Digestive and Liver Disease 56 (2024) 2103–2110

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

Metabolic signatures of metabolic dysfunction-associated steatotic liver disease in severely obese patients

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ARTICLE INFO

Article history:
Received 6 February 2024
Accepted 13 May 2024
Available online 1 June 2024

Keywords:
Non-targeted metabolomics
non-alcoholic fatty liver disease (NAFLD)
hepatic fibrosis

ABSTRACT

Backround: Metabolic dysfunction-associated steatotic liver disease (MASLD) can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Still, most patients with MASLD die from cardiovascular diseases indicating metabolic alterations related to both liver and cardiovascular pathology.
Aims and Methods: The aim of this study was to assess biologic pathways behind MASLD progression from steatosis to metabolic dysfunction-associated steatohepatitis (MASH) using non-targeted liquid chromatography-mass spectrometry analysis in 106 severely obese individuals (78 women, mean age 47.7 7 ± 9.2 years, body mass index 41.8 ± 4.3 kg/m^2) undergoing laparoscopic Roux-en-Y gastric bypass.
Results: We identified several metabolites that are associated with MASLD progression. Most importantly, we observed a decrease of lysophosphatidylcholines LPC(18:2), LPC(18:3), and LPC(20:3) and increase of xanthine when comparing those with steatosis to those with MASH. We found that indole propionic acid and threonine were negatively correlated to fibrosis, but not with the metabolic disturbances associated with cardiovascular risk. Xanthine, ketoleucine, and tryptophan were positively correlated to lobular inflammation and ballooning but also with insulin resistance, and dyslipidemia, respectively. The results did not change when taking into account the most important genetic risk factors of MASLD.
Conclusions: Our findings suggest that there are several separate biological pathways, some of them independent of insulin resistance and dyslipidemia, associating with MASLD.

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously called non-alcoholic fatty liver disease (NAFLD), refers to a spectrum of liver diseases characterized by an excess accumulation of fat in the liver [1]. The disease presents primarily as steatosis in the liver characterized by the accumulation of triglycerides (TG) in more than 5 % of the liver cells [2]. Currently, MASLD is the most common cause of chronic liver disease, with an estimated global prevalence of 32 % [3]. In some subjects with MASLD, the disease can progress to metabolic dysfunction-associated steatohepatitis (MASH), previously known as non-alcoholic steatohepatitis (NASH), where the fat accumulation in the liver is accompanied by lobular inflammation and hepatocellular ballooning [4]. A recent meta-analysis reported prevalence of MASH as high as 16 % among patients with MASLD [5]. Furthermore, in some subjects, the development of scar tissue (i.e. fibrosis) occurs, which can further lead to liver cirrhosis. Ultimately the disease can lead to hepatocellular carcinoma (HCC) or death [6,7].

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https://doi.org/10.1016/j.dld.2024.05.015

The etiology of MASLD is multifactorial and is known to be linked to a complex gene-environment interaction leading to a dysfunctional metabolic state [8,9]. The significant risk factors include sedentary lifestyle, dietary factors, abnormal glucose metabolism, insulin resistance, type 2 diabetes (T2D), obesity, dyslipidemia, and gut microbial dysbiosis [8,10–13]. Single nucleotide polymorphisms, such as G allele at rs738409 in patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), T allele at rs641738 in membrane bound O-acyltransferase domain containing 7 (*MBOAT7*), T allele at rs780094 in glucokinase regulator (*GCKR*) and T allele at rs58542926 in transmembrane 6 superfamily member 2 (*TM6SF2*) also contribute to the risk of development and progression of MASLD [8,10–12]. However, the exact molecular events leading to both the development and progression of MASLD are still not fully understood.

Currently, MASLD can be diagnosed with non-invasive methods such as ultrasound or magnetic resonance imaging [14,15], but no reliable non-invasive method to detect those with MASH exists [16]. At the moment, there is no curative treatment for steatotic liver or steatohepatitis except for losing excess weight [17–21] or to exercise [22]. To be able to develop medical treatment options, it is crucial to understand the biological mechanisms behind the pathogenesis of MASLD. The use of non-targeted metabolite profiling could aid in providing a comprehensive view of the metabolic changes and biological pathways associated with the disease pathogenesis [23,24] and ultimately elucidating why some people develop steatosis while in others, the disease progresses to more aggressive steatohepatitis.

This study investigated the serum metabolite profiles across the spectrum of MASLD using non-targeted metabolomics in severely obese patients. Furthermore, the correlations of identified metabolites with specific histological features of MASLD and with metabolic parameters related to MASLD were also explored to further investigate the metabolic pathways specifically associated with MASLD.

2. Methods

2.1. Study population

All patients undergoing obesity surgery in Kuopio University Hospital are recruited into our ongoing Kuopio Obesity Surgery Study (KOBS) investigating the metabolic consequences of obesity surgery. Criteria for the surgery are either body mass index (BMI) greater than 40 kg/m^2 or greater than 35 kg/m^2 with a significant comorbidity (e.g., T2D or hypertension) and failure of previous conservative treatments including lifestyle changes and medications to alleviate obesity. A total of 106 individuals (78 women) with mean age 47.7 ± 9.2 years, BMI 41.2 ± 4.3 kg/m^2 undergoing laparoscopic Roux-en-Y gastric bypass (LRYGB) for the treatment of severe obesity were selected based on the availability of fasting serum samples and liver histological characterization based on the steatosis, activity, fibrosis (SAF) score [25].

Clinical characteristics, possible diseases, and the use of medications were recorded during an out-patient visit before the surgery. Exclusion criteria included alcohol consumption >20 g/day, chronic hepatitis B and C (HBV and HCV) and hemochromatosis. HBV and HCV were excluded using serology if transaminases values were elevated prior to surgery. All patients were instructed to follow a very low-calorie diet (VLCD) for 4 weeks before the operation to achieve some weight-loss and make the surgical operation technically easier. All blood samples were collected after 12 h of fasting. Plasma glucose, insulin and serum lipid levels were defined as previously described [26]. The study was performed in accordance with the Declaration of Helsinki. An informed written consent was obtained from all participants. The study protocol was approved by the Ethics Committee of the Northern Savo Hospital District (54/2005, 104/2008, 27/2010 and 1108/2018).

2.2. Liver histology

Wedge liver biopsies were obtained during the elective LRYGB using Harmonic ultrasound scalpel. One experienced liver pathologist assessed all the liver samples using the SAF scoring system. The SAF system evaluates liver cell injury and inflammation equally and describes ballooning grades on morphological and semi-quantitative basis [27]. It comprises of three semi-quantitively evaluated histological features of MASLD and especially steatosis and inflammation. In addition, it allows characterizing cases that do not meet the criteria of distinct simple steatosis or have for example also mild fibrosis [25].

2.3. Non-targeted metabolomics

2.3.1. Sample preparation and liquid chromatography-mass spectrometry (LC-MS) analysis

Serum samples were prepared and analyzed as previously described [28]. Briefly, 100 µL of the sample was added to 400 µL of acetonitrile followed by filter centrifugation at 16,000 *g* for 10 min using 0.2 µm 96-well plate PTFE filters. Aliquots of 2 µL from at least half of the samples were mixed in one tube and used as the quality control (QC) samples. Liquid chromatography-mass spectrometry (LC-MS) analysis in reversed phase and hydrophilic interaction chromatography was performed using a 1290 Infinity Binary ultra-performance liquid chromatography coupled with a 6540 UHD Accurate-Mass quadrupole time-of-flight MS (Agilent Technologies, Santa Clara, California, USA). After each chromatographic separation, the ionization was carried out using jet stream electrospray ionization in the positive and negative modes, yielding four data files per sample. The high accuracy mass spectrometry full scan data was collected over a mass range of 50 to 1600 amu. For compatibility with the spectral databases, the product ion spectrum (MS/MS) was collected with collision energies of 10, 20, and 40 V. QC samples were injected at the beginning of the analysis and after every 12 samples thereafter.

2.4. Data analysis

The raw data files from the instrument were converted to ABF format using Reifycs Abf Converter (https://www.reifycs.com/AbfConverter). Thereafter, MS-DIAL was employed for automated peak picking and alignment [29]. For the peak collection, *m/z* values between 50 and 1500, and all retention times were considered. The amplitude of minimum peak height was set at 2000. The peaks were detected using the linear weighted moving average algorithm. For the alignment of the peaks across samples, the retention time tolerance was 0.1 min. The other parameters were set according to Klåvus et al. [30].

Data pre-processing was performed using notame R package after combining the data from all four modes. Briefly, signals present in less than 80 % of the samples and with a detection rate less than 70 % of the pooled QC samples were excluded. Thereafter, they were corrected for intensity drift. After drift correction, QC samples were removed, and low-quality signals were flagged, and the missing values were imputed using simple imputation with value of 1 for all features.

After preprocessing, Welch's pairwise *t*-test was used for identifying features differing between SAF groups. For multivariate model analysis, a random forest model was chosen. Spearman's correlation was used to analyze the associations between the metabolites and metabolic and genetic features. Metabolites were

Table 1
Clinical characteristics and measurements of the study population.

	Normal liver	Steatosis	MASH	P[1]
Total n (female/%)	49 (39/79.6)	35 (22/62.9)	22 (17/77.3)	0.989*
Age (years)	47.39±9.32	46.91±9.30	49.64±8.86	0.434
BMI (kg/m^2)	41.68±4.29	41.54±4.55	42.26±3.84	0.665
T2D n (%)	5 (10.2)	10 (28.6)	13 (59.1)	<0.001
fS-total cholesterol (mmol/l)	4.00±1.47	4.22±1.22	3.51±1.61	0.313
fS-LDL cholesterol (mmol/l)	2.23±1.03	2.35±1.12	1.88±0.95	0.312
fS-HDL cholesterol (mmol/l)	1.19±0.47	1.10±0.31	1.06±0.52	0.202
fS-Triglycerides	1.28±0.68	1.54±0.81	1.26±0.77	0.771
fP-Glucose	5.69±1.66	6.77±2.03	6.59±1.91	0.023
fS-Insulin	14.31±7.94	21.55±14.69	19.71±9.21	0.017
PNPLA3 rs738409 (%)	CC 32 (76.2)	CC 21 (63.6)	CC12 (54.5)	0.409*
	CG 8 (19.0)	CG 11 (33.3)	CG 9 (40.9)	
	GG 2 (4.8)	GG 1 (3.0)	GG 1 (4.5)	
MBOAT7 rs641738 (%)	CC 11 (26.8)	CC 15 (45.5)	CC 14 (63.6)	0.068*
	CT 19 (46.3)	CT 12 (36.4)	CT 4 (18.2)	
	TT 11 (26.8)	TT 6 (18.2)	TT 4 (18.2)	
TM6SF2 rs58542926 (%)	CC 38 (92.7)	CC 25 (75.8)	CC 19 (86.4)	0.274*
	CT 3 (7.3)	CT 7 (21.2)	CT 3 (13.6)	
	TT 0 (0.0)	TT 1 (3.0)	TT 0 (0.0)	

BMI, body mass index; MASH; metabolic dysfunction-associated steatohepatitis, fS, fasting serum; fP, fasting plasma; T2D, type 2 diabetes. Values are means ± standard deviation or number and percentage.
[1] One-way ANOVA,.
* Chi-square test.

chosen for the correlation analysis based on results of Welch's pairwise *t*-test.

2.5. Metabolite identification

All molecular features with a raw p-value less than 0.05 and with an abundance higher than 10,000 were chosen for annotation. Using MS-DIAL [29], the exact *m/z*, retention time, and MS/MS fragmentation patterns of the features were compared against the in-house standard library. Further, additional searches in online MS spectral databases were also performed [31–33]. Additionally, MS-FINDER Version 3.52 was used to characterize the unknown metabolite features [34].

2.6. Visualisations

All multivariate analyses were performed in R version 4.2.1. Partial least square discriminant analysis (PLS-DA) and principal component analysis (PCA) were performed on all the good quality features in the raw data after removing the quality control samples using mixOmics package [35]. Heatmaps representing the Spearman's correlation of the annotated metabolites with clinical parameters and selected genetic polymorphisms were plotted. Volcano plots of the fold change and p-values were created for each pairwise comparison.

3. Results

3.1. Clinical characteristics of the study population

The study cohort included a total of 106 severely obese patients. Their demographic features and clinical parameters according to the SAF score are shown in Table 1. There were no statistically significant differences in sex distribution, age, BMI, and serum lipids between the SAF groups. However, there were less subjects with T2D in those with normal liver compared to those with steatosis and MASH ($p < 0.001$).

3.2. Serum metabolomic profiles differed across the MASLD spectrum

The PLS-DA revealed that patients with steatosis and MASH differed from those with normal liver (Supplement Figure 1). Steatosis and MASH groups did not have any clear differences in PLS-DA. Moreover, PCA did not reveal any distinct separation based on the sex (Supplementary Figure 2). Using pairwise *t*-test, 7 metabolites were found to be significantly different between those with normal liver and those with steatosis (Fig. 1a, Supplement Figure 3, and Supplementary Table 1). There were 6 individual metabolites that clearly discriminated between individuals with steatosis and MASH (Fig. 1b for all, Fig. 2 box plots for annotated metabolites, and Supplementary Table 1). All of the metabolites differing between these two groups except xanthine were lower in those with MASH compared to those with steatosis (Fig. 1b, Fig. 2, and Supplementary Table 1). Interestingly, none of the annotated metabolites which differed between steatosis and MASH showed significant difference between normal liver and steatosis (Fig. 1a, Fig. 2, and Supplementary Table 1). A total of 18 metabolites were different between normal liver and MASH as shown in Fig. 1c, Supplement Figure 3 and Supplementary Table 1.

We selected all those 24 metabolites, which were significantly different in pairwise *t*-tests between normal liver, steatosis, and MASH to further analyze their correlations (Spearman) with histological characteristics of MASH (Fig. 3, left panel). From the metabolites that differed between normal liver and MASH and steatosis and MASH, indole propionic acid (IPA) and threonine correlated negatively with fibrosis (Fig. 3). In addition, lysophosphatidylcholines LPC(18:2), LPC(18:3) and LPC(20:3) correlated negatively with lobular inflammation (Fig. 3). Xanthine correlated positively with lobular inflammation and ballooning (Fig. 3).

As MASLD is closely associated with metabolic syndrome, we further explored the correlations between differential metabolites and clinical markers of insulin resistance and dyslipidemia to rule out the possibility that the difference in metabolite concentration between steatosis and MASH could be primarily linked to metabolic disturbances related to cardiovascular risk (Fig. 3, right panel). We used K- means clustering to group the metabolites after the correlation analysis. Cluster 1 contained xanthine and phosphatidylcholine PC(32:1), both of which were positively linked to MASH. PC(32:1) correlated positively with insulin and total cholesterol while xanthine associated positively with LDL and triglycerides and negatively with HDL (Fig. 3). Cluster 2 contained several LPCs and lysophosphatidylethanolamine LPE(20:4)

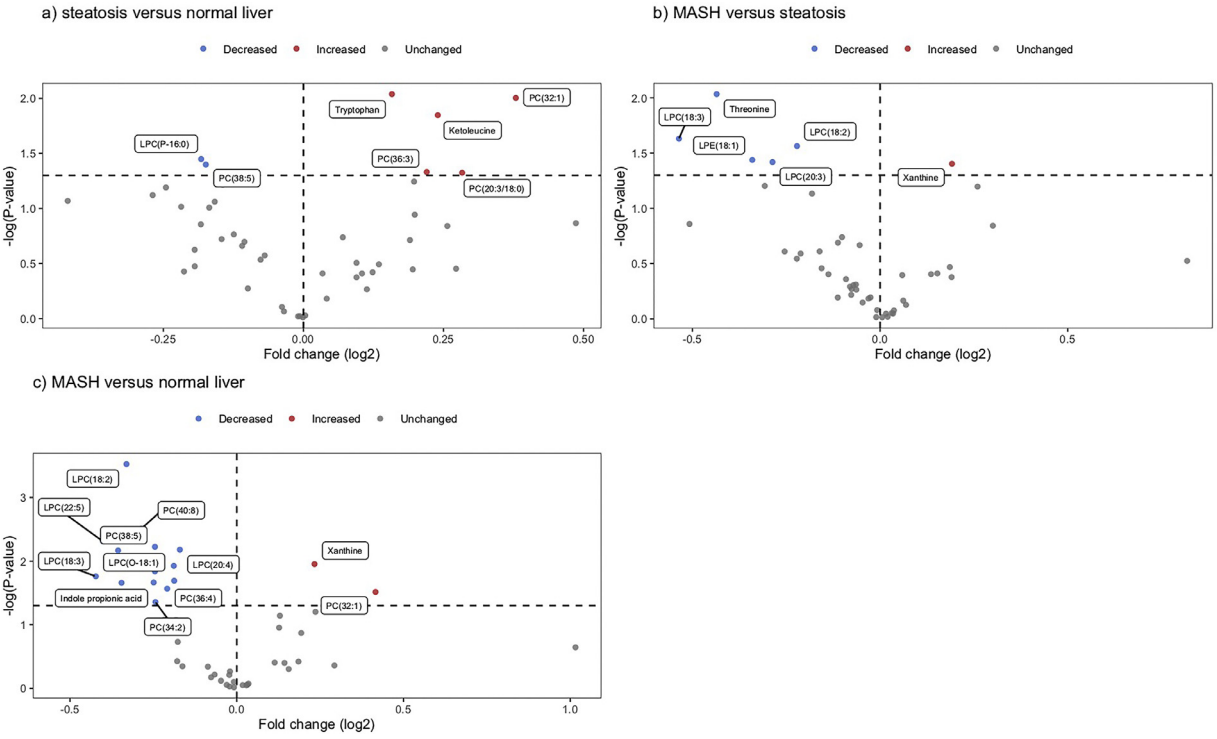

Fig. 1. Annotated serum metabolites across MASLD spectrum. X axis represents the log 2 fold change of each identified metabolite (Identification level 1 and 2), and Y axis represents the -log10 transformation of the p values. Increased metabolites are depicted in red, decreased in blue, and non-significant in grey. The raw p-value <0.05 was considered significant. a) Normal liver versus steatosis b) steatosis versus MASH c) normal liver versus MASH, PC; phosphatidylcholine, LPC; lyso-PC, LPE; lysophosphatidylethanolamine.

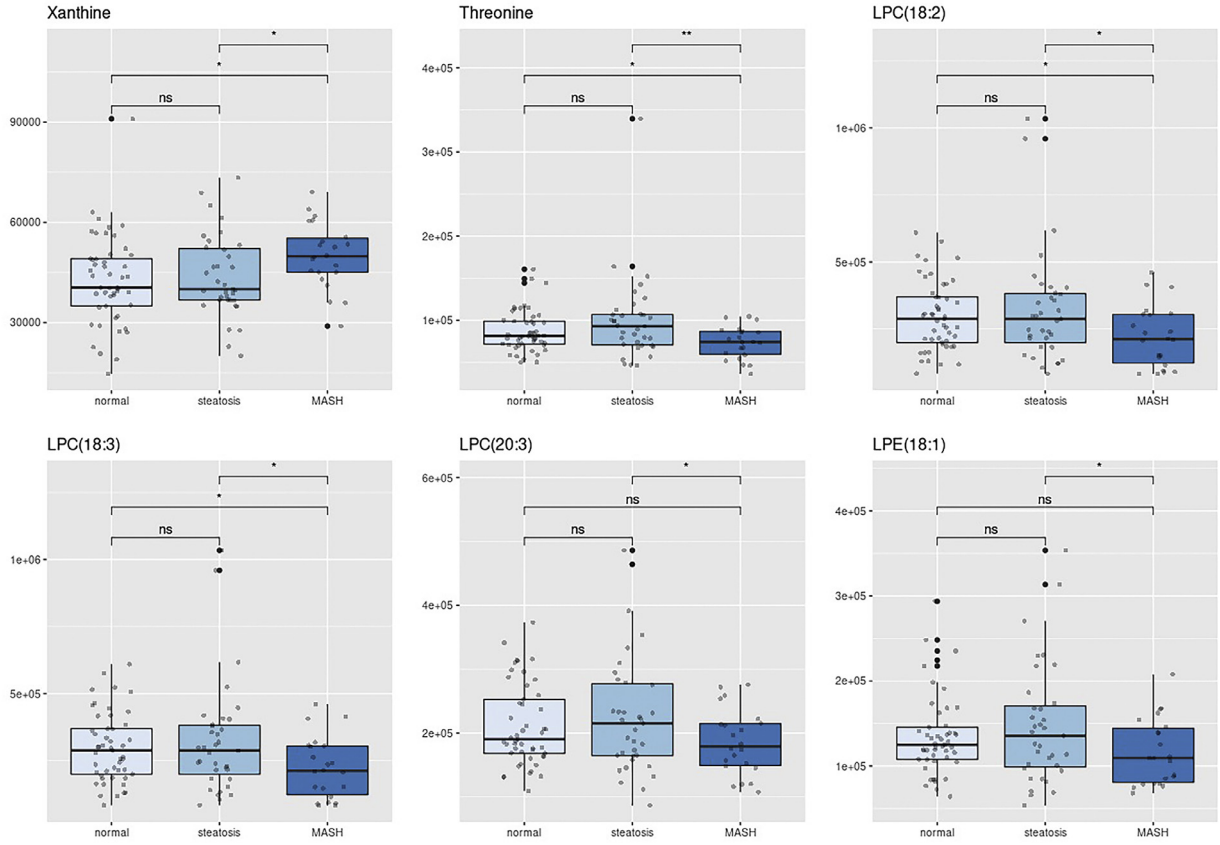

Fig. 2. Boxplots representing the metabolites that differed significantly between steatosis and MASH. Welch's *t*-test used for statistics. LPC; lysophosphatidylcholine, LPE; lysophospatidylethanolamine.

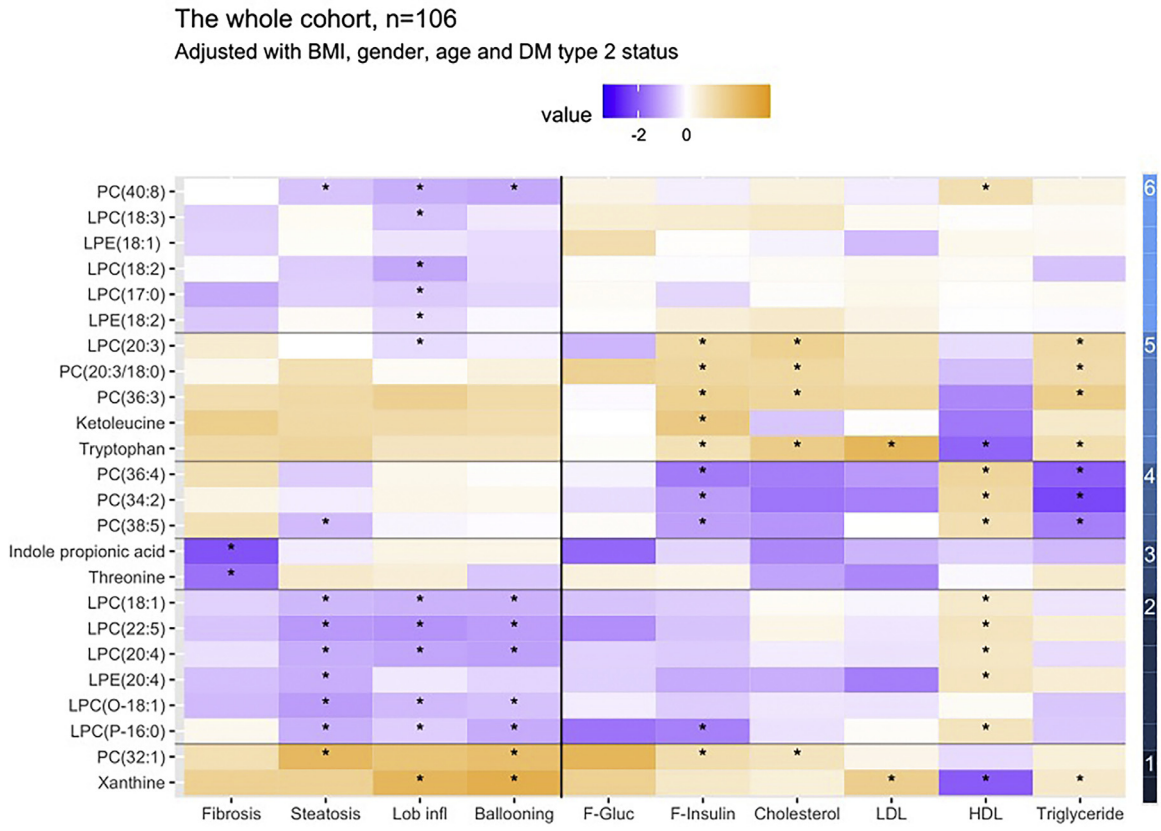

Fig. 3. Heatmap representing the Spearman's correlation (adjusted for age, gender, BMI and T2D status) and K means clustering between significantly different metabolites across MASLD spectrum (row-wise) and detailed liver histology (left side of the panel) as well as metabolic disturbances with K means clustering (right side of the panel). The color of the cells indicates the strength of the relationship (r_s). Statistically significant correlations ($p < 0.05$) are marked with an asterisk (*).BMI, body mass index; DM type 2; type 2 diabetes mellitus, PC; phosphatidylcholine, LPC; lyso-PC, LPE; lysophospatidylethanolamine, IPA; indole propionic acid, Lob infl; lobular inflammation, fP-Gluc; fasting plasma glucose level, fS-insulin; fasting serum insulin level, Col; serum total cholesterol level, LDL; low-density lipoprotein concentration in serum, HDL; high-density lipoprotein concentration in serum, Trigly; serum triglyceride level.

which were negatively associated with steatosis and MASH and positively with HDL (Fig. 3). Cluster 3 included IPA and threonine which were negatively associated with fibrosis, but interestingly did not correlate with any of the metabolic markers (Fig. 3). Cluster 6 contained PC(40:8) and several LPCs and LPE(18:1) and (18:2), which correlated negatively with liver inflammation, but did not correlate with metabolic markers (Fig. 3). The correlation analyses conducted by sex revealed that most LPCs in clusters 2 and 6 correlated negatively with lobular inflammation in females, whereas among males, they displayed a negative correlation with steatosis (Supplementary Figure 4a and 4b).

Clusters 4 and 5 represent metabolites that could be linked to metabolic phenotypes related to MASH, but not to all the histological characteristics of MASH. In cluster 4, there were several PCs that correlated negatively with fasting insulin and triglycerides and positively with HDL (Fig. 3). Cluster 5 represented the insulin resistant group in which tryptophan, ketoleucine, and some PCs correlated positively with fasting insulin, cholesterol and triglyceride levels (Fig. 3).

Similarly, we conducted the correlation analysis with several known genetic risk variants of MASLD (Supplement Figure 5). Overall, the correlations were much weaker between genotypes and metabolites as compared to those between e.g., histology and metabolites. Xanthine correlated negatively with *MBOAT7* rs641738 and LPC(18:3), LPE(18:1), and PC(20:3/18:0) with *PNPLA3* rs738409 (Supplement Figure 3). Adjusting the principal correlation analysis with *PNPLA3* rs738409, *TM6SF2* rs58542926 or *MBOAT7* rs641738, risk genotypes did not alter the results significantly (Supplement Figures 6–8).

4. Discussion

Our aim was to explore metabolic differences across MASLD spectrum especially focusing on their interplay with metabolic disturbances related to cardiovascular risk. The reason for this approach is that metabolites, which associate both with liver disease and metabolic disturbances might be mostly reflecting the metabolic disturbances associated with MASLD and are not reflecting the disease progression. Using metabolomics approach in severely obese patients, we found several metabolites, which associate with MASLD, but reflect different processes in the liver and whole-body metabolism. Importantly we also found metabolites, which were linked to liver disease, but not to metabolic disturbances.

In this study, IPA and threonine (Cluster 4) correlated negatively with liver fibrosis, which is the most important prognostic feature in MASLD as it associates independently with long-term outcomes and mortality in patients with MASLD [36]. IPA level was higher in patients with normal liver compared to those with MASH. IPA is produced from tryptophan by gut microbiota by the indole pathway [37,38] and it has been suggested to be beneficial/protective metabolite to cardiovascular disease [39], obesity, T2D, MASLD, and liver fibrosis [40]. In addition, tryptophan and kyneurine pathway (another major pathway for tryptophan metabolism) metabolites have been associated with disadvantageous effects to cardiovascu-

lar health [41,42]. Indeed, in our study tryptophan correlated with dyslipidemia. As IPA is produced by the gut microbiota, unfavorable alterations in gut microbiota could lead to a decrease in the production of IPA [43]. IPA is known to inhibit the production of endotoxins in the bowel and reduce the levels of proinflammatory cytokines in macrophages to repress inflammation in hepatocytes [43]. Overall, our results support findings about the hepatoprotective role of IPA.

We observed decreased levels of threonine in those with MASH compared to those with steatosis and those with normal liver, and threonine correlated also negatively with fibrosis. Threonine is an essential amino acid, which is mostly used as a substrate for protein synthesis or tricarboxylic acid cycle [44]. Previously circulating threonine levels have been reported to be unchanged in relation to MASLD progression in humans [45,46]. However, dietary threonine supplementation has been reported to reduce weight and improve insulin sensitivity in obese mice [47]. Threonine deficiency in nutrition led to upregulation of genes related to fatty acid and triglyceride synthesis and downregulation of genes related to fatty acid oxidation and triglyceride transport in ducks [48]. Our results suggest that threonine deficiency might have a role in MASLD pathogenesis in humans, because it was decreased in those with MASH and fibrosis. However, further studies are warranted to verify this, especially since our study population contained several patients with T2D, which could have affected the results. However, threonine seems to reflect liver fibrosis in MASLD.

Xanthine was increased in study participants with MASH when compared to those with steatosis. Not surprisingly, it also had a positive correlation with lobular inflammation and ballooning. Xanthine correlated positively also with dyslipidemia, highlighting its reported association with increased risk of developing hyperlipidemia and cardiovascular disease as reported earlier [49,50]. A possible mechanism for this is that xanthine is metabolized to uric acid, and it can cause oxidative stress in the liver [49]. Increase of uric acid is associated with increased TG content in the liver, increased hepatic fatty acid synthesis [51] and inducing inflammation via nuclear factor-KB [49]. Xanthine oxidase is proposed to be the link between hyperuricemia and MASLD [52]. Xanthine is a product of fructose metabolism in the liver and previous studies have linked excess fructose intake to MASLD [49,53]. Unfortunately, we were not able to measure serum fructose levels in this cohort.

LPCs are suggested to be important mediators of hepatic lipotoxicity [54]. In line with that, LPCs have been reported to be increased in liver in those with MASH [55,56], but the results in plasma and serum samples in patients with MASLD have been contradictory [57–59]. In our cohort, LPCs were present in clusters 2 and 6 which were negatively associated with MASH. This suggests that these LPCs may have a hepatoprotective role. Accordingly, blood levels of LPC(18:1), LPC(18:2), and LPC(20:3) have been reported to be lower in those with normal liver compared to those with steatosis or MASH [58,60].

In addition, cluster 2 contained plasmalogens, which have earlier been reported to be either increased or decreased in patients with MASH compared to those with steatosis [61]. In this study LPC(O-18:1) and LPC(P-16:0) correlated negatively with steatosis, ballooning, and lobular inflammation. LPC(O-18:1) was decreased in patients with MASH compared to those with normal liver and LPC(P-16:0) in patients with steatosis compared to those with normal liver. Decrease of circulating LPEs in patients with steatosis and MASH compared to healthy controls has previously been suggested as a biomarker and a therapeutic target for MASLD [57,62]. LPE(18:1), LPE(18:2) and LPE(20:4) have been reported to be lower in patients with steatosis and MASH compared to those with healthy liver [63]. Our results are in line with these observations. Interestingly, LPCs and LPE(20:4) in cluster 2 were negatively associated with inflammation, steatosis and ballooning in liver and positively with HDL, possibly reflecting favorable signs in lipoprotein metabolism. Cluster 6 did not correlate with any metabolic markers and only with lobular inflammation, which proposes that LPCs and LPEs could participate in different pathways regulating liver cell metabolism. Interestingly, in sex-specific correlation analyses these metabolites associated with steatosis and partly with ballooning in men and lobular inflammation in women. MASLD leads to cirrhosis and HCC more often in women, especially in elder women when the protective effect of estrogen is lost, and our results support the differences in MASLD based on sex [64]. However, our study size was limited, and thus further studies on this topic are needed.

Several genetic polymorphisms have also been associated with MASLD [8], but they did not have a major effect on our results. The G allele at rs738409 at the *PNPLA3* gene is the most significant and common gene variant associated with MASLD [65]. *PNPLA3* gene regulates lipid metabolism in hepatocytes [66] and the polymorphism increases the liver fat content and the risk of liver fibrosis [10]. In this study, PCs were decreased in liver in those who were homozygous for risk variant of *PNPLA3* [67]. LPCs are synthesized from PCs [68] and PCs can be synthetized from LPEs by methylation, which is an alternative route for the Kennedy pathway [69]. The negative correlations of LPC(18:3), LPE(18:1), and PC(20:3/18:0) with *PNPLA3* might therefore indicate that PNPLA3rs738409 genotype has an effect on PC synthesis via the Kennedy pathway. *MBOAT7* rs641738 gene variant also takes part in lipid metabolism in the liver regulating cell membrane phospholipids by decreasing toll-like receptor signaling [70]. Xanthine, which was increased in patients with MASLD and MASH compared to those with normal liver, also correlated negatively with *MBOAT7* rs641738 variant. Over-activation of toll-like receptors has been linked to more severe metabolic dysfunction in MASLD [70–73]. The observed negative correlation of xanthine with *MBOAT7*rs641738 could explain or reflect some of the decreased risk of cardiovascular disease associated with *MBOAT7*rs641738 variant [74].

There are several limitations to this study. First, all the patients in this study were severely obese. Therefore, the findings of this study might not be generalizable to overweight, normal, or lean individuals. However, our cohort offers a well-defined platform to study the whole spectrum of MASLD. Moreover, the participants followed VLCD for four weeks prior to sample collection. Rapid weight loss induced by VLCD has been connected to lower levels of serum glucose, choline and lipoproteins [75]. This could have some effect on results. Nevertheless, key metabolic differences between the study groups were observed in this study, and although four-week VLCD could reduce liver fat content, its effect on inflammation and fibrosis is weaker [76]. Furthermore, the sample size for this study was limited and therefore the results from this study need to be validated in larger cohorts. Additionally, the lack of complete MS/MS spectral libraries for annotation of all significant metabolites limited the metabolomics analysis, leaving a substantial number of features non-annotated. This calls for further research to investigate also these non-annotated metabolites and their role in MASLD. Moreover, it is important to note that this is a cross-sectional study and further research is needed on how the metabolite profile changes during progression of MASLD.

In conclusion, using metabolomics, we were able to identify several metabolites from different metabolic pathways associating with MASLD which may provide further understanding about the pathogenesis of the disease. Furthermore, identified metabolites had distinct associations with histological characteristics of MAFLD progressions and metabolic disturbances reflecting dyslipidemia and glucose intolerance. Importantly, we were able to link

several metabolites to the liver disease, but not to metabolic disturbances suggesting that the biological processes these metabolites represent may have divergent roles.

Author contributions

V.M. and J.P. designed the study and collected the clinical data. M.L. supervised the mass spectrometry analysis. A.F.B was involved in the pre-processing, statistical analysis, and annotation of the metabolomics data. K.H. supervised the metabolomics analysis. S.P. performed the statistical analysis of the metabolomics data. V.K. conducted the histological analysis of the liver biopsies. P.K. performed the LRYGB and collected the liver biopsies. A.F.B. and S.P. drafted the first version of the manuscript under the supervision of V.M, K.H., and J.P. All the authors have critically read, reviewed, and approved the final version of the manuscript.

Conflict of interest

The authors have no relevant conflict of interest to disclose. Ambrin Farizah Babu and Kati Hanhineva are employed by Afekta Technologies Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The mass spectrometry laboratory is supported by Biocenter Finland and Biocenter Kuopio.

Funding

This work was supported by the ITN Marie Curie BestTreat-Building a Gut Microbiome Engineering Toolbox for In Situ Therapeutic Treatments for Non-alcoholic Fatty Liver Disease (grant number 813781, A.F.B). K.H. is supported by Research Council of Finland (grant no. 321716) and ERA-NET NEURON (grant no. 334814). Kuopio Obesity Surgery Study was supported by the Finnish Diabetes Research Foundation, Kuopio University Hospital Project grant (EVO/VTR grants 2005-2021), the Academy of Finland grant (Contract no. 138006), the Finnish Cultural Foundation, and the University of Eastern Finland Spearhead Funding (J.P.). V.M. was supported by the Mary and Georg C. Ehrnrooth Foundation, Finnish Medical Foundation, Sigrid Jusélius Foundation, and Kuopio University Hospital Project grant (VTR grants).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dld.2024.05.015.

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