Plasma and Liver Metabolomic Profiling in Hispanic and Caucasian Subjects with Nonalcoholic Fatty Liver Disease

By

TAGREED ABDULHALEM MAZI
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutritional Biology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Valentina Medici, Chair

Angela Zivkovic

Kimber Stanhope

Committee in Charge

2022

Acknowledgment

First and foremost, all praise be to Allah the most gracious and glorious for the infinite grace, for blessing me with such an accomplishment. Next, I am filled with gratitude to many who believed in my potentials and supported me. Although words cannot express my appreciation, I must try.

I would like to thank my advisor and Dissertation Committee chair, Valentina Medici for mentorship, continuous support, and genuine advice. Thank you for handing over great projects, and for facilitating collaborations with outstanding scholars. I appreciate the independence level you allowed while always being available. These immense opportunities along with your guidance were essential for my professional development. The early times of COVID-19 pandemic were the most difficult, I am beyond thankful for the support and regular check-ins as we were distanced.

I am grateful to my Dissertation Committee members, Angela Zivkovic, and Kimber Stanhope. Thank you for your enthusiasm, flexibility, and valuable feedback. I appreciate the encouragement that always came in much needed times. Beside work on my dissertation, I was fortunate to interact with you on other levels. Angela, I very much enjoyed my teaching experience of NUT11. Thank you for the opportunity and for the support I received. Kimber, I had the honor to co-author papers with you, I appreciate these invaluable nurturing exposures.

To my Qualifying Exam Committee chair, Nancy Keim, thank you for your profound belief in my work, for all the support and guidance I received while preparing for the exam and after. To committee members, Jon Ramsey, Sheri Zidenberg-Cherr, Carolyn Slupsky and Souvik Sarkar, I am deeply grateful for your insightful suggestions that were essential to refine my research proposal.

I would also like to extend my sincere thanks to John Newman and Kamil Borkowski, I appreciate your invaluable gifts of time and patience. John, thank you for being welcoming and for facilitating resources. Kamil, thank you for the dedicated assistance with data analysis. I also appreciate you both for going above and beyond while I navigated analysis options and alternatives. Such immense support was a source of encouragement, and your valuable feedback on my analysis and writing enriched my perspectives. The fact that two chapters of this dissertations are peer-reviewed publications with both of

you as co-authors is a true privilege. I would not forget to recognize the effort of Rémy Bosviel and Sili Fan, thank you for the instrumental assistance in developing a preliminary statistical pipeline.

Profound thanks to my lab group. To Dorothy Kieffer for assisting in subjects' recruitment; to Noreene Shibata for the attentive feedback and edits on my writings; to Gaurav Vilas Sarode for advice, assistance, and most, for friendship.

I owe acknowledgement to King Saud University and administration in Riyadh-Saudi Arabia, to The Graduate Group in Nutritional biology at UC-Davis for funds that supported my graduate studies. As a recipient of The Mar and The Rucker Family Fellowships, I would like to acknowledge both families and representatives for the generous support. A special thanks go to Robert Rucker. I truly appreciate your constant check-in and encouragement, especially during the unprecedented time of COVID-19.

I am deeply indebted to my family. To my beloved parents, I am beyond appreciative! Thank you for your unconditional love and support, and for setting such an example of faith, hard work ethics and perseverance that are an inspiration to me, especially, while accomplishing this endeavor. To my dear husband, Ghassan. I commend you for surviving my graduate school journey. I appreciate your selfless support and motivation through all of this. Thank you for being present, and for bearing to uproot our family across the other side of the globe. To my beloved parents-in-law, thank you for your continuous support and encouragement, and for always keeping us in your thoughts and prayers.

To my precious kids, Jude, Abdulrahman, Lama, and Sara, you have been through it all! May God bless you always and forever. I am appreciative for many things, but most is your sweet smiles and hugs that were my supreme recharge. Big thanks to my dearest siblings, with no exception, for the unwavering logistics and moral support from the long-distance wake-up calls to flying-in to be by my side. A special thanks to Suhail, for being the coolest brother and uncle ever! For all the help and assistance through all my trials and tribulations. Last, but not least, to all my friends, here in Davis and beyond. You all have supported me in my pursuit at many levels. Thank you!

Table of Content:

Abstract

Background/Objective: Nonalcoholic fatty liver disease affects 30% of the world adult population. It comprises a spectrum of liver histopathological presentation that varies from hepatocellular lipid accumulation, or nonalcoholic fatty liver (NAFL), to nonalcoholic steatohepatitis (NASH). Mechanism(s) of NAFLD onset and progression are modulated by a multilayered interaction between genetics, environmental factors, and comorbidities. In the US., the Hispanic population presents with higher rate of NAFLD, and more advanced histological scores compared to other ethnicities. However, the underlying metabolic drivers of these observations are not clear. Previous metabolomic studies revealed that NAFLD is associated with several metabolic dysregulations in lipid, carbohydrate, and amino acid metabolism. Also, alterations in polyunsaturated fatty acids (PUFA) and downstream oxidized lipids as oxylipins (OXLs) and endocannabinoids (eCBs) are reported to distinguish different stages of NAFLD. However, is not clear if ethnicity-related metabolomic differences exist in NAFLD. The identification of ethnicity-related metabolomic differences will expand current knowledge with on mechanism(s) of NAFLD onset and progression. It can also identify unshared mechanism(s) that may explain the observed ethnicity-related disparity in NAFLD rate and severity. The objective of this "proof of principle" analyses is to explore metabolomic profiles of a group of Hispanic (HIS) and Caucasian (CAU) subjects with NAFLD undergoing bariatric surgery **Methods**: We compared metabolomic profiles in a group of obesity NAFL-HIS and NAFL-CAU subjects to ethnicity-matched lean and healthy control subjects. We also compared the profiles of subjects with NASH to NASH-free (0-NASH) subjects. For this we profiled plasma and liver samples using untargeted, semi-quantitative metabolomic approaches for metabolites related to primary metabolism using gas chromatography/time-of-flight mass spectrometry (GC-TOF MS); complex lipids using charged surface hybrid liquid chromatography/quadrupole time of flight mass spectrometry (CSH-QTOF MS/MS); choline and related metabolites using hydrophilic interaction liquid chromatography/quadrupole time of flight mass spectrometer (HILIC-QTOF MS/MS). We also performed a targeted and quantitative lipidomic analysis to profile for plasma fatty acids and related downstream lipid mediators using ultra-high-performance liquid chromatography-electrospray ionization-

tandem mass spectrometry (UPLC-ESI-MS/MS). **Results**: Findings from current analyses reveal ethnicity-related metabolic perturbation, independent of obesity. Specifically, lean, and healthy HIS subjects showed higher plasma profiles of triglycerides, acylcarnitines, free fatty acids and downstream lipid mediators. Our finding also shows that in NAFL there are ethnicity-related differences in plasma profiles of fatty acids and acylcarnitines. In specific, NAFL-CAU was characterized by higher arachidonic acid, while NAFL-HIS showed lower n-3 PUFA. With the progression to NASH, comparison within ethnicity groups indicated that the hepatic lipidomic profile in NASH-HIS was characterized higher levels of free fatty acids and lipotoxic lipids, suggesting lipotoxicity is involved in the progression of NASH. We also observed a higher hepatic triglyceride with signs of impaired mitochondrial β-oxidation. Remarkably, the plasma OXLs and eCBs profiles discriminated ethnicities with NASH, independent of histological severity. In specific, NASH-HIS was characterized with lower arachidonic acid derived OXLs, and findings suggest a downregulated lipoxygenase(s) and upregulated soluble epoxide hydrolase(s) activities. **Conclusion**: The analyses presented in this dissertation highlights the existence of ethnicity-related metabolomic variations in NAFLD that could potentially modulate disease risk and severity in HIS. It also indicates that ethnicity-specific lipidomic signature can distinguish NASH, which needs to be verified in larger studies.

Chapter 1

Literature Review

1. Definitions

Described first in 1980, [1], nonalcoholic fatty liver disease (NAFLD) is a range of abnormal histopathological presentations that include hepatocellular triglycerides (TGs) accumulation, also known as steatosis or nonalcoholic fatty liver (NAFL), necrosis and inflammation, or nonalcoholic steatohepatitis (NASH), which can advance to cirrhosis and hepatocellular cancer [2,3]. NAFL is defined by imaging or histology as the presence of hepatocellular lipid droplets, accounting for >5% the total liver weight; absence of hepatocellular injury, defined by hepatocyte ballooning, mild inflammation with or without fibrosis; after excluding secondary causes of hepatic fat accumulation. Other causes of hepatocellular fat accumulation include excessive alcohol consumption, (defined as the intake of >14 drinks/week in women or >21 drinks/week in men, or a daily intake of >10 g/day of alcohol); the use of steatogenic medication (e.g., corticosteroids, mipomersen, lomitapide, methotrexate, amiodarone); starvation; total parenteral nutrition; other coexisting liver disease etiologies, such as viral or autoimmune hepatitis, hemochromatosis, and Wilson disease [3]. On the other side, NASH is defined as the presence of NAFL in combination with histological evidence of inflammation and hepatocytes injury with or without fibrosis [3].

2. Epidemiology

According to a recent global meta-analysis that included 8.5 million subjects from 22 countries, the worldwide prevalence of NAFLD was estimated to be 25% of the general population, and this prevalence is expected to rise parallel to the metabolic syndrome (MetS) pandemic [4]. NAFLD is highly prevalent in the Middle East 32% and South America 31%, followed by the U.S. 24%, and with the rate lowest in Africa 14% [4]. When investigating NAFLD prevalence among subjects with multiple comorbidities, as obesity and type 2 diabetes mellitus (T2DM), the prevalence of NAFLD was 50% in this high-risk group compared to 16% in the general population, independent of ethnicity [5]. The prevalence of NASH in the general population is estimated to be between 2 to 6%. In morbidly obese subjects, this rate increases to 59%, whereas it is lower, being 7 to 30%, in low-risk subjects who undergoing liver biopsy without clinical suspicion [4].

3. Risk factors

3.1. *The metabolic syndrome*

The MetS is a cluster of metabolic derangements that include hypertension (HTN); low level of high-density lipoprotein-cholesterol (HDL-C) or dyslipidemia; high level of TGs or hypertriglyceridemia; elevated glucose level or T2DM; and visceral adiposity [6]. Clinically, MetS is defined as the existence of three or more of the for mentioned abnormal metabolic manifestations [6]. NAFLD is strongly associated with all components of MetS, including T2DM, dyslipidemia, TGs, and obesity [7,8]. MetS affects about 43% of subjects with NAFLD and 71% of subjects with NASH [4], with about 90% of NAFLD subjects presenting with at least one component of MetS [9-11]. Owing to this strong correlation, it is argued that NAFLD is the hepatic presentation of the MetS. However, evidence indicate that this link is bidirectional, as components of MetS can predict NAFLD onset and progression [12-14], and NAFLD may precede the development of MetS [15-17].

There are data suggesting that the effect of MetS on the risk of NAFLD may be influenced by ethnicity. In a study based on data from the U.S. Third National Health and Nutrition Examination Survey (1988–1994) and employing confirmatory factor analysis to challenge the notion that NAFLD is the hepatic continuum of MetS, a basic model constructed with components of MetS exhibited strong goodness-of-fit, whereas the addition NAFL decreased the model fit statistics [18]. When analysis was repeated by ethnicity, the addition of NAFL resulted in higher model fit for Hispanics and non-Hispanic Blacks compared to Whites, indicating that the association between MetS and NAFLD may be influenced by ethnicity [18]. In support of this evidence, findings from the NASH-Clinical Research Network (NASH-CRN) indicated that the effect of IR on the risk of NASH was modified by ethnicity, where IR was a strong predictor of NASH in non-Hispanics whites, but not in Hispanics [19].

3.2. Type 2 diabetes mellitus and insulin resistance

In patients with T2DM, the estimated worldwide prevalence of NAFLD and NASH was 55.5% and 37.3%, respectively [20]. It is estimated that about 23% of NAFLD and 44% of NASH subjects present with T2DM [4]. Compared to non-diabetic, subjects with T2DM are at higher risk for the

progression to NASH [21], fibrosis, and hepatocellular carcinoma [22]. This strong link between NAFLD and T2DM was found to be reciprocal, as results from a recent meta-analysis including studies of nondiabetic subjects show that NAFLD diagnosis increases the risk for T2DM [23].

Insulin resistance (IR), a condition characterized by impaired glucose disposal in the periphery, including muscle and adipose tissues [24,25], is thought to be a key player in the pathology of NAFLD [26,27]. In subjects with NAFL and NASH compared to healthy control and under euglycemic-clamp test, the rate of glucose disposal in response to glucose infusion was decreased and the ability to suppress adipose tissue lipolysis in response to insulin was impaired, indicating a state of peripheral IR that was evident at the level of skeletal muscle and adipose tissues [28]. Hepatic IR, or the inability of insulin to suppress hepatic glucose production in fasted state, with subsequent hyperglycemia and hyperinsulinemia [29], was also reported in NAFLD with T2DM [30] and in non-diabetic subjects [31]. The metabolic effect of IR state in the context of NAFLD is discussed in section 6.

3.3. Obesity

It was estimated that about 51% of NAFLD subjects and 82% of NASH subjects are obese [4]. Moreover, obesity was found to increase the risk of NAFLD by 3-fold compared to none-obese subjects [32-34]. In the U.S., NAFLD was estimated to affect 5 to 10% of non-obese subjects [35], and this rate increases to 95% in morbidly obese subjects [36]. The distribution of adiposity was found more determinant to NAFLD risk than amount of adiposity. This was demonstrated with a strong linear association between visceral adiposity and NAFLD [37-40], which could be explained by IR, which is associated with visceral adiposity [24,25]. The implications of obesity in the development and progression of NAFLD are discussed in section 6.

3.4. Dyslipidemia

Dyslipidemia affects about 43% of subjects with NAFLD and 71% of subjects with NASH [4]. In NAFLD, dyslipidemia mainly manifests as elevated TGs, elevated low-density-lipoprotein cholesterol (LDL-C) count or particle size, and decreased HDL-C [41,42]. The implications of dyslipidemia in the development and progression of NAFLD are discussed in section 6.

3.5. Hypertension

NAFLD is strongly associated with HTN [43,44], and arterial stiffness was found to correlate with NAFDL diagnosis, severity [45], and progression to fibrosis [46]. According to a global analysis, HTN was prevalent among 39% of subjects with NAFLD and 68% of subjects with NASH [4]. This link between NAFLD and HTN was shown to be bidirectional, as HTN increased the risk of NAFLD onset and progression, NAFLD was also found to be a strong predictor for HTN [47].

3.6. Age and sex

NAFLD diagnosis occurs more frequently after the fourth decade of life [20]. Advanced age was found to be a predictor for NAFLD development and progression to NASH [48]. This may be attributed to the fact that most NAFLD-related risk factors increase with age, as MetS, T2DM, and HTN [49,50]. The prevalence of NAFLD was reported to be higher in males compared to females [51-53]. In female, NAFLD prevalence increases significantly after the age of 50, and was found higher in postmenopausal women, compared to premenopausal, suggesting a protective role of female hormones [54-56]. The risk of NAFLD progression to fibrosis was higher in males compared to females [57,58]. Up to date, the mechanisms explaining this sex-related differences in NAFLD prevalence are not clear.

3.7. Genetics

Genome-wide association studies and exome-wide association studies revealed an association of multiple genes variants with risk of NAFLD development and progression. The identified genes were involved in the regulation of lipid and carbohydrate metabolism, mitochondrial function, insulin signaling, oxidative stress, inflammation, immune response, and fibrogenesis [59-61].

The rs738409 [C] > [G] variant of the patatin-like phospholipase domain-containing gene (*PNPLA3*), with the substitution of isoleucine to methionine at position 148 was associated with the full spectrum of NAFLD [62-68]. This association was also established in studies that included multiple ethnicities cohorts [68-73] and found to be independent of age, sex, T2DM [67], total or visceral adiposity [62,74], IR and dyslipidemia [75]. The potential mechanism by which the *PNPLA3* variant is involved in the pathogenesis of NAFLD is discussed in section 6.

The rs58542926 [C] > [T] variant of the transmembrane 6 superfamily member 2 (TM6SF2), with glutamate to lysine substitution at residue167 was associated with an increased hepatic TGs content [76-78] and a higher risk of NASH [79]. This gene variant was also associated with a marked reduction in apoB-100, very-low density lipoprotein (VLDL) particle count and size, as well as in the VLDL and LDL TGs and cholesterol content [77,78,80]. The *TM6SF2* gene encodes a trans-membrane protein that regulates the lipidation and secretion of VLDL and cholesterol synthesis [76,78,80,81]. The rs58542926 variant of *TM6SF2* results in a loss-of-function of the enzymatic activity with consequent NAFL due to reduced lipidation and/or secretion of VLDL [59].

The single nucleotide polymorphism in glucokinase regulator (GCKR) at rs780094 was associated with NAFLD in multiple ethnicities [73,82-85]. The *GCKR* gene encodes a protein that functions as a negative regulator of glucokinase, a hepatic enzyme responsible for glucose uptake [86,87]. The missense mutation impairs the protein's function, results in increased hepatic glucose uptake and malonyl Co-A production, which serves as substrate for hepatic *de novo* lipogenesis (DNL), a process that inhibits mitochondrial β-oxidation of fatty acids [88].

In a group of high-risk subjects of European descent, the rs641738 [C] > [T] gene variant of the membrane bound o-acyltransferase domain-containing 7 (*MBOAT7*) was found to be associated with a higher risk of NAFL and NASH [89], and with an increased risk of progression to hepatocellular carcinoma in NAFLD patients without cirrhosis [90]. The *MBOAT7* gene encodes for the enzyme lysophosphatidylinositol acyltransferase 1, responsible for incorporating arachidonic acid (AA, 20:4n-6) and other polyunsaturated fatty acids (PUFAs) into phospholipids (PLs) in remodeling pathways [91]. The rs641738 [C] > [T] variant is associated with a down-regulation of *MBOAT* gene transcripts and protein levels, with impaired incorporation of AA (20:4n-6) in PLs [89,92].

3.8. Diet

3.8.1. Hyper-caloric diet

A hyper-caloric diet may induce NAFLD by promoting obesity, which is a major risk factor for NAFLD [32-34]. However, NAFLD can occur in a smaller rate in non-obese subjects [35], suggesting the involvement of mechanisms that are not exclusively related to obesity. However, the link between

caloric-content and NAFLD may be influenced by weight status and dietary composition. In morbidly obese subjects, there was a lack of association between caloric content and the NAFLD spectrum, while the risk of inflammation was associated with a higher intake of carbohydrates and lower intake of fat [93]. Also, in animal models, NAFLD can be induced by caloric-restricted diet containing high sucrose or high fructose in the absence of weight gain [94,95].

3.8.2. Fructose and fructose-containing sugars

In experimental animals, dietary fructose, sucrose, or high-fructose corn syrup are commonly used to induce NAFL and NASH [95-98]. In humans, multiple observational studies pointed toward a strong association between fructose and fructose-containing sugar intake and the risk of NAFLD development and progression [93,99-104]. The intake of fructose and fructose-containing sugar was also associated with the development of unfavorable metabolic outcomes strongly linked to NAFLD, including dyslipidemia and HTN [105,106]. However, the effect of fructose-containing sugar on the development of NAFLD is controversial. There is a lack of large, long-term studies to assess this relationship. Nevertheless, in short-term setting, this relation may be confounded by excessive dose, caloric content, and weight status [107]. Results from one meta-analyses that inspected the short-term effect ($\leq$ 10 weeks) of fructose or sucrose intake on hepatic fat content, DNL, liver enzymes in normal and overweight subjects concluded that there is lack of evidence supporting the role of isocaloric or hypocaloric fructose or sucrose as cause of NAFLD [108]. Another meta-analysis that included normal and overweight subjects concluded that short-term hypercaloric and high-fructose diets increased hepatic fat content by 54%, while the intake of fructose with isocaloric diet did not induce NAFLD [109].

3.8.3. Fatty acids and cholesterol

In animals, a diet high in saturated fatty acids (SFAs) [110,111] and PUFAs deficiency was associated with development of NAFL [112-115]. In obese subjects with NAFLD, the diagnosis of NASH was associated with a high dietary intake of cholesterol [116], high SFAs and PUFAs dietary intake [99,116]. An excess dietary n-6 with reduced n-3 PUFAs was associated with NAFLD [117,118], and an increased SFA:PUFA ratio was associated with NASH [99,116]. Moreover, results

from two meta-analysis showed that n-3 PUFAs supplementation alleviated hepatic lipid content [119,120], with no effect on NASH histological outcomes [119]. Another meta-analysis concluded that n-3 PUFAs supplementation significantly reduced plasma/serum alanine aminotransferases (ALT), aspartate aminotransferases (ASL) and TGs level, however, with marginal reduction in hepatic fat content. In the same study, a dose-response analysis showed that an intake of 1.0 g/day of eicosapentanoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n- 3) predicted a 2.74% decrease of hepatic fat content and 9.97 mg/dL reduction in TGs levels [121]. Monounsaturated fatty acids (MUFA) intake, mainly as component of the Mediterranean diet [122], has been shown to be effective in reducing hepatic fat content, liver enzymes, and circulating lipid profile in subjects with NAFLD [123,124].

3.9. Ethnicity

In the U.S., disparity in NAFLD prevalence is reported, with Hispanics being impacted at higher prevalence [4,52,125-128] and severity [5,19,129]. Furthermore, this disparity trend was consistent when comparing Hispanic to non-Hispanic children and adolescents [130], suggesting that the ethnical disparity of NAFLD prevalence is independent of age [131]. However, the ethnical disparity in NAFLD prevalence was more profound in the general population, than in high-risk groups with other existing co-morbidities. In a study that examined NAFLD prevalence in high-risk cohort with obesity and T2DM, NAFLD prevalence rate was 50% and not associated with ethnicity [5]. However, in the same study, the risk of NASH among subjects with NAFLD was highest in Hispanics and lowest in African Americans, with no significant difference between ethnicities in fibrosis scores [5].

Gender disparity in the distribution of NAFLD according to ethnic group was also reported, as NAFLD in Caucasians was more prevalent in men than in women. However, this difference was not observed in Hispanic and African American [132]. Up to date, the mechanisms underpinning these ethnic disparities are not clear. Potential factors contributing to the higher rate of NAFLD in Hispanics are discussed in section 7.

4. Natural history

Both NAFL and NASH are recognized as progressive liver conditions that increase the risk for developing advanced liver disease and mortality [46,57,133-139]. It is estimated that about 30% of individuals with NAFL will eventually develop NASH [140-142]. Compared to subjects with NAFL, NASH patients are at increased risk for developing liver and non-liver-related complications [46]. About 40-50% of patients with NASH will develop fibrosis [133], with an estimated average progression of 0.09 fibrosis stages per year [4].

Mortality in NAFLD patients is mainly due cardiovascular disease, followed by non-liver malignancy, and then liver-related deaths, which is primary due to hepatocellular carcinoma [4,57,134,135]. Despite the low rate of liver-related mortality, it corresponds to 20,000,000 estimated deaths among subjects with NAFLD [4,143]. When NASH is present, this risk of liver-related mortality increases by 5 to 10-fold [144]. In another analysis, the presence of NASH with fibrosis was associated with lower survival, and this was not true for NAFL alone [145]. In the U.S., about 10% of NASH subjects receive liver transplantation [146]. NASH is considered a major indication for liver transplant [3] and was projected to be main indication in the U.S. by 2020 [146].

5. NAFLD diagnosis and treatment

Early diagnosis of NASH is determinant for its prognosis. Specifically, the identification of NASH in the pre-fibrotic stage can significantly improve the response to therapy [147]. Liver biopsy and histological confirmation are the gold standard to establish NAFLD diagnosis. On histological examination, the principal features of NAFLD are microvascular steatosis with or without the presence of lobular inflammation, peri-sinusoidal fibrosis, and hepatocellular ballooning [148]. The NAFLD activity score (NAS) is commonly used to establish a histopathological diagnosis and characterization of NASH, and it accounts for steatosis, hepatocellular ballooning, and lobular inflammation [3]. The NASH-CRN scoring system is commonly used to determine the stage of fibrosis [148]. In general, liver biopsy is an invasive procedure, subjected to risk of complications [149,150] and inter-observer variability of pathology interpretation [151].

NAFLD can also be identified and diagnosed by various abdominal imaging modalities. These include ultrasound, computed tomography, magnetic resonance elastography, and transient elastography. These imaging techniques vary according to sensitivity, specificity, and ability to quantify steatosis, necroinflammatory activity, and fibrosis [147]. The disadvantages of these imaging modalities include the high costs, radiation exposure, and limited applicability for morbidly obese patients, inability to detect low-moderate grades of fibrosis or to characterize necroinflammatory activity [152].

Despite the epidemic and significance of NAFLD, there are no FDA-approved treatments [153,154]. Clinical approaches to manage NAFLD are mainly directed to manage the concurrent components of MetS. Effective approaches include lifestyle changes, dietary modifications and increased physical activity aimed to body weight reduction [155-157]. However, lifestyle interventions are hard to comply to [158]. Bariatric surgery is indicated for s subset of subjects [159], with the benefits of reversal of NAFLD shown in long term follow up studies [160].

6. Pathophysiology

Steatosis develops when fatty acids availability exceeds the liver's disposal capacity [161]. Specifically, when the hepatic uptake of non-esterified fatty acids (NEFAs) from the circulation and lipid synthesis form DNL, outweighs the disposal rate, mainly by β-oxidation of FA and export of TGs with VLDL particles, the net result is hepatocellular lipid accumulation [162].

The mechanism of NAFLD progression was previously explained by the "two hits" hypothesis [163]. Hepatic lipid accumulation composes the "first hit". As hepatic lipid accumulation overwhelms the disposal and storage capacity, excess lipids will induce oxidative stress, inflammation, the "second hit", with ultimate progression to NASH [163]. Later, the "multiple hit" model was proposed to explain the heterogeneous presentation and disease course of NAFLD, and to include a multi-layered interaction between host genetics, environmental factors, and microbiome [164]. In the" multiple hit" model, following the accumulation of hepatocellular lipids, a cascade of metabolic insults orchestrates multiple cellular and systemic responses, including oxidative stress and lipid peroxidation, lipotoxicity, glucotoxicity, inflammation, endoplasmic reticular stress, mitochondrial dysfunction,

apoptosis, and intestinal dysbiosis, that will collectively alter lipid metabolism, induce hepatocytes injury, and provoke NAFLD development and progression [161,165-167].

Within the context of obesity and MetS, IR is considered a component of the 'multiple hits' in the pathogenesis of NAFLD [26,27]. IR is characterized by impaired glucose disposal in peripheral tissues [24,25] and results in impaired suppression of adipose tissue lipolysis, a state known as adipose tissue dysfunction, with consequent increased efflux of NEFA [168]. Adipose tissue and skeletal muscle IR were reported in NAFLD subjects compared to healthy controls [169,170]. In NAFLD patients, the insulin-mediated inhibition of adipose tissue lipolysis was found impaired and plasma NEFAs was found persistently elevated [28,171-173]. Moreover, in patients with NAFLD, adipose tissue lipolysis was identified as the major source of fatty acids in hepatocellular TGs store [174,175]. The uncontrolled lipolysis of visceral adipose tissue may further exacerbate the state of IR with the release of pro-inflammatory cytokines, which in turn triggers peripheral tissues inflammation and impair insulin signaling [176,177].

The increased flux of NEFA into the circulation is efficiently cleared by hepatic uptake [178]. Data on hepatic β-oxidation of fatty acids are inconsistent with reported increase [28,179] and decrease in NAFLD subjects compared to controls under basal conditions [180]. An impaired metabolic flexibility, or the inability to alternate between fat and carbohydrate oxidation as a source of energy during insulin infusion was also reported in NAFLD [181] and is thought to be an adaptive mechanism against excess hepatic free fatty acids (FFAs) buildup [182].

Hepatic IR, or the inability of insulin to suppress hepatic glucose production in fasted state [29], was reported in NAFLD subjects with [30] and without diabetes [31,169,170]. The extent of adipose, hepatic and skeletal muscle IR was comparable to that in T2DM patients, independently from the degree of steatosis [181]. Hepatic IR is accompanied by uncontrolled hepatic glucose production and a compensatory hyperinsulinemia, both stimulating hepatic DNL [183,184]. Accordingly, DNL was reported to be 3-fold higher in subjects with NAFLD, compared with healthy controls [175,185]. The ability of insulin to suppress hepatic DNL in fasted state was impaired in NAFLD subjects and hepatic DNL was comparable in fed and fasted states, this indicate metabolic inflexibility, which is

believed to contribute to the development of NAFL [174]. The ability of insulin to suppress VLDL production was impaired in NAFLD [186] and NAFL was found associated with increased production of TG-rich VLDL [187,188], which contributes to the development of hypertriglyceridemia and dyslipidemia.

Hepatic TGs accumulation is thought to be a protective mechanism from the metabolic burden of increased hepatic fatty acids availability [182,189,190]. However, this concept is questioned [191], as evidence from longitudinal studies show that substantial number of subjects with NAFL, in the absence of necroinflammation and ballooning, may progress to advanced fibrosis over a relatively short period of time [136,137]. Moreover, a role for hepatocellular lipids in mediating inflammation is proposed, as indicated by an up-regulation of genes involved in inflammatory response in subjects with NAFL [192].

Evidence indicates a potential role of specific lipid species in histological changes observed during NAFLD progression, and the involvement of mechanisms related to TGs, diglycerides (DGs), and PLs remodeling in NAFLD progression. In a recent study using Mass Spectrometry (MS) imaging techniques to study spatial distribution of lipids in liver tissue, it was found that in both animal models and humans, there was a loss of hepatic lipid zonation and marked remodeling of PLs with NAFLD progression, with enrichment of AA (20:4n-6) acid in PLs located in the pericentral hepatocytes zone, potentially to serve as a source for eicosanoid synthesis. In the same study, the transcript levels of lysophosphatidylcholine Acyltransferase 2 (*LPCAT2)* and cytosolic phospholipases A2 (*cPLA2*) involved in membrane remodeling; arachidonate 15-lipoxygenase (*ALOX15*) involved in eicosanoid production were increased [193]. In support of this evidence, genome-wide association studies identified a robust association of *PNPLA3* G allele with the full spectrum of NAFLD [62-68]. In hepatocytes, the PNPLA3 protein is thought to be involved in lipid remodeling, as it was shown to have a transacylase activity that facilitates the direct fatty acid transfer between lipids [194], and an acyltransferase activity catalyzing the conversion of lysophosphatidic acid to phosphatidic acid [195]. Also, in vitro studies showed the PNPLA3 protein modulates the activity of adipose TG lipase (ATGL) or Patatin Like Phospholipase Domain Containing 2 (PNPLA2) via sequestering the cofactor,

comparative gene identification-58, which is essential for ATGL function [196-198]. The net effect of PNPLA3 and ATGL activity is to induce the hydrolysis of fatty acid ester bonds of glycerolipids, with a high preference for oleic acid (OA,18:1n-9) among other fatty acyl moieties to be incorporated into complex lipids as TGs, DGs, and phosphatidylcholine [199-201]. The *PNPLA3* I148M variant results in a loss-of-function of the enzymatic activity, impairs the ATGL activity with consequent TGs accumulation, altered saturation of hepatocellular lipids [196-199,202-204], and impairment of hydrolysis and incorporation of OA (18:1n-9) moiety from TGs pool [205]. In hepatic stellate cells, the PNPLA3 protein is involved in retinol metabolism with potential role in fibrogenesis. The PNPLA3 has a retinyl-palmitate lipase activity that promotes the release of retinol from hepatic stellate cells [206,207]. The *PNPLA3* I148M variant results in reduced retinol release and bioavailability, which promotes inflammation and fibrogenesis [206,208].

Collectively, the combination of increased hepatocellular lipid retention and dysregulation in lipid remodeling, increases the demand on the liver to handle metabolic burden from excess energy substrates and may direct lipids to pathways that generate hepatotoxic lipid species, also known as lipotoxicity [209-211]. Lipotoxicity correlates with the severity of NAFLD [212] and induces production of reactive oxygen species and lipid peroxidation [213], as well as stress cellular-response and apoptosis [212,214]. Up to date, the lipotoxic lipid specie(s) promoting hepatocellular injury and the NASH phenotype are not identified. However, *in vitro* and *in vivo* evidence indicate the SFA, palmitate (PA,16:0) [110,215,216], DGs and ceramides (CERs) [217-219], lysophosphatidylcholines [220], and free cholesterol [221,222] as lipotoxic species with potential role in NAFLD.

7. Factors contributing to the high prevalence and severity of NAFLD in Hispanics

Multiple factors can come into interplay to explain the reported ethnic disparities in NAFLD rate and severity, these factors include the presence of comorbidities as MetS; genetic predisposition; environmental factors such as diet, socioeconomic status, and inaccessibility to health care [126,127,223]. The prevalence of MetS and its components is higher in Hispanics compared to non-Hispanic whites and African American [224]. Hispanics are also reported to have higher body mass index (BMI), visceral adiposity, and IR for BMI compared to other ethnicities [225-227].The

prevalence of T2DM is higher in Hispanics compared to non-Hispanic whites, however, comparable to that of African Americans [127,228]. Plasma TGs levels are significantly lower in African Americans compared to Hispanics and Caucasians, who have comparable TGs profile [229]. However, the extent by which these variations may explain the observed disparity in NAFLD prevalence and severity is unclear.

The homozygous carriers of the rs738409 [G] allele in *PNPLA3* gene, discussed in detail in section 3.7 and 6, has the highest frequency in Hispanics compared to other ethnicities. In one study, the frequency of the I148M variant was reported to be 49%, 23%, and 17% in Hispanics, Whites, and African Americans, respectively [62]. A following study reported the allelic frequency in a group of subjects with biopsy-confirmed NAFLD to be 91% and 70% in Hispanics and non-Hispanics, respectively [67]. Similar findings have been replicated in following studies [72,73,230].

Other environmental and life-style factors include diet, physical activity and socioeconomic status, based on data from the NASH-CRN, and compared to non-Hispanic Whites, Hispanics with NASH were younger, consumed higher amount of carbohydrates and less fat, performed less physical activity, and came from lower socio-economical background [19]. The acculturation of the Hispanics to the U.S. dietary habits and lifestyle was reported with a change in diet with successive generations of Hispanics in which fruit and vegetable intake decreased and sugar-sweetened beverages intake increased in third-generation Hispanics compared to first-generation [231]. Acculturation to American society was also found to be associated with a more sedentary lifestyle, as multiple studies have reported lower physical activity among Hispanics as compared with other ethnicities [232] [233].

8. Metabolomics for the study of NAFLD

Metabolomics is the comprehensive characterization of small molecules, or metabolites, in a biological matrix by applying analytical chemistry methods [234]. The high-throughput range of detected metabolites reflects substrates, intermediates, and products of metabolic processes that are direct product of gene-environment (exposome) interactions. Therefore, metabolomics has the advantage of better reflecting the phenotype, compared to genomics and proteomics [235,236]. Metabolomic approaches are with diverse scientific applications. The ability to predict a given

phenotype with a metabolomic signature is a common application of biomarker discovery. Characterizing changes in metabolomic profile between two disease states, or exposures, may provide insights into altered metabolic pathways to pin out mechanisms and potential therapeutic targets [234,236].

Mass spectrometry is a commonly used method with high sensitivity and the ability to detect metabolites in very low abundance. It includes gas chromatography-mass spectrometry (GC-MS) which characterizes thermally stable, volatile metabolites and liquid chromatography-mass spectrometry (LC-MS) which can profile more polar metabolites with lower thermal stability and higher relative molecular mass [237,238].

Previous metabolomic profiling studies on liver biopsies revealed that NAFLD is associated with defects in the tricarboxylic acid cycle (TCA) resulting in metabolic perturbations including alterations in amino acids, lipids, and bile acids profiles [239,240], impaired antioxidant capacity, and PLs composition [240]. In situ analysis of tissue lipidomic composition and distribution revealed that there are differential distribution of lipids within the liver lobule, and the progression of NAFL to NASH impacts lipid zonation and, possibly, remodeling [193,241-243]. Last, the employment of metabolomic for biomarker discovery enabled the identification of a plasma lipidomic signature that can distinguish NASH from NAFL [244-248].

9. Justifications and aims

NAFLD is a major health concern and a heavy economic burden on health systems. It is a frequent indication for liver transplant [3]. It is also associated with high risk of morbidity and mortality [249,250]. In the U.S., the annual cost of NAFLD is estimated to be 103 billion dollars [249]. The Hispanic population is a fast-growing population and expected to account for 30% of the U.S. population by 2060 [251]. Alongside, the economic impact of NAFLD is expected to inflate. The pathophysiology of NAFLD is not clearly understood, especially with regard to the progression from NAFL to NASH. Ethnicity-related disparity in NAFLD prevalence and severity are reported with Hispanics being affected disproportionally at higher rate compared to other ethnicities [5,125-127]. To date, the biological mechanism(s) underlying these observations is not clear. The characterization of

metabolomic profile associated with a given phenotype is a valid way to understand its biochemical underpinning. Within this context, metabolomics offers a valuable tool to expand our understanding of NAFLD etiology and pathophysiology. To our knowledge, ethnicity was not addressed before in a metabolomic profiling study conducted in NAFLD.

The standard method for establishing NAFLD diagnosis, liver biopsy, is invasive and subjected to limitations [149,150]; and a clinically feasible, non-invasive biomarker to characterize NASH is an unmet necessity. Moreover, with histopathological changes occurring with progression from NAFL to NASH, little is known about the correlation of liver histological features with liver tissue or plasma metabolome in NAFLD subjects. Most of the available literature focuses on plasma or serum, with few available data on liver metabolites.

A guiding principle for our methodology is that there are biochemical differences between Hispanic and Caucasian subjects with NAFLD that could explain, at least in part, the ethnicity-related disparity observed in NAFLD prevalence and progression. In this dissertation, as a "proof of principle", the objective is to explore metabolomics profiles of a group of Hispanic and Caucasian bariatric surgery subjects with NAFLD. In specific, we conducted this study to: (i) characterize liver and plasma metabolomic profiles by ethnicity groups; (ii) determine the role of specific biochemical pathway(s) involved in NAFLD in the study groups; (iii) identify if ethnicity-specific panel of metabolites characterizes NASH.

Results of this work will expand our understanding of mechanism(s) underlying NAFLD onset and progression and shed light on potential unshared mechanism(s) for NAFLD between ethnicities. Our results will provide bases for hypothesis generation for future research aiming to find therapeutic targets and to identify ethnicity- specific metabolomic signatures that differentiate stages of NAFLD, which with future validation, can aid in clinical practices.

10. Chapters overview

Lipid species as DGs, CERs, and FFAs are thought to be key factors in the progression to NASH [240]. Previous literature identified alterations in plasma FFAs and lipid composition in patients with NAFL vs. NASH [244,248]. The comparison of lipidomic profiles in relation to ethnicity in NAFLD

may provide insights into potential lipotoxic lipid specie(s) and pathway(s) that are unshared between ethnicities. In chapter two, published in [252], we conducted an untargeted lipidomic profiling of plasma and liver tissues for metabolites related to primary metabolism, including fatty acids and complex lipids; choline and related metabolites in a group of Hispanic and Caucasian bariatric surgery subjects with NAFLD. Our findings revealed ethnicity-related differences in plasma profiles, independent of obesity, with Hispanics showing higher plasma TGs, acylcarnitines, and FFAs. Also, in NASH, there were ethnicity-related differences in hepatic profile with Hispanics having higher FFAs, TGs and lysophospholipids. Our findings suggest that impaired mitochondrial β-oxidation and lipotoxicity are involved in the progression of NASH in Hispanics.

In chapter three, published in [253], we examined a targeted and quantitative lipidomic profiling of plasma oxylipins in a group of Hispanic and Caucasian bariatric surgery subjects with NAFLD. Oxylipins, including eicosanoids, prostaglandins, and leukotrienes, are biologically active lipids with implication in various pathological conditions, including diabetes, cardiovascular disease and cancer [254,255]. The study of oxylipins in NAFLD is a relatively new area [256]. Investigating oxylipins in NAFLD with regards to ethnicity can identify potential role of dietary PUFAs and down-stream inflammatory-mediators in NAFLD progression. Findings indicate that in NAFLD, there are ethnicity-related differences in plasma profiles with Hispanic having lower long chain PUFAs, independent of histological severity. Also, when comparing between ethnicity groups, differences in plasma lipid mediators distinguished ethnicities with NASH. Importantly, our findings point out to a lower lipoxygenase(s) activities and higher soluble epoxide hydrolase(s) activities in Hispanics with NASH.

11. References

1. Ludwig, J.; Viggiano, T.R.; Mcgill, D.B.; Oh, B. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. In Proceedings of the Mayo Clinic Proceedings, 1980; pp. 434-438.
2. Chalasani, N.; Younossi, Z.; Lavine, J.E.; Diehl, A.M.; Brunt, E.M.; Cusi, K.; Charlton, M.; Sanyal, A.J. The diagnosis and management of non‐alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* **2012**, *55*, 2005-2023,

3. Chalasani, N.; Younossi, Z.; Lavine, J.E.; Charlton, M.; Cusi, K.; Rinella, M.; Harrison, S.A.; Brunt, E.M.; Sanyal, A.J. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* **2018**, *67*, 328-357,
4. Younossi, Z.M.; Koenig, A.B.; 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. Rich, N.E.; Oji, S.; Mufti, A.R.; Browning, J.D.; Parikh, N.D.; Odewole, M.; Mayo, H.; Singal, A.G. Racial and Ethnic Disparities in Nonalcoholic Fatty Liver Disease Prevalence, Severity, and Outcomes in the United States: A Systematic Review and Meta-analysis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2018**, *16*, 198-210.e192, doi:10.1016/j.cgh.2017.09.041
6. Grundy, S.M.; Brewer Jr, H.B.; Cleeman, J.I.; Smith Jr, S.C.; Lenfant, C. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation* **2004**, *109*, 433-438,
7. Wainwright, P.; Byrne, C.D. Bidirectional relationships and disconnects between NAFLD and features of the metabolic syndrome. *International journal of molecular sciences* **2016**, *17*, 367,
8. Lonardo, A.; Ballestri, S.; Marchesini, G.; Angulo, P.; Loria, P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Digestive and Liver Disease* **2015**, *47*, 181-190,
9. Green, R.M. NASH—hepatic metabolism and not simply the metabolic syndrome. *Hepatology* **2003**, *38*, 14-17,
10. Chitturi, S.; Farrell, G.C. Etiopathogenesis of nonalcoholic steatohepatitis. In Proceedings of the Seminars in liver disease, 2001; pp. 027-042.
11. Vanni, E.; Bugianesi, E.; Kotronen, A.; De Minicis, S.; Yki-Järvinen, H.; Svegliati-Baroni, G. From the metabolic syndrome to NAFLD or vice versa? *Digestive and liver Disease* **2010**, *42*, 320-330,
12. Zhang, T.; Zhang, C.; Zhang, Y.; Tang, F.; Li, H.; Zhang, Q.; Lin, H.; Wu, S.; Liu, Y.; Xue, F. Metabolic syndrome and its components as predictors of nonalcoholic fatty liver disease in a northern urban Han Chinese population: a prospective cohort study. *Atherosclerosis* **2015**, *240*, 144-148,
13. Sorrentino, P.; Terracciano, L.; D'angelo, S.; Ferbo, U.; Bracigliano, A.; Vecchione, R. Predicting fibrosis worsening in obese patients with NASH through parenchymal fibronectin, HOMA-IR, and hypertension. *American Journal of Gastroenterology* **2010**, *105*, 336-344,
14. Hamaguchi, M.; Kojima, T.; Takeda, N.; Nakagawa, T.; Taniguchi, H.; Fujii, K.; Omatsu, T.; Nakajima, T.; Sarui, H.; Shimazaki, M. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Annals of internal medicine* **2005**, *143*, 722-728,
15. Adams, L.A.; Waters, O.R.; Knuiman, M.W.; Elliott, R.R.; Olynyk, J.K. NAFLD as a risk factor for the development of diabetes and the metabolic syndrome: an eleven-year follow-up study. *American Journal of Gastroenterology* **2009**, *104*, 861-867,
16. Targher, G.; Byrne, C.D.; Lonardo, A.; Zoppini, G.; Barbui, C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: a meta-analysis. *Journal of hepatology* **2016**, *65*, 589-600,
17. Zhang, Y.; Zhang, T.; Zhang, C.; Tang, F.; Zhong, N.; Li, H.; Song, X.; Lin, H.; Liu, Y.; Xue, F. Identification of reciprocal causality between non-alcoholic fatty liver disease and metabolic syndrome by a simplified Bayesian network in a Chinese population. *BMJ open* **2015**, *5*, e008204,

18. Smits, M.M.; Ioannou, G.N.; Boyko, E.J.; Utzschneider, K.M. Non‐alcoholic fatty liver disease as an independent manifestation of the metabolic syndrome: results of a US national survey in three ethnic groups. *Journal of gastroenterology and hepatology* **2013**, *28*, 664-670,
19. Bambha, K.; Belt, P.; Abraham, M.; Wilson, L.A.; Pabst, M.; Ferrell, L.; Unalp‐Arida, A.; Bass, N.; Group, N.S.C.R.N.R. Ethnicity and nonalcoholic fatty liver disease. *Hepatology* **2012**, *55*, 769-780,
20. Younossi, Z.M.; Golabi, P.; de Avila, L.; Paik, J.M.; Srishord, M.; Fukui, N.; Qiu, Y.; Burns, L.; Afendy, A.; Nader, F. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of hepatology* **2019**, *71*, 793-801,
21. Forlani, G.; Giorda, C.; Manti, R.; Mazzella, N.; De Cosmo, S.; Rossi, M.C.; Nicolucci, A.; Di Bartolo, P.; Ceriello, A.; Guida, P. The burden of NAFLD and its characteristics in a nationwide population with type 2 diabetes. *Journal of diabetes research* **2016**, *2016*,
22. Portillo-Sanchez, P.; Bril, F.; Maximos, M.; Lomonaco, R.; Biernacki, D.; Orsak, B.; Subbarayan, S.; Webb, A.; Hecht, J.; Cusi, K. High prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus and normal plasma aminotransferase levels. *The Journal of Clinical Endocrinology & Metabolism* **2015**, *100*, 2231-2238,
23. Ballestri, S.; Zona, S.; Targher, G.; Romagnoli, D.; Baldelli, E.; Nascimbeni, F.; Roverato, A.; Guaraldi, G.; Lonardo, A. Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome. Evidence from a systematic review and meta‐analysis. *Journal of gastroenterology and hepatology* **2016**, *31*, 936-944,
24. Lebovitz, H.E.; Banerji, M.A. Point: visceral adiposity is causally related to insulin resistance. *Diabetes care* **2005**, *28*, 2322-2325,
25. Janochova, K.; Haluzik, M.; Buzga, M. Visceral fat and insulin resistance--what we know? *Biomedical Papers of the Medical Faculty of Palacky University in Olomouc* **2019**, *163*,
26. Choudhury, J.; Sanyal, A.J. Insulin resistance and the pathogenesis of nonalcoholic fatty liver disease. *Clinics in liver disease* **2004**, *8*, 575-594, ix,
27. Bugianesi, E.; McCullough, A.J.; Marchesini, G. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology* **2005**, *42*, 987-1000,
28. Sanyal, A.J.; Campbell–Sargent, C.; Mirshahi, F.; Rizzo, W.B.; Contos, M.J.; Sterling, R.K.; Luketic, V.A.; Shiffman, M.L.; Clore, J.N. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* **2001**, *120*, 1183-1192,
29. Gastaldelli, A.; Miyazaki, Y.; Pettiti, M.; Buzzigoli, E.; Mahankali, S.; Ferrannini, E.; DeFronzo, R.A. Separate contribution of diabetes, total fat mass, and fat topography to glucose production, gluconeogenesis, and glycogenolysis. *The Journal of Clinical Endocrinology & Metabolism* **2004**, *89*, 3914-3921,
30. Gastaldelli, A.; Cusi, K.; Pettiti, M.; Hardies, J.; Miyazaki, Y.; Berria, R.; Buzzigoli, E.; Sironi, A.M.; Cersosimo, E.; Ferrannini, E. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* **2007**, *133*, 496-506,
31. Marchesini, G.; Brizi, M.; Bianchi, G.; Tomassetti, S.; Bugianesi, E.; Lenzi, M.; McCullough, A.J.; Natale, S.; Forlani, G.; Melchionda, N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* **2001**, *50*, 1844-1850,

32. Li, L.; Liu, D.W.; Yan, H.Y.; Wang, Z.Y.; Zhao, S.H.; Wang, B. Obesity is an independent risk factor for non‐alcoholic fatty liver disease: evidence from a meta‐analysis of 21 cohort studies. *Obesity Reviews* **2016**, *17*, 510-519,
33. Zimmermann, E.; Gamborg, M.; Holst, C.; Baker, J.L.; Sørensen, T.I.; Berentzen, T.L. Body mass index in school-aged children and the risk of routinely diagnosed non-alcoholic fatty liver disease in adulthood: a prospective study based on the Copenhagen School Health Records Register. *BMJ open* **2015**, *5*, e006998,
34. Miyake, T.; Kumagi, T.; Hirooka, M.; Furukawa, S.; Koizumi, M.; Tokumoto, Y.; Ueda, T.; Yamamoto, S.; Abe, M.; Kitai, K. Body mass index is the most useful predictive factor for the onset of nonalcoholic fatty liver disease: a community-based retrospective longitudinal cohort study. *Journal of gastroenterology* **2013**, *48*, 413-422,
35. Younossi, Z.M.; Stepanova, M.; Negro, F.; Hallaji, S.; Younossi, Y.; Lam, B.; Srishord, M. Nonalcoholic fatty liver disease in lean individuals in the United States. *Medicine* **2012**, *91*, 319-327,
36. Lonardo, A.; Bellentani, S.; Argo, C.K.; Ballestri, S.; Byrne, C.D.; Caldwell, S.H.; Cortez-Pinto, H.; Grieco, A.; Machado, M.V.; Miele, L. Epidemiological modifiers of non-alcoholic fatty liver disease: Focus on high-risk groups. *Digestive and Liver Disease* **2015**, *47*, 997-1006,
37. Kim, D.; Park, B.J.; Kim, W.; Jung, Y.J.; Kim, Y.J.; Yoon, J.-H.; Lee, H.-S. S1913 Visceral Fat As a Strong and Independent Risk Factor of Nonalcoholic Fatty Liver Disease. *Gastroenterology* **2008**, *134*, A-782-A-783,
38. Park, B.J.; Kim, Y.J.; Kim, D.H.; Kim, W.; Jung, Y.J.; Yoon, J.H.; Kim, C.Y.; Cho, Y.M.; Kim, S.H.; Lee, K.B. Visceral adipose tissue area is an independent risk factor for hepatic steatosis. *Journal of gastroenterology and hepatology* **2008**, *23*, 900-907,
39. Hamaguchi, M.; Kojima, T.; Itoh, Y.; Harano, Y.; Fujii, K.; Nakajima, T.; Kato, T.; Takeda, N.; Okuda, J.; Ida, K. The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. *The American journal of gastroenterology* **2007**, *102*, 2708,
40. van der Poorten, D.; Milner, K.L.; Hui, J.; Hodge, A.; Trenell, M.I.; Kench, J.G.; London, R.; Peduto, T.; Chisholm, D.J.; George, J. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. *Hepatology* **2008**, *48*, 449-457,
41. DeFilippis, A.P.; Blaha, M.J.; Martin, S.S.; Reed, R.M.; Jones, S.R.; Nasir, K.; Blumenthal, R.S.; Budoff, M.J. Nonalcoholic fatty liver disease and serum lipoproteins: the Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis* **2013**, *227*, 429-436,
42. Chatrath, H.; Vuppalanchi, R.; Chalasani, N. Dyslipidemia in patients with nonalcoholic fatty liver disease. In Proceedings of the Seminars in liver disease, 2012; pp. 022-029.
43. López-Suárez, A.; Guerrero, J.M.R.; Elvira-González, J.; Beltrán-Robles, M.; Cañas-Hormigo, F.; Bascuñana-Quirell, A. Nonalcoholic fatty liver disease is associated with blood pressure in hypertensive and nonhypertensive individuals from the general population with normal levels of alanine aminotransferase. *European journal of gastroenterology & hepatology* **2011**, *23*, 1011-1017,
44. Qian, L.-Y.; Tu, J.-F.; Ding, Y.-H.; Pang, J.; Che, X.-D.; Zou, H.; Huang, D.-S. Association of blood pressure level with nonalcoholic fatty liver disease in nonhypertensive population: Normal is not the new normal. *Medicine* **2016**, *95*,

45. Aneni, E.C.; Oni, E.T.; Martin, S.S.; Blaha, M.J.; Agatston, A.S.; Feldman, T.; Veledar, E.; Carvalho, J.A.; Santos, R.D.; Nasir, K. Blood pressure is associated with the presence and severity of nonalcoholic fatty liver disease across the spectrum of cardiometabolic risk. *Journal of hypertension* **2015**, *33*, 1207-1214,
46. Singh, S.; Allen, A.M.; Wang, Z.; Prokop, L.J.; Murad, M.H.; Loomba, R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. *Clinical Gastroenterology and Hepatology* **2015**, *13*, 643-654. e649,
47. Lonardo, A.; Nascimbeni, F.; Mantovani, A.; Targher, G. Hypertension, diabetes, atherosclerosis and NASH: cause or consequence? *Journal of hepatology* **2018**, *68*, 335-352,
48. Argo, C.K.; Northup, P.G.; Al-Osaimi, A.M.; Caldwell, S.H. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *Journal of hepatology* **2009**, *51*, 371-379,
49. Kraja, A.T.; Borecki, I.B.; North, K.; Tang, W.; Myers, R.H.; Hopkins, P.N.; Arnett, D.; Corbett, J.; Adelman, A.; Province, M.A. Longitudinal and age trends of metabolic syndrome and its risk factors: the Family Heart Study. *Nutrition & metabolism* **2006**, *3*, 41,
50. Devers, M.C.; Campbell, S.; Simmons, D. Influence of age on the prevalence and components of the metabolic syndrome and the association with cardiovascular disease. *BMJ Open Diabetes Research and Care* **2016**, *4*, e000195,
51. Pan, J.-J.; Fallon, M.B. Gender and racial differences in nonalcoholic fatty liver disease. *World journal of hepatology* **2014**, *6*, 274,
52. Browning, J.D.; Szczepaniak, L.S.; Dobbins, R.; Nuremberg, P.; Horton, J.D.; Cohen, J.C.; Grundy, S.M.; Hobbs, H.H. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **2004**, *40*, 1387-1395,
53. Pramfalk, C.; Pavlides, M.; Banerjee, R.; McNeil, C.A.; Neubauer, S.; Karpe, F.; Hodson, L. Sex-specific differences in hepatic fat oxidation and synthesis may explain the higher propensity for NAFLD in men. *The Journal of Clinical Endocrinology & Metabolism* **2015**, *100*, 4425-4433,
54. Yang, J.D.; Abdelmalek, M.F.; Pang, H.; Guy, C.D.; Smith, A.D.; Diehl, A.M.; Suzuki, A. Gender and menopause impact severity of fibrosis among patients with nonalcoholic steatohepatitis. *Hepatology* **2014**, *59*, 1406-1414,
55. Eguchi, Y.; Hyogo, H.; Ono, M.; Mizuta, T.; Ono, N.; Fujimoto, K.; Chayama, K.; Saibara, T. Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *Journal of gastroenterology* **2012**, *47*, 586-595,
56. Fan, J.-G.; Zhu, J.; Li, X.-J.; Chen, L.; Li, L.; Dai, F.; Li, F.; Chen, S.-Y. Prevalence of and risk factors for fatty liver in a general population of Shanghai, China. *Journal of hepatology* **2005**, *43*, 508-514,
57. Ong, J.P.; Pitts, A.; Younossi, Z.M. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. *Journal of hepatology* **2008**, *49*, 608-612,
58. Bedogni, G.; Miglioli, L.; Masutti, F.; Castiglione, A.; Croce, L.S.; Tiribelli, C.; Bellentani, S. Incidence and natural course of fatty liver in the general population: the Dionysos study. *Hepatology* **2007**, *46*, 1387-1391,
59. Eslam, M.; Valenti, L.; Romeo, S. Genetics and epigenetics of NAFLD and NASH: clinical impact. *Journal of hepatology* **2018**, *68*, 268-279,

60. Daly, A.K.; Ballestri, S.; Carulli, L.; Loria, P.; Day, C.P. Genetic determinants of susceptibility and severity in nonalcoholic fatty liver disease. *Expert review of gastroenterology & hepatology* **2011**, *5*, 253-263,
61. Dongiovanni, P.; Romeo, S.; Valenti, L. Genetic factors in the pathogenesis of nonalcoholic fatty liver and steatohepatitis. *BioMed research international* **2015**, *2015*,
62. Romeo, S.; Kozlitina, J.; Xing, C.; Pertsemlidis, A.; Cox, D.; Pennacchio, L.A.; Boerwinkle, E.; Cohen, J.C.; Hobbs, H.H. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nature genetics* **2008**, *40*, 1461,
63. Dongiovanni, P.; Donati, B.; Fares, R.; Lombardi, R.; Mancina, R.M.; Romeo, S.; Valenti, L. PNPLA3 I148M polymorphism and progressive liver disease. *World journal of gastroenterology: WJG* **2013**, *19*, 6969,
64. Singal, A.G.; Manjunath, H.; Yopp, A.C.; Beg, M.S.; Marrero, J.A.; Gopal, P.; Waljee, A.K. The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. *The American journal of gastroenterology* **2014**, *109*, 325,
65. Chalasani, N.; Guo, X.; Loomba, R.; Goodarzi, M.O.; Haritunians, T.; Kwon, S.; Cui, J.; Taylor, K.D.; Wilson, L.; Cummings, O.W. Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. *Gastroenterology* **2010**, *139*, 1567-1576. e1566,
66. Sookoian, S.; Pirola, C.J. 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*, 1883-1894,
67. Rotman, Y.; Koh, C.; Zmuda, J.M.; Kleiner, D.E.; Liang, T.J.; CRN, N. The association of genetic variability in patatin‐like phospholipase domain‐containing protein 3 (PNPLA3) with histological severity of nonalcoholic fatty liver disease. *Hepatology* **2010**, *52*, 894-903,
68. Liu, Y.-L.; Patman, G.; Leathart, J.; Piguet, A.-C.; Burt, A.; Dufour, J.-F.; Day, C.; Daly, A.; Reeves, H.; Anstee, Q. Carriage of the PNPLA3 rs738409 C> G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma. *Journal of hepatology* **2014**, *61*, 75-81,
69. Sookoian, S.; Castaño, G.O.; Burgueño, A.L.; Gianotti, T.F.; Rosselli, M.S.; Pirola, C.J. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. *Journal of lipid research* **2009**, *50*, 2111-2116,
70. Kawaguchi, T.; Sumida, Y.; Umemura, A.; Matsuo, K.; Takahashi, M.; Takamura, T.; Yasui, K.; Saibara, T.; Hashimoto, E.; Kawanaka, M. Genetic polymorphisms of the human PNPLA3 gene are strongly associated with severity of non-alcoholic fatty liver disease in Japanese. *PloS one* **2012**, *7*,
71. Kitamoto, T.; Kitamoto, A.; Yoneda, M.; Hyogo, H.; Ochi, H.; Nakamura, T.; Teranishi, H.; Mizusawa, S.; Ueno, T.; Chayama, K. Genome-wide scan revealed that polymorphisms in the PNPLA3, SAMM50, and PARVB genes are associated with development and progression of nonalcoholic fatty liver disease in Japan. *Human genetics* **2013**, *132*, 783-792,
72. Wagenknecht, L.E.; Palmer, N.D.; Bowden, D.W.; Rotter, J.I.; Norris, J.M.; Ziegler, J.; Chen, Y.D.I.; Haffner, S.; Scherzinger, A.; Langefeld, C.D. Association of PNPLA3 with non‐alcoholic fatty liver disease in a minority cohort: the Insulin Resistance Atherosclerosis Family Study. *Liver International* **2011**, *31*, 412-416,

73. Palmer, N.D.; Musani, S.K.; Yerges‐Armstrong, L.M.; Feitosa, M.F.; Bielak, L.F.; Hernaez, R.; Kahali, B.; Carr, J.J.; Harris, T.B.; Jhun, M.A. Characterization of European ancestry nonalcoholic fatty liver disease‐associated variants in individuals of African and Hispanic descent. *Hepatology* **2013**, *58*, 966-975,
74. Speliotes, E.K.; Butler, J.L.; Palmer, C.D.; Voight, B.F.; Consortium, G.C.t.M.; CRN, N.; Hirschhorn, J.N. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. *Hepatology* **2010**, *52*, 904-912,
75. Kantartzis, K.; Peter, A.; Machicao, F.; Machann, J.; Wagner, S.; Königsrainer, I.; Königsrainer, A.; Schick, F.; Fritsche, A.; Häring, H.-U. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. *Diabetes* **2009**, *58*, 2616-2623,
76. Luukkonen, P.K.; Zhou, Y.; Haridas, P.N.; Dwivedi, O.P.; Hyötyläinen, T.; Ali, A.; Juuti, A.; Leivonen, M.; Tukiainen, T.; Ahonen, L. Impaired hepatic lipid synthesis from polyunsaturated fatty acids in TM6SF2 E167K variant carriers with NAFLD. *Journal of hepatology* **2017**, *67*, 128-136,
77. Kozlitina, J.; Smagris, E.; Stender, S.; Nordestgaard, B.G.; Zhou, H.H.; Tybjærg-Hansen, A.; Vogt, T.F.; Hobbs, H.H.; Cohen, J.C. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. *Nature genetics* **2014**, *46*, 352,
78. Mahdessian, H.; Taxiarchis, A.; Popov, S.; Silveira, A.; Franco-Cereceda, A.; Hamsten, A.; Eriksson, P.; van't Hooft, F. TM6SF2 is a regulator of liver fat metabolism influencing triglyceride secretion and hepatic lipid droplet content. *Proceedings of the National Academy of Sciences* **2014**, *111*, 8913-8918,
79. Sookoian, S.; Castaño, G.O.; Scian, R.; Mallardi, P.; Fernandez Gianotti, T.; Burgueño, A.L.; San Martino, J.; Pirola, C.J. Genetic variation in transmembrane 6 superfamily member 2 and the risk of nonalcoholic fatty liver disease and histological disease severity. *Hepatology* **2015**, *61*, 515-525,
80. Kim, D.S.; Jackson, A.U.; Li, Y.K.; Stringham, H.M.; Kuusisto, J.; Kangas, A.J.; Soininen, P.; Ala-Korpela, M.; Burant, C.F.; Salomaa, V. Novel association of TM6SF2 rs58542926 genotype with increased serum tyrosine levels and decreased apoB-100 particles in Finns. *Journal of lipid research* **2017**, *58*, 1471-1481,
81. Li, T.-T.; Li, T.-H.; Peng, J.; He, B.; Liu, L.-S.; Wei, D.-H.; Jiang, Z.-S.; Zheng, X.-L.; Tang, Z.-H. TM6SF2: A novel target for plasma lipid regulation. *Atherosclerosis* **2018**, *268*, 170-176,
82. Speliotes, E.K.; Yerges-Armstrong, L.M.; Wu, J.; Hernaez, R.; Kim, L.J.; Palmer, C.D.; Gudnason, V.; Eiriksdottir, G.; Garcia, M.E.; Launer, L.J. Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. *PLoS genetics* **2011**, *7*,
83. Santoro, N.; Zhang, C.K.; Zhao, H.; Pakstis, A.J.; Kim, G.; Kursawe, R.; Dykas, D.J.; Bale, A.E.; Giannini, C.; Pierpont, B. Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents. *Hepatology* **2012**, *55*, 781-789,
84. Petta, S.; Miele, L.; Bugianesi, E.; Camma, C.; Rosso, C.; Boccia, S.; Cabibi, D.; Di Marco, V.; Grimaudo, S.; Grieco, A. Glucokinase regulatory protein gene polymorphism affects liver fibrosis in non-alcoholic fatty liver disease. *PLoS One* **2014**, *9*,
85. Zain, S.M.; Mohamed, Z.; Mohamed, R. A common variant in the glucokinase regulatory gene rs780094 and risk of nonalcoholic fatty liver disease: A meta‐analysis. *Journal of gastroenterology and hepatology* **2015**, *30*, 21-27,

86. Rees, M.G.; Wincovitch, S.; Schultz, J.; Waterstradt, R.; Beer, N.L.; Baltrusch, S.; Collins, F.S.; Gloyn, A.L. Cellular characterisation of the GCKR P446L variant associated with type 2 diabetes risk. *Diabetologia* **2012**, *55*, 114-122, doi:10.1007/s00125-011-2348-5
87. Santoro, N.; Caprio, S.; Pierpont, B.; Van Name, M.; Savoye, M.; Parks, E.J. Hepatic de novo lipogenesis in obese youth is modulated by a common variant in the GCKR gene. *The Journal of Clinical Endocrinology & Metabolism* **2015**, *100*, E1125-E1132,
88. Foster, D.W. Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. *The Journal of clinical investigation* **2012**, *122*, 1958-1959,
89. Mancina, R.M.; Dongiovanni, P.; Petta, S.; Pingitore, P.; Meroni, M.; Rametta, R.; Borén, J.; Montalcini, T.; Pujia, A.; Wiklund, O. The MBOAT7-TMC4 variant rs641738 increases risk of nonalcoholic fatty liver disease in individuals of European descent. *Gastroenterology* **2016**, *150*, 1219-1230. e1216,
90. Donati, B.; Dongiovanni, P.; Romeo, S.; Meroni, M.; McCain, M.; Miele, L.; Petta, S.; Maier, S.; Rosso, C.; De Luca, L. MBOAT7 rs641738 variant and hepatocellular carcinoma in non-cirrhotic individuals. *Scientific reports* **2017**, *7*, 1-10,
91. D'Souza, K.; Epand, R.M. Enrichment of phosphatidylinositols with specific acyl chains. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **2014**, *1838*, 1501-1508,
92. Luukkonen, P.K.; Zhou, Y.; Hyötyläinen, T.; Leivonen, M.; Arola, J.; Orho-Melander, M.; Orešič, M.; Yki-Järvinen, H. The MBOAT7 variant rs641738 alters hepatic phosphatidylinositols and increases severity of non-alcoholic fatty liver disease in humans. *Journal of hepatology* **2016**, *65*, 1263-1265,
93. Solga, S.; Alkhuraishe, A.R.; Clark, J.M.; Torbenson, M.; Greenwald, A.; Diehl, A.M.; Magnuson, T. Dietary composition and nonalcoholic fatty liver disease. *Digestive diseases and sciences* **2004**, *49*, 1578-1583,
94. Roncal-Jimenez, C.A.; Lanaspa, M.A.; Rivard, C.J.; Nakagawa, T.; Sanchez-Lozada, L.G.; Jalal, D.; Andres-Hernando, A.; Tanabe, K.; Madero, M.; Li, N. Sucrose induces fatty liver and pancreatic inflammation in male breeder rats independent of excess energy intake. *Metabolism* **2011**, *60*, 1259-1270,
95. Schultz, A.; Neil, D.; Aguila, M.B.; Mandarim-de-Lacerda, C.A. Hepatic adverse effects of fructose consumption independent of overweight/obesity. *International journal of molecular sciences* **2013**, *14*, 21873-21886,
96. Sánchez-Lozada, L.G.; Mu, W.; Roncal, C.; Sautin, Y.Y.; Abdelmalek, M.; Reungjui, S.; Le, M.; Nakagawa, T.; Lan, H.Y.; Yu, X. Comparison of free fructose and glucose to sucrose in the ability to cause fatty liver. *European journal of nutrition* **2010**, *49*, 1-9,
97. Ackerman, Z.; Oron-Herman, M.; Grozovski, M.; Rosenthal, T.; Pappo, O.; Link, G.; Sela, B.-A. Fructose-induced fatty liver disease: hepatic effects of blood pressure and plasma triglyceride reduction. *Hypertension* **2005**, *45*, 1012-1018, doi:10.1161/01.HYP.0000164570.20420.67
98. Collison, K.S.; Maqbool, Z.M.; Inglis, A.L.; Makhoul, N.J.; Saleh, S.M.; Bakheet, R.H.; Al‐Johi, M.A.; Al‐Rabiah, R.K.; Zaidi, M.Z.; Al‐Mohanna, F.A. Effect of dietary monosodium glutamate on HFCS‐induced hepatic steatosis: expression profiles in the liver and visceral fat. *Obesity* **2010**, *18*, 1122-1134,

99. Toshimitsu, K.; Matsuura, B.; Ohkubo, I.; Niiya, T.; Furukawa, S.; Hiasa, Y.; Kawamura, M.; Ebihara, K.; Onji, M. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. *Nutrition* **2007**, *23*, 46-52,
100. Zelber-Sagi, S.; Nitzan-Kaluski, D.; Goldsmith, R.; Webb, M.; Blendis, L.; Halpern, Z.; Oren, R. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *Journal of hepatology* **2007**, *47*, 711-717,
101. Ouyang, X.; Cirillo, P.; Sautin, Y.; McCall, S.; Bruchette, J.L.; Diehl, A.M.; Johnson, R.J.; Abdelmalek, M.F. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *Journal of hepatology* **2008**, *48*, 993-999,
102. Lim, J.S.; Mietus-Snyder, M.; Valente, A.; Schwarz, J.-M.; Lustig, R.H. The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. *Nature reviews Gastroenterology & hepatology* **2010**, *7*, 251,
103. Abdelmalek, M.F.; Suzuki, A.; Guy, C.; Unalp‐Arida, A.; Colvin, R.; Johnson, R.J.; Diehl, A.M.; Network, N.S.C.R. Increased fructose consumption is associated with fibrosis severity in patients with nonalcoholic fatty liver disease. *Hepatology* **2010**, *51*, 1961-1971,
104. Mosca, A.; Nobili, V.; De Vito, R.; Crudele, A.; Scorletti, E.; Villani, A.; Alisi, A.; Byrne, C.D. Serum uric acid concentrations and fructose consumption are independently associated with NASH in children and adolescents. *Journal of hepatology* **2017**, *66*, 1031-1036,
105. Zhang, Y.H.; An, T.; Zhang, R.C.; Zhou, Q.; Huang, Y.; Zhang, J. Very high fructose intake increases serum LDL-cholesterol and total cholesterol: a meta-analysis of controlled feeding trials. *The Journal of nutrition* **2013**, *143*, 1391-1398,
106. Te Morenga, L.A.; Howatson, A.J.; Jones, R.M.; Mann, J. Dietary sugars and cardiometabolic risk: systematic review and meta-analyses of randomized controlled trials of the effects on blood pressure and lipids. *The American journal of clinical nutrition* **2014**, *100*, 65-79,
107. Sievenpiper, J.L.; de Souza, R.J.; Kendall, C.W.; Jenkins, D.J. Is fructose a story of mice but not men? *Journal of the Academy of Nutrition and Dietetics* **2011**, *111*, 219-220,
108. Chung, M.; Ma, J.; Patel, K.; Berger, S.; Lau, J.; Lichtenstein, A.H. Fructose, high-fructose corn syrup, sucrose, and nonalcoholic fatty liver disease or indexes of liver health: a systematic review and meta-analysis. *The American journal of clinical nutrition* **2014**, *100*, 833-849,
109. Chiu, S.; Sievenpiper, J.; De Souza, R.; Cozma, A.; Mirrahimi, A.; Carleton, A.; Ha, V.; Di Buono, M.; Jenkins, A.; Leiter, L. Effect of fructose on markers of non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of controlled feeding trials. *European journal of clinical nutrition* **2014**, *68*, 416-423,
110. Wang, D.; Wei, Y.; Pagliassotti, M.J. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* **2006**, *147*, 943-951,
111. Marí, M.; Caballero, F.; Colell, A.; Morales, A.; Caballeria, J.; Fernandez, A.; Enrich, C.; Fernandez-Checa, J.C.; García-Ruiz, C. Mitochondrial free cholesterol loading sensitizes to TNF-and Fas-mediated steatohepatitis. *Cell metabolism* **2006**, *4*, 185-198,
112. Werner, A.; Havinga, R.; Bos, T.; Bloks, V.W.; Kuipers, F.; Verkade, H.J. Essential fatty acid deficiency in mice is associated with hepatic steatosis and secretion of large VLDL particles. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2005**, *288*, G1150-G1158,

113. Keim, N.L.; Mares-Perlman, J.A. Development of hepatic steatosis and essential fatty acid deficiency in rats with hypercaloric, fat-free parenteral nutrition. *The Journal of nutrition* **1984**, *114*, 1807-1815,
114. Goheen, S.C.; Larkin, E.C.; Rao, G.A. Severe fatty liver in rats fed a fat-free ethanol diet, and its prevention by small amounts of dietary arachidonate. *Lipids* **1983**, *18*, 285-290,
115. Sekiya, M.; Yahagi, N.; Matsuzaka, T.; Najima, Y.; Nakakuki, M.; Nagai, R.; Ishibashi, S.; Osuga, J.-i.; Yamada, N.; Shimano, H. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* **2003**, *38*, 1529-1539,
116. Musso, G.; Gambino, R.; De Michieli, F.; Cassader, M.; Rizzetto, M.; Durazzo, M.; Fagà, E.; Silli, B.; Pagano, G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* **2003**, *37*, 909-916,
117. Araya, J.; Rodrigo, R.; Videla, L.A.; Thielemann, L.; Orellana, M.; Pettinelli, P.; Poniachik, J. Increase in long-chain polyunsaturated fatty acid n− 6/n− 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clinical science* **2004**, *106*, 635-643, doi:10.1042/CS20030326
118. Simopoulos, A.P. Importance of the omega-6/omega-3 balance in health and disease: evolutionary aspects of diet. In *Healthy agriculture, healthy nutrition, healthy people*; Karger Publishers: 2011; Volume 102, pp. 10-21.
119. Musa-Veloso, K.; Venditti, C.; Lee, H.Y.; Darch, M.; Floyd, S.; West, S.; Simon, R. Systematic review and meta-analysis of controlled intervention studies on the effectiveness of long-chain omega-3 fatty acids in patients with nonalcoholic fatty liver disease. *Nutrition reviews* **2018**, *76*, 581-602,
120. Lu, W.; Li, S.; Li, J.; Wang, J.; Zhang, R.; Zhou, Y.; Yin, Q.; Zheng, Y.; Wang, F.; Xia, Y. Effects of omega-3 fatty acid in nonalcoholic fatty liver disease: a meta-analysis. *Gastroenterology research and practice* **2016**, *2016*,
121. Guo, X.-f.; Yang, B.; Tang, J.; Li, D. Fatty acid and non-alcoholic fatty liver disease: Meta-analyses of case-control and randomized controlled trials. *Clinical Nutrition* **2018**, *37*, 113-122,
122. Bach-Faig, A.; Berry, E.M.; Lairon, D.; Reguant, J.; Trichopoulou, A.; Dernini, S.; Medina, F.X.; Battino, M.; Belahsen, R.; Miranda, G. Mediterranean diet pyramid today. Science and cultural updates. *Public health nutrition* **2011**, *14*, 2274-2284,
123. Moosavian, S.P.; Arab, A.; Paknahad, Z. The effect of a Mediterranean diet on metabolic parameters in patients with non-alcoholic fatty liver disease: A systematic review of randomized controlled trials. *Clinical Nutrition ESPEN* **2019**,
124. Saeed, N.; Nadeau, B.; Shannon, C.; Tincopa, M. Evaluation of Dietary Approaches for the Treatment of Non-Alcoholic Fatty Liver Disease: A Systematic Review. *Nutrients* **2019**, *11*, 3064,
125. Lazo, M.; Hernaez, R.; Eberhardt, M.S.; Bonekamp, S.; Kamel, I.; Guallar, E.; Koteish, A.; Brancati, F.L.; Clark, J.M. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988–1994. *American journal of epidemiology* **2013**, *178*, 38-45,
126. Schneider, A.L.; Lazo, M.; Selvin, E.; Clark, J.M. Racial differences in nonalcoholic fatty liver disease in the US population. *Obesity* **2014**, *22*, 292-299,
127. Saab, S.; Manne, V.; Nieto, J.; Schwimmer, J.B.; Chalasani, N.P. Nonalcoholic Fatty Liver Disease in Latinos. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2016**, *14*, 5-12; quiz e19-10, doi:10.1016/j.cgh.2015.05.001

128. Tota-Maharaj, R.; Blaha, M.J.; Zeb, I.; Katz, R.; Blankstein, R.; Blumenthal, R.S.; Budoff, M.J.; Nasir, K. Ethnic and sex differences in fatty liver on cardiac computed tomography: the multi-ethnic study of atherosclerosis. In Proceedings of the Mayo Clinic Proceedings, 2014; pp. 493-503.
129. Mohanty, S.R.; Troy, T.N.; Huo, D.; O'Brien, B.L.; Jensen, D.M.; Hart, J. Influence of ethnicity on histological differences in non-alcoholic fatty liver disease. *Journal of hepatology* **2009**, *50*, 797-804,
130. Marzuillo, P.; del Giudice, E.M.; Santoro, N. Pediatric fatty liver disease: role of ethnicity and genetics. *World journal of gastroenterology: WJG* **2014**, *20*, 7347,
131. Szanto, K.B.; Li, J.; Cordero, P.; Oben, J.A. Ethnic differences and heterogeneity in genetic and metabolic makeup contributing to nonalcoholic fatty liver disease. *Diabetes, metabolic syndrome and obesity: targets and therapy* **2019**, *12*, 357,
132. Browning, J.D.; Szczepaniak, L.S.; Dobbins, R.; Horton, J.D.; Cohen, J.C.; Grundy, S.M.; Hobbs, H.H. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **2004**, *40*, 1387-1395,
133. Ekstedt, M.; Franzen, L.E.; Mathiesen, U.L.; Thorelius, L.; Holmqvist, M.; Bodemar, G.; Kechagias, S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* **2006**, *44*, 865-873, doi:10.1002/hep.21327
134. Angulo, P.; Bugianesi, E.; Bjornsson, E.S.; Charatcharoenwitthaya, P.; Mills, P.R.; Barrera, F.; Haflidadottir, S.; Day, C.P.; George, J. Simple noninvasive systems predict long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology* **2013**, *145*, 782-789. e784, doi:10.1053/j.gastro.2013.06.057
135. Rafiq, N.; Bai, C.; Fang, Y.; Srishord, M.; McCullough, A.; Gramlich, T.; Younossi, Z.M. Long-term follow-up of patients with nonalcoholic fatty liver. *Clinical Gastroenterology and Hepatology* **2009**, *7*, 234-238,
136. McPherson, S.; Hardy, T.; Henderson, E.; Burt, A.D.; Day, C.P.; Anstee, Q.M. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *Journal of hepatology* **2015**, *62*, 1148-1155,
137. Pais, R.; Charlotte, F.; Fedchuk, L.; Bedossa, P.; Lebray, P.; Poynard, T.; Ratziu, V.; Group, L.S. A systematic review of follow-up biopsies reveals disease progression in patients with non-alcoholic fatty liver. *Journal of hepatology* **2013**, *59*, 550-556,
138. Mcpherson, S.; Pais, R.; Valenti, L.; Schattenberg, J.M.; Dufour, J.-F.; Tsochatzis, E.; Francque, S.; Hardy, T.; Boyle, M.; Tiniakos, D. Further delineation of fibrosis progression in NAFLD: evidence from a large cohort of patients with sequential biopsies. *Journal of Hepatology* **2017**, *66*, S593-S593,
139. Wong, V.W.-S.; Wong, G.L.-H.; Choi, P.C.-L.; Chan, A.W.-H.; Li, M.K.-P.; Chan, H.-Y.; Chim, A.M.-L.; Yu, J.; Sung, J.J.-Y.; Chan, H.L.-Y. Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. *Gut* **2010**, *59*, 969-974,
140. Carr, R.M.; Oranu, A.; Khungar, V. Nonalcoholic Fatty Liver Disease: Pathophysiology and Management. *Gastroenterology clinics of North America* **2016**, *45*, 639-652, doi:10.1016/j.gtc.2016.07.003
141. Fazel, Y.; Koenig, A.B.; Sayiner, M.; Goodman, Z.D.; Younossi, Z.M. Epidemiology and natural history of non-alcoholic fatty liver disease. *Metabolism* **2016**, *65*, 1017-1025, doi:10.1016/j.metabol.2016.01.012

142. Calzadilla Bertot, L.; Adams, L.A. The Natural Course of Non-Alcoholic Fatty Liver Disease. *Int J Mol Sci* **2016**, *17*, doi:10.3390/ijms17050774
143. Rinella, M.; Charlton, M. The globalization of nonalcoholic fatty liver disease: prevalence and impact on world health. *Hepatology* **2016**, *64*, 19-22,
144. Musso, G.; Gambino, R.; Cassader, M.; Pagano, G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Annals of medicine* **2011**, *43*, 617-649,
145. Angulo, P.; Kleiner, D.E.; Dam-Larsen, S.; Adams, L.A.; Bjornsson, E.S.; Charatcharoenwitthaya, P.; Mills, P.R.; Keach, J.C.; Lafferty, H.D.; Stahler, A.; 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*, 389-397.e310, doi:10.1053/j.gastro.2015.04.043
146. Charlton, M.R.; Burns, J.M.; Pedersen, R.A.; Watt, K.D.; Heimbach, J.K.; Dierkhising, R.A. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* **2011**, *141*, 1249-1253,
147. Iqbal, U.; Perumpail, B.J.; Akhtar, D.; Kim, D.; Ahmed, A. The epidemiology, risk profiling and diagnostic challenges of nonalcoholic fatty liver disease. *Medicines* **2019**, *6*, 41,
148. Kleiner, D.E.; Brunt, E.M.; Van Natta, M.; Behling, C.; Contos, M.J.; Cummings, O.W.; Ferrell, L.D.; Liu, Y.C.; Torbenson, M.S.; Unalp‐Arida, A. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **2005**, *41*, 1313-1321,
149. Golabi, P.; Sayiner, M.; Fazel, Y.; Koenig, A.; Henry, L.; Younossi, Z.M. Current complications and challenges in nonalcoholic steatohepatitis screening and diagnosis. *Expert review of gastroenterology & hepatology* **2016**, *10*, 63-71,
150. Sumida, Y.; Nakajima, A.; Itoh, Y. Limitations of liver biopsy and non-invasive diagnostic tests for the diagnosis of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World journal of gastroenterology: WJG* **2014**, *20*, 475,
151. Juluri, R.; Vuppalanchi, R.; Olson, J.; Unalp, A.; Van, M.N.; Cummings, O.W.; Tonascia, J.; Chalasani, N. Generalizability of the nonalcoholic steatohepatitis Clinical Research Network histologic scoring system for nonalcoholic fatty liver disease. *Journal of clinical gastroenterology* **2011**, *45*, 55-58,
152. Ferraioli, G.; Tinelli, C.; Dal Bello, B.; Zicchetti, M.; Filice, G.; Filice, C.; Group, L.F.S. Accuracy of real‐time shear wave elastography for assessing liver fibrosis in chronic hepatitis C: a pilot study. *Hepatology* **2012**, *56*, 2125-2133,
153. Lomonaco, R.; Sunny, N.E.; Bril, F.; Cusi, K. Nonalcoholic fatty liver disease: current issues and novel treatment approaches. *Drugs* **2013**, *73*, 1-14,
154. Musso, G.; Gambino, R.; Cassader, M.; Pagano, G. A meta‐analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* **2010**, *52*, 79-104,
155. Dyson, J.; Anstee, Q.; McPherson, S. Republished: non-alcoholic fatty liver disease: a practical approach to treatment. *Postgraduate medical journal* **2015**, *91*, 92-101,
156. Dyson, J.; Day, C. Treatment of non-alcoholic fatty liver disease. *Digestive diseases* **2014**, *32*, 597-604,
157. Thoma, C.; Day, C.P.; Trenell, M.I. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *Journal of hepatology* **2012**, *56*, 255-266,

158. Hallsworth, K.; Avery, L.; Trenell, M.I. Targeting lifestyle behavior change in adults with NAFLD during a 20-min consultation: summary of the dietary and exercise literature. *Current gastroenterology reports* **2016**, *18*, 11,
159. Musso, G.; Cassader, M.; Gambino, R. Non-alcoholic steatohepatitis: emerging molecular targets and therapeutic strategies. *Nature reviews Drug discovery* **2016**, *15*, 249,
160. Lassailly, G.; Caiazzo, R.; Buob, D.; Pigeyre, M.; Verkindt, H.; Labreuche, J.; Raverdy, V.; Leteurtre, E.; Dharancy, S.; Louvet, A. Bariatric surgery reduces features of nonalcoholic steatohepatitis in morbidly obese patients. *Gastroenterology* **2015**, *149*, 379-388,
161. Friedman, S.L.; Neuschwander-Tetri, B.A.; Rinella, M.; Sanyal, A.J. Mechanisms of NAFLD development and therapeutic strategies. *Nature medicine* **2018**, *24*, 908-922,
162. Meex, R.C.; Watt, M.J. Hepatokines: linking nonalcoholic fatty liver disease and insulin resistance. *Nature Reviews Endocrinology* **2017**, *13*, 509,
163. Day, C.P.; James, O.F. Steatohepatitis: a tale of two "hits"? **1998**.
164. Alonso, C.; Fernández-Ramos, D.; Varela-Rey, M.; Martínez-Arranz, I.; Navasa, N.; Van Liempd, S.M.; Trueba, J.L.L.; Mayo, R.; Ilisso, C.P.; de Juan, V.G. Metabolomic identification of subtypes of nonalcoholic steatohepatitis. *Gastroenterology* **2017**, *152*, 1449-1461. e1447, doi:10.1053/j.gastro.2017.01.015
165. Yu, J.; Marsh, S.; Hu, J.; Feng, W.; Wu, C. *The Pathogenesis of Nonalcoholic Fatty Liver Disease: Interplay between Diet, Gut Microbiota, and Genetic Background*; 2016; Volume 2016, p. 13.
166. Wree, A.; Broderick, L.; Canbay, A.; Hoffman, H.M.; Feldstein, A.E. From NAFLD to NASH to cirrhosis—new insights into disease mechanisms. *Nature reviews Gastroenterology & hepatology* **2013**, *10*, 627,
167. Machado, M.V.; Diehl, A.M. Pathogenesis of nonalcoholic steatohepatitis. *Gastroenterology* **2016**, *150*, 1769-1777,
168. Bugianesi, E.; Moscatiello, S.; Ciaravella, M.; Marchesini, G. Insulin resistance in nonalcoholic fatty liver disease. *Current pharmaceutical design* **2010**, *16*, 1941-1951,
169. Fabbrini, E.; Magkos, F.; Mohammed, B.S.; Pietka, T.; Abumrad, N.A.; Patterson, B.W.; Okunade, A.; Klein, S. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proceedings of the National Academy of Sciences* **2009**, *106*, 15430-15435,
170. Lomonaco, R.; Ortiz‐Lopez, C.; Orsak, B.; Webb, A.; Hardies, J.; Darland, C.; Finch, J.; Gastaldelli, A.; Harrison, S.; Tio, F. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. *Hepatology* **2012**, *55*, 1389-1397,
171. Bugianesi, E.; Gastaldelli, A.; Vanni, E.; Gambino, R.; Cassader, M.; Baldi, S.; Ponti, V.; Pagano, G.; Ferrannini, E.; Rizzetto, M. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* **2005**, *48*, 634-642,
172. Kotronen, A.; Juurinen, L.; Tiikkainen, M.; Vehkavaara, S.; Yki–Järvinen, H. Increased liver fat, impaired insulin clearance, and hepatic and adipose tissue insulin resistance in type 2 diabetes. *Gastroenterology* **2008**, *135*, 122-130,
173. Seppälä-Lindroos, A.; Vehkavaara, S.; Häkkinen, A.-M.; Goto, T.; Westerbacka, J.; Sovijärvi, A.; Halavaara, J.; Yki-Järvinen, H. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *The Journal of Clinical Endocrinology & Metabolism* **2002**, *87*, 3023-3028,

174. Donnelly, K.L.; Smith, C.I.; Schwarzenberg, S.J.; Jessurun, J.; Boldt, M.D.; Parks, E.J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *The Journal of clinical investigation* **2005**, *115*, 1343-1351,
175. Lambert, J.E.; Ramos–Roman, M.A.; Browning, J.D.; Parks, E.J. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology* **2014**, *146*, 726-735,
176. Guilherme, A.; Virbasius, J.V.; Puri, V.; Czech, M.P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature reviews Molecular cell biology* **2008**, *9*, 367-377,
177. Tana, C.; Ballestri, S.; Ricci, F.; Di Vincenzo, A.; Ticinesi, A.; Gallina, S.; Giamberardino, M.A.; Cipollone, F.; Sutton, R.; Vettor, R. Cardiovascular risk in non-alcoholic fatty liver disease: mechanisms and therapeutic implications. *International journal of environmental research and public health* **2019**, *16*, 3104,
178. Bergman, R. Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein? *Diabetologia* **2000**, *43*, 946-952,
179. Dasarathy, S.; Yang, Y.; McCullough, A.J.; Marczewski, S.; Bennet, C.; Kalhan, S.C. Elevated hepatic fatty acid oxidation, high plasma fibroblast growth factor 21, and fasting bile acids in nonalcoholic steatohepatitis. *European journal of gastroenterology & hepatology* **2011**, *23*, 382,
180. Croci, I.; Byrne, N.M.; Choquette, S.; Hills, A.P.; Chachay, V.S.; Clouston, A.D.; O'Moore-Sullivan, T.M.; Macdonald, G.A.; Prins, J.B.; Hickman, I.J. Whole-body substrate metabolism is associated with disease severity in patients with non-alcoholic fatty liver disease. *Gut* **2013**, *62*, 1625-1633,
181. Brouwers, B.; Schrauwen-Hinderling, V.B.; Jelenik, T.; Gemmink, A.; Havekes, B.; Bruls, Y.; Dahlmans, D.; Roden, M.; Hesselink, M.K.; Schrauwen, P. Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes. *Clinical Science* **2017**, *131*, 1905-1917,
182. Gastaldelli, A. Insulin resistance and reduced metabolic flexibility: cause or consequence of NAFLD? *Clinical Science* **2017**, *131*, 2701-2704,
183. Adiels, M.; Olofsson, S.-O.; Taskinen, M.-R.; Borén, J. Overproduction of very low–density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arteriosclerosis, thrombosis, and vascular biology* **2008**, *28*, 1225-1236,
184. Sanders, F.W.; Griffin, J.L. De novo lipogenesis in the liver in health and disease: more than just a shunting yard for glucose. *Biological Reviews* **2016**, *91*, 452-468,
185. Diraison, F.; Moulin, P.; Beylot, M. Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. *Diabetes & metabolism* **2003**, *29*, 478-485,
186. Poulsen, M.K.; Nellemann, B.; Stødkilde-Jørgensen, H.; Pedersen, S.B.; Grønbæk, H.; Nielsen, S. Impaired insulin suppression of VLDL-triglyceride kinetics in nonalcoholic fatty liver disease. *The Journal of Clinical Endocrinology & Metabolism* **2016**, *101*, 1637-1646,
187. Choi, S.H.; Ginsberg, H.N. Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance. *Trends in Endocrinology & Metabolism* **2011**, *22*, 353-363,
188. Fabbrini, E.; Mohammed, B.S.; Magkos, F.; Korenblat, K.M.; Patterson, B.W.; Klein, S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology* **2008**, *134*, 424-431,

189. Koliwad, S.K.; Streeper, R.S.; Monetti, M.; Cornelissen, I.; Chan, L.; Terayama, K.; Naylor, S.; Rao, M.; Hubbard, B.; Farese, R.V. DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *The Journal of clinical investigation* **2010**, *120*, 756-767,
190. Yamaguchi, K.; Yang, L.; McCall, S.; Huang, J.; Yu, X.X.; Pandey, S.K.; Bhanot, S.; Monia, B.P.; Li, Y.X.; Diehl, A.M. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* **2007**, *45*, 1366-1374,
191. Adams, L.A.; Ratziu, V. Non-alcoholic fatty liver–Perhaps not so benign. *Journal of hepatology* **2015**, *62*, 1002-1004,
192. Chiappini, F.; Barrier, A.; Saffroy, R.; Domart, M.-C.; Dagues, N.; Azoulay, D.; Sebagh, M.; Franc, B.; Chevalier, S.; Debuire, B. Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Laboratory investigation* **2006**, *86*, 154-165,
193. Hall, Z.; Bond, N.J.; Ashmore, T.; Sanders, F.; Ament, Z.; Wang, X.; Murray, A.J.; Bellafante, E.; Virtue, S.; Vidal‐Puig, A. Lipid zonation and phospholipid remodeling in nonalcoholic fatty liver disease. *Hepatology* **2017**, *65*, 1165-1180,
194. Jenkins, C.M.; Mancuso, D.J.; Yan, W.; Sims, H.F.; Gibson, B.; Gross, R.W. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *Journal of Biological Chemistry* **2004**, *279*, 48968-48975,
195. Kumari, M.; Schoiswohl, G.; Chitraju, C.; Paar, M.; Cornaciu, I.; Rangrez, A.Y.; Wongsiriroj, N.; Nagy, H.M.; Ivanova, P.T.; Scott, S.A. Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase. *Cell metabolism* **2012**, *15*, 691-702,
196. BasuRay, S.; Wang, Y.; Smagris, E.; Cohen, J.C.; Hobbs, H.H. Accumulation of PNPLA3 on lipid droplets is the basis of associated hepatic steatosis. *Proceedings of the National Academy of Sciences* **2019**, *116*, 9521-9526,
197. BasuRay, S.; Smagris, E.; Cohen, J.C.; Hobbs, H.H. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. *Hepatology* **2017**, *66*, 1111-1124,
198. Wang, Y.; Kory, N.; BasuRay, S.; Cohen, J.C.; Hobbs, H.H. PNPLA3, CGI‐58, and inhibition of hepatic triglyceride hydrolysis in mice. *Hepatology* **2019**, *69*, 2427-2441,
199. Ruhanen, H.; Perttilä, J.; Hölttä-Vuori, M.; Zhou, Y.; Yki-Järvinen, H.; Ikonen, E.; Käkelä, R.; Olkkonen, V.M. PNPLA3 mediates hepatocyte triacylglycerol remodeling. *Journal of lipid research* **2014**, *55*, 739-746,
200. Huang, Y.; Cohen, J.C.; Hobbs, H.H. Expression and characterization of a PNPLA3 isoform (I148M) associated with nonalcoholic fatty liver disease. *Journal of Biological Chemistry* **2011**, jbc. M111. 290114,
201. Pérez-Chacón, G.; Astudillo, A.M.; Balgoma, D.; Balboa, M.A.; Balsinde, J. Control of free arachidonic acid levels by phospholipases A2 and lysophospholipid acyltransferases. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2009**, *1791*, 1103-1113,
202. Donati, B.; Motta, B.M.; Pingitore, P.; Meroni, M.; Pietrelli, A.; Alisi, A.; Petta, S.; Xing, C.; Dongiovanni, P.; Del Menico, B. The rs2294918 E434K variant modulates patatin‐like phospholipase domain‐containing 3 expression and liver damage. *Hepatology* **2016**, *63*, 787-798,

203. Mitsche, M.A.; Hobbs, H.H.; Cohen, J.C. Patatin-like phospholipase domain–containing protein 3 promotes transfers of essential fatty acids from triglycerides to phospholipids in hepatic lipid droplets. *Journal of Biological Chemistry* **2018**, *293*, 6958-6968,
204. Smagris, E.; BasuRay, S.; Li, J.; Huang, Y.; Ka‐man, V.L.; Gromada, J.; Cohen, J.C.; Hobbs, H.H. Pnpla3I148M knockin mice accumulate PNPLA3 on lipid droplets and develop hepatic steatosis. *Hepatology* **2015**, *61*, 108-118,
205. Huang, Y.; Cohen, J.C.; Hobbs, H.H. Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. *Journal of Biological Chemistry* **2011**, *286*, 37085-37093,
206. Pirazzi, C.; Valenti, L.; Motta, B.M.; Pingitore, P.; Hedfalk, K.; Mancina, R.M.; Burza, M.A.; Indiveri, C.; Ferro, Y.; Montalcini, T. PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells. *Human molecular genetics* **2014**, *23*, 4077-4085,
207. Bruschi, F.V.; Claudel, T.; Tardelli, M.; Caligiuri, A.; Stulnig, T.M.; Marra, F.; Trauner, M. The PNPLA3 I148M variant modulates the fibrogenic phenotype of human hepatic stellate cells. *Hepatology* **2017**, *65*, 1875-1890,
208. Mondul, A.; Mancina, R.M.; Merlo, A.; Dongiovanni, P.; Rametta, R.; Montalcini, T.; Valenti, L.; Albanes, D.; Romeo, S. PNPLA3 I148M variant influences circulating retinol in adults with nonalcoholic fatty liver disease or obesity. *The Journal of nutrition* **2015**, *145*, 1687-1691,
209. Cusi, K. Role of insulin resistance and lipotoxicity in non-alcoholic steatohepatitis. *Clinics in liver disease* **2009**, *13*, 545-563,
210. Ibrahim, S.H.; Kohli, R.; Gores, G.J. Mechanisms of lipotoxicity in NAFLD and clinical implications. *Journal of pediatric gastroenterology and nutrition* **2011**, *53*, 131,
211. Mota, M.; Banini, B.A.; Cazanave, S.C.; Sanyal, A.J. Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. *Metabolism-Clinical and Experimental* **2016**, *65*, 1049-1061,
212. Feldstein, A.E.; Canbay, A.; Angulo, P.; Taniai, M.; Burgart, L.J.; Lindor, K.D.; Gores, G.J. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* **2003**, *125*, 437-443,
213. Begriche, K.; Igoudjil, A.; Pessayre, D.; Fromenty, B. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* **2006**, *6*, 1-28,
214. Kusminski, C.M.; Shetty, S.; Orci, L.; Unger, R.H.; Scherer, P.E. Diabetes and apoptosis: lipotoxicity. *Apoptosis* **2009**, *14*, 1484-1495,
215. Hirsova, P.; Ibrabim, S.H.; Gores, G.J.; Malhi, H. Lipotoxic lethal and sublethal stress signaling in hepatocytes: relevance to NASH pathogenesis. *Journal of lipid research* **2016**, *57*, 1758-1770,
216. Pfaffenbach, K.T.; Gentile, C.L.; Nivala, A.M.; Wang, D.; Wei, Y.; Pagliassotti, M.J. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate-mediated cell death. *American Journal of Physiology-Endocrinology and Metabolism* **2010**, *298*, E1027-E1035,
217. Perry, R.J.; Samuel, V.T.; Petersen, K.F.; Shulman, G.I. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature* **2014**, *510*, 84-91,

218. Luukkonen, P.K.; Zhou, Y.; Sädevirta, S.; Leivonen, M.; Arola, J.; Orešič, M.; Hyötyläinen, T.; Yki-Järvinen, H. Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *Journal of hepatology* **2016**, *64*, 1167-1175,
219. Mauer, A.S.; Hirsova, P.; Maiers, J.L.; Shah, V.H.; Malhi, H. Inhibition of sphingosine 1-phosphate signaling ameliorates murine nonalcoholic steatohepatitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2017**, *312*, G300-G313,
220. Han, M.S.; Park, S.Y.; Shinzawa, K.; Kim, S.; Chung, K.W.; Lee, J.-H.; Kwon, C.H.; Lee, K.-W.; Lee, J.-H.; Park, C.K. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. *Journal of lipid research* **2008**, *49*, 84-97,
221. Ioannou, G.N.; Boyko, E.J.; Lee, S.P. The prevalence and predictors of elevated serum aminotransferase activity in the United States in 1999–2002. *The American journal of gastroenterology* **2006**, *101*, 76,
222. Asgharpour, A.; Cazanave, S.C.; Pacana, T.; Seneshaw, M.; Vincent, R.; Banini, B.A.; Kumar, D.P.; Daita, K.; Min, H.-K.; Mirshahi, F. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. *Journal of hepatology* **2016**, *65*, 579-588,
223. Carrion, A.F.; Ghanta, R.; Carrasquillo, O.; Martin, P. Chronic liver disease in the Hispanic population of the United States. *Clinical Gastroenterology and Hepatology* **2011**, *9*, 834-841,
224. Ford, E.S.; Giles, W.H.; Dietz, W.H. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *Jama* **2002**, *287*, 356-359,
225. Wang, J.; Thornton, J.C.; Burastero, S.; Shen, J.; Tanenbaum, S.; Heymsfield, S.B.; Pierson Jr, R.N. Comparisons for body mass index and body fat percent among Puerto Ricans, blacks, whites and Asians living in the New York City area. *Obesity research* **1996**, *4*, 377-384,
226. Weston, S.R.; Leyden, W.; Murphy, R.; Bass, N.M.; Bell, B.P.; Manos, M.M.; Terrault, N.A. Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology* **2005**, *41*, 372-379,
227. Casas, Y.G.; Schiller, B.C.; DeSouza, C.A.; Seals, D.R. Total and regional body composition across age in healthy Hispanic and white women of similar socioeconomic status. *The American journal of clinical nutrition* **2001**, *73*, 13-18,
228. Ferdinand, K.C.; Nasser, S.A. Racial/ethnic disparities in prevalence and care of patients with type 2 diabetes mellitus. *Current medical research and opinion* **2015**, *31*, 913-923,
229. Foster, T.; Anania, F.A.; Li, D.; Katz, R.; Budoff, M. The prevalence and clinical correlates of nonalcoholic fatty liver disease (NAFLD) in African Americans: the multiethnic study of atherosclerosis (MESA). *Digestive diseases and sciences* **2013**, *58*, 2392-2398,
230. Romeo, S.; Huang-Doran, I.; Baroni, M.G.; Kotronen, A. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. *Curr Opin Lipidol* **2010**, *21*, 247-252, doi:10.1097/mol.0b013e328338ca61
231. Allen, M.L.; Elliott, M.N.; Morales, L.S.; Diamant, A.L.; Hambarsoomian, K.; Schuster, M.A. Adolescent participation in preventive health behaviors, physical activity, and nutrition: differences across immigrant generations for Asians and Latinos compared with Whites. *American Journal of Public Health* **2007**, *97*, 337-343, doi:10.2105/AJPH.2005.076810

232. Unger, J.B.; Reynolds, K.; Shakib, S.; Spruijt-Metz, D.; Sun, P.; Johnson, C.A. Acculturation, physical activity, and fast-food consumption among Asian-American and Hispanic adolescents. *Journal of community health* **2004**, *29*, 467-481,
233. Crespo, C.J.; Smit, E.; Andersen, R.E.; Carter-Pokras, O.; Ainsworth, B.E. Race/ethnicity, social class and their relation to physical inactivity during leisure time: results from the Third National Health and Nutrition Examination Survey, 1988–1994. *American journal of preventive medicine* **2000**, *18*, 46-53,
234. Wishart, D.S. Emerging applications of metabolomics in drug discovery and precision medicine. *Nature reviews Drug discovery* **2016**, *15*, 473,
235. Patti, G.J.; Yanes, O.; Siuzdak, G. Innovation: Metabolomics: the apogee of the omics trilogy. *Nature reviews Molecular cell biology* **2012**, *13*, 263,
236. Johnson, C.H.; Ivanisevic, J.; Siuzdak, G. Metabolomics: beyond biomarkers and towards mechanisms. *Nature reviews Molecular cell biology* **2016**, *17*, 451-459,
237. Krone, N.; Hughes, B.A.; Lavery, G.G.; Stewart, P.M.; Arlt, W.; Shackleton, C.H. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). *The Journal of steroid biochemistry and molecular biology* **2010**, *121*, 496-504,
238. Drexler, D.M.; Reily, M.D.; Shipkova, P.A. Advances in mass spectrometry applied to pharmaceutical metabolomics. *Analytical and bioanalytical chemistry* **2011**, *399*, 2645-2653,
239. Kalhan, S.C.; Guo, L.; Edmison, J.; Dasarathy, S.; McCullough, A.J.; Hanson, R.W.; Milburn, M. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism-Clinical and Experimental* **2011**, *60*, 404-413,
240. García-Cañaveras, J.C.; Donato, M.T.; Castell, J.V.; Lahoz, A. A Comprehensive Untargeted Metabonomic Analysis of Human Steatotic Liver Tissue by RP and HILIC Chromatography Coupled to Mass Spectrometry Reveals Important Metabolic Alterations. *Journal of Proteome Research* **2011**, *10*, 4825-4834, doi:10.1021/pr200629p
241. Wattacheril, J.; Seeley, E.H.; Angel, P.; Chen, H.; Bowen, B.P.; Lanciault, C.; Caprioli, R.M.; Abumrad, N.; Flynn, C.R. Differential intrahepatic phospholipid zonation in simple steatosis and nonalcoholic steatohepatitis. *PloS one* **2013**, *8*,
242. Debois, D.; Bralet, M.-P.; Le Naour, F.; Brunelle, A.; Laprévote, O. In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF-SIMS imaging. *Analytical chemistry* **2009**, *81*, 2823-2831,
243. Le Naour, F.; Bralet, M.-P.; Debois, D.; Sandt, C.; Guettier, C.; Dumas, P.; Brunelle, A.; Laprevote, O. Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. *PLoS One* **2009**, *4*,
244. Loomba, R.; Quehenberger, O.; Armando, A.; Dennis, E.A. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. *J Lipid Res* **2015**, *56*, 185-192, doi:10.1194/jlr.P055640
245. Gorden, D.L.; Myers, D.S.; Ivanova, P.T.; Fahy, E.; Maurya, M.R.; Gupta, S.; Min, J.; Spann, N.J.; McDonald, J.G.; Kelly, S.L. Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *Journal of lipid research* **2015**, *56*, 722-736,
246. Orešič, M.; Hyötyläinen, T.; Kotronen, A.; Gopalacharyulu, P.; Nygren, H.; Arola, J.; Castillo, S.; Mattila, I.; Hakkarainen, A.; Borra, R.J. Prediction of non-alcoholic fatty-liver disease and liver fat content by serum molecular lipids. *Diabetologia* **2013**, *56*, 2266-2274,

247. Puri, P.; Baillie, R.A.; Wiest, M.M.; Mirshahi, F.; Choudhury, J.; Cheung, O.; Sargeant, C.; Contos, M.J.; Sanyal, A.J. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **2007**, *46*, 1081-1090,
248. Puri, P.; Wiest, M.M.; Cheung, O.; Mirshahi, F.; Sargeant, C.; Min, H.K.; Contos, M.J.; Sterling, R.K.; Fuchs, M.; Zhou, H. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **2009**, *50*, 1827-1838,
249. Younossi, Z.M.; Blissett, D.; Blissett, R.; Henry, L.; Stepanova, M.; Younossi, Y.; Racila, A.; Hunt, S.; Beckerman, R. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology* **2016**, *64*, 1577-1586,
250. Lonardo, A.; Nascimbeni, F.; Targher, G.; Bernardi, M.; Bonino, F.; Bugianesi, E.; Casini, A.; Gastaldelli, A.; Marchesini, G.; Marra, F. AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions. *Digestive and Liver Disease* **2017**, *49*, 471-483,
251. Colby, S.L.; Ortman, J.M. Projections of the size and composition of the US population: 2014 to 2060: Population estimates and projections. **2017**,
252. Mazi, T.A.; Borkowski, K.; Newman, J.W.; Fiehn, O.; Bowlus, C.L.; Sarkar, S.; Matsukuma, K.; Ali, M.R.; Kieffer, D.A.; Wan, Y.Y.; et al. Ethnicity-specific alterations of plasma and hepatic lipidomic profiles are related to high NAFLD rate and severity in Hispanic Americans, a pilot study. *Free Radic Biol Med* **2021**, *172*, 490-502, doi:10.1016/j.freeradbiomed.2021.06.024
253. Mazi, T.A.; Borkowski, K.; Fiehn, O.; Bowlus, C.L.; Sarkar, S.; Matsukuma, K.; Ali, M.R.; Kieffer, D.A.; Wan, Y.-J.Y.; Stanhope, K.L. Plasma oxylipin profile discriminates ethnicities in subjects with Non-alcoholic steatohepatitis: An exploratory analysis. *Metabolites* **2022**, *12*, 192,
254. Wu, T. Cyclooxygenase-2 in hepatocellular carcinoma. *Cancer treatment reviews* **2006**, *32*, 28-44,
255. Jump, D.B.; Depner, C.M.; Tripathy, S.; Lytle, K.A. Potential for Dietary ω-3 Fatty Acids to Prevent Nonalcoholic Fatty Liver Disease and Reduce the Risk of Primary Liver Cancer–. *Advances in Nutrition* **2015**, *6*, 694-702,
256. Yetukuri, L.; Katajamaa, M.; Medina-Gomez, G.; Seppänen-Laakso, T.; Vidal-Puig, A.; Orešič, M. Bioinformatics strategies for lipidomics analysis: characterization of obesity related hepatic steatosis. *BMC systems biology* **2007**, *1*, 12,

Chapter 2

Untargeted metabolomic profiling

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a continuum of liver pathology that includes steatosis (NAFL), hepatocellular inflammation and ballooning, or nonalcoholic steatohepatitis (NASH) and cirrhosis [1]. It affects 25% of the general population and up to 95% of individuals with medically complicated obesity [2,3]. Based on the strong correlations of NAFLD with metabolic syndrome and its components, NAFLD is argued to be its hepatic manifestation [4,5]. In the U.S., disparity in NAFLD prevalence is reported, with Hispanics (HIS) being impacted at higher rate and severity compared to other ethnicities [6-11]. To date, the biological background underlying this disparity remains unclear.

NAFL develops when fatty acid availability, from the hepatic uptake of free fatty acids (FFA) and *de novo* lipogenesis (DNL), outweighs the utilization and disposal capacity, mainly by synthesis of triglycerides (TG) and other lipids, mitochondrial β-oxidation and export as very low-density lipoprotein [12]. Mechanisms of NAFLD progression are explained by the "multiple hit" model, in which lipid overload, the first hepatic hit, overwhelms the disposal, utilization and storage capacity. This results in a cascade of metabolic insults including oxidative stress, lipotoxicity, endoplasmic reticular stress, mitochondrial dysfunction and apoptosis, collectively, provoking hepatocyte injury, inflammation and disease progression [12,13].

Mitochondrial function is altered in NAFLD and hepatic β-oxidation of fatty acids is reported to be both increased and decreased [14-16]. This inconsistency is thought to reflect a process of mitochondrial adaptation to compensate for the increased availability of FFAs, with an initial increase in β-oxidation, tricarboxylic acid cycle and oxidative phosphorylation [14,15,17]. Eventually, the metabolic capacity and antioxidant defenses are overwhelmed, resulting in hepatic oxidative stress and diverting FFAs to the production of hepatotoxic lipids [18,19]. It is thought that accumulation and generation of hepatotoxic lipids is critical to NAFLD progression [20]. As mentioned above, lipotoxicity is considered a component of the "multiple hit" and may result from the accumulation of hepatotoxic lipids or intermediates, such as saturated fatty acids (SFA), ceramides (Cer) and

lysophosphatidylcholine (LPC) [21-24]. Lipotoxicity can also result from a deficiency or imbalance in lipids essential for cellular integrity and function, including polyunsaturated fatty acids (PUFA) [24].

Previous metabolomic analysis of liver tissue and plasma have shown that NAFLD is associated with derangements of lipid, carbohydrate, and amino acid metabolism [25-29]. Dysregulation in one-carbon metabolism, including alterations in choline, betaine, methionine and in the universal methyl donor, S-Adenosyl-L-methionine (SAMe), is also implicated in NAFLD development and NASH severity, as shown from animal studies [30]. In humans, a recent metabolomic analysis identified a subtype of NAFLD subjects characterized by impaired one-carbon metabolism with NASH progression [31]. However, to our knowledge, no previous metabolomic profiling study has addressed ethnicity-related variations with regards to NAFLD.

The objective of this study was to investigate metabolomic profiles in a group of Hispanic (HIS) and White Caucasian (CAU) with obesity and biopsy-confirmed NAFLD. The identification of metabolomic differences between HIS and CAU subjects with NAFLD may provide a new research direction pointing towards ethnicity-specific changes as potential drivers for the disparity observed in NAFLD rate and progression. To this end, we employed untargeted metabolomic profiling of primary metabolism, complex lipids, choline and related metabolites in a group of Hispanic (NAFL-HIS) and Caucasian (NAFL-CAU) subjects with obesity and NAFLD of comparable histological presentations, as compared to a group of ethnicity-matched lean healthy control subjects. We also compared liver and plasma metabolomic profiles in a group of NASH (NASH-HIS) and (NASH-CAU) subjects with similar NAFLD Activity Score (NAS), to a group of BMI-matched NASH-free (0-NASH) subjects in both ethnicities.

2. Subjects and methods

1.1. *Subjects*

Liver biopsies (n =17) and plasma samples (n =15) were retrieved from the biobank repository at the Division of Gastroenterology and Hepatology, UC Davis Medical Center. Samples were collected from bariatric surgery patients who self-identified ethnicity as either HIS or CAU. Included subjects were both males and females; age range 18−75 years; with class II and III obesity (body mass index

(BMI) > 35.0 kg/m^2); no previous diagnosis of acute or chronic diseases except for obesity or type 2 diabetes based on medical history. Exclusion criteria were diagnosis of secondary causes of chronic liver disease based on medical history, including viral and autoimmune hepatitis, HIV, hemochromatosis, alpha 1 anti-trypsin deficiency, Wilson disease and drug-induced liver disease; excessive alcohol intake (defined as >20 g/day for women and >30 g/day for men); and subjects with advanced fibrosis (stage >2). Healthy control subjects (HC; *n* =22) were recruited via public postings. Both male and female subjects were included; age between 18−75 years; BMI between 18 and 25 kg/m^2; no current or previous diagnosis of chronic diseases; absence of signs of acute diseases.

For subjects with NAFLD, demographic, anthropometric and clinical data were collected retrospectively using data recorded within 7 days of bariatric surgery procedure. For HC subjects, demographic data were self-reported and anthropometric measurements were collected during one in-clinic visit. All subjects were consented with a signed form and the study protocol was approved by the Institutional Review Board at the University of California, Davis (protocol # 856052).

1.2. *Biological samples*

Liver tissue samples were collected by intraoperative core biopsy during bariatric surgery (time of collection is not available). For each subject, one sample was submitted in 10% formalin to the Department of Pathology for routine diagnostic interpretation. A second sample was flash-frozen in liquid nitrogen immediately after harvest and subsequently transferred and stored at −80 °C until analysis. Blood samples were collected by veno-puncture in EDTA-containing pre-cooled tubes after a 10-hour overnight fasting on the day of operation for NAFL group or in clinic for HC (time of collection is not available). Samples were centrifuged and stored at −80 °C until analysis.

1.3. *Histopathology*

Specimens received by the Department of Pathology were processed as per routine protocol. After diagnostic interpretation was completed, biopsy slides were retrieved and scored using the NASH Clinical Research Network (NASH-CRN) histology scoring system, which includes steatosis, hepatocellular ballooning, lobular inflammation, and fibrosis, by a gastrointestinal pathologist blinded to the subject's clinical data. The NAFLD Activity Score (NAS) and fibrosis score were tabulated

[32]. NASH diagnosis was determined by incorporating the histology scores into a diagnostic algorithm [33].

1.4. *Untargeted metabolomics*

Untargeted, semi-quantitative metabolomic profiling for plasma and liver samples was performed at the UC Davis West Coast Metabolomics Center. Samples were extracted using methanol: methyl tert-butyl ether (MTBE):water as previously described [34]. To profile metabolites related to primary metabolism including carbohydrates, sugar phosphates and amino acids, the aqueous phase was dried and subjected to trimethylsilation and methoximation and analyzed by gas chromatography/time-of-flight mass spectrometry (GC-TOF MS) [35]. Spectral data were processed and annotated using BinBase [36]. To profile FFAs and complex lipids, including mono-, di- and TGs, cholesteryl ester (CE), phospholipids (PL), sphingolipids (SL), the organic phase was dried, resuspended and analyzed by charged surface hybrid liquid chromatography/quadrupole time of flight mass spectrometry (CSH-QTOF MS/MS) [37]. Data collected in both positive and negative ion mode and processed using MassHunter (Agilent Technologies, Inc., Santa Clara, CA, http://www.agilent.com). Lipids were identified based on MS/MS fragmentation patterns using Lipidblast software [38]. To profile acylcarnitine, choline, betaine, S-Adenosyl-L-methionine (SAMe) and related metabolites, aqueous phase was analyzed by the biogenic amines platform using hydrophilic interaction liquid chromatography/quadrupole time of flight mass spectrometer (HILIC-QTOF MS/MS) [37]. Data were collected in both positive and negative mode. MS-DIAL was used for data processing [39]. For annotations, the three-levels of compound annotation by the Metabolomics Standards Initiative (MSI) was employed [40]. Metabolites with MSI level 1 annotation (i.e., an MS/MS spectral library with retention time and precursor mass) were excluded form statistical analyses. All mass spectra are available at Massbank of North America (http://mona.fiehnlab.ucdavis.edu). The study details are available on The Metabolomics Workbench (http://www.metabolomicsworkbench.org), ID number (ST000977).

In this study, fatty acids and complex lipids are described by name and lipid class, respectively, followed by number of (carbons, double bounds) of the fatty acyl moiety (i.e., linoleic acid (18:2n6)

and TG(50-52:0-6)). The index of fatty acid desaturase-1(FADS1, Δ-5 desaturase) was estimated as the product to precursor ratio or arachidonic acid (20:4n6)/dihomo-γ-linolenic acid (20:3n6). Stearoyl-CoA desaturase (SCD1, or Δ-9 desaturase) was estimated as palmitoleic acid (16:1n7)/palmitic acid (16:0). The hepatic relative ratio of free n6 to n3 PUFAs was calculated as arachidonic acid (20:4n6)/eicosapentaenoic acid (20:5n3) and arachidonic acid (20:4n6)/docosahexaenoic acid (22:6n3).

1.5. *Statistical analysis:*

Statistical analysis was performed using JMP Pro 14.1 (SAS Institute Inc., Cary, NC; http://www.jmp.com). Unknowns and metabolites and with MSI level 1 identification from HILIC-QTOF MS/MS platform were excluded. Outliers were detected and excluded using the robust Huber M test and missing data were imputed using multivariate normal imputation if greater than 70% complete. Metabolites with more than 30% missing data were excluded. The Johnson's transformation was used to attain normal data distributions for statistical assessment.

Metabolite means were calculated using Log normalized data, fold changes were calculated as the mean of NAFL/HC for comparison between NAFL and HC or NASH/0-NASH for comparison between NASH and 0-NASH. Students' *t*-test was used to compare means. Differences were considered likely at $p < 0.05$, and possible if the p-value was ≥ 0.05 and < 0.1.

To examine if ethnicity-specific differences exist in NAFLD and to evaluate the interaction of ethnicity x health status (i.e., NASH or NAFL), full factorial analysis of covariates (ANCOVA) was employed. This model included ethnicity, health status, ethnicity x health status interaction as fixed effects, and age and sex as covariates. Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons using a $q = 0.2$ was performed, given the pilot nature and small sample number of this study [41]. To reduce data dimensionality and facilitate visualization, metabolites were clustered using the JMP implementation of the SAS VARCLUS procedure, a principal component analysis (PCA)-based clustering algorithm, with cluster components calculated as the linear sum of all variables in a cluster.

For NAFL vs. HC comparisons and to focus on ethnicity-specific differences, we only clustered metabolites with p <0.1 for ethnicity x NAFL interaction. Next, cluster components were subjected to partial least square-discriminant analysis (PLS-DA) separately in each ethnicity with leave-one-out cross validation (LOOCV) [42]. A variable importance in projection (VIP) score of >1 was set as a threshold for variable selection [43]. Because the PLS-DA model was constructed using reduced set of data to highlight only ethnicity- specific differences between NAFL and HC, the VIP scores should not be directly compared between the two analyses.

For NASH vs. 0-NASH comparisons and to focus on ethnicity-specific differences, we clustered metabolites with p <0.05 for the NASH effect within ethnicity groups or with p <0.05 for the ethnicity x NASH interaction. To further examine the effect of ethnicity between NASH and 0-NASH, cluster components were reevaluated using *t*-test for group comparison within ethnicity and ANCOVA to check on interaction (ethnicity x NASH).

To examine the association between metabolomic profiles and liver histological scoring, Spearman's rank correlations was performed. To correct for multiple testing, Benjamini-Hochberg FDR adjustment was performed with q =0.2 [41].

1.6. *Over representation analysis*

To provide an overview on the general changes in metabolomic profile, we employed ChemRICH [44], a statistical enrichment analysis based on chemical similarity. This approach clusters metabolites into non-overlapping chemical groups using Tanimoto substructure chemical similarity coefficients and calculates cluster *p*-values using the Kolmogorov–Smirnov test.

1.7. *Pathway enrichment analysis*

Pathway analysis was performed using MetaboAnalyst (McGill University, Quebec, CA; http://metaboanalyst.ca) [45]. Metabolites with differential alterations between ethnicity in NASH (p <0.05) were compared against pathway-associated metabolite sets from Kyoto Encyclopedia of Genes and Genomes (KEGG) [46]. Fisher's Exact test was used to assess over-representation and the relative betweenness centrality was used for topology analysis.

3. Results

1.8. *Subject characteristics*

The clinical and histological features for NAFL and NASH subjects are presented in Table 1 and Table 2. When comparing between ethnicities, both NAFL and NASH groups showed no difference in age, BMI and other clinical parameters including histology NAS scores.

In NAFL groups, the mean age was 45 ± 16 and 50 ± 16 years in HIS and CAU, respectively (p >0.05); 29% of HIS and 30% of CAU had moderate to severe steatosis; 43% of HIS and 50% of CAU were diagnosed with NASH and exhibited various degrees of lobular inflammation. One NAFL-CAU subject had portal/periportal fibrosis, or stage 1A. The mean NAS score for NAFL subjects was 2.4 ± 2.4 and 2.9 ± 1.5 for HIS and CAU, respectively (p >0.05). When compared to HC, BMI was 80% higher in NAFL-HIS and 70% higher in NAFL-CAU. In NASH subjects, the mean NAS score was 4.7 ± 0.58 and 4 ± 1 for HIS and CAU, respectively (p >0.05). Compared to 0-NASH, both ethnicities showed no difference in age, BMI or other clinical parameters.

1.9. *Ethnicity-specific differences in plasma metabolome in NAFL*

Given the low sample size and broad analysis in this study, we first evaluated NAFL-dependent changes by chemical classes separately in each ethnicity, effectively reducing the number of comparisons (Fig. 1). In both ethnicities, when compared to corresponding HC, NAFL was associated with lower plasma phosphatidylcholines (PC), unsaturated LPCs, galactosyl Cers, CEs and amino acids. Specific to CAU, NAFL showed higher levels of ACs and unsaturated FFAs, driven by elevated PUFAs including linoleic acid (18:2n6), α-linolenic acid (18:3n3), eicosadienoic acid (20:2n6) and MUFAs, heptadecenoic acid (17:1n7) and physeteric acid (14:1n7). Also, NAFL-CAU showed a reduction in many PLs including ether-linked PLs (Table S1). These differences in ACs and in unsaturated fatty acids were not evident in the NAFL-HIS group, which was characterized by alterations in sphingomyelins (SM) and xanthines, with higher level hydroxybutyrates, trimethyl ammonium compounds and sugar alcohols (Table S2).

To further examine this ethnicity-associated divergence in metabolomic profile, ANCOVA was performed with interaction (ethnicity x NASH). Of the 940 metabolites detected, 46 plasma

metabolites (4.9%) were found altered (p <0.05) in NAFL between ethnicities with an additional 59 metabolites showing a p-value between 0.05 and <0.1. However, none of these passed the FDR-based multiple comparisons adjustment. Although the probability of differences was low and the number of findings was close to that expected by random chance, due to the strong class-specific changes and the exploratory nature of this study, we proceeded with a characterization of metabolite differences between the groups.

The variable clustering approach reduced the data to 21 cluster components. To highlight the apparent ethnicity-specific metabolic differences between NAFL and HC groups, these cluster components were projected using a PLS-DA (Fig. 2). In CAU, 11 of the 21 clusters contributed to the discrimination of NAFL and HC in CAU with VIP scores >1. These clusters were represented by ether-linked PL(32-40:0-5) (cluster 8 and 15), PE(18-36:0-1) and PC(34-36:0-3) (cluster 3) which were lower in NAFL-CAU, and PC(38:4-5) (cluster 20) which appeared higher. Also, NAFL-CAU showed higher abundance of short to medium length acylcarnitines (i.e. AC(2-14:0-2); (cluster 2 and 7) and in non-esterified FFAs (cluster 2), including the SFAs myristic acid (14:0) and margaric acid (17:0), oleic acid (18:1n9); the PUFAs linoleic acid (18:2n6) and α-linolenic acid (18:3n3). Similar trends were observed for docosahexaenoic acid (22:6n3) and eicosapentaenoic acid (20:5n3) (cluster 21), but with VIP <1. Also, NAFL-CAU showed higher levels of the intestinal microbiota-related, trimethylamine N-oxide (TMAO) and indole-3-acetate (cluster 5) and lower levels of the purine-related metabolites, adenosine and guanine (cluster 16 and 13).

The differences in ACs and in non-esterified FFAs were not evident in NAFL-HIS, as compared to corresponding HC. Instead, 9 of the 21 clusters contributed to the discrimination of NAFL and HC with VIP scores >1. These clusters indicated lower levels of PC(40:6-7) (cluster 9) and higher levels of PC(38:4) (cluster 19) and ether-linked PL (36:1-4) (cluster 4 and 19). There was also evidence of higher abundance of metabolites related to purine metabolism, xanthine and hypoxanthine (cluster 11) and in the endocannabinoid-like stearoylethanolamide (cluster 17), with differences in multiple other organic compounds (cluster 6, 11, 12, 14, 16). Because the PLS-DA was generated using a reduced number of metabolites (with ethnicity interaction p <0.1), the VIP scores should not

be compared directly between the two models. Details on ANCOVA, variable clustering and PLS-DA results are shown in Table S3.

Together, results suggest metabolomic variation between NAFL and HC in CAU with alterations in PLs, ACs, non-esterified FFAs and some organic compounds, while in NAFL-HIS, changes were limited to PLs and some organic compounds. In addition, plasma FFA and AC profiles appear differentially altered with NAFL between ethnicity groups. These findings are depicted by the unsupervised PCA performed on cluster components (Fig. S1), showing complete separation of NAFL-CAU from HC-CAU, indicating these two groups are metabolically distinct, while there was a partial overlap between NAFL-HIS and HC-HIS, suggesting less metabolic variations between these groups.

1.10. *High acylcarnitines, triglycerides and unsaturated fatty acids distinguish HIS from CAU in HC*

The limited differences seen between NAFL and HC in HIS may reflect metabolic dysregulations in lean healthy HIS. To examine this, we compared plasma metabolomic profile in HC between ethnicities. Chemical similarity analysis clearly showed that in lean healthy subjects, HIS presented with higher ACs, TGs and unsaturated FFAs (Fig 3 and Table S4). HC-HIS had higher level (p <0.05) of the FFAs, margaric acid (17:0), oleic acid (18:1n9), linoleic acid (18:2n6), with similar trends in a number of other fatty acids. In addition, HC-HIS presented higher TG(51-58:1-9), DG(36-38:2-6), AC(2-16:0-1), altered PLs profiles, lower levels of the amino acids, glycine and alanine and the purine-related metabolites, adenosine, and hypoxanthine (Table S5).

1.11. *Ethnicity-specific alteration in hepatic FFAs profile characterizes NASH*

To examine differences in hepatic metabolomic profile associated with the progression to NASH, we stratified NAFL group into 0-NASH and NASH. Due to the limited sample size and breadth of current analysis, we first examined NASH-dependent changes in each ethnicity by chemical classes. Next, to examine ethnicity-specific metabolomic alterations in NASH, ANCOVA was performed with interaction (ethnicity x NASH) followed by variable clustering for data reduction, given the strong class-specific changes observed, the small sample size and the exploratory nature of this pilot.

Upon examining liver metabolomic profile, ChemRich analysis (Fig. 4) showed that NASH was associated with higher unsaturated FFAs in HIS, driven by heptadecenoic acid (17:1n7); lower unsaturated PCs concurrent with higher levels in unsaturated LPCs; lower levels of unsaturated PEs with an elevation in many ether-linked PLs; lower ACs with higher TGs and DGs. To a lesser impact, HIS-NASH showed alteration in organic compounds, including lower level of several amino acids and related derivatives and in purine nucleosides and adenosine (Table S6). NASH in CAU showed higher SFAs, mainly capric acid (10:0) and lauric acid (12:0); lower levels of many unsaturated FFAs, PE, phosphatidylserines and in organic compounds including trimethyl ammonium compounds and dipeptides (Table S7).

ANCOVA showed 26 liver metabolites, (2.8%) of detected metabolites being altered at a $p < 0.05$ between ethnicities in case of NASH with an additional 33 metabolites showing a *p*-value between 0.05 and <0.1. However, these did not survive FDR-correction. Regardless, we proceeded with the characterization of the composition of these metabolites and their distribution between the groups.

Variable clustering performed on metabolites with $p < 0.05$ for the NASH effect within ethnicity groups and with $p < 0.05$ for the ethnicity x NASH interaction collapsed the data into 32 cluster components. Comparison of cluster components between ethnicities (Fig. 5) suggested that with NASH, clusters 3, 4, 6, 7, 8, 16, 18, 22, 23, 24 were differentially altered with tendencies shown for cluster 2, 9, 13, 14, 26. Cluster descriptions, correlations, means and *p*-values for cluster components and individual metabolites are detailed in Table S8.

NASH appeared associated with differential changes in the hepatic FFA profiles between ethnicities. Among the altered clusters was cluster 18 ($p < 0.05$ for interaction of ethnicity x NASH). This cluster is composed of the MUFAs, heptadecenoic acid (17:1n7), oleic acid (18:1n9) and eicosenoic acid (20:1n9); and the PUFAs, linoleic acid (18:2n6), α-linolenic acid (18:3n3). These FFAs were higher with NASH in HIS with an opposite trend seen in CAU. Similar trends were observed for arachidonic acid (20:4n6) and eicosapentanoic acid (20:5n3) (cluster 14), however, this cluster showed tendency for interaction (ethnicity x NASH). The MUFAs and PUFAs in cluster 14 and 18 correlated with LPC(16:1) and LPC(20:5).

Hepatic LPC and LPE profiles appeared to be differentially altered between ethnicities in NASH with higher levels seen in HIS and lower levels in CAU and interaction shown for LPC(16-20:1-5) (clusters 18, 8, 25) and LPE(18-20:0-4) (cluster 16 and 23), and with tendency shown for LPC(16-22:1-6) (cluster 2, 14). For both ethnicities, NASH had lower levels of almost all hepatic PCs and PEs. This trend differed between ethnicities in HIS for PC(32-37:1-3) and PE(34-36:2-4) (cluster 4), with apparent alterations in some ether-linked PLs (cluster 9 and 23) and SM(d33-36:1-2) (cluster 4).

There also appeared to be a lower level of hepatic ACs in NASH cases. However, a differentially lower ACs with interaction (ethnicity x NASH) was shown in HIS for AC(10-18:0-2) (cluster 22, 6) with tendency for AC(2:0) (cluster 26). In both ethnicities, NASH showed higher hepatic TGs, with differentially higher (p <0.05 in NASH between ethnicities) seen for TG(44-58:0-2) (cluster 2) and tendency shown for TG(48-50:0) (cluster 7, 4) observed in HIS. Some organic compounds of unspecific classification (cluster 3, 13, 14, 8) were also differentially altered in NASH between ethnicities.

Collectively, these findings suggest ethnicity-related differences in hepatic metabolomic profile with the progression to NASH, with markedly increased hepatic FFAs, TGs; lower levels of ACs and changes in several PLs seen in NASH-HIS. A heatmap illustrating variations in plasma and liver FFAs profile with NASH is shown in Fig. 6. Additionally, we performed pathway analysis using hepatic metabolites seen to be different between ethnicity group in NASH (raw p <0.05). These results support our cluster analysis with pathways related to unsaturated fatty acid metabolism found altered (FDR-adjusted (p <0.2), reflecting alteration in linoleic acid (18:2n6), eicosapentaenoic acid (20:5), oleic acid (18:1n9) and several PLs (Fig 7 and Table S9).

1.12. *Ethnicity-specific alteration in plasma metabolites characterizing NASH*

When examining NASH-dependent changes by chemical classes in plasma and in comparison to 0-NASH groups, ChemRich analysis (Fig. 8) showed that NASH-HIS had higher unsaturated TGs and SMs (Table S10) and NASH-CAU had higher Cers and SMs (Table S11).

To examine ethnicity-specific metabolomic alterations in NASH, we performed ANCOVA with interaction (ethnicity x NASH) followed by variable clustering for data reduction. Results showed 18

(1.9%) of detected plasma metabolites appeared different at a p <0.05 in NASH between ethnicities, with 13 additional metabolites showing a p-value between 0.05 and <0.1. These differences did not survive FDR-correction. Cluster analysis reduced data dimensionality into 25 clusters. Comparison of cluster components (Fig. 9) showed that clusters 4, 5, 17, 20, 25 were differentially altered with NASH between ethnicities with tendencies shown for cluster 7, 11, 12, 13, 16. Cluster descriptions, correlations, means and p-values for cluster components and individual metabolites are detailed in Table S12.

With NASH in HIS, the plasma TGs profile was differentially altered (p <0.05 for ethnicity x NASH interaction) with higher levels of TG(50-52:0-6) (cluster 4) and tendency for TG(54-56:1-2) (cluster 7). Compared to respective 0-NASH, plasma levels of CEs, PCs, and PEs were higher in NASH. Ethnicity-specific differences included CE(16:1) (cluster 11), which was higher in CAU; LPE(18:2) (cluster 4) and ether-linked PLs (cluster 5 and 25), which were higher in HIS.

Differences in FFA profiles were not observed in plasma between NASH and 0-NASH. Both ethnicities showed lower plasma AC(10-14:1-2) (cluster 22) with NASH, however, HIS had a differentially lower (p <0.05 for ethnicity x NASH interaction) 3-hydroxybutyric acid (cluster 5). NASH was also associated with specific plasma amino acid profile with interaction (ethnicity x NASH) seen in HIS with higher alanine (cluster 25). Also, differential or with tendency for interaction are alterations in some organic compounds (cluster 4, 7, 12, 13, 15, 16, 17, 20 and 25).

Together, these findings reveal that ethnicity-related differences associated with NASH in plasma mainly affected TGs and some PLs in HIS, with altered organic compounds seen in both ethnicities.

1.13. *Correlations of metabolomic profiles with liver histology*

Correlation analyses were performed on hepatic metabolites with steatosis and lobular inflammation, two histological features relevant to NASH diagnosis. In NASH-CAU, lobular inflammation correlated positively with metabolites including the eicosanoid, 20-hydroxyarachidonic acid; and negatively with MUFA(16-18:1); TG(50:0) (r =-0.6, raw p <0.05). Hepatic steatosis correlated negatively with metabolites including MUFA(20-24:1); arachidonic acid (20:4n6); choline

and S-Adenosyl-L-homocysteine. It correlated positively some SFA(9-12:0), TG(49:0) (r >-0.6, raw p <0.05).

In NASH-HIS, hepatic inflammation correlated negatively with metabolites including AC(5-20:0-4); purine related metabolites, adenosine, inosine, hypoxanthine; SAMe; Cer(d40:1-2), and PC(33-40:1-7). It correlated positively with DG(34-36:1-3); TG(44-60:0-4); the MUFA(17-24:1); PUFA, α-linolenic acid (18:3n3), linoleic acid (18:2n6), eicosadienoic acid (20:2n6); and LPC(18-20:0-5) (r >0.7, raw p <0.05). These metabolites retained significance after FDR-adjustments. Hepatic steatosis correlated positively with TG(48-58:2-8) and negatively with some organic compounds (r >0.7, raw p <0.05) (Table S13). Conversely, hepatic steatosis and lobular inflammation scores correlated with many plasma metabolites, but these did not pass FDR-adjustment (Table S14).

4. Discussion

Hispanics are impacted with higher prevalence and progression rate of NAFLD [6-11]. However, the biological background for this disparity is not clear. To investigate metabolic variations associated with NAFL in these groups, we examined plasma and liver metabolomic profiles with respect to ethnicity in a group of HIS and CAU subjects with obesity and liver biopsy-characterized NAFL. Results revealed several ethnicity-related metabolomic distinctions and the major findings are: 1) In NAFL, as compared to HC, the plasma FFAs and ACs profile are differentially altered between ethnicities; 2) HIS present with signs of metabolic perturbation independent of obesity; 3) In NASH, comparing to BMI and histology-matched NASH-free subjects, the hepatic profile was differentially altered between ethnicities with HIS showing higher abundance FFAs and lipotoxic lipids.

In NAFLD, peripheral insulin resistance contributes to elevated plasma FFAs as the insulin-mediated suppression of adipose tissue lipolysis is impaired [47,48]. In the current study, NAFL groups were obese with features of metabolic syndrome. However, our results show discrepancies in plasma profiles between ethnicities. In NAFL-CAU, there were higher plasma FFAs, as compared to HC. This is consistent with insulin resistance, increased adipose tissue efflux and is in line with previous lipidomic analysis in NAFLD subject [24,29,49]. NAFLD is also characterized by impaired metabolic flexibility and plasma ACs are reported to be altered [25,50]. In NAFL-CAU, we confirmed

an elevation in many ACs species, including short chain AC(2-5:0) and long chain AC(10-14:0-2). AC(3-5) are derived from amino acids, even-chain AC(4-20) reflect incomplete fatty acid β-oxidation and AC(2:0) is the product of complete β-oxidation and may originate from glucose metabolism [51]. Altered plasma ACs profile indicates dysregulations in substrate oxidation across all tissues.

Unexpectedly, elevations in plasma FFAs and in ACs profiles were not marked in NAFL-HIS, which can be explained by the higher ACs and unsaturated fatty acids seen in lean and healthy HIS, as compared to CAU counterparts. We also observed higher plasma TGs in HC-HIS, a feature often associated with insulin resistance and obesity [52]. Hypertriglyceridemia is reported to be more frequent in overweight and obese HIS, compared to other ethnicities [53-56]. However, our findings indicate that this derangement along with a higher FFAs, ACs, altered PLs and amino acids profiles are independent of obesity and suggest early signs of metabolic perturbation in HC-HIS. Given that NAFLD results from a complex interplay between environmental factors, host genetics, intestinal dysbiosis and comorbidities [13], it is possible that such metabolic derangements increase the predisposition of HIS to NAFLD and/or its associated risk factors and are worth further investigation.

Our findings also show lower ether-linked PLs in NAFL-CAU. Although these differences were not evident in NAFL-HIS, a lower abundance of many ether-linked PLs were observed in HC-HIS, compared to HC-CAU. In NAFLD, a reduction in ether-linked PLs correlated with disease severity [29]. Beside a role in maintaining bio membrane fluidity and integrity, ether-linked PLs are thought to ameliorate NAFLD by modulating fatty acid oxidation and an antioxidant capacity [57,58].

With the progression from NAFL to NASH, our findings show differences in hepatic lipidomic profile between ethnicities, despite comparable NAS scores. Remarkably, NASH-HIS subjects had higher levels of hepatic FFAs with ethnicity interaction (p <0.05) shown for the MUFA heptadecenoic acid (17:1n), oleic acid (18:1n9), eicosenoic acid (20:1n9); and in the PUFA, α-linolenic acid (18:3n3), linoleic acid (18:2n6). Results from hepatic pathway analysis corroborate our metabolomic findings and show unsaturated fatty acid metabolism pathways differentially altered with NASH between ethnicities. These ethnicity- related alterations in hepatic FFAs suggest variations in

processes including hepatic uptake of FFAs, DNL, PLs turnover and hepatic unsaturated fatty acid metabolism, which are worth further examination.

Upregulated hepatic FFA clearance may explain the observed elevation of hepatic FFAs in NASH-HIS. In fact, tracer studies in NAFLD show that 60% of hepatic lipid content originates from adipose tissue lipolysis, indicating a major contribution of the liver's uptake to hepatic FFAs profile [59]. In support, in both animals and humans, the expression and membrane localization for fatty acid translocase CD36 are increased in NASH [60].

Hepatic TGs accumulation is thought to be a protective adaptive mechanism from the lipotoxic effect of FFAs [61-63]. However, this notion is questioned as in subjects with early NAFL and no histological evidence of inflammation, the expression of several hepatic inflammatory genes were upregulated [64]. In NASH, DNL was reported to be increased in comparison to NAFL [65]. This process generates SFA(14-16:0), which are converted to MUFA via stearoyl-CoA desaturase (SCD1, or Δ-9 desaturase) and further esterified to form DGs and TGs [66]. In our study, NASH in both ethnicities showed no differences in the estimated hepatic Δ-9 desaturase index (results not shown). NASH also had higher level of TGs, but more profound in HIS with a trend of higher DGs. The higher levels of TGs, DGs and MUFA observed in NAHS-HIS suggest increased DNL. In NASH-HIS, lobular inflammation correlated positively with TG(44-60:0-4) (r >0.7, p <0.05), DG(34-36:1-3) (r >0.7, p <0.000) and the MUFAs, heptadecenoic acid (17:1n7), oleic acid (18:1n9), eicosenoic acid (20:1n9), and nervonic acid (24:1n9) (r >0.8, p <0.00) suggesting an involvement of TGs, DGs, MUFAs with NASH in HIS. Interestingly, in NASH-CAU a negative correlation (r >-0.6, p <0.05) was observed between hepatocellular inflammation and heptadecenoic acid (17:1n7), oleic acid (18:1n9), with tendency shown for palmitoleic acid (16:1n7). While, when tested individually, MUFAs are shown to be less lipotoxic compared than SFAs, it has been shown that treatment of HepG2 cells and human primary hepatocytes with a mixture of five SFAs and MUFAs as observed in NASH, exhibited higher toxicity compared to the mixture reported in normal liver and NAFL [21,24]. Therefore, the effect of MUFAs may be dependent factors including proportion and composition.

Also in our samples, plasma TGs were differentially higher in NASH-HIS, which suggests upregulated hepatic export or impaired peripheral clearance.

An upregulated PL turnover can contribute to elevated unsaturated fatty acids. In our results, the strong correlation found between the altered MUFAs, PUFAs and many lysophospholipid species (Table S8) suggests a shared biochemical pathway. As part of hepatic lipid remodeling, phospholipase A_2 catalyzes the breakdown of membrane PL to form lysophospholipids and release unsaturated fatty acids. In NASH, the transcript level of cytosolic phospholipases A_2 (*cPLA*$_2$) is upregulated [67]. Also, NASH is markedly lower in hepatic PCs content and higher in LPCs [24,28,68]. Our results are in line with the decreased hepatic PCs and PEs profile seen in NASH in both ethnicities, with more marked reductions seen in NAHS-HIS. However, the hepatic LPC and LPE profile showed differential alterations by ethnicity with higher levels observed in HIS and lower levels in CAU. Together, the higher abundance of hepatic PUFA, lower level of PC and higher LPC seen in NASH-HIS support an upregulated hepatic PL turnover. The depletion in PC or alteration in PC to PE ratio disrupts the function and integrity of cellular, mitochondrial and lipid droplets bio-membranes and results in cellular stress, apoptosis and inflammation [69]. *In vitro* evidence shows a lipotoxic effect of LPC by altering mitochondrial function and activating apoptosis [23,70,71]. In HIS-NASH, lobular inflammation correlated positively with LPC(16-20:0-5) and the PUFA, linoleic acid (18:2n6), α-linolenic acid (18:3n3) and eicosadienoic acid (20:2n6); and negatively with PC(32-40:1-7) and PE(34-38:2-6) (r >0.8, p <0.006), which are also in support of a role of upregulated PLs turnover in NASH pathogenesis.

A decrease in hepatic fatty acid desaturase-1(FADS1, Δ-5 desaturase) is reported with NASH [24]. This is thought to induce preferential increase in n6 PUFAs flux at the expense of n3 PUFAs. Accordingly, NAFLD progression is characterized by de-enrichment of long-chain PUFAs with a shift towards a higher n6 to n3 PUFAs ratio seen across multiple lipid classes [27,28]. In the current study, we estimated Δ-5 desaturase and the relative ratio of n6 to n3 PUFAs from hepatic FFAs and results show no differences with NASH for both ethnicities (not shown).

Also, the progression to NASH was associated with reduction in most hepatic ACs in both ethnicities with differentially lower AC(12-18: 0-2) seen in HIS. We also found the hepatic AC(5-18:0-4) correlated negatively with lobular inflammation in NASH-HIS. In plasma, 3-hydroxybutyric acid was found differentially reduced in NASH-HIS. 3-hydroxybutyric (or β-hydroxybutyrate) is a liver-produced ketone body from acetyl-CoA derived from mitochondrial β-oxidation of fatty acid [72]. Together, these findings suggest a role of mitochondrial β-oxidation dysfunction in both ethnicities with NASH.

Alterations in hepatic amino acid, choline, methionine and SAMe profiles were associated with NASH but not different between ethnicities. The dysregulations in hepatic unsaturated fatty acid metabolism associated with NASH progression were not reflected in plasma. This, along with the limited correlations of plasma metabolomic profile with liver histological scores, leads us to conclude plasma profile is not an optimal predictor of hepatic histological differences relevant to NASH.

Our data may be used for hypotheses generation. Diet is a modulator of PUFAs status [73]. In this study, we did not account for the role of diet or the treatment with lipid-lowering drugs to the ethnicity- related differences observed in NAFL and NASH. Since performing a liver biopsy without clinical indication is ethically questionable, we could not confirm that our HC subjects are free from liver pathology. It might have been interesting to genotype our subjects for *PNPLA3* as the G allele is robustly associated with NAFLD and the homozygous rs738409 [G] variant presents with high frequency in HIS [74-76]. Due to sample size, cross-sectional and proof-of-concept nature, our findings need to be confirmed in larger and mechanistic settings. In particular, based on selected metabolites with ethnicity interaction (p <0.05) (Table S15), the minimum sample size required to verify plasma findings in lean healthy subjects is 45 subjects per group. To verify alterations in hepatic lipidomic profile, a minimum of 24 and 12 subjects would be required for each arm in CAU and HIS, respectively.

To our knowledge, this is the first metabolomic profiling addressing ethnicity in NAFLD. Our NAFL patients are biopsy-characterized with comparable BMI, clinical and histological presentations; with almost all plasma and liver samples obtained from the same subjects. Our findings provide

preliminary evidence supporting ethnicity-related variations in NAFLD pathogenesis and highlight signs of metabolic perturbations in HIS independent of obesity and other components of metabolic syndrome. With the progression to NASH, our data suggest ethnicity-related alterations in hepatic unsaturated fatty acids metabolism and points toward potential involvement of lipotoxicity in its mechanisms. We postulate that such alterations predispose HIS to higher rate of NAFLD and/or add a "hit" component when coexisting with metabolic syndrome to act as a driver for the advanced NASH presentation seen in HIS.

5. Funding

This work was supported by The National Institutes of Health (NIH)-West Coast Metabolomic Center (WCMC) grant number U24 DK097154, by R01 DK104770 (to V.M.), by 1R01 HL09133 (to P.H.) and 1R01 HL107256 (to P.H.). Additional support was provided by The United States Department of Agriculture (USDA) project 2032-51530-025-00D (to J.W.N). The USDA is an equal opportunity provider and employer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, the WCMC or the USDA.

This chapter was published in Free Radical Biology and Medicine; Vol number *172*; Mazi, T.A., Borkowski, K., Newman, J.W., Fiehn, O., Bowlus, C.L., Sarkar, S., Matsukuma, K., Ali, M.R., Kieffer, D.A., Wan, Y.J.Y. and Stanhope, K.L.; Ethnicity-specific alterations of plasma and hepatic lipidomic profiles are related to high NAFLD rate and severity in Hispanic Americans, a pilot study; Page No. 490-502; Copyright Elsevier (2021). The use of this manuscript is granted upon permission.

6. References

1. Chalasani, N.; Younossi, Z.; Lavine, J.E.; Diehl, A.M.; Brunt, E.M.; Cusi, K.; Charlton, M.; Sanyal, A.J. The diagnosis and management of non-alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* **2012**, *55*, 2005-2023,
2. Younossi, Z.M.; Koenig, A.B.; 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,
3. Lonardo, A.; Bellentani, S.; Argo, C.K.; Ballestri, S.; Byrne, C.D.; Caldwell, S.H.; Cortez-Pinto, H.; Grieco, A.; Machado, M.V.; Miele, L. Epidemiological modifiers of non-alcoholic fatty liver disease: Focus on high-risk groups. *Digestive and Liver Disease* **2015**, *47*, 997-1006,

4. Lonardo, A.; Ballestri, S.; Marchesini, G.; Angulo, P.; Loria, P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Digestive and Liver Disease* **2015**, *47*, 181-190,
5. Kim, C.H.; Younossi, Z.M. Nonalcoholic fatty liver disease: a manifestation of the metabolic syndrome. *Cleve Clin J Med* **2008**, *75*, 721-728,
6. Browning, J.D.; Szczepaniak, L.S.; Dobbins, R.; Nuremberg, P.; Horton, J.D.; Cohen, J.C.; Grundy, S.M.; Hobbs, H.H. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **2004**, *40*, 1387-1395,
7. Lazo, M.; Hernaez, R.; Eberhardt, M.S.; Bonekamp, S.; Kamel, I.; Guallar, E.; Koteish, A.; Brancati, F.L.; Clark, J.M. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988–1994. *American journal of epidemiology* **2013**, *178*, 38-45,
8. Saab, S.; Manne, V.; Nieto, J.; Schwimmer, J.B.; Chalasani, N.P. Nonalcoholic Fatty Liver Disease in Latinos. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2016**, *14*, 5-12; quiz e19-10, doi:10.1016/j.cgh.2015.05.001
9. Mohanty, S.R.; Troy, T.N.; Huo, D.; O'Brien, B.L.; Jensen, D.M.; Hart, J. Influence of ethnicity on histological differences in non-alcoholic fatty liver disease. *Journal of hepatology* **2009**, *50*, 797-804,
10. Bambha, K.; Belt, P.; Abraham, M.; Wilson, L.A.; Pabst, M.; Ferrell, L.; Unalp-Arida, A.; Bass, N.; Group, N.S.C.R.N.R. Ethnicity and nonalcoholic fatty liver disease. *Hepatology* **2012**, *55*, 769-780,
11. Rich, N.E.; Oji, S.; Mufti, A.R.; Browning, J.D.; Parikh, N.D.; Odewole, M.; Mayo, H.; Singal, A.G. Racial and Ethnic Disparities in Nonalcoholic Fatty Liver Disease Prevalence, Severity, and Outcomes in the United States: A Systematic Review and Meta-analysis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2018**, *16*, 198-210.e192, doi:10.1016/j.cgh.2017.09.041
12. Friedman, S.L.; Neuschwander-Tetri, B.A.; Rinella, M.; Sanyal, A.J. Mechanisms of NAFLD development and therapeutic strategies. *Nature medicine* **2018**, *24*, 908-922,
13. Yu, J.; Marsh, S.; Hu, J.; Feng, W.; Wu, C. *The Pathogenesis of Nonalcoholic Fatty Liver Disease: Interplay between Diet, Gut Microbiota, and Genetic Background*; 2016; Volume 2016, p. 13.
14. Dasarathy, S.; Yang, Y.; McCullough, A.J.; Marczewski, S.; Bennet, C.; Kalhan, S.C. Elevated hepatic fatty acid oxidation, high plasma fibroblast growth factor 21, and fasting bile acids in nonalcoholic steatohepatitis. *European journal of gastroenterology & hepatology* **2011**, *23*, 382,
15. Sanyal, A.J.; Campbell–Sargent, C.; Mirshahi, F.; Rizzo, W.B.; Contos, M.J.; Sterling, R.K.; Luketic, V.A.; Shiffman, M.L.; Clore, J.N. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* **2001**, *120*, 1183-1192,
16. Croci, I.; Byrne, N.M.; Choquette, S.; Hills, A.P.; Chachay, V.S.; Clouston, A.D.; O'Moore-Sullivan, T.M.; Macdonald, G.A.; Prins, J.B.; Hickman, I.J. Whole-body substrate metabolism is associated with disease severity in patients with non-alcoholic fatty liver disease. *Gut* **2013**, *62*, 1625-1633,
17. Sunny, N.E.; Parks, E.J.; Browning, J.D.; Burgess, S.C. Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell metabolism* **2011**, *14*, 804-810,
18. Koliaki, C.; Szendroedi, J.; Kaul, K.; Jelenik, T.; Nowotny, P.; Jankowiak, F.; Herder, C.; Carstensen, M.; Krausch, M.; Knoefel, W.T. Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell metabolism* **2015**, *21*, 739-746,

19. Pérez-Carreras, M.; Del Hoyo, P.; Martín, M.A.; Rubio, J.C.; Martín, A.; Castellano, G.; Colina, F.; Arenas, J.; Solis-Herruzo, J.A. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* **2003**, *38*, 999-1007,
20. Alkhouri, N.; Dixon, L.J.; Feldstein, A.E. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. *Expert review of gastroenterology & hepatology* **2009**, *3*, 445-451,
21. Malhi, H.; Bronk, S.F.; Werneburg, N.W.; Gores, G.J. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *Journal of Biological Chemistry* **2006**, *281*, 12093-12101,
22. Mauer, A.S.; Hirsova, P.; Maiers, J.L.; Shah, V.H.; Malhi, H. Inhibition of sphingosine 1-phosphate signaling ameliorates murine nonalcoholic steatohepatitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2017**, *312*, G300-G313,
23. Han, M.S.; Park, S.Y.; Shinzawa, K.; Kim, S.; Chung, K.W.; Lee, J.-H.; Kwon, C.H.; Lee, K.-W.; Lee, J.-H.; Park, C.K. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. *Journal of lipid research* **2008**, *49*, 84-97,
24. Chiappini, F.; Coilly, A.; Kadar, H.; Gual, P.; Tran, A.; Desterke, C.; Samuel, D.; Duclos-Vallée, J.-C.; Touboul, D.; Bertrand-Michel, J. Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. *Scientific reports* **2017**, *7*, 46658,
25. Kalhan, S.C.; Guo, L.; Edmison, J.; Dasarathy, S.; McCullough, A.J.; Hanson, R.W.; Milburn, M. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism-Clinical and Experimental* **2011**, *60*, 404-413,
26. García-Cañaveras, J.C.; Donato, M.T.; Castell, J.V.; Lahoz, A. A Comprehensive Untargeted Metabonomic Analysis of Human Steatotic Liver Tissue by RP and HILIC Chromatography Coupled to Mass Spectrometry Reveals Important Metabolic Alterations. *Journal of Proteome Research* **2011**, *10*, 4825-4834, doi:10.1021/pr200629p
27. Araya, J.; Rodrigo, R.; Videla, L.A.; Thielemann, L.; Orellana, M.; Pettinelli, P.; Poniachik, J. Increase in long-chain polyunsaturated fatty acid n− 6/n− 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clinical science* **2004**, *106*, 635-643, doi:10.1042/CS20030326
28. Puri, P.; Baillie, R.A.; Wiest, M.M.; Mirshahi, F.; Choudhury, J.; Cheung, O.; Sargeant, C.; Contos, M.J.; Sanyal, A.J. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **2007**, *46*, 1081-1090,
29. Puri, P.; Wiest, M.M.; Cheung, O.; Mirshahi, F.; Sargeant, C.; Min, H.K.; Contos, M.J.; Sterling, R.K.; Fuchs, M.; Zhou, H. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **2009**, *50*, 1827-1838,
30. da Silva, R.P.; Eudy, B.J.; Deminice, R. One-Carbon Metabolism in Fatty Liver Disease and Fibrosis: One-Carbon to Rule Them All. *The Journal of nutrition* **2020**, *150*, 994-1003,
31. Alonso, C.; Fernández-Ramos, D.; Varela-Rey, M.; Martínez-Arranz, I.; Navasa, N.; Van Liempd, S.M.; Trueba, J.L.L.; Mayo, R.; Ilisso, C.P.; de Juan, V.G. Metabolomic identification of subtypes of nonalcoholic steatohepatitis. *Gastroenterology* **2017**, *152*, 1449-1461. e1447, doi:10.1053/j.gastro.2017.01.015
32. Kleiner, D.E.; Brunt, E.M.; Van Natta, M.; Behling, C.; Contos, M.J.; Cummings, O.W.; Ferrell, L.D.; Liu, Y.C.; Torbenson, M.S.; Unalp-Arida, A.; et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **2005**, *41*, 1313-1321, doi:10.1002/hep.20701

33. Bedossa, P.; Poitou, C.; Veyrie, N.; Bouillot, J.L.; Basdevant, A.; Paradis, V.; Tordjman, J.; Clement, K. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* **2012**, *56*, 1751-1759,
34. Matyash, V.; Liebisch, G.; Kurzchalia, T.V.; Shevchenko, A.; Schwudke, D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *Journal of lipid research* **2008**, *49*, 1137-1146,
35. Fiehn, O. Metabolomics by gas chromatography–mass spectrometry: Combined targeted and untargeted profiling. *Current protocols in molecular biology* **2016**, *114*, 30.34. 31-30.34. 32,
36. Skogerson, K.; Wohlgemuth, G.; Barupal, D.K.; Fiehn, O. The volatile compound BinBase mass spectral database. *BMC bioinformatics* **2011**, *12*, 321,
37. Funk, R.S.; Singh, R.K.; Becker, M.L. Metabolomic Profiling to Identify Molecular Biomarkers of Cellular Response to Methotrexate In Vitro. *Clinical and translational science* **2020**, *13*, 137-146,
38. Kind, T.; Liu, K.-H.; Lee, D.Y.; DeFelice, B.; Meissen, J.K.; Fiehn, O. LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nature methods* **2013**, *10*, 755,
39. Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; VanderGheynst, J.; Fiehn, O.; Arita, M. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nat Methods* **2015**, *12*, 523-526, doi:10.1038/nmeth.3393
40. Schymanski, E.L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H.P.; Hollender, J. Identifying small molecules via high resolution mass spectrometry: communicating confidence. **2014**.
41. Benjamini, Y.; Yekutieli, D. Quantitative trait loci analysis using the false discovery rate. *Genetics* **2005**, *171*, 783-789, doi:10.1534/genetics.104.036699
42. Westerhuis, J.A.; Hoefsloot, H.C.; Smit, S.; Vis, D.J.; Smilde, A.K.; van Velzen, E.J.; van Duijnhoven, J.P.; van Dorsten, F.A. Assessment of PLSDA cross validation. *Metabolomics* **2008**, *4*, 81-89,
43. Chong, I.-G.; Jun, C.-H. Performance of some variable selection methods when multicollinearity is present. *Chemometrics and intelligent laboratory systems* **2005**, *78*, 103-112,
44. Barupal, D.K.; Fiehn, O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. *Scientific reports* **2017**, *7*, 1-11,
45. Xia, J.; Wishart, D.S. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current protocols in bioinformatics* **2016**, *55*, 14.10. 11-14.10. 91,
46. Kanehisa, M.; Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research* **2000**, *28*, 27-30,
47. Kotronen, A.; Juurinen, L.; Tiikkainen, M.; Vehkavaara, S.; Yki–Järvinen, H. Increased liver fat, impaired insulin clearance, and hepatic and adipose tissue insulin resistance in type 2 diabetes. *Gastroenterology* **2008**, *135*, 122-130,
48. Seppälä-Lindroos, A.; Vehkavaara, S.; Häkkinen, A.-M.; Goto, T.; Westerbacka, J.; Sovijärvi, A.; Halavaara, J.; Yki-Järvinen, H. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *The Journal of Clinical Endocrinology & Metabolism* **2002**, *87*, 3023-3028,
49. Zhou, Y.; Oresic, M.; Leivonen, M.; Gopalacharyulu, P.; Hyysalo, J.; Arola, J.; Verrijken, A.; Francque, S.; Van Gaal, L.; Hyotylainen, T.; et al. Noninvasive Detection of Nonalcoholic Steatohepatitis Using Clinical Markers and Circulating Levels of Lipids and Metabolites. *Clin Gastroenterol Hepatol* **2016**, *14*, 1463-1472 e1466, doi:10.1016/j.cgh.2016.05.046

50. Brouwers, B.; Schrauwen-Hinderling, V.B.; Jelenik, T.; Gemmink, A.; Havekes, B.; Bruls, Y.; Dahlmans, D.; Roden, M.; Hesselink, M.K.; Schrauwen, P. Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes. *Clinical Science* **2017**, *131*, 1905-1917,
51. Koves, T.R.; Ussher, J.R.; Noland, R.C.; Slentz, D.; Mosedale, M.; Ilkayeva, O.; Bain, J.; Stevens, R.; Dyck, J.R.; Newgard, C.B. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism* **2008**, *7*, 45-56,
52. Beshara, A.; Cohen, E.; Goldberg, E.; Lilos, P.; Garty, M.; Krause, I. Triglyceride levels and risk of type 2 diabetes mellitus: a longitudinal large study. *Journal of Investigative Medicine* **2016**, *64*, 383-387,
53. Mitchell, B.D.; Stern, M.P.; Haffner, S.M.; Hazuda, H.P.; Patterson, J.K. Risk factors for cardiovascular mortality in Mexican Americans and non-Hispanic whites: the San Antonio Heart Study. *Am. J. Epidemiol.* **1990**, *131*, 423-433,
54. Aguilar-Salinas, C.A.; Tusie-Luna, T.; Pajukanta, P. Genetic and environmental determinants of the susceptibility of Amerindian derived populations for having hypertriglyceridemia. *Metabolism: clinical and experimental* **2014**, *63*, 887-894,
55. Christian, J.B.; Bourgeois, N.; Snipes, R.; Lowe, K.A. Prevalence of severe (500 to 2,000 mg/dl) hypertriglyceridemia in United States adults. *The American journal of cardiology* **2011**, *107*, 891-897,
56. Fan, W.; Philip, S.; Granowitz, C.; Toth, P.P.; Wong, N.D. Residual Hypertriglyceridemia and Estimated Atherosclerotic Cardiovascular Disease Risk by Statin Use in US Adults With Diabetes: National Health and Nutrition Examination Survey 2007–2014. *Diabetes Care* **2019**, *42*, 2307-2314,
57. Dean, J.M.; Lodhi, I.J. Structural and functional roles of ether lipids. *Protein & Cell* **2018**, *9*, 196-206,
58. Jang, J.E.; Park, H.S.; Yoo, H.J.; Baek, I.J.; Yoon, J.E.; Ko, M.S.; Kim, A.R.; Kim, H.S.; Park, H.S.; Lee, S.E. Protective role of endogenous plasmalogens against hepatic steatosis and steatohepatitis in mice. *Hepatology* **2017**, *66*, 416-431,
59. Donnelly, K.L.; Smith, C.I.; Schwarzenberg, S.J.; Jessurun, J.; Boldt, M.D.; Parks, E.J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *The Journal of clinical investigation* **2005**, *115*, 1343-1351,
60. Zhao, L.; Zhang, C.; Luo, X.; Wang, P.; Zhou, W.; Zhong, S.; Xie, Y.; Jiang, Y.; Yang, P.; Tang, R. CD36 palmitoylation disrupts free fatty acid metabolism and promotes tissue inflammation in non-alcoholic steatohepatitis. *Journal of hepatology* **2018**, *69*, 705-717,
61. Koliwad, S.K.; Streeper, R.S.; Monetti, M.; Cornelissen, I.; Chan, L.; Terayama, K.; Naylor, S.; Rao, M.; Hubbard, B.; Farese, R.V. DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *The Journal of clinical investigation* **2010**, *120*, 756-767,
62. Yamaguchi, K.; Yang, L.; McCall, S.; Huang, J.; Yu, X.X.; Pandey, S.K.; Bhanot, S.; Monia, B.P.; Li, Y.X.; Diehl, A.M. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* **2007**, *45*, 1366-1374,
63. Gastaldelli, A. Insulin resistance and reduced metabolic flexibility: cause or consequence of NAFLD? *Clinical Science* **2017**, *131*, 2701-2704,
64. Chiappini, F.; Barrier, A.; Saffroy, R.; Domart, M.-C.; Dagues, N.; Azoulay, D.; Sebagh, M.; Franc, B.; Chevalier, S.; Debuire, B. Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Laboratory investigation* **2006**, *86*, 154-165,

65. Lawitz, E.J.; Coste, A.; Poordad, F.; Alkhouri, N.; Loo, N.; McColgan, B.J.; Tarrant, J.M.; Nguyen, T.; Han, L.; Chung, C. Acetyl-CoA carboxylase inhibitor GS-0976 for 12 weeks reduces hepatic de novo lipogenesis and steatosis in patients with nonalcoholic steatohepatitis. *Clinical Gastroenterology and Hepatology* **2018**, *16*, 1983-1991. e1983,
66. Liu, X.; Strable, M.S.; Ntambi, J.M. Stearoyl CoA desaturase 1: role in cellular inflammation and stress. *Advances in nutrition* **2011**, *2*, 15-22,
67. Hall, Z.; Bond, N.J.; Ashmore, T.; Sanders, F.; Ament, Z.; Wang, X.; Murray, A.J.; Bellafante, E.; Virtue, S.; Vidal-Puig, A. Lipid zonation and phospholipid remodeling in nonalcoholic fatty liver disease. *Hepatology* **2017**, *65*, 1165-1180,
68. Chiappini, F.; Desterke, C.; Bertrand-Michel, J.; Guettier, C.; Le Naour, F. Hepatic and serum lipid signatures specific to nonalcoholic steatohepatitis in murine models. *Scientific reports* **2016**, *6*, 31587,
69. van der Veen, J.N.; Kennelly, J.P.; Wan, S.; Vance, J.E.; Vance, D.E.; Jacobs, R.L. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. **2017**.
70. Kakisaka, K.; Cazanave, S.C.; Fingas, C.D.; Guicciardi, M.E.; Bronk, S.F.; Werneburg, N.W.; Mott, J.L.; Gores, G.J. Mechanisms of lysophosphatidylcholine-induced hepatocyte lipoapoptosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2012**, *302*, G77-G84,
71. Hollie, N.I.; Cash, J.G.; Matlib, M.A.; Wortman, M.; Basford, J.E.; Abplanalp, W.; Hui, D.Y. Micromolar changes in lysophosphatidylcholine concentration cause minor effects on mitochondrial permeability but major alterations in function. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2014**, *1841*, 888-895,
72. Newman, J.C.; Verdin, E. Ketone bodies as signaling metabolites. *Trends in Endocrinology & Metabolism* **2014**, *25*, 42-52,
73. Wood, K.E.; Mantzioris, E.; Gibson, R.A.; Ramsden, C.; Muhlhausler, B. The effect of modifying dietary LA and ALA intakes on omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) status in human adults: a systematic review and commentary. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **2015**, *95*, 47-55,
74. Rotman, Y.; Koh, C.; Zmuda, J.M.; Kleiner, D.E.; Liang, T.J.; CRN, N. The association of genetic variability in patatin-like phospholipase domain-containing protein 3 (PNPLA3) with histological severity of nonalcoholic fatty liver disease. *Hepatology* **2010**, *52*, 894-903,
75. Wagenknecht, L.E.; Palmer, N.D.; Bowden, D.W.; Rotter, J.I.; Norris, J.M.; Ziegler, J.; Chen, Y.D.I.; Haffner, S.; Scherzinger, A.; Langefeld, C.D. Association of PNPLA3 with non-alcoholic fatty liver disease in a minority cohort: the Insulin Resistance Atherosclerosis Family Study. *Liver International* **2011**, *31*, 412-416,
76. Palmer, N.D.; Musani, S.K.; Yerges-Armstrong, L.M.; Feitosa, M.F.; Bielak, L.F.; Hernaez, R.; Kahali, B.; Carr, J.J.; Harris, T.B.; Jhun, M.A. Characterization of European ancestry nonalcoholic fatty liver disease-associated variants in individuals of African and Hispanic descent. *Hepatology* **2013**, *58*, 966-975,

7. Tables:

Table 1. Demographic, clinical, and histological characteristics of study subjects.

	Hispanics		Caucasians		*p*-value		
	NAFL	HC	NAFL	HC	a	b	c
Liver, n (F/M)	7 (5/2)	-	10 (5/5)	-			
Plasma, n (F/M)	7 (5/2)	14 (6/8)	8 (4/4)	8 (4/4)			
Age (Yrs.)	45 ± 16	43 ± 14	50 ± 16	44 ± 12	0.818	0.413	0.55
BMI (Kg/m^2)	47 ± 7	26 ± 2	42 ± 7	25 ± 3	<.0001	<.0001	0.28
DM, yes (%)	3 (42.9)	-	4 (40)	-	-	-	0.913
FBG (mmol/L)	93 ± 5	-	94 ± 13	-	-	-	0.88
Cholesterol(mg/dL)	168 ± 29	-	173 ± 33	-	-	-	0.952
TG (mg/dL)	112 ± 37	-	139 ± 65	-	-	-	0.42
HDL (mg/dL)	44 ± 7	-	41 ± 8	-	-	-	0.403
LDL (mg/dL)	102 ± 23	-	104 ± 33	-	-	-	0.855
HbA1c (%)	6.1 ± 0.7	-	6.2 ± 0.8	-	-	-	0.67
AST (U/L)	27 ± 12	-	31 ± 19	-	-	-	0.499
ALT (U/L)	34 ± 18	-	31 ± 10	-	-	-	0.752
Platelet	308 ± 58	-	292 ± 100	-	-	-	0.506
NAS	2.43 ± 2.40	-	3 ± 1.5	-	-	-	0.653
NASH, yes (%)	6 (60)	-	5 (50)	-	-	-	0.708
Steatosis (%)							
< 5%	3 (42.8)	-	2 (20)	-	-	-	0.452
5 to ≤ 33%	2 (28.6)	-	5 (50)	-	-	-	
34 to ≤ 66 %	2 (28.6)	-	2 (20)	-	-	-	
> 66%	0 (0)	-	1 (10)	-	-	-	
Inflammation (%)							
None	3 (42.9)	-	1 (10)	-	-	-	0.763
Mild	1 (14.3)	-	6 (60)	-	-	-	
Moderate	2 (28.6)	-	3 (30)	-	-	-	
Severe	1 (14.3)	-	0 (0)	-	-	-	
Ballooning (%)							
None	4 (57)	-	5 (50)	-	-	-	0.772
Few	3 (42.9)	-	5 (50)	-	-	-	
Many	0 (0)	-	0 (0)	-	-	-	
Fibrosis (%)							
None	7 (100)	-	9 (90)	-	-	-	0.998
1A	0 (0)	-	1 (10)	-	-	-	
2	0 (0)	-	0 (0)	-	-	-	
4	0 (0)	-	0 (0)	-	-	-	

General characteristics are shown as percent (for categorical data) and mean ± SEM (for nominal data). Group comparisons were performed by chi-square test (categorical) or t- test (nominal). (a) NAFL-HIS vs. HC-HIS; (b) NAFL-CAU vs. HC-CAU; (c) NAFL-HIS vs. NAFL-CAU. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low density lipoprotein; NAS, the NAFLD Activity Score; TG, triglycerides.

Table 2. Demographic, clinical, and histological characteristics of subjects with NASH.

	Hispanics		Caucasians		*p*-value	
	0 NASH	NASH	0 NASH	NASH	a	b
Liver, n (F/M)	4(3/1)	3(2/1)	5(3/2)	5(2/3)		
Plasma, n (F/M)	4(3/1)	3(2/1)	5(3/2)	3(1/2)		
Age (Yrs.)	45 ± 21	46 ± 10	54 ± 19	45 ± 14	0.872	0.405
BMI (Kg/m^2)	47.4 ± 7.9	45.7 ± 6.7	39.9 ± 4.4	44.4 ± 3.8	0.787	0.406
FBG (mmol/L)	93 ± 5	94 ± 4	98 ± 15	90 ± 10	0.873	0.379
TG (mg/dL)	105 ± 39	121 ± 40	120 ± 80	157 ± 50	0.59	0.35
AST (U/L)	20 ± 3	36 ± 14	21 ± 3	40 ± 23	0.064	0.0276
ALT (U/L)	23 ± 9	48 ± 18	25 ± 2.2	38 ± 11	0.0431	0.0246
Steatosis	12 ± 19	22.7 ± 9.3	15.6 ± 15.5	32.2 ± 21.8	0.197	0.1474
NAS	0.75 ± 1.1	4.7 ± 0.6	1.8 ± 1	4 ± 1	0.004	0.017
Inflammation (%)						
None	3(75)	0(0)	1(20)	0(0)	0.998	0.323
Mild	1(25)	0(0)	3(60)	3(60)		
Moderate	0(0)	2(66.7)	1(20)	2(40)		
Severe	0(0)	1(33.3)	0(0)	0(0)		
Ballooning (%)						
None	4(100)	0(0)	5(100)	0(0)	<0.0001	<0.0001
Few	0(0)	3(100)	0(0)	5(100)		
Many	0(0)	0(0)	0(0)	0(0)		
Fibrosis (%)						
None	4(100)	3(100)	5(100)	4(80)	1	0.998
1A	0(0)	0(0)	0(0)	1(20)		
2	0(0)	0(0)	0(0)	0(0)		
3	0(0)	0(0)	0(0)	0(0)		
4	0(0)	0(0)	0(0)	0(0)		

General characteristics are shown as percent (for categorical data) and mean ± SEM (for nominal data). Comparison was performed by *t*- test (nominal) or chi-square test (categorical). (a) NASH-HIS vs. 0-NASH-HIS; (b) NASH-CAU vs. 0-NASH-CAU. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBG, fasting blood glucose; NAS, the NAFLD Activity Score; NASH, Nonalcoholic steatohepatitis; 0-NASH, Nonalcoholic steatohepatitis-free; TG, triglycerides.

8. Figures

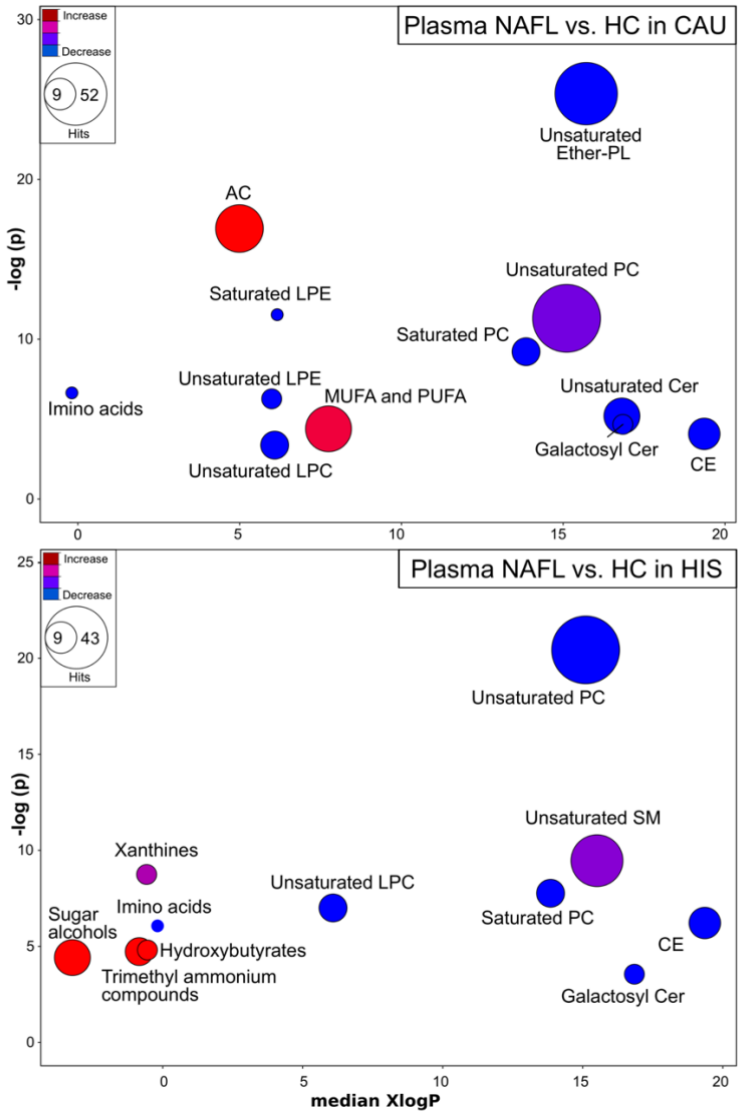

Figure 1. Plasma metabolites altered by chemical class in NAFL, compared to HC in both ethnicities. Chemical similarity enrichment analysis (ChemRICH) and enrichment statistics plot for NAFL vs. HC in CAU (top panel) and HIS (bottom panel). Each cluster represents altered chemical class of metabolites (p <0.05). Cluster sizes represent the total number of metabolites. Cluster's color represents the directionality of metabolite differences: red – higher in NAFL; blue – lower in NAFL. Colors in between refer to mixed population of metabolites manifesting both higher and lower levels in NAFL when compared to the control. The x-axis represents the cluster order on the chemical similarity tree. The plot y-axis shows chemical enrichment p-values calculated using Kolmogorov–Smirnov test. Only clusters with p <0.05 are shown. FDR-adjustment q =0.2, and clusters with FDR-adjusted p ≥0.2 are shown in gray. (n, NAFL-HIS =7, HC-HIS =14; NAFL-CAU =8, HC-CAU =8). The detailed ChemRICH results are shown in (Table S1 and S2). AC, Acylcarnitines; CAU, White Caucasian; Cer, Ceramides; CE, Cholesteryl ester; DG, Diglycerides; Ether-PL, Ether-linked phospholipids; HIS, Hispanic; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamines; MUFA, Monounsaturated fatty acid; PC, Phosphatidylcholines; PUFA, Polyunsaturated fatty acids; SFA, Saturated fatty acids; SM, Sphingomyelins; NAFL, Steatosis; TG, Triglycerides.

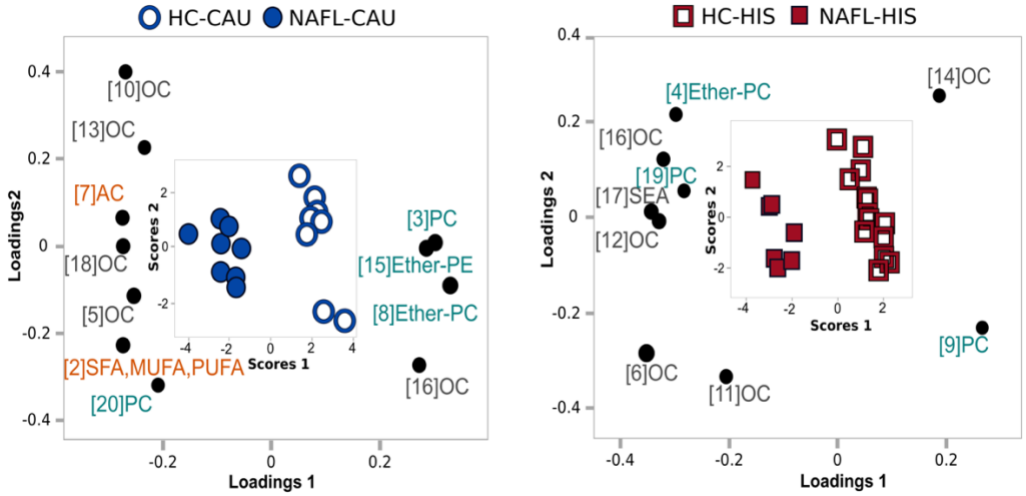

Figure 2. Supervised clustering model illustrating potential ethnicity-specific variations in plasma observed between NAFL vs. HC in both ethnicities. Metabolites with p <0.1 for interaction (ethnicity x NAFL) from ANCOVA were clustered and cluster components were subjected to partial least square-discriminant analysis (PLS-DA) separately in each ethnicity. Only clusters of variable importance in projection (VIP)>1 are illustrated. A combined loading and score plot for a) NAFL-CAU vs. HC-CAU; b) NAFL-HIS vs. HC-HIS. The model was validated with leave-one-out cross validation (LOOCV). The (Q^2), (R^2X) and (R^2Y) is 0.817, 0.285 and 0.918; 0.816, 0.204 and 0.911 for CAU and HIS, respectively. The details on metabolites and clusters components are shown in (Table S3). AC, Acylcarnitines; CAU, White Caucasian; Ether-PC, Ether-linked phosphatidylcholines; Ether-PE, Ether-linked phosphatidylethanolamines; HC, Healthy control; HIS, Hispanic; MUFA, Monounsaturated fatty acid; OC, Organic compounds; PC, Phosphatidylcholines; PUFA, Polyunsaturated fatty acids; NAFL, Steatosis; SEA, Stearoyl ethanolamine; SFA, Saturated fatty acids.

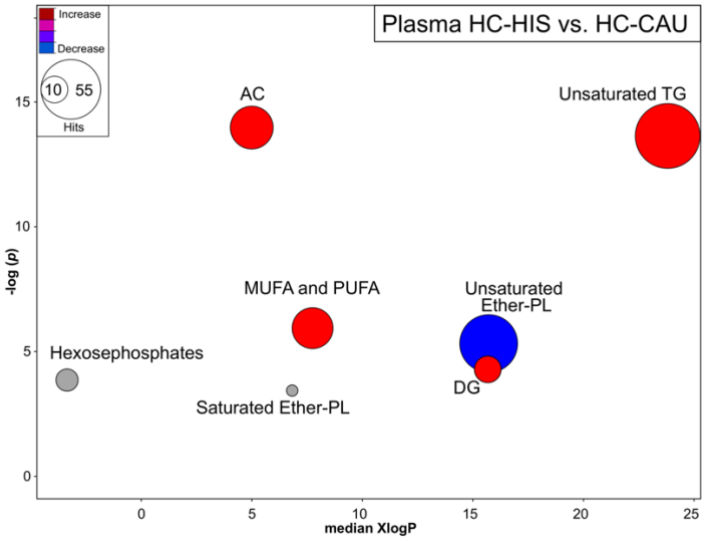

Figure 3. Plasma metabolites altered by chemical class in lean healthy control subjects between ethnicities. Chemical similarity enrichment analysis (ChemRICH) highlighting plasma metabolomic changes observed in HC-HIS, compared to HC-CAU. Each cluster represents altered chemical class of metabolites (p <0.05). Cluster sizes represent the total number of metabolites. Cluster color represents the directionality of metabolite differences: red – higher in HC-HIS; blue – lower in HC-HIS. Colors in between refer to mixed population of metabolites manifesting both higher and lower levels in HC-HIS when compared to the HC-CAU. The x-axis represents the cluster order on the chemical similarity tree. The plot y-axis shows chemical enrichment p-values calculated using Kolmogorov–Smirnov test. Only clusters with p <0.05 are shown. FDR-adjustment q =0.2, and clusters with FDR-adjusted p ≥0.2 are shown in gray. (n, HC-HIS =14, HC-CAU =8) The detailed ChemRICH results are shown in (Table S4). AC, Acylcarnitines; CAU, White Caucasian; DG, Diglycerides; Ether-PC, Ether-linked phospholipids; HIS, Hispanic; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acids; TG, Triglycerides.

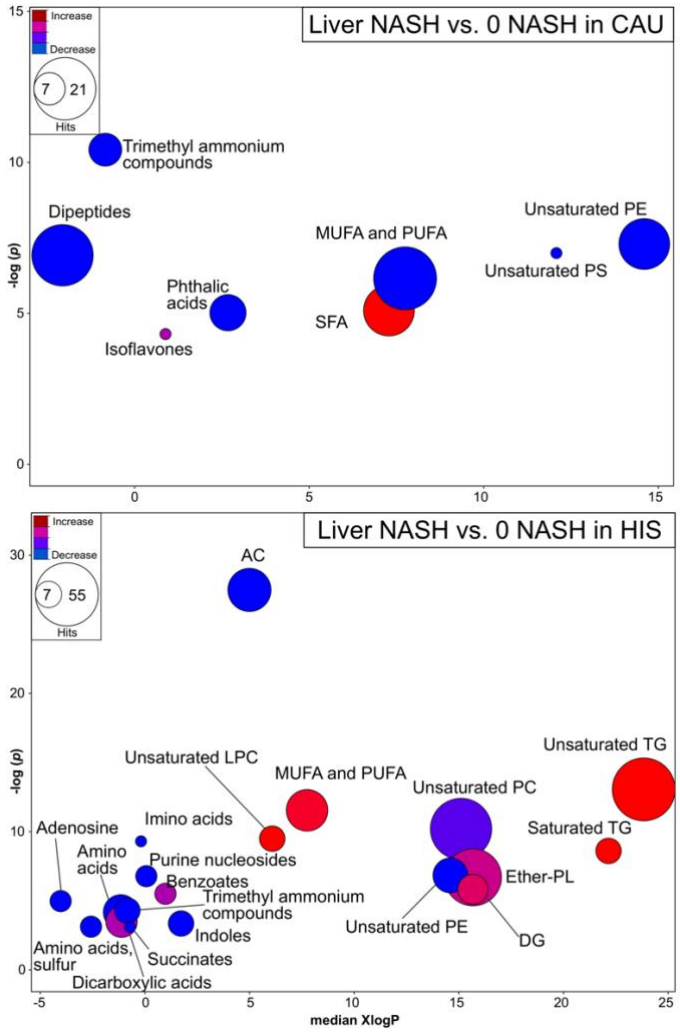

Figure 4. Liver metabolites altered by chemical class in NASH, compared to NASH -free subjects, in both ethnicities. Chemical similarity enrichment analysis (ChemRICH) illustrating hepatic metabolite class altered in NASH compared to 0-NASH in both ethnicities. ChemRICH enrichment statistics plot, illustrating liver alterations in NASH vs. 0-NASH in CAU (top panel) and HIS (bottom panel). Each cluster represents altered chemical class of metabolites (p <0.05). Cluster sizes represent the total number of metabolites. Cluster's color represents the directionality of metabolite differences: red – higher in NASH; blue – lower in NASH. Colors in between refer to mixed population of metabolites manifesting both higher and lower levels in NASH when compared to the 0-NASH. The x-axis represents the cluster order on the chemical similarity tree. The plot y-axis shows chemical enrichment p-values calculated using Kolmogorov–Smirnov test. Only clusters with p <0.05 are shown. FDR-adjustment q =0.2, and clusters with FDR-adjusted p ≥0.2 are shown in gray. (n, 0 NASH-HIS =4, NASH-HIS =3; 0 NASH-CAU =5, NASH-CAU =5). The detailed ChemRICH results are shown in (Table S6 and S7). AC, Acylcarnitines; CAU, White Caucasian; Cer, Ceramides; CE, Cholesteryl ester; DG, Diglycerides; Ether-PL, Ether-linked phospholipids; HIS, Hispanic; LPC, Lysophosphatidylethanolamines; MUFA, Monounsaturated fatty acid; NASH, Nonalcoholic steatohepatitis; 0-NASH, Nonalcoholic steatohepatitis-free; PC, Phosphatidylecholine; PE, Phosphatidylethanolamine; PUFA, Polyunsaturated fatty acids; SFA, Saturated fatty acids; TG, Triglycerides.

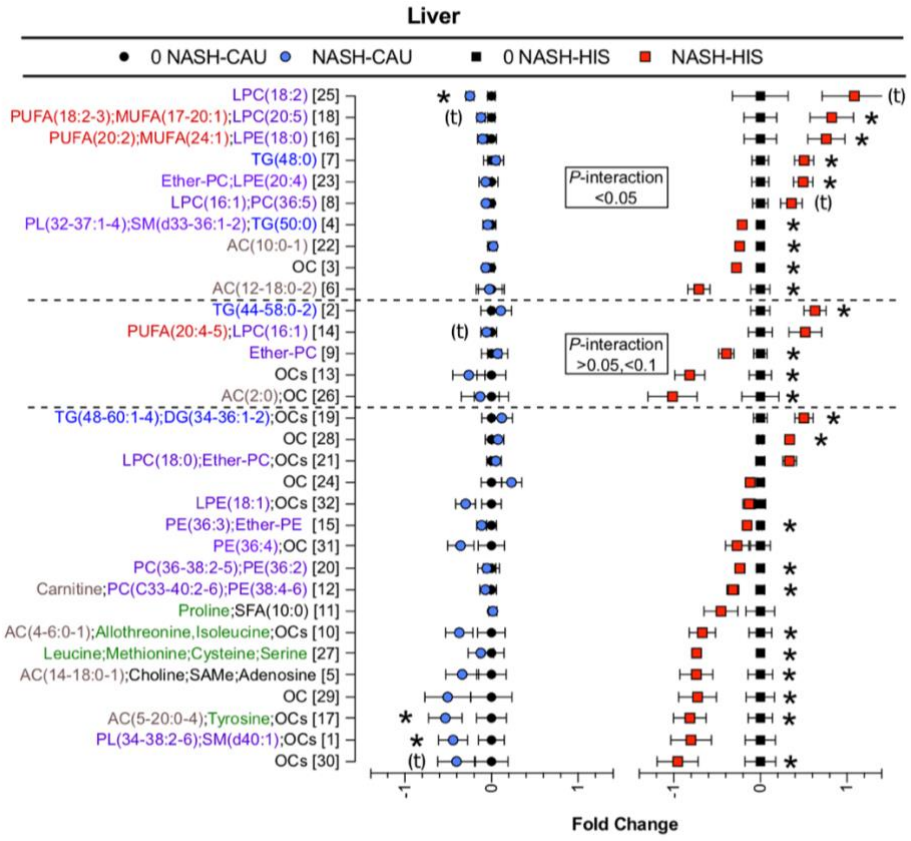

Figure 5. Ethnicity-related variations in liver metabolomic profile in NASH vs. 0-NASH. Results of *t*-test and ANCOVA (ethnicity x NASH) interaction comparison performed on plasma variable cluster components between NASH and 0-NASH in both ethnicities. Clustering was performed on metabolites that were different (p <0.05) within groups (NASH vs. 0-NASH) or between ethnicity groups (interaction (NASH x ethnicity)). Data are presented as the fold change from 0-NASH; error bars represent standard error. Metabolite clusters are ranked in order of p-value for (interaction (NASH x ethnicity) and labeled by a number and a description of representative metabolite. For complex lipids, lipid class is followed by number of (carbons, double bounds) of the fatty acyl moiety. Clusters with interaction (p <0.05) or tendency for interaction (p =0.05 to < 0.1) are marked with the dashed line. Clusters showing ethnicity-related differences in NASH (p <0.05) or tendency for interaction (p=0.05 to <0.1) are marked with the dashed line. Clusters affected by NASH within ethnicity (p <0.05) are denoted with (*); clusters with tendency (p=0.05 to <0.1) are denoted with (*t*). (*n*, 0 NASH-HIS =4, NASH-HIS =3; 0 NASH-CAU =5, NASH-CAU =5). The details on metabolites and clusters components are shown in (Table S8). AC, Acylcarnitines; CAU, White Caucasian; DG, diglycerides; Ether-PC, Ether-linked phosphatidylcholines; Ether-PE, Ether-linked phosphatidylethanolamines; HIS, Hispanic; MUFA, Monounsaturated fatty acid; NASH, Nonalcoholic steatohepatitis; 0-NASH, Nonalcoholic steatohepatitis-free; OC, Organic compounds; PC, Phosphatidylcholines; PE, Phosphatidylethanolamine; PL, Phospholipids; PUFA, Polyunsaturated fatty acids; NAFL, Steatosis; SAMe, S-Adenosyl-L-methionine; SFA, Saturated fatty acids; SM, sphingomyelins; TG, triglycerides.

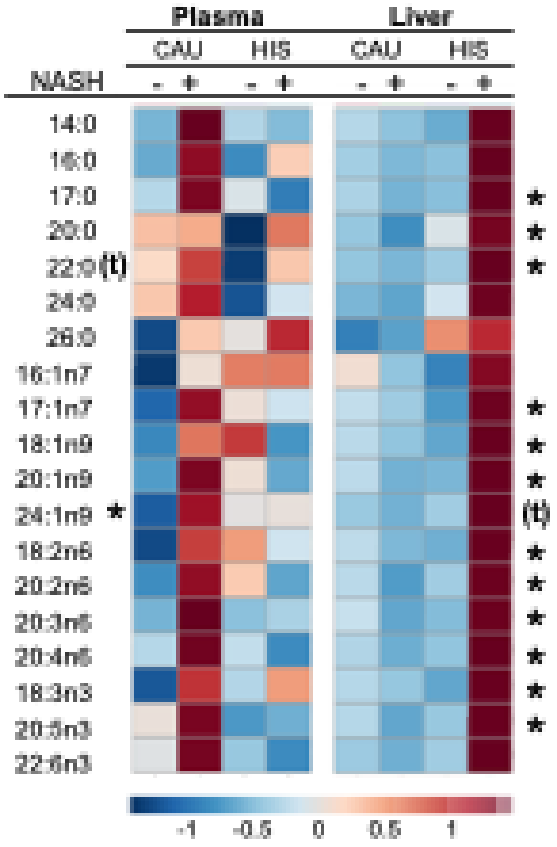

Figure 6. Heat-map for plasma and liver free fatty acids with fold change and *p*-values in NASH vs. 0 NASH in both ethnicities. Group means are shown with normalized data converted to z-scores. Fatty acids showing ethnicity-related differences (p <0.05 on interaction NASH x ethnicity) in are denoted by (*), and with tendency for interaction (p =0.05 to <0.1) are denoted with (*t*). Mean direction of change is indicated by color and intensity, with red representing increased values, and blue representing decreased values. CAU, White Caucasian; HIS, Hispanics; NASH, Nonalcoholic steatohepatitis.

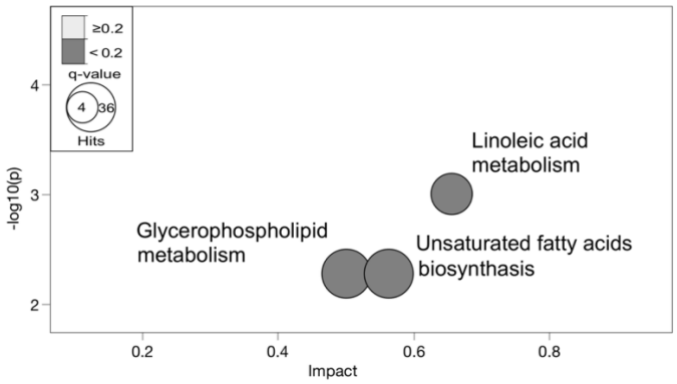

Figure 7. Hepatic metabolic pathways differentially altered with NAHS between ethnicities. Metabolites with differential alterations between ethnicity in NASH (p <0.05) were compared against pathway-associated metabolites sets from Kyoto Encyclopedia of Genes and Genomes (KEGG) [46]. Metabolic pathways significantly altered are shown as nodes. The (y-axis) represents the p-values as determined by Fisher's Exact test. The (x-axis) represents the impact of pathways as determined by the relative betweenness centrality-topology analysis. The size of the node represents the total hit number of hits. (n, 0 NASH-HIS=4, NASH-HIS=3; 0 NASH-CAU=5, NASH-CAU=5). Detailed pathway analysis statistics are shown in (Table S9).

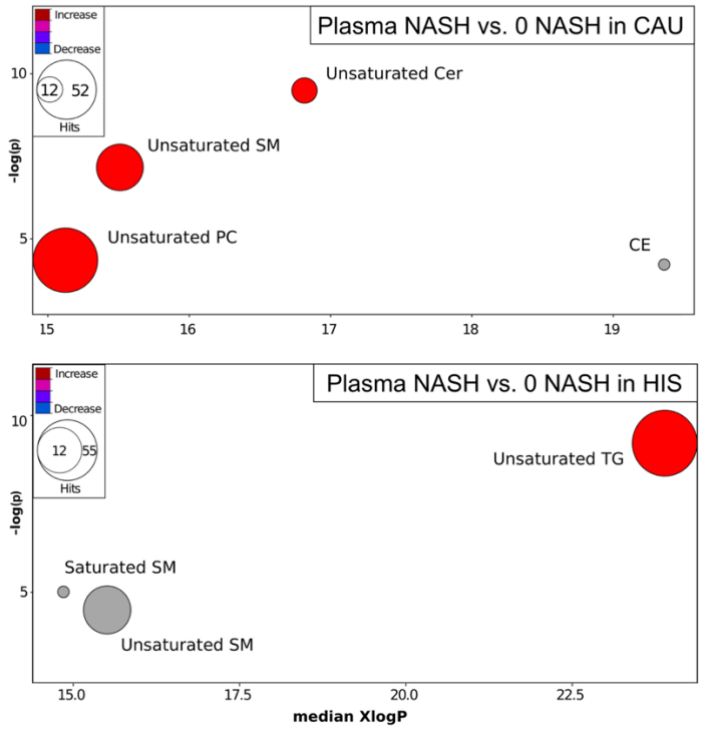

Figure 8. Plasma metabolites altered by chemical class in NASH, compared to NASH -free subjects, in both ethnicities. Chemical Similarity Enrichment Analysis (ChemRICH) of illustrating plasma metabolite class altered in NASH compared to 0-NASH in both ethnicities. ChemRICH enrichment statistics plot, illustrating plasma alterations in NASH vs. 0-NASH in CAU (top panel) and HIS (bottom panel). Each cluster represents altered chemical class of metabolites (p <0.05). Cluster sizes represent the total number of metabolites. Cluster's color represents the directionality of metabolite differences: red – higher in NASH; blue – lower in NASH. Colors in between refer to mixed population of metabolites manifesting both higher and lower levels in NASH when compared to the 0-NASH. The x-axis represents the cluster order on the chemical similarity tree. The plot y-axis shows chemical enrichment p-values calculated using Kolmogorov–Smirnov test. Only clusters with p <0.05 are shown. FDR-adjustment q=0.2, and clusters with FDR-adjusted p ≥0.2 are shown in gray. (n, 0 NASH-HIS=4, NASH-HIS=3; 0 NASH-CAU=5, NASH-CAU=3). The detailed ChemRICH results are shown in (Table S10 and S11). CAU, White Caucasian; Cer, Ceramides; CE, Cholesteryl ester; HIS, Hispanic; SM, Sphingomyelins; NASH, Nonalcoholic steatohepatitis; PC, Phosphatidylcholine; TG, Triglycerides.

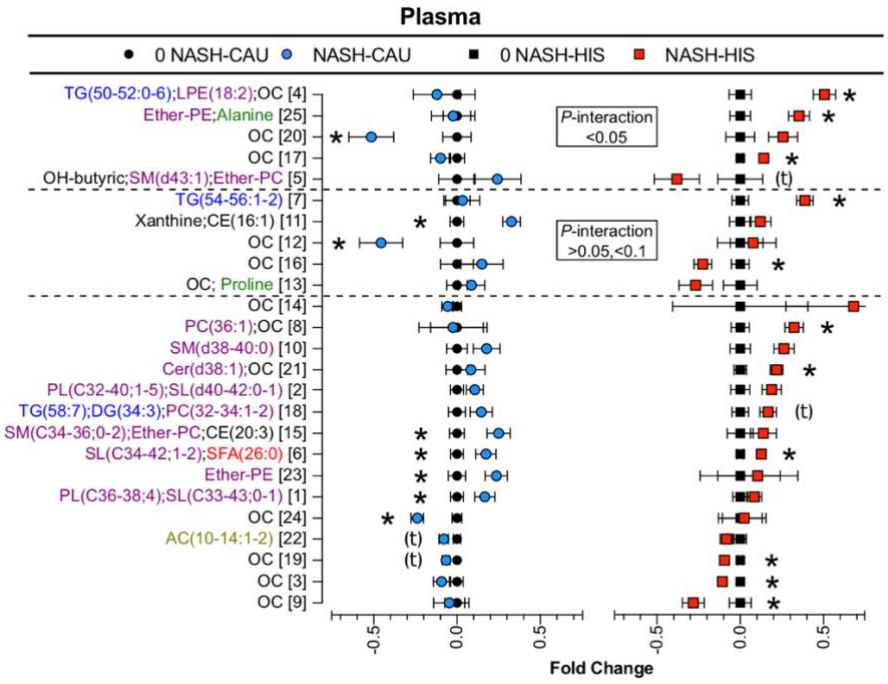

Figure 9. Ethnicity-related differences in plasma metabolomic profile in NASH vs. 0-NASH. Results of *t*-test and ANCOVA (ethnicity x NASH) interaction comparison performed on plasma variable cluster components between NASH and 0-NASH in both ethnicities. Clustering was performed on metabolites that were different (p <0.05) within groups (NASH vs. 0-NASH) or between ethnicity groups (interaction (NASH x ethnicity)). Data are presented as the fold change from 0-NASH; error bars represent standard error. Metabolite clusters are ranked in order of p-value for (interaction (NASH x ethnicity) and labeled by a number and a description of representative metabolite. For complex lipids, lipid class is followed by number of (carbons, double bounds) of the fatty acyl moiety. Clusters showing ethnicity-related differences in NASH (p <0.05) or tendency for interaction (p=0.05 to <0.1) are marked with the dashed line. Clusters affected by NASH within ethnicity (p <0.05) are denoted with (*); clusters with tendency (p=0.05 to <0.1) are denoted with (*t*). (*n*, 0 NASH-HIS=4, NASH-HIS=3; 0 NASH-CAU=5, NASH-CAU=3). The details on metabolites and clusters components are shown in (Table S12). AC, Acylcarnitines; CAU, White Caucasian; CE, cholesteryl ester; DG, diglycerides; Ether-PC, Ether-linked phosphatidylcholines; Ether-PE, Ether-linked phosphatidylethanolamines; HIS, Hispanic; MUFA, Monounsaturated fatty acid; NASH, Nonalcoholic steatohepatitis; 0-NASH, Nonalcoholic steatohepatitis-free; OC, Organic compounds; PC, Phosphatidylcholines; PL, Phospholipids; PUFA, Polyunsaturated fatty acids; NAFL, Steatosis; SFA, Saturated fatty acids; SM, sphingomyelins; TG, triglycerides.

9. Supplemental materials

Table S1. ChemRich enrichment statistics for plasma comparisons of obese with NAFL vs. lean healthy control in White Caucasian.

Table S2. ChemRich enrichment statistics for plasma comparisons of obese with NAFL vs. lean healthy control in Hispanic.

Table S3. Untargeted semi-quantification data table for comparisons of obese with NAFL (NAFL) vs. lean healthy control (HC) in Hispanic (HIS) and White Caucasian (CAU).

Table S4. ChemRich enrichment statistics for plasma comparisons of healthy control Hispanic vs. White Caucasian.

Table S5. Untargeted semi-quantification data table for comparisons of healthy control subjects between ethnicities.

Table S6. ChemRich enrichment statistics for liver comparisons of NASH vs. 0-NASH in Hispanic.

Table S7. ChemRich enrichment statistics for liver comparisons of NASH vs. 0-NASH in White Caucasians.

Table S8. Untargeted semi-quantification data table for comparisons of liver metabolomic profiles in NASH vs. 0-NASH in both ethnicities.

Table S9. Results from pathway analysis performed on liver metabolites of interaction with ethnicity in NASH. Metabolites with (*p*-value <0.05) on (ethnicity x NASH) from ANCOVA were mapped against pathway-associated metabolites sets from Kyoto Encyclopedia of Genes and Genomes (KEGG).

Table S10. ChemRich enrichment statistics for plasma comparisons of NASH vs. 0-NASH in Hispanics.

Table S11. ChemRich enrichment statistics for plasma comparisons of NASH vs. 0-NASH in White Caucasian.

Table S12. Untargeted semi-quantification data table for comparisons for plasma metabolomic profiles in NASH vs. 0-NASH in both ethnicities.

Table S13. Spearman's rank correlation analysis performed on liver metabolites and histological features in NASH.

Table S14. Spearman's rank correlation analysis performed on plasma metabolites and histological features in NASH.

Table S15. The minimum sample size required to detect differences between group means with 80% power and 95% confidence level was calculated. Calculation is based on selected metabolites with ethnicity interaction (p <0.05) for (a) Lean healthy comparison, (b) NASH comparison.

Figure S1. Natural clustering of study groups illustrating variations in plasma metabolomic profile

Chapter 3

Targeted oxylipins and endocannabinoid profiling

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver condition affecting one in four adults worldwide and this rate increases with coexisting components of metabolic syndrome [1]. Its histological presentation includes hepatocellular steatosis, or non-alcoholic fatty liver (NAFL) with a range of necroinflammation with or without fibrosis. When hepatocellular damage and ballooning are present, this is clinically defined as non-alcoholic steatohepatitis (NASH) [2]. The pathogenesis of NAFLD is not fully elucidated. Whereas its onset involves an interplay between genetics and environmental factors with coexisting comorbidities, the progression to NASH appears to be provoked by multiple or parallel hits including oxidative stress and inflammation [3,4]. Oxidative stress modulates insulin signaling, lipid metabolism, inflammation, and fibrogenesis, and many oxidative stress biomarkers have been associated with NAFLD severity [5,6]. In the U.S., the risk and severity of NAFLD vary among ethnic/racial groups, with Hispanics (HIS) being affected disproportionately and presenting more frequently with advanced inflammation and fibrosis compared to other ethnicities [7-9]. The metabolic drivers underlying this disparity are not clear.

Polyunsaturated fatty acid (PUFAs) are bioactive lipids and precursors to inflammatory lipid mediators including oxylipins (OXLs) and endocannabinoids (eCBs). OXLs are produced from PUFAs by mono- and dioxygenases, including lipoxygenases (e.g., 5-LOX, 12-LOXs, and 15-LOXs); cyclooxygenases (i.e., COX-1 and -2), and a variety of cytochrome P450s (CYPs) [10]. PUFAs can also undergo non-enzymatic oxygenation mediated by free radicals and the rate of this production is increased under oxidative stress [11]. In general, OXLs from n-3 PUFAs have anti-inflammatory or less pro-inflammatory effect compared to those derived from n-6 PUFAs [12,13]. The fatty acid ethanolamides (i.e., N-acylethanolamides), one class of eCB, are synthesized by complex interactions of lipases and fatty acid amide hydrolase from PUFAs and membrane associated precursors [14]. Collectively, these lipids work through receptor-mediated mechanisms and likely contribute to NAFLD by modulating processes including lipogenesis, inflammation, and mitochondrial β-oxidation [10,15].

Previous lipidomic analyses showed that NAFLD is associated with dysregulated PUFAs metabolism [16-20]. Alterations in circulating OXL and eCB profiles are reported in NAFLD and other liver pathologies. In fact, numerous lipid mediators have been shown to predict NAFL or NASH [18,21-26]. However, metabolomic profiling in NAFLD with regards to ethnicity is limited. Our prior semi-quantitative lipidomic profiling study indicated ethnicity-specific differences in plasma PUFA profiles in subjects with NAFL, with higher abundance of linoleic acid (LA) and α-linolenic acid (ALA) seen in Caucasians (CAU) compared to ethnicity-matched lean subjects [27]. In the same study, the progression to NASH was characterized by ethnicity-specific differences in hepatic lipidomic profiles with higher levels of saturated and unsaturated fatty acids seen in NASH-HIS. Ethnicity was not previously addressed in OXL and eCB profiling efforts. Examining such lipidomic differences among ethnicities may shed light on potential mechanisms modulating the disparity in NAFLD prevalence and severity.

The objective of this "proof-of-concept" study is to examine ethnicity-related changes in PUFAs and their downstream inflammatory mediators in a group of subjects with obesity and biopsy-confirmed NAFL and NASH. We employed targeted lipidomic analysis of plasma PUFAs, OXLs, and the N-acylethanolamides class of eCBs to compare HIS and CAU subjects with medically complicated obesity to ethnicity-matched lean healthy controls (HC). Profiles in subjects diagnosed with NASH were also compared to ethnicity and BMI-matched participants without NASH (0-NASH). In addition, we conducted a secondary analysis including prospectively collected subjects to compare OXL profile between ethnicities in NASH.

2. Subjects and methods

2.1. *Subjects and samples*

In this retrospective/ prospective cohort study (Figure S4), all subjects self-reported ethnicity as either HIS or CAU. HC subjects (n = 22) were recruited via public posts. Plasma and liver samples form bariatric surgery patients with medically complicated obesity were retrieved from the biobank repository of the Division of Gastroenterology and Hepatology, UC Davis Medical Center. The primary cohort (n = 18) consisted of subjects with NAFL and various degrees of necroinflammation.

Only subjects with NASH were included in the secondary analysis (n = 9) and this cohort was expanded with prospectively collected subjects diagnosed with NASH (n = 20). Subject inclusion and exclusion criteria and details on data collection are described elsewhere [25]. Briefly, plasma samples were collected preoperatively after an overnight fast, and liver tissue samples were collected by biopsy performed during bariatric surgery. Liver histopathological evaluations were performed in a blinded fashion in the UC Davis Medical Center Department of Pathology, and samples were scored according to the NASH Clinical Research Network (NASH-CRN) histology system, The NAFLD Activity Score (NAS) and fibrosis scores were calculated [26]. NASH diagnosis was determined using a diagnostic algorithm based on steatosis, inflammation, and fibrosis scores [27]. All subjects were consented and the Institutional Review Board at the University of California, Davis approved the study protocol (# 856052).

2.2. *Plasma targeted lipidomic analysis*

Quantitative lipidomic profiling of PUFAs, OXLs and NAEs was performed by ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) (S2), as previously described [28]. Briefly, plasma samples were enriched with deuterated surrogates, isolated by liquid/liquid extraction, and separated and quantified by UPLC-ESI-MS/MS. In the primary analysis, ESI-polarity switching facilitated the simultaneous detection of eCBs (positive mode) and oxylipins and PUFAs (negative mode) on an API 6500 QTRAP (AB Sciex, Framingham, MA, USA). Metabolites were quantified against authentic analytical standards with 6-to-10-point calibration curves and calculated concentrations were corrected for analytical surrogate recovery. This method detected 5 PUFAs and 66 lipid mediators, including 10 eCBs and 46 OXLs.

The secondary analysis was performed on an API 4000 QTrap (AB Sciex) and restricted to the negative mode electrospray ionization to increase the power of the OXLs discovery. This approach detected 46 OXLs and two nitrolipids. Details on the analysis protocols and reported data are available on the Metabolomics Workbench (http://www.metabolomicsworkbench.org, accessed on 06/13/2021), ID numbers (ST000977 and ST001845). Analyses were carried out at the UC Davis

West Coast Metabolomics Center. In this manuscript, abbreviations used for OXLs and eCBs follow standard consensus and are detailed with lipid identifiers in Table S6.

2.3. *Statistical analysis*

Statistical analyses were performed using JMP Pro 14.1 (SAS Institute Inc., Cary, NC; http://www.jmp.com, accessed on 03/10/2021). Outliers were identified and excluded using "robust Huber M test". Lipids with >30% missing data were excluded. Missing data were imputed by "multivariate normal imputation". Data normality was achieved by Johnson's transformation. After data processing, and to determine if subjects with stage 4 fibrosis (n = 2) are biological outliers, we employed principal component analysis (PCA). As a result, no outliers were detected (Figure S5).

Non-normalized data were used to calculate metabolite geometric means. Fold change (FC) was calculated for each ethnicity separately as (A − B)/B where A is the mean of (NAFL or NASH) and B is the mean of (HC or 0-NASH). A FC >0 indicates an increase and <0 indicates decrease and ± 20% FC was set as a threshold. Student's *t*-test of Johnson normalized data was used to examine differences between (NAFL vs. HC) and (0-NASH vs. NASH) in each ethnicity. Full factorial analysis of covariates (ANCOVA) was employed to evaluate the interaction of ethnicity × health status. This model included ethnicity (HIS or CAU), health status (NAFL or HC; 0-NASH or NASH), ethnicity x health status interaction as fixed effects, with age and sex as covariates. To check any effect of fibrosis or advance NAS score on the differences observed, we repeated the analysis on a subset of histology-matched subjects (n = 5 HIS and n = 5 CAU). Pathways/network visualization with fold change and *p*-values were plotted using Cystoscope 3.8.2 (https://cytoscape.org, accessed on 05/22/2021). [29]. Mean differences were considered likely at p <0.05. To adjust for false discovery rate (FDR), Benjamini-Hochberg FDR correction was performed [30]. A q = 0.2 was set as a threshold, given the pilot nature and small sample size of the study.

For the secondary analysis, raw data were auto-scaled to correct for batch effect (Figure S6) [31]. Lipids affected by batch were excluded (three lipids) and data were normalized by Johnson's transformation. Partial least square-discriminant analysis (PLS-DA) was performed to discriminate ethnicities in NASH subjects with leave- one-out cross validation (LOOCV) [32]. An R^2 and Q^2 > 0.5

are acceptable values to indicate reliability of the model in explaining differences between groups [33]. A variable importance in projection (VIP) score of >1.0 was set as a threshold for variable selection. To check any effect of advanced fibrosis, we repeated the analysis after excluding subjects with fibrosis grade 3 and 4 (n = 3 HIS and n = 4 CAU).

3. Results

3.1. *Subject characteristics*

The clinical and histological features of NAFL subjects from the primary cohort are presented in Table 1. The mean age in NAFL and HC, respectively, was 47 ± 15 and 43 ± 14 in HIS; 50 ± 18 and 44 ± 12 in CAU (n.s). The mean BMI in HIS was 46 ± 6 in NAFL and 26 ± 2 in HC (p-value < 0.05); in CAU, the mean BMI was 42 ± 8 in NAFL and 25 ± 3 in HC (p-value < 0.05). Within NAFL group, the mean NAS score was 3 ± 3 and 3 ± 1 for HIS and CAU, respectively (n.s). No difference in clinical and histological parameters was found between ethnicities. In subjects with NASH compared to 0-NASH, the mean NAS score was 5 ± 2 and 4 ± 1 for HIS and CAU, respectively (n.s) (data not shown).

The secondary cohort included NASH subject with various degrees of necroinflammation and fibrosis (Table 2). When comparing NASH-HIS and NASH-CAU, no difference was found with BMI and other clinical and histological parameters. The mean NAS score was 5 ± 2 and 5 ± 1 for NASH-HIS and NASH-CAU, respectively (n.s).

3.2. *Ethnicity-related rlterations in plasma PUFAs and lipid mediator profiles characterize NAFL*

We examined differences in plasma fatty acids and lipid mediators between NAFL and HC (Figure 1 and Table S1). Compared to corresponding HC, 25 (38% of total detected) and 7 (11%) lipid levels were different in NAFL-HIS and NAFL-CAU, respectively (FDR-adjusted p < 0.2). Ethnicity-specific changes observed in NAFL, with interaction (ethnicity x NAFL), include 8 lipids (15%) and 2 enzymatic ratios (raw p-Interaction < 0.05) but did not survive FDR-correction (q = 0.2). To rule out any effect of histological severity on the differences observed between ethnicities, the analysis was repeated on a subset of histology-matched subjects (Table S2). As a result, 12 lipids

(19%) and one enzymatic ratio were found altered (raw *p*-Interaction < 0.05), with 3 lipids, i.e., ALA, LA, and 9-hydroperoxyoctadecadienoic acid (-HpODE) surviving FDR correction.

When compared to HC, there were overlapping differences seen in NAFL for both ethnicities as well as ethnicity-specific differences (Figure S1). In both ethnicities, NAFL showed higher levels of several eCBs derived from PUFA and other fatty acids. The 18 carbon (C18) PUFAs, ALA and LA showed similar higher trend with higher levels of downstream fatty acid alcohols, hydroperoxide, ketones, epoxides, and vicinal diols. Specific to NAFL-HIS, there were differentially higher levels of LA-triols (i.e., trihydroxyoctadecaenoic acids (TriHOMEs) (raw *p*-Interaction < 0.05). On histology-matched analysis, higher TriHOMEs and LA-epoxide, 12(13)-epoxyoctadecenoic acid (-EpOME) levels were found significant (raw *p*-Interaction < 0.05). Of note, the n-6 to n-3 ratio was higher, however, with no ethnicity x NAFL interaction. In NAFL-CAU, there was differentially higher ALA and LA and its hydroperoxide, 9-HpODE (raw *p*-Interaction < 0.05). On histology-matched analysis, these lipids retained significance with LA, and 9-HpODE passing the FDR-threshold.

The 20 carbon (C20) and longer chain PUFAs (LC-PUFA) showed opposite trends with higher levels in NAFL-CAU and lower in NAFL-HIS. The ratio of docosahexaenoic acid (DHA)/ eicosapentanoic acid (EPA) + ALA was found lower in both ethnicities (FDR-adjusted *p*-value). In HIS, alcohols, ketones, thromboxane derived from arachidonic acid (AA) were higher, however with no interaction (ethnicity x NAFL). DHA and its vicinal diol, 19,20-dihydroxydocosapentaenoic acid (-DiHDoPA), levels were differentially lower (raw *p*-Interaction < 0.05) with tendency shown for lower EPA. On histology-matched analysis, lower levels of these lipids were found significant (raw *p*-Interaction < 0.05). Specific to CAU, there was differentially higher AA and its vicinal diol, 14,15-dihydroxyeicosatrienoic acid (-DiHETrE) that remained significant after histology adjustment (raw *p* < 0.05). While these findings show common alterations seen in NAFL for both ethnicities, they also highlight ethnicity-specific changes. This includes a divergence in LC-PUFA profile, mainly with lower EPA and DHA seen in HIS. Although these differences did not pass FDR adjustment, histology-matched analysis yielded consistent and stronger differences, suggesting ethnicity-specific

differences characterized NAFL, independently of liver histology severity. It also suggests that fibrosis may weaken the differences between ethnicities.

3.3. *Ethnicity-related differences in plasma PUFAs and lipid mediators' independent of obesity*

Ethnicity-specific differences in plasma lipidome within lean HC were examined (Figure S2 and Table S1). Among the differences observed, HIS had higher LA (raw $p < 0.05$), ALA, 9-HpODE, and TriHOMEs levels (FDR-adjusted *p*-value), and lower AA-derived prostaglandin, PGE2 (FDR-adjusted *p*-value) levels. These findings indicate alterations in plasma PUFAs and lipid mediator profiles in HIS independent of obesity.

3.4. *The progression to NASH is characterized by ethnicity-related alterations in plasma PUFAs and lipid mediator profiles*

We examined differences in plasma fatty acids and lipid mediators between 0-NASH and NASH (Figure 2 and Table S3). Compared to corresponding 0-NASH, 7 (11% of total detected) and 6 (9%) lipids were found different in NASH-HIS and NASH-CAU, respectively (raw *p*-value <0.05). None passed the FDR-correction threshold. There were differentially altered lipids by ethnicity group (raw *p*-Interaction < 0.05), including 11 lipids (17%) and two enzymatic ratios. Three of these lipids and the two enzymatic ratios passed the FDR-correction threshold.

With the progression from 0-NASH to NASH, less marked differences in plasma PUFA profile were observed. Compared to 0-NASH, NASH-HIS showed a trend for lower plasma PUFAs, only affecting AA (raw *p*-value < 0.05). There was a trend for higher C18-PUFA derived alcohols, triol, epoxides and vicinal diols, with TriHOMEs being differentially higher (raw *p*-Interaction < 0.05). We also observed a trend for lower LC-PUFA derived lipid mediators, mainly affecting AA-alcohols, 5-hydroxyeicosatetraenoic acid (-HETE); thromboxane (TXB2); and prostaglandin (PGE2), with 5-HETE and TXB2 found differentially changed between ethnicities (raw *p*-Interaction < 0.05). Also, the oleic acid (OA)-derived N-oleoyl glycine levels were lower with NASH (raw *p*-Interaction < 0.05).

In NASH-CAU, there was a trend of higher C18, LC-PUFAs and downstream lipid mediators. Interaction (ethnicity × NASH) was shown with higher 9-HpODE, TXB2, and in EPA-epoxide,

17(18)-epoxyeicosatetraenoic acid (-EpETE) (raw *p*-Interaction < 0.05). There was an opposite trend for C18-PUFA derived vicinal diols that were higher in HIS and lower in CAU, compared to corresponding 0-NASH, with interaction (ethnicity x NASH) shown for 12,13- and 15,16-dihydroxyoctadecadienoic acid (-DiHODE) (raw *p*-Interaction < 0.05). Multiple sEH enzymatic indices were higher in HIS and lower in CAU, including 9_10-dihydroxyoctadecenoic acid (-DiHOME)/9(10)- epoxyoctadecenoic acid (-EpOME) and 9_10-DiHODE)/9(10)-epoxyoctadecadienoic acid (-EpODE) (raw *p*-Interaction <0.05). Also, with NASH, there were higher levels of many eCBs, including dihomo-γ-linolenoylethanolamide, palmitoleoylethanolamide, palmitoleoylethanolamide, oleoyl-ethanolamide and N-oleoylglycine (raw *p*-Interaction < 0.05). Although PUFA changes are less marked in NASH, trends are consistent with changes seen in NAFL and support divergence in LC-PUFA profiles. It also highlights ethnicity-related differences in OXLs and eCBs associated with NASH progression. Given that NASH groups in both ethnicities had comparable NAS scores, this suggests the ethnicity-related differences observed with NASH are not likely driven by histological severity.

3.5. *Plasma OXLs profile discriminates between ethnicities with NASH*

A supervised PLS-DA was performed including all profiled lipids to examine if plasma OXLs profile can discriminate between ethnicities with NASH. The model demonstrated separation between HIS and CAU with 22 (49%) contributing lipids having VIP > 1. This indicates differences in OXLs profile characterizes HIS and CAU with NASH (Figure S3 and Table S4). The Q^2 and R^2 for the model were 0.62 and 0.72, respectively, indicating a fair reliability of the model. Of note, an overlap between ethnic groups was observed and subjects within this area shared advanced fibrosis (grade 3 and 4), indicating that HIS and CAU subjects with NASH and advanced fibrosis share similar plasma OXLs profile. Also, it suggests that advanced fibrosis may be attenuating the multivariate model. Therefore, we repeated the analysis after excluding subjects with advance fibrosis (Figure 3 and Table S4). As a result, the model exhibited complete separation between ethnicities in NASH with 20 (44%) lipids contributing to this difference (VIP > 1.0). The Q^2, R^2 were 0.99 and 0.98, respectively, indicating optimal prediction and reliability of the multivariate model.

In subjects with NASH and mild to moderate fibrosis, OXLs profiles showed opposite direction of change between ethnicities in some AA-derived mediators, with TXB2, 15-keto PGE2, 5-HETE and 5,6 DiHETrE being lower in HIS compared to CAU. Many OXLs derived via LOX pathway and/or auto-oxidative routes were lower in NASH-HIS, including TriHOMEs, 9- and 13-keto-octadecadienoic acid (-KODE), 9- and 13- hydroxyoctadecatrienoic acid (-HOTE), and 5-HETE. Some CYP-derived OXLs were lower in NASH-HIS, as 9(10)-EpOME, 12(13) EpOME and 12(13)-EpODE. Multiple sEH enzymatic indices were found higher in NASH-HIS, compared to NASH-CAU, including 12,13-DiHOME/12(13)-EpOME; 9,10-DiHOME/9(10)-EpOME; 17_18-DiHETE/17(18)-EpETE along with higher levels of the vicinal diol, 14,15-DiHETrE. These findings further confirm lower AA-derived OXLs in HIS with NASH, which are also characterized by lower LOX and higher sEH-derived lipid mediators compared to CAU. They also indicate that plasma OXL profiles can discriminate between ethnicities in NASH.

4. Discussion

This study is the first to examine targeted plasma PUFA, OXL, and eCB profiles with regards to ethnicity in a group of HIS and CAU subjects with obesity and biopsy-diagnosed NAFL and NASH. Our findings indicate that: (1) NAFL and NASH are characterized by ethnicity-related differences in plasma PUFA profiles, independent of histological severity; (2) Ethnicity-related differences in plasma OXLs profiles characterize NASH, independent of histological scores; (3) Plasma PUFA profile is altered in apparently healthy HIS, independent of obesity.

The hepatic and serum/plasma PUFA profiles are dysregulated in NAFLD [16-20]. Our results expand on these findings and show ethnicity-related differences in plasma PUFA profile in NAFL and NASH. With NAFL, both ethnicities showed higher ALA and LA levels, which were pronounced in CAU but not in HIS. This can be attributed to differences in the levels of these PUFAs in lean and healthy subjects, as HIS showed higher levels compared to their CAU counterpart. There was also a divergence in LC-PUFA profiles between ethnicities. CAU showed higher levels mainly affecting AA, while HIS displayed lower levels mainly affecting DHA and EPA. Consistent trends were shown with the progression from 0-NASH to NASH with arachidonic almost reaching significance

(*p*-Interaction = 0.07). This is in line with our previous untargeted profiling in NAFL done on the same subjects showing higher C18-PUFAs in CAU, and a trend for lower LC-PUFA with tendencies shown for DHA and EPA in HIS [27]. The lack of significant difference in LC-PUFA in our previous analysis may be due to the semi-quantitative nature and the clustering statistical approach. Also in agreement with our current finding is the lower serum/plasma DHA and EPA levels reported in obese HIS, compared to non-HIS [34,35]. Together, this implies diminished plasma LC-PUFA characterizes obese HIS with NAFL and NASH. As diet affects circulating and tissue PUFA levels [36], the ethnicity-dependent differences in dietary intake of n-3 PUFAs which is reportedly lower in HIS could be responsible for these observed changes [35,37]. While we did not account for diet, our findings suggest a possible etiological role for it as we observed higher linoleic and ALA levels independent of obesity in lean healthy HIS compared to CAU. Beside diet, genetic variants in cluster region of fatty acid desaturases (FADS) can predict LC-PUFA serum/blood levels [38,39]. Single nucleotide polymorphisms (SNP) in FADS1 and FADS2, which encode fatty acid desaturases, were robustly associated with NAFLD [40,41]. Lower Δ-5 desaturase levels are reported in both NAFL and NASH [16,17,20]. Notably, SNPs in FADS that are associated with insufficient LC-PUFA biosynthesis present with high frequency in Amerindians, a subgroup of HIS [42]. However, genotype was not examined in current study, and both ethnicities had lower estimated Δ-5 desaturase activity with NAFL. Therefore, diet and/or genetic factors may contribute to the observed ethnicity-related PUFA alterations but need further assessment.

Other key findings include the ethnicity-related differences in OXLs and eCBs profiles. The COX pathway exerts pro-inflammatory effects as it catalyzes the conversion of AA to prostaglandin PGE2, thromboxane TXB2, and other fatty acid alcohols [10]. In animal models of NASH, the expression and activity of COX-2 were upregulated, and its inhibition ameliorated NAFL and NASH [43,44]. Previously, high TXB2 and PGE2 levels were reported in subjects with NAFL and NASH [22]. Findings in NASH-CAU from our secondary analysis are consistent with this literature, as TXB2 and 15-Keto PGE2 discriminated between ethnicities with NASH with higher levels in CAU and lower levels in HIS. When comparing within ethnicities, the progression from 0-NASH to NASH in

HIS was marked with a trend for lower AA, almost reaching statistical significance (*p*-Interaction = 0.07) and downstream OXLs with TXB2 being differentially lower (*p*-Interaction < 0.05). These findings suggest ethnicity-related alterations in AA metabolism and downstream COX-derived OXLs in NASH.

Animal studies indicate a role for LOX pathways in NAFL and inflammation [45,46]. LOX pathways lead to the synthesis of fatty acid alcohols, ketones, hydroperoxides, and the specialized pro-resolving mediators (SPMs). With possible exceptions, n-6 PUFA derived alcohols are pro-inflammatory [10]. Under oxidative stress, PUFAs can also undergo auto-oxidation to form alcohols, ketones, hydroperoxides [11]. Previous studies reported higher LOX and auto-oxidation metabolites in NAFL and increased AA metabolites via LOX with the progression to NASH [18,22,23,41]. In our results, compared to control groups, NAFL and NASH in both ethnicities presented higher alcohols and ketones derived from C18-PUFAs, indicating an upregulated LOX pathway(s). In NAFL, we observed a positive correlation between some fatty acid alcohols and the oxidative stress markers, F2-isoprostanes and 9-HETE (Table S5), implying a contribution of non-enzymatic auto-oxidation. Interestingly, our secondary analysis showed many LOX derived OXLs being higher in NASH-CAU compared to NASH-HIS, with a similar trend found for the oxidative stress marker, 9-HETE (VIP = 0.98). Together, while LOX and oxidative pathways are upregulated with NAFL in both ethnicities, the magnitude of these alterations is lesser in HIS with NASH, compared to CAU. Based on this finding, we reasoned that LOX, and possibly oxidative stress, may be pivotal for NASH severity in CAU, and to a lesser extent in HIS.

CYP enzymes catalyze the synthesis of fatty acids epoxides and alcohols. In general, fatty acid alcohols are pro-inflammatory, and epoxides are anti-inflammatory and transient, and are hydrolyzed by the action of sEH to form inactive or less active vicinal diols [10,47]. A role for sEH in NAFLD progression is indicated by animal studies, showing that sEH inhibition improves NAFL, NASH, and fibrosis [47]. In subjects with NASH, compared to NAFL, AA derived vicinal diols are higher [22]. Our results show, with the progression to NASH, an ethnicity-dependent opposite trend for vicinal diols derived from C18-PUFA, which were higher in HIS and lower in CAU. Some of these vicinal

diols and sEH enzymatic indices showed interaction (ethnicity × NASH) and were found higher in NAHS-HIS and lower in NASH-CAU, compared corresponding 0-NASH. This may suggest higher activity of sEH in NASH-HIS. Our secondary analysis also shows higher ratios of multiple sEH enzymatic indices in HIS compared to CAU, and lower C18-PUFA epoxides possibly due higher hydrolysis rate. NASH-CAU showed higher levels of many PUFA epoxides, compared to NASH-HIS, indicating upregulated CYP pathway(s) and/or less hydrolysis. Together, our finding highlights ethnicity-related differences in sEH activity that was higher in HIS with NASH.

Extensive evidence from animal studies indicates a role for eCB system in NAFL, mitochondrial dysfunction and inflammation and fibrosis [48,49]. In NAFL, both ethnicities had higher levels of several eCBs. However, with the progression to NASH, many eCBs were higher in CAU and lower in HIS as compared to corresponding 0-NASH (raw *p*-Interaction < 0.05). We also observed levels of the OA-derived mediators N-oleoyl glycine, and oleoylethanolamide. These observations could not be examined in our secondary analysis as we detected limited numbers of eCBs and did not profile for fatty acids. Nevertheless, this may indicate ethnicity-related variations in eCBs profiles and OA metabolism with NAFLD in HIS that need to be further examined.

Our findings corroborate the epidemiological evidence indicating ethnicity as one variable affecting the association between PUFAs and cardiometabolic risks [29,45,46]. In fact, the observed ethnicity-related alterations may be relevant to NAFLD severity. EPA and DHA modulate hepatic fatty acid oxidation, de novo lipogenesis, redox balance and inflammation via direct interaction with nuclear receptors and transcription factors [50]. These LC-PUFAs are also precursors to potent SPMs which drive inflammatory resolution [10]. Also, the pro-inflammatory cascade of AA via COX is necessary for the biosynthesis of SPMs and initiating inflammatory resolution [13,51]. Therefore, a diminished level of these PUFAs may abolish anti-steatogenic and anti-inflammatory mechanisms. Likewise, a higher sEH activity may result in deactivation of anti-inflammatory PUFA epoxides [10,47]. Interestingly, our findings suggest that upregulated LOX pathway(s) may be imperative to NASH severity in CAU with a lesser extent in HIS. Collectively, we postulate that the observed ethnicity-related changes translate to the more advanced NASH histological presentation seen in HIS.

Of note, these changes are independent of fibrosis or NAS scores, in fact, histology adjustment resulted in stronger differences in both analyses, implying that subjects with advanced fibrosis may share similar lipidomic profile.

Our findings have clinical/diagnostic implications. Given liver biopsy risks and limitations [52], there is an ongoing search for noninvasive biomarker for NAFLD, with multiple biomarkers have been recently proposed including betatrophin and fetuin-A [53,54]. Also, several AA- derived OXLs were shown to predict NASH including higher levels of 5- and 15-HETE, PGE2, and some vicinal diols [18,22]. While our findings in NASH-CAU show trends consistent with current literature, findings in HIS indicate otherwise. Ethnicity-related differences in plasma metabolomic profile have been reported before in diabetes, Alzheimer's disease, and bladder cancer [55-57]. We propose that ethnicity-specific plasma signature may characterize NASH. In fact, utilizing ethnicity-related variations in plasma lipidomic profile may be instrumental for the enhanced precision of such diagnostic tools. If further verified, it will serve as a much-needed non-invasive tool aiding in clinical practice for early detection of NASH in both HIS and CAU populations. It can also pave the road for examination of ethnicity-specific lipidomic signatures in other ethnicities as the Asian and African American populations. On another note, a role of EPA and DHA supplementations in improving NAFLD and its risk factors is supported by clinical evidence [58,59]. Also, growing data indicate the utility of sEH inhibitors in NASH treatment [42,57,58]. Therefore, evaluating these interventions for NASH treatment seems warranted, particularly in the HIS population.

This "proof-of-concept" analysis is based on a small, single-center study. The limited sample size may have compromised the correction for multiple testing in the primary analysis. However, findings from the secondary analysis were consistent and the multivariate model is validated for overfitting and predictability. Other strengths include biopsy-characterized NAFL and NASH and analysis adjusted for BMI and histology. While NAFLD prevalence is reported to be higher in males compared to females [60-62], we could not examine sex differences due to small sample size. However, we did adjust for sex as a covariate.

In conclusion, we performed targeted lipidomic profiling for PUFAs and related lipid mediators with regards to ethnicity. Results show ethnicity-related divergence in LC-PUFA and downstream OXLs profiles with NAFL and NASH progression, independent of histological scores. Our secondary analysis indicates that in NASH and compared to CAU, HIS are characterized by lower levels of AA derived OXLs, lower LOX with an upregulated sEH pathway(s). These lipidomic differences may be relevant to the ethnicity-related disparity reported in NAFLD rate and severity and are worth further investigations. Our findings suggest ethnicity-specific lipidomic signature may characterize NASH. Although preliminary, these novel observations support the need for larger validation studies.

5. Funding

The National Institutes of Health (NIH)-West Coast Metabolomic Center (WCMC) grant number U24 DK097154, R01 DK104770 (to V.M.), 1R01 HL09133 (to P.H.) and 1R01 HL107256 (to P.H.). The U.S. Department of Agriculture (USDA) project 2032-51530-025-00D (to J.W.N). The USDA is an equal opportunity provider and employer. The content is exclusively the responsibility of the authors and does not represent the official views of NIH, WCMC or USDA.

This article was published in Metabolites; Vol number 12; Issue number 2; Mazi, T.A., Borkowski, K., Fiehn, O., Bowlus, C.L., Sarkar, S., Matsukuma, K., Ali, M.R., Kieffer, D.A., Wan, Y.J.Y., Stanhope, K.L. and Havel, P.J.; Plasma oxylipin profile discriminates ethnicities in subjects with Non-alcoholic steatohepatitis: An exploratory analysis; Page No. 192; Copyright retained by the authors (2022).

6. References

1. Younossi, Z.M.; Koenig, A.B.; 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,
2. Chalasani, N.; Younossi, Z.; Lavine, J.E.; Charlton, M.; Cusi, K.; Rinella, M.; Harrison, S.A.; Brunt, E.M.; Sanyal, A.J. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* **2018**, *67*, 328-357, doi:10.1002/hep.29367
3. Yu, J.; Marsh, S.; Hu, J.; Feng, W.; Wu, C. The pathogenesis of nonalcoholic fatty liver disease: interplay between diet, gut microbiota, and genetic background. *Gastroenterology research and practice* **2016**, *2016*, doi:doi.org/10.1155/2016/2862173

4. Tilg, H.; Moschen, A.R. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* **2010**, *52*, 1836-1846, doi:10.1002/hep.24001
5. Chen, Z.; Tian, R.; She, Z.; Cai, J.; Li, H. Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radical Biology and Medicine* **2020**, *152*, 116-141,
6. Rolo, A.P.; Teodoro, J.S.; Palmeira, C.M. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free radical biology and medicine* **2012**, *52*, 59-69,
7. Lazo, M.; Hernaez, R.; Eberhardt, M.S.; Bonekamp, S.; Kamel, I.; Guallar, E.; Koteish, A.; Brancati, F.L.; Clark, J.M. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988–1994. *American journal of epidemiology* **2013**, *178*, 38-45,
8. Rich, N.E.; Oji, S.; Mufti, A.R.; Browning, J.D.; Parikh, N.D.; Odewole, M.; Mayo, H.; Singal, A.G. Racial and Ethnic Disparities in Nonalcoholic Fatty Liver Disease Prevalence, Severity, and Outcomes in the United States: A Systematic Review and Meta-analysis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2018**, *16*, 198-210.e192, doi:10.1016/j.cgh.2017.09.041
9. Mohanty, S.R.; Troy, T.N.; Huo, D.; O'Brien, B.L.; Jensen, D.M.; Hart, J. Influence of ethnicity on histological differences in non-alcoholic fatty liver disease. *Journal of hepatology* **2009**, *50*, 797-804,
10. Gabbs, M.; Leng, S.; Devassy, J.G.; Monirujjaman, M.; Aukema, H.M. Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Advances in Nutrition* **2015**, *6*, 513-540, doi:10.3945/an.114.007732
11. Roy, J.; Le Guennec, J.-Y.; Galano, J.-M.; Thireau, J.; Bultel-Poncé, V.; Demion, M.; Oger, C.; Lee, J.C.-Y.; Durand, T. Non-enzymatic cyclic oxygenated metabolites of omega-3 polyunsaturated fatty acid: Bioactive drugs? *Biochimie* **2016**, *120*, 56-61, doi:10.1016/j.biochi.2015.06.010
12. Dennis, E.A.; Norris, P.C. Eicosanoid storm in infection and inflammation. *Nature Reviews Immunology* **2015**, *15*, 511-523, doi:10.1038/nri3859
13. Serhan, C.N. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *The FASEB Journal* **2017**, *31*, 1273-1288, doi:10.1096/fj.201601222R
14. Mallat, A.; Teixeira-Clerc, F.; Deveaux, V.; Manin, S.; Lotersztajn, S. The endocannabinoid system as a key mediator during liver diseases: new insights and therapeutic openings. *British journal of pharmacology* **2011**, *163*, 1432-1440, doi:doi: 10.1111/j.1476-5381.2011.01397.x
15. Tam, J.; Liu, J.; Mukhopadhyay, B.; Cinar, R.; Godlewski, G.; Kunos, G. Endocannabinoids in liver disease. *Hepatology* **2011**, *53*, 346-355, doi:10.1002/hep.24077
16. Puri, P.; Baillie, R.A.; Wiest, M.M.; Mirshahi, F.; Choudhury, J.; Cheung, O.; Sargeant, C.; Contos, M.J.; Sanyal, A.J. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **2007**, *46*, 1081-1090,
17. Chiappini, F.; Coilly, A.; Kadar, H.; Gual, P.; Tran, A.; Desterke, C.; Samuel, D.; Duclos-Vallée, J.-C.; Touboul, D.; Bertrand-Michel, J. Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. *Scientific reports* **2017**, *7*, 46658, doi:10.1038/srep46658
18. Puri, P.; Wiest, M.M.; Cheung, O.; Mirshahi, F.; Sargeant, C.; Min, H.K.; Contos, M.J.; Sterling, R.K.; Fuchs, M.; Zhou, H. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **2009**, *50*, 1827-1838,

19. Kalhan, S.C.; Guo, L.; Edmison, J.; Dasarathy, S.; McCullough, A.J.; Hanson, R.W.; Milburn, M. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism-Clinical and Experimental* **2011**, *60*, 404-413,
20. Walle, P.; Takkunen, M.; Männistö, V.; Vaittinen, M.; Lankinen, M.; Kärjä, V.; Käkelä, P.; Ågren, J.; Tiainen, M.; Schwab, U. Fatty acid metabolism is altered in non-alcoholic steatohepatitis independent of obesity. *Metabolism* **2016**, *65*, 655-666, doi:doi: 10.1016/j.metabol.2016.01.011
21. Maciejewska, D.; Ossowski, P.; Drozd, A.; Ryterska, K.; Jamioł-Milc, D.; Banaszczak, M.; Kaczorowska, M.; Sabinicz, A.; Raszeja-Wyszomirska, J.; Stachowska, E. Metabolites of arachidonic acid and linoleic acid in early stages of non-alcoholic fatty liver disease—A pilot study. *Prostaglandins & other lipid mediators* **2015**, *121*, 184-189, doi:10.1016/j.prostaglandins.2015.09.003
22. Loomba, R.; Quehenberger, O.; Armando, A.; Dennis, E.A. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. *J Lipid Res* **2015**, *56*, 185-192, doi:10.1194/jlr.P055640
23. Feldstein, A.E.; Lopez, R.; Tamimi, T.A.-R.; Yerian, L.; Chung, Y.-M.; Berk, M.; Zhang, R.; McIntyre, T.M.; Hazen, S.L. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Journal of lipid research* **2010**, *51*, jlr. M007096, doi:10.1194/jlr.M007096
24. Gorden, D.L.; Myers, D.S.; Ivanova, P.T.; Fahy, E.; Maurya, M.R.; Gupta, S.; Min, J.; Spann, N.J.; McDonald, J.G.; Kelly, S.L. Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *Journal of lipid research* **2015**, *56*, 722-736,
25. Kimberly, W.T.; O'Sullivan, J.F.; Nath, A.K.; Keyes, M.; Shi, X.; Larson, M.G.; Yang, Q.; Long, M.T.; Vasan, R.; Peterson, R.T. Metabolite profiling identifies anandamide as a biomarker of nonalcoholic steatohepatitis. *JCI insight* **2017**, *2*, doi:doi: 10.1172/jci.insight.92989
26. Zelber-Sagi, S.; Azar, S.; Nemirovski, A.; Webb, M.; Halpern, Z.; Shibolet, O.; Tam, J. Serum levels of endocannabinoids are independently associated with nonalcoholic fatty liver disease. *Obesity* **2017**, *25*, 94-101, doi:10.1002/oby.21687
27. Mazi, T.A.; Borkowski, K.; Newman, J.W.; Fiehn, O.; Bowlus, C.L.; Sarkar, S.; Matsukuma, K.; Ali, M.R.; Kieffer, D.A.; Wan, Y.Y.; et al. Ethnicity-specific alterations of plasma and hepatic lipidomic profiles are related to high NAFLD rate and severity in Hispanic Americans, a pilot study. *Free Radic Biol Med* **2021**, *172*, 490-502, doi:10.1016/j.freeradbiomed.2021.06.024
28. Pedersen, T.L.; Gray, I.J.; Newman, J.W. Plasma and serum oxylipin, endocannabinoid, bile acid, steroid, fatty acid and nonsteroidal anti-inflammatory drug quantification in a 96-well plate format. *Analytica Chimica Acta* **2021**, *1143*, 189-200,
29. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research* **2003**, *13*, 2498-2504, doi:10.1101/gr.1239303
30. Benjamini, Y.; Yekutieli, D. Quantitative trait loci analysis using the false discovery rate. *Genetics* **2005**, *171*, 783-789, doi:10.1534/genetics.104.036699
31. Berg, R. Hoefsloot HCJ van den, Westerhuis JA, Smilde AK, Werf MJ, van der: Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics* **2006**, *7*, 142,

32. Westerhuis, J.A.; Hoefsloot, H.C.; Smit, S.; Vis, D.J.; Smilde, A.K.; van Velzen, E.J.; van Duijnhoven, J.P.; van Dorsten, F.A. Assessment of PLSDA cross validation. *Metabolomics* **2008**, *4*, 81-89,
33. Eriksson, L.; Byrne, T.; Johansson, E.; Trygg, J.; Vikström, C. *Multi-and megavariate data analysis basic principles and applications*; Umetrics Academy: 2013; Volume 1.
34. Murphy, R.A.; Devarshi, P.P.; Ekimura, S.; Marshall, K.; Mitmesser, S.H. Long-chain omega-3 fatty acid serum concentrations across life stages in the USA: an analysis of NHANES 2011–2012. *BMJ open* **2021**, *11*, e043301, doi:10.1136/bmjopen-2020-043301
35. Steffen, B.; Steffen, L.; Tracy, R.; Siscovick, D.; Jacobs, D.; Liu, K.; He, K.; Hanson, N.; Nettleton, J.; Tsai, M. Ethnicity, plasma phospholipid fatty acid composition and inflammatory/endothelial activation biomarkers in the Multi-Ethnic Study of Atherosclerosis (MESA). *European journal of clinical nutrition* **2012**, *66*, 600-605, doi:10.1038/ejcn.2011.215
36. Wood, K.E.; Mantzioris, E.; Gibson, R.A.; Ramsden, C.; Muhlhausler, B. The effect of modifying dietary LA and ALA intakes on omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) status in human adults: a systematic review and commentary. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **2015**, *95*, 47-55, doi:10.1016/j.plefa.2015.01.001
37. Ramírez-Silva, I.; Villalpando, S.; Moreno-Saracho, J.E.; Bernal-Medina, D. Fatty acids intake in the Mexican population. Results of the National Nutrition Survey 2006. *Nutrition & metabolism* **2011**, *8*, 33, doi:10.1186/1743-7075-8-33
38. Schaeffer, L.; Gohlke, H.; Müller, M.; Heid, I.M.; Palmer, L.J.; Kompauer, I.; Demmelmair, H.; Illig, T.; Koletzko, B.; Heinrich, J. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. *Human molecular genetics* **2006**, *15*, 1745-1756, doi:doi: 10.1093/hmg/ddl117
39. Koletzko, B.; Lattka, E.; Zeilinger, S.; Illig, T.; Steer, C. Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children. *The American journal of clinical nutrition* **2011**, *93*, 211-219, doi:10.3945/ajcn.110.006189
40. Wang, L.; Athinarayanan, S.; Jiang, G.; Chalasani, N.; Zhang, M.; Liu, W. Fatty acid desaturase 1 gene polymorphisms control human hepatic lipid composition. *Hepatology* **2015**, *61*, 119-128, doi:10.1002/hep.27373
41. Vernekar, M.; Amarapurkar, D.; Joshi, K.; Singhal, R. Gene polymorphisms of desaturase enzymes of polyunsaturated fatty acid metabolism and adiponutrin and the increased risk of nonalcoholic fatty liver disease. *Meta Gene* **2017**, *11*, 152-156,
42. Yang, C.; Hallmark, B.; Chai, J.C.; O'Connor, T.D.; Reynolds, L.M.; Wood, A.C.; Seeds, M.; Chen, Y.-D.I.; Steffen, L.M.; Tsai, M.Y. Impact of Amerind ancestry and FADS genetic variation on omega-3 deficiency and cardiometabolic traits in Hispanic populations. *Communications biology* **2021**, *4*, 1-12, doi:10.1038/s42003-021-02431-4
43. Cheng, Q.; Li, N.; Chen, M.; Zheng, J.; Qian, Z.; Wang, X.; Huang, C.; Xu, S.; Shi, G. Cyclooxygenase-2 promotes hepatocellular apoptosis by interacting with TNF-α and IL-6 in the pathogenesis of nonalcoholic steatohepatitis in rats. *Digestive diseases and sciences* **2013**, *58*, 2895-2902, doi:10.1007/s10620-013-2823-6

44. Chen, J.; Liu, D.; Bai, Q.; Song, J.; Guan, J.; Gao, J.; Liu, B.; Ma, X.; Du, Y. Celecoxib attenuates liver steatosis and inflammation in non-alcoholic steatohepatitis induced by high-fat diet in rats. *Molecular medicine reports* **2011**, *4*, 811-816, doi:10.3892/mmr.2011.501
45. Lazic, M.; Inzaugarat, M.E.; Povero, D.; Zhao, I.C.; Chen, M.; Nalbandian, M.; Miller, Y.I.; Cherñavsky, A.C.; Feldstein, A.E.; Sears, D.D. Reduced dietary omega-6 to omega-3 fatty acid ratio and 12/15-lipoxygenase deficiency are protective against chronic high fat diet-induced steatohepatitis. *PloS one* **2014**, *9*, e107658, doi:10.1371/journal.pone.0107658
46. López-Parra, M.; Titos, E.; Horrillo, R.; Ferré, N.; González-Périz, A.; Martínez-Clemente, M.; Planagumà, A.; Masferrer, J.; Arroyo, V.; Clària, J. Regulatory effects of arachidonate 5-lipoxygenase on hepatic microsomal TG transfer protein activity and VLDL-triglyceride and apoB secretion in obese mice. *Journal of lipid research* **2008**, *49*, 2513-2523, doi:10.1194/jlr.M800101-JLR200
47. Warner, J.; Hardesty, J.; Zirnheld, K.; McClain, C.; Warner, D.; Kirpich, I. Soluble Epoxide Hydrolase Inhibition in Liver Diseases: A Review of Current Research and Knowledge Gaps. *Biology* **2020**, *9*, 124, doi:10.3390/biology9060124
48. Jourdan, T.; Djaouti, L.; Demizieux, L.; Gresti, J.; Vergès, B.; Degrace, P. CB1 antagonism exerts specific molecular effects on visceral and subcutaneous fat and reverses liver steatosis in diet-induced obese mice. *Diabetes* **2010**, *59*, 926-934, doi:10.2337/db09-1482
49. Osei-Hyiaman, D.; Liu, J.; Zhou, L.; Godlewski, G.; Harvey-White, J.; Jeong, W.-i.; Bátkai, S.; Marsicano, G.; Lutz, B.; Buettner, C. Hepatic CB 1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. *The Journal of clinical investigation* **2008**, *118*, 3160-3169, doi:doi: 10.1172/JCI34827
50. de Castro, G.S.; Calder, P.C. Non-alcoholic fatty liver disease and its treatment with n-3 polyunsaturated fatty acids. *Clinical nutrition* **2018**, *37*, 37-55, doi:10.1016/j.clnu.2017.01.006
51. Musso, G.; Gambino, R.; Cassader, M.; Paschetta, E.; Sircana, A. Specialized proresolving mediators: enhancing nonalcoholic steatohepatitis and fibrosis resolution. *Trends in pharmacological sciences* **2018**, *39*, 387-401, doi:10.1016/j.tips.2018.01.003
52. Golabi, P.; Sayiner, M.; Fazel, Y.; Koenig, A.; Henry, L.; Younossi, Z.M. Current complications and challenges in nonalcoholic steatohepatitis screening and diagnosis. *Expert review of gastroenterology & hepatology* **2016**, *10*, 63-71,
53. Sonmez, A.; Dogru, T.; Ercin, C.N.; Genc, H.; Celebi, G.; Gurel, H.; Tapan, S.; Cicek, A.F.; Barcin, C.; Haymana, C. Betatrophin Levels Are Related to the Early Histological Findings in Nonalcoholic Fatty Liver Disease. *Metabolites* **2021**, *11*, 425,
54. Dogru, T.; Kirik, A.; Gurel, H.; Rizvi, A.A.; Rizzo, M.; Sonmez, A. The Evolving Role of Fetuin-A in Nonalcoholic Fatty Liver Disease: An Overview from Liver to the Heart. *International Journal of Molecular Sciences* **2021**, *22*, 6627,
55. Vardarajan, B.; Kalia, V.; Manly, J.; Brickman, A.; Reyes-Dumeyer, D.; Lantigua, R.; Ionita-Laza, I.; Jones, D.P.; Miller, G.W.; Mayeux, R. Differences in plasma metabolites related to Alzheimer's disease, APOE ε4 status, and ethnicity. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* **2020**, *6*, e12025, doi:10.1002/trc2.12025
56. Vantaku, V.; Donepudi, S.R.; Piyarathna, D.W.B.; Amara, C.S.; Ambati, C.R.; Tang, W.; Putluri, V.; Chandrashekar, D.S.; Varambally, S.; Terris, M.K. Large-scale profiling of serum metabolites in

African American and European American patients with bladder cancer reveals metabolic pathways associated with patient survival. *Cancer* **2019**, *125*, 921-932, doi:10.1002/cncr.31890

57. van Valkengoed, I.G.; Argmann, C.; Ghauharali-van der Vlugt, K.; Aerts, J.M.; Brewster, L.M.; Peters, R.; Vaz, F.M.; Houtkooper, R.H. Ethnic differences in metabolite signatures and type 2 diabetes: a nested case–control analysis among people of South Asian, African and European origin. *Nutrition & diabetes* **2017**, *7*, 1-8,
58. Musa-Veloso, K.; Venditti, C.; Lee, H.Y.; Darch, M.; Floyd, S.; West, S.; Simon, R. Systematic review and meta-analysis of controlled intervention studies on the effectiveness of long-chain omega-3 fatty acids in patients with nonalcoholic fatty liver disease. *Nutrition reviews* **2018**, *76*, 581-602,
59. Lu, W.; Li, S.; Li, J.; Wang, J.; Zhang, R.; Zhou, Y.; Yin, Q.; Zheng, Y.; Wang, F.; Xia, Y. Effects of omega-3 fatty acid in nonalcoholic fatty liver disease: a meta-analysis. *Gastroenterology research and practice* **2016**, *2016*, 1459790, doi:10.1155/2016/1459790
60. Pan, J.-J.; Fallon, M.B. Gender and racial differences in nonalcoholic fatty liver disease. *World journal of hepatology* **2014**, *6*, 274,
61. Browning, J.D.; Szczepaniak, L.S.; Dobbins, R.; Nuremberg, P.; Horton, J.D.; Cohen, J.C.; Grundy, S.M.; Hobbs, H.H. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **2004**, *40*, 1387-1395,
62. Pramfalk, C.; Pavlides, M.; Banerjee, R.; McNeil, C.A.; Neubauer, S.; Karpe, F.; Hodson, L. Sex-specific differences in hepatic fat oxidation and synthesis may explain the higher propensity for NAFLD in men. *The Journal of Clinical Endocrinology & Metabolism* **2015**, *100*, 4425-4433,

7. Tables

Table 1. Demographic, clinical, and histological characteristics of study subjects in primary analysis.

	NAFL-HIS	NAFL-CAU	*p*-Value *
Plasma, *n* (F/M)	10 (7/3)	8 (4/4)	-
Age (Yrs.)	47 ± 15	50 ± 18	0.6
DM, yes (%)	4 (40)	4 (50)	1
FBG mmol/L	101 ± 14	94 ± 13	0.3
Cholesterol (mg/dL)	172 ± 28	166 ± 34	0.7
TG (mg/dL)	135 ± 79	129 ± 70	0.8
HDL (mg/dL)	44 ± 6	43 ± 8	0.7
LDL (mg/dL)	107 ± 24	99 ± 35	0.4
HbA1c (%)	6 ± 1	6 ± 1	0.7
AST (U/L)	31 ± 13	26 ± 9	0.6
ALT (U/L)	40 ± 27	31 ± 11	0.9
Platelet	280 ± 84	302 ± 110	0.8
NAS	3 ± 3	3 ± 1	0.7
Steatosis (%)			
<5%	4 (40)	1 (12.5)	0.9
5 to ≤33%	3 (30)	5 (62.5)	
34 to ≤66 %	2 (20)	2 (25)	
>66%	1 (10.0)	0 (0)	
Inflammation (%)			
None	3 (30)	1 (12)	0.6
Mild	1 (10)	4 (50)	
Moderate	4 (40)	3 (38)	
Severe	2 (20)	0 (0)	
Ballooning (%)			
None	4 (40)	5 (62)	0.3
Few	5 (50)	3 (38)	
Many	1 (10)	0 (0)	
Fibrosis (%)			
None	7 (70)	7 (88)	0.3
1A	0 (0)	1 (12)	
2	1 (10)	0 (0)	
4	2 (20)	0 (0)	

General characteristics of NAFL group in both ethnicities shown as percent (for categorical data) and mean ± SEM (for nominal data). Comparisons were performed by *t*-test (nominal) or chi-square test (categorical). (*) NAFL-HIS vs. NAFL-CAU.

Table 2. Demographic, clinical, and histological characteristics of NASH subjects in secondary analysis.

	NASH-HIS	NASH-CAU	*p*-Value *
Plasma, *n* (F/M)	12 (9/3)	17 (11/6)	-
Age (Yrs.)	49 ± 8	50 ± 13	0.8
BMI (Kg/m^2)	41 ± 8	37 ± 7	0.6
NAS	5 ± 2	5 ± 1	0.9
Steatosis (%)			
<5%	2 (17)	0 (0)	0.4
5 to ≤33%	4 (33)	7 (41)	
34 to ≤66 %	3 (25)	4 (24)	
>66%	3 (25)	6 (35)	
Inflammation (%)			
None	0 (0)	0 (0)	0.1
Mild	2 (17)	3 (18)	
Moderate	6 (50)	13 (76)	
Severe	4 (33)	1 (6)	
Ballooning (%)			
None	0 (0)	0 (0)	0.6
Few	8 (67)	13 (76)	
Many	4 (33)	4 (24)	
Fibrosis (%)			
None	5 (41)	4 (23)	0.8
1a, b, c	2 (17)	6 (35)	
2	2 (17)	3 (18)	
3	2 (17)	2 (12)	
4	1 (8)	2 (12)	

General characteristics of subjects included in the secondary analysis shown as percent (for categorical data) and mean ± SEM (for nominal data). Comparisons were performed by *t*-test (nominal) or chi-square test (categorical). (*) NASH-HIS vs. NASH-CAU.

8. Figures

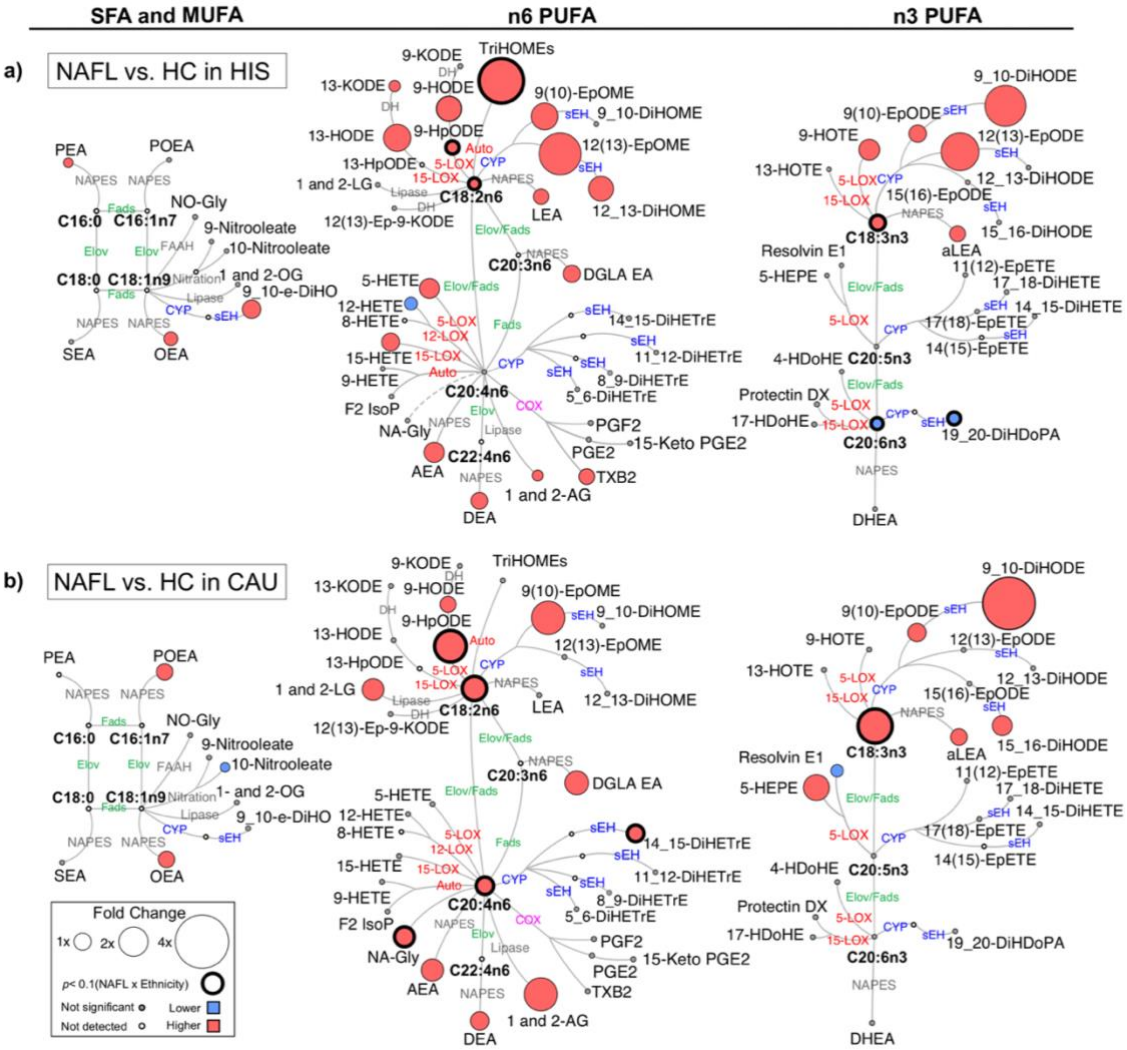

Figure 1. Differences in plasma polyunsaturated fatty acids (PUFAs) and lipid mediators between non-alcoholic fatty liver (NAFL) compared to healthy control (HC) in primary cohort. Metabolic network for (a) Hispanic (HIS); (b) Caucasian (CAU) illustrating saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs), including n-3 and n-6 PUFAs with pathways of oxylipins and endocannabinoids synthesis. Node size represents fold changes, calculated as (HC − NAFL)/HC. Node's color represents the directionality of differences: higher in NAFL (red); lower in NAFL (blue); no change (grey). Shown are only lipids with differences between NAFL vs. HC (*t*-test raw $p < 0.05$) and/or with interaction (ethnicity x NAFL) (ANCOVA raw $p < 0.05$). Lipids with interaction (ethnicity x NAFL) (ANCOVA raw $p < 0.05$) are marked with a solid circle. Means and *p*-values are detailed in Table S1. Fatty acids are described by number of carbons and double bounds of the fatty acyl moiety (i.e., C18:2n6). NAFL (n = 10 HIS and 8 CAU); HC (n = 14 HIS and 8 CAU). ADH, alcohol dehydrogenase; AEA, arachidonoyl ethanolamine; AG, arachidonoyl glycerol; DEA, docosatetraenyl ethanolamide; DGLEA, dihomo-gamma-linolenoyl ethanolamide; DH, dehydrogenase; DHEA, docosahexaenoyl ethanolamide; DiHDoPA, dihydroxydocosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHO, dihydroxyoctadecanoic acid; DiHODE, dihydroxyoctadecadienoic acid; DiHOME, dihydroxyoctadecenoic acid; Elov, fatty acid elongase; Ep-KODE, epoxyoxooctadecenoic acid; EpETE, epoxyeicosatetraenoic acid; EpODE, epoxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; F2-IsoP, F2 isoprostanes; FAAH, fatty acid amide hydrolase; Fads, fattyaciddesaturase; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTE, hydroxyoctadecatrienoic acid; HpODE, hydroperoxyoctadecadienoic acid; KETE, keto-eicosatetraenoic; KODE, keto-octadecadienoic acid;

LEA, Linoleyl ethanolamine; LG, linoleoylglycerol; LOX, lipoxygenase; NA-Gly, arachidonylglycine; NAPES, N-acylphosphatidyl ethanolamine-specific; NO-Gly; OEA, oleoyl ethanolamine; OG, oleoylglycerol; PEA, palmitoyl ethanolamine; PGE, prostaglandin E; PGF, prostaglandin F; POEA, palmitoleoyl ethanolamide; SEA, stearoyl ethanolamide; sEH, soluble epoxide hydrolase; TriHOME, trihydroxyoctadecaenoic acid; TXB, thromboxane.

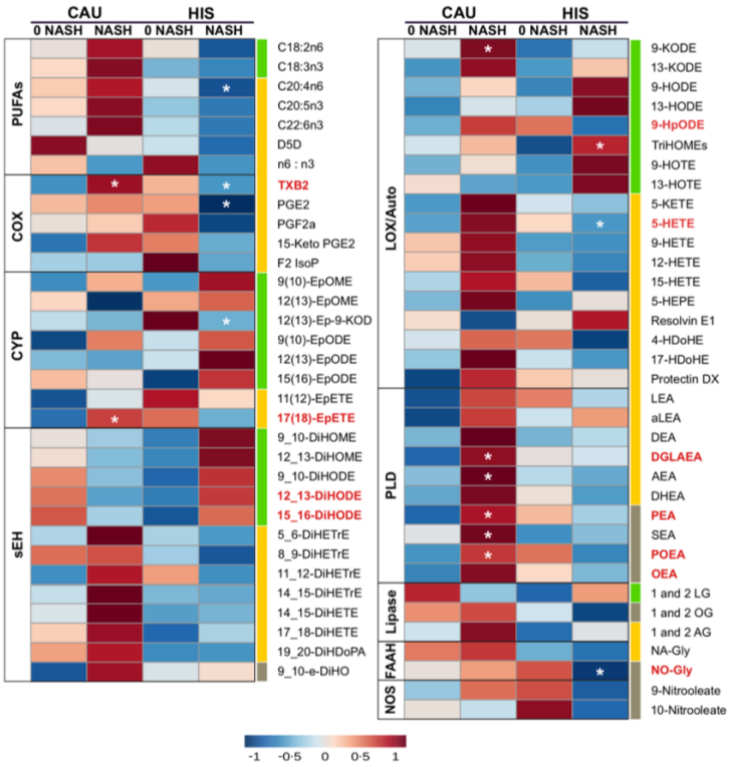

Figure 2. Heatmap illustrating fold changes and differences in plasma polyunsaturated fatty acids (PUFAs) and lipid mediators between non-alcoholic steatohepatitis (NASH) compared to NASH-free (0-NASH) in primary cohort. Fold changes are indicated by color and intensity, with red indicating an increase, and blue indicating a decrease. Lipids different in NASH vs. 0-NASH (t-test raw $p < 0.05$) are noted by (*). Ethnicity-related differences, or interaction (ethnicity x NASH) (ANCOVA raw $p < 0.05$) are marked with bold red color. C18 PUFAs and related lipids are marked with "green"; long chain-PUFAs and related lipids are marked with "yellow"; saturated fatty acids (SFA) and monounsaturated fatty acid (MUFA) related lipids are marked with "grey". Means and p-values are detailed in Table S3. Fatty acids are described by number of carbons and double bounds of the fatty acyl moiety (i.e., C18:2n6). NASH (n = 6 Hispanic (HIS) and 3 Caucasian (CAU); 0-NASH (n = 4 HIS and 5 CAU). ADH, alcohol dehydrogenase; AEA, arachidonoyl ethanolamine; AG, arachidonoyl glycerol; DEA, docosatetraenyl ethanolamide; DGLEA, dihomo-gamma-linolenoyl ethanolamide; DH, dehydrogenase; DHEA, docosahexaenoyl ethanolamide; DiHDoPA, dihydroxydocosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHO, dihydroxyoctadecanoic acid; DiHODE, dihydroxyoctadecadienoic acid; DiHOME, dihydroxyoctadecenoic acid; Elov, fatty acid elongase; Ep-KODE, epoxyoxooctadecenoic acid; EpETE, epoxyeicosatetraenoic acid; EpODE, epoxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; F2-IsoP, F2 isoprostanes; FAAH, fatty acid amide hydrolase; Fads, fattyaciddesaturase; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTE, hydroxyoctadecatrienoic acid; HpODE, hydroperoxyoctadecadienoic acid; KETE, keto-eicosatetraenoic; KODE, keto-octadecadienoic acid; LEA, Linoleyl ethanolamine; LG, linoleoylglycerol; LOX, lipoxygenase; NA-Gly, arachidonylglycine; NAPES, N-acylphosphatidyl ethanolamine-specific; NO-Gly; OEA, oleoyl ethanolamine; OG, oleoylglycerol; PEA, palmitoyl ethanolamine; PGE, prostaglandin E; PGF, prostaglandin F; POEA, palmitoleoyl ethanolamide; SEA, stearoyl ethanolamide; sEH, soluble epoxide hydrolase; TriHOME, trihydroxyoctadecaenoic acid; TXB, thromboxane

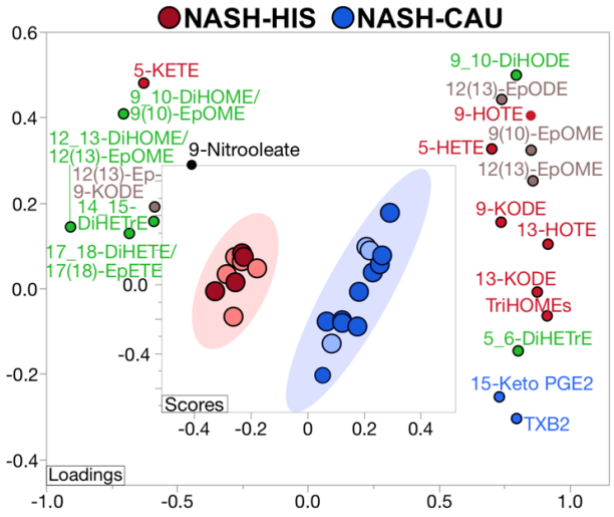

Figure 3. Supervised multivariate clustering model demonstrating ethnicity specific oxylipins profile discriminates Hispanic (HIS) and Caucasian (CAU) with non-alcoholic steatohepatitis (NASH) without advanced fibrosis. Score plot for NASH-HIS (red) vs. NASH-CAU (blue) when excluding stage 3 and 4 fibrosis. Light color represents subjects from primary cohort and dark color represents secondary cohort. The model was performed on all profiled lipids. Lipid mediators are colored according to metabolizing enzyme pathways, lipoxygenase, and autoxidation (red); cytochrome p450 epoxygenase (brown); and soluble epoxide hydrolase (green); cyclooxygenase (blue); other (black). The model was validated with leave-one-out cross validation. The Q^2 and R^2 are 0.99 and 0.98, respectively. Details on variable importance into the projection scores are shown in Table S4. NASH (*n* = 9 HIS and 13 CAU). DiHETE, dihydroxyeicosatetraenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHODE, dihydroxyoctadecadienoic acid; DiHOME, dihydroxyoctadecenoic acid; Ep-KODE, epoxyoxooctadecenoic acid; EpETE, epoxyeicosatetraenoic acid; EpODE, epoxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; HETE, hydroxyeicosatetraenoic acid; HOTE, hydroxyoctadecatrienoic acid; KETE, keto-eicosatetraenoic; KODE, keto-octadecadienoic acid; PGE, prostaglandin E; TriHOME, trihydroxyoctadecaenoic acid; TXB, thromboxane.

9. Supplementary materials

Table S1: Targeted quantification data table for comparisons of HIS and CAU obese subjects with NAFL vs. lean HC.

Table S2: Demographic, clinical, and histological characteristics of histology-matched subjects. Table S3: Targeted quantification data table for comparisons of HIS and CAU with NASH vs. 0-NASH.

Table S4: Targeted quantification data table for comparisons of HIS and CAU with NASH in the secondary analysis.

Table S5: Pearson's correlation analysis between LOX metabolites and markers of oxidative stress.

Table S6: Details on detected lipid class and identifiers.

Figure S1: Venn-Diagram illustrating overlapping and unshared differences in polyunsaturated fatty acids (PUFAs) and lipid mediators between non-alcoholic fatty liver (NAFL) compared to healthy control (HC) in both ethnicities.

Figure S2: Differences in plasma PUFA and lipid mediators between Hispanic (HIS) and Caucasian (CAU) in lean healthy subjects.

Figure S3: Supervised multivariate clustering model demonstrating ethnicity specific oxylipins profile discriminates Hispanic (HIS) and Caucasian (CAU) with non-alcoholic steatohepatitis (NASH) with advanced fibrosis.

Figure S4: Flow chart illustrating subject recruitment details for primary and secondary analysis. Figure S5: Principal component analysis (PCA) illustrating outliers before (left) and after (right) data normalization.

Figure S6: Principal component analysis (PCA) illustrating the unsupervised clustering of samples from primary (red) and secondary cohort (green) shown before (left) and after (right) batch correction.