Low sphingolipid levels predict poor survival in patients with alcohol-related liver disease

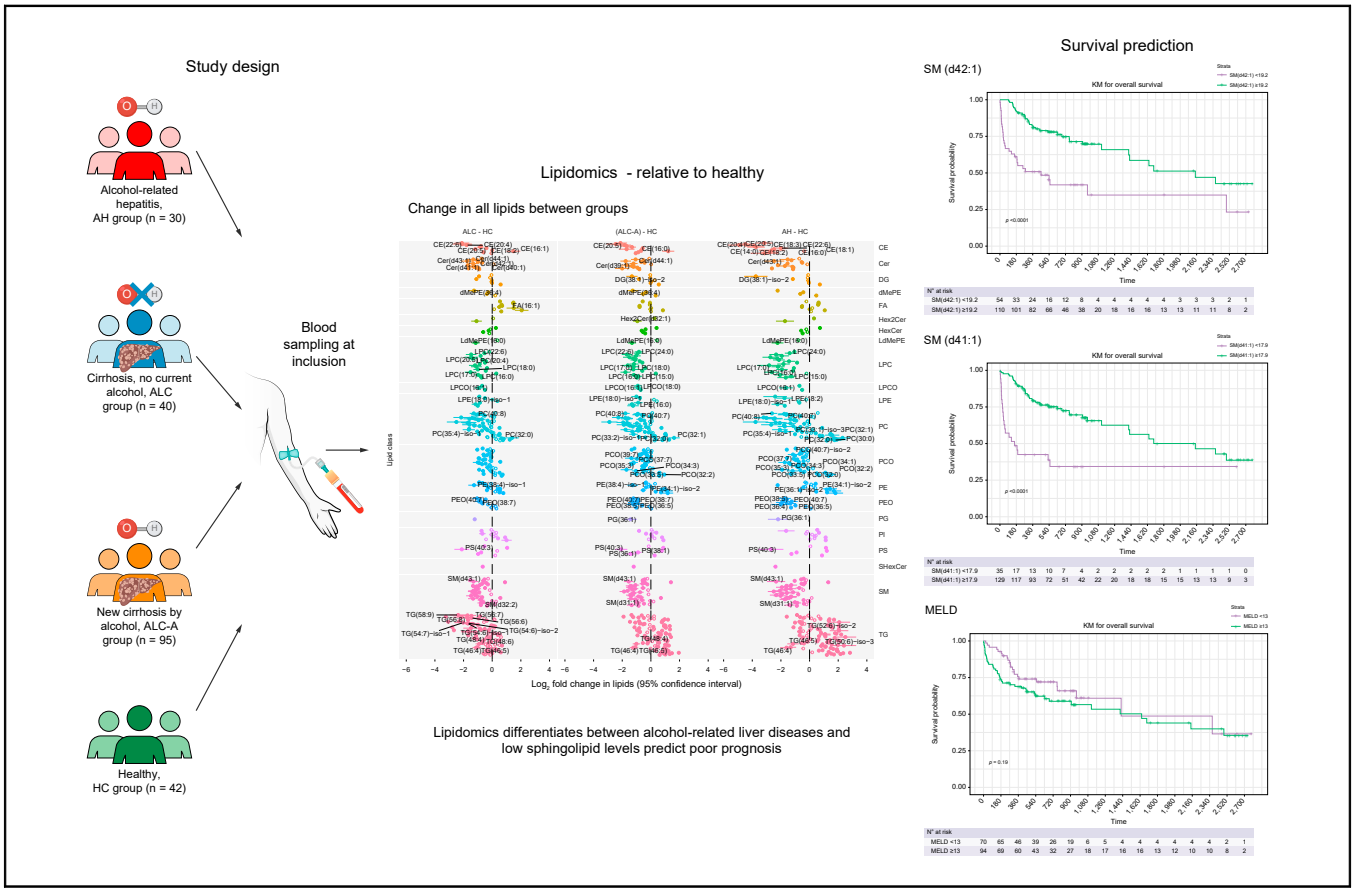
Authors

Thit Mynster Kronborg, Qian Gao, Kajetan Trošt, Henriette Ytting, Malene Barfod O'Connell, Mikkel Parsberg Werge, Mira Thing, Lise Lotte Gluud, Ole Hamberg, Søren Møller, Thomas Moritz, Flemming Bendtsen, Nina Kimer

Correspondence

thit.mynster.kronborg@regionh.dk (T.M. Kronborg).

Graphical abstract



Highlights

- Triglycerides and free fatty acids differed between cirrhosis groups.
- Total bile acids are increased in alcohol-related liver diseases.
- Low levels of sphingomyelins (d42:1) and (d41:1) predicted mortality in alcohol-related liver diseases.

Impact and implications

Lipidomics has the potential to diagnose and risk stratify patients with liver diseases. Lipidomics differed between patients with alcohol-related hepatitis and alcohol-related cirrhosis with and without recent alcohol use. Furthermore, lipidomics could predict short-term mortality and might be suitable as a prognostic tool in the future.

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Research article

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Low sphingolipid levels predict poor survival in patients with alcohol-related liver disease

Thit Mynster Kronborg,[1,*] Qian Gao,[2,†] Kajetan Trošt,[2,†] Henriette Ytting,[1,3] Malene Barfod O'Connell,[1] Mikkel Parsberg Werge,[1] Mira Thing,[1] Lise Lotte Gluud,[1] Ole Hamberg,[4] Søren Møller,[3,5] Thomas Moritz,[2] Flemming Bendtsen,[1] Nina Kimer[1]

[1]*Gastro Unit, Medical Division, University Hospital Hvidovre, Hvidovre, Denmark;* [2]*Novo Nordisk Foundation Centre for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;* [3]*Department of Clinical Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark;* [4]*Medical Department, University Hospital of Zealand, Koege, Denmark;* [5]*Centre for Functional and Diagnostic Imaging and Research, Department of Clinical Physiology and Nuclear Medicine, Hvidovre Hospital, Hvidovre, Denmark*

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Background & Aims: Alcohol-related hepatitis (AH) and alcohol-related cirrhosis are grave conditions with poor prognoses. Altered hepatic lipid metabolism can impact disease development and varies between different alcohol-related liver diseases. Therefore, we aimed to investigate lipidomics and metabolomics at various stages of alcohol-related liver diseases and their correlation with survival.

Methods: Patients with newly diagnosed alcohol-related cirrhosis, who currently used alcohol (ALC-A), stable outpatients with decompensated alcohol-related cirrhosis with at least 8 weeks of alcohol abstinence (ALC), and patients with AH, were compared with each other and with healthy controls (HC). Circulating lipids and metabolites were analysed using HPLC and mass spectrometry.

Results: Forty patients with ALC, 95 with ALC-A, 30 with AH, and 42 HC provided plasma. Lipid levels changed according to disease severity, with generally lower levels in AH and cirrhosis than in the HC group; this was most pronounced for AH, followed by ALC-A. Nine out of 10 free fatty acids differed between cirrhosis groups by relative increases of 0.12–0.66 in ALC compared with the ALC-A group (p <0.0005). For metabolomics, total bile acids increased by 19.7, 31.3, and 80.4 in the ALC, ALC-A, and AH groups, respectively, compared with HC (all p <0.0001). Low sphingolipid ([d42:1] and [d41:1]) levels could not predict 180-day mortality (AUC = 0.73, p = 0.95 and AUC = 0.73, p = 0.95) more accurately than the model for end-stage liver disease score (AUC = 0.71), but did predict 90-day mortality (AUC $_{d42:1}$ = 0.922, AUC $_{d41:1}$ = 0.893; p $_{d42:1}$ = 0.005, p $_{d41:1}$ = 0.007) more accurately than the MELD score AUC_{MELD} = 0.70, p_{MELD} = 0.19).

Conclusions: Alcohol-related severe liver disease is characterised by low lipid levels progressing with severity of liver disease, especially low sphingomyelins, which also associate to poor prognoses.

Impact and implications: Lipidomics has the potential to diagnose and risk stratify patients with liver diseases. Lipidomics differed between patients with alcohol-related hepatitis and alcohol-related cirrhosis with and without recent alcohol use. Furthermore, lipidomics could predict short-term mortality and might be suitable as a prognostic tool in the future.

Clinical Trials Registration: Scientific Ethics Committee of the Capital Region of Denmark, journal no. H-21013476.

Introduction

Excess alcohol intake impairs liver metabolism and is associated with alcohol-related liver diseases (ALDs) such as steatohepatitis, alcohol-related hepatitis (AH), fibrosis, and cirrhosis, all of which can increase mortality.[1]

Patients with AH typically receive a grave prognosis, with a 3-month mortality as high as 30–50%.[2–4] Often, patients with AH have established cirrhosis at the time of diagnosis, which markedly worsens their prognosis and indicates a phenotypic overlap.[3,5] Progression to cirrhosis is likely with continued alcohol abuse,[6] whereas patients with ALD who discontinue alcohol consumption have a lower risk of mortality and decompensation, indicating the benefits of abstinence.[7–9]

Alcohol-induced steatohepatitis is the initial step on the disease spectrum, and the adipose tissue appears to play an important role in disease progression, including disruption of the beta-oxidation of fatty acids in the mitochondria.[10–12] Disturbances in the hepatic lipid metabolism, cause intra- and extracellular accumulation of lipids, leading to lipotoxicity observed in various liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic steatohepatitis, and cirrhosis.[13] Lipidomics characterises lipid molecular species, their structure, and their biological role and represents a comprehensive overview of the

Keywords: Alcohol-related cirrhosis; Alcohol-related hepatitis; Lipidomics; Triglycerides; Free fatty acids.

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† Shared second authorship.

* Corresponding author. Address: Hvidovre University Hospital, Gastro Unit, Medical Division, 360, Kettegaard alle 30, 2650 Hvidovre, Denmark. Tel.: +45 23 498 946.

E-mail address: thit.mynster.kronborg@regionh.dk (T.M. Kronborg).

integrated lipid metabolism at a specific disease stage.[14] Recently, a distinct lipid depletion was observed in blood and liver tissue from patients with early ALD, with the magnitude of depletion correlating with liver-related events.[15]

Medium polarity metabolites, including bile acids (BAs), amino acids, and specified free fatty acids (FFAs), collectively known as the metabolome, are disrupted in ALD and may have an impact on outcomes in ALD.[16]

A few human studies investigating lipidomics and metabolomics in steatohepatitis and alcohol-related cirrhosis have been reported.[17,18] To our knowledge, the impact of the lipidomes and metabolomes on severity of liver diseases and on prognosis in patients with advanced liver disease, including alcohol-related hepatitis has not been made. We hypothesise that lipid metabolism is altered at various stages of ALD and might play a role in mortality. Furthermore, lipidomics may be influenced by alcohol consumption.

We aimed to identify lipids with differentiating and prognostic value for 180-day and 90-day survival in patients with ALD. In addition, we aimed to investigate the impact of alcohol on lipidomics and metabolomics in patients with ALD and to compare them with healthy individuals.

Patients and methods

Study participants

This comparative study was approved by the Scientific Ethics Committee of the Capital Region of Denmark, journal no. H-21013476.

Participants with AH (AH group) were recruited from a clinical trial conducted between September 2015 and May 2018 at the Department of Intestinal Failure and Liver Diseases, Rigshospitalet, Copenhagen, Denmark (EudraCT: 2014-02264-33).[19] All participants donated blood during diagnostic and investigative procedures, including biological material for future research. AH was defined as an alcohol intake of > 3 units of alcohol (36 g) per day for > 3 months or > 10 units per day for > 1 month, rapid development of jaundice (within 14 days), and plasma bilirubin > 80 μmol/L.[19]

Participants with alcohol-related cirrhosis and no use of alcohol within at least the past 8 weeks (ALC group) were recruited from another clinical trial (EudraCT: 2012-002890-71). This study was a double-blinded, randomised, controlled trial conducted between February 2013 and December 2015 at the Gastro Unit, Medical Division, Hvidovre University, Hvidovre, Denmark.[20] All participants in the ALC and AH groups underwent liver vein catheterisation with local anaesthesia as previously described.[20]

Participants with newly diagnosed alcohol-related cirrhosis and active alcohol use (ALC-A group) were recruited from a prospective cohort study (Scientific Ethics Committee, journal no.: H-19024348). Participants had an alcohol intake of > 14 units (168 g) per week within 1 month before their inclusion in the study, as reported by patients, their relatives or assessed by recruiting personnel and questionnaires. Participants were recruited at the Gastro Unit, Medical Division, Hvidovre University Hospital between October 2019 and March 2022. Patients underwent transjugular liver vein catheterisation as part of routine clinical management.

A healthy control group (HC group) without liver disease was recruited from a prospective cohort study (Scientific Ethics Committee, journal no.: H-17029039). The HC group had no comorbidities and had a normal Fibroscan (CAP and median) and routine biochemistry, including liver function tests and lipids. Two participants in the HC group had a BMI of 31, the rest had a BMI < 30. Participants were enrolled between September 2019 and June 2021.

All study participants gave their informed consent before donating blood for storage in a biobank. Performance of lipidomics and metabolomics was approved by the Scientific Ethics Committee of the Capital Region of Denmark in October 2021.

Blood sampling

Blood was sampled from a peripheral vein after acceptance into a study cohort. Routine biochemical analyses were performed according to standard operating procedures. EDTA plasma for the lipidomics was centrifuged, pipetted, and stored at -80 °C until analysis. All samples were thawed and aliquoted at once, followed by analysis of all samples placed in random on analysis plates. Analyses were performed blinded to clinical outcomes.

Plasma preparation for lipidomics analysis

Lipidomics were analysed according to existing procedures[21,22] and were modified for the purposes of the specific sample set and instrumentation. Samples were analysed randomly, without knowledge of any disease or clinical outcomes. For a detailed description, see the Supplementary material.

Plasma preparation for metabolomics analysis

Analyses of metabolomics were performed as described previously[21,22] and modified for the purposes of the specific project using plasma as the study matrix. For a detailed description, see the Supplementary material.

Instrumental analysis

Chromatographic separation was performed using an Agilent 1290 Infinity II ultra-HPLC system, UHPLC (Agilent, Waldbronn, Germany).

For details about the chromatographic separation of lipidomics and metabolomics and mass detection, see the Supplementary material.

Statistical analysis

Continuous variables are presented as means (SD, standard deviation) if normally distributed and as medians (IQR), if non-normally distributed. Categorical variables are reported as n (%). Differences among groups were tested using Welch ANOVA for normally distributed variables, the Kruskal–Wallis test for non-normally distributed variables, and the X^2 test for categorical variables.

To evaluate the associations between biochemical parameters and the hepatic venous pressure gradient (HVPG) with lipids and metabolites, we used Spearman's correlation. The 50 lipids with the highest hazard ratios (HRs), and all metabolites with significant HRs, were selected for analysis.

Lipids and metabolite levels were compared among groups using weighted least squares (WLS) and principal component analysis (PCA). Lipidomics and metabolomics data were log2-transformed before analysis. All concentrations were measured as the peak area of chromatography peaks for each lipid and metabolite, providing semi-quantitative measures among groups.

Log-rank test was used to compare mortality rates in the groups. To evaluate the associations between model for end-stage liver disease (MELD), biochemical parameters, lipids and

metabolites with overall and liver-specific mortality, we used multivariate Cox models and adjusted for baseline age and sex. Lipidomics and metabolomics data were log2-transformed and scaled before analysis. To evaluate the parameters for discriminating between survival and death, a receiver operating characteristic (ROC) curve was computed, and the area under the ROC curve (AUC) was calculated. The Youden index was used to determine optimal cut-offs for survival of 180 days. The Youden index is the difference between the true positive rate and false positive rate, and the maximising point in the index was chosen as the cut-off. The difference between ROC curves was assessed using the DeLong test.

To explore the optimal combination of lipids to discriminate between survival and death, the Best Subset Selection method was applied to optimise the predictive performance of Cox models.[23] The procedure was performed on different subsets of lipidomics data (all lipids, sphingomyelins [SMs], ceramides [Cers], SMs and Cers, SM + Cer + MELD, SM[d42:1] and SM [d41:1]). The maximum number of items in combinations were set to five. ROC curves with AUCs were calculated. The difference between the resulting ROC curves and the ROC curve for SM (d42:1) was tested using the DeLong test.

A two-tailed value of p <0.05 was considered statistically significant and was corrected for multiple testing using the Benjamini–Hochberg procedure.

All statistical analyses were performed in R (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

The study population comprised 30 patients with AH (AH group), 40 patients with alcohol-related cirrhosis without current alcohol use (ALC group), and 95 patients with alcohol-related cirrhosis and active alcohol use (ALC-A group). Forty-two healthy individuals served as a control group (HC group).

The mean age of the patients was 55.8 years (range: 24–80) and 65 (31%) were female. Table 1 summarises HVPG and biochemical characteristics of the study population, as well as comorbidities and outcomes of the patient groups. No patient was diagnosed with invasive cancer at inclusion, but two patients in the ALC-A group were diagnosed with hepatocellular carcinoma (HCC) shortly after and died within 180 days. MELD scores increased significantly between the ALC group and ALC-A and AH groups (Fig. S1).

Survival analysis

The liver-related and overall mortality was registered for 165 patients. One patient was lost to follow up (change of habitant country); 66 patients died during follow up: 36 of liver-related causes (55.4%), nine (13.8%) of causes not associated with liver disease, and 20 (30.8%) of unknown causes. Liver-related causes were spontaneous bacterial peritonitis, terminal liver failure, bleeding oesophageal varices, hepatorenal syndrome and HCC in cirrhotic liver. Causes not associated with liver diseases were trauma causing cerebral haemorrhage, cancer (non-HCC), heart failure, and colon diverticulitis with abscess and perforation. Survival curves for overall and liver-specific survival are presented in Fig. S2. The Kaplan–Meier curves show crossovers among groups, indicating that hazard ratios were not constant over time. To compare the ratios of different follow-up periods, we used a Cox model with a step function for 0–180, 181–1,080, 1,081–2,160, and >2,160 days of survival. Our main focus was on 180-day survival. The AH group had a significantly lower probability of survival compared with the ALC group in the first 180 days (p = 0.008). No difference was observed between the ALC and ALC-A groups (p = 0.18).

The lipid species with the biggest relative changes between groups in the lipidomics analysis were also those best able to predict overall mortality. Higher levels of SM and Cer were especially associated with a lower risk of all-cause death (Fig. 1).

To further explore the ability of lipids to predict all-cause mortality, ROC curves were computed, and compared with MELD scores.

The predicting performance of all lipids and metabolites analysed showed significant results for 90-day survival. The top three were SM(d42:1), triglyceride (TG)(48:4), and SM(d41:1), performing higher AUCs and lower p values. TGs are more easily affected by diet, and SMs are more likely to reflect functional changes, why we chose to focus on the two SMs.[24,25]

SM(d42:1) and SM(d41:1) showed a tendency of better ability to predict 180-day survival (AUC = 0.73 and 0.73) compared with MELD (AUC = 0.71) and significantly better ability to predict 90-day survival (AUC = 0.92 and 0.89; p = 0.005 and 0.008, respectively) than MELD scores (AUC = 0.70). When leaving out the AH group, the SMs were still better predictors compared with MELD, with SM(d42:1) giving an $AUC_{180\ days}$ = 0.63 and SM(d41:1) showing $AUC_{180\ days}$=0.64, and significantly better prediction of 90-day mortality by $AUC_{90\ days}$ = 0.90 for SM(d42:1) and $AUC_{90\ days}$ = 0.84 for SM(d41:1). The inter-group comparisons showed non-significant changes in terms of survival between the AH and the ALC-A group for both SM(d42:1) and SM(d41:1) (Table 2). Survival at 720 and 1,800 days showed non-significant associations. The optimal cut-off point was calculated based on 180-day survival and is shown in the Kaplan–Meier plots in Fig. 2, where SM(d42:1) and SM(d41:1) below the cut-off points (19.2 and 17.9) indicated a higher risk of mortality. We found no differences in survival between the ALC and ALC-A groups; hence, Kaplan–Meier curves were prepared for the AH group alone and for the ALC and ALC-A groups together. Both curves show significant associations between low SM(d42:1) and SM(d41:1) and mortality (Fig. S3). On the contrary, Kaplan–Meier curves for the MELD scores of the AH group alone and the cirrhosis groups pooled showed only non-significant associations (Fig. S4).

Adding MELD scores to the prognostic models using SM(d42:1) or SM(d41:1) did not improve their ability to predict survival/mortality; as such, we continued to use separate models.

Combining SM(d42:1) and SM(d41:1) to predict 90-day mortality was not superior to SM(d42:1) alone (AUC = 0.92). Furthermore, combining SM and Cer lipids in models with up to five lipids showed similar AUCs (AUC = 0.93–0.94), with non-significant differences from SM(d42:1) (p values: 0.46–0.66) in predicting 90-day mortality. Adding MELD score to the analyses had no impact on the AUCs mentioned above.

Influence of cancer

Two patients in the ALC-A cohort were diagnosed with malignancy shortly after inclusion. However, their lipid and metabolite profiles were similar to the remaining cohort (Fig. S5A and B). These patients were not excluded from the analyses.

Lipidomics

Six lipid groups

We compared the relative changes in log2 fold changes in 21 lipid groups (376 distinct lipids) from all patient groups with the

Table 1. Patient characteristics.

	Total	Cirrhosis without current alcohol use, ALC group	Cirrhosis with current alcohol use, ALC-A group	Alcohol-related hepatitis, AH group	*p* value, patient groups compared	Healthy control group, HC group	*p* value, all groups compared
N	207	40	95	30		42	
Male	142 (68.6)	33 (82.5)	62 (65.3)	20 (66.7)	0.128	27 (64.3)	0.213
Age	57 (49–64)	55 (51–60)	61 (55–67)	51 (47–61)	<0.001	48 (34–57)	<0.001
Use of alcohol		**None within the past 8 weeks**	**Daily alcohol use until the time of sampling**	**>36 g/day for >3 months or >120 g/day for >1 month**		**Below official recommendations†**	
Hb, mmol/L	7.50 (6.43; 8.47)	7.60 (7.00; 8.35)	7.10 (5.95; 7.70)	6.55 (6.10; 7.50)	0.002	8.90 (8.50; 9.30)	<0.001
Platelets, 10^9/L	177 (119; 234)	141 (115; 204)	153 (109; 234)	183 (98; 217)	0.718	227 (187; 261]	<0.001
Sodium, mmol/L	136 (133; 139)	136 (134; 138)	135 (131; 137)	135 (130; 139)	0.299	140 (139; 141)	<0.001
Creatinine, μmol/L	65 (55; 84)	62 (57; 77)	59 (49; 78)	76 (56; 100)	0.037	74 (66; 88)	<0.001
Albumin, g/L	27 (21; 34)	32 (28; 34)	24 (21; 28)	21 (17; 25)	<0.001	38 (36; 41)	<0.001
INR	1.4 (1.1; 1.8)	1.3 (1.2; 1.4)	1.4 (1.2; 1.8)	2.0 (1.8; 2.8)	<0.001	1.0 (1.0; 1.1)	<0.001
ALT, U/L	30.50 (21.25; 44.00)	24.00 (18.50; 32.00)	37.00 (24.00; 53.50)	44.00 (36.00; 66.00)	<0.001	21.00 (17.00; 25.75)	<0.001
Bilirubin, μmol/L	28 (12; 84.5)	18.5 (13; 34)	34 (18.5; 84)	345 (267; 444.5)	<0.001	10 (7; 15]	<0.001
CRP, mg/L	8.0 (2.0; 27.8)	5.0 (2.0; 7.0)	18.5 (5.6; 41.0)	28.5 (13.5; 41.5)	<0.001	0.7 (0.6; 1.3)	<0.001
In-hospital at inclusion	106 (51.2)	0 (0.0)	76 (80.0)	30 (100.0)		0 (0.0)	
MELD, score	12 (8; 19)	11 (9; 13)	14 (10; 19)	28 (23; 31)	<0.001	7 (6; 7)	<0.001
Child–Pugh					<0.001		<0.001
A	11 (8.1)	0 (0.0)	11 (11.6)	–		–	
B	78 (57.8)	34 (85.0)	44 (46.3)	–		–	
C	46 (34.1)	6 (15.0)	40 (42.1)	–		–	
GAHS				10 (9; 11)			
Ascites	103 (49.8)	40 (100)	43 (45.3)	20 (66.7)		0 (0)	–
HVPG, mm Hg	15 (12; 18)	16 (13; 18)	14 (10; 16)	18 (13; 19)	0.007	2 (1; 2)	<0.001
Diabetes, type I and II (%)	21 (10.3)	6 (15.0)	12 (12.6)	3 (11.5)	0.904	0 (0.0)	0.096
Heart disease (%)	14 (6.9)	4 (10.0)	10 (10.5)	0 (0)	0.204	0 (0)	0.058
Hypertension (%)	18 (8.9)	3 (7.5)	10 (10.5)	4 (16.0)	0.556	1 (2.4)	0.245
Hypercholesteraemia (%)	8 (5.1)	1 (2.5)	6 (6.3)	1 (4.5)	0.649	–	0.654
Statin use (%)	30 (14.9)	4 (10.0)	22 (23.2)	2 (8.0)	0.073	2 (4.8)	0.017
Cause of death (%)					0.144		<0.001
Liver-related causes	36 (55.4)	11 (68.8)	14 (45.2)	11 (61.1)		0 (0.0)	
Other causes	9 (13.8)	2 (12.5)	7 (22.6)	0 (0.0)		0 (0.0)	
Unknown	20 (30.8)	3 (18.8)	10 (32.3)	7 (38.9)		0 (0.0)	
Status (%)					0.014		
Death (follow-up of ≥180 days)	65 (31.4)	16 (40.0)	31 (32.6)	18 (60.0)		NA	

All data are presented as medians (IQR), or n (%). The *p* values comparing patient groups and *p* values comparing all groups are listed. Welch ANOVA was used for normal distributed variables, Kruskal–Wallis test for non-normal distributed variables and X^2 test for categorical variables. †<24 g/day for men and <12 g/day for women. ALAT, alanine aminotransferase; CRP, C-reactive protein; DM, diabetes mellitus; GAHS, Glasgow alcoholic hepatitis score; Hb, haemoglobin; HVPG, hepatic venous pressure gradient; INR, international normalised ratio; MELD, model for end-stage liver disease.

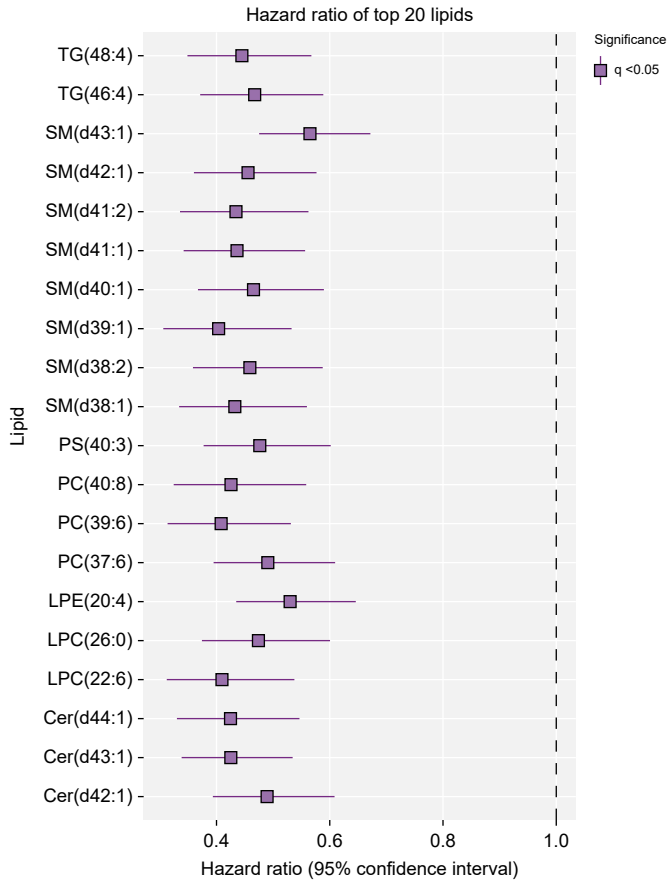


Fig. 1. Hazard ratio of all-cause mortality of the top 20 most significant lipids (95% CIs) for all participants pooled (n = 207). HRs were calculated from Cox proportional hazards models adjusted for baseline age and sex. Higher levels of the lipids are associated with a lower HR of all-cause mortality. Cer, ceramide; HR, hazard ratio; LPC, lysophosphatidylcholine; LPE, lysephosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride.

healthy control group (Fig. 3A), and a PCA analysis was performed to compare levels of lipids between groups (Fig. S5). All relative level changes from the analyses are reported in Supplementary Data File 1. The changes were similar across all three patient groups and included lower levels of SMs, Cers, lysophosphatidylcholines (LPCs), and cholesterol esters (CEs), also when adjusting for MELD score (Fig. 3B). Phosphatidylcholines (PCs) and phosphatidylcholine-ethers (PCOs) showed a mixed trend where smaller lipids with fewer double bonds were increased and larger lipids with more double bonds were decreased. The changes varied among the patient groups, with the most pronounced changes in the AH group, followed by the ALC-A group, and least in the ALC group.

Cers were strikingly different in all patient groups compared with the healthy group, whereas inter-patient group differences were less common (Table S1). The lowest levels were observed in the AH group compared with the ALC-A group and the ALC group, the latter of which had the highest levels among the three patient groups. Significantly changed lipids were more numerous in the SM, LPC, and CE classes among all patient groups, with the fewest differences found between the ALC and ALC-A groups. Inter-group comparisons of all lipid classes in the patient groups are shown in Fig. 3C).

Triglycerides and free fatty acids

Compared with HC, most TGs were at lower levels in the ALC group and higher in the ALC-A and AH groups. Nine of the 10 FFAs in the ALC group were higher than in the HC group, with relative increases ranging from 0.1 to 3.16 (p values from <0.0001 to 0.025). TGs and FFAs showed multiple differences among patients (Fig. 3C), interestingly also between the two cirrhosis groups. The ALC group also showed increases relative to all other groups, with eight or nine increased FFAs in all comparisons (nine FFA increased by 0.12–0.66 compared with the ALC-A group, p values <0.0001 to 0.0005). Differences in TGs and FFAs between the groups are shown as volcano plots in Fig. 4, along with a few other significantly different lipid molecules, and are listed in Table S1. Correlations between lipids, biochemical parameters, and HVPG for all study participants are shown as a heatmap in Fig. S6A. Correlations between lipids and MELD among groups are shown in Fig. S6B.

Metabolomics

All relative changes from the metabolomic analysis are reported in Supplementary Data File 2.

Bile acids

BAs in plasma from all patient groups and their comparison to HCs are shown in Fig. 5A. Most BAs were increased in patients and were positively associated with their MELD score (a heat map of the correlations between biochemical parameters, HVPG, and BAs is shown in Fig. S7), except deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA), which had lower levels. Non-conjugated BAs did not differ between groups. UDCA was lower in the ALC-A and the AH group compared to HC, whereas DCA was lower only in the AH group (Table 3 and Fig. 5A).

Glycine-conjugated BAs increased in all of the patient groups as compared with the HCs; a greater increase was observed in taurine-conjugated BAs (Fig. 5 and Table 3). We found only minor differences between the ALC group and ALC-A group for all BAs, except for significant differences in glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA) and UDCA, with the ACL-A group having higher levels of GCDCA and GCA and lower levels of UDCA. The AH group had higher levels of most BAs than the ALC-A group and ALC group (Table 3 and Fig. 5B). Total BAs were significantly higher in all patient groups than in the HC group (increased levels by 19.7 in the ALC, 31.3 in ALC-A, and 80.4 in the AH group), with p values <0.001. Total differences in BAs are presented in Fig. S8.

Other metabolites

The ALC group had higher levels of a wide range of FFAs such as palmitoleic acid, oleic acid and elaidate (see Fig. S9 for the top 20 most significant metabolites). This was not the case in the ALC-A or the AH group. Biliverdin, a by-product of haemoglobin breakdown, was significantly higher in the AH group. No metabolite was significantly relatively changed in all group comparisons (Table S2), but multiple single significant differences were found. The smallest differences were found between the AH and ALC-A groups and between the ALC and ALC-A groups.

Table 2. Relative changes of SM(d42:1) and SM(d41:1) according to survival among groups.

	AH group *vs.* HC group	AH group *vs.* ALC group	AH group *vs.* ALC-A group	ALC group *vs.* HC group	ALC-A group *vs.* ALC group	ALC-A group *vs.* HC group
SM(d41:1)	-0.74 (-0.8; -0.65) *p* <0.0001	-0.43 (-0.58; -0.23) *p* <0.0001	-0.27 (-0.45; -0.03) n.s.: *p* = 0.1068	-0.54 (-0.62; -0.45) *p* <0.0001	-0.22 (-0.36; -0.04) *p* = 0.0048	-0.64 (-0.7; -0.57) *p* <0.0001
SM(d42:1)	-0.59 (-0.69; -0.45) *p* <0.0001	-0.24 (-0.43; 0.00) *p* = 0.0224	-0.13 (-0.34; 0.15) n.s.: *p* = 0.3014	-0.45 (-0.54; -0.35) *p* <0.0001	-0.13 (-0.27; 0.03) n.s.: *p* = 0.0852	-0.52 (-0.6; -0.43) *p* <0.0001

p values adjusted for multiple testing. AH, alcohol-related hepatitis; ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use; HC, healthy control; n.s., non significant.

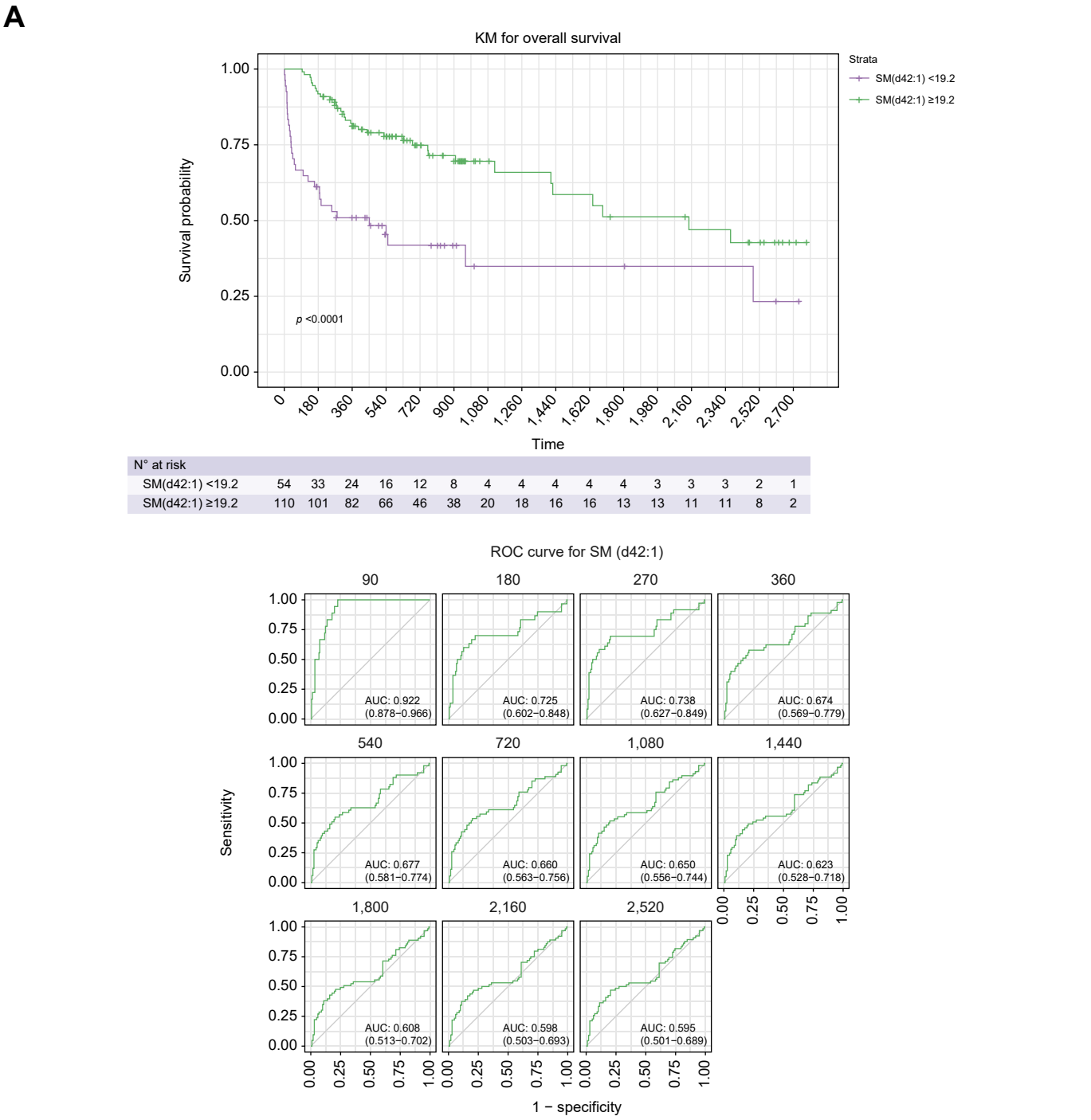
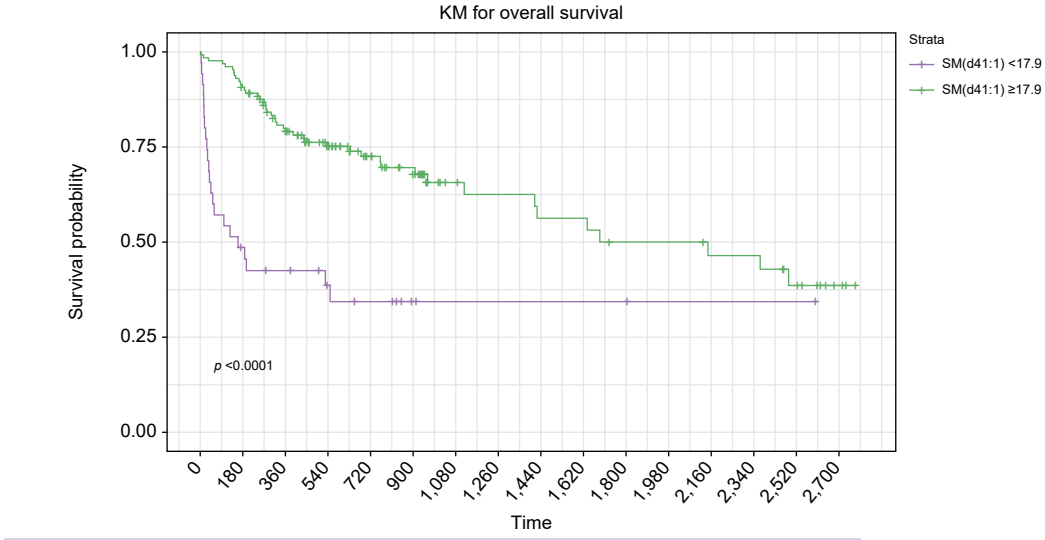


Fig. 2. Survival prediction using SM(d42:1), SM(d41:1) and MELD score. Kaplan–Meier curves for ALC, ALC-A, and AH groups pooled with an optimal cut-off point based on 180-day survival. Curves illustrate SM levels <19.2 and ≥19.2 in (A), <17.9 and ≥17.9 in (B), and MELD score <13 and ≥13 in (C). AH, alcohol-related hepatitis; ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use; KM, Kaplan–Meier; MELD, model for end-stage liver disease; ROC, receiver operating characteristic; SM, sphingomyelin.

B



N° at risk																
SM(d41:1) <17.9	35	17	13	10	7	4	2	2	2	2	2	1	1	1	1	0
SM(d41:1) ≥17.9	129	117	93	72	51	42	22	20	18	18	15	15	13	13	9	3

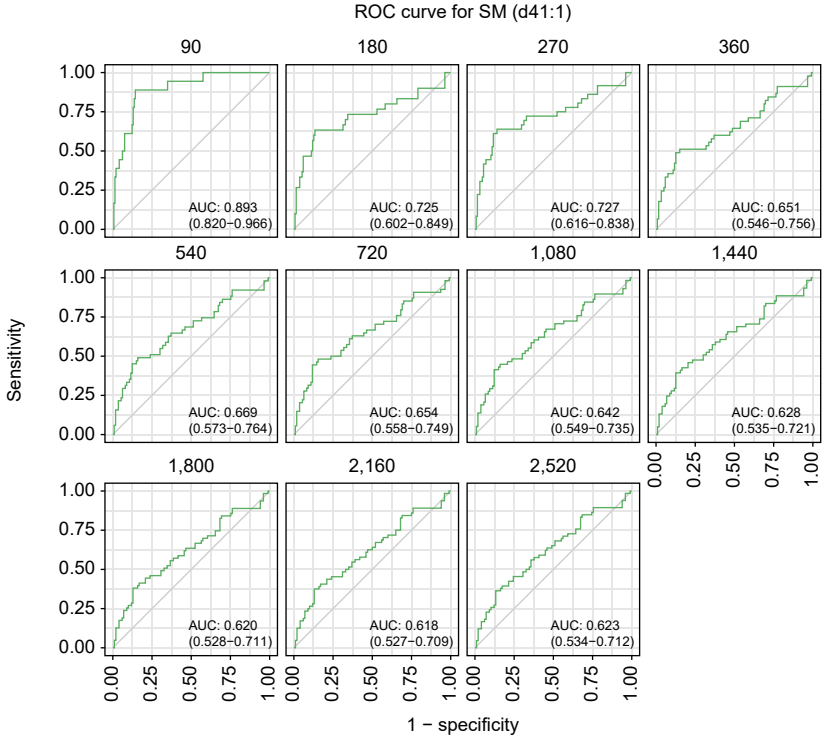


Fig. 2 (*continued*).

Discussion

The current study presents numerous lipidomic and metabolomic differences between patients with ALD and healthy individuals, with gradual relative differences among the patient groups, progressing with the severity of the liver disease. The most striking findings were the potential of the lipidomics and metabolomics to differentiate among patient groups, where FFAs were higher in the ALC group than in all other groups, the diversity in TGs and the capability of the sphingomyelins SM(d42:1) and SM(d41:1) to predict mortality. Pronounced changes in lipidomics were observed in the AH group, followed by ALC-A, and least of all in ALC.

Lipidomics has the potential to reveal biomarkers for disease and the risk of developing disease, including NAFLD, and to show the effects of alcohol on brain tissue.[26–29] In particular, it has been suggested that elevated TGs and low PCs could be important biomarkers for predicting the presence of NAFLD, indicating a role of TGs and PCs in liver disease.[30] When TGs are metabolised, FFAs are released and converted back to TGs in hepatic cells and transported via the blood as very low-density lipoproteins to

C

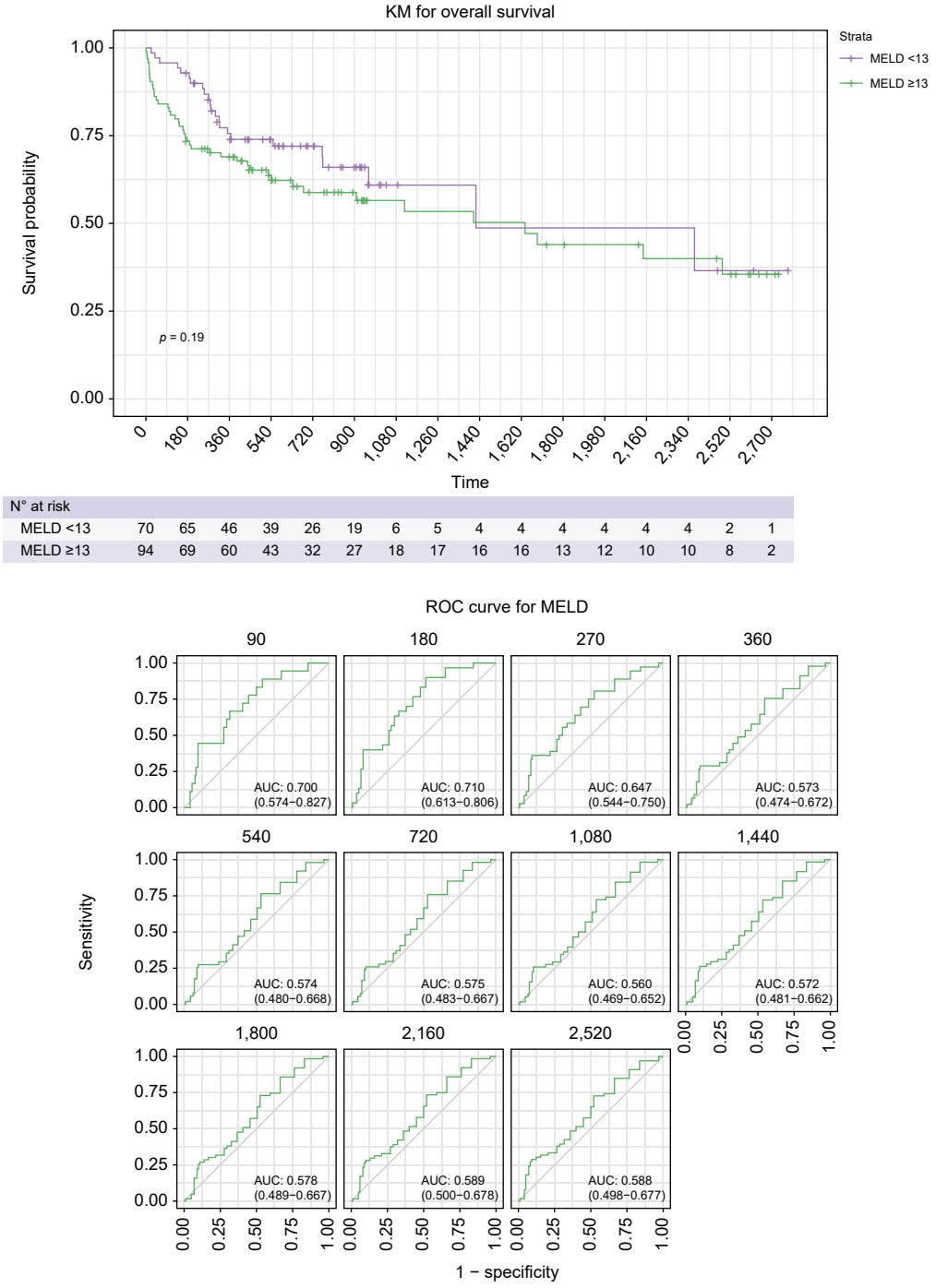


Fig. 2 (*continued*).

avoid fat accumulation. Alcohol disrupts this balance by promoting lipogenesis and leads to an increase of intracellular FFAs.[11,13] The liver is able to reverse this process completely when alcohol is removed through abstinence,[11] but chronic alcohol consumption exacerbates lipolysis of the adipose tissue, probably by impairing insulin sensitivity, which results in reduced body fat mass despite increases in hepatic lipid accumulation.[31]

TGs were lower in the ALC group than in the ALC-A and the AH groups, and the AH group showed higher TGs in all comparisons, as described previously.[32]

FFAs were higher in patients with cirrhosis without recent alcohol intake (ALC group) than in patients with cirrhosis with recent alcohol intake (ALC-A), AH patients, and healthy participants. In contrast, Rachakonda *et al.*[10] found higher levels of FFAs in serum samples from patients with AH than in cirrhosis. Differences in methods (especially participant criteria) and study designs might explain discrepancies such as these. The ALC group had the highest levels of FFAs, comparable to those observed in type 2 diabetes, obesity, hepatic steatosis, and several cardiovascular diseases. Increased FFAs contribute to the

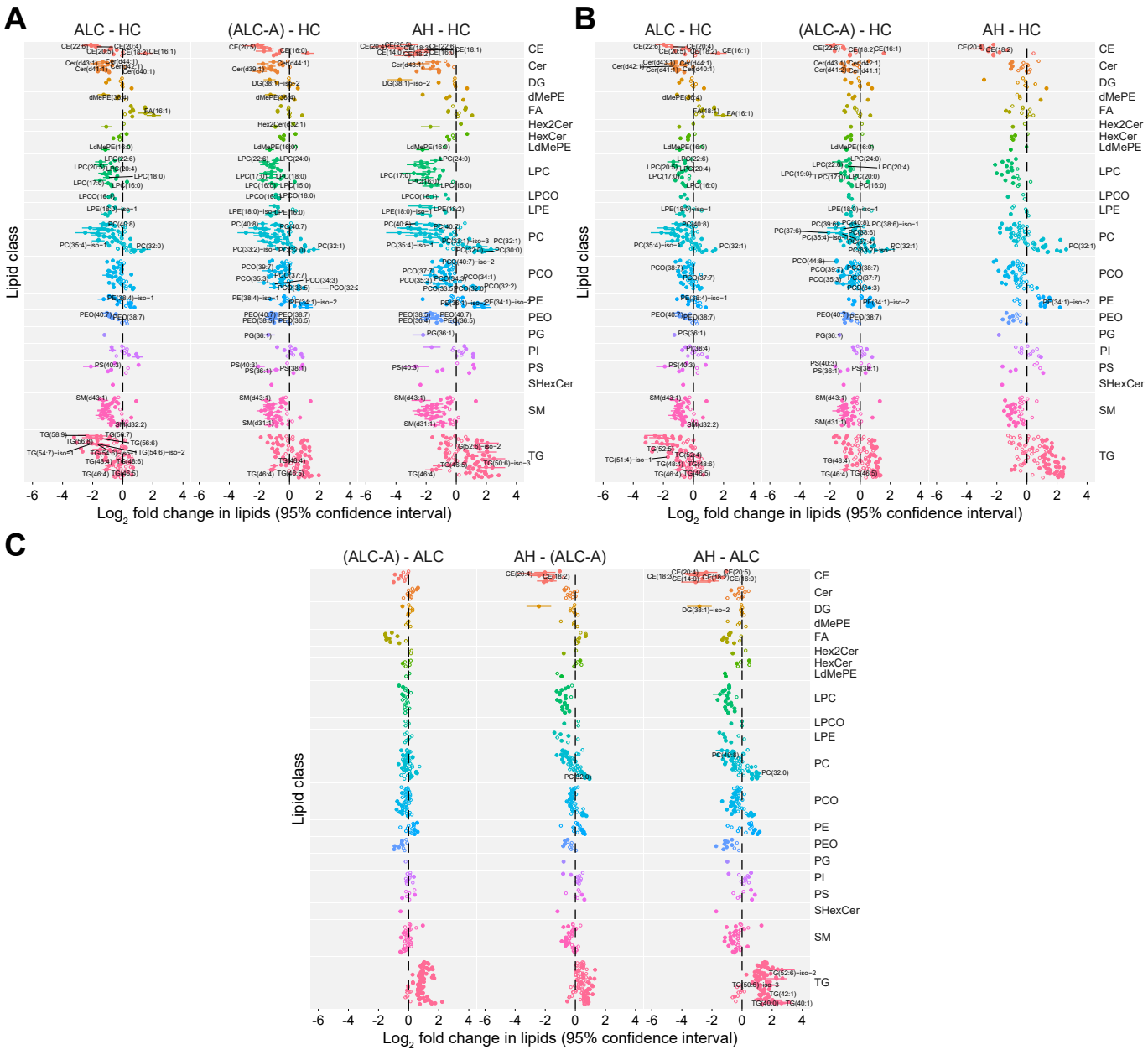


Fig. 3. Plasma lipidomics (21 lipid classes). Analysis between (A) groups with alcohol-related liver disease and healthy individuals, (B) groups with alcohol-related liver disease and healthy individuals adjusted for MELD, and (C) between groups with alcohol-related liver disease. Relative changes in lipids derived from plasma. Changes are calculated from weighted least squares regression coefficients. The Benjamini–Hochberg procedure was used to correct for multiple testing. ○ = non-significant; ● = p <0.05; p <1e^{-10}. AH, alcohol-related hepatitis; ALC, cirrhosis without current alcohol use, ALC-A, cirrhosis with current alcohol use, CE, cholesterol ester; Cer, ceramide; DG, diacylglycerol; dMePE, dimethylphosphatidylethanolamine; FA, free fatty acid; HC, healthy control group; Hex2Cer, dihexosyl ceramide; HexCer, hexosyl ceramide; LdMePE, lysodimethylphosphatidylethanolamine; LPC, lysophosphatidylcholine; LPCO, lysophosphatidylcholine ether; LPE, lysephosphatidylethanolamine; PC, phosphatidylcholine; PCO, phosphatidylcholine ether; PE, phosphatidylethanolamine; PEO, phosphatidylethanolamine ether; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; sHexCer, sulfatides hexosyl ceramide, SM, sphingomyelin, TG, triglyceride.

development of atherosclerosis by endothelial cell apoptosis, thereby increasing the risk of cardiovascular events.[33]

Interestingly, the use of statins and the number of patients with diabetes and hypercholesterolaemia were low and evenly distributed among our groups. Hence, diabetes and use of statins cannot explain the differences within patient groups and between patients and HC. These findings require further investigation of the underlying mechanisms, and potentially harmful effects, of dysregulated TGs and FFAs.[33]

The differences in TGs, FFAs, and the diversity in lipid metabolites indicate a distinct pathology for stable cirrhosis, and is likely accompanied by a different risk profile, compared with patients with newly diagnosed cirrhosis and active use of alcohol. Israelsen *et al.*[21] reported the acute effects of

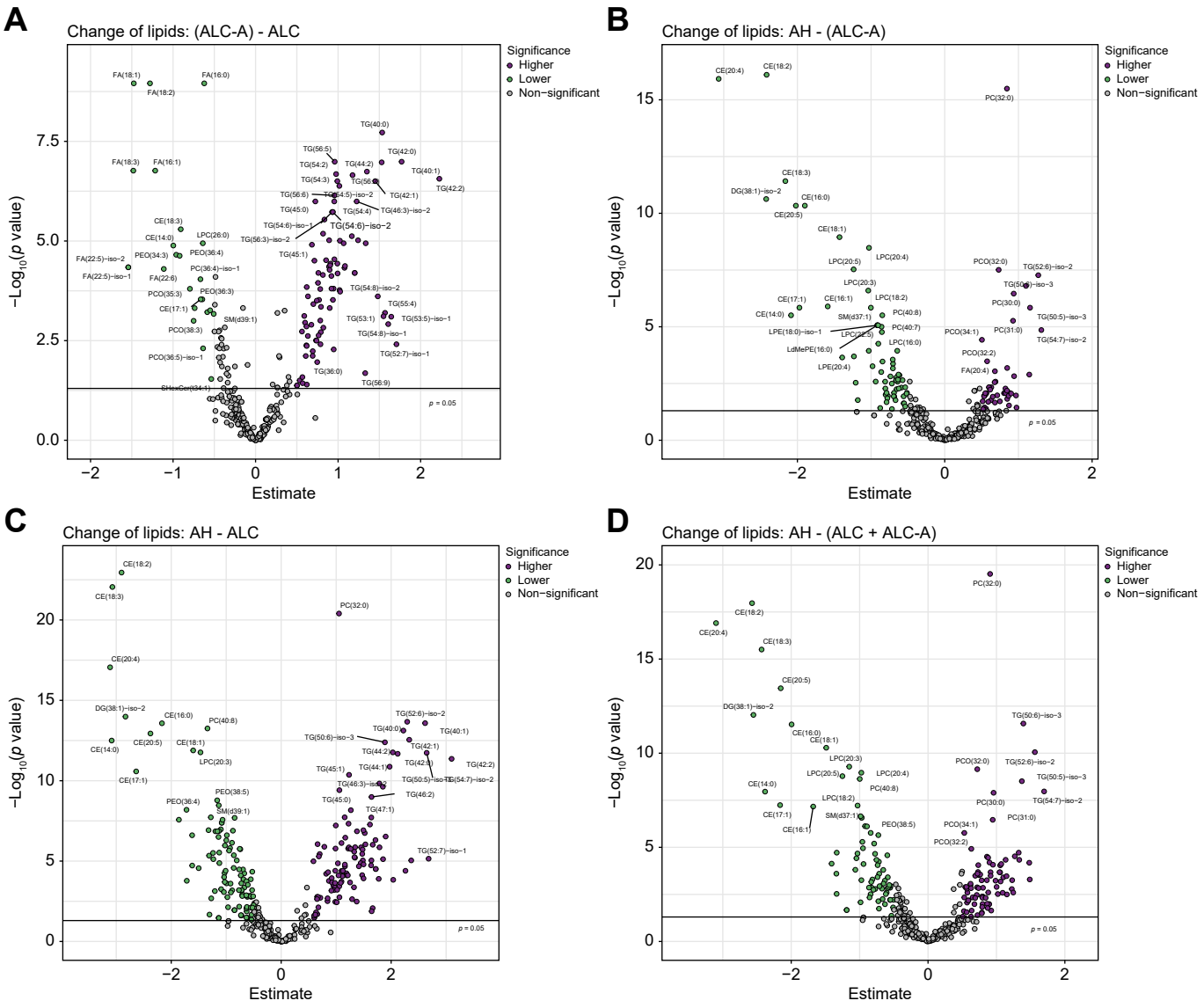


Fig. 4. Lipid differences among groups. Volcano plot of distinct differences among the ALC-A and ALC groups (A). Levels in the ALC-A groups compared with levels in the ALC group. Estimates were calculated as log2 fold changes between groups. Red dots indicate an increase in the ALC-A group compared with the ALC group, whereas blue dots indicate decreases in the ALC-A group compared with the ALC group. (B) Distinct differences among the AH and ALC-A groups, (C) distinct differences among the AH and ALC groups, (D) distinct differences among the AH and ALC-A + ALC groups. AH, alcohol-related hepatitis; ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use; CE, cholesterol ester; DG, diacylglycerol; FA, free fatty acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PCO, phosphatidylcholine ether; TG, triglyceride.

alcohol in patients with ALD, where LPC and FFA decreased and TGs increased, corresponding with our results in the ALC-A group. Our finding of high FFAs in the most stable patient group supports the theory of the sudden effect of alcohol on lipid metabolism and a long-term redistribution of TGs as FFAs.

Metabolomics has already shown its potential for differential diagnoses and a prognostic value for AH *vs.* decompensated cirrhosis with suspected AH.[34,35] Recently, metabolomics also improved clinical prediction models of decompensation and death in patients with compensated cirrhosis and portal hypertension.[36] Our findings of elevated taurine agree with Brandl *et al.*,[37] who found higher levels of taurine and glycine conjugates in patients with AH than in patients with excessive alcohol consumption and healthy controls.

A metabolic phenotype of AH with several metabolites separating AH from alcohol-related cirrhosis with 100% accuracy has been described in a previous study,[38] but our results were not able to confirm this observation. Nonetheless, we did find low levels of numerous lipid metabolites, suggesting impaired cell membrane modelling, especially in AH. Furthermore, the findings of the highest level of metabolomic discrepancy between the AH and ALC group underline the diverse metabolomic landscape in ALD and encourage further exploration.

We found all BAs to be elevated in patient groups *vs.* HC, which concurs with other studies of cirrhosis that have observed

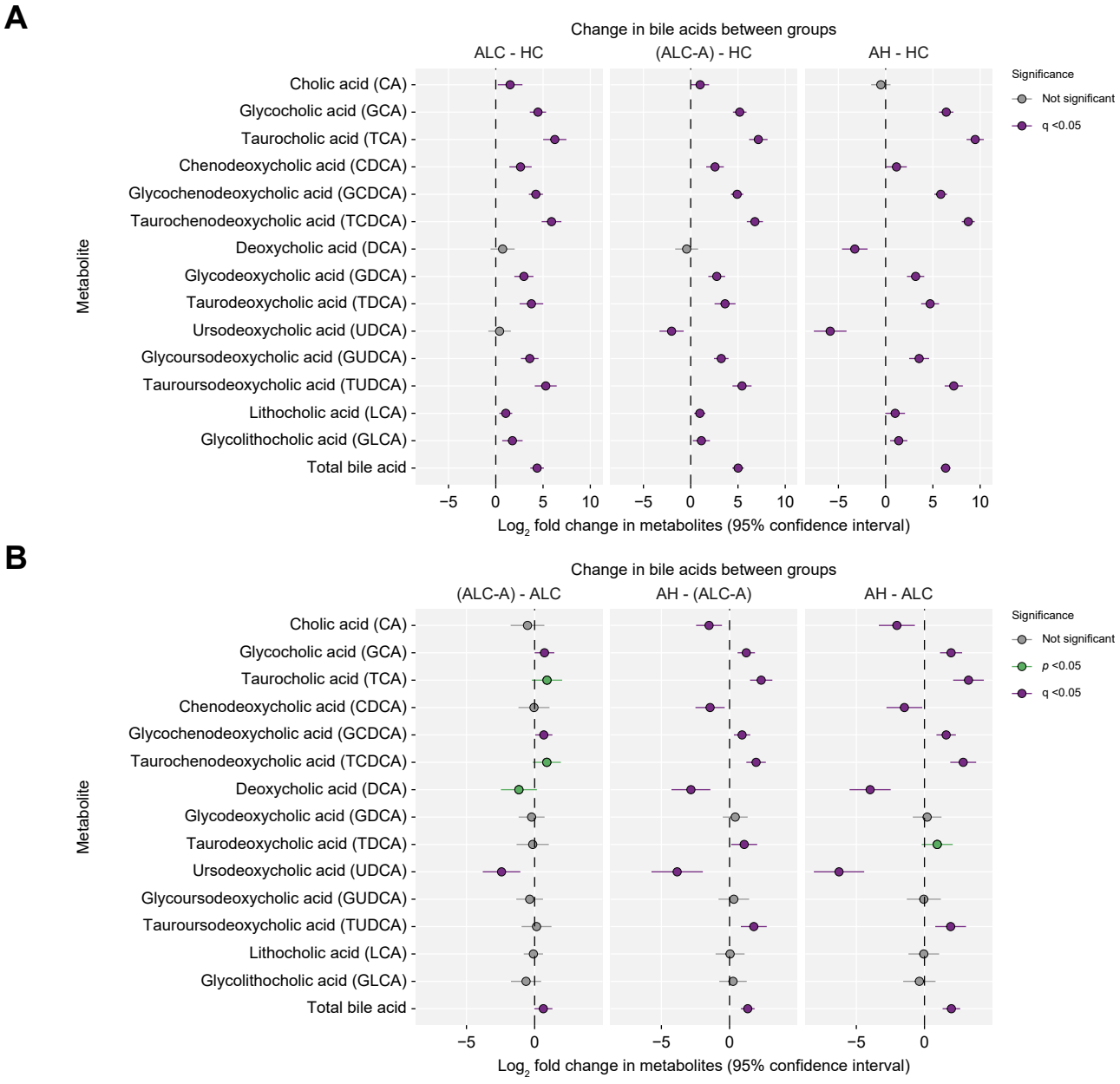


Fig. 5. Bile acid metabolomics. Bile acid metabolomics in (A) patients (ALC, ALC-A, AH) compared with healthy individuals and (B) comparisons between patient groups. Relative changes in bile acids derived from plasma. Changes are calculated from weighted least squares regression coefficients. The Benjamini–Hochberg procedure was used to correct for multiple testing. *q* values are *p* values adjusted for multiple testing. AH, alcohol-related hepatitis; ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use.

correlations between BAs and disease severity.[39–43] Significant elevations of specific BAs in acute decompensation compared to compensated cirrhosis have also been described, including increases in GCDCA and GCA, that agree with our findings in the cirrhosis groups and could indicate that increased GCDCA and GCA are correlated with disease severity.[43]

Patients with low levels of SMs and Cers had significantly worse short-term survival, which was true for pooled patient groups, the AH group alone, and the cirrhosis groups when pooled, as shown in Fig. S8. SMs are a multifunctional group of phospholipids located in cell membranes, which during sphingomyelinase activity, are changed to Cer.[44] The relationship between SM and Cer, and the activity of enzymes processing sphingolipids, have been described elsewhere as crucial in multiple disease mechanisms, including liver diseases. When SM levels decrease and Cer levels increase, it seems correlated to steatosis and fibrosis formation in NAFLD.[44] Recent research has found reduced levels of SM (including SM[d41:1]) and Cers which also predicted mortality and liver related events in patients with alcohol-related fibrosis.[15] The present study

Table 3. Relative differences in bile acids.

Bile acid	AH *vs.* HC	AH *vs.* ALC	AH *vs.* ALC-A	ALC *vs.* HC	ALC-A *vs.* ALC	ALC-A *vs.* HC
Cholic acid	-0.3 (-0.65; 0.43) n.s.: 0.2963	**-0.75 (-0.9; -0.39) *p* = 0.0008**	**-0.65 (-0.82; -0.32) *p* = 0.0009**	**1.86 (0.15; 6.08) *p* = 0.0093**	-0.3 (-0.7; 0.64) n.s.: 0.452	**0.99 (0.03; 2.87) *p* = 0.0197**
Deoxycholic acid	**-0.9 (-0.96; -0.73) *p* <0.0001**	**-0.94 (-0.98; -0.82) *p* <0.0001**	**-0.86 (-0.95; -0.62) *p* <0.0001**	0.65 (-0.32; 3) n.s.: 0.2166	-0.55 (-0.82; 0.12) n.s.: 0.0756	-0.26 (-0.68; 0.72) n.s.: 0.4665
Glycocheno-deoxycholic acid	**55.99 (34.55; 90.35) *p* <0.0001**	**2.01 (0.84; 3.94) *p* <0.0001**	**0.89 (0.25; 1.86) *p* = 0.0005**	**17.92 (10.3; 30.69) *p* <0.0001**	**0.59 (0.03; 1.47) *p* = 0.016**	**29.17 (18.17; 46.47) *p* <0.0001**
Glycocholic acid	**84.17 (49.18; 143.58) *p* <0.0001**	**2.87 (1.2; 5.79) *p* <0.0001**	**1.34 (0.5; 2.64) *p* <0.0001**	**21.03 (11.11; 39.08) *p* <0.0001**	**0.65 (0; 1.72) *p* = 0.0239**	**35.39 (21.07; 58.99) *p* <0.0001**
Glycodeoxycholic acid	**8.01 (3.78; 15.99) *p* <0.0001**	0.15 (-0.45; 1.38) n.s.: 0.6818	0.33 (-0.29; 1.51) n.s.: 0.4813	**6.87 (2.9; 14.89) *p* <0.0001**	-0.14 (-0.56; 0.66) n.s.: 0.7379	**5.75 (2.64; 11.53) *p* <0.0001**
Glycolithocholic acid	**1.61 (0.38; 3.93) *p* = 0.0006**	-0.23 (-0.66; 0.75) n.s.: 0.5446	0.19 (-0.41; 1.39) n.s.: 0.6408	**2.39 (0.6; 6.17) *p* = 0.0004**	-0.35 (-0.7; 0.39) n.s.: 0.3648	**1.19 (0.17; 3.1) *p* = 0.0054**
Glycourso-deoxycholic acid	**10.67 (4.61; 23.28) *p* <0.0001**	-0.03 (-0.59; 1.3) n.s.: 0.9406	0.24 (-0.43; 1.7) n.s.: 0.6769	**11.08 (5.33; 22.04) *p* <0.0001**	-0.22 (-0.61; 0.53) n.s.: 0.694	**8.39 (4.51; 15.03) *p* <0.0001**
Lithocholic acid	**1.01 (-0.03; 3.14) *p* = 0.0374**	-0.03 (-0.56; 1.11) n.s.: 0.9406	0.03 (-0.51; 1.15) n.s.: 0.9502	**1.08 (0.29; 2.35) *p* = 0.0004**	-0.06 (-0.42; 0.53) n.s.: 0.9609	**0.96 (0.29; 1.95) *p* = 0.0005**
Taurocheno-deoxycholic acid	**426.71 (264.74; 687.39) *p* <0.0001**	**6.16 (2.71; 12.84) *p* <0.0001**	**2.86 (1.35; 5.33) *p* <0.0001**	**58.71 (27.91; 122.32) *p* <0.0001**	0.86 (-0.09; 2.8) n.s.: 0.0581	**109.93 (60.32; 199.69) *p* <0.0001**
Taurocholic acid	**711.38 (376.81; 1342.22) *p* <0.0001**	**8.38 (3.3; 19.46) *p* <0.0001**	**4.00 (1.83; 7.82) *p* <0.0001**	**74.96 (31.43; 176.93) *p* <0.0001**	0.88 (-0.13; 3.07) n.s.: 0.0756	**141.53 (71.37; 279.68) *p* <0.0001**
Taurodeoxy-cholic acid	**25.12 (12.53; 49.43) *p* <0.0001**	0.91 (-0.13; 3.21) n.s.: 0.0651	**1.11 (0.09; 3.08) *p* = 0.0147**	**12.67 (4.74; 31.56) *p* <0.0001**	-0.09 (-0.6; 1.06) n.s.: 0.8545	**11.4 (4.74; 25.77) *p* <0.0001**
Taurourso-deoxycholic acid	**146.76 (74.87; 286.79) *p* <0.0001**	**2.78 (0.73; 7.27) *p* <0.0001**	**2.43 (0.78; 5.63) *p* <0.0001**	**38.05 (16.49; 86.15) *p* <0.0001**	0.1 (-0.48; 1.36) n.s.: 0.8509	**42.04 (20.34; 85.82) *p* <0.0001**
Ursodeoxycholic acid	**-0.98 (-0.99; -0.94) *p* <0.0001**	**-0.99 (-1; -0.95) *p* <0.0001**	**-0.93 (-0.98; -0.74) *p* <0.0001**	0.32 (-0.42; 2.02) n.s.: 0.4501	**-0.81 (-0.93; -0.52) *p* <0.0001**	**-0.75 (-0.9; -0.4) *p* = 0.0002**
Total bile acids	**80.38 (55.28; 116.69) *p* <0.0001**	**2.92 (1.51; 5.13) *p* <0.0001**	**1.52 (0.77; 2.58) *p* <0.0001**	**19.73 (11.61; 33.09) *p* <0.0001**	**0.56 (-0.02; 1.47) *p* = 0.0321**	**31.29 (20.15; 48.3) *p* <0.0001**

Negative values indicate that the first group have lower levels than the second group. Estimated changes are adjusted for age and sex. Comparisons are calculated by weighted least squares, and log_2-transforned. ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use; AH, alcohol-related hepatitis; HC, healthy control group.

supports these findings in patients with more severe alcohol-related liver disease. However, the mechanisms of SM metabolism have not yet been fully elucidated with regard to liver disease.

The present results are similar to those of Gao *et al.*,[45] who predicted 30-day survival among patients with AH by high acyl-carnitines levels and low SM, Cer, and cholesterol levels. In addition, three distinct sphingomyelins (C20:2, C18:1, and OH) and PCs were found to be lower in alcohol-related cirrhosis compared with healthy individuals.[46] Interestingly, sphingolipids were also suppressed in acutely decompensated cirrhosis, as were Cers.[47,48] Reduced Cers were inversely proportional to the severity of cirrhosis with various causes, with low Cer-24 independently associated with overall survival.[49] Sphingolipids also seem to differ between patients with acute-on-chronic liver failure in acutely decompensated cirrhosis.[47] On the contrary, sphingolipids (including ceramides) were increased in extracellular vesicles in patients with AH compared with healthy individuals, patients with heavy drinking patterns, end-stage liver disease, and decompensated alcohol-related cirrhosis.[50] Serum SM(d36:0) is more abundant in patients with an alcohol use disorder and is an indicator of progressive ALD, as opposed to non-progressive liver disease.[51]

We found that SM, Cer, and cholesterol groups have predictive value for survival models in patients with AH and alcohol-related cirrhosis. SMs, in particular, was associated with survival and proved superior to using the MELD score.

The present study has several limitations. We did not establish a validation cohort to verify our findings, but a healthy group was included, providing important comparison observations. The study compared patient groups from prior unmatched studies, which might have led to bias as metabolism is affected by age, nutrition, and BMI.[52,53] When using data from former studies, participants are pre-selected by prior trial inclusion criteria, which may introduce selection bias. Alcohol use was registered at baseline with a small risk of recall bias of patients. We have no data about alcohol use or alcohol interventions in the follow-up period, which could have influenced survival in all or any of the groups. Many patients died in their homes, with an unknown cause of death, which may underestimate the liver-related mortality. MELD scores differed significantly between groups, indicating various stages of the disease severity affecting the prognoses. However, relative changes in lipids remained, when adjusting for MELD. Using MELD for AH patients was once uncommon, but nowadays, seems more useful in predicting severity and short-term mortality than the Maddrey Discriminant Function.[54,55] Omics analyses should be repeated in more extensive studies to validate profiles along the spectrum of severity of ALD, and to establish correlations to current scores of disease severity, such as MELD and Child–Pugh. Various confounding factors, such as comorbidities and lifestyle, could have influenced our results; however, the numbers of patients with cancer, diabetes, hypercholesterolaemia, and statin use were low. Clinically relevant adjustment analyses may be applied in future studies. Our statistical analyses did not differentiate between the stages of decompensation in the ALC-A group, which could impact lipids and metabolites.[43,56]

Nevertheless, our study provides insights into the complete mapping of lipidomics and metabolomics in ALD. Our findings indicate significant changes in lipid metabolism in ALD: we found increased lipolysis even in the alcohol-abstinent group, along with distinct changes in metabolites and BAs. These results should encourage further studies of treatment targets and prognostic markers for people with ALD.

SM and Cer groups were superior to MELD scores when predicting short-term survival, suggesting they could have a prognostic application in ALD. The specific lipids SM(d42:1) and SM(d41:1) seem especially promising.

In conclusion, lipidomic levels are lower in patients with ALD and could be used to distinguish between different stages of liver injury. Metabolomics and lipidomics offer valuable prognostic information and could ultimately be used to help predict the development of complications and individuals' disease courses.

Abbreviations

AH, alcohol-related hepatitis; AH group, group of patients with alcohol-related hepatitis; ALAT, alanine amino transferase; ALC, group of patients with alcohol-related cirrhosis, no current alcohol use; ALC-A, group of patients with newly diagnosed alcohol-related cirrhosis, current or recent alcohol use; ALD, alcohol-related liver disease; BAs, bile acids; Cers, ceramides (a group of sphingomyelins); CEs, cholesterol esters; CRP, C-reactive protein; DCA, deoxycholic acid; DTEA, decatrienoic acid; FFA, free fatty acids; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GUDCA, glycol-ursodeoxycholic acid; HC, healthy control group; HCC, hepatocellular carcinoma; HRs, hazard ratio; HVPG, hepatic venous pressure gradient; INR, international normalised ratio; LPC, lysophosphatidylcholine; LPE, lysephosphatidylethanolamine; MELD, model for end-stage liver disease; NAFLD, non-alcoholic fatty liver disease; PC, phosphatidylcholines; PCA, principal component analysis; PCO, phosphatidylcholine-ethers; PS, phosphatidylserine; ROC, receiver operating characteristic; SM, sphingomyelin; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TGs, triglycerides; UDCA, ursodeoxycholic acid; WLS, weighted least squares.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualised and designed the study: NK, FB. Recruited participants and carried out the investigations: NK, TMK, HY, MBOC, MPW, MT, LLG, OH. Curated the data and conducted the lipidomics and metabolomics: KT. Carried out the statistical analyses of lipidomics and metabolomics data: QG. Interpreted the results: NK, FB, SM, TM and TMK. Access to all data and reviewed and approved the final manuscript: all authors.

Data availability statement

All data are provided in the manuscript and supplementary data files.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100953.

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Author names in bold designate shared co-first authorship

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