


Changes in the metabolic profile of human male postmortem frontal cortex and cerebrospinal fluid samples associated with heavy alcohol use

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Funding information

Finnish Foundation for Cardiovascular Research; Seventh Framework Programme, Grant/Award Number: 201668; VTR funding; Finnish Foundation for Alcohol Studies

Abstract

Heavy alcohol use is one of the top causes of disease and death in the world. The brain is a key organ affected by heavy alcohol use. Here, our aim was to measure changes caused by heavy alcohol use in the human brain metabolic profile. We analyzed human postmortem frontal cortex and cerebrospinal fluid (CSF) samples from males with a history of heavy alcohol use ($n = 74$) and controls ($n = 74$) of the Tampere Sudden Death Series cohort. We used a nontargeted liquid chromatography mass spectrometry-based metabolomics method. We observed differences between the study groups in the metabolite levels of both frontal cortex and CSF samples, for example, in amino acids and derivatives, and acylcarnitines. There were more significant alterations in the metabolites of frontal cortex than in CSF. In the frontal cortex, significant alterations were seen in the levels of neurotransmitters (e.g., decreased levels of GABA and acetylcholine), acylcarnitines (e.g., increased levels of acylcarnitine 4:0), and in some metabolites associated with alcohol metabolizing enzymes (e.g., increased levels of 2-piperidone). Some of these changes were also significant in the CSF samples (e.g., elevated 2-piperidone levels). Overall, these results show the metabolites associated with neurotransmitters, energy metabolism and alcohol metabolism, were altered in human postmortem frontal cortex and CSF samples of persons with a history of heavy alcohol use.

KEYWORDS

alcohol, alcoholism, brain, metabolomics, microbiota

1 | INTRODUCTION

Heavy alcohol use is among the top five causes of disease, disability, and death worldwide, accounting for 5% of global disease burden and

nearly 10% of global deaths among populations aged 15–49 years.¹ The brain is one of the essential organs influenced by alcohol use, and many alterations in the structure and function of the brain have been associated with alcohol use.^{2,3}

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Moreover, heavy alcohol use is associated with an altered circulating metabolic profile (for a review, see Voutilainen and Kärkkäinen⁴). For example, increased levels of glutamate, tyrosine, and phosphatidylcholine diacyls, and decreased levels of glutamine, serotonin, and phosphatidylcholine acyl-alkyls have been associated with heavy alcohol use.^{5–8} Furthermore, some of these changes in the circulating metabolic profile have been linked to structural changes in the white and grey matter.^{8,9} Moreover, in rats, it has been shown that alcohol exposure alters the brain metabolic profile.¹⁰ In living humans, *in vivo* ¹H magnetic resonance spectroscopy can be used to measure metabolites. Although this method has shown alterations in brain metabolite levels in individuals with alcohol use disorder, for example, decreased levels of choline containing compounds, the method is limited to only measuring a handful of metabolites and does not give a good picture of whole metabolic profile level changes (for a review, see Meyerhoff¹¹). Similarly, there are reports on changes in many metabolite classes, for example, ethanolamines, steroids, and neurotransmitters, in human postmortem brains measured with targeted methods.^{12–14}

However, to our knowledge, there is no published measurement of changes in the whole human postmortem brain metabolic profile associated with heavy alcohol use to get an overall view of altered metabolic processes. Therefore, our aim here was to measure changes in the human brain metabolic profile associated with heavy alcohol use by performing a nontargeted metabolomics analysis on human postmortem frontal cortex and cerebrospinal fluid samples.

2 | METHODS AND MATERIALS

2.1 | Subjects

We used samples from the Tampere Sudden Death Series (TSDS) cohort, which was collected from forensic medicine autopsies of people who suffered out of hospital death in the area of the Pirkanmaa Hospital District during 2010–2014. Frontal cortex (Brodmann area 9) and CSF samples, a portion extracted via syringe, were collected from a total of 700 subjects, of which we selected 74 heavy alcohol users and 74 controls, stored at -80°C until use. Selection of the subjects was based on autopsy reports and medical records. Inclusion criteria for the heavy alcohol use group was diagnosis of alcohol-related diseases (ICD-10 codes F10.X, G31.2, G62.1, G72.1, I42.6, K70.0–K70.4, K70.9, and K86.0) or signs of heavy alcohol use in clinical or laboratory findings (e.g., increased levels of alcohol use biomarkers gamma-glutamyl transferase, mean corpuscular volume, and carbohydrate-deficient transferrin). Lack of these findings was inclusion criteria for the control group, most of whom had died due to cardiovascular system diseases. Causes of death and general background characteristics are shown in Table 1. We only used samples from males, because there were only nine suitable female cases with a history of heavy drinking.

2.2 | Metabolomics analysis

Tissue samples were weighed, and 80% methanol was added (v/v H₂O, LC-MS Ultra CHROMASOLV®, Fluka) to a ratio of 1,000- μl

TABLE 1 Background characteristics

	Control (n = 74)		Alcohol (n = 74)		p
PMI (days, mean \pm SD)	5.6	± 2.6	5.4	± 2.1	0.6432 ^a
Age (years, mean \pm SD)	57	± 13	56	± 11	0.6184 ^a
BMI (mean \pm SD)	30.1	± 6.4	27.7	± 7.5	0.0377 ^a
Brain (grams, mean \pm SD)	1,515	± 134	1,469	± 144	0.0490 ^a
Smoking (n, %) ^c	24	55%	27	71%	0.1242 ^b
Liver cirrhosis (n, %)	1	1%	14	19%	0.0046 ^b
CSF alcohol (‰, mean \pm SD) ^d	0.07	± 0.28	0.85	± 0.70	<0.0001 ^a
Urine alcohol (‰, mean \pm SD) ^e	0.29	± 0.55	1.00	± 0.65	0.0045 ^a
Cause of death (n, %)					<0.0001 ^b
Alcohol/drug poisoning	0	0%	16	22%	
Alcohol-related disease	0	0%	29	39%	
Cardiovascular disease	56	76%	8	11%	
Other disease	10	14%	8	11%	
Suicide	0	0%	5	7%	
Accident	7	9%	4	5%	
Unknown	1	1%	4	5%	

Abbreviations: BMI, body mass index; CSF, cerebrospinal fluid; p, p value; PMI, post-mortem interval.

^aWelch's t test.

^b χ^2 test.

^cSmoking status known only from 82 subjects (44 controls and 38 heavy alcohol users).

^dCSF alcohol concentration measured from 76 subjects (34 controls and 42 heavy alcohol users).

^eUrine alcohol concentration measured from 30 subjects (17 controls and 13 heavy alcohol users).

solvent/100-mg tissue. Extraction was facilitated by breaking the tissue with a Teflon-coated plastic stick followed by water sonication for 10 min (BRAUSON GWB 2200). For CSF samples, 100 μ l of the sample was added to 400 μ l of acetonitrile (VWR International, LC-MS grade) and mixed by pipette. Samples were then vortexed and centrifuged (Eppendorf centrifuge 5,804 R, 13,000 rpm, 5 min at 4°C). Supernatant was then collected and filtered into high-performance liquid chromatography (HPLC) bottles through an Acrodisc CR 4-mm (0.45 μ m) filter. Quality control samples were made by taking 5 μ l of solution from each sample. The samples were analyzed using an UHPLC-qTOF-MS system (Agilent Technologies, Waldbronn, Karlsruhe, Germany) that consisted of a 1,290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6,540 UHD accurate-mass qTOF spectrometer. Details of the method have been previously published.¹⁵ In brief, two different chromatographic techniques were used, that is, reversed phase (RP, Zorbax Eclipse XDB-C18, particle size 1.8 μ m, dimensions of 2.1 \times 100 mm, Agilent Technologies, USA) and hydrophilic interaction (HILIC, Acquity UPLC BEH Amide 1.7 μ m, 2.1 \times 100 mm, Waters, Ireland) chromatography and data acquired at both positive (+) and negative (-) polarity. RP chromatography: column oven temperature was set to 50°C, and the flow rate to 0.4 ml/min gradient elution with water (eluent A) and methanol (eluent B) both containing 0.1% (v/v) of formic acid. Gradient profile for RP separations was: 0–10 min: 2% B \rightarrow 100% B; 10–14.5 min: 100% B; 14.5–14.51 min: 100% B \rightarrow 2% B; 14.51–16.5 min: 2% B. HILIC: column oven temperature was set to 45°C, flow rate 0.6 ml/min, gradient elution with 50% v/v ACN in water (eluent A) and 90% v/v ACN in water (eluent B), both containing 20-mM ammonium formate (pH 3). The gradient profile for HILIC separations was: 0–2.5 min: 100% B, 2.5–10 min: 100% B \rightarrow 0% B; 10–10.01 min: 0% B \rightarrow 100% B; 10.01–12.5 min: 100% B. The sample tray was kept at 4°C during analysis. MassHunter Acquisition B.07.00 (Agilent Technologies) software was used for data acquisition. The quality control samples were injected at the beginning of the analysis as well as every 12th injection. The analysis order of the samples was randomized. We used three different collision-induced dissociation voltages (10, 20, and 40 eV) for the data-dependent tandem mass spectrometry (MSMS) analysis. The mass spectrometry data processing was performed using MS-DIAL ver. 3.40.¹⁶ The peak picking and peak alignment parameters were as follows: mass range from 40 to 1,000 (HILIC) or 1,600 (RP) Da, MS tolerance 0.005 Da, MS2 tolerance 0.01 Da, minimum peak width 10 scans, minimum peak height 10,000 (selected based on background noise level). Peaks needed to be detected in at least 70% of samples from one study group to be included in the final data matrix. Drift correction of the metabolomics data was done using results from the QC sample, according to a previously published protocol.¹⁵ In brief, the molecular features were corrected for the drift pattern caused by the LC-MS procedures using regularized cubic spline regression, fit separately for each feature on the QC samples. The smoothing parameter was chosen from an interval between 0.5 and 1.5 using leave-one-out cross validation to prevent overfitting. After the drift correction, feature quality was assessed, and low-quality features were flagged. Features were kept if their RSD* was

below 20% and their D-ratio below 40%. In addition, features with classic RSD, RSD* and basic D-ratio all below 10% were kept. This additional condition prevents the flagging of features with very low values in all but a few samples. Missing values were imputed using random forest imputation. QC samples were removed prior to imputation to prevent them from affecting imputation.

Metabolite identification was focused on statistically significantly altered molecular features. Metabolite identification was based on exact mass, MSMS fragmentation, and retention time. Identifications were ranked according to common guidelines.¹⁷ Metabolites in Level 1 were matched to the mass, retention time, and MS/MS spectra of fragmented ions from the in-house library built of chemical standards using the same instrument and experimental conditions. Level 2 includes metabolites matching the exact mass and MSMS spectra from public libraries (METLIN, Lipidmaps, and Human Metabolome DataBase were used) or in the case of lipids, the built-in MS-DIAL library version 3.40. In Level 3, only the chemical group of the compound (but not the exact compound) is identifiable. Level 4 indicates unidentified compounds.

2.3 | Statistical analysis

We used Welch's *t* test (continuous variables) and χ^2 test (binomial variables) to evaluate differences between the study groups in background characteristics. We used Welch's *t* test and Cohen's *d* effect sizes to evaluate differences between the study groups in the metabolomics data. To account for multiple testing, we adjusted the α level by the number of principal components needed to explain 95% of the variation in the data. For correlations, we used Spearman's correlations. We used JASP (JASP team 2019, version 0.10) for statistical analyses.

3 | RESULTS

Based on background information, the heavy alcohol user group had lower brain weight and lower body mass index (BMI) when compared with controls (Table 1). Moreover, as expected, the heavy alcohol use group had more cases of liver cirrhosis, as well as higher CSF and urine alcohol concentrations at time of death. It should be noted that smoking information was available for only 85 subjects (57%). When evaluated from the point of metabolomics data, there was no significant difference between study groups in the level of cotinine (a metabolite of nicotine, *p* values in frontal cortex and CSF were 0.1370 and 0.1251, respectively).

In the principal component analysis, 96 latent variables were needed to explain 95% of variance in the metabolomics data. Therefore, to account for multiple testing, we reduced the α level to 0.0005. Molecular features with *p* values between 0.0005 and 0.05 were considered to be trends. There were more significantly altered molecular features in the frontal cortex samples between the study groups than in the CSF samples. We detected 2,471 molecular features in the frontal cortex samples, of which 939 had *p* values below

0.05 and of these, 208 were below the multiple testing adjusted α level of 0.0005. For CSF samples, we found 2,107 molecular features, of which 327 had p values below 0.05, and of these, 33 had p values below the multiple testing adjusted α level of 0.0005 (Figure 1).

Identified metabolites that had a p value < 0.05 when comparing study groups are shown in Tables S1 (frontal cortex) and S2 (CSF). Overall, we were able to identify more altered metabolites from the frontal cortex samples than from the CSF samples. Of note is that the MSMS spectrum of 2-piperidone was similar to 5-aminovalerate [M-OH⁺], but the retention time was different (RP positive mode retention time for 2-piperidone was 2.1 min, and 4.3 min for 5-aminovalerate). All measured molecular features are shown in Table S4.

In the frontal cortex samples, after correction for multiple testing, we observed significantly increased levels of 2-piperidone ($d = 1.03$), carnosine ($d = 0.74$), acetylcarnitine 20:1 ($d = 0.72$), 3-hydroxyisovaleric acid ($d = 0.71$), and acetylcarnitine 4:0 ($d = 0.68$), and significantly decreased levels of GABA ($d = -0.81$), acetylcholine ($d = -0.71$), cystine ($d = -0.63$), glutaryl carnitine ($d = -0.60$), aspartic acid ($d = -0.59$), and 25-hydroxycholesterol ($d = -0.59$, Figure 2). Furthermore, in the CSF samples, after correction for multiple testing, we observed significantly increased levels of 2-piperidone ($d = 1.04$, similar to frontal cortex), aspartyl-proline (Asp-Pro, $d = 0.95$), ethyl hydrogen sulfate (found in beer, $d = 0.88$), 3-hydroxyisovaleric acid ($d = 0.76$, similar to frontal cortex), and leucic acid ($d = 0.65$, Figure 2).

There were some correlations between the measured metabolite levels and postmortem interval (PMI), age at time of death and BMI, which are shown in Tables S1 and S2. Of the statistically significantly altered metabolites, there were no significant correlations between the metabolite levels and PMI, age, and BMI, after correction for

multiple testing, with the exception of acetylcholine and BMI ($r = 0.30$, $p = 0.00019$), 25-hydroxycholesterol and age at the time of death ($r = -0.37$, $p < 0.00001$).

Correlations between frontal cortex and CSF levels of metabolites, which had p values below 0.05 in both measured sample types, are shown in Table S3. There were significant correlations between frontal cortex and CSF samples in many of the altered metabolites, including 2-piperidone and 3-hydroxyisovaleric acid.

4 | DISCUSSION

In the present study, we found differences in the metabolic profile of human postmortem brain and CSF samples associated with a history of heavy alcohol use compared with controls. This is in line with previous studies showing that heavy alcohol use is associated with an altered metabolic profile in blood, urine, and other organs (for a review, see Voutilainen and Kärkkäinen⁴).

We observed decreased levels of GABA in the frontal cortex samples of heavy alcohol users. Results from previous studies using human brain in vivo ¹H magnetic resonance spectroscopy in persons with alcohol use disorder have produced mixed results, with some studies showing low GABA levels,¹⁸ whereas other studies have reported no significant changes in GABA levels.^{19,20} These differences could be related to populations and timing of the analysis (e.g., subjects' intoxication status, whether subjects are in withdrawal of abstinence and for how long). Previous postmortem brain studies have reported decreased levels of GABA-A receptors and GABA transporters in persons with alcohol dependence.^{21,22} Overall, the present results support the view that the GABAergic system is altered

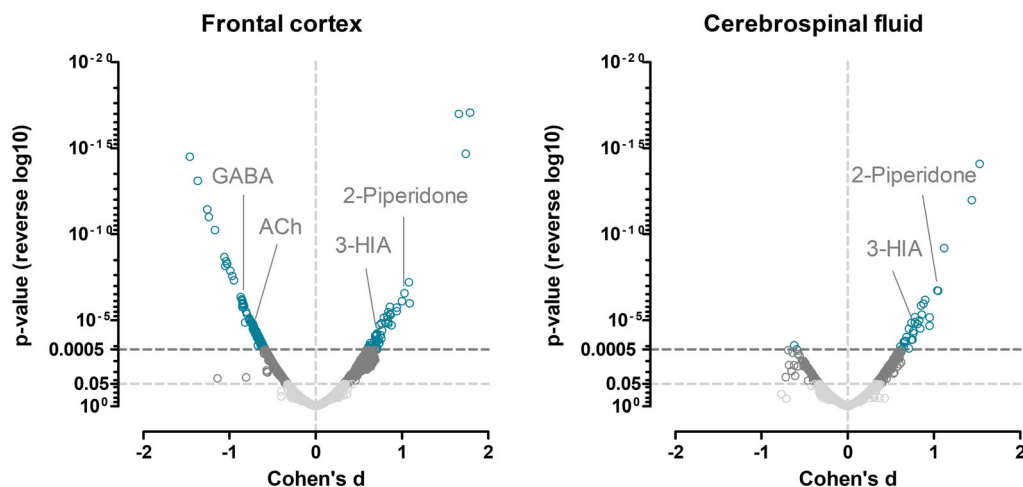
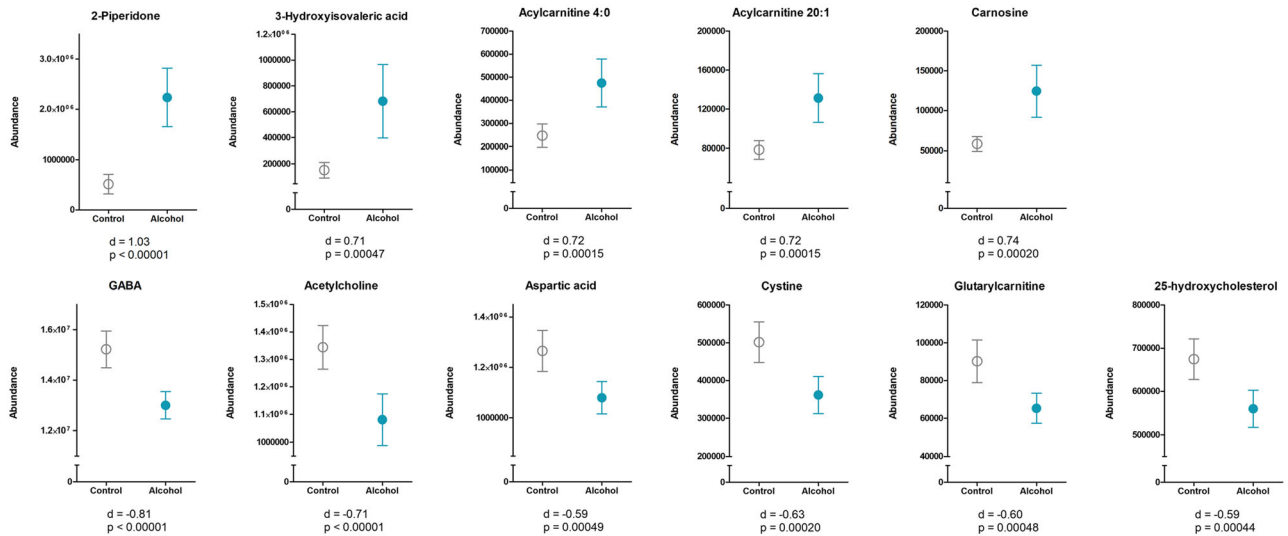


FIGURE 1 Volcano plots of metabolic profile changes in the frontal cortex and cerebrospinal fluid samples. Ninety-six latent variables were needed to explain 95% of variance in the data in the principal component analysis. Therefore, to account for multiple testing, we reduced the α level to 0.0005. In the frontal cortex and cerebrospinal fluid (CSF) samples, a total of 2,471 and 2,107 molecular features were measured, respectively. Of these, 939 (frontal cortex) and 327 (CSF) had p values below 0.05, and 208 (frontal cortex) and 33 (CSF) molecular features had p values below the multiple testing adjusted α level of 0.0005. Not all molecular features could be identified. Key significantly changed identified metabolites are highlighted: increased levels of 2-piperidone and 3-hydroxyisovaleric acid (3-HIA) were seen in the heavy drinking group in both frontal cortex and CSF samples, whereas decreased levels of neurotransmitters gamma-aminobutyric acid (GABA) and acetylcholine (ACh) were observed only in the frontal cortex

Frontal cortex



CSF

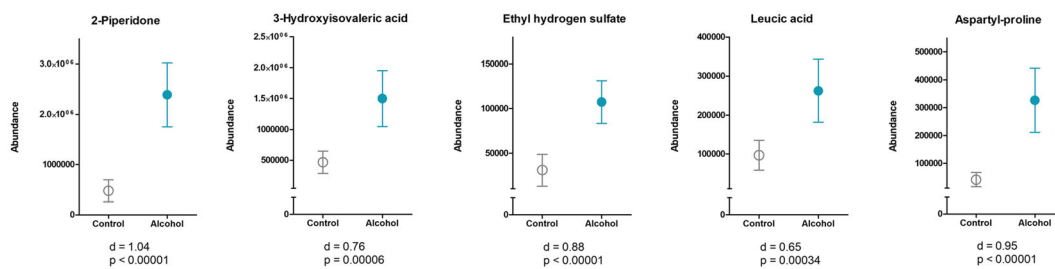


FIGURE 2 Significantly altered metabolites. Heavy alcohol use was associated with significant (corrected α level = 0.0005 to account for multiple testing) differences in metabolite levels in both postmortem frontal cortex and cerebrospinal fluid (CSF) samples when compared with controls. High 2-piperidone and 3-hydroxyisovaleric acid levels were observed in the heavy alcohol use group in both frontal cortex and CSF samples. Mean ion abundance and 95% confidence intervals are shown. Legend: *d*, Cohen's *d* effect size; GABA, gamma-aminobutyric acid; *p*, *p* value from Welch's *t* test

in persons with a history of heavy alcohol use associated with, for example, development of dependence and increased risk of seizures during withdrawal.

Furthermore, we observed increased levels of 2-piperidone (δ -valerolactam) in the frontal cortex and CSF samples of heavy alcohol users. 2-Piperidone is a metabolite of cadaverine via intermediate 1-piperideine, which can also be metabolized to a GABA analog 5-aminovalerate.²³ 2-Piperidone is a substrate for cytochrome P450-2E1 (CYP2E1), which metabolizes 2-piperidone to 6-hydroxy-2-piperidone.²⁴ 2-Piperidone has been suggested to be a biomarker of CYP2E1 activity.²⁴ Therefore, high 2-piperidone levels observed in the present study could be associated with alcohol-induced inhibition of 2-piperidone metabolism by blocking its access to the CYP2E1 enzyme. CYP2E1 has an important role in alcohol-induced changes in the function of the brain because it produces acetaldehyde, which has been associated with the behavioral effects of alcohol.²⁵ Further research is needed to understand the possible

biological role of 2-piperidone in the brain and investigate if 2-piperidone levels are also increased in the plasma or urine in heavy alcohol users.

Moreover, the cholinergic system of the brain is considered to be important in the development of alcohol dependence.²⁶ We observed significantly decreased levels of acetylcholine and a trend in decreased choline levels in the frontal cortex samples, which is in line with a previous *in vivo* ¹H magnetic resonance spectroscopy study where decreased levels of choline containing molecules were measured in persons with alcohol use disorder.²⁰ In contrast, these postmortem brain results show a trend towards increased levels of many lysophosphatidylcholines (lysoPCs) and phosphocholine (Table S1). This indicates that the choline balance has shifted towards lysoPCs, compared with free choline or acetylcholine. LysoPCs can increase NF κ B activation²⁷ and the recruitment of microglia,²⁸ and increased levels have been reported in animal models of brain damage²⁹ and in human postmortem samples from persons with Alzheimer's disease.³⁰

Furthermore, in the present study, we observed significantly decreased levels of aspartic acid and trends toward decreased levels of both *N*-acetylaspartate (NAA) and *N*-acetylaspartylglutamic acid (NAAG) in postmortem frontal cortex brain samples (Table S1). This is in line with previous studies showing decreased NAA peaks (consisting of both NAA and NAAG in magnetic resonance spectroscopy) in frontal cortex and white matter in association with heavy alcohol use.^{20,31–37} Decreased aspartic acid levels could be associated with alcohol caused ketosis,³⁸ and NAA and NAAG levels are considered to be a sign of decreased neuronal viability and/or integrity.^{39–41} However, NAA is also found in myelin, and low NAA levels have been associated with myelin damage.⁴² Therefore, the present results of decreased aspartic acid, NAA, and NAAG levels could be a sign of gray and white matter damage in the frontal cortex in the heavy alcohol users.

Many changes were seen in lipid metabolism in the frontal cortex and CSF samples especially in levels of acylcarnitines (AC), phosphatidylcholines (PC), lysoPCs, phosphatidylethanolamines (PE), and lysoPEs (Tables S1 and S2). Altered circulating lipid levels have previously been reported in persons with alcohol use disorder.^{5,43,44} Altered lipid metabolism in the frontal cortex could be linked to altered energy metabolism in general in these samples, as seen also in other markers of altered energy metabolism like NAA and NAAG. Moreover, altered phospholipid levels could be associated with commonly reported alterations in the brain structure seen in persons with a history of heavy alcohol use,² indicated here as reduced brain weight, because phospholipids are major components of cell membranes.

High 3-hydroxyisovaleric acid levels observed in both frontal cortex and CSF samples in the heavy alcohol use group could be associated with a combination of biotin deficiency and smoking.⁴⁵ 3-Hydroxyisovaleric acid is a byproduct of the leucine degradation pathway made by the biotin-dependent enzyme 3-methylcrotonyl-Co A carboxylase.⁴⁵

The main limitation of the present study is that PMI could influence the results because many metabolic processes continue after death. However, after correcting for multiple testing, all metabolites with significant differences between the study groups did not significantly correlate with PMI. This indicates that PMI is likely not causing the differences between the study groups of significantly altered metabolites. Other possible confounding factors include that differences in medical diagnoses (there are no healthy controls in post-mortem studied) and used medications have been different between the cases and controls. For example, the metabolomics analysis showed that the controls used more metoprolol and the cases more nordiazepam, which could affect the seen results, for example, the decreased GABA levels (Table S1). Moreover, future studies should also analyze samples from females and different brain regions, because alcohol use could influence the metabolic profile differently in different genders and brain regions.

In conclusion, we show here that postmortem frontal cortex and CSF samples from persons with a history of heavy alcohol use have an altered metabolic profile when compared with samples from control subjects. Most alterations could be associated with neurotransmitter and energy metabolism, but metabolites associated with gut

microbiota were also altered. Further studies are needed in other brain regions and with preclinical models to understand the spatial and temporal aspects of how heavy alcohol use alters the brain metabolic profile with connection to alcohol-related diseases.

ACKNOWLEDGMENTS

We thank Miia Reponen for the excellent technical assistance with the mass spectrometry analyses. This study is funded by the Finnish Foundation for Alcohol Studies (OK), VTR funding (JT and PJK), European Union 7th Framework Program Grant 201668 for AtheroRemo Project (PJK), and the Finnish Foundation for Cardiovascular Research (PJK). The authors want to thank Biocenter Finland and Biocenter Kuopio for supporting their core LC-MS laboratory facility.

AUTHOR CONTRIBUTIONS

OK and EK were responsible for the metabolomics study concept and design. PJK, MM, and EK designed the collection of the TSDS cohort. ML and SA supervised the mass spectrometry analysis. MK did preprocessing of the metabolomics data. OK did statistical analysis and interpretation of the findings and drafted the manuscript. JT, PJK, KH, and EK provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved the final version for publication.

CONFLICT OF INTEREST

OK and KH are founders of Afekta Technologies Ltd., a company offering metabolomics analysis services. Other authors report no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Kärkkäinen O, Kokla M, Lehtonen M, et al. Changes in the metabolic profile of human male postmortem frontal cortex and cerebrospinal fluid samples associated with heavy alcohol use. *Addiction Biology*. 2021; e13035. <https://doi.org/10.1111/adb.13035>