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HEPATOBILIARY

Sphingolipids Are Depleted in Alcohol-Related Liver Fibrosis

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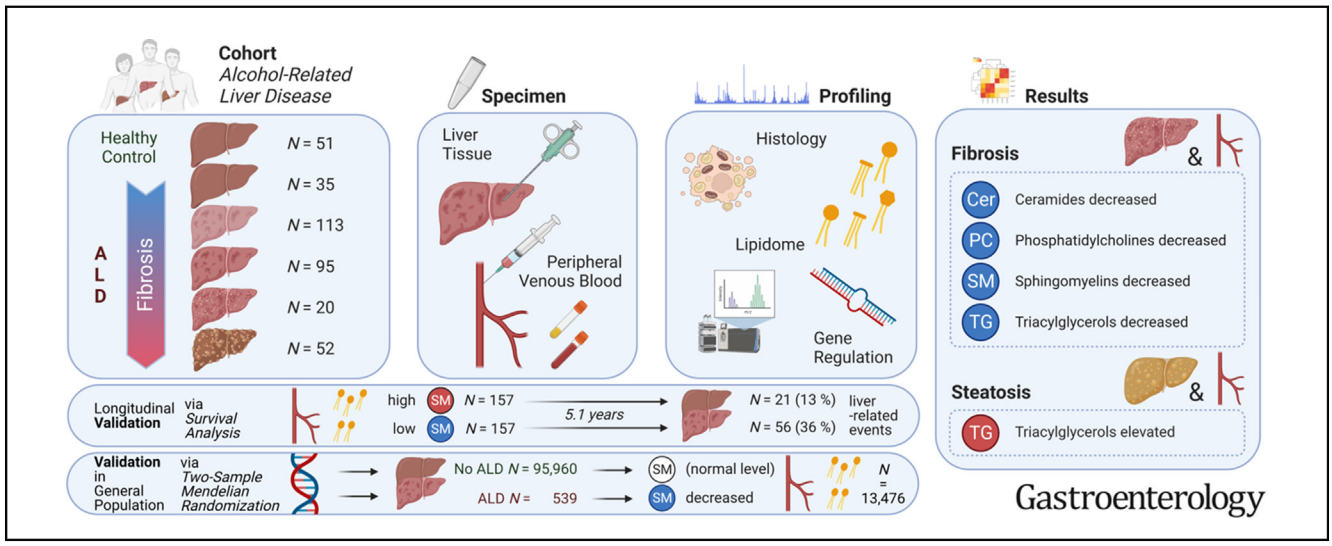

BACKGROUND & AIMS: Alcohol disturbs hepatic lipid synthesis and transport, but the role of lipid dysfunction in alcohol-related liver disease (ALD) is unclear. In this biopsy-controlled, prospective, observational study, we characterized the liver and plasma lipidomes in patients with early ALD. **METHODS:** We performed mass spectrometry–based lipidomics of paired liver and plasma samples from 315 patients with ALD and of plasma from 51 matched healthy controls. We associated lipid levels with histologic fibrosis, inflammation, and steatosis with correction for multiple testing and adjustment for confounders. We further investigated sphingolipid regulation by means of quantitative real-time polymerase chain reaction sequencing of microRNA, prediction of liver-related events, and tested causality with Mendelian randomization. **RESULTS:** We detected 198 lipids in the liver and 236 lipids in the circulation from 18 lipid classes. Most sphingolipids (sphingomyelins and ceramides) and phosphocholines were co–down-regulated in both liver and plasma, where lower abundance correlated with higher fibrosis stage. Sphingomyelins showed the most pronounced negative correlation to fibrosis, mirrored by negative correlations in both liver and plasma with hepatic inflammation. Reduced sphingomyelins predicted future liver-related events. This seemed to be characteristic of "pure ALD," as sphingomyelin levels were higher in patients with concomitant metabolic syndrome and ALD/nonalcoholic fatty liver disease overlap. Mendelian randomization in FinnGen and UK Biobanks indicated ALD as the cause of low sphingomyelins, and alcohol use disorder did not correlate with genetic susceptibility to low sphingomyelin levels. **CONCLUSIONS:** Alcohol-related liver fibrosis is characterized by selective and progressive lipid

depletion in liver and blood, particularly sphingomyelins, which also associates with progression to liver-related events.

Keywords: Fatty Liver; Fibrosis; Metabolomics; Alcoholic Liver Disease.

Alcohol is the leading cause of cirrhosis worldwide, with half of all global deaths from cirrhosis attributed to excess drinking. One in 5 people with harmful use of alcohol exhibit progressive liver fibrosis.[1,2] The molecular basis of the development and progression of alcohol-related liver disease (ALD) is largely unknown.[3,4] Hence, there is an urgent need to advance our understanding of the biochemical and pathophysiological changes in the liver and circulation during the progression of ALD.

Alcohol-induced lipid dysfunction is well known, driven in part by stress to the endoplasmic reticulum, which controls lipid and cholesterol synthesis.[5,6] Consequently, alcohol impairs hepatocyte fatty acid (FA) oxidation and lipid transport and increases lipogenesis, resulting in steatosis.[6] Furthermore, exposure to alcohol leads to hepatocellular stress, resulting in caspase activation, degradation of the cytoskeleton, and subsequent apoptosis and ballooning—a hallmark of steatohepatitis.[7,8]

Triglycerides and FAs represent the major lipid classes accumulating in hepatocytes during the formation of steatosis, but most are inert and not lipotoxic.[9] Considering that alcohol disrupts the global hepatic lipid metabolism, a comprehensive lipidomics catalogue may be important to understanding lipid dysfunction in ALD.[10] Lipidomics studies of nonalcoholic fatty liver disease (NAFLD) indicate that bioactive lipids, such as phosphocholines (PCs) and sphingolipids, which control cell integrity and function, play a major role in lipotoxicity.[11] Human case-control studies in NAFLD have found that disease severity correlates with PCs and sphingolipids.[12–14] One such study found a negative correlation between sphingomyelins (SMs) and nonalcoholic steatohepatitis (NASH),[12] and another found elevated SMs in NASH vs controls, but decreased SMs in cirrhosis vs controls.[14] Crucially, only 2 case-control studies have been published in ALD, concerning patients with cirrhosis and alcoholic hepatitis.[15,16]

Consequently, there is a need for investigations into the global lipidome across the spectrum of alcohol-related liver fibrosis. In this study, we aimed to describe differences in the levels of individual lipids across lipid classes in liver tissue and plasma according to liver fibrosis stage, inflammation grade, and steatosis score in a cohort study of biopsy-controlled ALD compared with matched healthy controls. Then we examined key sphingolipid findings in depth by testing their prognostic relevance, the causality of the association by a Mendelian randomization study, their correlation with concomitant metabolic syndrome, and the microRNA (miRNA) regulation of genes involved in sphingolipid metabolism.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Alcohol is known to induce abnormal lipid metabolism, not only in the liver but also in circulation.

NEW FINDINGS

Sphingolipid levels are reduced in alcohol-related liver disease in both liver and plasma, with increasing severity of fibrosis and inflammation. This marks an elevated risk of progression to liver-related events.

LIMITATIONS

Further studies are warranted to understanding the mechanisms underlying the depletion of the sphingolipid pathway and whether the depletion is specific to alcohol-related liver disease.

CLINICAL RESEARCH RELEVANCE

Sphingomyelins are the lipid class that is affected earliest in fibrosis in both liver and in plasma. Their depletion is associated with liver-related events during follow-up, primarily in patients with moderate and severe fibrosis. Further research is warranted to evaluate the prognostic potential.

BASIC RESEARCH RELEVANCE

Alcohol-related liver disease has a causal effect on low blood sphingomyelin level. Moreover, sphingolipids levels are lower in "pure" alcohol-related liver disease than in disease with a metabolic overlap.

Methods

We conducted a prospective observational cohort study of patients with ALD and matched, healthy control subjects. Both studies received approval from the Danish data protection agency (13/8204, 16/3492) and the Ethics Committee for Region of Southern Denmark (ethical ID S-20120071, S-20160021, S-20170087, and S-20160006G). The ALD cohort has been described in detail previously.[17–19] All participants gave written, informed consent before inclusion. Further details on methodology can be found in the Supplementary Material.

Patients and Healthy Controls

We recruited consecutive patients with a history of chronic, excessive alcohol use for at least 1 year (>14 units/wk for women and >21 units/wk for men). Additional inclusion criteria were age 18–75 years and informed consent to undergo a liver biopsy. We excluded patients in case of decompensated cirrhosis, competing liver disease, alcoholic hepatitis, mechanical bile duct obstruction, any debilitating disease with an

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Abbreviations used in this paper: **ALD, alcohol-related liver disease; ANCOVA, analysis of covariance; ANI, alcohol-related liver disease to nonalcoholic fatty liver disease index; Cer, ceramide; FA, free fatty acid; HexCer, hexocyl-ceramide; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PC, phosphocholine; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SNP, single-nucleotide polymorphism; TG, triacylglycerol.**

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expected survival of <1 year, or inability to comply with the study protocol.

We matched 51 healthy controls with patients with ALD on the basis of sex, age, and body mass index. Exclusion criteria were prior excessive use of alcohol, any daily drinking, average weekly alcohol intake exceeding 7 units for women and 14 units for men, binge drinking (≥5 units at a time), known liver disease of any etiology, any long-term medication, or any self-reported chronic disease discovered during inclusion or occurring within 6 months after inclusion.

Study Investigations

All investigations were performed on the same day, after a 10-minute rest, and preceded by an overnight fast. Investigations included medical history; current and prior alcohol use; and metabolic risk factors, characterized by blood pressure, body mass index, glycated hemoglobin A1c, plasma triglycerides, cholesterols, and homeostatic model assessment of insulin resistance. We categorized patients as abstinent if they did not consume alcoholic beverages for at least 7 days leading up to inclusion.

We performed percutaneous liver biopsy in patients with ALD. One pathologist evaluated biopsy quality (≥10 mm length and ≥5 portal tracts or presence of regenerative nodules); scored fibrosis stage according to the Kleiner score (F0–F4); and used the NASH Clinical Research Network Activity Score for semi-quantitative evaluation of steatosis (S0–S3), hepatocyte ballooning (B0–B2), and lobular inflammation (I0–I3).[20] For a composite measure of inflammatory activity, we used the sum of lobular inflammation and ballooning (0–5).

Follow-up outcomes data were acquired by manual search of nationwide electronic health records.[19] We defined a *liver-related event* as any of the following: alcoholic hepatitis, varices needing treatment, variceal bleeding, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, hepatocellular carcinoma, hepatorenal syndrome, upper gastrointestinal bleeding, or jaundice due to liver failure.

Plasma Lipidomics

We performed untargeted lipidomics on 500 μL lithium-heparin plasma, centrifuged at 2000*g* and stored at −80°C immediately after sampling. We used internal standards according to protocols at Steno Diabetes Center Copenhagen.[21] We identified lipids by ultra-high-performance liquid chromatography (1290 Infinity UHPLC System) coupled with quadrupole time-of-flight mass spectrometry (6550 Q-ToF; Agilent Technologies, Santa Clara, CA), which separates molecules on the basis of their polarity, after which lipids can be classified on the basis of their size and degree of unsaturation.[22] The untargeted lipidomics method has been validated previously across 31 European laboratories using the National Institute of Standards and Technology Standard Reference Material of human plasma metabolites.[23]

Liver Lipidomics

Liver biopsy samples were prepared according to previously established methods and modified to suit the experimental design and analytical instruments.[24] We used 5 mm of liver tissue, snap-frozen in liquid nitrogen immediately after biopsy. Each tissue sample were transferred from −80°C into Covaris TT05 tissue tubes, immerged into liquid nitrogen, and crushed with Covaris CryoPrep impactor CP02. Finally, 2 mg of the powdered liver tissue underwent chloroform-methanol extraction, with the exact mass used for normalization. Finally, we performed the identical instrumental analysis as for plasma lipidomics. We performed quality control of individual measurements using lipidomics of pooled extracts.

Preprocessing, Quality Control, and Postprocessing of Lipidomics Data

We used MZmine 2 to preprocess the lipidomics data and a pipeline in R software for quality control and postprocessing. We normalized the lipid measurements according to spiked-in internal standards and batch-corrected the values. Lipids with >20% missing values were omitted from subsequent analysis and remaining missing values were imputed using the k-nearest neighbor algorithm. We standardized all data variables to zero mean and unit variance for the inference of standardized effect sizes.

Data Analysis

First, we tested liver and plasma lipids separately, correlating the individual lipid species in each lipid class to fibrosis stage, using analysis of covariance (ANCOVA) coupled with pairwise post-hoc tests with no liver fibrosis (F0) as the reference. We controlled for potential confounders by adjusting all ANCOVA regression models for sex, age, body mass index, glycated hemoglobin A1c, homeostasis model assessment for insulin resistance, systolic blood pressure, use of statins, and abstinence from alcohol at the time of inclusion. Those lipid species in which the ANCOVA showed an association with fibrosis were displayed in heatmaps. We repeated these analyses for inflammation grade and steatosis score. We used chord diagrams to integrate all of these associations.[25]

Next, we analyzed lipid classes for overrepresentation or enrichment. Overrepresentation tests whether there are more affected lipids from a particular class than expected on average; ranging from −100%, when all lipid species in a class correlates negatively, to 100%, when all species correlate positively.

Finally, we demonstrated individual lipid species from the lipid classes with the earliest class-wide aberrations as markers of systematic pathophysiological changes measurable in plasma. We picked the lipid class with first class-wide aberration in plasma for fibrosis, inflammation, and steatosis, each. From these classes we selected the lipid species with the highest ANCOVA *F*-statistic summed over liver and plasma. We further tested the lipid with the strongest association to fibrosis for its association with liver-related events during follow-up by means of Cox proportional hazards model adjusted for potential confounders, as with ANCOVA. Finally, we visualized liver-related events using the Kaplan-Meier estimator in the entire cohort, as well as in subgroups defined by sex and fibrosis stage.

We reported standardized effect sizes per SD increase to facilitate comparability across studies.[26] We corrected all results for multiple testing over the lipidome using the Benjamini-Hochberg method. We analyzed the data with R software (version 4.2.0) using the packages lipidomeR,[27] circlize, gplots, ggplot2, survival, and survminer.

Sphingolipid Regulation and Causality

According to the lipidome-wide results, we further investigated SM aberrations by means of several analyses, all described in detail in the Supplementary Methods.

First, we used miRNA in circulation and liver to investigate the regulation of SM degradation and synthesis pathways.

Second, we studied causality between ALD and blood SM with 2-sample Mendelian randomization meta-analysis. We used the MR-Base platform[28] to accessing biobank data and in calculating the analysis. Only class-level data of SM were available in the biobank. We selected single-nucleotide polymorphisms (SNPs) as genetic instruments at $P < 10^{-5}$ and used the inverse variance–weighted estimator[29] to test the causal association. We assessed the stability of the estimate with the leave-one-out procedure, thus evaluating whether any of the individual SNPs has a disproportionate influence on the estimate.

We addressed the following questions: First, does ALD cause a change in the SM level? Second, does NAFLD cause a change in the SM level? Third, does alcohol addiction cause a change in the SM level?

Data on ALD (n = 539 cases and n = 95,960 controls) and NAFLD (n = 272 cases and n = 96,227 controls) were available in the FinnGen Biobank (R2). Data on ever addicted to alcohol were available from the UK Biobank[30] (n = 2778 cases and n = 3736 controls; Neale Laboratory, R2). Data on total blood SM were available in a cross-European study on metabolite quantitative trait loci[31] (n = 13,476).

Third, prior studies on SMs in NAFLD, metabolic syndrome, and type 2 diabetes had shown increases in SMs and ceramides (Cers) in patients compared with controls. We therefore investigated this discrepancy with our findings by means of evaluating the lipidomic fingerprint in the patients with ALD with metabolic syndrome and we calculated the ALD/NAFLD index (ANI) to study lipid levels in the patients with more "pure ALD" (ANI > 0.0) vs ALD/NAFLD overlap (ANI < 0.0).[32] In sensitivity analysis, we repeated the ANI analysis in subgroups of individual fibrosis stages F0–F4.

Results

Patients and Healthy Controls

We included 315 patients with ALD and 51 matched healthy controls between 2013 and 2018 (Table 1). We assessed steatosis, ballooning, and lobular inflammation in liver tissue from 312 participants with ALD, whereas 3 biopsies showed regenerative nodules diagnostic of cirrhosis, but not enough tissue for reliable inflammation or steatosis scoring. We acquired a lipidomic profile of the liver in 301 of the participants, whereas 10 biopsies were used for adjustment of the method and 4 liver samples did not contain enough material for lipidomics. Seventy-seven participants with ALD (24%) experienced a liver-related event during a median of 5.1 years of follow-up.

We measured 198 lipids in the liver and 236 lipids in the circulation coming from 18 lipid families or classes: Cers, diacylglycerols, free FAs, hexocyl-ceramides (HexCers), lactocyl-Cers, lyso-PCs, alkyl or alkenyl ether lyso-PCs, lysophosphatidylethanolamines, PCs, alkyl or alkenyl ether PCs, phosphatidylethanolamines, alkyl or alkenyl ether phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidylserines, sulfatide HexCer, SMs, and triacylglycerols (TGs) (see Supplementary Tables 1 and 2 for lists of all identified lipids).

Integrative Analysis of Liver Histology and the Lipidomes

Only the lipidome of patients with ALD with no fibrosis were comparable with matched healthy controls (Supplementary Figure 1). Aberrations in phospholipids and SMs dominated the lipidome in fibrosis in the liver (Figure 1, *left* and Supplementary Figure 2) and in plasma (Figure 1, *right* and Supplementary Figure 3). In inflammatory activity, half of the aberrated liver lipids were phospholipids and sphingolipids and the other half were TGs (Supplementary Figure 4), rendering the liver lipidome of inflammation a crossover between fibrosis and steatosis. In plasma, however, hardly any TGs were aberrated in inflammation (Supplementary Figure 5). As expected, TGs dominated the liver and plasma lipidomes in steatosis (Supplementary Figures 6 and 7). The total number of molecular lipids associated with liver health was greater in the liver lipidome compared with the plasma lipidome (Figure 1).

Next, we studied at what specific stages of ALD the aberrations emerged at a lipid class level using overrepresentation (ie, enrichment) analysis. In the liver (Figure 2, *left*), 25%–50% of all SMs and PCs associated negatively with the late stages of fibrosis, and more than half of lactocyl-Cers and minor phospholipid classes correlated positively. The majority of glycerolipids (diacylglycerols and TGs) were elevated early in inflammation and steatosis. In plasma (Figure 2, *right*), the most obvious lipid class aberrations were negatively correlated SMs and Cers from moderate fibrosis. Similarly, inflammatory activity was associated with early depletion of SMs. SMs were the only plasma lipid class with an overrepresentation of affected lipids—a depletion—in all lesion types.

In the lipidomic integrative analysis, we identified 34, 3, and 11 lipid species simultaneously associated with liver fibrosis, inflammation, or steatosis, respectively, in both liver and plasma (Supplementary Figure 8). The 34 co-dysregulated lipids in fibrosis were sphingolipids (Cers and SMs), PCs, and TGs. Figure 3 shows 3 of these co-dysregulated lipid species, namely the most strongly affected species from the class that was systematically affected earliest in fibrosis, inflammation, and steatosis, each: SM(d41:1) in fibrosis, SM(d40:1) in inflammation, and TG(50:2) in steatosis. As a result of the selection process (see Methods for details), these 3 lipids are part of a lipid class that is systematically associated with histologic disease severity, dysregulated early in the disease, and co-dysregulated in liver and plasma.

Sphingomyelin SM(d41:1) and Liver-Related Events

SM(d41:1) levels were highly predictive of a liver-related event (Figure 4). The risk of liver-related events increased 46% with an SD decrease in plasma SM(d41:1)

Table 1. Characteristics of 315 Patients With Alcohol-Related Liver Disease and 51 Healthy Controls Matched for Age, Sex, and Body Mass Index

Characteristic	ALD	Healthy controls	*P* value
n	315	51	—
Male, n (%)	239 (76)	39 (77)	1.0
Age, *y*, mean ± SD	55 ± 11	57 ± 9	.11
Body mass index, kg/m^2, mean ± SD	27 ± 5	26 ± 4	.36
Abstinent at inclusion,[a] n (%)	155 (49)	2 (4)	<.001
Diabetes,[b] n (%)	45 (14)	0	<.001
Arterial hypertension,[c] n (%)	96 (31)	0	<.001
Statin treatment, n (%)	64 (20)	0	<.001
HbA1c, *mmol/mol*, mean ± SD	37 ± 10	35 ± 4	.14
Fasting blood glucose, *mmol/L*, mean ± SD	6.8 ± 2.2	5.6 ± 0.5	<.001
HOMA-IR,[d] mean ± SD	4.9 ± 7.76	—	—
Total cholesterol, *mmol/L*, mean ± SD	5.2 ± 2.0	5.5 ± 0.8	.26
LDL cholesterol, *mmol/L*, mean ± SD	3.0 ± 1.4	3.5 ± 0.7	.027
HDL cholesterol, *mmol/L*, mean ± SD	1.4 ± 0.5	1.5 ± 0.5	.16
Total triglycerides, *mmol/L*, mean ± SD	1.6 ± 1.2	1.2 ± 0.6	.01
MELD score, median (IQR)	6 (1)	6 (1)	.040
Biopsy scores,[d,e] n (%)			
Fibrosis stage, F0/F1/F2/F3/F4, n (%)	35/113/95/20/52 (11/36/30/6/17)	—	—
Ballooning, 0/1/2, n (%)	158/94/60 (51/30/19)	—	—
Lobular inflammation, 0/1/2/3, n (%)	81/133/71/27 (26/42/23/9)	—	—
Steatosis, 0/1/2/3, n (%)	148/76/62/26 (48/24/20/8)	—	—

NOTE. Between-group differences calculated by Student t test for normal-distributed variables, by Kruskal-Wallis rank sum test for ordinal variables, and by Fisher test for categorical variables.
HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein; MELD, model of end-stage liver disease.
[a]Most of the healthy controls reported low-level social drinking (average 3 drinks/wk, interquartile range [IQR], 5). We defined abstinence as not drinking any alcoholic beverages in the week up to inclusion. All patients with ALD had a history of excess drinking, with a mean of 11–20 years of excessive drinking and median 20 units/d (IQR, 10–30) at the height of overuse.
[b]Diabetes was defined as taking antidiabetic medication and/or HbA1C exceeding 48 mmol/mol and/or self-reported diabetes.
[c]Arterial hypertension was defined as taking antihypertensive medication and/or resting blood pressure >130/>85 mm Hg systolic/diastolic and/or self-reported arterial hypertension.
[d]We did not calculate HOMA-IR or perform a liver biopsy in the healthy control subjects.
[e]Three liver biopsies showed regenerative nodules characteristic of cirrhosis, but not enough tissue for reliable scoring of steatosis, ballooning, or lobular inflammation.

after adjustment for the clinical covariates ($P < .001$). When dividing the cohort according to the median of plasma SM(d41:1), the lower half had 170% more liver-related follow-up events than the upper half (56 vs 21 events among the 157 participants in each of the 2 strata) (Figure 4, *right*). We observed a similar prognostic ability of liver SM(d41:1) (Figure 4, *left*).

The association remained significant in liver as well as in plasma when stratifying for sex (Supplementary Figure 9). In a subgroup analysis of fibrosis stages, only fibrosis stage F3 in plasma remained significant ($P = .011$; Supplementary Figure 10). Patients with fibrosis stage F2 in the low-SM(d41:1) group also experienced more events than the high-SM(d41:1) group, especially during the first 3 years of follow-up ($P = .36$). In liver, individual fibrosis stages were not significant, but there were more events in the low SM(d41:1) half in all 5 fibrosis stages (binomial test, $P = .031$).

MicroRNA Regulation of Sphingolipid Biosynthesis and Metabolism

As SMs and Cers, the major sphingolipid classes, exhibited the strongest co-dysregulation in liver and plasma, we further explored miRNA regulation of their biosynthesis and metabolism pathways using Kyoto Encyclopedia of Genes and Genomes pathways and PathWalks.

Three circulating miRNAs targeting 21 genes in the sphingolipid pathways, miR-21-5p, miR-24-3p, and mir-

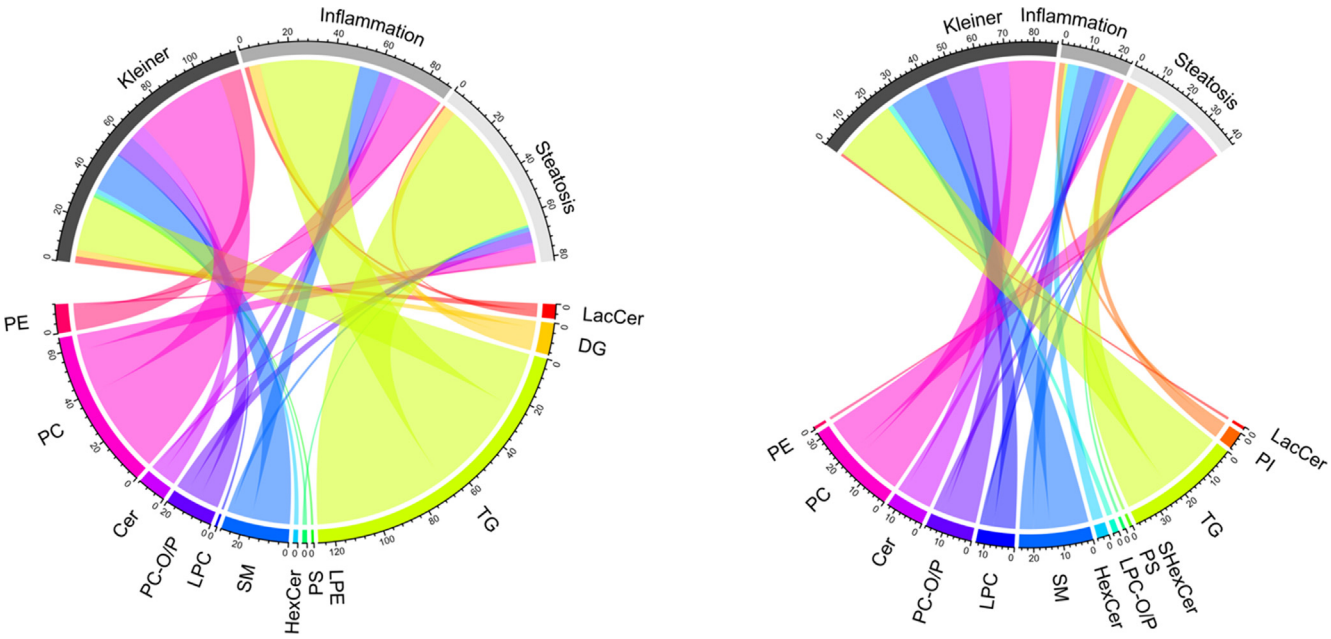

Figure 1. *Chord diagrams* for integrative analysis of the lipidomic changes in the liver (*left*) and plasma (*right*) according to the 3 main types of histologic changes in the liver (ie, Kleiner fibrosis stage, inflammatory activity, and steatosis score). Lipid classes are shown in distinct *colors* and the width of the *line* between a lipid class and a histologic liver measure indicates the respective number of significant associations ($P < .05$) detected by the ANCOVA. For example, 116 hepatic lipids are significantly associated with fibrosis stage, most of which are PCs, followed by SMs. Lipids are shown in the *bottom half* of the *circle* and liver lesions are shown in the *top half* of the *circle*. Results are based on liver samples from 301 people and plasma samples from 315 people. Results are with adjustment to clinical covariates and correction for multiple testing. DG, diacylglycerol; LacCer, lactocyl-ceramide; LPC, lyso-phosphocholine; LPC-O/P, alkyl or alkenyl ether lyso-phosphocholine; LPE, lyso-phosphatidylethanolamine; PC-O/P, alkyl-acyl phosphocholine; PE, phosphatidylethanolamines; PI, phosphatidylinositol; PS, phosphatidylserine; SHexCer, sulfatide hexocyl-ceramide.

146a-5p, were significantly up-regulated with more severe ALD fibrosis ($P < .05$; Supplementary Figures 11 and 12). This was out of a total of 44 measured miRNAs known to regulate the sphingolipid metabolism pathway. In liver tissue, however, we only detected miR-21-5p of the 3 miRNAs. Expression of miR-21-5p in liver did not mirror the expression in plasma. In contrast, livers with advanced fibrosis showed slightly, but significantly, lower expression of miR-21-5p (3% lower, $P < .05$; Supplementary Figure 11).

Causality Between Alcohol-Related Liver Disease and Sphingomyelin Depletion

We investigated the effect of ALD on SM level in the general population, independent of the present cohort. In data from FinnGen Biobank, 13 SNPs were associated with the risk of ALD at $P < 10^{-5}$ (for names, see Figure 5, *right*). These SNPs were used as instrumental variables in the calculation that simulates a randomized trial. Half of the SNPs belong to the B3GAT2, CCDC73, CDH18, LINC02668, PTP4A3, SERGEF, and SRP14-AS1 genes, whereas the remaining 7 SNPs are in noncoding regions. Data on the association of these SNPs with blood SM level were available in a cross-European study on metabolite quantitative trait loci.[31] Although the lipid profiles in the present cohort go down to the molecular species level, as of now the same level of detail is not available in genome-wide association studies. Therefore, we used total SM level in this external validation analysis.

Genetic susceptibility to ALD was associated with genetic susceptibility to lower SM level in the blood (inverse variance–weighted estimate of –0.032; $P = .0058$) (Figure 5, *left*). In other words, we detected a causal association between ALD and low SM level in the blood. The detected inverse association was stable in a leave-one-out analysis, in which we omitted 1 SNP at a time and recalculated the causal estimate (Figure 5, *right*).

Unlike between ALD and SM, we did not confirm an association between NAFLD and SM or alcohol dependency and SM (Supplementary Results). Moreover, we did not confirm an association in the other direction, from SM to ALD; that ALD causes SM was the only viable causal association (Supplementary Figure 13).

Alcohol-Related Liver Disease and Nonalcoholic Fatty Liver Disease Overlap

Finally, low SM levels were characteristic of pure ALD, not of ALD-NAFLD overlap. First, SM and Cer levels in plasma showed a positive correlation with presence of the metabolic syndrome (Figure 2, *right*). Second, we found higher levels of SMs in patients with a negative ANI (ANI $<$ 0.0), indicative of ALD-NAFLD overlap, and patients with ANI $\geq$ 0.0 ("pure ALD") were more likely to have higher levels of PCs (Figure 6). The ANI pattern was highly

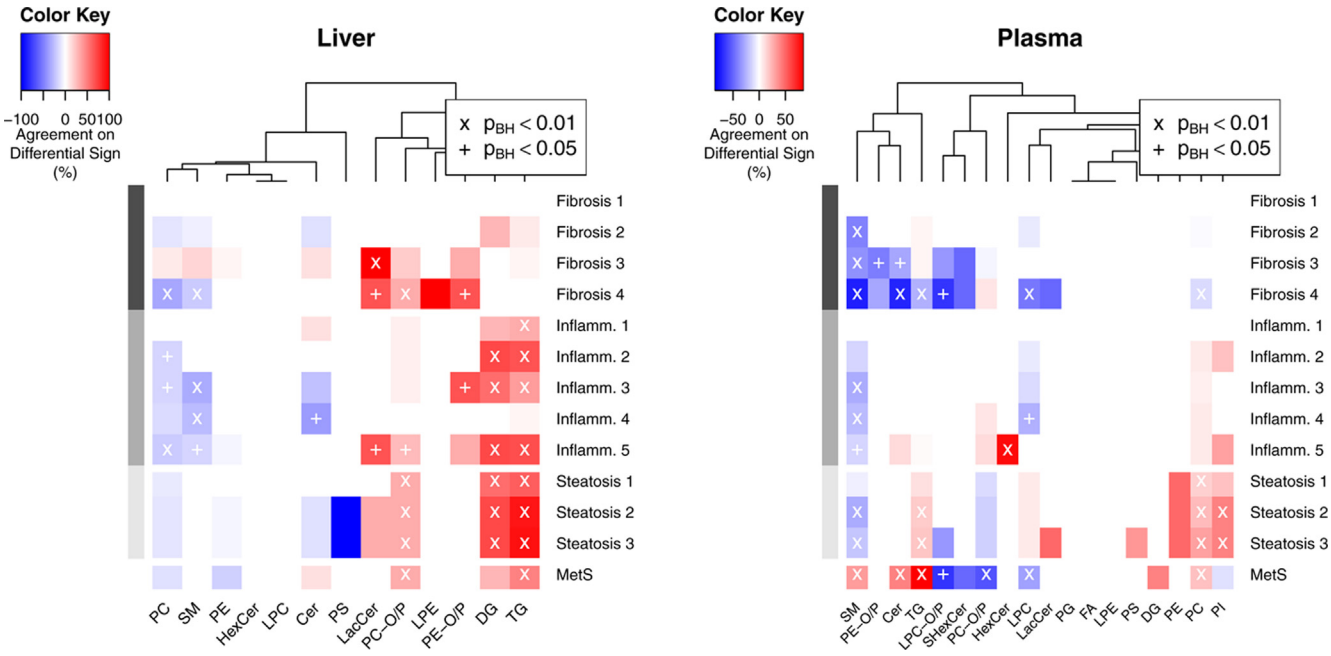

Figure 2. Overrepresentation analysis of lipid classes associated with ALD histologic severity in the liver lipidome (*left*) and plasma lipidome (*right*). The overrepresentation, or enrichment, shows the proportion of lipid species per lipid class (*columns*) that exhibit a significant correlation with liver histology (*rows*). Statistical significance is indicated by "x" ($P < .01$) or "+" ($P < .05$), both after adjustment to clinical covariates and correction for multiple testing. The *depth of the color* shows the proportion of lipids with a significant association in the class, and the *color* shows the sign of the associations (*red*: positive; *blue*: inverse). For instance, a significant proportion of all measured SMs were decreased in cirrhosis patients (fibrosis stage F4, Kleiner 4), as indicated by the *asterisk* in the *blue square* in the "SM" column and "Kleiner 4" *row* of the *left-side subfigure*. The results are based on liver samples from 301 people and plasma samples from 315 people. DG, diacylglycerols; LacCer, lactocyl-ceramides; LPC, lyso-phosphocholines; LPC-O/P, alkyl or alkenyl ether lyso-phosphocholines; LPE, lyso-phosphatidylethanolamines; PC-O/P, alkyl-acyl phosphocholines; PE, phosphatidylethanolamines; PG, phosphatidylglycer-ols; PI, phosphatidylinositols; PS, phosphatidylserines; SHexCer, sulfatide hexocyl-ceramides.

preserved at specific fibrosis stages in plasma (Supplementary Figure 14). Overall, the distinction between pure ALD and ALD-NAFLD overlap was more extensive in plasma, both in the full cohort and in the specific fibrosis stages.

Discussion

In this study, we reported that ALD is associated with comprehensive changes in the liver and plasma lipidomes across the full spectrum of histologic changes in the liver. The 2 major sphingolipid classes—SMs and Cers—exhibit a progressive depletion in liver fibrosis, mirrored by a progressive depletion in hepatic inflammation. This depletion is independent of metabolic comorbidities, current alcohol intake, and use of lipid-lowering drugs. In contrast, liver steatosis is characterized by elevated TGs and diacylglycerols.

SM(d41:1)—the lipid most tightly correlated with fibrosis in plasma and in liver—predicted 5-year risk of liver-related events. The prognostic potential was carried mostly by more liver-related events in patients with moderate and severe fibrosis with low SM(d41:1) levels vs high levels. Mendelian randomization suggested that ALD causes low SM levels and not vice versa. This causal link is present in ALD, but not in alcohol dependence. Similarly, the causal link does not seem present in NAFLD: both presence of the metabolic syndrome and a negative ANI indicative of ALD-NAFLD overlap correlated with higher SM levels. Finally, our analyses indicated aberrations in liver and circulating miRNAs, which control sphingolipid metabolism pathways.

Lipidomics—the systematic study of the molecular lipid profiles—has so far been used to describe diseases associated with metabolic dysfunction, although studies are emerging in ALD.[14,33,34] Our results are unexpected because they seem to conflict with the existing literature on NAFLD and ALD. Lipidomics studies in rodent models found that elevated sphingolipids associate with steatosis and steatohepatitis, and that Cer inhibition improves steatosis, attenuates insulin resistance, and protects against obesity.[11,35–38] Two studies on bariatric surgery patients found increased Cer in the livers of patients with NAFLD with insulin resistance and NASH.[39,40] However, a large biopsy-controlled study found that patients with NASH had significantly lower levels of SMs and PCs than patients with simple steatosis.[12]

We found that metabolic syndrome correlated positively with sphingolipids in our study, and that patients with ANI < 0.0 (ALD-NAFLD overlap) had higher SMs and Cers and lower PCs in both liver and plasma. When controlling for fibrosis, the plasma lipidome correlations with ANI remained stable at all stages of fibrosis. In liver, the pattern

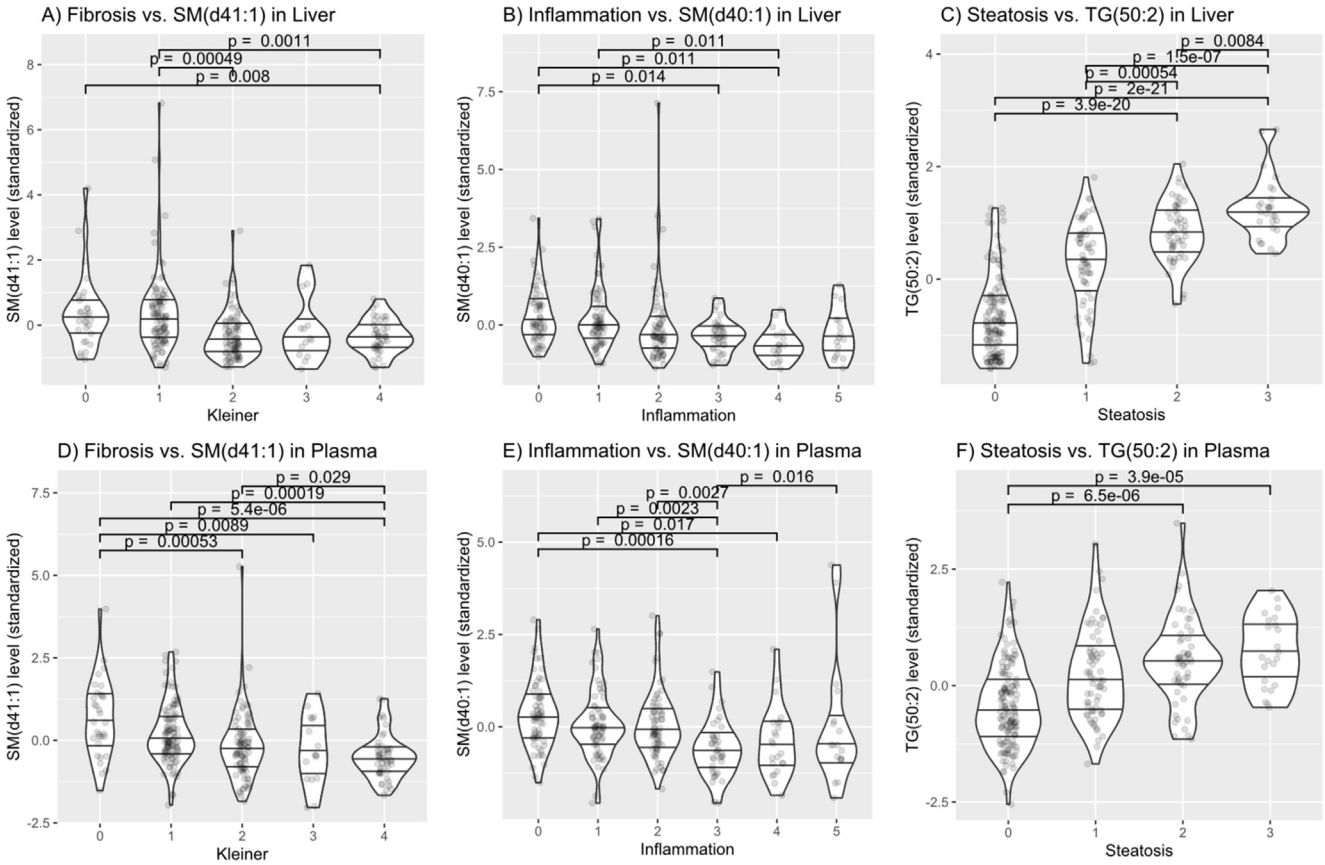

Figure 3. *Violin plots* of individual lipid species from lipid classes that had the earliest class-wide aberration of plasma levels in fibrosis (SMs; *left*), inflammation (SMs; *middle*), and steatosis (TGs; *right*). Individual lipids with strongest aberration over both liver and plasma were selected from these consistently affected classes. The quartiles of the levels of 3 individual lipid species are shown in the liver (*top*; n = 301) and in plasma (*bottom*; n = 315). SM(d41:1), SM(d40:1) and TG(50:2) were associated with fibrosis stage (*left*), inflammatory activity (*center*), and steatosis score (*right*), respectively. Observations are standardized to zero mean and unit variance for comparability. *P* values are after adjustment to clinical covariates and Benjamin-Hochberg correction for multiple testing.

was seen, but not significant, at early stages in PCs and at late stages in sphingolipids. These findings reveal heterogeneity in the lipidomic fingerprint of ALD, ranging from pure ALD to ALD-NAFLD overlap. However, comparison with a true NAFLD cohort without evidence of excessive drinking is needed to determine whether SM depletion could be used to distinguish between ALD and NAFLD.

Moreover, several short phospholipids and TGs were elevated with more severe fibrosis, unlike the rest of the class, which could indicate changes in de novo lipogenesis or loss of enzymatic function.[41] Lipid research in ALD has focused mostly on triglycerides, as they are the major lipid class accumulating in hepatocytes.[9] Previous reports on sphingolipids in ALD contrast with our findings; 1 case-control study found that lower Cers, but higher SMs, correlated with ALD cirrhosis, although neither with statistical significance.[15] Another case-control study found increased levels of Cers in patients with ALD with cirrhosis.[42] A rodent study linked alcohol intake to sphingolipid accumulation,[43] and a case-control study found increased activity of acid sphingomyelinase—the enzyme that hydrolyses SM into Cer—in patients hospitalized for alcohol detoxification.[44] However, as our Mendelian randomization showed, alcohol dependence does not cause low sphingolipid levels.

The depletion of sphingolipids in fibrosis was more notable in plasma than in liver. In line with our findings, a marked reduction in serum SMs was recently reported in acute decompensated cirrhosis.[45] In another recent study on severe fibrosis, the circulating levels of Cers were reduced in 12 people with cirrhosis out of a total of 75 people with chronic hepatitis C virus.[46] Taken together, these data elucidate the progressive depletion and disruption of the sphingolipid pathway in ALD fibrosis, especially in the circulation, but also in the liver.

Women generally have higher blood sphingolipid levels compared with men at the average age of the cohort.[47] In spite of the physiological differences in the lipid levels between men and women, we observed the same overall pattern of ALD, including the depletion of sphingolipids, in women and men alike.

There is evidence showing sphingolipid production by the gut microbiome, especially bacteroides via serine palmitoyltransferase activity.[48] In a study of the metagenomes

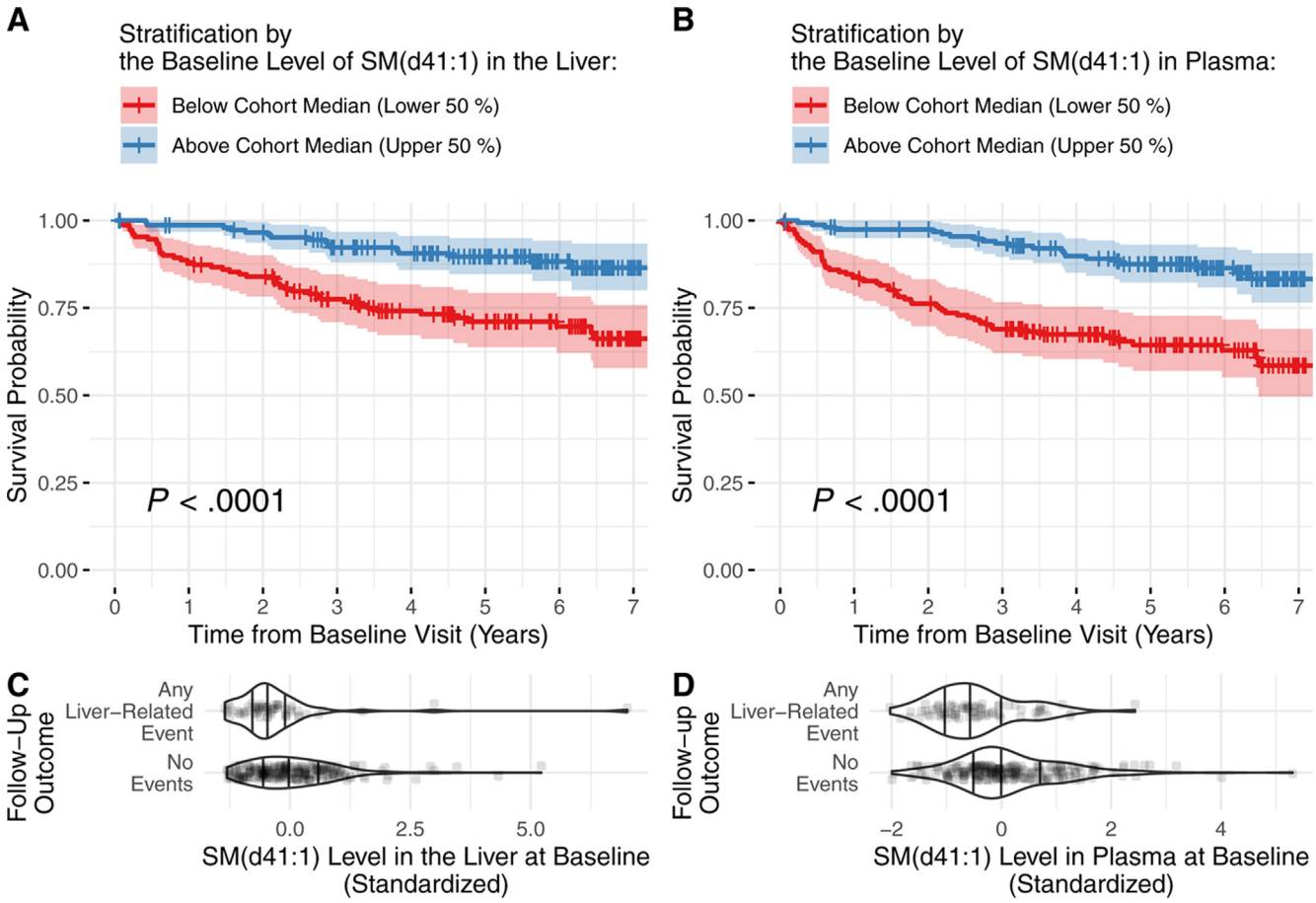

Figure 4. Survival curves for SM(d41:1) as a potential prognostic biomarker for prediction of liver-related events. *Top*: Kaplan-Meier survival curves and their 95% CIs for progression to liver-related events in the participants above (*blue*) and below (*red*) the median plasma level of the lipid SM(d41:1) at baseline in liver (*left*) and in plasma (*right*). *Bottom*: Violin plots showing baseline levels of SM(d41:1) in liver (*left*) and plasma (*right*) in patients with or without liver-related events during follow-up. Observations are standardized to zero mean and unit variance for comparability. SM(d41:1) levels in the event group are approximately 1 quartile lower compared with the event-free group. SMs are the lipid class in plasma that is affected class-wide earliest in fibrosis. Over both liver and plasma, SM(d41:1) is the individual lipid from SMs with the strongest aberration.

of 72 patients with alcohol dependence syndrome and 27 patients with alcohol-related cirrhosis, the relative abundance of bacteroides overall was unchanged, although 9 individual species were significantly decreased in patients with cirrhosis vs dependence.[49]

In our study, lactocyl-Cers and HexCers (also sphingolipids) were increased with fibrosis and inflammation, respectively, suggesting the possibility of Cer degeneration via the galactosylceramide synthase route.[50] Inhibition of another degeneration route via the acid ceramidase has been shown to ameliorate fibrosis in mice by means of activating hepatic stellate cells.[51] However, administration of the short-chained C6-Cer restored the lipid disbalance in a mouse model of NASH.[52]

Sphingolipids are not merely proinflammatory or lipotoxic. Rather, Cers, SMs, and their end-product sphingosine-1-phosphate (S1P) are readily convertible via the same enzymatic pathways, and aberrations in 2 points in the pathway may have opposing effects[11,35]; although Cers and SMs are associated with apoptosis and growth arrest, S1P has been ascribed to proinflammatory activity as well as to cell survival, growth, and proliferation.[11] Similarly, the role of Cers and SMs as signaling molecules differs from their effects in the cell nucleus or intracellular organelles (Supplementary Figure 15).

We propose that progressive ALD causes an inflammatory cascade that increases breakdown, or utilization, of Cers and SMs, ultimately leading to their depletion. However, our cohort study was not designed for an in-depth exploration of the possible mechanisms of sphingolipid depletion. Three circulating miRNAs known to regulate sphingolipid metabolism were up-regulated, while only 1 of those, miR-21-5p, was detected in liver tissue at lower expressions in patients with advanced fibrosis. Hepatic miR-21 likely protects the liver against acute alcohol injury.[53] Although the net effect of the aberrations is not known, our results indicate a reduction in tissue-repair capacity in the cirrhotic liver. Elevated circulating miR-21 has, in contrast to hepatic miR-21, been linked to increased liver fibrosis and inflammation.[53]

Two drugs are known to modulate the Cer–SM–S1P pathway. Suramin inhibits the S1P receptor 3. It appears to

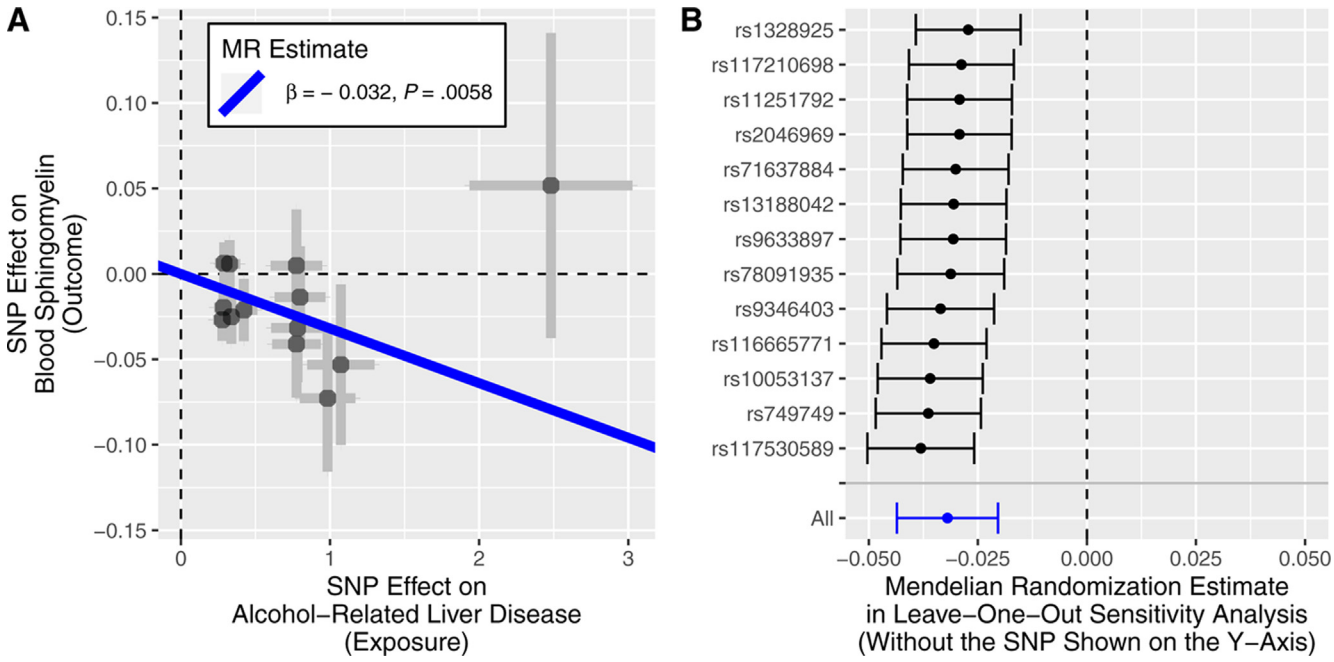

Figure 5. Test of causal association between ALD and blood SM level. *Left*: 2-sample Mendelian randomization (MR) meta-analysis indicates that genetic predisposition to ALD (*x-axis*) is inversely associated with genetic predisposition to high SM level in blood (*y-axis*), suggesting that ALD causes a decrease in the blood SM level. *Right*: The causal association was stable in a leave-one-out sensitivity analysis of the ALD risk SNPs (*rows*).

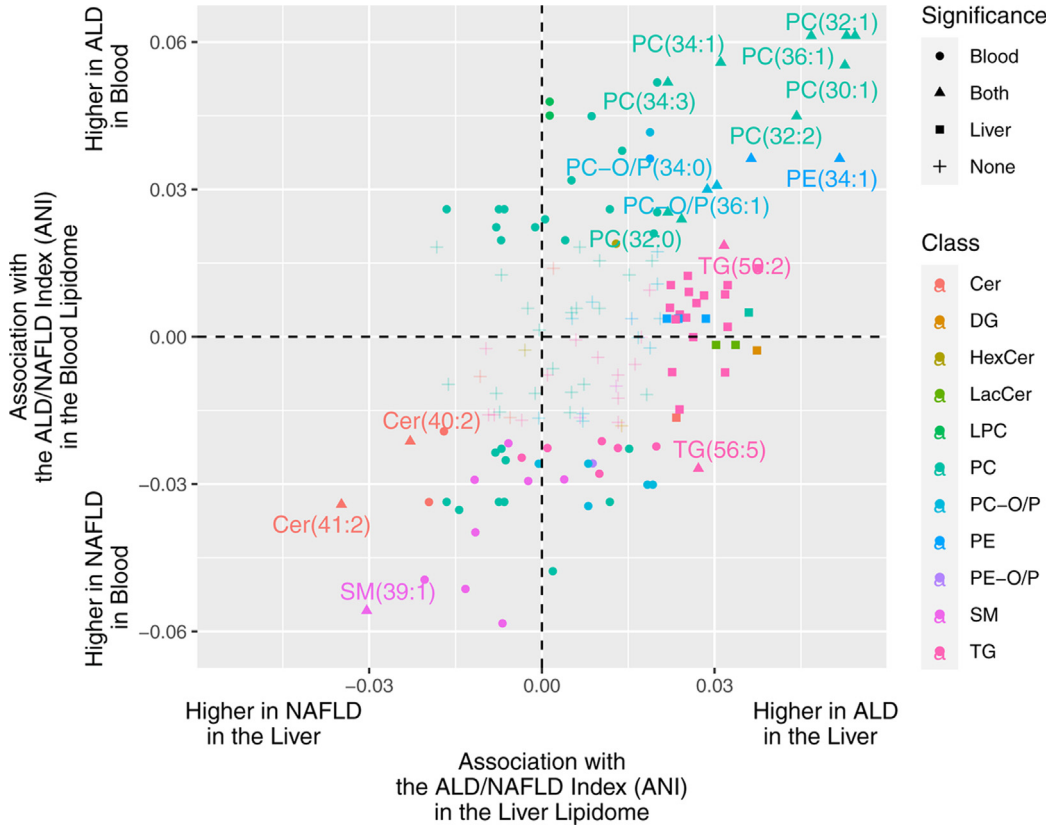

Figure 6. Associations between the ANI and levels of lipids detected in both liver and plasma. Lipids in the *lower left quadrant* are higher in liver and plasma in patients with ANI < 0, indicating ALD-NAFLD overlap-type liver disease. Lipids in the *upper right quadrant* are higher in both liver and plasma in patients with ANI > 0, indicating "pure ALD." Few lipids showed positive correlation with ANI > 0 in liver, but with ANI < 0 in plasma (*right lower quadrant*) or vice versa with ANI < 0 in liver, but ANI > 0 in plasma (*left upper quadrant*). Lipid species with a significant correlation in both liver and plasma are shown as *triangles* and labeled as *Class* (carbon length:number of double bonds). Lipid species that are associated with ANI only in the liver or plasma are labeled as *squares* or circles, respectively. PC-O/P, alkyl-acyl phosphocholine; PE, phosphatidylethanolamine.

attenuate bile duct ligation–induced fibrosis, but it also promotes inflammation.[54] Fingolimod is an S1P receptor modulator. In a mouse model of NAFLD, it decreased steatosis but increased inflammation and had no effect on fibrosis.[55] Dietary sphingolipids have been shown to diminish intestinal absorption of cholesterol, FAs, and TGs, and reduce hepatic lipid uptake.[56]

The main limitation of our study was the lack of mechanistic validation. However, we sought to validate our findings by investigating the regulation of genes involved in the sphingolipid pathways and by testing the association to follow-up outcomes, as well as by studying the causality of the association in external cohorts with Mendelian randomization. Another limitation was the coverage of lipids and lipid classes. We have aimed to report lipids that are representative of the lipid classes in human samples. This means that very-low-concentrated species may not have been covered. However, we did cover the entire size range of physiological SMs and most of the Cers. Although more challenging to detect, lower-concentrated species may also be relevant for disease pathophysiology, and thus further research is warranted. Future mass-spectrometry methods may be able to cover a wider range of lipid species and classes than what is currently possible, thereby shedding more detailed light on the role of individual lipids according to their length and saturation.

Advantages of our study were the large number of deep-phenotyped participants covering the full spectrum of histologic lesions, the careful adjustment to potential confounders, the interlaboratory-validated lipidomics method, and the follow-up data, together with the possibility of studying causality by Mendelian randomization in independent cohorts.

In conclusion, global lipidomics analyses revealed that alcohol-related liver fibrosis is associated with a progressive depletion of SMs, Cers, and phosphatidylcholines in the circulation and in the liver. This indicates a pro-fibrotic and pro-inflammatory mechanism, which could be characteristic of ALD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2023.02.023.

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Conflicts of interest
These authors disclose the following: Maja Thiele has received speaking fees from Norgine, EchoSens, Siemens Healthcare, and Tillotts Pharma, and consulting fee from GE Healthcare. Peter Rossing has received honoraria to Steno Diabetes Center Copenhagen from teaching and consultancy for Astellas, Astra Zeneca, Boehringer Ingelheim, Bayer, MSD, Gilead, Novo Nordisk, Sanofi, Vlfor, and Eli Lilly. Jonel Trebicka has received speaking and/or consulting fees from Gore, Bayer, Alexion, MSD, Gilead, Intercept, Norgine, Grifols, Versantis, and Martin Pharmaceutical. The remaining authors disclose no conflicts.

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Data Availability
The full study data sets are available from the authors on request to Odense Patient Data Exploratory Network (open@rsyd.dk)—a research infrastructure unit at Odense University Hospital—with reference to project ID OP_040. The study protocol, standard operating procedures, and patient information are also available on request. The data must not be processed for purposes other than statistical and scientific studies. Data are available on request for researchers who have acquired the required legal permissions from the Danish Data Protection Agency.