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# Utility of Metabolomic Biomarkers to Identify Nonalcoholic Fatty Liver Disease in Liver Transplant Recipients

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**Background.** Nonalcoholic fatty liver disease (NAFLD) is a rising indication for liver transplantation (LT). Identification of NAFLD recurrence and those at risk for more progressive disease after LT remains elusive as the diagnosis requires biopsy, which is invasive and impractical for serial monitoring. We therefore aimed to identify metabolites in the blood associated with recurrent NAFLD that could potentially be used for detection and monitoring. **Methods.** This cross-sectional pilot study included 37 LT recipients who underwent simultaneous liver biopsy and plasma collection for metabolomic analysis. Metabolic profiles were compared between patients with recurrent NAFLD, normal liver (negative control), and acute rejection (rejection control). **Results.** Univariate analysis revealed 14 metabolites that were significantly altered in patients with recurrence of NAFLD compared with negative controls and 19 compared with rejection controls ( $P < 0.05$ ). In addition, metabolomic profiling identified 16 metabolites that distinguished nonalcoholic fatty liver versus nonalcoholic steatohepatitis. Metabolite class trends among patients with recurrent NAFLD following LT were consistent with prior metabolomics data in patients with NAFLD in the non-LT setting. **Conclusions.** In conclusion, we identified candidate metabolites that could be used in the clinical setting to noninvasively identify recurrent NAFLD and differentiate NAFL from the more progressive nonalcoholic steatohepatitis. Further investigation with a larger sample size is warranted to validate these results.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver injury worldwide. NAFLD is composed of a spectrum of histologically defined stages of liver injury ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), in which inflammation and hepatocellular injury can ultimately lead to fibrosis, cirrhosis, and hepatocellular carcinoma. NASH is currently the most common indication for liver transplantation (LT) in women and adults over the age of 65 and is on pace to become the most common indication for LT overall in the wake of rising rates of metabolic syndrome and declining rates of hepatitis C virus.<sup>1,2</sup>

With survival rates increasing in the LT population, allograft-associated NAFLD is likely to impact an increasing number of LT recipients in the future. Studies have demonstrated evidence of recurrent or de novo hepatic steatosis in 10%–100%, and NASH in up to 36% of patients at 3–5 y post-LT.<sup>3–6</sup> Despite high rates of NAFLD recurrence, a paucity of diagnostic tools are available for reliable diagnosis and serial monitoring. Even more so than NASH before transplant, the development of post-LT NASH and its progression to advanced fibrosis can occur without concomitant liver enzyme abnormalities in the posttransplant setting.<sup>7</sup> High rates of recurrence in patients transplanted for NASH cirrhosis are linked to baseline metabolic dysregulation exacerbated by

the additive impact of immunosuppressive agents. Although liver enzymes may not reliably indicate allograft disease, the abnormal metabolic milieu can be reflected in altered blood metabolite profiles, which may facilitate the identification of recipients with or at high risk for recurrent NASH.

Currently, noninvasive detection of allograft NAFLD is limited to the identification of steatosis on liver imaging, although liver biopsy is needed to differentiate NAFL from NASH, identify the presence of mild to moderate fibrosis, and differentiate NAFLD from most other causes of liver dysfunction. Liver biopsy has drawbacks that limit routine utilization, such as invasiveness, cost, and susceptibility to sampling error or interpretation variability.<sup>8,9</sup> For these reasons, liver biopsy is neither a feasible nor desirable option for screening or for serial monitoring in most patients at risk for recurrent NASH. Imaging modalities such as transient elastography (TE) are widely used in the nontransplant setting to identify advanced fibrosis, although TE has limited accuracy in diagnosing NASH or lesser degrees of fibrosis.<sup>10</sup> In the LT population, the accuracy of TE to characterize disease recurrence, especially NAFLD, is even more limited.<sup>11</sup>

Biomarker panels, including metabolites, proteins, and nucleic acids, are all being investigated as a supplement to or replacement for liver biopsy. Current panels used to detect NAFLD are generally derived from direct and indirect measures of steatosis (eg, Fatty Liver Index and SteatoTest), NASH (eg, NASH test and OWLiver), fibrosis (eg, FIB-4 index, NAFLD fibrosis score, FibroTest, and ELF), or some combination of the above. These biomarker panels have shown promise in the nontransplant setting, although none have been validated for use in post-LT allograft-associated NAFLD.<sup>12-18</sup> Little is known about whether NASH in the posttransplant state has a unique biomarker signature because of altered metabolism from immunosuppressive agents, native graft disease, and increased insulin resistance.<sup>19</sup>

The aim of this novel exploratory study was to use an unbiased metabolomics approach to identify candidate metabolites capable of differentiating NAFLD from normal liver (NL) and other causes of liver dysfunction following LT. Once identified, future validation and confirmatory studies could lead to a biomarker panel for noninvasive monitoring of NAFLD recurrence.

## MATERIALS AND METHODS

### Study Design and Population

This was a cross-sectional study conducted at Northwestern Memorial Hospital (Chicago, IL) and included 37 LT recipients. Participants were recruited at presentation for medically indicated liver biopsy (n=26) or protocol-directed surveillance liver biopsy (n=11). Because of the unreliability of noninvasive tools to detect NAFLD recurrence in those transplanted for NASH, protocol liver biopsy was instituted in 2015 to monitor for recurrence in all patients transplanted for NASH  $\geq 5$  y post-LT. Recipients transplanted for NASH or a nonviral, nonalcohol-related indication were recruited at time of medically indicated liver biopsy. Transplant indication and resulting histology of the 37 included LT recipients is outlined in Table 1. Participants were stratified into 4 cohorts based on biopsy histology: (1) NAFL, (2) NASH, (3) NL, and (4) rejection control (RC). Normal and rejection biopsies were included to assess the ability of metabolite

**TABLE 1.**

**Participant transplant indication and cohort stratification based on post-LT histology**

Transplant indication	Biopsy histology				
	NAFLD	NASH	NL	RC	Total
NASH	8	5	7	9	29
Autoimmune	—	—	—	3	3
Miscellaneous non-NASH, nonautoimmune	—	—	—	5	5
Total	8	5	7	17	37

LT, liver transplantation; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NL, normal liver; RC, rejection control.

profiles to distinguish disease recurrence from normal histology as well as those with acute inflammation. Participants with histology not consistent with the previous 4 groups or clinical suspicion of alcohol contributing significantly to liver disease were excluded. The study was conducted according to guidelines and approved by the Institutional Review Board at Northwestern University.

### Sample Collection

Liver biopsy was obtained using a 16-gauge needle under guidance of ultrasound per clinical protocol. Liver biopsy specimens were immediately fixed in formalin and processed following standard institutional guidelines. All diagnoses were established histologically by trained liver pathologists. The histopathologic diagnosis of NASH was made by the presence of steatosis, lobular inflammation, and hepatocellular ballooning. NAFL diagnosis was defined as having steatosis  $\geq 5\%$  on liver biopsy in the absence of findings consistent with NASH.<sup>20</sup>

Clinical laboratory data were obtained under fasting conditions at the time of liver biopsy or the most recent value available before liver biopsy if the former were unavailable (Table 2). All blood specimens used in the metabolomic analysis were collected under fasting conditions immediately before liver biopsy. Supernatant aliquots were stored in a  $-80^{\circ}\text{C}$  freezer until being packaged with dry ice and shipped to One Way Liver Metabolomics (Derio, Spain) for metabolomic analysis.

### Metabolomic Analysis

Plasma samples were analyzed using ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS) to elucidate metabolomic profiles. Metabolite extraction process, chromatographic separation, and mass spectrometric detection conditions for each platform follow the procedure described by Barr et al.<sup>21</sup> Quality control procedures were used to ensure high-quality data for analyses. This study used 3 UHPLC-MS platforms to cover a wide range of metabolites in the plasma sample—broadly characterized into (1) fatty acids, bile acids, steroids, and lysoglycerophospholipids; (2) glycolipids, glycerophospholipids, sterol lipids, and sphingolipids; and (3) amino acids (AAs).

Data were preprocessed using the TargetLynx application manager for MassLynx 4.1 software (Waters Corp., Milford, MA).<sup>14</sup> Intra-batch and interbatch normalization was performed by inclusion of multiple internal standards and pool calibration response correction, following the procedure described by Martinez-Arranz et al.<sup>22</sup>

**TABLE 2.****Sociodemographic and clinical characteristics of study participants stratified by post-LT biopsy histology**

	Normal liver	NAFL/NASH	Rejection control
Count	7	13	17
Age, mean (SD)	66.2 (7.4)	64.4 (6.4)	56.3 (16.6)
Female sex, n (%)	3 (42.9)	6 (46.2)	6 (35.3)
Race/ethnicity, n (%)			
Non-Hispanic White	6 (85.7)	11 (84.6)	12 (70.6)
Hispanic	1 (14.3)	2 (15.4)	5 (29.4)
D from LT to biopsy (SD)	2002.9 (1320.6)	2344.2 (1200.5)	1752.2 (2097.8)
BMI, mean (SD)	32.5 (6.4)	35.2 (6.2)	27.7 (4.1)
Hypertension, n (%)	5 (71.4)	7 (53.8)	8 (47.1)
Diabetes mellitus, n (%)	4 (57.1)	8 (61.5)	7 (41.2)
Laboratory data, mean (SD)			
HBA1C (%)	7.0 (1.4)	6.9 (2.3)	6.3 (0.9)
ALT (U/L)	65.6 (64.4)	48.5 (50.9)	201.4 (160.4)
AST (U/L)	51.4 (54.5)	34.3 (23.4)	153.6 (135.2)
ALP (U/L)	126.7 (94.0)	112 (62.2)	368.4 (401.4)
TB (U/L)	0.6 (0.2)	0.62 (0.28)	2.1 (4.0)
Total cholesterol (mg/dL)	158.9 (54.5)	154.3 (54.2)	165.3 (34.9)
Triglycerides (mg/dL)	218.4 (126.4)	166.5 (91.4)	132.1 (47.0)
HDL-C (mg/dL)	38.3 (18.2)	38.8 (10.3)	49.6 (25.4)
LDL-C (mg/dL)	72.3 (45.6)	76.7 (39.6)	89.2 (26.6)
Immunosuppression, n (%) <sup>a</sup>			
Tacrolimus	5 (71.4)	9 (69.2)	13 (81.3)
Sirolimus	1 (14.3)	1 (7.7)	0 (0)
Everolimus	0 (0)	0 (0)	1 (6.3)
Cyclosporine agent	1 (14.3)	2 (15.4)	4 (25)
Mycophenolic acid	6 (85.7)	4 (30.8)	12 (75)
Corticosteroid	2 (28.6)	2 (15.4)	6 (37.5)
None	0 (0)	1 (7.7)	0 (0)
Cardiometabolic medications, n (%)			
Insulin	3 (23.1)	4 (57.1)	5 (29.4)
GLP-1	0 (0)	1 (14.3)	0 (0)
Sulfonylurea	0 (0)	0 (0)	1 (6.0)
Statin	5 (38.5)	3 (42.9)	3 (17.6)
CCB	6 (46.2)	4 (57.1)	8 (47.1)
Beta-blocker	6 (46.2)	5 (71.4)	6 (35.3)
ACE-I/ARB	4 (30.8)	1 (14.3)	5 (29.4)

None of these characteristics were statistically significantly different between groups.

<sup>a</sup>Immunosuppression data were not available for 1 participant (RC cohort).

ACE-I, angiotensin converting enzyme inhibitor; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CCB, calcium channel blocker; GLP-1, glucagon-like peptide 1; HBA1C, hemoglobin A1C; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LT, liver transplantation; NAFL/NASH, nonalcoholic fatty liver/nonalcoholic steatohepatitis; TB, total bilirubin.

### Statistical Analysis

Principal component analysis (PCA) was performed using SIMCA-P+ software package (version 14.1 Umetrics, Sweden). Univariate analyses were also performed calculating group percentage changes and *P* values using the Wilcoxon signed-rank test. Given the size limitation of the present study, no normality test of the distributions has been applied and instead a nonparametric test (Wilcoxon signed-rank) was utilized in order not to require any assumption about the distribution of the observed data. The following comparisons were made (1) NAFLD versus NL, (2) NAFLD versus RC, (3) RC versus NL, (4) NAFL versus NASH, and (5) fibrosis versus no fibrosis. These calculations were performed using the statistical software package R v.3.4.1 (R Development Core Team, 2017; <http://cran.r-project.org>).

A hierarchical clustering algorithm based on metabolite ion intensity was used to visualize the differences in metabolite signatures between samples, as well as the Ward's minimum variance method as agglomeration method. Metabolite data followed a normalization procedure that consisted of mean-centering and division by the SD of each variable. The maximum of the average of the individual silhouette widths was calculated for the clusters. Cluster analyses were calculated with SciPy library.<sup>23</sup> Heatmaps were realized with the Seaborn library v0.11.1.

## RESULTS

### Patient Characteristics

In total, 37 samples were included in the analysis and stratified into the following cohorts: (1) NL (n=7), (2) NAFL (n=8), (3) NASH (n=5), and (4) RC (n=17). All participants in the NL and NAFLD cohorts were transplanted for NASH cirrhosis, whereas the majority of patients in the RC cohort were transplanted for NASH as outlined in Table 1. The RC participants included in this study had generally higher aminotransferase levels compared with those with NL or NAFLD on liver biopsy [alanine aminotransferase (ALT) of 201.4 versus 65.6 versus 48.5 U/L, respectively]. Even though ALT and aspartate aminotransferase (AST) differences between cohorts were not statistically significant, this trend was likely due to many of the RC participants receiving liver biopsy for a clinical cause (such as elevated liver enzymes), whereas many of the patients with NAFLD histology had screening biopsies in the absence of clinical abnormalities. In addition, the RC cohort had a dramatically higher alkaline phosphatase (ALP) and total bilirubin level compared with the NL and NAFLD cohorts. However, it is worth noting that 1 participant in the RC cohort had a profound elevation in cholestatic markers (ALP 942, total bilirubin 17) and dramatically skewed the clinical characteristics in this cohort. Interestingly, those with recurrent NAFLD had trended toward lower ALT, AST, and triglyceride (TG) than those with a normal post-LT biopsy. However, neither this difference nor any of the remaining data presented in Table 2, including history of diabetes, triglycerides, and cardiometabolic medications, had a statistically significant difference between groups.

### Metabolomic Dataset and Sample Clustering

Plasma sample analysis resulted in the detection of 463 metabolites across 3 UHPLC-MS platforms (Table S1, SDC, <http://links.lww.com/TXD/A370>, <http://links.lww.com/TXD/A369>). Using methods described previously,<sup>21</sup> 29 of these metabolites were found to be outside their corresponding linear detection range and were not used for statistical analysis. The remaining 434 metabolites were included in the multivariate and univariate data analyses.

Multivariate PCA demonstrated clustering of samples as depicted by the score plots in Figure 1. Initial analysis demonstrated samples clustered according to the cause of transplant, as demonstrated by Figure 1A. The volcano plot in Figure 1B demonstrates the divergence in metabolic profiles when mapped by transplant cause (NASH versus non-NASH) and on further analysis, the majority of the differences were seen in the diglyceride and TG metabolite classes. Additionally, samples were plotted according to post-LT histology, which showed that RCs seemed to separate from other cohorts in certain component analyses, as shown by Figure 1C with the second component, t[2] (Figure 1C). To further investigate the relationship between

transplant cause and clustering of samples, we generated a PCA model using only patients transplanted for NASH and stratified by histology (Figure 1D), which resulted in a random distribution of samples. This suggested that the underlying transplant indication had a stronger influence on intergroup variability than the post-LT biopsy phenotype.

### Hierarchical Analysis

The hierarchical clustering analyses presented in Figures 2 to 4 demonstrate heatmaps of all detected metabolites and grouped in similar fashion to the PCA analyses in Figure 1. Figure 2B shows how through hierarchical analysis, there appears to be distinct patterns of metabolite concentration based on transplant cause (NASH versus other) when only analyzing metabolites that had a statistically significant difference between groups. However, when all metabolites are analyzed as in Figures 2A, 3, and 4, there is no clear hierarchy or pattern of clustering by histological pattern evident beyond the subgroup level.

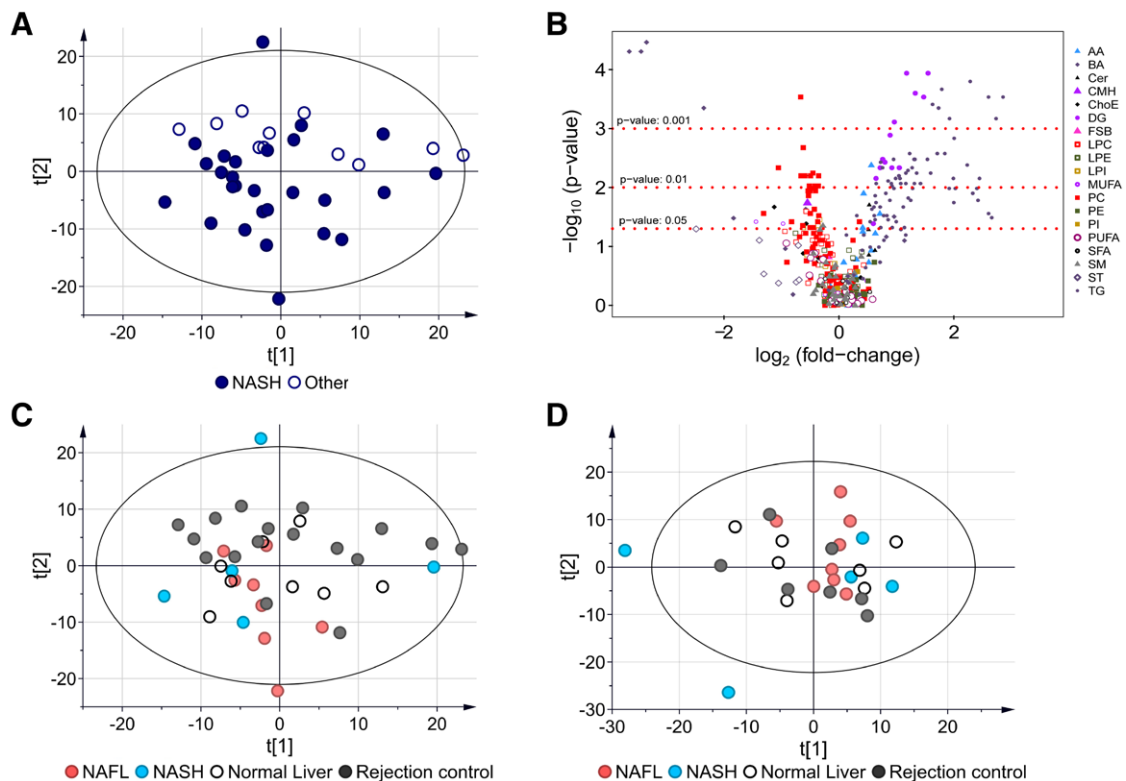
### NAFLD Versus Control Groups

Multiple analyses were performed using univariate methodology comparing the various cohorts. Results are summarized in a heat map of the recurrent NAFLD versus all other patient control comparisons (Figure 5). Grouped data used to generate Figure 5 can be found in the Table S2 (SDC, <http://links.lww.com/TXD/A371>, <http://links.lww.com/TXD/A369>). For all comparisons, there were multiple metabolites that could

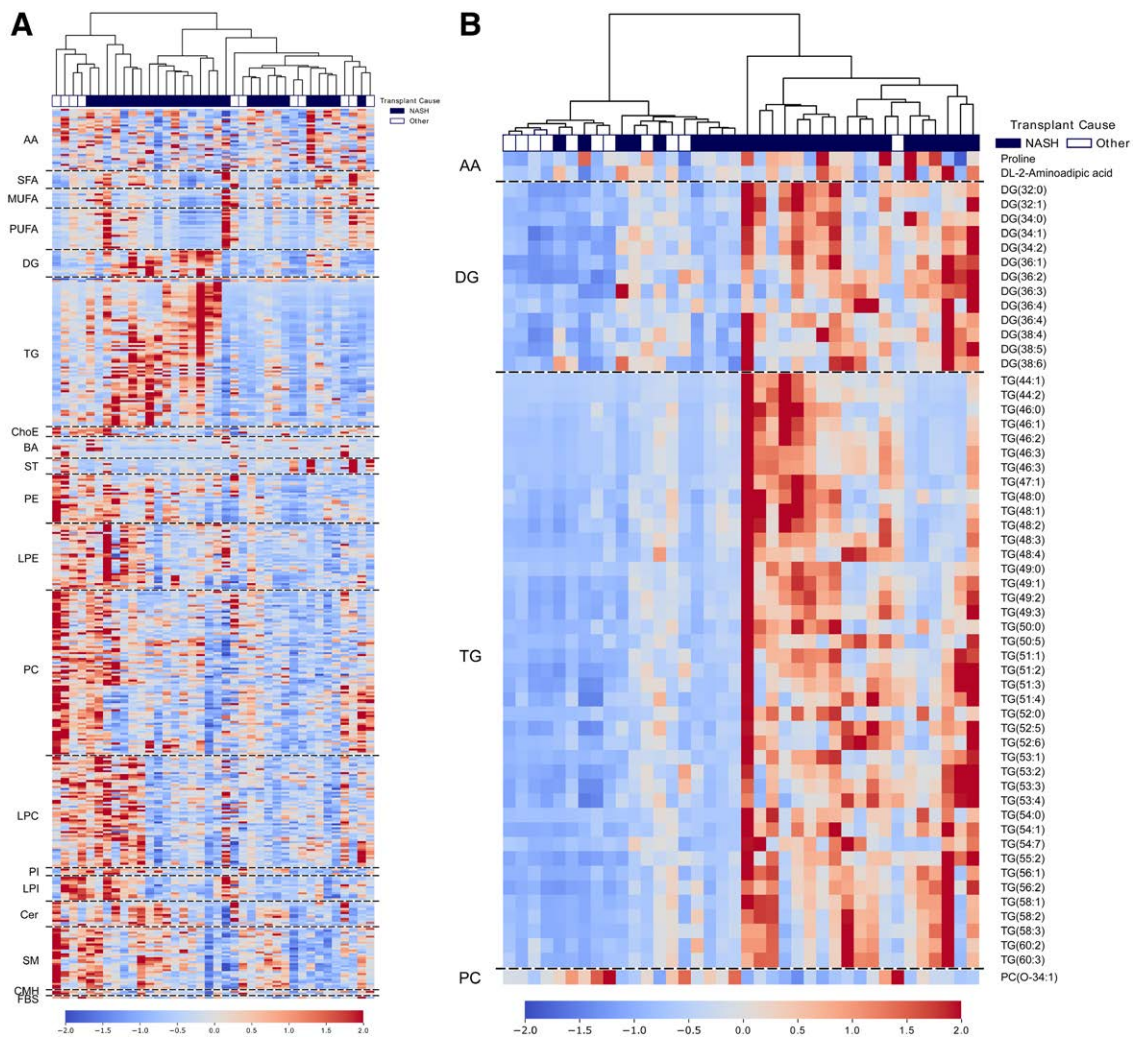
distinguish recurrent NAFLD from rejection or negative control groups. When comparing recurrent NAFLD to a cohort of both RC and NL samples, triglycerides, and free fatty acids (FFAs) composed a large proportion of the significantly different metabolites and tended to be at higher concentration among NAFLD samples. Many of these triglycerides were saturated, as shown by the carbon plot of Figure 6.

When comparing metabolite profiles between NAFLD and NL histology in patients transplanted for NASH, 14 metabolites were significantly altered. These were a mix of TG, sphingomyelins (SMs), AAs, bile acids, phosphatidylethanolamines (PEs), and phosphatidylcholines (PCs) (Table 3). The bile acids generated the greatest fold-change between groups, whereas PC(32:1) was the most significantly altered metabolite based on Wilcoxon signed-rank test analysis ( $P=0.006$ ). Class-specific trends are notable for saturated TGs trending toward significantly higher concentrations in those with NAFLD on post-LT histology ( $P=0.079$ ).

When the RC cohort was compared with NL among patients transplanted for NASH, 9 metabolites were found to have significantly different concentrations. Sphingomyelins made up 3 out of the 9 metabolites, which were otherwise fairly diverse in terms of metabolite class changes (Table 4). In the analysis of recurrent NAFLD versus NL or rejection on histology, saturated triglycerides and FFAs were more likely than their unsaturated counterparts to be at significantly elevated concentrations in those with recurrent NAFLD (Figure 5).



**FIGURE 1.** Multivariate analyses of samples depicted in scatter plots. (A) PCA analysis showed separation in  $t[2]$  between patients transplanted for NASH and patients transplanted for other primary diagnoses; (B) Volcano plot representation indicating the  $-\log_{10}(P \text{ value})$  and  $\log_2(\text{fold-change})$  for the comparison between patients transplanted for NASH and patients transplanted for other primary diagnoses per metabolite; (C) PCA analysis demonstrated slight separation of rejection control samples from other phenotypes in  $t[2]$  domain; (D) PCA analysis demonstrated a random distribution of samples when analyzing only those transplanted for NASH. AA, amino acid; BA, bile acid; Cer, ceramide; CMH, monohexosylceramide; ChoE, cholesteryl ester; DG, diglycerides; FSB, free sphingoid base; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MUFA, monounsaturated-FA; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; ST, steroid; TG, triglycerides.



**FIGURE 2.** (A) Heatmap representation of the hierarchical clustering of the serum metabolomic profile from patients according to the transplant cause: NASH or other. The hierarchical clustering is based on the optimum average silhouette width. Each data point corresponds to the relative ion abundance of a given metabolite (vertical axis) in an individual patient's serum, following a normalization procedure of mean-centering and division by the SD of each variable. The color scale indicates the relative abundance of each metabolite (blue, low abundance; red, high abundance). (B) Heatmap representation of the hierarchical clustering of the serum metabolites discriminating ( $P < 0.01$ ) patients according to the transplant cause: NASH or other. The hierarchical clustering is based on the optimum average silhouette width, splitting the patients into 2 groups: 1 cluster enriched in patients transplanted due to NASH and a cluster enriched in patients transplanted due to other causes. Each data point corresponds to the relative ion abundance of a given metabolite (vertical axis) in an individual patient's serum, following a normalization procedure of mean-centering and division by the SD of each variable. The color scale indicates the relative abundance of each metabolite (blue, low abundance; red, high abundance). AA, amino acid; BA, bile acid; Cer, ceramide; CMH, monohexosylceramide; ChoE, cholesteryl ester; DG, diglycerides; FSB, free sphingoid base; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MUFA, monounsaturated-FA; NASH, nonalcoholic steatohepatitis; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; ST, steroid; TG, triglycerides.

Nineteen metabolites were significantly altered between those with NAFLD recurrence compared with RCs and were predominately made up of PEs, AAs, and bile acids (Table 5). Notable metabolite class trends include a significantly higher concentration of total AAs ( $P = 0.038$ ) and a nearly significant elevation in bile acids ( $P = 0.055$ ) among RCs compared with those with recurrent NAFLD on histology. The elevation in bile acids was driven by bile acids conjugated to glycine ( $P = 0.01$ ) and taurine ( $P = 0.03$ ). Triglycerides and FFAs tended to be elevated in those with NAFLD recurrence compared with RCs, although these changes were not statistically significant.

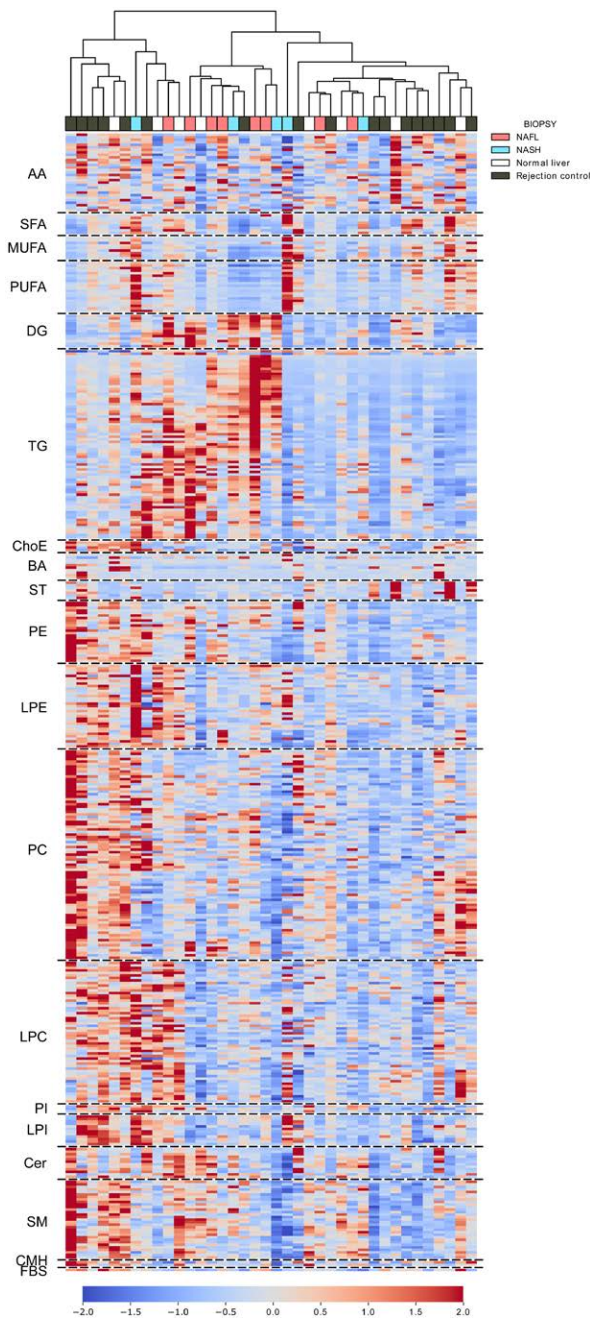
#### NAFL Versus NASH

Sixteen metabolites were found to be significantly altered between LT recipients with recurrent NAFL compared with

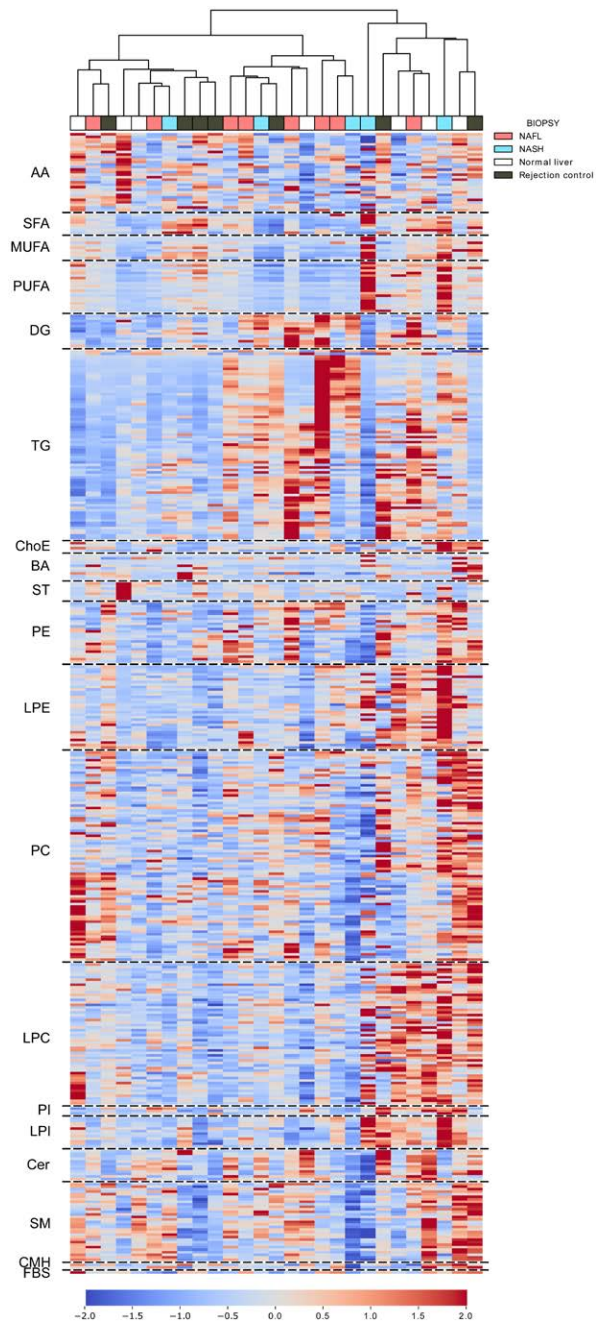
those with recurrent NASH on liver biopsy (Table 6). The most differentially expressed chemical class was phosphatidylcholines, with 10 of these lipids significantly decreased in the NASH cohort. The remaining metabolites consisted of AAs, sterols, PEs, and SMs. Among metabolite classes, both saturated and unsaturated FFAs trended toward higher concentrations in those with NASH on post-LT histology and phosphatidylcholines trended toward lower concentrations in those with NASH on post-LT histology, although no class trends reached statistical significance.

#### Identification of Fibrosis

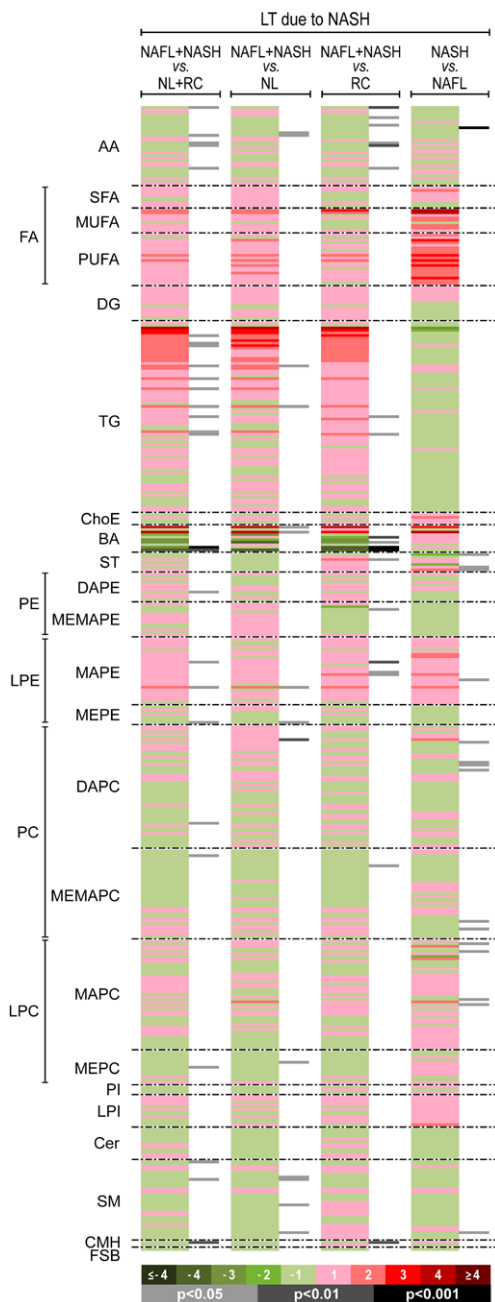
When assessing patients transplanted for NASH with any-stage fibrosis on liver biopsy ( $n = 8$ ) compared with those without fibrosis ( $n = 6$ ), no metabolites were significantly altered in this small sample pool.



**FIGURE 3.** Heatmap representation of the hierarchical clustering of serum metabolomic profiles from patients according to the type of recurrence after the liver transplant: rejection control, NL, NAFL, and NASH. The hierarchical clustering is based on the optimum average silhouette width. Each data point corresponds to the relative ion abundance of a given metabolite (vertical axis) in an individual patient's serum, following a normalization procedure of mean-centering and division by the SD of each variable. The color scale indicates the relative abundance of each metabolite (blue, low abundance; red, high abundance). AA, amino acid; BA, bile acid; Cer, ceramide; CMH, monohexosylceramide; ChoE, cholesteryl ester; DG, diglycerides; FSB, free sphingoid base; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MUFA, monounsaturated-FA; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NL, normal liver; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; ST, steroid; TG, triglycerides.



**FIGURE 4.** Heatmap representation of the hierarchical clustering of the serum metabolomic profiles in patients transplanted due to NASH according to the type of recurrence after the liver transplant: rejection control, NL, NAFL, and NASH. The hierarchical clustering is based on the optimum average silhouette width. Each data point corresponds to the relative ion abundance of a given metabolite (vertical axis) in an individual patient's serum, following a normalization procedure of mean-centering and division by the SD of each variable. The color scale indicates the relative abundance of each metabolite (blue, low abundance; red, high abundance). NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NL, normal liver. AA, amino acid; BA, bile acid; Cer, ceramide; CMH, monohexosylceramide; ChoE, cholesteryl ester; DG, diglycerides; FSB, free sphingoid base; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MUFA, monounsaturated-FA; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NL, normal liver; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; ST, steroid; TG, triglycerides.



**FIGURE 5.** Heatmap representation of metabolite alterations in NAFLD vs control cohort comparisons. Represents binary comparisons of patients with recurrence to NAFL or NASH vs NL or RC cohorts. Colored section depicts the relative fold-change ( $\log_2$ ) in circulating levels of metabolites among various comparisons with green or red coloring representing metabolites that were reduced or increased, respectively. Gray/black bars indicate significant changes (light-gray,  $t$ -test  $P < 0.05$ ; dark-gray,  $P < 0.01$ ; black,  $P < 0.001$ ). LT, liver transplantation; AA, amino acid; BA, bile acid; Cer, ceramide; CMH, monohexosylceramide; ChoE, cholesteryl ester; DAPE, diacyl-phosphatidylethanolamine; DG, diglycerides; FSB, free sphingoid base; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LT, liver transplantation; MAPE, monoacyl-phosphatidylethanolamine; MEPE, monoether-phosphatidylethanolamine; MEMAPE, 1-ether,2-acyl-phosphatidylethanolamine; MUFA, monounsaturated-FA; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NL, normal liver; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; RC, rejection control; SFA, saturated fatty acid; SM, sphingomyelin; ST, steroid; TG, triglycerides.

## OWLiver

When the OWLiver assay, which was previously validated in the non-LT population, was used on available serum samples ( $n = 18$ ) from this study, it was found to not be statistically predictive for patients with development of NASH post-LT.

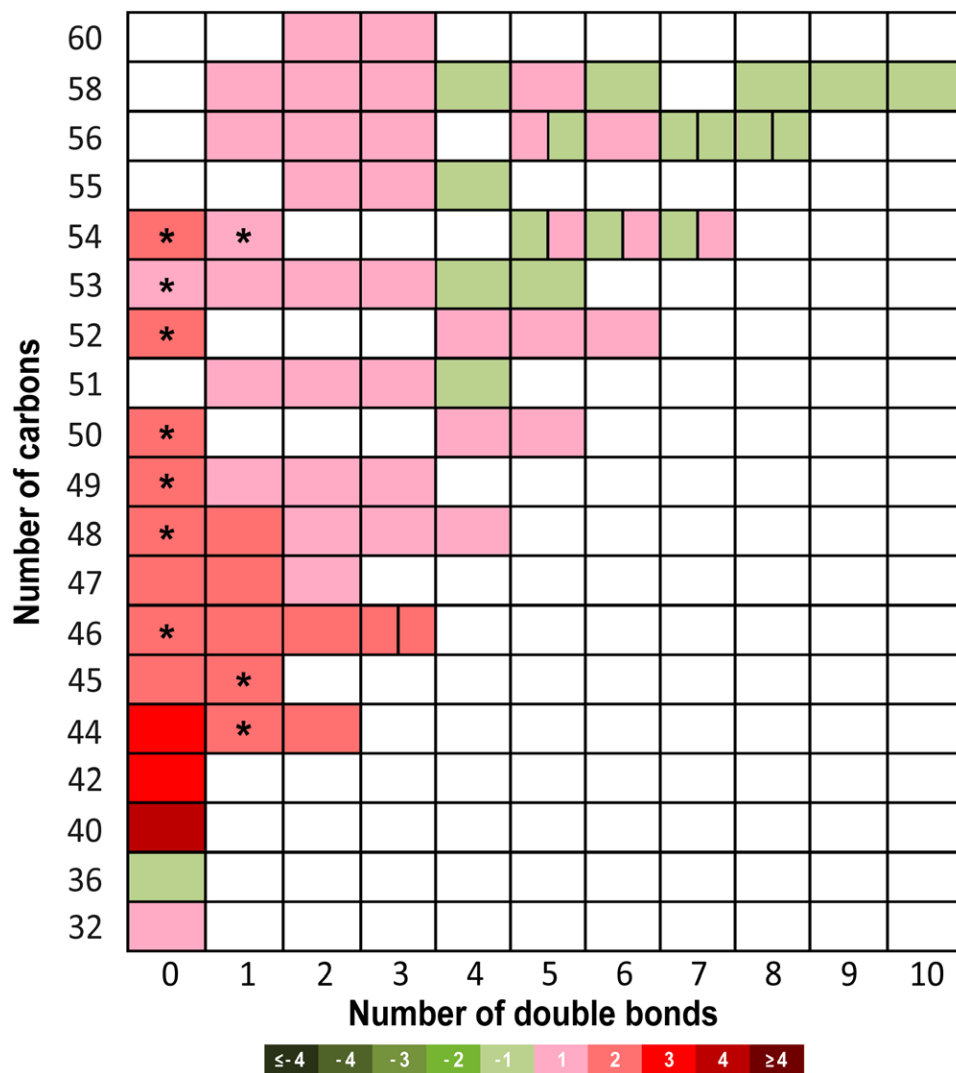
## DISCUSSION

This is the first known study evaluating metabolomic biomarkers to identify posttransplant NASH recurrence. Our findings suggest that unique metabolomic signatures may be able to identify NAFLD recurrence following liver transplant and in particular identify those with NASH who are most likely to develop significant graft injury in the future.

Our analysis demonstrated distinct metabolite profiles that may be able to distinguish between several clinically relevant cohorts including recurrent NAFLD versus normal histological controls, recurrent NAFL versus recurrent NASH, and recurrent NAFLD versus RCs. Among the 14 metabolites that differentiated recurrent NAFLD from normal controls, no specific metabolite class predominated. However, several class-specific trends were clearly evident, which can be visualized in the heatmap (Figure 5). Triglycerides and FFAs tended to be at relatively higher concentrations in the NAFLD compared with NL cohort, which aligns with prior NAFLD metabolomic data in the nontransplant setting and known role of alterations of de novo lipogenesis in NAFLD.<sup>24-26</sup>

Within the NAFLD cohort, there was a distinct metabolite profile between those with NAFL and those with NASH on post-LT histology. Of the 16 significantly altered metabolites, 10 of them were phosphatidylcholines and all were at lower concentrations in those with recurrent NASH on post-LT biopsy. Previous studies have similarly demonstrated a relative deficiency of phosphatidylcholines in patients with NASH compared with NAFL histology in the non-LT population.<sup>21,27</sup> Lyso-phosphatidylcholines correlate with insulin sensitivity and inversely with inflammation in patients with NAFLD.<sup>28</sup> Proposed mechanisms include activation of G protein-coupled receptors leading to inhibition of reactive oxygen species production.<sup>29,30</sup> Additionally in this study, saturated FFAs trended toward higher concentrations in those with recurrent NASH compared with those with recurrent NAFL, and although this trend did not reach statistical significance, it does align with what is seen in the nontransplant NASH population. In the non-LT population, saturated FFAs contribute to a more lipotoxic environment leading to inflammation and hepatocyte apoptosis through oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction.<sup>31</sup> Our study suggests that phosphatidylcholines and FFAs are logical metabolite classes to investigate further as specific biomarkers of NASH or those at high risk of recurrence in the LT population.

The consistency in metabolite class trends among our data in the liver transplant population and prior NAFLD metabolomics research in the non-LT setting supports the theory that recurrent NAFLD develops in much the same way as de novo NAFLD. However, when we assessed the OWLiver NASH assay in the post-LT population, which had previously been validated in the non-LT setting, the assay was unable to reliably distinguish NASH. This suggests that despite having many similarities in metabolite class trends, there are likely



**FIGURE 6.** Heatmap representation of carbon and double bond content in NAFLD compared with negative and rejection controls. The color code represents the magnitude of metabolite concentration difference between the NAFLD and NL/RC cohorts as represented by  $\log_2(\text{fold-change})$ . A positive (or colored red) fold-change indicates a higher concentration in the NAFLD cohort, whereas green indicates a lower concentration relative to the NL/RC cohort. The y-axis denotes the number of carbons and the x-axis the number of double bonds. Wilcoxon signed-rank test  $P$  values: \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . NAFLD, nonalcoholic fatty liver disease; NL, normal liver; RC, rejection control.

distinct metabolic differences that warrant a unique assay developed for the post-LT population. Metabolomic signatures are likely influenced by the unique context of LT. Further research with larger populations is warranted given the size limitations of this study preclude robust conclusions about specific metabolites.

We included a rejection cohort in our analysis in hopes that this cohort could serve as a generalizable proxy for acute inflammation. Although acute rejection was the inflammatory control phenotype used in this study, future studies would need to be performed to better understand if this generalizes to other phenotypes that may be more clinically relevant. Our results demonstrated that recurrent NAFLD and rejection cohorts had distinct metabolite profiles, with 9 specific metabolites significantly altered. In addition, participants with rejection had statistically significant elevations in total AAs and bile acids compared with those with recurrent NAFLD. It is notable that most of the patients in the RC group had elevated aminotransferase levels relative to other cohorts and that earlier forms of rejection, which may have less pronounced aminotransferase

elevations, could be underrepresented in this study. Because of our small sample size, the performance of this metabolomics assay to differentiate early rejection from NASH could not be assessed; however, it should be explored in future studies.

Interestingly, unsupervised PCA demonstrated clustering of samples when plotting by indication for transplant (NASH versus non-NASH), suggests the metabolic milieu of patients who have had NASH is distinct from those who have not. However, with the hierarchical clustering algorithm, clustering by transplant cause was only evident when metabolites with a statistically significant plasma concentration difference were included (Figure 2B), and not when all metabolites were included in analysis (Figure 2A). It is also worth noting that since every patient transplanted for a cause other than NASH had acute rejection on biopsy, it is difficult to confidently attribute this association to the indication for transplant rather than the inflammation itself. For instance, Figure 1C appears to show separation between patients with histological evidence of acute rejection from other groups, which supports the hypothesis that multivariate separation is due to



**TABLE 3.****Significantly altered circulating metabolites in the comparison NAFLD vs normal liver among patients transplanted for NASH**

Metabolite	Mean (altered)	Mean (control)	Fold-change	Log <sub>2</sub> (fold-change)	P
PC(0-20:0/0:0)	0.222	0.338	0.656	-0.608	0.038
SM(36:2)	0.980	1.432	0.684	-0.547	0.025
SM(d18:2/20:0)	0.898	1.291	0.696	-0.524	0.020
Cystine—double charge	6.116	8.520	0.718	-0.478	0.031
SM(32:1)	2.209	3.076	0.718	-0.478	0.038
SM(33:1)	2.000	2.753	0.726	-0.461	0.025
Arginine	0.335	0.431	0.777	-0.364	0.031
PE(P-20:0/0:0)	0.350	0.444	0.788	-0.344	0.046
PC(32:1)	5.799	3.044	1.905	0.930	0.006
TG(52:0)	9.766	3.763	2.596	1.376	0.046
TG(48:0)	6.044	2.260	2.674	1.419	0.046
PE(0:0/16:1)	2.141	0.603	3.551	1.828	0.025
Chenodeoxycholic acid	6.416	0.515	12.454	3.639	0.046
Cholic acid	5.033	0.334	15.045	3.911	0.025

A positive log<sub>2</sub>(fold-change) value indicates a higher concentration in the NAFLD cohort, whereas a negative value indicates a lower concentration relative to the NL cohort. *P* were calculated using the Wilcoxon signed-rank test.

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NL, normal liver; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TG, triglycerides.

**TABLE 4.****Significantly altered circulating metabolites in the comparison rejection control vs normal liver among patients transplanted for NASH**

Metabolite	Mean (altered)	Mean (control)	Fold-change	Log <sub>2</sub> (fold-change)	P
TG(56:8)	4.006	8.056	0.497	-1.008	0.026
SM(36:2)	0.890	1.432	0.621	-0.687	0.038
SM(18:2/20:0)	0.875	1.291	0.678	-0.561	0.011
SM(38:1)	1.359	1.825	0.745	-0.425	0.011
TG(32:0)	0.944	0.865	1.091	0.126	0.026
PC(0-18:2/20:4)	2.190	1.360	1.610	0.687	0.026
PE(18:1e/22:6)	3.274	1.611	2.031	1.023	0.017
Taurochenodeoxycholic acid	6.691	1.590	4.209	2.073	0.011
Glycocholic acid	5.643	1.314	4.293	2.102	0.011

A positive log<sub>2</sub>(fold-change) value indicates a higher concentration in the RC cohort, whereas a negative value indicates a lower concentration relative to the NL cohort. *P* values were calculated using the Wilcoxon signed-rank test.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; NASH, nonalcoholic steatohepatitis; NL, normal liver; RC, rejection control; SM, sphingomyelin; TG, triglycerides.

histological phenotype and not transplant cause. To explore this further, we performed PCA analysis (Figure 1D) and hierarchical analysis (Figure 4) only in patients transplanted for NASH and with this population there did not appear to be clustering by histological phenotype. It is possible that by removing patients transplanted for a cause other than NASH the sample size of the analysis became too small to appreciate statistically significant changes. Further research is warranted to investigate this further as multivariate analysis, especially analysis of only participants transplanted for NASH, is severely limited by small sample size and interpretation of results should take this into account.

The data from this study supports the diagnostic potential of metabolomic biomarkers for the post-LT population in 2 situations, (1) as a noninvasive method for serial monitoring and surveillance since recurrent NAFL/NASH are often not accompanied by significant liver enzyme perturbations and (2) as a diagnostic tool in the setting of elevated liver enzymes to distinguish NAFL/NASH recurrence from other causes of inflammation. Although the clinical profile of acute rejection is distinct from recurrent NAFLD, which typically

does not have profound alterations in liver biochemistries, metabolomic screening could be more useful in earlier or less severe forms of rejection, in which biochemical abnormalities are less marked. Increasing numbers of patients are being transplanted for NASH cirrhosis and recurrence of disease is often insidious. Liver enzymes are often not elevated, even in the setting of recurrent NASH with advanced fibrosis and serial protocol liver biopsies are not a tenable solution for the identification of those with recurrent NASH and fibrosis underscoring the need for noninvasive biomarkers in the post-LT NASH population. Suppression of inflammation due to immunosuppressive medications in the post-LT setting may contribute to the absence of significant enzyme elevations in the context of recurrent NASH, even at advanced stages. Through these applications, metabolomic biomarkers could limit the need for liver biopsy and thereby minimize costs, procedural risks, and improve patient satisfaction and outcomes. Patients with an early diagnosis of recurrent NAFLD could be monitored and targeted for more intense lifestyle modification or pharmacologic therapy, including multiple drugs currently being developed for the treatment of NASH

**TABLE 5.**  
Significantly altered circulating metabolites in the comparison NAFLD vs RC

Metabolite	Mean (altered)	Mean (control)	Fold-change	Log <sub>2</sub> (fold-change)	P
Tauroursodeoxycholic acid	1.080	14.872	0.073	-3.783	0.0002
Taurochenodeoxycholic acid	0.814	6.691	0.122	-3.040	0.0001
Glycoursodeoxycholic acid	2.249	10.153	0.222	-2.174	0.016
Glycocholic acid	2.288	5.643	0.406	-1.302	0.006
PE(18:1e/22:6)	1.865	3.274	0.570	-0.812	0.031
PC(0-18:0/18:2)	0.657	1.022	0.642	-0.639	0.046
Tyrosine	0.541	0.795	0.681	-0.553	0.046
Glucosylceramide(d18:1/16:0)	1.029	1.444	0.713	-0.488	0.002
Methionine	0.656	0.901	0.728	-0.459	0.010
Phenylalanine	0.388	0.528	0.735	-0.444	0.025
Serine	0.250	0.338	0.741	-0.433	0.020
Asparagine	0.666	0.850	0.783	-0.352	0.006
Kynurenine	1.332	1.634	0.815	-0.295	0.031
PE(20:4/0:0)	1.249	0.832	1.501	0.586	0.002
TG(53:0)	1.749	1.098	1.593	0.672	0.038
PE(22:5/0:0)	1.667	0.948	1.758	0.814	0.031
PE(22:5/0:0) (isomer)	1.774	0.885	2.005	1.004	0.020
TG(54:1)	8.027	3.776	2.126	1.088	0.031
Isomer androsterone sulfate	0.290	0.126	2.301	1.202	0.038

A positive log<sub>2</sub>(fold-change) value indicates a higher concentration in the NAFLD cohort, whereas a negative value indicates a lower concentration relative to the RC cohort. *P* values were calculated using the Wilcoxon signed-rank test.

NAFLD, nonalcoholic fatty liver disease; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RC, rejection control; TG, triglycerides.

**TABLE 6.**  
Significantly altered circulating metabolites in the comparison NASH vs NAFL

Metabolite	Mean (altered)	Mean (control)	Fold-change	Log <sub>2</sub> (fold-change)	P
Androsterone sulfate	0.110	0.430	0.255	-1.970	0.012
PC(0:0/17:0)	0.200	0.374	0.534	-0.904	0.029
PC(P-17:0/20:4)	1.114	1.981	0.563	-0.830	0.029
PC(P-16:0/20:4)	0.818	1.407	0.581	-0.783	0.019
PC(17:1/18:1)	1.946	3.309	0.588	-0.766	0.012
SM(18:2/20:0)	0.659	1.031	0.640	-0.644	0.042
PC(15:0/18:2)	2.071	3.199	0.647	-0.628	0.042
PC(0:0/15:0)	0.281	0.432	0.651	-0.619	0.029
PC(15:0/0:0)	0.252	0.380	0.663	-0.592	0.042
PC(17:0/18:1)	2.871	4.153	0.691	-0.533	0.042
PC(17:0/0:0)	0.223	0.316	0.704	-0.506	0.042
Lysine	0.371	0.481	0.771	-0.375	0.001
PC(16:0/19:1)	1.926	2.395	0.804	-0.314	0.042
Isomer pregn-5-ene-3,20-diol sulfate	0.490	0.280	1.751	0.808	0.019
PE(22:5/0:0)	2.056	1.140	1.803	0.850	0.019
Pregnenolone sulfate	0.720	0.270	2.669	1.416	0.042

A positive log<sub>2</sub>(fold-change) value indicates a higher concentration in the NASH cohort, whereas a negative value indicates a lower concentration relative to the NAFL cohort. *P* values were calculated using the Wilcoxon signed-rank test.

NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

that are available for off label use or likely to be approved in the future for a NASH indication.

The most important limitation of this study is its small number of observations and large number of variables analyzed, thereby increasing the potential for type I errors. However, general metabolomic trends seen in the present study align with prior research in the nontransplant setting, which supports the validity of the presented observations. This cross-sectional study was not intended to provide definitive conclusions regarding the distinct metabolomic profiles of recurrent NAFLD or NASH specifically. Rather, it was

meant to be hypothesis generating through the identification of candidate pathways that may be differentially impacted in the clinical scenarios presented here. This exploratory design was further strengthened by using an unbiased metabolomic approach, which allowed measurement of all possible metabolites without preselecting features a priori.

In summary, we identified potential biomarkers of allograft-associated liver disease and provided preliminary evidence that recurrent NAFLD following LT is associated with a similar circulating metabolomic profile to NAFLD in the non-LT population. If future studies with larger sample sizes are able

to validate the observations presented here, these biomarkers could be used to diagnose and stage recurrent NAFLD through noninvasive testing and allow for serial assessments to monitor and potentially alter disease course.

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