, the impact of current investigational agents on lean disease is unclear. Of interest, inhibition of ileal bile acid uptake led to resolution of steatohepatitis in a mouse model (F. Chen et al. 2019), while liraglutide, a glucagon-like peptide- 1 (GLP-1) analogue improved liver histology in lean patients (Ipsen et al. 2018).

Conclusion

A significant proportion of patients with NAFLD are lean, however this entity remains poorly characterized and understood. While these patients demonstrate distinct pathophysiological mechanisms culminating in similar liver histology to obese patients,

individuals with lean NAFLD remain at risk of developing hepatic and extra-hepatic complications. Targeted studies are required to further clarify lean NAFLD pathogenesis and to develop appropriate management approaches.

Figures and legends:

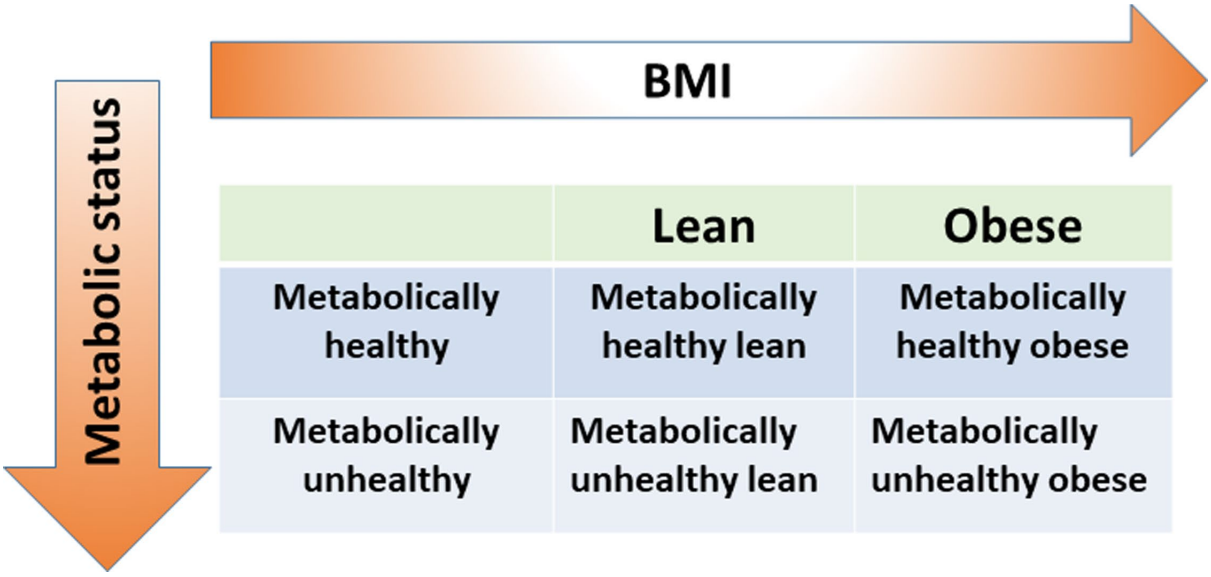


Figure 1: Relationship between body mass index (BMI) and metabolic health status. Subjects who are obese by BMI criteria can be metabolically healthy, while a proportion of those who have a normal BMI are metabolically unhealthy. The term "metabolic health" refers to the metabolic health status of an individual and is a composite of a number of metabolic indicators. A metabolically healthy individual has a low risk of impending cardiometabolic disease. Metabolic health is related to, but not the same as, the absence of metabolic syndrome. Currently there are different subsets of variables that define "metabolic health", with no universal consensus(Maclagan and Tu 2015).

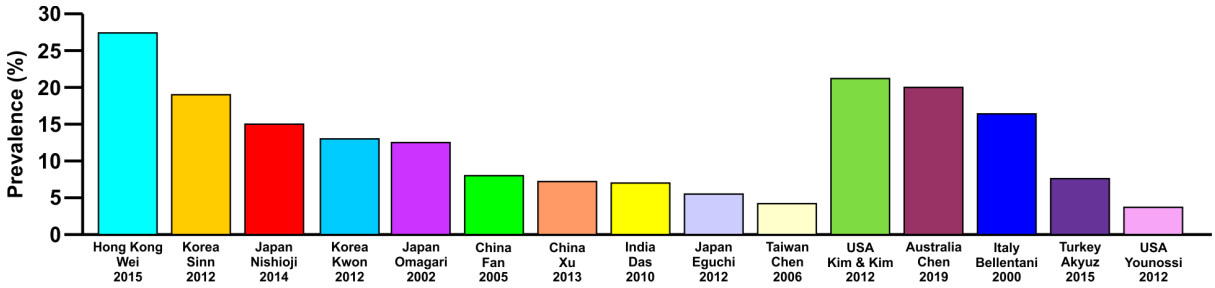


Figure 2: Prevalence of NAFLD in lean patients in the published literature. In these studies, BMI < 25 Kg/m^2 was used as a cut-off for definition of leanness in most of the studies.

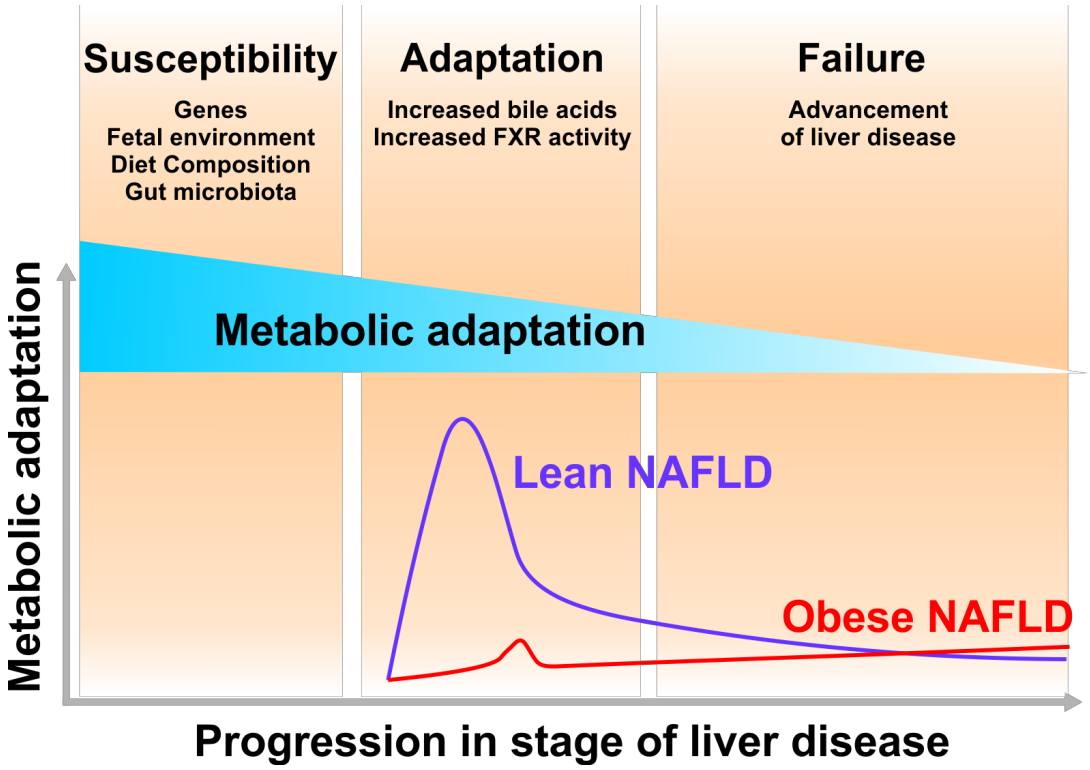


Figure 3: Metabolic adaptation in patients with NAFLD. Conceptually, the evolution of NAFLD in lean patients can be divided in three stages: 1. Subjects at high risk of NAFLD have increase susceptibility likely from genetic factors, the foetal microenvironment, dietary intake including its composition, changes in the epigenetic code during the intrauterine period and early life, as well as changes in gut microbiota; 2. in lean patients with NAFLD, there is a phase of adaptation through increasing bile acids, FXR activity and potentially other mechanisms; 3. with advancement of disease, individuals with lean NAFLD have a failure of metabolic adaptation brought about by the interaction of various and complex systemic processes.

References

1. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nature Reviews Gastroenterology & Hepatology 2018;15:11-20.
2. Chen F, Esmaili S, Rogers G, Bugianesi E, Petta S, Marchesini G, Bayoumi A, et al. Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation. Hepatology 2019.
3. Leung JCF, Loong TCW, Wei JL, Wong GLH, Chan AWH, Choi PCL, Shu SST, et al. Histological Severity and Clinical Outcomes of Nonalcoholic Fatty Liver Disease in Nonobese Patients. Hepatology 2017;65:54-64.
4. Dela Cruz AC, Bugianesi E, George J, Day CP, Liaquat H, Charatcharoenwitthaya P, Mills PR, et al. Characteristics and Long-Term Prognosis of Lean Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 2014;146:S909-S909.
5. Hagstrom H, Nasr P, Ekstedt M, Hammar U, Stal P, Hultcrantz R, Kechagias S. Risk for development of severe liver disease in lean patients with nonalcoholic fatty liver disease: A long-term follow-up study. Hepatol Commun 2018;2:48-57.
6. Sinn DH, Kang D, Choi SJ, Paik SW, Guallar E, Cho J, Gwak GY. Lean non-alcoholic fatty liver disease and development of diabetes: a cohort study. European Journal of Endocrinology 2019;181:185-192.
7. Feldman A, Eder SK, Felder TK, Kedenko L, Paulweber B, Stadlmayr A, Huber-Schonauer U, et al. Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver. American Journal of Gastroenterology 2017;112:102-110.
8. Eslam M, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. Journal of Hepatology 2018;68:268-279.
9. Petta S, Valenti L, Tuttolomondo A, Dongiovanni P, Pipitone RM, Camma C, Cabibi D, et al. Interferon Lambda 4 rs368234815 TT >delta G Variant Is Associated With Liver Damage in Patients With Nonalcoholic Fatty Liver Disease. Hepatology 2017;66:1885-1893.
10. Eslam M, Hashem AM, Leung R, Romero-Gomez M, Berg T, Dore GJ, Chan HLK, et al. Interferon-lambda rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease. Nature Communications 2015;6.
11. Wong VWS, Wong GLH, Chan RSM, Shu SST, Cheung BHK, Li LS, Chim AML, et al. Beneficial effects of lifestyle intervention in non-obese patients with non-alcoholic fatty liver disease. Journal of Hepatology 2018;69:1349-1356.
12. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. N Engl J Med 2018;378:e34.
13. Johnson NA, Sachinwalla T, Walton DW, Smith K, Armstrong A, Thompson MW, George J. Aerobic Exercise Training Reduces Hepatic and Visceral Lipids in Obese Individuals Without Weight Loss. Hepatology 2009;50:1105-1112.
14. Ipsen DH, Rolin B, Rakipovski G, Skovsted GF, Madsen A, Kolstrup S, Schou-Pedersen AM, et al. Liraglutide Decreases Hepatic Inflammation and Injury in Advanced Lean Non-Alcoholic Steatohepatitis. Basic & Clinical Pharmacology & Toxicology 2018;123:704-713.
15. Maclagan LC, Tu JV. Using the concept of ideal cardiovascular health to measure population health: a review. Current Opinion in Cardiology 2015;30:518-524.

9.1.3 Nature Reviews Gastroenterology and Hepatology

Chen, F, Younossi, ZM, Anstee, QM, Rinella, M, Wong, VWS, George, J, Eslam, M; *The role of metabolic health and metabolic adaptation in lean NAFLD;* invited review and submitted for publication in Nature Reviews Gastroenterology and Hepatology 28 January 2020; manuscript no: NRGH-19-237V1

The role of metabolic health and metabolic adaptation in lean MAFLD

Fei Chen[1], Zobair M. Younossi[2], Quentin M Anstee[3], Mary Rinella[4], Vincent Wai-Sun Wong[5], Jacob George[1], Mohammed Eslam[1]

[1] Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney

[2] Center for Liver Diseases, Department of Medicine, Inova Fairfax Hospital, Falls Church, VA, USA

[3] Institute of Translational & Clinical Research, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom; Newcastle NIHR Biomedical Research Centre, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom.

[4] Department of Gastroenterology and Hepatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA.

[5] Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong

Competing interest's statement: The authors declare no competing interests for this manuscript.

Acknowledgements: ME and JG are supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney; a National Health and Medical Research Council of Australia (NHMRC) Program Grant (APP1053206, APP1149976) and Project grants (APP1107178 and APP1108422). FC is supported by the Commonwealth government of Australia Research Training Program (RTP) scholarship and the Westmead Institute of Medical Research Top-up grant. QMA is supported by the EPoS (Elucidating Pathways of Steatohepatitis) consortium funded by the Horizon 2020 Framework Program of the European Union under Grant Agreement 634413, the LITMUS (Liver Investigation: Testing Biomarker Utility in Steatohepatitis) consortium funded by the IMI2 H2020 Framework Program of the European Union under Grant Agreement 777377 and the Newcastle NIHR Biomedical Research Centre.

Corresponding Author

Jacob George: Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Westmead 2145, NSW, Australia, Ph: 61-2-88907705; Fx 61-2-96357582. Email: jacob.george@sydney.edu.au

Mohammed Eslam: Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Westmead 2145, NSW, Australia, Ph: 61-2-88907705. Email: mohammed.eslam@sydney.edu.au

Word count: 6,213 words.

Abstract

Metabolic associated fatty liver disease (MAFLD) affects between 20-30% of the population in many countries; its incidence and prevalence have risen in parallel with rising rates of obesity and type 2 diabetes. MAFLD is a leading cause for end-stage liver disease, cancer and liver transplantation, as well as for death from associated extrahepatic consequences including cardiometabolic disease. However, there is growing evidence that not all obese individuals suffer from the metabolic consequences of obesity, including MAFLD. Conversely, not all lean people are metabolically healthy compared to their obese counterparts and a significant proportion develop fatty liver disease, referred to as lean MAFLD. The clinical characteristics, natural history and pathophysiology of this subgroup of patients is poorly characterised. In this review we describe the epidemiology and natural history of MAFLD and outline the concepts of metabolic health and metabolic adaptation that can be used as a framework to understand the development of MAFLD, including in lean people. It is hoped this will lead to a better understanding of the entity and result in improved methods for diagnosis and management.

Introduction

The global rise in the prevalence of obesity and type 2 diabetes has been the impetus for the growth in non-communicable chronic diseases that places a considerable burden on health systems, both in developed and developing nations(Agbim et al. 2019). Worldwide, the prevalence of overweight and obesity has risen between 1980 to 2013 in both men (from 28.8% to 36.9%) and women (from 29.8% to 38.0%) and is continuing to rise at pandemic rates(Bluher 2019; Ng et al. 2014). Similarly, the prevalence of overweight and obesity in children and adolescents has increased in both developing (up to 12.9% in boys and 13.9% in girls) and developed countries (up to 23.8% in boys and 22.6% in girls)(Ng et al. 2014). Nearly a third of adults in the USA are obese (defined as having a body mass index (BMI) of more than 30 kg/m^2)(Ng et al. 2014), while at least 50% of men in Tonga and women in Kuwait, Kiribati, Libya, Qatar and Samoa are overweight or obese (defined as having a BMI of more than 25 kg/m^2 or $30kg/m^2$, respectively)(Ng et al. 2014).

These increases are driven by a combination of excess energy intake relative to expenditure, nutritionally imbalanced diets, and a sedentary lifestyle with increased physical inactivity. The societal changes are an outcome of increased urbanisation, access to energy dense foods in rural and urban areas and increased availability of labour saving devices and technologies(Bluher 2019; Popkin 2006). A recent study estimated the prevalence of insufficient physical activity at around 23.3% in 2010(Hallal et al. 2012). Alarmingly, a more recent report on worldwide trends in physical inactivity between 2001-2016 suggested that the prevalence of physical inactivity has not altered since 2001, with

the rate being twice as high in high income countries, and rising over time(Guthold et al. 2018).

One of the non-communicable diseases with well-described adverse clinical outcomes that has risen in parallel with obesity is metabolic associated fatty liver disease (MAFLD) (formerly known as NAFLD), which affects about 20-30% of the population(Eslam et al. 2019; Sarin et al. 2020; Z. Younossi et al. 2018a). MAFLD is a leading cause for end-stage liver disease, cancer and liver transplantation, with an estimated 20 million likely to eventually die from liver disease(Bellentani 2017; Le et al. 2017; Z. Younossi et al. 2018b). Using Global Burden of Disease dataset, it has been shown that MAFLD is fastest growing global driver of cirrhosis and liver cancer(Paik et al. 2019). In addition, MAFLD represents a significant economic burden to society that reduces quality of life including through increased symptoms of fatigue and decreased mental well-being. This affects how well a person is able to function in their daily activities (Sayiner et al. 2016). In the United States, patients with MAFLD are reported to have higher annual health care expenditure ($19,390 versus $5,567) with higher rates of unemployment (55% versus 30%) and disability related unemployment (30.5% versus 6.6%) compared to those without chronic liver disease. In Europe, MAFLD is estimated to have an annual cost of about €35billion (from €354 to €1,163 per patient; highest in patients aged 45-65) (Stepanova et al. 2017; Z. M. Younossi et al. 2016b). Although MAFLD and its subtype of NASH have been considered "asymptomatic" diseases, they are associated with significant impairment pf patient reported outcomes(Golabi et al. 2016). In fact, as NASH progresses this negative impact on PROs become even more pronounced(Huber et al. 2019; Z. M. Younossi et al. 2019b; Z. M. Younossi et al. 2019a).

MAFLD classically presents in close association with metabolic syndrome or one of its components, including obesity, hypertension, type 2 diabetes mellitus and dyslipidaemia. Some regard excess liver fat deposition as the hepatic manifestation of the metabolic syndrome (Chalasani et al. 2012; Z. M. Younossi et al. 2016a). Attesting to this link, a 2016 meta-analysis of 21 cohort studies found obesity to be associated with a 3.5-fold increased risk of developing MAFLD, with a dose-dependent relationship to BMI(L. Li et al. 2016). Similarly, dual biopsy natural history studies indicate that incident type 2 diabetes and hypertension are key factors linked to disease progression (McPherson et al. 2015; Singh et al. 2015). In addition to high prevalence, increasing number of components of metabolic syndrome also increases the risk for mortality in MAFLD(Golabi et al. 2018). Finally, it is important to note that MAFLD is not only associated with adverse liver related outcomes (cirrhosis, HCC and liver mortality) but also extrahepatic manifestation such as CVD, extra-hepatic cancers, CKD, Osteoarthritis, obstructive sleep apnea, gallbladder disease and psoriasis(Z. M. Younossi 2019).

Routine assessment of obesity has thus become part of clinical practice with several methods used for its assessment. They include anthropometric methods such as calculating the body mass index (BMI), waist/hip ratio (WHR), densitometry measurements and imaging (Han et al. 2006). BMI and WHR when done together and correctly, provide a rapid and inexpensive measure to estimate body fat and body fat distribution. However, there are inter-operator variations and the cut-offs for different ethnic backgrounds have not been fully defined (Han et al. 2006). Importantly, BMI, the single most frequently used measure of adiposity cannot adequately distinguish lean body mass from fat mass(Agbim et al. 2019). Densitometry measurements and imaging techniques on the other hand can be

used in small-scale studies and provides better information about lean body mass and visceral fat depot distribution. However, these techniques are expensive, time-consuming, involves exposure to radiation, and are not suitable for population level data gathering (Han et al. 2006).

The outcome from many of the reported studies reveals a remarkable and less well-understood paradox that not all obese patients (as defined by BMI) suffer from the metabolic disturbances related to obesity, including MAFLD. Conversely, not all lean people are metabolically healthy compared to their obese counterparts. The concept of "metabolically healthy obesity" present in up to 30% of all obese individuals refers to those with no evidence of metabolic or cardiovascular complications(Wildman et al. 2008). At the other end of the spectrum, 20% of the normal weight adult population are considered metabolically unhealthy with a higher risk for developing MAFLD and greater than a 3-fold higher all-cause mortality and/or cardiovascular event rate compared to those who have normal weight and are metabolically healthy(Das and Chowdhury 2013; Stefan et al. 2017; Z. M. Younossi et al. 2012). This subset of lean individuals with MAFLD is often referred to as lean MAFLD, or as MAFLD arising in lean individuals. Lean MAFLD is usually defined as fatty liver disease in people with a BMI less than 25 kg/m^2 in reports from Caucasian populations and less than 23 kg/m^2 in studies from Asia (D. Kim and Kim 2017). The existence, pathogenesis and long-term outcomes of lean MAFLD are still debated due to inconsistencies in the literature, the heterogeneity of studies and the lack of generally accepted criteria for its definition(A. C. e. a. Dela Cruz 2014; Fracanzani et al. 2011; D. Kim and Kim 2017; J. C. Leung et al. 2017a; Sookoian and Pirola 2017; Z. M. Younossi et al. 2012).

In this review, we describe the epidemiology and natural history of MAFLD and outline concepts of metabolic health and metabolic adaptation that can be used as a framework to understand the development of MAFLD, including disease in lean people. It is hoped that these concepts will lead to a better understanding of the entity and result in improved methods for diagnosis and management.

Lean MAFLD

Epidemiology

The first population study describing lean MAFLD was conducted in Korea in 2004, where lean MAFLD was present in 23.4% of the non-obese population with associated metabolic disorders (H. J. Kim et al. 2004). Since then, lean MAFLD has been described in several Asian and Caucasian reports. (**Figure 1 and Supplementary Table 1**) These data indicate that there are patients with fatty liver who are lean by BMI criteria and secondly that disease prevalence is between 5 - 26% in Asian and 7 - 20% in Western populations (Younes and Bugianesi 2019). In one study, up to 75% of patients with MAFLD and significant liver disease prevalence was shown to have normal BMI in a rural Indian population (Das et al. 2010). However, owing to the lack of a widely accepted definition of "lean" across studies, as well as the heterogeneity in MAFLD diagnostic criteria, the current data suffers from many limitations.

Histological characteristics

The histological characteristics of MAFLD vary between ethnic groups irrespective of BMI classification. A recent systematic review from cross sectional studies shows that liver fibrosis stage is significantly lower in lean compared to overweight/obese MAFLD(Sookoian and Pirola 2018). Similarly, the MAFLD activity scores and presence

of steatohepatitis are lower compared to overweight/obese patients, although there was substantial heterogeneity in the results (Sookoian and Pirola 2018). In contrast, some studies however have reported a more severe histological picture in lean patients with higher rates of advanced fibrosis, ballooning and lobular inflammation, as well as greater steatohepatitis compared to their non-lean counterparts (Denkmayr et al. 2018; Q. Wang et al. 2019).

Genetic contribution

Several gene loci are associated with the risk of MAFLD development and progression; some have been studied after stratification to lean versus non-lean. The most widely examined variant is the *PNPLA3* isoleucine to methionine substitution at position 148 (rs738409 C>G encoding for *PNPLA3* I148M) that induces loss of function of the enzymatic hydrolase activity resulting in entrapment of triglycerides and retinyl esters in lipid droplets within hepatocytes and hepatic stellate cells. The polymorphism consequently leads to liver lipid retention and injury with the end result being fibrosis development and hepatocellular carcinoma (Eslam et al. 2018a; Eslam and George 2020; Liu et al. 2014a). The allele frequency of rs738409 C>G appears to be higher in Asian patients with lean- compared to obese MAFLD, although in a study of Western lean patients, no difference in the risk allele frequency was observed (Fracanzani et al. 2017; Wei et al. 2015). Nevertheless, many reports have demonstrated independent associations of the *PNPLA3* risk allele with steatohepatitis development and higher stages of fibrosis (stage 2 or more) in lean patients (Fracanzani et al. 2017; Wei et al. 2015).

Another variant strongly implicated with MAFLD is the rs58542926 C>T variant in the transmembrane 6 superfamily member 2 (*TM6SF2*) gene which encodes an amino acid

substitution (E167K) involved in the enrichment of triglycerides to apolipoprotein B100 in the pathway for very low density lipoprotein (VLDL) secretion by hepatocytes (Eslam et al. 2016b; Liu et al. 2014b). Carriers of the mutation have higher liver triglyceride content and lower circulating lipoproteins resulting in a greater risk for MAFLD but a lower risk of cardiovascular disease (Eslam et al. 2018a; Kahali et al. 2015). In a recent study, carriers of the risk variant had significantly more endotoxemia (Pang et al. 2017). A report comparing obese to lean MAFLD patients reported higher rates of carriage of the rs58542926 C>T allele in the latter(Fracanzani et al. 2017; Q. Wang et al. 2019).

Recently, the membrane bound O-acyltransferase domain-containing 7 (*MBOAT7*) rs641738 C>T variant was found to be associated with the risk of MAFLD, inflammation and fibrosis, as well as risk of progression to hepatocellular carcinoma (HCC) (Thabet et al. 2016; Thabet et al. 2017). This protein is involved in remodelling of phosphatidylinositol with arachidonic acid as part of the Land's cycle. The rs641738 C>T variant results in downregulation of MBOAT7 at the mRNA and protein level, which reduces the level of phosphatidyl-inositol containing arachidonic acid in hepatocytes and in the circulation(Mancina et al. 2016). In a large study from Austria, there was no significant difference in carriage of the *MBOAT7* rs641738 C>T allele between lean and obese MAFLD patients (Denkmayr et al. 2018). Finally, interferon (IFN) lambda 3/4 variants, initially described to be associated with severity of hepatitis and fibrosis progression in hepatitis C virus infection, has now been recognised to be associated with liver damage in patients with MAFLD, with more profound effect on lean individuals (Eslam et al. 2015b; Petta et al. 2017b).

Prognosis

In contrast to studies that examine the prevalence and presentation of lean MAFLD, data on its long-term prognosis have been scarce and conflicting. Some reports suggest that clinical events and prognosis are worse in the obese compared to the lean MAFLD population, with higher cardiovascular events and death (Fracanzani et al. 2011; J. C. Leung et al. 2017a). One recent study with a median follow up of 49 months reported a clinical event rate of 11.9% in obese compared to 8.3% in the lean MAFLD population (J. C. Leung et al. 2017a). However a study in 2014 by Delacruz et al. looking at the long-term prognosis of lean patients with MAFLD and a median follow up of 11 years has challenged this finding (A. C. e. a. Dela Cruz 2014). This international cohort study included 483 patients with biopsy-proven disease and suggested that the median survival free of liver transplantation was in fact lower in those who were lean compared to obese. This occurred despite having a better metabolic profile and less advanced liver fibrosis (A. C. e. a. Dela Cruz 2014). This result was supported by another report of 646 Swedish patients with biopsy proven MAFLD and a median 19.9 years follow up where although patients with lean disease did not have increased mortality, they had an increased risk for the development and progression to severe liver disease compared to obese patients (hazard ratio 2.69, $p = 0.007$) (Hagstrom et al. 2018).

While lean MAFLD reflects the hepatic manifestation of a metabolically unhealthy normal weight, studies involving other organ systems also indicate that individuals with a metabolically unhealthy phenotype may suffer a worse prognosis despite a normal BMI. Studies of diabetes mellitus in underweight or normal weight people suggest a distinct, albeit less well characterized pathophysiology to disease in the overweight/obese

population, with higher mortality rates(George et al. 2015). Similarly, metabolic health (as measured by the number of components of metabolic syndrome) has been shown to be a stronger predictor for myocardial dysfunction than simply BMI or fat mass alone (Dobson et al. 2016).

Explaining MAFLD pathogenesis in lean individuals: metabolic health and metabolic adaptation

Metabolic health – not all fat is the same

The most widely accepted definition of metabolic health is the absence of insulin resistance, no evidence of subclinical inflammation as determined by high sensitivity C-reactive protein (CRP), together with only one component of the metabolic syndrome according to the Adult Treatment Panel III criteria (Table 1)(Lorenzo et al. 2007; Wildman et al. 2008).

Table 1. Clinical parameters used for the diagnosis of metabolic health

Systemic inflammation	hs-CRP level < 0.1mg/L
Insulin resistance	HOMA-IR < 5.13
Plus only **one** (or none) of the following components:	
Clinical parameter	**Criteria for metabolic abnormality**
Blood pressure	Systolic/diastolic blood pressure ≥ 130/85 mmHg or anti-hypertensive drug use
Triglyceride level	Fasting triglyceride level ≥ 150mg/dL (or ≥ 1.7 mmol/L)
HDL-C level	HDL-C level < 40mg/dL (or < 1.0 mmol/L) in men or < 50mg/dL (or < 1.3 mmol/L) in women or use of lipid lowering medication
Glucose level	Fasting glucose level ≥ 100mg/dL (or ≥ 5.6 mmol/L) or use of anti-diabetic medication

Metabolic health is defined as absence of systemic inflammation with only one (or none) other component of metabolic syndrome. Abbreviations: hs-CRP, high sensitivity C-

reactive protein; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model of insulin resistance.

Mechanisms for variation in metabolic health

While it is not entirely clear as to the underlying mechanisms explaining individual variation in metabolic health, the term "adiposopathy" or defective/sick adipose tissue has been introduced. Adiposopathy governs an individual's cardiometabolic risk, above and beyond BMI alone(H. E. Bays 2011) and refers to the pathogenic enlargement of fat cells and fat tissue, resulting in anatomic and functional disturbances leading to altered lipid metabolism, adipose inflammation and adverse clinical outcomes(H. E. Bays et al. 2008).

However, given that adipose tissue is not a single, functionally uniform organ, it is not only how fat is stored (adipocyte proliferation versus adipocyte hypertrophy) that matters, but where the fat is stored (visceral versus subcutaneous, upper body versus lower body) and the type of fat (brown versus white). The 'where' and 'type' of adiposity has a greater impact on an individual's metabolic health than total fat mass(Iacobini et al. 2019). Thus, visceral and subcutaneous adipose tissues differ with regards to their contribution to metabolic risk. Visceral adipose tissue (VAT) as well as ectopic fat in or around the liver, heart and skeletal muscle lipid content (intramyocellular) has been linked to impaired glucose homeostasis, insulin resistance and cardiovascular disease(Lim and Meigs 2013). Generally, subcutaneous adipose tissue (SAT) is believed to confer significantly lower metabolic risk. However the distribution of SAT seems to matter with lower body SAT (gluteofemoral body fat) being characteristic of metabolically healthy individuals and aa lower risk for metabolic diseases (Goodpaster et al. 2005; Manolopoulos et al. 2010). In contrast, and upper body subcutaneous fat, the primary source of circulating free fatty acids

and hence an important determinant of insulin resistance and metabolic impairment. This has been demonstrated in several disease states associated with accumulation of upper body fat, including Cushing's syndrome, lipodystrophy and human immunodeficiency virus associated lipodystrophy (Ebbert and Jensen 2013; J. J. Lee et al. 2017) (**Figure 2**).

The adipocyte responds to positive energy balance through adipocyte hypertrophy as well as adipocyte hyperplasia (i.e. recruitment and proliferation of adipocyte precursors). Adipose tissue expandability and the increase in fat mass, especially SAT expansion, has been linked in previous studies to metabolic improvement and protection from type 2 diabetes(J. Y. Kim et al. 2007; McLaughlin et al. 2011). Whereas SAT expansion protects from metabolic risk, expansion of VAT or limited expansion of SAT is strongly associated with insulin resistance due to its hyperlipolytic state that is resistant to the anabolic actions of insulin, thereby producing larger amounts of circulating free fatty acids(Despres and Lemieux 2006; O'Connell et al. 2010). Although both SAT and VAT sizes correlate with the degree of fatty liver, only VAT size is related to metabolic health and progression from hepatic steatosis to fibrosis(O'Connell et al. 2010). Previous studies have shown that surgical removal of abdominal SAT through liposuction does not improve insulin resistance in obese individuals, whereas transplantation of SAT into the abdominal cavity results in improved insulin sensitivity and glucose metabolism. This supports the notion that differences in metabolic health appear to be reflected by the "fitness" of SAT, while dysfunctional SAT (adiposopathy) is characteristic of the metabolically unhealthy state (Iacobini et al. 2019; Klein et al. 2004; Tran et al. 2008).

Factors that influence metabolic health (adiposopathy)

Ethnic differences

Racial/ethnic differences impact fat distribution and obesity and through this, metabolic health. For example, data from population datasets of the Framingham Heart Study (FHS) and the Jackson Heart study (JHS) as well as the National Health and Nutrition Examination Survey (NHANES) reveal that age-adjusted adult obesity prevalence is higher in Hispanics and non-Hispanic blacks compared to non-Hispanic whites and Asians(Agbim et al. 2019). Plasma adiponectin levels, an adipocytokine associated with cardiometabolic health is higher in non-Hispanic whites compared to non-Hispanic blacks and higher in women than men (Jiang et al. 2016).

Correspondingly, studies of fat distribution between ethnic groups suggest that when total body fat is controlled, persons of South Asian and Chinese ancestry have more VAT than Europeans, with the impact of body weight gain more detrimental in Asians compared to non-Hispanic whites, Hispanics and non-Hispanic blacks. Each one unit increase in BMI is thus associated with a higher risk of hypertension and diabetes in Asians(Agbim et al. 2019; R. J. Wong et al. 2014). As a result, the WHO has proposed a BMI cut-off as a trigger for public health action for Asians, with a BMI of $\geq$ 23 kg/m^2 as increased risk, $\geq$27.5 kg/m^2 as high risk, $\geq$32.5 kg/m^2 as higher than high risk and $\geq$37.4 kg/m^2 as the highest risk, as they have higher body fat percentage at a lower BMI compared with non-hispanic whites(WHO 2004). While the underlying mechanism(s) governing these ethnic differences remain unclear, the increased risk of cardiometabolic disorders seen in lower obesity prevalence ethnicities suggest that metabolic health plays a greater role than BMI alone, and might explain why lean MAFLD exists.

Environmental factors

Lifestyle habits clearly contribute to the heterogeneity of metabolic health between individuals. Population based cross-sectional studies have also shown that metabolically healthy obesity is more prevalent in younger and female adults, and that these individuals are more likely to exercise and less likely to smoke or drink heavily (Goday et al. 2016; Matheson et al. 2012). The possible mechanisms governing this difference may lie in how individuals modulate whole body energy metabolism as evidenced by the fact that concurrent physical activity increases fatty acid oxidation during high calorie intake periods (S. R. Smith et al. 2000). In addition, a healthy diet and lifestyle is associated with a lower fasting respiratory quotient (which assesses nutrient utilization using indirect calorimetry by measuring the ratio between carbon dioxide production and oxygen consumption) and an increased ability to extract energy from fat (Pujia et al. 2016). Other lifestyle factors contributing to cardio metabolic health risk include sleep duration and sleep quality factors (Koren and Taveras 2018). A study in China for example found that patients with MAFLD had a shorter duration of sleep compared to healthy controls (C. Li et al. 2019). The most commonly used definition of a healthy lifestyle includes the incorporation of four healthy habits which include moderate alcohol intake, not smoking, 30 minutes of daily exercise and eating five or more servings of vegetables and fruits a day (Matheson et al. 2012).

In the first study on the lifestyle of patients with different types of MAFLD, total caloric intake as well as carbohydrate, protein, fat and iron intake was similar between lean and non-lean MAFLD patients compared to healthy controls (C. Li et al. 2019). Another study demonstrated that the percentage of carbohydrate energy intake is higher in lean MAFLD

patients compared to healthy controls (Kwak et al. 2018). This suggests that an unhealthy diet in those with lean MAFLD further contributes to their adverse metabolic health status.

Genetic factors

Considerable inter-individual variation exists with regards to the metabolic risk for a given BMI. Evidence for a role for genetics in determining how an individual responds to excess energy dates back over 25 years ago where a study involving 12 pairs of identical twins demonstrated variations in weight gain and fat distribution among the pairs in response to overfeeding(Bouchard et al. 1990). In that study, monozygotic young male twins were overfed a total of 84,000 kCal over 100-days. There were significant similarities within each pair with respect to weight gain, changes in regional fat distribution and amount of abdominal visceral fat, but about six times as much variance between pairs (Bouchard et al. 1990)

The waist hip ratio used as a surrogate measure of regional fat distribution is estimated to have a heritable contribution of up to 60%, independent of the risk for overall obesity (Schleinitz et al. 2014). In the era of genome wide association studies (GWAS), many loci have been identified that regulate obesity and control body fat distribution, as well as the metabolic profile of excess adiposity (i.e. metabolically healthy obesity versus metabolically unhealthy obesity)(Iacobini et al. 2019). As an example, a single nucleotide polymorphism (SNP) near the *MC4R* (melanocortin 4 receptor) gene that is involved in insulin resistance and obesity is one of the major loci associated with waist circumference (J. C. Chambers et al. 2008). A meta-analysis of GWAS in 2010 uncovered 13 loci associated with WHR adjusted for BMI; the known association signal at *LYPLAL1* involved in lipase activity was confirmed, with effect sizes reaching 0.059 per risk allele in

women (Heid et al. 2010). Many of these loci are associated with metabolic traits such as fasting glucose, insulin, adiponectin levels and BMI, as well as with diseases such as type 2 diabetes, hypertension and coronary artery disease(Kilpelainen et al. 2011; Schleinitz et al. 2014). Equally, several gene variants have been associated with a lower risk of metabolic abnormalities despite having a BMI in the obese range(Yaghootkar et al. 2016).

Interestingly, the same genetic variants seem to share similar patterns of metabolic trait association with the monogenic lipodystrophy phenotype, including lower BMI, higher VAT to SAT ratio, impaired insulin sensitivity and increased risk of type 2 diabetes (Iacobini et al. 2019; Yaghootkar et al. 2014). This suggests that unlike in specific altered fat distribution phenotypes like lipodystrophy where there is a clear genetic mutation involved, the common type of obesity and leanness is polygenic in nature and influences fat distribution (visceral versus subcutaneous) and metabolic traits, with individual variants having low effect sizes and with further modulation by factors such as epigenetic, environmental and biologic factors (Schleinitz et al. 2014).

Although there have been no specific studies looking into the genetic variations in lean MAFLD patients, the studies above shed light into the heritability of adiposity and fat distribution, which may partly explain the underlying pathophysiology of lean MAFLD.

Epigenetic factors

Despite advances in genetic analyses to identify polymorphisms associated with waist hip ratio and fat distribution, the reported variants only explain a small proportion of the phenotypic variance and genetic heritability(Eslam et al. 2018a). Therefore, other factors linking genetic to environmental risks such as epigenetics need to be considered (Hardy and Mann 2016). Epigenetic changes link an individual's genetic background with

environmental (exposure) cues and are dynamically regulated throughout an individual's lifespan(Hardy and Mann 2016). Known epigenetic modifiers include DNA methylation, histone modifications and chromatin remodeling, and non-coding RNAs (Hardy and Mann 2016).

Strong adipose tissue-specific gene expression patterns in early development seem to be preserved from one pre-adipocyte to the next over several generations, suggesting the existence of yet unknown mechanisms to maintain these expression profiles over time(Schleinitz et al. 2014). Genome-wide methylation analysis using methylated DNA immunoprecipitation sequencing of eight different adipose depots in three pig breeds displaying different fat levels despite living in a comparable environment demonstrated functionally relevant methylation differences between different adipose depots. Visceral adipose tissue which carries the highest metabolic risk was associated with impaired inflammatory and immune responses(M. Li et al. 2012). Whether similar differences occur in humans is not yet known.

Several human studies support a role for epigenetics in the regulation of fat distribution. The expression of leptin, an important adipokine involved in regulation of energy homeostasis primarily in adipocytes, is positively correlated to adiposity. The overall expression and tissue distribution of leptin is influenced to a degree by the DNA methylation pattern at the leptin promoter, which is determined during embryogenesis, and remains stable despite alterations in leptin expression levels in white adipose tissue during changes in body weight(Marchi et al. 2011). In another recent study, altered DNA methylation at the *IGF2/H19* locus as a result of an adverse in-utero environment was associated with changes in subcutaneous fat measures, but not visceral or central

adiposity(Huang et al. 2012). This suggests an important role for epigenetics in determining fat distribution from very early life.

Studies involving several species have demonstrated that oscillations in intrauterine and early postnatal nutritional, metabolic and hormonal environments increase the susceptibility to develop metabolic disorders and diseases in later life(Plagemann 2004). For example, maternal gestational diabetes and early postnatal overfeeding have been associated with fetal and neonatal hyperinsulinemia (Plagemann 2004). Furthermore, maternal nutrition during pregnancy contributes to perinatal programming of the genome which has an influence on fetal body composition and fat distribution, and ultimately the risk of obesity and metabolic diseases later in life(Blumfield et al. 2012). This 'embryonic or fetal programming' suggests that metabolic health and adiposopathy is trans-generational (H. Bays and Scinta 2015).

Overall the metabolic health of an individual plays a large part in the pathogenesis of MAFLD, is partly determined in the early stages of life, and remains stable throughout a life, despite variations in environmental stimuli.

The world within – Gut microbiota, bile acids and the enterohepatic circulation

Aside from calorie content, the nutrient composition of a person's diet plays an important role in overall physiological responses including the gut microbiota profile, adiposity and insulin resistance. About 10-100 trillion micro-organisms composed of bacteria, fungi, archae and viruses reside inside or on the body. The majority of these microbial symbionts (collectively known as the microbiota) reside within the digestive tract (Turnbaugh et al. 2007). Four main phyla of bacteria make up the human microbiome: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria, although the Firmicutes and

Bacteroidetes make up about more than 90% of the gut microbiota species (Turnbaugh et al. 2007).

Diet exerts a dominant effect on microbiota composition, irrespective of the host genotype (Carmody 2015). Studies have also shown that long-term dietary habits are strongly associated with specific enterotype clustering of microbiota, where a *Bacteroides* enterotype is associated with a diet rich in animal fat and protein and the *Prevotella* enterotype with carbohydrate rich diets (De Filippo et al. 2010; Wu et al. 2011). Furthermore, although alterations in diet can lead to changes in microbiota composition within 24 hours, an individual's enterotype identity is only affected by their long-term dietary habits (Wu et al. 2011). Common to all published studies in the literature on the role of microbiota in MAFLD and lean MAFLD, is that there are measurable differences in the microbiome diversity and composition between different stages of MAFLD and steatohepatitis compared to healthy controls (B. Wang et al. 2016; Wieland et al. 2015).

In the gut, in addition to their roles in energy harvest of nutrients and regulation of mucosal permeability and inflammation, the microbiome also plays important roles in bile acid physiology, through the conversion of primary to secondary bile acids (Tremaroli and Backhed 2012). Bile acids (BA) are steroid molecules synthesized in the liver from cholesterol through the actions of about 15 enzymes. The primary bile acids chenodeoxycholate (CDCA) and cholate (CA) are synthesized from cholesterol, mostly through the classical pathway, initiated by the rate-limiting enzyme cytochrome P450 cholesterol 7α-hydroxylase (CYP7A1)(Arab et al. 2017). A small proportion of the BA pool (between 3% to 18%) is synthesized through an alternative pathway initiated by cytochrome P450 27α-hydroxylase (CYP27A1)(Khalid et al. 2015). Primary BAs are then

conjugated into their taurine or glycine conjugates and excreted into bile where they assist in fat emulsification and absorption. Primary bile acids undergo conversion to the secondary bile acids deoxycholate (DCA), lithocolic acid (LCA) and ursodeoxycholic acid (UDCA) by intestinal bacteria and are mostly reabsorbed in the distal ileum via the enterohepatic circulation (Arab et al. 2017; Khalid et al. 2015). The conversion of primary to secondary bile acids requires an initial deconjugation by bile salt hydrolase (BSH) before downstream modifications by 7-alpha dehydroxylase to produce deoxycholic acid (DCA) and lithocholic acid (LCA) or by 7-alpha hydroxysteroid dehydrogenase to produce ursodeoxycholic acid (UDCA) (Jiao et al. 2017; Ridlon et al. 2006). BSH activity is present in all major gut bacterial species, however the conversion of primary to secondary bile acids by 7-dehydroxylation is restricted to bacteria with bile acid inducible genes. These include those belonging to the genera *Clostridium* (clusters XIVa and XI), *Eubacterium, Blautia, Ruminococcaceae* and *Lachnospiraceae*, all of which belong to the *Firmicutes* phylum (Wahlstrom et al. 2016a; Yokota et al. 2012).

Alteration in the gut microbiota profile therefore, concert with an individual's genetic make-up and dietary intake, contributes to the propensity to develop metabolic diseases including obesity and MAFLD through regulation of bile acid composition which eventually affects lipid and glucose metabolism (Dabke et al. 2019) (**Figure 3**).

Besides their role in the digestion and absorption of fat and fat soluble vitamins, bile acids are signalling molecules involved in the regulation of lipid and glucose metabolism, as well as inflammatory modulators in the liver and several other tissues (Arab et al. 2017; Chavez-Talavera et al. 2017; Khalid et al. 2015). These actions are mediated through binding specific bile acid receptors, including members of the farsenoid X receptor (FXR),

pregnane X receptor (PXR), Vitamin D receptor and Takeda G protein coupled receptor 5 (TGR5) families (Arab et al. 2017).

The binding of bile acids to FXR in ileocytes trigger transcription and production of Fibroblast Growth Factor 19 (FGF-19), which is then transported to the liver where it binds to the tyrosine kinase FGF receptor 4 (FGFR4)/beta Klotho complex(Khalid et al. 2015). This activates c-Jun N terminal-kinases 1/2 signalling and subsequently down regulates CYP7A1 the key P450 enzyme in the classical bile acid synthesis pathway (Khalid et al. 2015). FXR can be stimulated by most bile acids although at varying potency, with CDCA displaying the highest potency, followed by DCA and CA, then LCA (Khalid et al. 2015). In addition to regulating bile acid synthesis, FGF-19 plays a significant role in glucose and cholesterol homeostasis by promoting hepatic glycogen storage, fatty acid beta oxidation and decreasing hepatic lipogenesis, as well as playing a role in liver regeneration/ repair (Arab et al. 2017; Chavez-Talavera et al. 2017; Khalid et al. 2015). BA can also activate TGR5 receptors with different potencies (LCA>DCA>CDCA>CA) in the enteroendocrine L cells along the gastrointestinal tract (mostly in the colon) which subsequently induces preproglucagon gene expression and glucose like peptide-1 (GLP-1) secretion (Chavez-Talavera et al. 2017). Both FXR and TGR5 are also expressed in pancreatic β-cells where they have positive effects on the synthesis and secretion of insulin, in response of glucose intake, suggesting a crucial role for BA in glucose homeostasis (Chavez-Talavera et al. 2017).

FXR and TGR5 receptors are also differentially expressed in adipocytes, FXR in white, and TGR5 in brown adipocytes, respectively, as well as in certain immune-inflammatory cells in adipose tissue(E. P. Broeders 2015). In adipocytes, FXR regulates the

differentiation and functions of adipocytes and promotes peroxisome proliferator-activated receptor-γ (PPARγ) activity which interferes with the Wnt/β-catenin pathway, while TGR5 activates the thyroid hormone receptor to uncouple mitochondrial function and increase thermogenesis in brown adipose tissue, which further contributes to their anti-inflammatory and insulin-sensitizing effects (Abdelkarim et al. 2010; Watanabe et al. 2006). The enzymes involved in bile acid synthesis are controlled tightly in response to changing metabolic conditions and metabolic alterations, along with chronic low-grade inflammation, which are characteristics of meta-inflammatory disorders such as obesity, type 2 diabetes and MAFLD(Chavez-Talavera et al. 2017; Xie et al. 2015).

Therefore, the interplay between an individual's lifestyle factors, combined with their microbiota and bile acid profile, shaped in part by their dietary composition and genetic as well as epigenetic backgrounds, has a significant impact on an individual's overall metabolic health. This in turn governs the risk for metabolic disorders, including MAFLD.

Relationship between metabolic health and MAFLD

Given that metabolic health status (defined as per **Table 1**) is an integral aspect of MAFLD pathophysiology, several studies have investigated the relationship between metabolic health and MAFLD. In these, the risk of developing steatohepatitis and significant fibrosis increases progressively as the number of metabolic risk factors increases(Ampuero et al. 2018). Consistently, a cross-sectional study of more than 1,000 patients with biopsy proven MAFLD demonstrated that metabolic health has a greater impact on the risk of NASH development, significant fibrosis, atherogenic dyslipidaemia and kidney dysfunction than obesity or BMI alone(Ampuero et al. 2018). That study also found a similar risk for steatohepatitis and fibrosis in a metabolically unhealthy group, regardless of their body

weight, suggesting that metabolic health has a greater impact on the severity of liver disease than BMI, possibly through unfavourable body fat distribution (and/or as yet unknown factors) with a long but important period of subclinical systemic inflammation(Ampuero et al. 2018). Similar findings have been demonstrated in Asian and Mexican populations (Gutierrez-Grobe et al. 2017; M. K. Lee et al. 2015; Sung et al. 2014).

Despite these data, metabolically healthy obesity cannot be considered entirely benign as it carries almost double the risk of steatohepatitis compared to individuals who are metabolically healthy and normal weight(Sung et al. 2014). This implies that healthy obesity (acting through subclinical or as yet be discovered impacts on metabolic health) perhaps represents a "honeymoon phase" that in some individuals eventually progresses to a metabolically unhealthy obese state (Kramer et al. 2013). Conversely, the presence of MAFLD can promote (or at least be associated with) the conversion of an individual's metabolic health from metabolically healthy to metabolically unhealthy, independent of age, sex, BMI, lifestyle factors, individual components of metabolic syndrome and insulin resistance. The effect is greater in those with a lower BMI and body fat mass compared to those with high BMI and body fat mass(Hwang et al. 2019).

Metabolic adaptation

The human body has great capacity to maintain body weight homeostasis through effects on food intake and energy expenditure. The ability of the body to increase or decrease energy expenditure beyond the obligatory energy costs of depositing and maintaining new tissues, digesting food, moving and maintaining body mass, without any change in body mass is defined as metabolic adaptation(Johannsen et al. 2019). Adaptation is achieved

through a fine balance of regulatory systems through the interaction of hormones, chemokine signals and the neuroendocrine axis (Johannsen et al. 2019). In response to certain nutrition and/or physical activity conditions, several cytokines or peptides secreted from muscles (myokines), adipose tissue (adipokines) and liver (hepatokines) engage in cross-talk to maintain energy homeostasis by governing lipid and glucose metabolism as well as by mediating local and systemic inflammation. Any perturbations in the systems involved results in loss of metabolic adaptation, resulting in abnormal expansion of adipose tissue and obesity, hepatic fat accumulation, and insulin resistance(Oh et al. 2016).

In addition, the enterohepatic circulation including bile acids (BA) and their metabolites, as well as gut microbiota play important roles in metabolic adaptation which occurs in part due to genetic and developmental influences(Wahlstrom et al. 2016a). A number of early experiments involving protein overfeeding have shown large variations in weight gain among nonrelated subjects but high correlation within twin pairs(Bouchard et al. 1990). Further, studies have shown that the change in energy expenditure was due to a change in non-exercise activity thermogenesis(Diaz et al. 1992; Leibel et al. 1995). This concept of metabolic adaptation may explain why some individuals appear to be obesity resistant while others gain weight easily when challenged with caloric abundance.

Impact of metabolic adaptation on lean MAFLD pathogenesis

Given the complex and multifactorial pathogenesis of MAFLD(Buzzetti et al. 2016) and knowing that not all obese people have MAFLD and not all MAFLD patients are obese(Younes and Bugianesi 2019), how an individual adapts to an unfavorable set of metabolic circumstances will govern when he/she will manifest fatty liver disease. This

adaptive ability is the capability of the body to increase or decrease energy expenditure beyond obligatory energy requirements without any change in body mass.

Thus, an individual may respond to increased dietary cholesterol or caloric intake with appropriate metabolic adaptation to maintain body weight, or they may have complete loss of metabolic adaptation, resulting in weight gain, with increased adiposity/adiposopathy and hepatic fat accumulation. In other individuals, increased caloric intake may only result in partial loss of metabolic adaptation, where the outcome is lean MAFLD. In this scenario, as outlined in **Figure 4** as an example, increased dietary cholesterol in the context of perturbed metabolic adaptive capacity (shaped by their background genetic, epigenetic and gut microbiota profile), associates with some metabolic adaptation through increased production of bile acids, especially secondary bile acids and increased FXR activity to maintain body weight and serum cholesterol levels(F. Chen et al. 2019). This metabolic adaptation as reflected by higher FXR activity (measured through FGF-19 levels) and lower C4 levels (bile acid synthetic marker) may explain why these patients have better liver histology, at least in the earlier stages of their disease(F. Chen et al. 2019). Interestingly, with regard to nutrients, other studies have suggested that patients with lean MAFLD have higher dietary cholesterol and lower dietary polyunsaturated fatty acids (PUFAs are associated with lower hepatic TNFα and improved insulin resistance) intake compared to non-lean MAFLD patients and healthy controls(Enjoji et al. 2012; Yasutake et al. 2009a). Similar findings have been demonstrated in animal models where feeding a cholesterol rich diet results in steatohepatitis without obesity or insulin resistance(Kainuma et al. 2006). In addition, dietary cholesterol itself, rather than hepatic steatosis is associated

with the risk of progression to hepatic inflammation in murine models(Wouters et al. 2008).

BMI – A marker of maladaptation?

Given that metabolic health and metabolic adaptive capacity have an impact on the risk for and the progression of MAFLD, equally BMI may be a less robust predictor of MAFLD outcomes. In this context, BMI is thus perhaps better considered a marker of maladaptation. Consideration in future therefore needs be given to classifying MAFLD based on metabolic health and adaptive responses rather than measures of BMI. How this is quantified remains an open question.

Clinical implications and future directions – MAFLD is not a single disease entity

In this review, we have presented data on the complex pathophysiology of MAFLD focusing mainly on lean MAFLD, a distinct subset with poor metabolic health but better metabolic adaptation. Therefore, lean and non-lean MAFLD represents one of the best examples of disease heterogeneity, and the wide spectrum of disease. An obvious implication is that future clinical trials should stratify patients into lean and non-lean, as the subgroups have a different underlying patho-biology and drivers, and likely differences in outcome. In addition, given the importance of metabolic health to MAFLD pathophysiology, classification of patients based on their metabolic health status warrants further attention.

Conclusion

Lean MAFLD presents as a unique phenotype of patients with fatty liver disease with distinct characteristics. Metabolic health status plays a major role in the development of

MAFLD and among lean individuals with the disease, their genetic, epigenetic, gut microbiota and bile acid profiles, enterohepatic circulation and lifestyle factors explain their phenotype despite a normal BMI. The distinct and better adaptation of lean patients allows them to respond to adverse metabolic inputs to maintain lean body weight despite an increase in cardiometabolic risk. Whether or not this partial metabolic adaptation is preserved in the long run and what triggers the switch to maladaptation with disease progression remains to be elucidated.

References

Abdelkarim, Mouaadh, et al. (2010), 'The Farnesoid X Receptor Regulates Adipocyte Differentiation and Function by Promoting Peroxisome Proliferator-activated Receptor-γ and Interfering with the Wnt/β-Catenin Pathways', *Journal of Biological Chemistry,* 285 (47), 36759-67.

Agbim, U., et al. (2019), 'Ethnic Disparities in Adiposity: Focus on Non-alcoholic Fatty Liver Disease, Visceral, and Generalized Obesity', *Curr Obes Rep,* 8 (3), 243-54.

Alam, S., et al. (2014), 'Clinical, anthropometric, biochemical, and histological characteristics of nonobese nonalcoholic fatty liver disease patients of Bangladesh', *Indian J Gastroenterol,* 33 (5), 452-7.

Albaugh, V. L., et al. (2019), 'Role of Bile Acids and GLP-1 in Mediating the Metabolic Improvements of Bariatric Surgery', *Gastroenterology,* 156 (4), 1041-51 e4.

Ampuero, J., et al. (2018), 'The effects of metabolic status on non-alcoholic fatty liver disease-related outcomes, beyond the presence of obesity', *Aliment Pharmacol Ther,* 48 (11-12), 1260-70.

Angulo, 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), 389-97 e10.

Arab, J. P., et al. (2017), 'Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives', *Hepatology,* 65 (1), 350-62.

Asrani, S. K., et al. (2019), 'Burden of liver diseases in the world', *J Hepatol,* 70 (1), 151-71.

Bays, H. and Scinta, W. (2015), 'Adiposopathy and epigenetics: an introduction to obesity as a transgenerational disease', *Curr Med Res Opin,* 31 (11), 2059-69.

Bays, H. E. (2011), 'Adiposopathy is "sick fat" a cardiovascular disease?', *J Am Coll Cardiol,* 57 (25), 2461-73.

Bays, H. E., et al. (2008), 'Is adiposopathy (sick fat) an endocrine disease?', *Int J Clin Pract,* 62 (10), 1474-83.

Bedossa, P. (2016), 'Histological Assessment of NAFLD', *Dig Dis Sci,* 61 (5), 1348-55.

Bellentani, S. (2017), 'The epidemiology of non-alcoholic fatty liver disease', *Liver Int,* 37 Suppl 1, 81-84.

Bergman, Y. and Cedar, H. (2013), 'DNA methylation dynamics in health and disease', *Nat Struct Mol Biol,* 20 (3), 274-81.

Bernstein, B. E., Meissner, A., and Lander, E. S. (2007), 'The mammalian epigenome', *Cell,* 128 (4), 669-81.

Bhat, G., et al. (2013), 'Insulin resistance and metabolic syndrome in nonobese Indian patients with non-alcoholic fatty liver disease', *Trop Gastroenterol,* 34 (1), 18-24.

Bian, E. B., et al. (2013), 'DNA methylation: new therapeutic implications for hepatic fibrosis', *Cell Signal,* 25 (1), 355-8.

Bluher, M. (2019), 'Obesity: global epidemiology and pathogenesis', *Nat Rev Endocrinol,* 15 (5), 288-98.

Blumfield, M. L., et al. (2012), 'Dietary balance during pregnancy is associated with fetal adiposity and fat distribution', *Am J Clin Nutr,* 96 (5), 1032-41.

Bouchard, C., et al. (1990), 'The response to long-term overfeeding in identical twins', *N Engl J Med,* 322 (21), 1477-82.

Broeders, E. P. (2015), 'The bile acid chenodeoxycholic acid increases human brown adipose tissue activity', *Cell Metab.,* 22, 418-26.

Broeders, E. P. M., et al. (2015), 'The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity', *Cell Metabolism,* 22 (3), 418-26.

Browning, M. G., et al. (2019), 'Changes in Bile Acid Metabolism, Transport, and Signaling as Central Drivers for Metabolic Improvements After Bariatric Surgery', *Curr Obes Rep*.

Brunt, E. M., et al. (2011), 'Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings', *Hepatology,* 53 (3), 810-20.

Buzzetti, E., Pinzani, M., and Tsochatzis, E. A. (2016), 'The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD)', *Metabolism,* 65 (8), 1038-48.

Cani, P. D., et al. (2008), 'Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet-Induced Obesity and Diabetes in Mice', *Diabetes,* 57 (6), 1470-81.

Carmody, R. N. (2015), 'Diet dominates host genotype in shaping the murine gut microbiota', *Cell Host Microbe,* 17, 72-84.

Chalasani, N., 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), 1592-609.

Chambers, A. P., et al. (2011), 'Weight-independent changes in blood glucose homeostasis after gastric bypass or vertical sleeve gastrectomy in rats', *Gastroenterology,* 141 (3), 950-8.

Chambers, J. C., et al. (2008), 'Common genetic variation near MC4R is associated with waist circumference and insulin resistance', *Nat Genet,* 40 (6), 716-8.

Chavez-Talavera, O., et al. (2017), 'Bile Acid Control of Metabolism and Inflammation in Obesity, Type 2 Diabetes, Dyslipidemia, and Nonalcoholic Fatty Liver Disease', *Gastroenterology,* 152 (7), 1679-94 e3.

Chen, C. H., et al. (2006), 'Prevalence and risk factors of nonalcoholic fatty liver disease in an adult population of taiwan: metabolic significance of nonalcoholic fatty liver disease in nonobese adults', *J Clin Gastroenterol,* 40 (8), 745-52.

Chen, F., et al. (2019), 'Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation', *Hepatology*.

Cho, H. C. (2016), 'Prevalence and Factors Associated with Nonalcoholic Fatty Liver Disease in a Nonobese Korean Population', *Gut Liver,* 10 (1), 117-25.

Choo, J. M., Leong, L. E. X., and Rogers, G. B. (2015), 'Sample storage conditions significantly influence faecal microbiome profiles', *Scientific Reports,* 5.

Chu, H., et al. (2018), 'Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease', *Gut*.

Cole, A. J., et al. (2015), 'The Influence of Bariatric Surgery on Serum Bile Acids in Humans and Potential Metabolic and Hormonal Implications: a Systematic Review', *Curr Obes Rep,* 4 (4), 441-50.

Dabke, K., Hendrick, G., and Devkota, S. (2019), 'The gut microbiome and metabolic syndrome', *J Clin Invest,* 129 (10), 4050-57.

Das, K. and Chowdhury, A. (2013), 'Lean NASH: distinctiveness and clinical implication', *Hepatol Int,* 7 Suppl 2, 806-13.

Das, K., et al. (2010), 'Nonobese population in a developing country has a high prevalence of nonalcoholic fatty liver and significant liver disease', *Hepatology,* 51 (5), 1593-602.

De Filippo, C., et al. (2010), 'Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa', *Proc Natl Acad Sci U S A,* 107 (33), 14691-6.

Del Chierico, F., et al. (2017a), 'Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach', *Hepatology,* 65 (2), 451-64.

Del Chierico, F., et al. (2017b), 'Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach', *Hepatology,* 65 (2), 451-64.

Dela Cruz, A. C., et al. (2014), 'Characteristics and Long-Term Prognosis of Lean Patients With Nonalcoholic Fatty Liver Disease', *Gastroenterology,* 146 (5), S909-S09.

Dela Cruz, A. C. et al (2014), 'Characteristics and long-term prognosis of lean patients with nonalcoholic fatty liver disease', *Gastroenterology,* 146 (S-909).

Denkmayr, L., et al. (2018), 'Lean Patients with Non-Alcoholic Fatty Liver Disease Have a Severe Histological Phenotype Similar to Obese Patients', *J Clin Med,* 7 (12).

Despres, J. P. and Lemieux, I. (2006), 'Abdominal obesity and metabolic syndrome', *Nature,* 444 (7121), 881-7.

Diaz, E. O., et al. (1992), 'Metabolic response to experimental overfeeding in lean and overweight healthy volunteers', *Am J Clin Nutr,* 56 (4), 641-55.

Ding, C., Chan, Z. L., and Magkos, F. (2016), 'Lean, but not healthy: the "metabolically obese, normal-weight' phenotype', *Current Opinion in Clinical Nutrition and Metabolic Care,* 19 (6), 408-17.

Dobson, R., et al. (2016), 'Metabolically healthy and unhealthy obesity: differential effects on myocardial function according to metabolic syndrome, rather than obesity', *Int J Obes (Lond),* 40 (1), 153-61.

Dutia, R., et al. (2015), 'Temporal changes in bile acid levels and 12alpha-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes', *Int J Obes (Lond),* 39 (5), 806-13.

Duwaerts, C. C. and Maher, J. J. (2014), 'Mechanisms of Liver Injury in Non-Alcoholic Steatohepatitis', *Curr Hepatol Rep,* 13 (2), 119-29.

Ebbert, J. O. and Jensen, M. D. (2013), 'Fat depots, free fatty acids, and dyslipidemia', *Nutrients,* 5 (2), 498-508.

Eckel, N., et al. (2015), 'Characterization of metabolically unhealthy normal-weight individuals: Risk factors and their associations with type 2 diabetes', *Metabolism,* 64 (8), 862-71.

Enjoji, M., et al. (2012), 'Nutrition and nonalcoholic Fatty liver disease: the significance of cholesterol', *Int J Hepatol,* 2012, 925807.

Eslam, M. and George, J. (2016), 'Genetic and epigenetic mechanisms of NASH', *Hepatol Int,* 10 (3), 394-406.

--- (2020), 'Genetic contributions to NAFLD: leveraging shared genetics to uncover systems biology', *Nat Rev Gastroenterol Hepatol,* 17 (1), 40-52.

Eslam, M., Valenti, L., and Romeo, S. (2018a), 'Genetics and epigenetics of NAFLD and NASH: Clinical impact', *J Hepatol,* 68 (2), 268-79.

--- (2018b), 'Genetics and epigenetics of NAFLD and NASH: Clinical impact', *Journal of Hepatology,* 68 (2), 268-79.

Eslam, M., Sanyal, A. J., and George, J. (2019), 'Toward More Accurate Nomenclature for Fatty Liver Diseases', *Gastroenterology,* 157 (3), 590-93.

Eslam, M., et al. (2011), 'Use of HOMA-IR in hepatitis C', *J Viral Hepat,* 18 (10), 675-84.

Eslam, M., et al. (2016a), 'Diverse Impacts of the rs58542926 E167K Variant in TM6SF2 on Viral and Metabolic Liver Disease Phenotypes', *Hepatology,* 64 (1), 34-46.

Eslam, M., et al. (2016b), 'Diverse impacts of the rs58542926 E167K variant in TM6SF2 on viral and metabolic liver disease phenotypes', *Hepatology,* 64 (1), 34-46.
Eslam, M., et al. (2015a), 'Interferon-lambda rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease', *Nature Communications,* 6.
Eslam, M., et al. (2015b), 'Interferon-lambda rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease', *Nat Commun,* 6, 6422.
Eslam, M., et al. (2016c), 'FibroGENE: A gene-based model for staging liver fibrosis', *Journal of Hepatology,* 64 (2), 390-98.
Esteller, M. (2008), 'Epigenetics in Cancer', *N Engl J Med,* 358, 1148 - 59.
Estruch, R., et al. (2018), 'Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts', *N Engl J Med,* 378 (25), e34.
Fan, Y., et al. (2016), 'Hepatic Transmembrane 6 Superfamily Member 2 Regulates Cholesterol Metabolism in Mice', *Gastroenterology,* 150 (5), 1208-18.
Fang, S., et al. (2015), 'Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance', *Nature Medicine,* 21 (2), 71-77.
Feinberg, A. P. and Vogelstein, B. (1983), 'Hypomethylation distinguishes genes of some human cancers from their normal counterparts', *Nature,* 301 (5895), 89-92.
Feldman, A., et al. (2017a), 'Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver', *American Journal of Gastroenterology,* 112 (1), 102-10.
--- (2017b), 'Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver', *Am J Gastroenterol,* 112 (1), 102-10.
Feng, R. N., et al. (2014), 'Lean-non-alcoholic fatty liver disease increases risk for metabolic disorders in a normal weight Chinese population', *World J Gastroenterol,* 20 (47), 17932-40.
Ferslew, B. C., et al. (2015), 'Altered Bile Acid Metabolome in Patients with Nonalcoholic Steatohepatitis', *Dig Dis Sci,* 60 (11), 3318-28.
Fracanzani, A. L., et al. (2011), 'Risk of nonalcoholic steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease and low visceral adiposity', *J Hepatol,* 54 (6), 1244-9.
Fracanzani, A. L., et al. (2017), 'Liver and Cardiovascular Damage in Patients With Lean Nonalcoholic Fatty Liver Disease, and Association With Visceral Obesity', *Clin Gastroenterol Hepatol,* 15 (10), 1604-11 e1.
Francque, S. M., van der Graaff, D., and Kwanten, W. J. (2016), 'Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanisms and implications', *J Hepatol,* 65 (2), 425-43.
Fu, L., et al. (2004), 'Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes', *Endocrinology,* 145 (6), 2594-603.
George, A. M., Jacob, A. G., and Fogelfeld, L. (2015), 'Lean diabetes mellitus: An emerging entity in the era of obesity', *World J Diabetes,* 6 (4), 613-20.
Gitto, S., et al. (2018), 'Study of the Serum Metabolomic Profile in Nonalcoholic Fatty Liver Disease: Research and Clinical Perspectives', *Metabolites,* 8 (1).
Goday, A., et al. (2016), 'Prevalence and clinical characteristics of metabolically healthy obese individuals and other obese/non-obese metabolic phenotypes in a working population: results from the Icaria study', *BMC Public Health,* 16, 248.
Golabi, P., et al. (2018), 'Components of metabolic syndrome increase the risk of mortality in nonalcoholic fatty liver disease (NAFLD)', *Medicine,* 97 (13).
Golabi, P., et al. (2016), 'Non-alcoholic Fatty Liver Disease (NAFLD) is associated with impairment of Health Related Quality of Life (HRQOL)', *Health and Quality of Life Outcomes,* 14.

Gonzalez-Cantero, J., et al. (2018), 'Insulin resistance in lean and overweight non-diabetic Caucasian adults: Study of its relationship with liver triglyceride content, waist circumference and BMI', *PLoS One,* 13 (2), e0192663.

Goodpaster, B. H., et al. (2005), 'Obesity, regional body fat distribution, and the metabolic syndrome in older men and women', *Arch Intern Med,* 165 (7), 777-83.

Guerrero, R., et al. (2009), 'Ethnic differences in hepatic steatosis: an insulin resistance paradox?', *Hepatology,* 49 (3), 791-801.

Guthold, Regina, et al. (2018), 'Worldwide trends in insufficient physical activity from 2001 to 2016: a pooled analysis of 358 population-based surveys with 1·9 million participants', *The Lancet Global Health,* 6 (10), e1077-e86.

Gutierrez-Grobe, Y., et al. (2017), 'Less liver fibrosis in metabolically healthy compared with metabolically unhealthy obese patients with non-alcoholic fatty liver disease', *Diabetes Metab,* 43 (4), 332-37.

Hagstrom, H., et al. (2018), 'Risk for development of severe liver disease in lean patients with nonalcoholic fatty liver disease: A long-term follow-up study', *Hepatol Commun,* 2 (1), 48-57.

Hallal, Pedro C., et al. (2012), 'Global physical activity levels: surveillance progress, pitfalls, and prospects', *The Lancet,* 380 (9838), 247-57.

Han, T. S., Sattar, N., and Lean, M. (2006), 'ABC of obesity. Assessment of obesity and its clinical implications', *Bmj,* 333 (7570), 695-8.

Hardwick, R. N., et al. (2011), 'Variations in ATP-binding cassette transporter regulation during the progression of human nonalcoholic fatty liver disease', *Drug Metab Dispos,* 39 (12), 2395-402.

Hardy, T. and Mann, D. A. (2016), 'Epigenetics in liver disease: from biology to therapeutics', *Gut*.

Hardy, T., et al. (2016), 'Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease', *Gut*.

Harrison, Stephen A., et al. (2018), 'NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial', *The Lancet,* 391 (10126), 1174-85.

Heid, I. M., et al. (2010), 'Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution', *Nat Genet,* 42 (11), 949-60.

Huang, R. C., et al. (2012), 'DNA methylation of the IGF2/H19 imprinting control region and adiposity distribution in young adults', *Clin Epigenetics,* 4 (1), 21.

Huber, Y., et al. (2019), 'Health-related Quality of Life in Nonalcoholic Fatty Liver Disease Associates With Hepatic Inflammation', *Clinical Gastroenterology and Hepatology,* 17 (10), 2085-+.

Hwang, Y. C., Ahn, H. Y., and Park, C. Y. (2019), 'Association Between Nonalcoholic Fatty Liver Disease and Future Deterioration of Metabolic Health: A Cohort Study', *Obesity (Silver Spring),* 27 (8), 1360-66.

Iacobini, C., et al. (2019), 'Metabolically healthy versus metabolically unhealthy obesity', *Metabolism,* 92, 51-60.

Ioannou, G. N., et al. (2009), 'Association between dietary nutrient composition and the incidence of cirrhosis or liver cancer in the United States population', *Hepatology,* 50 (1), 175-84.

Ipsen, D. H., et al. (2018), 'Liraglutide Decreases Hepatic Inflammation and Injury in Advanced Lean Non-Alcoholic Steatohepatitis', *Basic & Clinical Pharmacology & Toxicology,* 123 (6), 704-13.

Jacobs, A., et al. (2016), 'An Overview of Mouse Models of Nonalcoholic Steatohepatitis: From Past to Present', *Curr Protoc Mouse Biol,* 6 (2), 185-200.

Jensen, T., et al. (2018), 'Fructose and sugar: A major mediator of non-alcoholic fatty liver disease', *J Hepatol,* 68 (5), 1063-75.

Jiang, Y., et al. (2016), 'Adiponectin levels predict prediabetes risk: the Pathobiology of Prediabetes in A Biracial Cohort (POP-ABC) study', *BMJ Open Diabetes Res Care,* 4 (1), e000194.

Jiao, N., et al. (2017), 'Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD', *Gut*.

Johannsen, D. L., et al. (2019), 'Metabolic adaptation is not observed after 8 weeks of overfeeding but energy expenditure variability is associated with weight recovery', *Am J Clin Nutr*.

Johnson, N. A., et al. (2009), 'Aerobic Exercise Training Reduces Hepatic and Visceral Lipids in Obese Individuals Without Weight Loss', *Hepatology,* 50 (4), 1105-12.

Kahali, B., et al. (2015), 'TM6SF2: catch-22 in the fight against nonalcoholic fatty liver disease and cardiovascular disease?', *Gastroenterology,* 148 (4), 679-84.

Kainuma, M., et al. (2006), 'Cholesterol-fed rabbit as a unique model of nonalcoholic, nonobese, non-insulin-resistant fatty liver disease with characteristic fibrosis', *J Gastroenterol,* 41 (10), 971-80.

Kakiyama, G., et al. (2013), 'Modulation of the fecal bile acid profile by gut microbiota in cirrhosis', *Journal of Hepatology,* 58 (5), 949-55.

Kalhan, S. C., et al. (2011), 'Plasma metabolomic profile in nonalcoholic fatty liver disease', *Metabolism,* 60 (3), 404-13.

Kazankov, K., et al. (2016), 'The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease', *Liver International,* 36 (10), 1549-57.

Kechagias, S., et al. (2015), 'Phosphatidylethanol Compared with Other Blood Tests as a Biomarker of Moderate Alcohol Consumption in Healthy Volunteers: A Prospective Randomized Study', *Alcohol Alcohol,* 50 (4), 399-406.

Khalid, Quratulain, Bailey, Ian, and Patel, Vinood B. (2015), 'Non-Alcoholic Fatty Liver Disease: The Effect of Bile Acids and Farnesoid X Receptor Agonists on Pathophysiology and Treatment', *Liver Research - Open Journal,* 1 (2), 32-40.

Kilpelainen, T. O., et al. (2011), 'Genetic variation near IRS1 associates with reduced adiposity and an impaired metabolic profile', *Nat Genet,* 43 (8), 753-60.

Kim, D. and Kim, W. R. (2017), 'Nonobese Fatty Liver Disease', *Clin Gastroenterol Hepatol,* 15 (4), 474-85.

Kim, H. J., et al. (2004), 'Metabolic significance of nonalcoholic fatty liver disease in nonobese, nondiabetic adults', *Arch Intern Med,* 164 (19), 2169-75.

Kim, J. Y., et al. (2007), 'Obesity-associated improvements in metabolic profile through expansion of adipose tissue', *J Clin Invest,* 117 (9), 2621-37.

Klass, D. M., et al. (2006), 'Biliary lipids, cholesterol and bile synthesis: different adaptive mechanisms to dietary cholesterol in lean and obese subjects', *Aliment Pharmacol Ther,* 23 (7), 895-905.

Klein, S., et al. (2004), 'Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease', *N Engl J Med,* 350 (25), 2549-57.

Kleiner, D. E., et al. (2005a), 'Design and validation of a histological scoring system for nonalcoholic fatty liver disease', *Hepatology,* 41 (6), 1313-21.

Kleiner, D. E., et al. (2005b), 'Design and validation of a histological scoring system for nonalcoholic fatty liver disease', *Hepatology,* 41 (6), 1313-21.

Kohli, R., et al. (2013), 'Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids', *J Clin Endocrinol Metab,* 98 (4), E708-12.

Kohli, R., et al. (2015), 'Bile Acid Signaling: Mechanism for Bariatric Surgery, Cure for NASH?', *Dig Dis,* 33 (3), 440-6.

Koren, D. and Taveras, E. M. (2018), 'Association of sleep disturbances with obesity, insulin resistance and the metabolic syndrome', *Metabolism,* 84, 67-75.

Kozlitina, J., et al. (2014), 'Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease', *Nat Genet,* 46 (4), 352-6.

Kramer, C. K., Zinman, B., and Retnakaran, R. (2013), 'Are metabolically healthy overweight and obesity benign conditions?: A systematic review and meta-analysis', *Ann Intern Med,* 159 (11), 758-69.

Kumar, R., et al. (2013), 'Clinicopathological characteristics and metabolic profiles of non-alcoholic fatty liver disease in Indian patients with normal body mass index: Do they differ from obese or overweight non-alcoholic fatty liver disease?', *Indian J Endocrinol Metab,* 17 (4), 665-71.

Kwak, J. H., et al. (2018), 'Lifestyle predictors of obese and non-obese patients with nonalcoholic fatty liver disease: A cross-sectional study', *Clin Nutr,* 37 (5), 1550-57.

Le, M. H., et al. (2017), 'Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States', *PLoS One,* 12 (3), e0173499.

Lee, J. J., et al. (2017), 'Upper Body Subcutaneous Fat Is Associated with Cardiometabolic Risk Factors', *Am J Med,* 130 (8), 958-66 e1.

Lee, M. K., et al. (2015), 'Metabolic Health Is More Important than Obesity in the Development of Nonalcoholic Fatty Liver Disease: A 4-Year Retrospective Study', *Endocrinol Metab (Seoul),* 30 (4), 522-30.

Legry, V., et al. (2017), 'Bile acid alterations are associated with insulin resistance, but not with NASH in obese subjects', *J Clin Endocrinol Metab*.

Leibel, R. L., Rosenbaum, M., and Hirsch, J. (1995), 'Changes in energy expenditure resulting from altered body weight', *N Engl J Med,* 332 (10), 621-8.

Leung, J. C., et al. (2017a), 'Histological severity and clinical outcomes of nonalcoholic fatty liver disease in nonobese patients', *Hepatology,* 65 (1), 54-64.

Leung, J. C. F., et al. (2017b), 'Histological Severity and Clinical Outcomes of Nonalcoholic Fatty Liver Disease in Nonobese Patients', *Hepatology,* 65 (1), 54-64.

Li, C., et al. (2019), 'Lean non-alcoholic fatty liver disease patients had comparable total caloric, carbohydrate, protein, fat, iron, sleep duration and overtime work as obese non-alcoholic fatty liver disease patients', *J Gastroenterol Hepatol,* 34 (1), 256-62.

Li, L., et al. (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), 510-9.

Li, M., et al. (2012), 'Co-methylated genes in different adipose depots of pig are associated with metabolic, inflammatory and immune processes', *Int J Biol Sci,* 8 (6), 831-7.

Lim, S. and Meigs, J. B. (2013), 'Ectopic fat and cardiometabolic and vascular risk', *Int J Cardiol,* 169 (3), 166-76.

Liu, Y. L., et al. (2014a), 'Carriage of the PNPLA3 rs738409 C>G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma', *J Hepatol,* 61 (1), 75-81.

Liu, Y. L., et al. (2014b), 'TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease', *Nat Commun,* 5, 4309.

Lorenzo, C., et al. (2007), 'The National Cholesterol Education Program - Adult Treatment Panel III, International Diabetes Federation, and World Health Organization definitions of the metabolic syndrome as predictors of incident cardiovascular disease and diabetes', *Diabetes Care,* 30 (1), 8-13.

Lozano, R., et al. (2012), 'Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010', *Lancet,* 380 (9859), 2095-128.

Ma, J., Zhou, Q., and Li, H. (2017), 'Gut Microbiota and Nonalcoholic Fatty Liver Disease: Insights on Mechanisms and Therapy', *Nutrients,* 9 (10).

Maclagan, L. C. and Tu, J. V. (2015), 'Using the concept of ideal cardiovascular health to measure population health: a review', *Current Opinion in Cardiology,* 30 (5), 518-24.

Mancina, R. M., et al. (2016), 'The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent', *Gastroenterology,* 150 (5), 1219-30 e6.

Mann, D. A. (2014), 'Epigenetics in liver disease', *Hepatology,* 60 (4), 1418-25.

Manolio, T. A., et al. (2009), 'Finding the missing heritability of complex diseases', *Nature,* 461 (7265), 747-53.

Manolopoulos, K. N., Karpe, F., and Frayn, K. N. (2010), 'Gluteofemoral body fat as a determinant of metabolic health', *Int J Obes (Lond),* 34 (6), 949-59.

Marchi, M., et al. (2011), 'Human leptin tissue distribution, but not weight loss-dependent change in expression, is associated with methylation of its promoter', *Epigenetics,* 6 (10), 1198-206.

Margariti, E., et al. (2012), 'Non-alcoholic fatty liver disease may develop in individuals with normal body mass index', *Ann Gastroenterol,* 25 (1), 45-51.

Martinez, I., et al. (2013), 'Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters', *Appl Environ Microbiol,* 79 (2), 516-24.

Matheson, E. M., King, D. E., and Everett, C. J. (2012), 'Healthy lifestyle habits and mortality in overweight and obese individuals', *J Am Board Fam Med,* 25 (1), 9-15.

Matthews, D. R., et al. (1985), 'Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia,* 28 (7), 412-9.

Mayo, R., et al. (2018), 'Metabolomic-based noninvasive serum test to diagnose nonalcoholic steatohepatitis: Results from discovery and validation cohorts', *Hepatol Commun,* 2 (7), 807-20.

McGlone, E. R., et al. (2019), 'Bile acid mediated changes after bariatric surgery: what can we learn from mouse models?', *Gastroenterology*.

McLaughlin, T., et al. (2011), 'Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity', *J Clin Endocrinol Metab,* 96 (11), E1756-60.

McPherson, S., et al. (2015), 'Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: Implications for prognosis and clinical management', *J Hepatol,* 62 (5), 1148-55.

Min, H. K., et al. (2012), 'Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic Fatty liver disease', *Cell Metab,* 15 (5), 665-74.

Mudaliar, S., et al. (2013), 'Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease', *Gastroenterology,* 145 (3), 574-82.e1.

Musso, G., et al. (2003), 'Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis', *Hepatology,* 37 (4), 909-16.

Neuschwander-Tetri, B. A., et al. (2015), 'Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial', *Lancet,* 385 (9972), 956-65.

Ng, Marie, 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), 766-81.

Niriella, M. A., et al. (2019), 'Lean non-alcoholic fatty liver disease (lean NAFLD): characteristics, metabolic outcomes and risk factors from a 7-year prospective, community cohort study from Sri Lanka', *Hepatol Int,* 13 (3), 314-22.

Nishioji, K., et al. (2015), 'Prevalence of and risk factors for non-alcoholic fatty liver disease in a non-obese Japanese population, 2011-2012', *J Gastroenterol,* 50 (1), 95-108.

O'Connell, J., et al. (2010), 'The relationship of omental and subcutaneous adipocyte size to metabolic disease in severe obesity', *PLoS One,* 5 (4), e9997.

Oh, K. J., et al. (2016), 'Metabolic Adaptation in Obesity and Type II Diabetes: Myokines, Adipokines and Hepatokines', *Int J Mol Sci,* 18 (1).

Paik, J. M., et al. (2019), 'Mortality Related to Nonalcoholic Fatty Liver Disease Is Increasing in the United States', *Hepatology Communications,* 3 (11), 1459-71.

Pang, J., et al. (2017), 'Significant positive association of endotoxemia with histological severity in 237 patients with non-alcoholic fatty liver disease', *Aliment Pharmacol Ther,* 46 (2), 175-82.

Pastore, A., et al. (2014), 'Plasma levels of homocysteine and cysteine increased in pediatric NAFLD and strongly correlated with severity of liver damage', *Int J Mol Sci,* 15 (11), 21202-14.

Pathak, P., et al. (2018), 'Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism', *Hepatology,* 68 (4), 1574-88.

Patti, M. E., et al. (2009), 'Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism', *Obesity (Silver Spring),* 17 (9), 1671-7.

Perez, M. J. and Briz, O. (2009), 'Bile-acid-induced cell injury and protection', *World J Gastroenterol,* 15 (14), 1677-89.

Petta, S., et al. (2017a), 'Interferon Lambda 4 rs368234815 TT >delta G Variant Is Associated With Liver Damage in Patients With Nonalcoholic Fatty Liver Disease', *Hepatology,* 66 (6), 1885-93.

--- (2017b), 'Interferon lambda 4 rs368234815 TT>deltaG variant is associated with liver damage in patients with nonalcoholic fatty liver disease', *Hepatology,* 66 (6), 1885-93.

Pierre, J. F., et al. (2019), 'Bile Diversion Improves Metabolic Phenotype Dependent on Farnesoid X Receptor (FXR)', *Obesity (Silver Spring),* 27 (5), 803-12.

Pierre, J. F., et al. (2016), 'Activation of bile acid signaling improves metabolic phenotypes in high-fat diet-induced obese mice', *Am J Physiol Gastrointest Liver Physiol,* 311 (2), G286-304.

Plagemann, A. (2004), ''Fetal programming' and 'functional teratogenesis': on epigenetic mechanisms and prevention of perinatally acquired lasting health risks', *J Perinat Med,* 32 (4), 297-305.

Popkin, B. M. (2006), 'Global nutrition dynamics: the world is shifting rapidly toward a diet linked with noncommunicable diseases', *Am J Clin Nutr,* 84 (2), 289-98.

Pournaras, D. J., et al. (2012), 'The Role of Bile After Roux-en-Y Gastric Bypass in Promoting Weight Loss and Improving Glycaemic Control', *Endocrinology,* 153 (8), 3613-19.
Pujia, A., et al. (2016), 'Individuals with Metabolically Healthy Overweight/Obesity Have Higher Fat Utilization than Metabolically Unhealthy Individuals', *Nutrients,* 8 (1).
Puri, P., et al. (2007), 'A lipidomic analysis of nonalcoholic fatty liver disease', *Hepatology,* 46 (4), 1081-90.
Puri, P., et al. (2017), 'The presence and severity of nonalcoholic steatohepatitis is associated with specific changes in circulating bile acids', *Hepatology*.
Ramirez-Perez, O., et al. (2017), 'The Role of the Gut Microbiota in Bile Acid Metabolism', *Ann Hepatol,* 16 (Suppl. 1: s3-105.), s15-s20.
Ridlon, J. M., Kang, D. J., and Hylemon, P. B. (2006), 'Bile salt biotransformations by human intestinal bacteria', *J Lipid Res,* 47 (2), 241-59.
Romeo, S., et al. (2008), 'Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease', *Nat Genet,* 40 (12), 1461-5.
Ruderman, N., et al. (1998), 'The metabolically obese, normal-weight individual revisited', *Diabetes,* 47 (5), 699-713.
Sachdev, S., et al. (2016), 'FGF 19 and Bile Acids Increase Following Roux-en-Y Gastric Bypass but Not After Medical Management in Patients with Type 2 Diabetes', *Obes Surg,* 26 (5), 957-65.
Sarin, S. K., et al. (2020), 'Liver diseases in the Asia-Pacific region: a Lancet Gastroenterology & Hepatology Commission', *Lancet Gastroenterol Hepatol,* 5 (2), 167-228.
Sayiner, M., et al. (2016), 'Assessment of health utilities and quality of life in patients with non-alcoholic fatty liver disease', *BMJ Open Gastroenterol,* 3 (1), e000106.
Schleinitz, D., et al. (2014), 'The genetics of fat distribution', *Diabetologia,* 57 (7), 1276-86.
Schnabl, B. and Brenner, D. A. (2014), 'Interactions Between the Intestinal Microbiome and Liver Diseases', *Gastroenterology,* 146 (6), 1513-24.
Schumacher, J. D., et al. (2020), 'Direct and Indirect Effects of Fibroblast Growth Factor (FGF) 15 and FGF19 on Liver Fibrosis Development', *Hepatology,* 71 (2), 670-85.
Schwimmer, J. B., et al. (2009), 'Heritability of nonalcoholic fatty liver disease', *Gastroenterology,* 136 (5), 1585-92.
Simonen, M., et al. (2013), 'Desmosterol in human nonalcoholic steatohepatitis', *Hepatology,* 58 (3), 976-82.
Singh, S., et al. (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), 643-54 e9.
Sinn, D. H., et al. (2019), 'Lean non-alcoholic fatty liver disease and development of diabetes: a cohort study', *European Journal of Endocrinology,* 181 (2), 185-92.
Smith, Gordon I., Mittendorfer, Bettina, and Klein, Samuel (2019), 'Metabolically healthy obesity: facts and fantasies', *Journal of Clinical Investigation,* 129 (10), 3978-89.
Smith, S. R., et al. (2000), 'Concurrent physical activity increases fat oxidation during the shift to a high-fat diet', *Am J Clin Nutr,* 72 (1), 131-8.
Sookoian, S. and Pirola, C. J. (2011), 'Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease', *Hepatology,* 53 (6), 1883-94.
--- (2017), 'Systematic review with meta-analysis: risk factors for non-alcoholic fatty liver disease suggest a shared altered metabolic and cardiovascular profile between lean and obese patients', *Aliment Pharmacol Ther,* 46 (2), 85-95.

--- (2018), 'Systematic review with meta-analysis: the significance of histological disease severity in lean patients with nonalcoholic fatty liver disease', *Aliment Pharmacol Ther,* 47 (1), 16-25.
Sookoian, S., et al. (2010), 'Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter', *Hepatology,* 52 (6), 1992-2000.
Speliotes, E. K., et al. (2011), 'Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits', *PLoS Genet,* 7 (3), e1001324.
Stefan, N., Schick, F., and Haring, H. U. (2017), 'Causes, Characteristics, and Consequences of Metabolically Unhealthy Normal Weight in Humans', *Cell Metab,* 26 (2), 292-300.
Stepanova, M., et al. (2017), 'Direct and Indirect Economic Burden of Chronic Liver Disease in the United States', *Clin Gastroenterol Hepatol,* 15 (5), 759-66.e5.
Stephenson, K., et al. (2018), 'Updates on Dietary Models of Nonalcoholic Fatty Liver Disease: Current Studies and Insights', *Gene Expr,* 18 (1), 5-17.
Sung, K. C., et al. (2014), 'Metabolically healthy obese subjects are at risk of fatty liver but not of pre-clinical atherosclerosis', *Nutr Metab Cardiovasc Dis,* 24 (3), 256-62.
Thabet, K., et al. (2017), 'The membrane-bound O-acyltransferase domain-containing 7 variant rs641738 increases inflammation and fibrosis in chronic hepatitis B', *Hepatology,* 65 (6), 1840-50.
Thabet, K., et al. (2016), 'MBOAT7 rs641738 increases risk of liver inflammation and transition to fibrosis in chronic hepatitis C', *Nat Commun,* 7, 12757.
Tobari, M., et al. (2018), 'Characteristics of non-alcoholic steatohepatitis among lean patients in Japan: Not uncommon and not always benign', *J Gastroenterol Hepatol*.
Trabelsi, M. S., et al. (2015), 'Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells', *Nat Commun,* 6, 7629.
Tran, T. T., et al. (2008), 'Beneficial effects of subcutaneous fat transplantation on metabolism', *Cell Metab,* 7 (5), 410-20.
Tremaroli, V. and Backhed, F. (2012), 'Functional interactions between the gut microbiota and host metabolism', *Nature,* 489 (7415), 242-9.
Turnbaugh, P. J., et al. (2007), 'The human microbiome project', *Nature,* 449 (7164), 804-10.
van der Poorten, D., et al. (2013), 'Hepatic fat loss in advanced nonalcoholic steatohepatitis: are alterations in serum adiponectin the cause?', *Hepatology,* 57 (6), 2180-8.
Van Herck, M. A., Vonghia, L., and Francque, S. M. (2017), 'Animal Models of Nonalcoholic Fatty Liver Disease-A Starter's Guide', *Nutrients,* 9 (10).
Van Rooyen, D. M., et al. (2011), 'Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis', *Gastroenterology,* 141 (4), 1393-403, 403 e1-5.
Vendhan, R., et al. (2014), 'Comparison of characteristics between nonobese and overweight/obese subjects with nonalcoholic fatty liver disease in a South Indian population', *Diabetes Technol Ther,* 16 (1), 48-55.
Wahlstrom, A., et al. (2016a), 'Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism', *Cell Metab,* 24 (1), 41-50.
--- (2016b), 'Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism', *Cell Metabolism,* 24 (1), 41-50.
Wang, B., et al. (2016), 'Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver Disease', *Sci Rep,* 6, 32002.
Wang, Qianyi, et al. (2019), 'Non-obese histologically confirmed NASH patients with abnormal liver biochemistry have more advanced fibrosis', *Hepatology International,* 13 (6), 766-76.

Watanabe, M., et al. (2011), 'Lowering Bile Acid Pool Size with a Synthetic Farnesoid X Receptor (FXR) Agonist Induces Obesity and Diabetes through Reduced Energy Expenditure', *Journal of Biological Chemistry,* 286 (30), 26913-20.

Watanabe, M., et al. (2006), 'Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation', *Nature,* 439 (7075), 484-9.

Wei, J. L., et al. (2015), 'Prevalence and Severity of Nonalcoholic Fatty Liver Disease in Non-Obese Patients: A Population Study Using Proton-Magnetic Resonance Spectroscopy', *Am J Gastroenterol,* 110 (9), 1306-14; quiz 15.

Werling, M., et al. (2013), 'Enhanced fasting and post-prandial plasma bile acid responses after Roux-en-Y gastric bypass surgery', *Scand J Gastroenterol,* 48 (11), 1257-64.

WHO, Expert Consultation (2004), 'Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies', *The Lancet,* 363 (9403), 157-63.

Wieland, A., et al. (2015), 'Systematic review: microbial dysbiosis and nonalcoholic fatty liver disease', *Aliment Pharmacol Ther,* 42 (9), 1051-63.

Wildman, R. P., et al. (2008), 'The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004)', *Arch Intern Med,* 168 (15), 1617-24.

Wong, R. J., et al. (2014), 'Ethnic disparities in the association of body mass index with the risk of hypertension and diabetes', *J Community Health,* 39 (3), 437-45.

Wong, V. W. S., et al. (2018), 'Beneficial effects of lifestyle intervention in non-obese patients with non-alcoholic fatty liver disease', *Journal of Hepatology,* 69 (6), 1349-56.

Wouters, K., et al. (2008), 'Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis', *Hepatology,* 48 (2), 474-86.

Wu, G. D., et al. (2011), 'Linking long-term dietary patterns with gut microbial enterotypes', *Science,* 334 (6052), 105-8.

Xie, G., et al. (2015), 'Profiling of serum bile acids in a healthy Chinese population using UPLC-MS/MS', *J Proteome Res,* 14 (2), 850-9.

Xu, Aimin, et al. (2003), 'The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice', *Journal of Clinical Investigation,* 112 (1), 91-100.

Yaghootkar, H., et al. (2014), 'Genetic evidence for a normal-weight "metabolically obese" phenotype linking insulin resistance, hypertension, coronary artery disease, and type 2 diabetes', *Diabetes,* 63 (12), 4369-77.

Yaghootkar, H., et al. (2016), 'Genetic Evidence for a Link Between Favorable Adiposity and Lower Risk of Type 2 Diabetes, Hypertension, and Heart Disease', *Diabetes,* 65 (8), 2448-60.

Yasutake, K., et al. (2009a), 'Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol', *Scand J Gastroenterol,* 44 (4), 471-7.

--- (2009b), 'Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: The significance of dietary cholesterol', *Scandinavian Journal of Gastroenterology,* 44 (4), 471-77.

Yilmaz, Y., et al. (2019), 'Growing burden of nonalcoholic fatty liver disease in Turkey: A single-center experience', *Turk J Gastroenterol,* 30 (10), 892-98.

Yokota, A., et al. (2012), 'Is bile acid a determinant of the gut microbiota on a high-fat diet?', *Gut Microbes,* 3 (5), 455-9.

Younes, R. and Bugianesi, E. (2019), 'NASH in Lean Individuals', *Semin Liver Dis,* 39 (1), 86-95.

Younossi, Z., et al. (2018a), 'Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention', *Nature Reviews Gastroenterology & Hepatology,* 15 (1), 11-20.
--- (2018b), 'Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention', *Nat Rev Gastroenterol Hepatol,* 15 (1), 11-20.
Younossi, Z. M. (2019), 'Non-alcoholic fatty liver disease - A global public health perspective', *Journal of Hepatology,* 70 (3), 531-44.
Younossi, Z. M., et al. (2016a), 'Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes', *Hepatology,* 64 (1), 73-84.
Younossi, Z. M., et al. (2012), 'Nonalcoholic fatty liver disease in lean individuals in the United States', *Medicine (Baltimore),* 91 (6), 319-27.
Younossi, Z. M., et al. (2016b), 'The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe', *Hepatology,* 64 (5), 1577-86.
Younossi, Z. M., et al. (2019a), 'Reduced Patient-Reported Outcome Scores Associate With Level of Fibrosis in Patients With Nonalcoholic Steatohepatitis', *Clin Gastroenterol Hepatol,* 17 (12), 2552-60 e10.
Younossi, Z. M., et al. (2019b), 'Reduced Patient-Reported Outcome Scores Associate With Level of Fibrosis in Patients With Nonalcoholic Steatohepatitis', *Clinical Gastroenterology and Hepatology,* 17 (12), 2552-+.
Zain, S. M., Mohamed, Z., and Mohamed, R. (2015), 'Common variant in the glucokinase regulatory gene rs780094 and risk of nonalcoholic fatty liver disease: a meta-analysis', *J Gastroenterol Hepatol,* 30 (1), 21-7.
Zaratiegui, M., Irvine, D. V., and Martienssen, R. A. (2007), 'Noncoding RNAs and gene silencing', *Cell,* 128 (4), 763-76.
Zeybel, M., et al. (2015), 'Differential DNA methylation of genes involved in fibrosis progression in non-alcoholic fatty liver disease and alcoholic liver disease', *Clin Epigenetics,* 7, 25.
Zhang, H., et al. (2009), 'Human gut microbiota in obesity and after gastric bypass', *Proc Natl Acad Sci U S A,* 106 (7), 2365-70.
Zhao, L., et al. (2018), 'A Glucagon-Like Peptide-1 Receptor Agonist Lowers Weight by Modulating the Structure of Gut Microbiota', *Front Endocrinol (Lausanne),* 9, 233.
Zhao, Z. H., et al. (2019), 'Indole-3-propionic acid inhibits gut dysbiosis and endotoxin leakage to attenuate steatohepatitis in rats', *Exp Mol Med,* 51 (9), 103.
Zhou, D. and Fan, J. G. (2019), 'Microbial metabolites in non-alcoholic fatty liver disease', *World J Gastroenterol,* 25 (17), 2019-28.
Zhou, D., et al. (2017), 'Sodium butyrate attenuates high-fat diet-induced steatohepatitis in mice by improving gut microbiota and gastrointestinal barrier', *World J Gastroenterol,* 23 (1), 60-75.
Zietak, M. (2016), 'Altered microbiota contributes to reduced diet-induced obesity upon cold exposure', *Cell Metab.,* 23, 1216-23.

Figures and legends

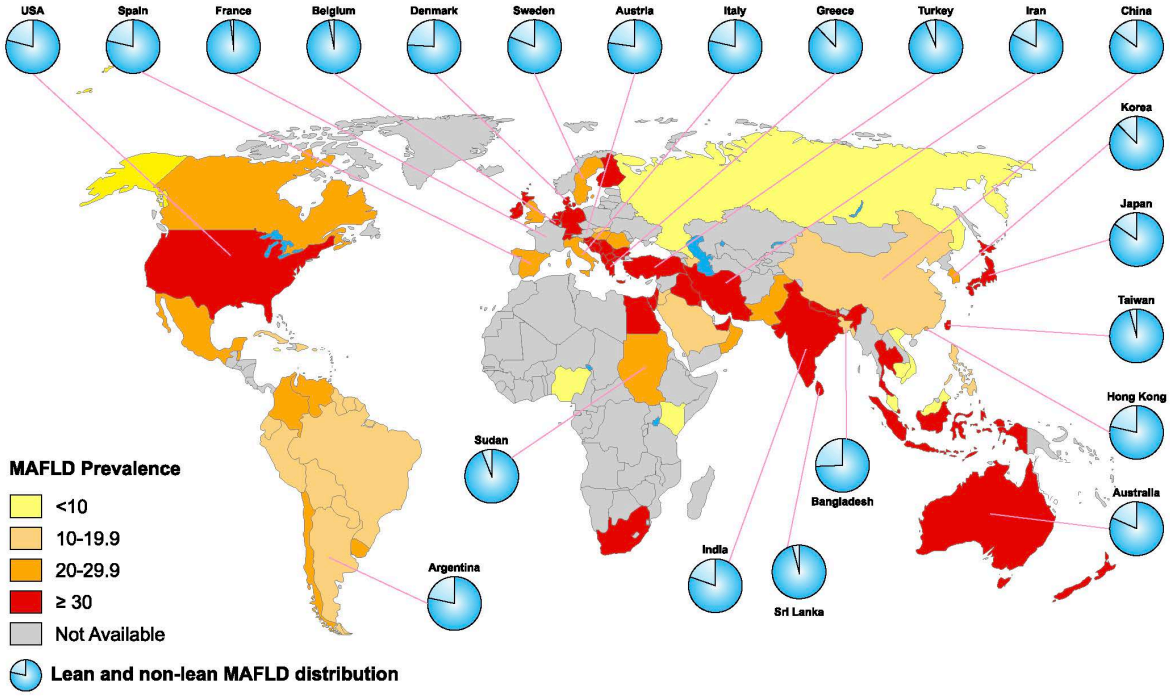


Figure 1. Worldwide prevalence of MAFLD and of lean MAFLD as a proportion of total MAFLD

Worldwide distribution of MAFLD with data on the prevalence of lean MAFLD (light blue; where available). Abbreviation: MAFLD – Metabolic associated fatty liver disease.

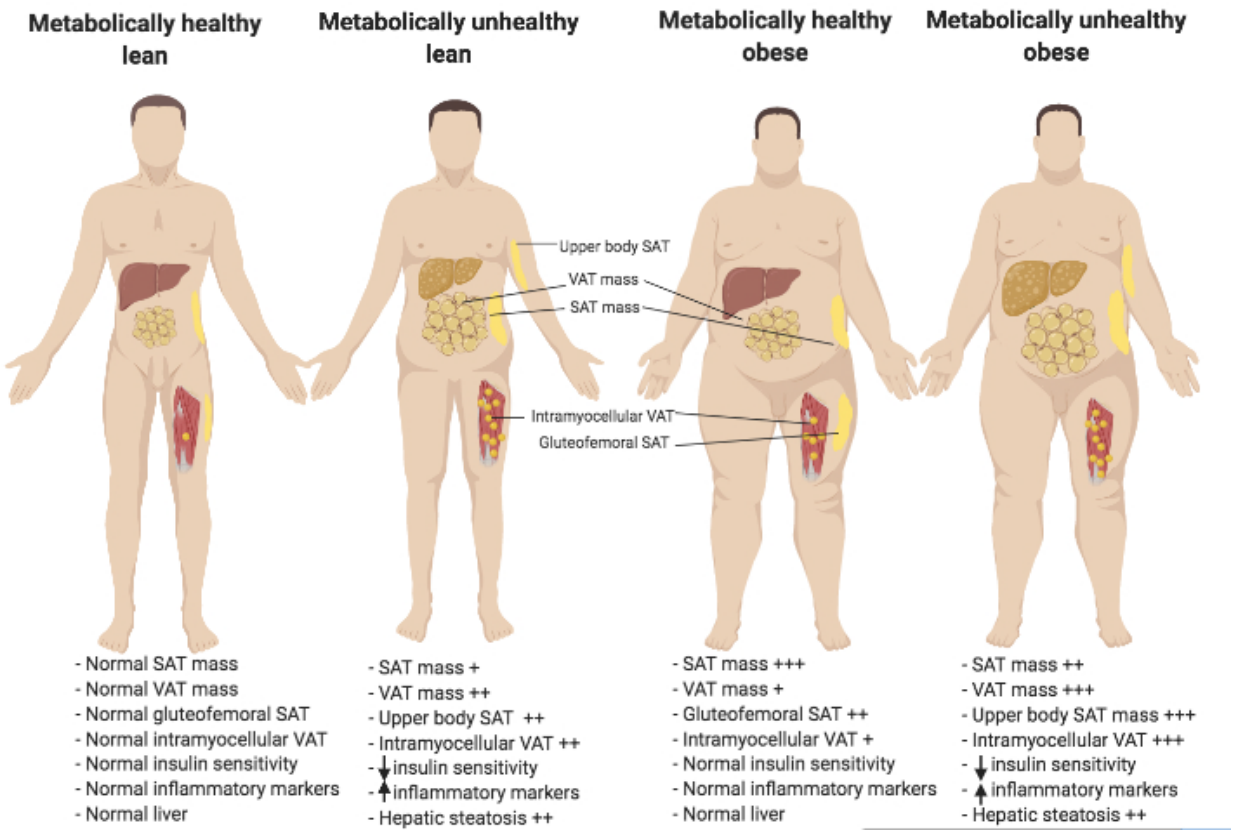


Figure 2. Adiposity phenotype based on metabolic health status and body weight.

The difference in fat depots, insulin sensitivity, inflammatory marker and hepatic fat content in individuals with metabolically healthy lean, metabolically unhealthy lean (lean MAFLD), metabolically healthy obese and metabolically unhealthy obese phenotypes. Abbreviations: SAT – Subcutaneous adipose tissue; VAT – Visceral adipose tissue; MAFLD – Metabolic associated fatty liver disease.

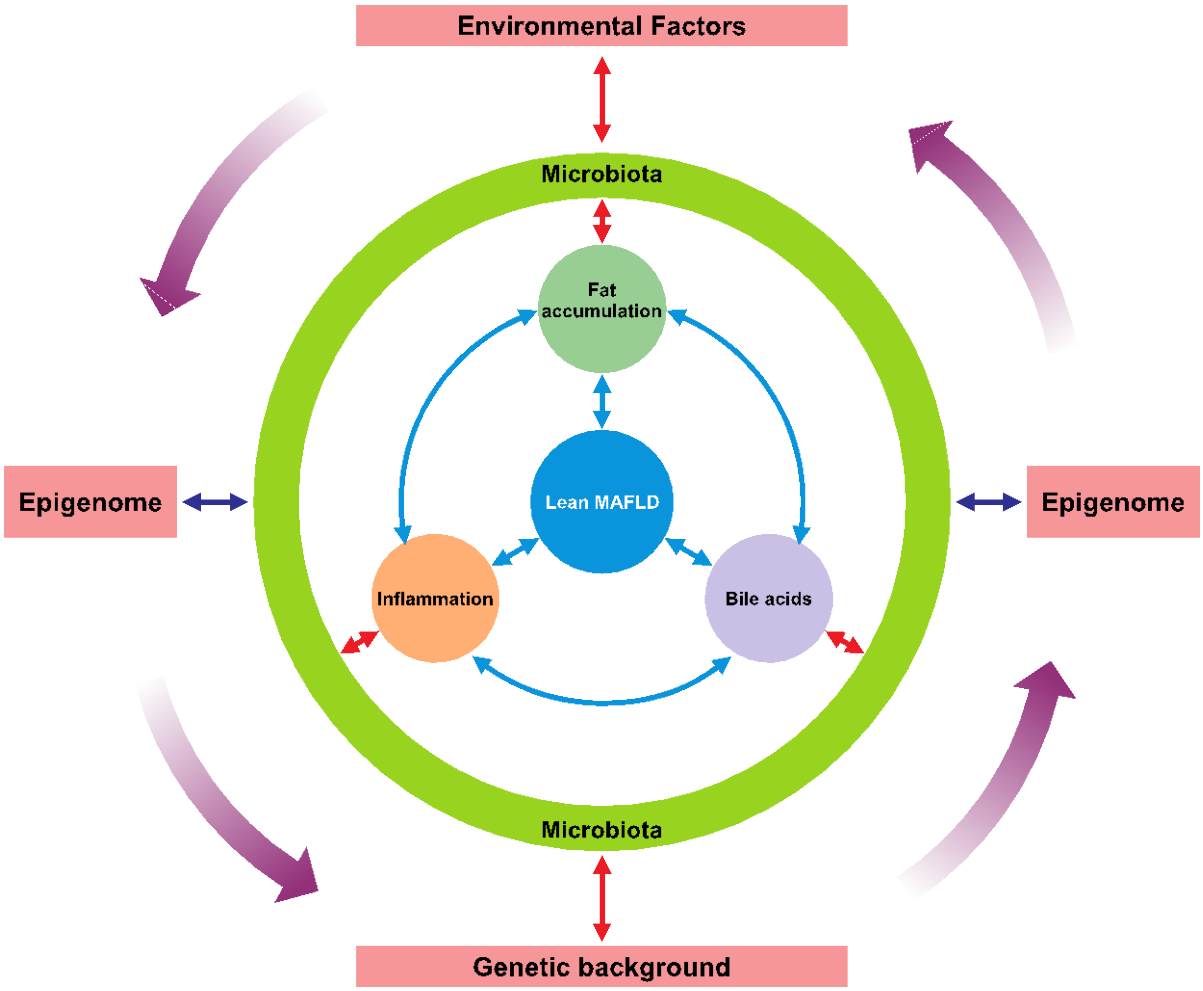


Figure 3. The interplay between factors affecting lean MAFLD pathogenesis. Environmental, genetic and epigenetic factors, along with the gut microbiota profile influence lean MAFLD pathogenesis through regulation of fat accumulation, inflammation and bile acid metabolism. Abbreviation: MAFLD – Metabolic associated fatty liver disease.

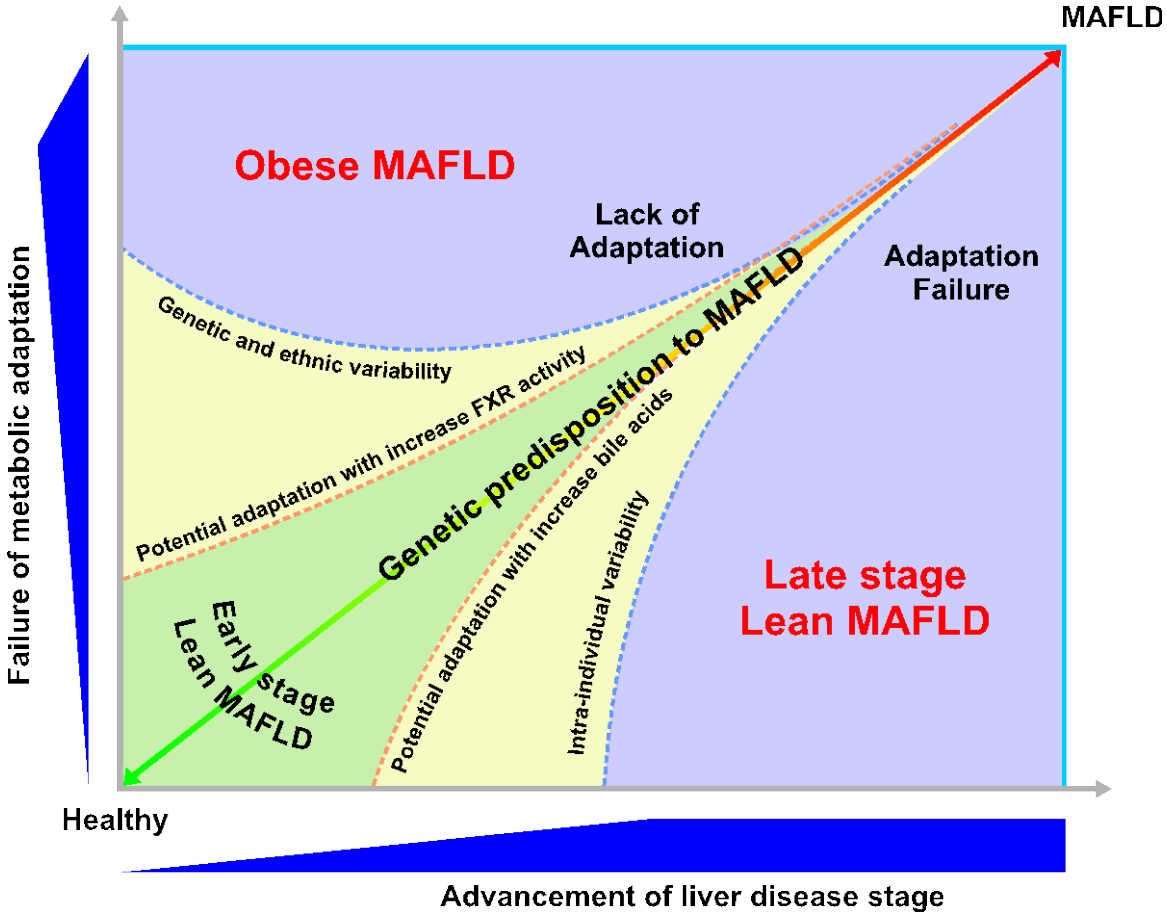


Figure 4. The role of metabolic adaptation in lean and obese MAFLD pathogenesis.

Schematic representation of the differences in metabolic adaptation between individuals with lean and obese MAFLD on a background of metabolic and genetic predisposition. In obese MAFLD patients, there is relatively poor metabolic adaptation resulting in adiposity and the development of liver disease. In contrast, among lean MAFLD patients there is partial metabolic adaptation at least in the early stages of the disease. In the example shown, this is through increased bile acid production and FXR activity (other mechanisms may also be operative). This results in an "obesity resistant" phenotype", which appears to be lost as the disease progresses. Abbreviations: MAFLD – Metabolic associated fatty liver disease; FXR – Farsenoid X receptor.

Supplementary Table 1. Summary of a selection of published literature on lean MAFLD

Author, year, country	Definition of lean MAFLD	Sample size	Main findings
Kim, HJ, 2004, Korea (H. J. Kim et al. 2004)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	74 lean and 106 non-lean MAFLD; 386 lean healthy and 202 non-lean healthy controls	Metabolic disorders are present in MAFLD subjects with normal weight
Chen, CH, 2006, Taiwan (C. H. Chen et al. 2006)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	61 lean and 291 non-lean MAFLD; 1383 lean healthy and 654 non-lean healthy controls	Hypertriglyceridaemia was related to MAFLD in non-obese subjects
Das, K, 2010, India (Das et al. 2010)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound, confirmed on CT	123 lean and 41 non-lean MAFLD; 1660 lean healthy and 87 non-lean healthy controls	Lean MAFLD is present in 75% of this predominantly non-obese population, with potentially significant liver disease
Younossi, 2012, USA (Z. M. Younossi et al. 2012)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	431 lean and 2061 non-lean MAFLD; 4026 lean healthy and 5095 non-lean healthy controls	Lean MAFLD patients (20.9%) are younger, have lower metabolic syndrome and is more common in females
Margariti, 2012, Greece (Margariti et al. 2012)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	19 lean and 143 non-lean MAFLD	Lean MAFLD patients (12%) have lower metabolic syndrome and higher ALT/AST than non-lean MAFLD
Bhat, 2013, India (Bhat et al. 2013)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	30 lean and 120 non-lean MAFLD	Lean MAFLD present in 20% of patients. Insulin resistance is common amongst patients with MAFLD, including lean MAFLD (80%)
Kumar, 2013, India (Kumar et al. 2013)	Lean BMI < 23 kg/m^2, biopsy proven MAFLD	27 lean and 141 non-lean MAFLD	Lean MAFLD patients (13.2%) have less severe histology and lower insulin resistance than non-lean MAFLD
Delacruz, 2014, Australia (A. C. e. a. Dela Cruz 2014)	Lean BMI < 25 kg/m^2, biopsy proven MAFLD	125 lean and 965 non-lean MAFLD	Lean MAFLD patients (11.5%) have higher mortality than patients with non-lean MAFLD despite presenting with healthier metabolic profile
Alam, 2014, India (Alam et al. 2014)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound, biopsy in some (220/465)	119 lean and 346 non-lean MAFLD	Lean MAFLD patients (25.6%) were metabolically and histologically similar to non-lean MAFLD patients, with similar rates of NASH and fibrosis
Feng, 2014, China (Feng et al. 2014)	Lean BMI < 24 kg/m^2, hepatic steatosis on liver ultrasound	134 lean and 764 non-lean MAFLD; 597 lean healthy and 284 non-lean healthy controls	Lean MAFLD patients (14.9%) had higher visceral adiposity index and comparable metabolic risk profile to non-lean MAFLD
Vendhan, 2014, India (Vendhan et al. 2014)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	48 lean and 125 non-lean MAFLD	Lean MAFLD patients (27.7%) had better metabolic profile but similar association to coronary artery disease as non-lean MAFLD

Wei, 2015, Hong Kong (Wei et al. 2015)	Lean BMI < 25 kg/m^2, liver fat assessed by proton-magnetic resonance spectroscopy	135 lean and 127 non-lean MAFLD	Lean MAFLD patients (19.3%) had similar intrahepatic triglyceride content, but lower cytokeratin-18 fragments and liver fibrosis. PNPLA3 G allele was more common in lean MAFLD.
Nishioji, 2015, Japan (Nishioji et al. 2015)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	411 lean and 394 non-lean MAFLD; 2285 lean healthy and 181 non-lean healthy controls	Lifestyle and metabolic factors (higher triglycerides and waist circumference) increases the risk of MAFLD, even in lean patients (15.2%)
Cho, 2016, Korea (Cho 2016)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	213 lean and 347 non-lean MAFLD; 1498 lean healthy controls	Lean MAFLD patients (12.4%) had higher proportion of females, lower insulin resistance and fewer metabolic risk factors than non-lean MAFLD
Feldman, 2017, Austria (Feldman et al. 2017b)	Lean BMI < 25 kg/m^2, hepatic steatosis on liver ultrasound	55 lean and 61 non-lean MAFLD; 71 lean healthy controls	Lean MAFLD patients (29.4%) had impaired glucose tolerance, low adiponectin concentrations and a distinct metabolic profile with increased PNPLA3 risk allele carriage
Leung, 2017, Hong Kong (J. C. Leung et al. 2017a)	Lean BMI < 25 kg/m^2, biopsy-proven MAFLD	72 lean and 235 non-lean MAFLD	Lean MAFLD patients (23.5%) had less severe disease and better prognosis than non-lean MAFLD. Hypertriglyceridaemia and high creatinine were associated with advanced liver disease in lean MAFLD
Fracanzani, 2017, Italy (Fracanzani et al. 2017)	Lean BMI < 25 kg/m^2, biopsy-proven MAFLD	143 lean and 526 non-lean MAFLD	Lean MAFLD patients (21.4%) had higher TM6SF2 risk allele carriage and lower metabolic syndrome, less NASH and lower fibrosis but thinner carotid intima compared to non-lean MAFLD
Sookoian, 2017, Argentina (Sookoian and Pirola 2018)	Systematic review, lean BMI ≤ 25 kg/m^2	493 lean and 2209 non-lean MAFLD	Lean patients tended to have milder histological features compared to non-lean MAFLD
Sookoian, 2017, Argentina (Sookoian and Pirola 2017)	Systematic review with meta-analysis, lean BMI ≤ 25 kg/m^2, hepatic steatosis on liver ultrasound	1966 lean and 5938 non-lean MAFLD; 9946 lean healthy and 6027 obese healthy controls	Lean MAFLD shared common altered metabolic and cardiovascular profile compared to non-lean MAFLD, although the effect is less severe in lean MAFLD
Hagstorm, 2017, Sweden (Hagstrom et al. 2018)	Lean BMI < 25 kg/m^2, biopsy proven MAFLD	123 lean, 335 overweight and 188 obese MAFLD	Lean MAFLD patients (19%) had lower fibrosis at better metabolic profile at baseline but increased risk of development of severe liver disease
Denkmayr, 2018, Austria (Denkmayr et al. 2018)	Lean BMI ≤ 25 kg/m^2, biopsy proven MAFLD	72 lean, 242 overweight and 150 obese MAFLD	Lean MAFLD patients (15.9%) had severe histological features similar to obese but more progressed than overweight MAFLD
Tobari, 2018, Japan (Tobari et al. 2018)	Lean BMI < 25 kg/m^2, biopsy proven MAFLD	116 lean, 173 overweight and 115 obese MAFLD	Advanced fibrosis was not associated with BMI but histological steatosis was more common in lean

			MAFLD
Li, 2019, China (C. Li et al. 2019)	Lean BMI < 24 kg/m^2, hepatic steatosis on liver ultrasound	84 lean and 85 non-lean MAFLD; 90 lean healthy and 92 non-lean healthy controls	Lean MAFLD patients had comparable total caloric, calorigenic nutrition, iron, sleep duration and overtime work as obese MAFLD
Niriella, 2019, Srilanka (Niriella et al. 2019)	Lean BMI < 23 kg/m^2, hepatic steatosis on liver ultrasound	120 lean and 816 non-lean MAFLD; 1206 healthy controls	Lean MAFLD patients (4%) had similar risk of developing metabolic comorbidities compared to non-lean MAFLD, with higher MAFLD associated with PNPLA3 incidence
Yilmaz, 2019, Turkey (Yilmaz et al. 2019)	Lean BMI < 25 kg/m^2, biopsy proven MAFLD	30 lean and 428 non-lean MAFLD	Lean MAFLD was present in 6.4% of the study sample, with metabolic syndrome present in 63% of the sample population
Wang, 2019, China (Q. Wang et al. 2019)	Lean BMI < 25 kg/m^2, biopsy confirmed MAFLD	36 lean and 48 non-lean MAFLD	Lean MAFLD patients (42.9%) have a female predominance and more advanced fibrosis compared to non-lean MAFLD patients

9.2 SUPPLEMENTARY PROTOCOLS

9.2.1 AMIDE protocol

AMIDE Method

Introduction

This method measures polar compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) in the negative ion mode. Positive mode can also be used. Analytes include amino acids, nucleotides, nucleosides, nucleotide triphosphates, high energy intermediates, organic acids, TCA cycle intermediates, bile acids and vitamins.

Materials

Chemicals:

Methanol (MeOH)	Thermo Fisher	FSBA456-4	Methanol OPTIMA LC/MS grade– 4L
Acetonitrile (ACN)	Thermo Fisher	FSBA955-4	Acetonitrile OPTIMA LC/MS grade– 4L
Water	Thermo Fisher	FSBW6-4	Water OPTIMA LC/MS grade– 4L
Ammonium Hydroxide	Sigma	17837-100ml	Fluka, puriss. p.a., ~98%
Ammonium Acetate	Sigma	73594-100G	Fluka
L-Phenylalanine-d_8 (98%)	CIL*	DLM-372-1	Isotopically labeled internal standard
Thymine-d_4			
Inosine-$15N_4$	Sigma		Isotopically labeled internal standard

*CIL = Cambridge Isotope Laboratories, Inc.

Equipment:

Pipettors	Eppendorf	various	Research plus
Multitube vortexer	Ratek	VM1	Vortex mixer (By CPC)
Centrifuge	Thermo Fisher	FRESCO 21	Centrifuge (By CPC)
Agilent Vial Rack	Agilent	5067-0243	Rack for 2 mL glass vials

Consumable supplies:

XBridge Amide column	Waters	186004868	XBridge Amide 4.6 x 100mm, 3.5 µm
Glass Vials	Waters	186000273	Clear screw-top vial, 100 per pack
Glass Vial Inserts	Waters	WAT072294DV	200 µL deactivated glass insert, 100 per pack
Vial Caps	Waters	186000274	Screw cap with bonded PTFE/silicone septa
Pipet tips	Eppendorf	various	Tips for Pipettes Research plus

Reagent Preparation

Amide Injection Solvent: Acetonitrile:Methanol (75:25, v:v)

1. Add 125 mL of methanol in a 500 mL glass bottle.
2. Add 375 mL of acetonitrile.
3. Store the solvent tightly-capped to prevent evaporation.

Mobile Phase A: (95:5 H_2): Acetonitrile (v:v) with 20mM Ammonium Acetate and 20mM Ammonium Hydroxide

1. Transfer 1.54g of ammonium acetate to 945 mL of HPLC grade water in a 1L glass bottle
2. Add 7mL of HPLC grade 10% ammonium hydroxide
3. Add 50mL of acetonitrile
4. 4. Confirm pH = 9.0 with pH paper. Store at room temperature, tightly capped. Store for up to 2 weeks.

Mobile phase B: 100% acetonitrile

1. Add 1000mL of HPLC grade acetonitrile in a 1L glass bottle
2. 2. Store at room temperature, tightly-capped. Store for up to 6 weeks.

Stock Solution of Internal Standards: (10 mM final concentrations)

1. Weigh out 17.32 mg of isotopically labeled reference standard L-Phenylalanine-d_8, 13.01 mg of thymine-d_4 and 12.52 mg of L-Valine-d_8 in a 15 mL screw cap vial.
2. Add 10 mL of methanol to yield a final concentration of 10 mM.
3. Store at -20°C, tightly-capped to prevent evaporation.

Amide IS-IS: Generic injection solvent with Amide Internal Standards: (10 mM of L-Phe-d_8 stock, 10 mM of thymine-d_4 stock, 10 mM of L-Valine-d_8 stock solution)

1. For 500 mL:
   100 µL of 10 mM stock solution of
   Phenylalanine-d_8, 100 µL of 10 mM thymine-d_4,
   and
   100 µL of 10 mM Valine-d_8
   fill bottle with 500 mL of Generic Injection Solvent.
2. Store at -4°C, tightly-capped to prevent evaporation

Standards Preparation for LC-MS/MS

Standards:

1. Add 30 μL of 500 μM analytical standard to 70 μL of Amide IS-SS solution for a final volume of 100 μL in a 0.6 mL microfuge tube to yield a final concentration of 100 μM.
2. Vortex the samples to mix both.
3. Pipet 70 μL of sample into a glass vial with glass insert. Cap each vial tightly and store at 4°C (or 10°C in the autosampler stack).

Sample preparation

Extraction for Routine samples for negative mode:

1. Transfer 20 uL of plasma into a 0.6mL microfuge tube, Add 80uL of amide IS-IS (cooled to -20^{0}C) for a final volume of 100uL.
2. Vortex the samples to promote protein precipitation
3. Centrifuge samples at 14000 rpm for 20 minutes at 4^{o}C
4. Transfer 75uL of supernatant into glass vial with inserts, taking care to avoid transferring protein pellet particles. Cap each vial tightly and store at -30^{o}C (or 10^{o}C in the autosampler stack)

For plasma spiked with standards:

1. Transfer each sample into a 0.6 mL microfuge tube.
2. Add 20 μL of 500 μM analytical standard to 30 μL of plasma to a final volume of 50 μL.
3. Vortex the samples to mix both.
4. Pipet 30 μL of the mixture and add to 70 μL of Generic IS-IS solution for a final volume of 100 μL in another 0.6 mL microfuge tube.
5. Vortex the samples to promote protein precipitation.
6. Centrifuge samples at 14000 rpm for 20 min at 4°C.

7. Pipet 75 µL of sample into a glass vial with glass insert, taking care to avoid transferring protein pellet particles. Cap each vial tightly and store at 4°C (or 10°C in the autosampler stack).

Analysis

Analyst 1.5.1 Acquisition Method Name: AMIDE_unsched_MRM.dam

Autosampler settings:

Autosampler:	Agilent 1260 Infinity Standard Autosmapler
Syringe:	50 µL
Needle Rinse 1:	75:25 HPLC Water:Acetonitrile
Needle Rinse 2:	Acetonitrile
Sample Stack Temperature	10°C

Cycle Name:	
Delay Time Column 1:	0 sec
Inject 2 Time:	1200 sec
Delay Time Column 2:	0 sec
Pre-clean with Solvent 2:	1
Pre Inject Delay:	500 ms
Post Inject Delay:	500 ms
Column Sample Volume:	10 µL
Filling Speed:	5 µL/sec
Injection Speed:	10 µL/sec
Post Clean with Solution 1:	2
Post Clean with Solution 2:	2
Valve Clean with Solution 1:	2
Valve Clean with Solution 2:	2
Replicate Count:	1

HLPC settings

HPLC: Agilent 1260 HPLC
Guard column: XBridge BEH Amide Van Guard Catridge, 3.5µm,
2.1x5mm Column: XBridge Amide, 3.5µm, 4.6 x 100mm
Flow rate: 0.250 – 0.500 mL/min
Column Sleeve: 25 cm column sleeve
Column Temp: 30°C
Injection Volume: 10 µL
Run Time: 16 minutes
Mobile Phase A: 95:5 H_2O:Acetonitrile (v:v) with 20mM Ammonium Acetate and 20mM Ammonium Hydroxide (pH 9.0)
Mobile Phase B: Acetonitrile
Needle Rinse 1: Water:Acetonitrile (75:25,
v:v) Needle Rinse 2: Acetonitrile

Step	Total Time (min)	Flow Rate (µL/min)	% A	% B
0	0.0	250	15	85
1	8.0	250	65	35
2	9.0	250	98	2

3	10.0	250	98	2
4	11.0	250	15	85
5	12.5	500	15	85
6	15.0	500	15	85
7	16.0	250	15	85

Mass Spectrometer settings

Mass Spectrometer: AB Sciex API-5500 QTrap triple quadrupole mass spectrometer Interface: Turboionspray, negative ionization mode
Scan Mode: Scheduled multiple reaction monitoring (MRM) MRM Window: 30 sec
Target Scan Time: 1.0 sec
Source Temp: 450°C
Ion Source position: Vertical: 1, Horizontal: 5 Parameters
CAD Gas: High CUR Gas: 25 Ion Spray (v): 4500
TEMP: 350 Gas 1: 33 Gas 2: 33
Exit Potential (EP): -10
Resolution Q1: Unit
Resolution Q3: Unit

Diverter Valve:

Valve: Valco Diverter Valve 10 port 2 position LTG WC027522 (Applied Biosystems), or equivalent
Valve Cable Assembly 2 position actuator, WC024740
Position A: Flow to waste
Position B: Flow to ion source

Negative Ionization Mode

	Q1	Q3	QTRAP 5500 (RT)	Metabolites	DP	EP	CE	CX	KEGG Identifier
1	146.993	100.9	12.68	2-hydroxy-2-methylbutyric acid (2H2MB)	-55	-10	-20	-13	HMDB01987
2	146.957	128.8	12.68	2-hydroxyglutarate (2-HG)	-55	-10	-18	-7	C02630
3	128.646	101	4.80	2-ketohexanoic acid (2-KH)	-105	-10	-14	-19	C00902
4	910.09	159	12.93	3-hydroxy-3-methylglutaryl coA (3H3MGcoA)	-165	-10	-108	-17	C00356
5	102.95	58.8	11.78	3-hydroxybutyrate (3-HB)	-30	-10	-14	-7	C01089
6	184.885	78.7	13.02	3-phosphoglycerate (3-PG)	-125	-10	-56	-39	C00597
7	190.952	146.8	11.07	5-hydroxyindoleacetic acid (5-HIAA)	-35	-10	-18	-21	C05635
8	425.576	78.6	13.02	ADP.1	-105	-10	-102	-29	C00008
9	157.078	42	11.50	Allantoin	-30	-10	-46	-7	C02350
10	505.894	158.9	13.08	ATP.1	-115	-10	-44	-17	C00002
11	505.894	78.9	13.08	ATP.2	-115	-10	-122	-9	C00002
12	465.163	96.7	3.59	Cholesteryl sulfate (CholSO4)	-220	-10	-94	-31	C18043

13	327.982	134.2	11.90	cAMP	-60	-10	-46	-13	C00575
14	407.275	343.4	11.00	Cholic acid	-160	-10	-48	-19	C00695
15	391.225	345.1	6.88	Deoxycholic acid (DCA)	-150	-10	-48	-19	C04483
16	116	74.3	13.10	Guanidoacetic acid (GAA)	-25	-10	-16	-11	C00581
17	131.011	84.9	5.23	Hydroxyisocaproic acid (HICA)	-65	-10	-22	-9	HMDB00746
18	134.971	91.8	11.14	Hypoxanthine	-5	-10	-24	-11	C00262
19	207.012	190	11.99	Kynurenine	-50	-10	-12	-27	C00328
20	132.932	114.9	12.78	Malate	-50	-10	-18	-13	C00149
21	130.005	88	11.75	N-Acetyl-L-Alanine	-30	-10	-16	-15	C01073
22	165.129	147.1	6.49	Phenyllactic	-40	-10	-18	-11	C05607
23	163.123	91.1	4.69	Phenylpyruvate	-30	-10	-16	-1	C00166
24	127.947	82.2	12.23	Pyroglutamic acid	-40	-10	-22	-11	C01879
25	242.949	42.1	11.06	Uridine	-75	-10	-44	-9	C00299
26	150.961	107.9	11.96	Xanthine	-20	-10	-24	-5	C00385
27	282.932	150.9	12.55	Xanthosine	-75	-10	-28	-15	C01762
28	203.939	159.8	11.75	Xanthurenic acid (XAN)	-50	-10	-22	-19	C02470
29	808.099	79.1	12.75	Acetyl-coA	-135	-10	-130	-41	C00024
30	345.905	78.8	12.78	AMP.1	-85	-10	-84	-21	C00020
31	345.905	134.2	12.78	AMP.2	-85	-10	-50	-13	C00020
32	242.991	42.2	11.85	Biotin	-50	-10	-56	-7	C00120
33	267.022	135.1	11.96	Inosine	-40	-10	-36	-21	C00294
34	204.819	170.8	6.70	Lipoate	-30	-10	-16	-35	C00725
35	174.999	131.8	13.12	N-carbomyl-aspartate	-45	-10	-18	-7	C00438
36	449.201	434.2	4.07	Phytonadione	-30	-10	-42	-25	C02059
37	283.094	182.9	3.78	Tropisetron (Drug)	-135	-10	-44	-29	D02130
38	88.83	42.94	11.64	Lactate	-34	-10	-17	-11	C00186
39	175.055	114.9	12.60	2-isopropylmalic acid (2-IPM)	-65	-10	-20	-13	C02504
40	99	71	6.89	2-methylacetoacetate (2-MAA)	-30	-10	-16	-19	HMDB03771
41	100.963	57.1	6.90	2-oxobutanoate (2-OB)	-60	-10	-12	-9	C00109
42	150.99	106.9	11.04	4-hydroxyphenylacetic acid (4-HPA)	-20	-10	-14	-17	C00642
43	138.881	78.7	6.30	Acetylphosphate (ACP)	-5	-10	-34	-9	C00227
44	425.576	134	13.02	ADP.2	-105	-10	-34	-13	C00008
45	128.959	85	12.50	Citraconic acid	-5	-10	-16	-11	C02226
46	611.072	306.1	13.08	Glutathione oxidized (GSSG)	-85	-10	-36	-17	C00127
47	167.044	123.1	6.57	Homogentisate	-40	-10	-18	-13	C00544
48	852.062	78.9	12.92	Malonyl-coA	-135	-10	-128	-9	C00083
49	147.187	59.1	11.55	Mevaloate (MEV)	-40	-10	-20	-9	C00418
50	121.652	78.1	11.18	Nicotinate	-55	-10	-18	-9	C00253

51	176.892	78.9	14.32 (wider MRM-60s)	Pyrophosphate	-35	-10	-46	-9	C00013
52	87	43	7.90 (wider MRM-60s)	Pyruvate	-25	-10	-16	-11	C00022
53	177.038	128.8	9.15	D-Glucolactone	-50	-10	-14	-33	C00198
54	440.076	174.8	12.75	Folate	-95	-10	-50	-11	C00504
55	130.996	87.9	12.61	Ureidopropionic acid	-40	-10	-14	-11	C02642
56	114.985	70.9	7.06	3-methyl-2-oxobutyrate (3M2OB)	-40	-10	-14	-9	C00141
57	159.059	59.1	12.63	2-oxodipate (2-OD)	-30	-10	-20	-5	C00322
58	223.023	206	5.94	3-hydroxykynurenine (3-HK)	-35	-10	-14	-13	C02794
59	172.821	128.9	13.00	Aconitate	-15	-10	-12	-19	C00417
60	160.002	116.1	12.97	Aminoadipidic acid (AAD)	-60	-10	-20	-7	C00956
61	174.999	115	12.74	Ascorbic acid	-50	-10	-18	-7	C00072
62	343.982	149.9	12.47	cGMP	-75	-10	-34	-27	C00942
63	212.933	79	12.88	Deoxyribose-phosphate (DRP)	-115	-10	-58	-9	C00672
64	338.925	78.8	13.19	Fruc-1,6-bP	-55	-10	-92	-9	C00354
65	259.089	78.7	13.14	Fruc-6-P	-65	-10	-84	-11	C00085
66	305.92	142.8	12.70	Glutathione reduced ss(GSSH)	-55	-10	-30	-19	C00051
67	89	59	6.98	D/L-Glyceraldehyde	-25	-10	-10	-5	C02426/C00577
68	181.944	79.8	12.80	Homocysteic acid (HCA)	-70	-10	-38	-13	C16511
69	190.913	72.9	13.04	Isocitrate	-35	-10	-36	-35	C00311
70	187.975	144	8.30	Kynurenic acid	-105	-10	-24	-15	C01717
71	114.531	70.982	13.15	Fumarate	-22	-10	-13	-10	C00122
72	154.936	111	7.04	Orotate	-25	-10	-16	-13	C00295
73	135.974	92.2	5.51	p-aminobenzoic acid (p-AB)	-80	-10	-16	-11	C00568
74	167.047	79	13.03	Phosphoenolpyruvate (PEP)	-10	-10	-46	-13	C00074
75	274.797	257.1	13.13	Saccharopine	-40	-10	-22	-19	C00449
76	383.063	133.9	13.07	S-adenosyl-L-homocysteine	-40	-10	-40	-21	C00021
77	866.034	158.6	12.89	Suc-coA	-180	-10	-90	-27	C00091
78	124	80	12.61	Taurine	-50	-10	-22	-55	C00245
79	497.727	79.8	6.90	Taurodeoxycholic acid (TDCA)	-280	-10	-108	-25	C05463
80	423.946	78.9	13.26	Thiamine pyrophosphate (TPP)	-50	-10	-80	-9	C00068
81	241.037	42.1	8.10	Thymidine	-80	-10	-60	-11	C00214
82	111.05	42.1	8.24	Uracil	-35	-10	-24	-11	C00106
83	182.983	149.9	6.94	3-methoxy-4-hydroxyphenylglycol (3M4HPG)	-50	-10	-20	-25	C05594
84	135.913	91.8	5.21	Anthranilic acid (AA)	-50	-10	-22	-27	C00108

85	289.046	271	13.20	Arginosuccinate	-75	-10	-18	-19	C03406
86	198.852	79.1	13.04	Erythrose 4-phosphate (E4P.1)	-50	-10	-62	-13	C00279
87	198.852	97	13.04	E4P.2	-50	-10	-16	-3	C00279
88	168.879	78.9	12.87	Glyceraldehyde 3-phosphate (G3P)	-5	-10	-40	-15	C00118
89	273.993	130.8	12.82	Glutarylcarnitine (C5-DC)	-30	-10	-18	-15	HMDB13130
90	102.945	59	12.77	Malonate	-15	-10	-14	-15	C00383
91	120.712	42.2	7.18	Niacinamide	-30	-10	-70	-9	C00153
92	130.974	86.9	12.72	Oxaloacetate	-40	-10	-16	-13	C00036
93	224.988	162.8	12.72	Prephenate	-40	-10	-12	-9	C00254
94	300.924	151	6.82	Quercetin	-80	-10	-34	-25	C00389
95	454.988	79.1	12.77	Riboflavin 5'-monophosphate (R5'MP.1)	-90	-10	-106	-9	C00061
96	454.988	96.8	12.76	R5'MP.2	-90	-10	-46	-15	C00061
97	605.994	78.7	12.89	UDP-N-acetyl-glucosamine	-140	-10	-128	-9	C00043
98	402.972	79	13.06	Uridine 5'-diphosphate (U5'dP)	-100	-10	-30	-15	C00015
99	123.78	76.9	13.03	Val-d8	-85	-10	-18	-13	
100	172.075	154.2	12.55	Phe-d8	-130	-10	-20	-9	
101	129.026	42.1	7.95	Thymine-d4	-100	-10	-30	-15	
102	258.988	78.9	13.32	Glucose-6-phosphate	-75	-10	-80	-9	C00092
103	116.72	72.98	13.43	Succinate	-24	-10	-17	-10	C00042
104	230.9	80	6.48	CSA (Camphor-10-sulfonic acid)	-170	-10	-40	-13	
105	664.42	158.893 (514, 649)	13.17	NADH	-110	-10	-69	-16	C00004
106	179.037	32	13.56	Glucose	-45	-10	-26	-15	C00031
107	663.606	78.9(158.9, 540.4)	13.19	NAD	-70	-10	-124	-11	C00003
108	743.939	158.919	13.39	NADPH	-88	-10	-77	-15	C00005
109	743.293	621.079	13.52	NADP.1	-72	-10	-27	-28	C00006
110	743.293	158.895	13.52	NADP.2	-72	-10	-55	-22	C00006
111	190.843	110.970	12.89	Citrate	-50	-10	-19	-12	
112	152.016	107.900	7.20	3-hydroxyanthralinic acid	-85	-10	-20	-11	

****FROM JOHN (We use HILIC method for +ve Ionization)**

Ionization mode	Q1 Precursor	Q3 Product	RT Retention time	eV Collision energy	Metabolite name gray boxes are isobaric	KEGG Identifier
+	61.1	44.2		25	Urea	C00086
+	62.1	44.2		12	ethanolamine	C00189
+	69	42.24		23	Imidazole	C01589
+	76	48		10	glycine_HILIC	
+	76.1	30.5		18	glycine	C00037

+	76.1	42		50	trimethylamine-N-oxide	
+	78	61		20	cysteamine	
+	89	72		12	putrescine	C00134
+	90.01	72		13	beta-alanine	
+	90.04	44.1		20	sarcosine	C00213
+	90.1	44.2		13	alanine	C00041
+	93	57		12	glycerol	
+	102	58		21	betaine aldehyde	C00576
+	104	60		21	choline	C00114
+	104.1	60		27	choline_HILIC	
+	104.01	69		22	4-aminobutyrate	C00334
+	104.02	58		21	dimethylglycine	C01026
+	104.1	87		17	GABA	
+	104.1	86		16	aminoisobutyric acid	
+	106	60		15	serine	C00065
+	112	95		26	histamine	
+	112.1	95		19	cytosine	C00380
+	114	44.2		19	Creatinine	C00791
+	116.1	70.1		13	proline	C00148
+	118	91		26	indole	C16074
+	118.02	58		36	betaine	C00719
+	118.1	55.2		13	valine	C00183
+	118.1	72		18	valine_HILIC	
+	118.1	58		41	betaine	
+	119.1	87		8	methyl-hydroxyisobutyric acid	
+	120	74		13	threonine	C00188
+	120.15	44.2		32	homoserine	C00263
+	121	94		25	purine	C00465
+	122.1	59.1		29	cysteine	C00491
+	123	80		30	niacinamide	
+	123.1	80		22	nicotinamide	C00153
+	126.1	68.1		29	1-methylhistamine	
+	126.2	44.1		31	taurine	
+	127.002	81		15	Imidazoleacetic acid	C05828
+	127.1	110		19	thymine	C00178
+	130	84		18	DL-Pipecolic acid	C00408
+	131.001	114		12	N-Acetylputrescine	C02714
+	132.004	68.2		19	hydroxyproline	C01157
+	132.1	86		13	leucine-isoleucine	C00123
+	132.1	90		17	creatine	
+	132.1	86.2		18	cis/trans hydroxyproline	
+	133	70		14	ornithine	C00077
+	133.4	70		30	ornithine_HILIC	
+	133.1	74		19	asparagine	C00152
+	133.1	115		12	N-carbomoyl-beta-alanine	
+	134	74		17	aspartate	C00049
+	136	119		26	adenine	C00147
+	136	90		20	homocysteine	
+	136.02	119.02		12	Methylcysteine	C00155
+	136.12	90.1		17	homocysteine	C00155
+	137.001	94		20	methylnicotinamide	C02918
+	138	120		18	anthranilic acid	

+	142	44		22	phosphoethanolamine	
+	142.1	95		20	histidinol	C00860
+	146	112		15	spermidine	C00315
+	146.1	87		21	acetylcholine	
+	146.2	72		22	spermidine	
+	147	67		32	lysine	C00047
+	147.1	84		25	lysine_HILIC	
+	147.1	84.1		17	glutamine	C00064
+	148	106		14	O-acetyl-L-serine	C00979
+	148.1	84.1		17	glutamate	C00025
+	150.1	133		12	methionine	C00073
+	150.1	61		31	methionine_HILIC	
+	152.2	110		20	guanine	C00242
+	153	135		9	xanthine	
+	153	108		16	cystamine	
+	154	136.2		18	3-hydroxyanthranilic acid	
+	156.1	110.1		14	histidine	C00135
+	159	116		11	allantoin	
+	160	55.3		21	2-Aminooctanoic acid	HMDB00991
+	162.1	103		20	carnitine	C00318
+	162.1	85		29	carnitine-HILIC	
+	163.1	85		29	glucose	
+	166	74		14	Methionine sulfoxide	HMDB02005
+	166.1	103		30	phenylalanine	C00079
+	166.1	120.2		19	phenylalanine_HILIC	
+	169	134		25	Pyridoxamine	C00534
+	170	134		24	pyridoxine	C00314
+	170.1	124		20	1-Methyl-Histidine	C01152
+	174.2	128		19	phenylalanine-d8	
+	175	115.1		16	N-acetyl-L-ornithine	C00437
+	175.02	60		16	arginine	C00062
+	175.1	70		32	arginine_HILIC	
+	176	159		14	citrulline	C00327
+	176	113.2		20	citrulline_HILIC	
+	177.05	74		19	N-carbamoyl-L-aspartate	C00438
+	177.1	80		34	cotinine	
+	177.1	160		18	serotonin	
+	180	162		12	glucosamine	C00329
+	182.1	77		39	tyrosine	C00082
+	182.5	136.1		19	tyrosine_HILIC	
+	184.001	125		23	Phosphorylcholine	C00588
+	186	88		12	3-phospho-serine	C01005
+	189.001	84.2		26	N6-Acetyl-L-lysine	C02727
+	189.002	84		23	Acetyllysine	C02727
+	189.1	130		17	N-acetyl-glutamine	HMDB06029
+	189.1	70		40	NMMA	
+	190.1	84.1		24	N-acetyl-glutamate	C00624
+	190.2	144		29	kynurenic acid	
+	192.3	146.2		18	5-HIAA	
+	202.1	129.1		19	spermine	C00750
+	203.2	129.3		20	spermine_HILIC	
+	203	70		24	Ng,NG-dimethyl-L-arginine	C03626

+	203.1	70.3		40	ADMA/SDMA	
+	204	85		19	Acetylcarnitine DL	C02571
+	204.4	85.1		28	C2-carnitine	
+	205	146		18	tryptophan	C00078
+	205.5	188.3		16	tryptophan_HILIC	
+	206	160		30	xanthurenate	
+	209	146		25	Kynurenine	C00328
+	218.4	85.1		28	C3-carnitine	
+	221.1	204		18	5-hydroxytryptophan	
+	222	138		18	N-acetyl-glucosamine	C00140
+	223	121		29	Flavone	C15608
+	223	134		13	cystathionine	C00542
+	225	208		30	3-hydroxy kynurenine	
+	227.1	110		33	carnosine	
+	228.1	112.1		15	2'-deoxycytidine	
+	231	216		27	Visnagin	
+	232.003	90		14	creatine	C00300
+	232.4	85.1		28	C4-butyryl-carnitines	
+	234.2	113.2		33	carnosine-d7	
+	235	176		22	5-methoxytryptophan	HMDB02339
+	241.002	74		32	Cystine	C00491
+	241.1	109.1		33	anserine	
+	243.1	127		16	thymidine	
+	244.1	112		14	cytidine	C00475
+	245.1	227		20	biotin	C00120
+	245.2	113.1		17	uridine	
+	246.5	85.1		27	C5-valeryl-carnitines	
+	248.4	85.1		28	C3-malonyl-carnitine	
+	252	136		22	deoxyadenosine	C00559
+	258.1	104		16	Glycerophosphocholine	C00670
+	259	110		24	acadesine	D02742
+	260	126		17	D-glucosamine-6-phosphate	C00352
+	260.1	162.1		17	D-glucosamine-1-phosphate	C03783
+	260.5	85.1		27	C6-carnitine	
+	262.4	85.1		28	C4-methylmalonyl-carnitine	
+	265	122		19	thiamine	C00378
+	267.2	190.3		27	atenolol	
+	268	88		31	S-ribosyl-L-homocysteine_pos	C03539
+	268.1	152		17	deoxyguanosine	C00212
+	268.15	136.1		29	adenosine	C00212
+	268.2	116.3		30	metoprolol	
+	274.5	85.1		27	C7-carnitine	
+	276.5	85.1		27	C5-glutaryl-carnitine	
+	281.8	150		27	1-Methyladenosine	C02494
+	284.1	135		35	guanosine	C00387
+	285.1	153		18	xanthosine	
+	288.5	85.1		27	C8-carnitine	
+	291	70		37	L-arginino-succinate	C03406
+	291.1	70		54	arginosuccinate_HILIC	
+	298	136		29	S-methyl-5-thioadenosine	C00170
+	298.002	166		24	7-methylguanosine	HMDB01107
+	302.5	85.1		27	C9-carnitine	

+	308	112		18	dCMP	C00239
+	308.1	162		21	glutathione	C00051
+	309.2	251		20	Warfarin	
+	316.6	85.1		27	C10-carnitine	
+	323	81		19	dTMP	C00364
+	324	112		18	CMP	C00055
+	325	97		14	UMP	C00105
+	330.3	136.2		30	cAMP	
+	332.1	136		23	dAMP	C00360
+	335	123		30	Nicotinamide ribotide	C00455
+	339	110		32	C9H15N4O8P	
+	344.6	85.1		31	C12-carnitine	
+	345.2	122		15	thiamine-phosphate	C01081
+	348.1	135		38	dGMP	C00362
+	348.15	136		23	AMP	C00020
+	348.2	62.3		37	anandamide	
+	349	137		21	IMP	C00130
+	355	250		20	S-adenosyl-L-methioninamine	C01137
+	357.9	139		20	Indomethacin	
+	364	152		21	GMP	C06193
+	365	97		13	xanthosine-5-phosphate	C00655
+	372.7	85.1		31	C14-carnitine	
+	377	243		26	riboflavin	C00255
+	385.1	136		21	S-adenosyl-L-homoCysteine_pos	C00021
+	399.1	250		15	S-adenosyl-L-methionine	C00019
+	400.7	85.1		35	C16-carnitine	
+	406.2	84		60	lisinopril	
+	407.2	100		30	carvedilol	
+	424.7	85.1		35	C18:2-carnitine	
+	426.7	85.1		35	C18:1-carnitine	
+	428.7	85.1		35	C18-carnitine	
+	442	295		18	folate	C00504
+	444.2	178		32	7,8-dihydrofolate	C00415
+	455.3	165.3		40	verapamil	
+	460.1	313.1		21	5-methyl-THF	C00440
+	475.2	100.2		45	sildenafil	
+	489.3	184.2		55	citicholine	
+	494.1	169.1		51	glyburide	
+	505.8	85.1		35	C24:4-carnitine	
+	525.5	352.8		31	Diiodothyronine	HMDB00582
+	540.9	85.1		35	C26-carnitine	
+	559.3	440.3		30	atorvastatin	
+	613	231		35	glutathione disulfide_pos	C00127
+	651.9	606.1		35	triiodothyronine	
+	664.1	428		32	NAD+_pos	C00003
+	666.1	514		28	NADH	C00004
+	678.3	147.3		52	cobalamin	
+	688	348		27	dephospho-CoA_pos	C00882
+	744.2	136		50	NADP+_pos	C00006
+	746.15	729		18	NADPH	C00005
+	768	261		39	coenzyme A_pos	C00010
+	777.8	732		35	thyroxine	

+	786	348		26	FAD	C00016
+	810	303		30	acetyl-CoA_pos	C00024
+	824.1	317.1		35	propionyl-CoA_pos	C00100
+	852	345		36	acetoacetyl-CoA_pos	C00332
+	854	347		28	malonyl-CoA_pos	C00083
+	868.1	361.1		40	succinyl-CoA_pos	C00091

9.2.2 HILIC protocol

HILIC Method

Introduction

This method measures polar compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) in the positive ion mode. Analytes include amino acids, nucleotides, neurotransmitters and selected medications and vitamins.

Materials

Chemicals:

Methanol (MeOH)	Thermo Fisher	FSBA456-4	Methanol OPTIMA LC/MS grade– 4L
Acetonitrile (ACN)	Thermo Fisher	FSBA955-4	Acetonitrile OPTIMA LC/MS grade– 4L
Water	Thermo Fisher	FSBW6-4	Water OPTIMA LC/MS grade– 4L
Formic Acid	Sigma	06440	Fluka, puriss. p.a., ~98%
L-Phenylalanine-d_8 (98%)	CIL*	DLM-372-1	Isotopically labeled internal standard
L-Valine-d_8 (98%)	Sigma	486027	Isotopically labeled internal standard

*CIL = Cambridge Isotope Laboratories, Inc.

Equipment:

Pipettors	Eppendorf	various	Research plus
Multitube vortexer	Ratek	VM1	Vortex mixer (By CPC)
Centrifuge	Thermo Fisher	FRESCO 21	Centrifuge (By CPC)
Agilent Vial Rack	Agilent	5067-0243	Rack for 2 mL glass vials

*ASP = Analytical Sales & Products

Consumable supplies:

HPLC HILIC column	Waters	186002015	Atlantis HILIC Silica 2.1 x 150mm, 3 μm
Glass Vials	Waters	186000273	Clear screw-top vial, 100 per pack
Glass Vial Inserts	Waters	WAT072294DV	200 μL deactivated glass insert, 100 per pack
Vial Caps	Waters	186000274	Screw cap with bonded PTFE/silicone septa
Pipet tips	Eppendorf	various	Tips for Pipettes Research plus

Reagent Preparation

Extraction Medium: Acetonitrile:Methanol:Formic Acid (75:25:0.2, v:v:v)

1. Add 1 mL of formic acid to 125 mL of methanol in a 500 mL glass bottle.

2. Add 375 mL of acetonitrile.
3. Store the solvent tightly-capped to prevent evaporation.

Mobile Phase A: 0.1% Formic acid, 10 mM Ammonium Formate

1. Transfer 0.631 g of Ammonium formate to 999 mL of HPLC grade water in a 1 L glass bottle.
2. Add 1 mL of HPLC grade formic acid.
3. Store at room temperature, tightly capped.

Mobile Phase B: 0.1% Formic acid in Acetonitrile

1. Add 1 mL of HPLC grade formic acid to 999 mL of HPLC grade acetonitrile in a 1 L glass bottle.
2. Store at room temperature, tightly-capped.

Stock Solution of Internal Standards: (10 mM of L-Valine-d_8 stock and 10 mM of L-Phe-d_8 stock solution)

1. Weigh out 17.32 mg of isotopically labeled reference standard (L-Phenylalanine-d_8) and 12.52 mg of L-Valine-d_8 in a 15 mL screw cap vial.
2. Add 10 mL of methanol to yield a final concentration of 10 mM.
3. Store at -20°C, tightly-capped to prevent evaporation.

HILIC IS-SS: Extraction Medium with Internal Standard:

1. Pipet 100 μL of the stock solution containing 10 mM L-Phenylalanine-d_8 and 10 mM L-Valine-d_8 into a 500 mL glass bottle containing 500 mL of Extraction Medium.
2. Final concentration of each internal standard is 0.002 mM. Store at -4°C, tightly-capped to prevent evaporation.

Master Mix of Reference Compounds: (0.2 μg/mL)

1. To validate the HPLC retention times and tandem MS/MS transitions of the target analytes, a single master mix can be made.
2. The final concentration of each analyte is 0.2 μg/mL, which corresponds to a 1:500 dilution from each standard's stock solution.
3. Store at -20°C, tightly-capped to prevent evaporation.

Calibration Curve:

1. A calibration curve for a specified analyte can be constructed from a stable isotope-labeled standard in pooled plasma, using serial dilutions.
2. Starting with the 1000 μg/mL stock solution, make serial dilutions of the standard in Extraction Medium to achieve a final concentration of the standard of 0, 0.05, 0.1, 0.5, 1, 10, 50 and 100 μg/mL in pooled plasma.

Standards Preparation for LC-MS/MS

Standards:

1. Add 20 μL of 500 μM analytical standard to 80 μL of HILIC IS-SS solution for a final volume of 100 μL in a 0.6 mL microfuge tube to yield a final concentration of 100 μM.
2. Vortex the samples to mix both.
3. Pipet 70 μL of sample into a glass vial with glass insert. Cap each vial tightly and store at 4°C (or 10°C in the autosampler stack).

Sample Preparation

Extraction:

1. Transfer 10uL of plasma into a 0.6mL microfuge tube. Add 90uL of HILIC IS-IS (cooled to -30°C) for a final volume of 100uL.
2. Vortex the samples to promote protein precipitation.
3. Centrifuge samples at 14000 rpm for 20 min at 4°c
4. Transfer 75uL of supernatant into glass vial with inserts, taking care to avoid transferring protein pellet particles. Cap each vial tightly and store at -30°C (or 10°C in the autosampler stack)

For making pooled plasma:

1. Transfer 20 μL of each plasma from different groups (preferably from the same study mice/patient) into a 0.6 mL microfuge tube. Vortex to mix the samples. Aliquot the required amount and stored the remains in -80°C.

2. Add 90 μL of HILIC IS-SS/ 70 μL of Amide IS-SS solution and 10 μL of the pre-mixed plasma/ 30 μL of the pre-mixed plasma for Amide method to a new 0.6 mL microfuge tube to make up a final volume of 100 μL.
3. Vortex the samples to promote protein precipitation.
4. Centrifuge samples at 14000 rpm for 20 min at 4°C.
5. Pipet 75 μL of sample into a glass vial with glass insert, taking care to avoid transferring protein pellet particles. Cap each vial tightly and store at -20°C (or 10°C in the autosampler stack).

For plasma spiked with standards:

1. Transfer each sample into a 0.6 mL microfuge tube.
2. Add 20 μL of 500 μM analytical standard to 30 μL of plasma to a final volume of 50 μL.
3. Vortex the samples to mix both.
4. Pipet 30 μL of the mixture and add to 70 μL of HILIC IS-SS solution for a final volume of 100 μL in another 0.6 mL microfuge tube.
5. Vortex the samples to promote protein precipitation.
6. Centrifuge samples at 14000 rpm for 10 min at 4°C.
7. Pipet 70 μL of sample into a glass vial with glass insert, taking care to avoid transferring protein pellet particles. Cap each vial tightly and store at 4°C (or 10°C in the autosampler stack).

Equilibration

Equilibration is required for brand new columns or when you switch columns. New HILIC columns must be equilibrated with 50:50 ACN:Water for 100 min at 0.25 ml/min followed by 40 minutes of the initial mobile phase conditions. If just switching between columns, then equilibrate with initial mobile phase conditions for 60 minutes.

Analysis

Analyst 1.5.1 Acquisition Method Name: HILIC_MRM_CNY_New.dam

Autosampler settings:

Autosampler:	Leap CTC Pal or equivalent system
Syringe:	50 μL
Needle Rinse 1:	75:25 HPLC Water:Acetonitrile
Needle Rinse 2:	Acetonitrile
Sample Stack Temperature	10°C

Cycle Name:	
Delay Time Column 1:	0 sec
Inject 2 Time:	1200 sec
Delay Time Column 2:	0 sec
Pre-clean with Solvent 2:	1
Pre Inject Delay:	500 ms
Post Inject Delay:	500 ms
Column Sample Volume:	10 μL
Filling Speed:	5 μL/sec
Injection Speed:	10 μL/sec
Post Clean with Solution 1:	2
Post Clean with Solution 2:	2
Valve Clean with Solution 1:	2
Valve Clean with Solution 2:	2

Replicate Count:	1

HLPC settings

HPLC: Agilent QTRAP 5500
Guard column: XBridge BEH Amide Van Guard Catridge, 3.5µm,
2.1x5mm Column: Atlantis HILIC Silica, 3µm, 2.1x150mm
Flow rate: 0.250 – 0.400 mL/min
Column Sleeve: 25 cm column sleeve
Column Temp: 30°C (can be modified to optimize separation and peak shape) Injection Volume: 10 µL
Run Time: 25 minutes
Mobile Phase A: 0.1% Formic acid in 10 mM Ammonium Formate (pH ~2.5) Mobile Phase B: 0.1% Formic Acid in Acetonitrile
Needle Rinse 1: Water:Acetonitrile (75:25, v:v) Needle Rinse 2: Acetonitrile

Step	Total Time (min)	Flow Rate (µL/min)	% A	% B
0	0.0	250	5	95
1	0.5	250	5	95
2	6.0	250	60	40
3	9.0	250	60	40
4	10.0	250	5	95
5	11.0	400	5	95
6	23.5	400	5	95
7	24.5	250	5	95
6	25.0	250	5	95

Mass Spectrometer settings

	Q1	Q3	QTRAP 5500 (RT)	Metabolites	DP	EP	CE	CX	KEGG/HMDB Identifier
1	76.018	30	10.14	Glycine	40	10	9	13	C00037
2	90.23	44.069	10.15	Alanine	41	10	16	8	C00041
3	116.095	70.04	10.49	Proline	51	10	24	13	C00148
4	118.076	72.03	9.87	Valine	61	10	16	15	C00183
5	120.099	74.055	10	Threonine	44	10	15	8	C00188
6	121.94	59.1	11.01	Cysteine	63	10	26	9	C00491
7	132.17	86.06	9.81	Isoleucine_Leucine	37	10	16	22	
8	132.17	69.01	9.63	Isoleucine	46	10	25	16	C00407
9	132.17	43	9.52	Leucine	46	10	44	10	C00123
10	134.14	73.97	10.23	Aspartate	85	10	19.5	12.5	C00049
11	147.14	84.007	11.97	Lysine	51	10	25	12	C00047
12	147.14	44	10.24	Glutamine_spec	51	10	73	10	C00064
13	150.088	61.072	9.55	Methionine	44	10	33	10	C00073
14	166.117	120.04	9.38	Phenylalanine	40	10	21	14	C00079
15	182.139	91.066	9.41	Tyrosine	41	10	40	10	C00082
16	182.1	136	9.80	Methionine sulfone (MetSul)	51	10	15	8	HMDB02005

17	196.12	100	9.01	Methylsulfone (MES)	140	10	31	25	
18	205.114	188.047	9.21	Tryptophan	43	10	15	12	C00078
19	133.15	74	10.2	Asparagine	56	10	22	10	C00152
20	203.069	70.3	12.12	ADMA/SDMA	41	10	41	12	C03626
21	118.013	58	11.09	Betaine	66	10	43	16	C00719
22	232.076	85.1	10.84	C4-butyrl-carnitine	51	10	29	4	
23	329.93	135.9	9.52	Adenosine 3',5'-cyclic phosphate (cAMP)	41	10	47	16	C00575
24	104.808	59.8	11.28	Choline	111	10	31	12	C00114
25	176.062	113.1	10.56	Citrulline	36	10	27	8	C00327
26	103.893	57.9	10.79	Gamma aminobutyric acid (GABA)	296	10	27	26	C00334
27	189.974	144	8.23	Kynurenic acid	36	10	29	16	C01717
28	132.92	70.1	11.48	Ornithine	46	10	25	8	C00077
29	177.086	159.9	9.14	Serotonin	1	10	19	16	C00978
30	125.901	44.1	9.26	Taurine	41	10	29	10	C00245
31	777.623	731.8	8.71	Thyroxine	71	10	43	42	C01829
32	651.783	605.8	8.71	Triidothyronine	31	10	31	16	C02465
33	75.87	58	10.81	Trimethylamine N-oxide (TMAO)	96	10	27	8	C01104
34	267.004	135	9.81	3-deaazadenosine	106	10	35	14	
35	204.066	85	11.72	Acetylcarnitine	46	10	31	16	C02571
36	147.059	88.2	11.3	Acetylcholine	51	10	21	14	C01996
37	162.106	60	11.54	Carnitine	41	10	23	8	C00318
38	120.086	74	9.73	L-Homoserine	51	10	17	10	C00263
39	189	130	9.33	N-acetylglutamine	46	10	19	12	C00624
40	189.036	70	11.67	N^G-monomethyl-L- arginine (L-NMMA)	56	10	35	14	C03884
41	379.391	105.2	4.98	2-Arachidonyl glycerol	151	10	61	20	C13856
42	305.298	77.1	5.01	Arachidonic acid	21	10	95	10	C00219
43	162.079	98.1	10.67	Aminoadipic acid (AAD)	1	10	21	14	C00956
44	268.002	136	8.30	Adenosine	26	10	29	18	C00212
45	202.013	70.3	10.25	DMGV	46	10	23	8	
46	90.092	72	10.66	beta-Alanine	51	10	11	10	C00099
47	131.928	62.8	8.95	Creatine	66	10	29	10	C00300
48	184.964	98.9	11.77	Phosphocholine	61	10	29	12	C00588
49	131.753	63.1	8.98	trans-hydroxyproline (t-HYP)	51	10	51	6	C01157
50	104.024	85.9	10.24	BAIBA	61	10	11	12	C05145
51	106.074	88.094	10.08	Serine	46	10	15	10	C00065
52	163.123	45.068	12.12	GlucosePos2	54	10	28	9	C00031
53	175.139	70.02	11.27	Arginine	43	10	32	10	C00062
54	126.1	80	0	Valine-d8	40	10	18	15	
55	174.2	128	0	Phe-d8	40	10	19	15	

56	126.002	85.1	11.61	1-methylhistamine	41	10	27	10	C05127
57	252.068	57	8.48	2'-deoxyadenosine	46	10	57	16	C00559
58	227.976	111.9	9.02	2'-deoxycytidine	71	10	21	52	C00881
59	191.966	146	5.25	5-hydroxyindoleacetic acid (5-HIAA)	61	10	27	24	C05635
60	158.926	117.9	11.56	Allantoin	41	10	17	12	C01551
61	241.033	109.2	12.67	Anserine	31	10	35	16	C01262
62	177.059	80.2	8.51	Cotinine	41	10	35	12	HMDB01046
63	152.907	65.1	7.80	Cystamine	51	10	33	10	
64	78.858	61	12.64	Cysteamine	16	10	29	8	C01678
65	243.977	112.1	8.5	Cytidine	71	10	29	28	C00475
66	112.004	95	9.19	Cytosine	101	10	23	10	C00380
67	92.83	75	12.47	Glycerol	46	10	15	10	C00116
68	112.921	68.1	12.59	Histamine	36	10	45	8	C00388
69	202.977	70.1	11.9	Spermine	56	10	37	10	C00750
70	265.916	122	12.96	Thiamine	36	10	29	14	C00378
71	242.985	127.2	6.27	Thymidine	26	10	29	6	C00214
72	244.982	113.1	6.84	Uridine	66	10	23	10	C00299
73	190.001	129.9	4.92	3-indolepropionic acid (3-IPA)	56	10	31	14	HMDB02302
74	120.977	94.2	7.99	Purine	56	10	31	14	C15587
75	169.988	151.9	9.75	Pyridoxine	36	10	21	18	C00314
76	377.047	243	7.71	Riboflavin	21	10	33	18	C00255
77	348.133	67	5.67	Anandamide	76	10	30	15	C11695
78	148.15	84	9.95	Glutamate	54	10	23	10	C00025
79	156.072	110	11.34	Histidine	49	10	21	12	C00135
80	224.996	208.1	9.35	3-hydroxykynurenine (3HK)	61	10	13	6	C02794
81	227.002	110	12.48	Carnosine	51	10	33	14	C00386
82	141.868	58	11.31	Phosphoethanolamine	291	10	37	6	C00346
83	400.141	358.3	7.58	Colchicine	116	10	31	16	HMDB15466

Mass Spectrometer: AB Sciex API-5500 QTrap triple quadrupole mass spectrometer Interface: Turboionspray, positive ionization mode
Scan Mode: Scheduled multiple reaction monitoring (MRM) sMRM Window: 30 sec
Target Scan Time: 1.0 sec
Source Temp: 450°C
Ion Source position: Vertical: 1, Horizontal: 5 Parameters
CAD Gas: High CUR Gas: 25 Ion Spray (v): 4500
TEMP: 350 Gas 1: 30
Gas 2: 30 Exit Potential (EP): 10 Resolution Q1: Unit
Resolution Q3: Unit

Diverter Valve:

Valve: Valco Diverter Valve 10 port 2 position LTG WC027522 (Applied Biosystems), or equivalent
Valve Cable Assembly 2 position actuator, WC024740 Position A: Flow to waste
Position B: Flow to ion source

Notes when integrating peaks:

Leucine and isoleucine in same window. Leucine is first and isoleucine is second peak. ADMA/SDMA. Integrate both peaks

Glutamate: 1st peak
Glutamine: 2nd peak

5 OH tryptophan: 1st peak
Cytosine: middle peak