

# Blood metabolomic profiling reveals new targets in the management of psychological symptoms associated with alcohol use disorder

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## Abstract

Alcohol use disorder (AUD) is a global health problem with limited therapeutic options. The biochemical mechanisms that lead to alcohol addiction are not yet fully understood, and in this respect, metabolomics represents a promising approach to decipher metabolic events related to AUD. The plasma metabolome contains a plethora of bioactive molecules that reflects the functional changes in host metabolism but also the impact of the gut microbiome and nutritional habits. In this study, we investigated the impact of chronic alcohol abuse, and of a three-week period of alcohol abstinence, on the blood metabolome (non-targeted LC-MS metabolomics analysis) in 96 patients diagnosed with severe alcohol use disorder (AUD). We found that the plasma levels of different lipids (lyso)phosphatidylcholines, long-chain fatty acids, short-chain fatty acids (i.e. 3-hydroxyvaleric acid) and bile acids were altered in AUD patients. In addition, several microbial metabolites, including indole-3-propionic acid, p-cresol sulfate, hippuric acid, pyrocatechol sulfate, and metabolites belonging to xanthine class (paraxanthine, theobromine and theophylline) were sensitive to alcohol abuse and alcohol withdrawal. 3-Hydroxyvaleric acid, caffeine metabolites (theobromine, paraxanthine and theophylline) and microbial metabolites (hippuric acid and pyrocatechol sulfate) were correlated with anxiety, depression and alcohol craving. Metabolomics analysis in post-mortem samples of frontal cortex and cerebrospinal fluid of those consuming a high level of alcohol revealed that those metabolites can be found also in brain tissue. Our data allow to for the identification of neuroactive metabolites, from interactions between food components and microbiota, which may represent new targets in the management of neuropsychiatric diseases such as AUD.

The study was registered at [clinicaltrials.gov](https://clinicaltrials.gov) under the identification number NCT03803709.

### eLife assessment

This study provides **valuable** insights and allows for hypothesis generation around diet-microbe-host interactions in alcohol use disorder. The strength of the evidence is **convincing**: the work is done in a rigorous manner and includes well-characterized and described human samples. Limitations include the cross-sectional study design, and the authors should clarify their experimental groups and definitions.

## Introduction

Alcohol use disorder (AUD) is a global health problem accounting for substantial difficulties for the individuals who consume, their related persons, and for the society. The biochemical mechanisms that lead to alcohol addiction are not yet fully understood, and in this respect, metabolomics represents an interesting approach to decipher metabolic events related to AUD. Improving our understanding of the pathology could lead to discovering potential novel targets for therapies (1 [↗](#)).

Alcohol consumption clearly leads to alterations of the circulating metabolome (1 [↗](#)–4 [↗](#)). For instance, changes in the levels of lipids (fatty acids, phosphatidylcholine, steroids) and amino acids (glutamine, tyrosine, alanine, serotonin, asparagine) are commonly observed and, interestingly, some changes in the blood metabolite profile precedes the emergence of alcohol use related diseases, such as lower levels of serotonin and asparagine (5 [↗](#)). Metabolomics studies conducted in rodent models of alcohol exposure have mostly targeted the liver tissue or urine (6 [↗](#)–8 [↗](#)). In human, plasma or serum metabolomics studies have tested the effects of low/moderate/excessive alcohol intake (9 [↗](#)) but are rarely performed in clinical populations of AUD patients, including analysis of central nervous system (CNS) tissues (10 [↗](#)). Furthermore, the methodology could differ across studies, some using nuclear magnetic resonance (NMR) while others using more sensitive mass spectrometry (MS) coupled with liquid or gas chromatography (LC or GC), leading to the detection of different metabolites.

The human blood metabolome consists of 1) small molecules that directly represent the functional changes in host metabolism, 2) metabolites produced by intestinal micro-organisms and 3) metabolites originating from nutrition of other exogenous sources like drugs (1 [↗](#), 11 [↗](#)). The aims of this study are multiple. First, we investigated the impact of severe AUD on the blood metabolome by non-targeted LC-MS metabolomics analysis. Second, we investigated the impact of a short-term alcohol abstinence on the blood metabolome followed by assessing the correlations between the blood metabolome and psychological symptoms developed in AUD patients. Last, we hypothesized that metabolites significantly correlated with depression, anxiety or alcohol craving could potentially have neuroactive properties, and therefore the presence of those neuroactive metabolites was confirmed in the central nervous system using post-mortem analysis of frontal cortex and cerebrospinal fluid of persons with a history of heavy alcohol use.

Our data bring new insights on xenobiotics- or microbial-derived neuroactive metabolites, which can represent an interesting strategy to prevent or treat psychiatric disorders such as AUD.

## Results

### Clinical characteristics of the study participants

Two cohorts of AUD patients (ALCOHOLBIS and GUT2BRAIN) were used in this study. Both groups of patients were similar in terms of age, gender, smoking and drinking habits and presented with high scores of depression, anxiety and alcohol craving at T1 ([Table 1](#)). The only differences between the cohorts resided in the number of previous withdrawal cures and the score of craving (compulsion sub-score) which were higher in the GUT2BRAIN cohort compared to ALCOHOLBIS cohort. Nevertheless, in both cohorts, the score of craving was high, as usually seen in other cohorts of AUD patients ([12](#)–[14](#)). Therefore, for the analysis of metabolomics data, both cohorts of AUD patients were combined, and compared to a group of healthy controls.

### Alterations in the plasma metabolome of AUD patients

The metabolomics analysis allowed for sorting out a total of 11,651 features from the four analytical modes of the plasma samples. An unsupervised PCA model of the plasma metabolomic profiles between healthy controls and AUD patients at the beginning of the withdrawal (T1) is shown in [Fig. 1a](#). In addition, the scores plot and the performance of a supervised sPLS-DA model are shown in [Figs. S1](#) and [S2](#), respectively. Between healthy controls and AUD T1, the annotated differential (Welch *t*-test  $q < 0.05$  and sPLS-DA VIP score  $> 2.0$ ) metabolites included compounds from several metabolite classes as indicated in [Figs. 1b](#), [1c](#) and [Table S3](#). Compared to healthy controls, the metabolic profiles of AUD patients were characterized by an increase in long-chain fatty acids, such as 16:1 (palmitoleic acid), 18:1 (octadecenoic acid) and 22:4 (docosatetraenoic acid) fatty acids and phospholipids holding these fatty acids. In addition, several drugs (like diazepam, trazodone) and metabolites with steroid backbone such as glycinated bile acids (glycohyodeoxycholic acid and glycochenodeoxycholic acid), steroid hormones and acylcarnitines were increased in the AUD group. We also observed a significant increase in vitamin B6 metabolite, 4-pyridoxic acid, nicotine metabolite cotinine, a hydroxy fatty acid 3-hydroxyvaleric acid and stress hormone cortisol. However, LPCs holding a saturated odd-chain (e.g. LPC 15:0 and LPC 17:0), polyunsaturated 18-carbon fatty acid or an ether bond (O-) containing lipid were consistently decreased in AUD compared to controls ([Fig. 1c](#)). Further, we also showed a decrease in furan fatty acids 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid (3-CMPFP) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), in a carotenoid compound and in several metabolites belonging to the family of xanthines. In addition, several amino-acid derived bacterial metabolites such as pipercolic acid, 3-indole propionic acid, p-cresol sulfate and hippuric acid were significantly decreased in AUD patients compared to controls.

We then conducted a correlational analysis between blood metabolites and alcohol consumption reported by the patients. Alcohol intake was positively correlated with annotated bile acids, steroids and drugs while xanthines (paraxanthine, theobromine and theophylline), odd-chain or ether-bond LPCs and bacterial metabolite hippuric acid, p-cresol sulfate, pyrocatechol sulfate, and 3-indole propionic acid showed negative correlations ([Fig. 1d](#)).

### Alcohol withdrawal shapes the plasma metabolome

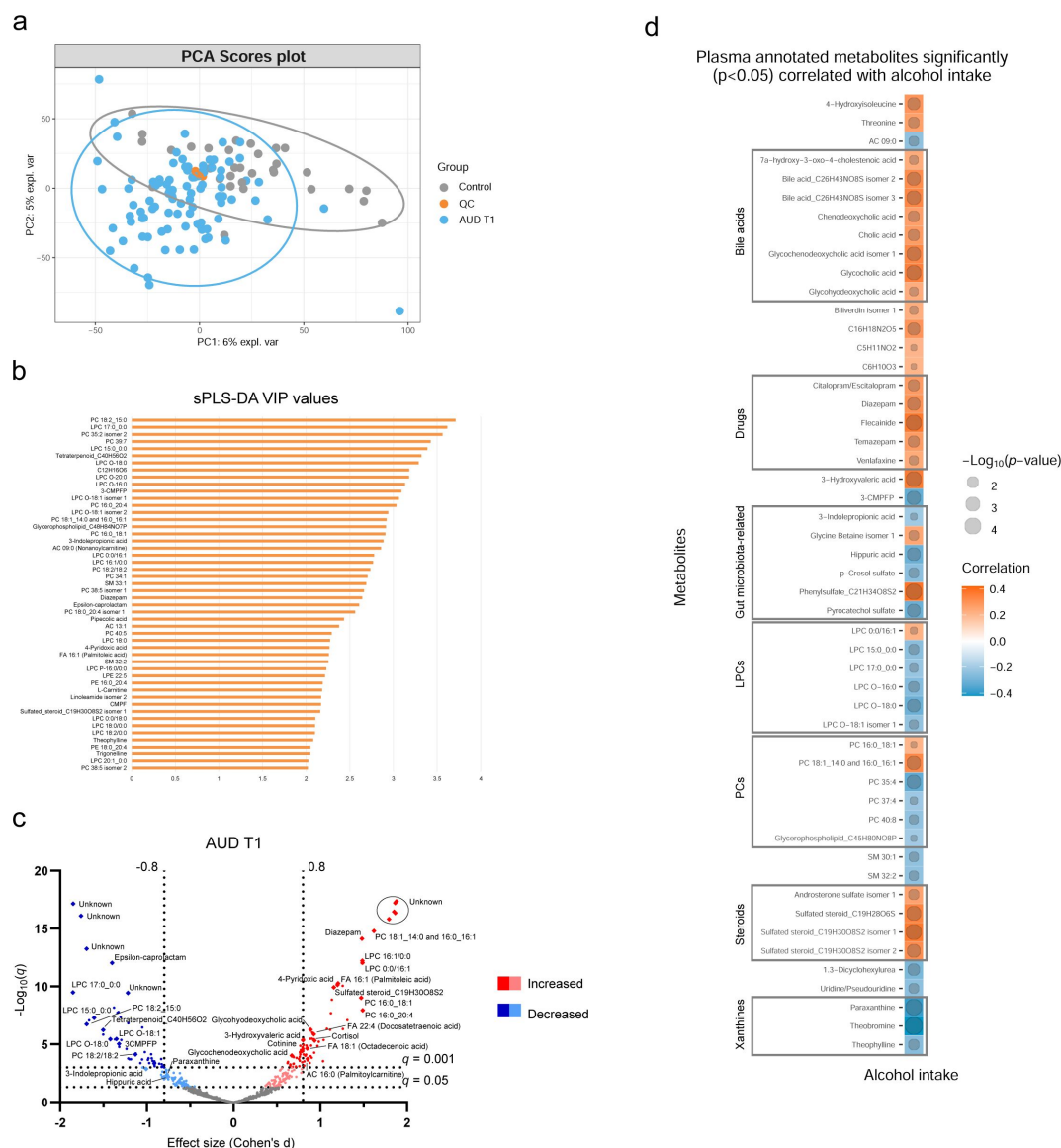
The score plot of the sPLS-DA in [Fig. 2a](#) shows a clear discrimination in plasma metabolomic profiles in the course of withdrawal in AUD patients. The sPLS-DA model performance and the unsupervised PCA model scores plot are shown in supplementary [Figs. S3](#) and [S4](#), respectively. Annotated metabolites discriminating AUD groups (paired *t*-test  $q < 0.05$ , sPLS-DA VIP score  $> 2.0$ ) before (T1) and after (T2) the 3-week withdrawal period included metabolites from a range of chemical classes ([Figs. 2b](#), [2c](#), [Table S3](#)). Apart from the metabolites belonging to the class of

|                                     | ALCOHOLBIS<br>cohort | GUT2BRAIN<br>cohort | All       | p-value            |
|-------------------------------------|----------------------|---------------------|-----------|--------------------|
| Number of subjects                  | 48                   | 48                  | 96        |                    |
| Age                                 | 46 ± 10              | 48 ± 9              | 47 ± 10   | 0.24               |
| <b>Gender</b>                       |                      |                     |           |                    |
| Men, n (%)                          | 34 (71%)             | 30 (62.5%)          | 64 (67%)  | 0.39 <sup>\$</sup> |
| Women, n (%)                        | 14 (29%)             | 18 (37.5%)          | 32 (33%)  |                    |
| <b>Smoking status</b>               |                      |                     |           |                    |
| Active smoker (%)                   | 77                   | 79                  | 78        | 0.83 <sup>\$</sup> |
| Non-smoker (%)                      | 23                   | 21                  | 22        |                    |
| <b>Alcohol history</b>              |                      |                     |           |                    |
| Alcohol consumption (g/day)         | 151 ± 112            | 139 ± 73            | 145 ± 94  | 0.54               |
| Duration of drinking habits (years) | 17 ± 10              | 16 ± 11             | 16 ± 11   | 0.76               |
| Number of withdrawal cures          | 1 ± 2                | 2 ± 2               | 1.8 ± 2.3 | 0.015*             |
| AUDIT score                         | 31 ± 7               |                     |           |                    |
| DSM5 score                          |                      | 8 ± 2               |           |                    |
| <b>Depression</b>                   |                      |                     |           |                    |
| Score at T1                         | 23 ± 11              | 26 ± 12             | 25 ± 12   | 0.26               |
| <b>Anxiety</b>                      |                      |                     |           |                    |
| Score at T1                         | 44 ± 11              | 46 ± 15             | 45 ± 13   | 0.27               |
| <b>Alcohol craving</b>              |                      |                     |           |                    |
| Total score at T1                   | 20 ± 7               | 25 ± 6              | 22 ± 7    | 0.00*              |
| Obsession score at T1               | 9 ± 5                | 11 ± 4              | 10 ± 4    | 0.26               |
| Compulsion score at T1              | 11 ± 3               | 14 ± 3              | 13 ± 3    | 0.00*              |

Results are means ± standard deviations. Independent t-tests to compare ALCOHOLBIS *versus* GUT2BRAIN cohorts. <sup>\$</sup>chi-square test for categorical variables; \*p < 0.05

**Table 1.**

**Clinical features of the study participants**



**Figure 1.**

## Chronic alcohol intake alters the plasma metabolome compared to healthy controls.

**a** Principal component analysis score plot of the plasma metabolomic features between healthy controls and persons with AUD at T1. QC samples are colored in orange. **b** Top 50 annotated metabolites ranked on their variable importance in projections (VIP) scores in the sPLS-DA model. **c** Volcano plot depicting the effect size (Cohen's D) and  $-\log_{10}$  transformed  $q$  values derived from Welch's  $t$ -test analysis of the metabolomic features different between healthy controls and persons with AUD at T1. **d** Annotated metabolites having significant association (Spearman  $p < 0.05$ ) with alcohol intake (g/day) in persons with AUD at T1. Circle size refers to the level of significance, blue gradient color to the strength of negative while red to the strength of positive correlation coefficients. 3-CMPFP 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; AC acylcarnitine; CLA conjugated linoleic acid; FA fatty acid; LPC lysophosphatidylcholine; LPE lysophosphatidylethanolamine; PC phosphatidylcholine; PE phosphatidylethanolamine; SM sphingomyelin.

bilirubins, the levels of identified metabolites that were significantly changed upon alcohol withdrawal (the major ones being indoles, acylcarnitines, glycerophospholipids and xanthines) came back towards to the levels observed in controls (Table S3). In the course of alcohol abstinence, we noted a significant decrease in 16-chain acylcarnitines, LPCs with 16- or 18-chain fatty acid tails excluding LPCs with ether bonds, retinol, tryptophyl-phenylalanine dipeptide and 3-hydroxyvaleric acid (**Fig. 2c**). On the contrary, LPCs with odd-chain fatty acid tails or ether bonds show a significant increase along with tryptophan derivatives 3-indoleacetic acid and 3-indolepropanoic acid and metabolites of the xanthine family such as theophylline, paraxanthine and trigonelline during alcohol abstinence. Moreover, **Fig. 2c** demonstrates that alcohol withdrawal shifted the AUD plasma metabolic fingerprint towards healthy controls.

## Correlations between blood metabolites and psychological symptoms

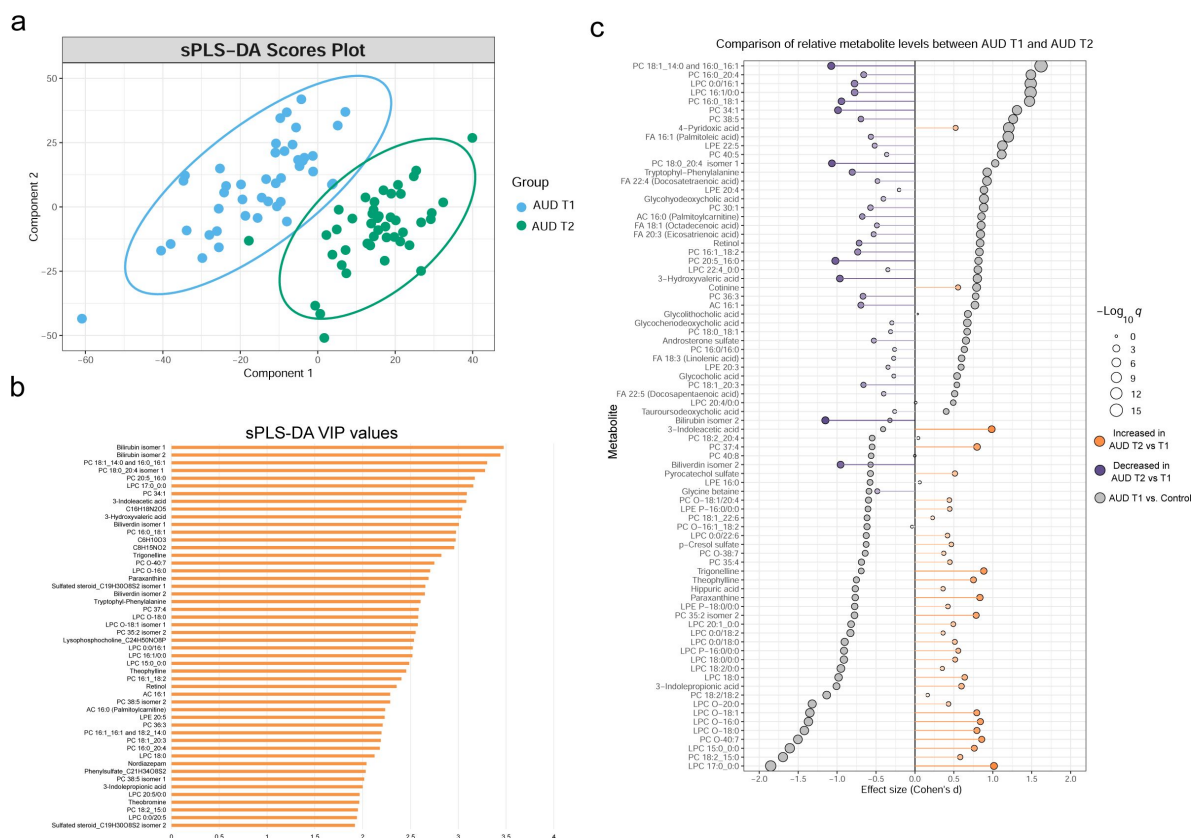
Correlation analysis shows that, at T1, 96 annotated features were significantly ( $p < 0.05$ ) correlated with psychological scores of anxiety, depression and alcohol craving (with sub-scores of obsession and compulsion) (**Fig 3**). Annotated bile acids, drugs, lysophosphoethanolamines (LPE), fatty acids, LPCs apart from LPCs with an ether-bond or 15:0 or 17:0 fatty acid tail were consistently positively correlated with psychological symptoms, and more particularly with the compulsive component of alcohol craving. Members of the xanthine family, pyrocatechol sulfate, a pentose sugar (mannose/fructose), hippuric acid, 1-methyl-pyridone-carboxamide, acylcarnitines with maximum of 10 carbons, creatinine and kynurenine were negatively correlated with psychological symptoms. Within the acylcarnitine metabolite class, an interesting pattern was observed, as the shorter chain-length acylcarnitines were consistently negatively correlated with the psychological parameters, and the long-chain ones demonstrated positive correlation.

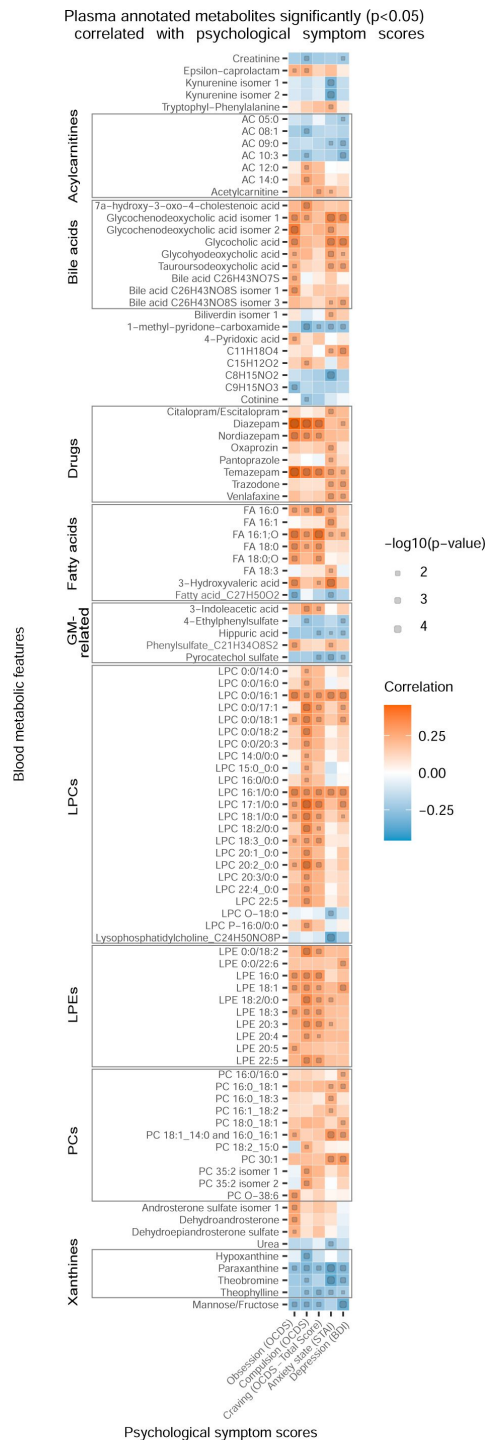
## Alterations in the brain metabolome of AUD patients

Based on the annotated significantly altered plasma metabolites, we conducted a targeted search in a metabolomics dataset consisting of CSF and frontal cortex samples collected from deceased individuals with a history of heavy alcohol use and control individuals. 79 and 74 of the annotated plasma metabolites were identified from the CSF and frontal cortex, respectively (**Fig. 4a**, Table S3). We looked specifically at metabolites significantly correlated with at least one psychological symptom. 3-Hydroxyvaleric acid, cotinine, theobromine and paraxanthine were indeed present in the CNS and significantly (Welch  $t$ -test  $p < 0.05$ ) different between heavy alcohol use and control groups in both frontal cortex (**Fig 4b**) and CSF (**Fig 4c**). Additional significantly altered metabolites found only in the frontal cortex were LPE 20:3, nordiazepam, PC 16:0\_18:1 and urea (**Fig. 4b**). In the CSF, the independent differential metabolites were FA 16:1 (palmitoleic acid), hippuric acid, LPCs 16:0 and 18:1, LPEs 16:0, 18:1 and 20:4 and pyrocatechol sulfate (**Fig. 4c**).

## Discussion

The circulating metabolome reflects the crosstalk between nutrition, microbiome and host metabolism (15), with diet and microbiome being the strongest determinants of the human blood metabolome (11,16). In this study, we showed the impact of chronic alcohol abuse, and the impact of a short-term abstinence, on the blood metabolome. We analyzed the correlations between blood metabolites and psychological symptoms, as well as the presence of identified metabolites in the CNS of individuals considered as heavy alcohol drinkers.

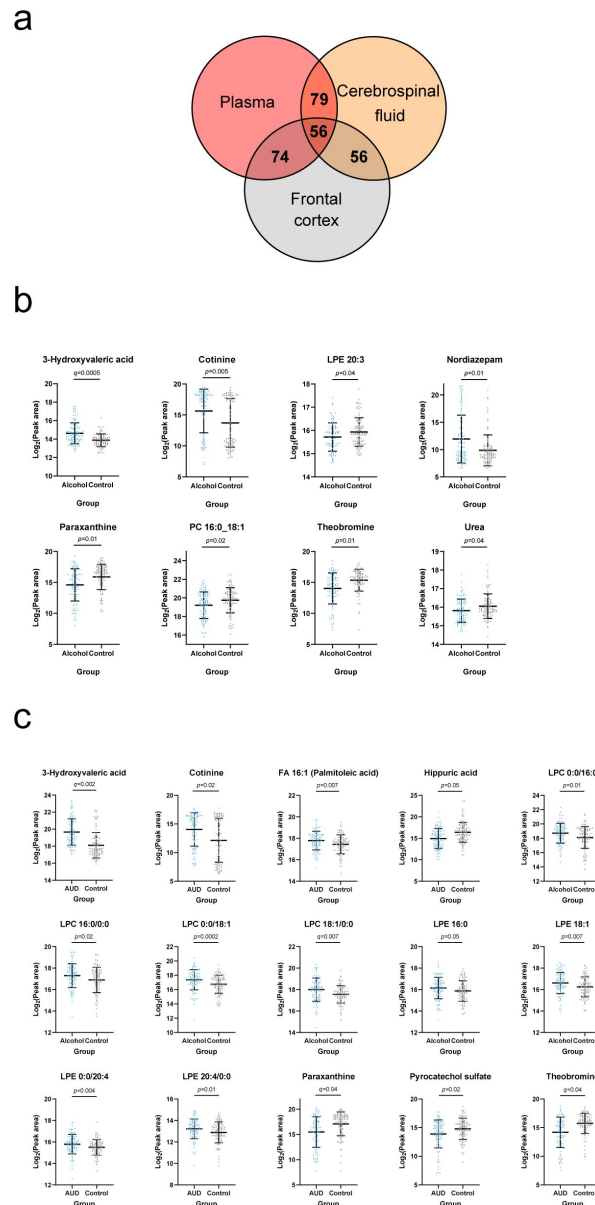




**Figure 3.**

### Plasma metabolites associated with psychological symptom scores.

Heatmap of the annotated metabolites having significant ( $p < 0.05$ ) Spearman correlation with one or more psychological symptom score of obsession, compulsion, alcohol craving, anxiety state or depression. Circle size refers to the level of significance, blue gradient color to the strength of negative while red to the strength of positive correlation coefficients. AC acylcarnitine; FA fatty acid; GM gut microbiota; LPC lysophosphatidylcholine; LPE lysophosphatidylethanolamine; PC phosphatidylcholine.



**Figure 4.**

### Overlapping metabolites within the plasma and brain metabolome.

**a** Venn diagram of the annotated plasma metabolites that are also present in the frontal cortex and cerebrospinal fluid metabolome. **b** Significantly altered ( $p < 0.05$ ) frontal cortex metabolites with a corresponding plasma metabolite showing an association with one or more psychological symptom score. **c** Significantly altered ( $p < 0.05$ ) cerebrospinal fluid metabolite with a corresponding plasma metabolite showing an association with one or more psychological symptom score. Data expressed as mean  $\pm$  SD with individual values shown. Statistical values derived from Welch's  $t$ -test comparing metabolomic features between control and alcohol groups.

## Impact of chronic alcohol abuse on the blood metabolome

In 2019, a review summarized the results obtained from 23 studies that have used a metabolomics approach for measuring changes in metabolite profiles in relation to alcohol use (1 [↗](#)). Changes in lipids have been highlighted as the most consistent changes across studies. Lipids are an integral part of cell membranes and signaling molecules in the body. PCs and LPCs have been suggested to form a new class of biomarkers for alcohol consumption (2 [↗](#)). For instance, in our study palmitoleic acid (FA 16:1) was largely increased in AUD patients compared to controls, and in other studies, this metabolite has likewise been significantly associated with alcohol consumption (17 [↗](#), 18 [↗](#)). Another clear observation in our study was the lower level of odd-chain lipids in AUD patients. Since the lipids containing FA 15:0 and FA 17:0 have been suggested to be products from bacterial metabolism (19 [↗](#), 20 [↗](#)), the existence of gut dysbiosis in AUD patients could explain the lower abundance of LPC 17:0 and LPC 15:0 (14 [↗](#)).

We found that some bile acids, sulphated steroids and 3-hydroxyvaleric acid were positively associated with the amount of alcohol consumed. Sulphated steroids and hydroxyvalerate have previously been associated with alcohol intake (21 [↗](#), 22 [↗](#)). Metabolites belonging to the xanthine family (theobromine, theophylline, paraxanthine) and microbial metabolites (hippuric acid, indole-3-propionic acid, p-cresol sulfate, pyrocatechol sulfate) were negatively correlated with alcohol consumption. Altogether, these results suggest that these metabolites are sensitive to alcohol exposure. Interestingly, these metabolites were also correlated with the severity of the psychological symptoms suggesting that they could play a role in the symptomatology of alcohol addiction.

## Effect of short-term alcohol abstinence on the blood metabolome

Since alcohol consumption is known to influence lipid metabolism, it was expected that a short-term alcohol abstinence could reverse or ameliorate lipidomic alterations. Indeed, we found that some phospholipids that were increased in AUD patients at baseline, such as PC 16:0\_18:1, PC18:1\_14:0 and 16:0\_16:1 as well as LPC 16:1, were downregulated during alcohol withdrawal to reach the levels of healthy controls after detox. On the other hand, LPC 15:0 and LPC 17:0 that were decreased in AUD patients at baseline, increased during alcohol withdrawal, but did not reach the levels of controls at the end of detox.

The metabolite that contributed the most to the differences observed with alcohol detoxification was bilirubin. While bilirubin was not statistically higher in AUD patients at baseline vs controls, we observed a significant reduction of this metabolite after a 3-week alcohol withdrawal. In a previous study, serum bilirubin was found to be associated with alcohol consumption, cigarette smoking and coffee consumption (23 [↗](#)). Interestingly, the caffeine metabolites belonging to the xanthine family such as paraxanthine, theophylline and theobromine that were decreased at baseline in AUD patients compared to controls, increased significantly during alcohol withdrawal to reach the levels of healthy controls. Changes in dietary intake of coffee, tea and chocolate during alcohol withdrawal could explain these results (data not shown). Also, the bacterial metabolites indole derivatives such as 3-indolepropionic acid and 3-indoleacetic acid increased during alcohol withdrawal to reach the levels of healthy controls. Intriguingly, 3-hydroxyvaleric acid significantly decreased during alcohol withdrawal and was found to be lower than healthy controls at the end of detoxification period.

Metabolites that remained significantly higher in AUD patients at the end of detoxification compared to controls are stress hormone cortisol, palmitoleic acid (FA 16:1), some bile acids, some drugs (diazepam, trazodone), vitB6 metabolite (4-pyridoxic acid, which is likely due to the fact that patients received vitamin B supplement during their hospital stay) and cotinine (nicotine metabolite that reveals the higher proportion of smokers in AUD patients compared to controls).

## Identification of blood metabolites with potential neuroactive properties

The metabolites belonging to the xanthine family (theobromine, paraxanthine and theophylline) are metabolites of caffeine produced upon cytochrome P450 dependent oxidation in the liver. They were all decreased in the blood of AUD patients at baseline and were negatively correlated with alcohol intake, alcohol craving, depression and anxiety. The decrease in caffeine metabolites has previously been described in the urine of AUD patients, that is linked to the increasing severity of alcoholic liver disease (24 [↗](#)).

Theobromine is the principle alkaloid found in cocoa beans and is responsible for the bitter taste of chocolate. It is known for its mood improving effect (25 [↗](#)). Like caffeine, theobromine is an inhibitor of brain adenosine receptors and phosphodiesterase. A study in rats showed that the antagonist of A2a adenosine receptor produced a reduction of ethanol reinforcement (26 [↗](#)), suggesting adenosine receptor as a potential target for the treatment of alcohol abuse. In a randomized, double-blind, placebo-controlled trial, the phosphodiesterase inhibitor pentoxifylline associated with escitalopram showed greater reduction of depression scores compared to patients receiving escitalopram alone (27 [↗](#)). In another study, Apremilast which is also a phosphodiesterase inhibitor, suppressed excessive alcohol drinking in AUD patients (28 [↗](#)). Paraxanthine has a psychostimulant effect and can modulate dopamine release in the striatum (29 [↗](#)). Interestingly, in 2017 a systematic review indicated that consumption of coffee, tea and cocoa could have protective effects against depression (30 [↗](#)).

Lipids, and mostly LPCs (except ether LPC derivatives) and LPEs were significantly and positively correlated with the compulsive component of alcohol craving. LPCs are secreted by the liver and are actively transported via the blood-brain barrier (BBB) and have been associated with pro-inflammatory events (31 [↗](#)). LPCs are also precursors of brain lysophosphatidic acid (LPA), which regulates glutamatergic transmission and cortical excitability within the CNS. Recently, LPA has been shown to induce hyperphagia following food restriction and this effect was dependent on hypothalamic agouti-related peptide (AgRP) neurons (32 [↗](#)). AgRP neurons have also been implicated in circuitry controlling non-feeding behavior, including those associated with reward, anxiety and compulsive disorders, more particularly in anorexia nervosa (33 [↗](#)). Therefore, we hypothesize that the positive correlation between peripheral LPC and compulsion for alcohol drinking found in AUD patients who have just been deprived of alcohol could be mediated by the effect of LPA on AgRP neurons. Consistent with that, postmortem brain tissues from patients consuming a high intake of alcohol showed increased levels of many LPCs (10 [↗](#)).

Circulating bile acids can reach the brain by crossing the BBB, either by simple diffusion or active transport. Some bile acids show neuroprotective effects (34 [↗](#)) while others are rather neurotoxic (35 [↗](#)). In Alzheimer disease patients, the levels of glycochenodeoxycholic acid was associated with worse cognition (36 [↗](#)). In our study, both primary (glycochenodeoxycholic acid and glycocholic acid) and secondary (glycohyodeoxycholic acid, tauroursodeoxycholic acid) bile acids were positively correlated with depression and anxiety in AUD patients.

3-Hydroxyvaleric acid, also called  $\beta$ -hydroxypentanoate, was significantly and positively correlated with anxiety and alcohol craving. This metabolite is formed from odd carbon fatty acids in the liver and can reach the brain. 3-hydroxyvaleric acid is a C5-ketone body and is a precursor of propionyl-CoA that refills intermediates of citric acid cycle and is useful for alternative energy fuel in the brain (37 [↗](#), 38 [↗](#)).

Other co-metabolites, i.e. produced by the gut microbiota and then processed by the liver, were negatively correlated with all psychological symptoms. Pyrocatechol sulfate is a phenolic compound derived from the gut microbiota, present in the CSF of mice, and implicated in synapse

formation and fear extinction learning (39). In Parkinson disease patients, the plasma level of pyrocatechol sulfate is decreased compared to controls (40). In our study, blood pyrocatechol sulfate was significantly and negatively correlated with all psychological symptoms of AUD patients (i.e. anxiety, depression and craving) suggesting a neuroprotective role of this metabolite. Interestingly, 4-ethylphenylsulfate, another gut-derived metabolite linked with neurodevelopment abnormalities, autism and anxiety behavior in mice (41–43) showed negative correlations with depression and the compulsive component of alcohol craving.

Hippuric acid, the glycine conjugate of benzoic acid has long been associated with the microbial degradation of specific dietary components, including polyphenolic compounds (like chlorogenic acid and catechin) found in fruits, vegetables, coffee and tea (44). Hippuric acid is indeed a host-microbe cometabolite (45). It is synthesized in the liver and in the renal cortex from the microbial metabolite benzoate. The plasma concentration of hippuric acid has been shown to be 17-fold higher in conventional mice compared with their germ-free counterparts suggesting a substantial contribution of the gut microbiota in its production (46). We showed that blood hippurate levels, that correlated negatively with anxiety, depression and craving, were decreased in AUD patients, as shown in ethanol-treated mice (6) and humans characterized by high alcohol intake and those with major depression (47,48). Urinary hippurate excretion is also decreased in depression, schizophrenia and autism spectrum disorders patients (44). A recent Mendelian randomization study including > 13,000 individuals from five European cohorts characterized for depression suggested that low hippuric acid levels in the circulation is part of the causal pathway leading to depression (49), which was consistent with a significant decrease of the dietary sources of hippuric acid including fresh fruits and vegetables in depressed patients (49).

Another way to support the neuroactive effects of the blood metabolites that are correlated with one or several psychological factors is to demonstrate their presence in the brain. We therefore conducted a targeted search in a database of post-mortem frontal cortex and CSF metabolomics analysis (10) and found that 3-hydrovaleric acid, caffeine metabolites (theobromine, paraxanthine and theophylline) and microbial metabolites (hippuric acid and pyrocatechol sulfate) that were correlated with anxiety, depression and alcohol craving in our plasma cohort were also present in the brain and in CSF, and the direction of their changes in the plasma (increase or decrease) mimicked changes in the central nervous system.

## Advantages and limitations of the study

Most of the studies assessing the impact of alcohol consumption on the blood metabolome were cross-sectional, and only included male participants (47). Here, we reported longitudinal data to assess the impact of a short-term alcohol abstinence on the blood metabolome, both in male and female AUD patients. In the study of Zhu et al (9), the AUD patients recruited were alcohol abstinent, but for various periods of abstinence. To avoid the bias of abstinence duration, our patients were enrolled in a rigorous and standardized manner, within 24 hours after the last drink. Furthermore, alcohol consumption was carefully evaluated with the time line follow back method, which allows precise calculation of the amount (and type) of alcohol consumed (50).

This study also presents some limitations. First, the metabolomics analysis was conducted with LC-MS while some important molecules, like lipoproteins, could have been measured with NMR-based methods. Combining NMR and MS-based methods could have covered a wider spectrum of metabolites. However, the non-targeted metabolic profiling with two different chromatographic methods and ionization polarities covers a wide range of metabolites ideal for our discovery-based approach. Large studies are usually required in metabolomics to observe small and medium size changes. Here, we included only 96 AUD patients, but they were all well characterized and received standardized therapies (for instance, vitB supplementation) during alcohol withdrawal.

The selection of the control group is always challenging in alcohol research. Here, the healthy subjects were matched for sex, age and BMI but not for smoking status or nutritional intake. Alcohol addiction is a major cause of malnutrition in developed countries and tobacco smoking is more prevalent in alcohol users compared to healthy subjects. These two main confounding factors, although being an integral part of the alcoholic pathology, are known to influence the blood metabolome (51 [↗](#)–53 [↗](#)).

## Conclusion

LC-MS metabolomics plasma analysis allowed for the identification of metabolites that were clearly linked to alcohol consumption, and reflected changes in metabolism, alterations of nutritional status, and gut microbial dysbiosis associated with alcohol intake. In particular the changes in lipid class involving odd-chain fatty acids and ether-bond lipids as well as compounds produced by gut microbiota seem to be the most prominent indicators of metabolic malfunction related to alcohol use disorder, and thus warrant further studies and targeted intervention. Also, the discovery of metabolites associated with behavioral and psychiatric traits related to AUD were of importance, and could be considered potential new therapeutic targets in the management of AUD, namely as adjuvants in the period of alcohol abstinence. The novelty of our work was to characterize the impact of chronic alcohol abuse on the blood metabolome, and the impact of a short-term alcohol abstinence in the same individuals, within a cohort that included both male and female patients. Intervention studies are needed in order to bring the proof of concept that nutritional interventions – namely the addition of specific lipids, or of nutrients modulating the gut microbiome - for example prebiotic dietary fibers - may be essential and so far underestimated components of alcohol withdrawal efficacy.

## Subjects and methods

### Study design and participants

A total of 96 AUD patients hospitalized for a 3-week detoxification program in the alcohol withdrawal unit at Cliniques Universitaires Saint-Luc, Brussels, Belgium were recruited. These patients belong to two different cohorts, namely ALCOHOLBIS (patients recruited in 2015 and 2019) and GUT2BRAIN (patients recruited in 2018-2019) (Table 1 [↗](#)). The severity of AUD was evaluated by a psychiatrist using the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria, fourth edition (DSM-IV) (ALCOHOLBIS cohort) or fifth edition (DSM-5) (GUT2BRAIN cohort). Patients were eligible if they had been drinking until the day of admission to the detoxification unit or the day before, and if they also did not suffer from inflammatory bowel disease, other chronic inflammatory diseases (such as rheumatoid arthritis) or cancer, nor from metabolic disorders such as obesity (BMI > 30 kg/m<sup>2</sup>), diabetes and bariatric surgery, or severe cognitive impairment (MMSE < 24). We also excluded subjects who had taken antibiotics, probiotics, or prebiotics in the 2 months prior to enrolment and those who were taking non-steroidal anti-inflammatory drugs or glucocorticoids within 1 month of inclusion. Patients with known cirrhosis or significant liver fibrosis (≥F2) detected by Fibroscan (>7.6 kPa) on the day of admission were also excluded from the study. No other psychiatric diseases, and no other addiction (except tobacco) have been diagnosed in these patients.

AUD patients were tested twice, on the day following their admission (T1) and on days 18–19 (T2) corresponding to the last days of the detoxification program. The patients of the GUT2BRAIN cohort were initially enrolled in a randomized, double-blind, placebo-controlled study assessing the impact of prebiotic fiber supplementation on the gut-liver-brain axis (54 [↗](#), 55 [↗](#)). For this reason, only biological and psychological data obtained at admission (T1), and before the

beginning of the prebiotic/placebo treatment, were considered. The patients of the ALCOHOLBIS cohort did not take part in any other clinical study during the 3-week hospitalization stay. Therefore, for this cohort, data obtained at both times of alcohol withdrawal (T1 and T2) were considered. Thirty-two healthy controls (13 from the GUT2BRAIN cohort and 19 from the ALCOHOLBIS cohort) matched for age, gender and BMI with no AUD (Alcohol use disorders test [AUDIT] score <8 in males and <7 in females) were also recruited using flyers posted in Brussel's public setting (Table S1). The inclusion/exclusion criteria were the same as for AUD patients except for the alcohol related items. Healthy controls and AUD patients were not matched for smoking status.

The study was approved by the "Comité d'éthique Hospitalo-facultaire Saint-Luc UCLouvain" (2017/04JUL/354 and 2014/31dec/614, identification number NCT03803709 at [ClinicalTrials.gov](https://clinicaltrials.gov)). The study has been carried out in accordance with The Code of Ethics of the World Medical Association and followed the ethical guidelines set out in the Declaration of Helsinki. All participants provided written informed consent in compliance with the European law 2001/20/CE guidelines.

For investigating presence of the potentially neuroactive metabolites in the CNS, we used metabolomics data from frontal cortex (Brodmann area 9) and CSF samples from the Tampere Sudden Death Study (TSDS) cohort, which have been described in detail elsewhere (10). TSDS was collected from forensic autopsies done in the area of the Pirkanmaa Hospital District during 2010-2015, a total of 700 subjects. Out of these we identified 97 heavy alcohol users based on autopsy reports and medical records (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 the clinical or laboratory findings, e.g., increased levels of gamma-glutamyl transferase, mean corpuscular volume, carbohydrate-deficient transferrin). Lack of these findings was inclusion criteria for the control group (n = 100), most of whom had died due to cardiovascular diseases (Table S2). Samples were stored at -80 °C until use.

## Assessment of psychological symptoms

AUD patients were tested for depression, anxiety and alcohol craving with self-reported questionnaires: the Beck Depression Inventory [BDI] (56), the State-Trait Anxiety Inventory (STAI form YA) (57), and the Obsessive-Compulsive Drinking Scale [OCDS] (58) (see supplemental material for details)

## Biological sampling

To avoid variation due to fasting state and circadian rhythm, blood samples were collected in all participants in the morning between 8:00 and 8:30 am after an overnight fasting, at T1 and T2. Blood was drawn in tubes containing EDTA as an anticoagulant. The samples were centrifuged at 1000 g for 15 min at 4°C and the plasma was frozen at -80°C until analysis.

## Nontargeted metabolomics analysis

Plasma sample preparation and LC-MS measurement were performed as previously detailed in Klåvus et al (59) (see supplemental material for details).

## Statistical analysis

R software version 4.0.3. was used for statistical analyses. Multivariate analyses, namely PCA for dimension reduction and sPLS-DA for group discrimination, were conducted by 'mixOmics' R package v. 6.14.1 (60). For the sPLS-DA model we used a cross-validation (CV) procedure of 10-fold CV repeated 50 times. Univariate analyses were conducted by 'notame' R package v. 0.2.1 (59). Significant features were shortlisted using Welch's and paired t-tests. All *p*-values were corrected using the Benjamini-Hochberg false discovery rate (FDR) to calculate the *q*-value. For all

tests,  $p$  and  $q$  values  $< 0.05$  were considered statistically significant. Visualizations were created by the previously mentioned R packages and GraphPad Prism v. 8.4.2. Correlation analyses were performed at T1 using R software version 3.6.1. Spearman coefficient was calculated and  $p$ -value  $< 0.05$  was considered statistically significant.

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## Author contributions

Conceptualized and designed the study: SL, CA, PS, PdT, NMD

Performed participant visits: SL, CA, GP, MP

Collected and provided the TSDS post-mortem samples: EK, PJK

Performed data analysis, interpreted the data and drafted the manuscript: SL, HA, CA, QL, PdT, SLa, AMN, OK, KH, NMD

Performed the analysis of the metabolomics data: HA, VK, OK, KH

Contributed to ethics application: SL, PS, PdT, NMD

All authors approved the final version of the manuscript.

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**Reviewer #1 (Public Review):**

Summary:

This work by Leclercq and colleagues performed metabolomics on biospecimens collected from 96 patients diagnosed with several types of alcohol use disorders (AUD). The authors discovered strong alterations in circulating glycerophospholipids, bile acids, and some gut

microbe-derived metabolites in AUD patients compared to controls. An exciting part of this work is that metabolomics was also performed in frontal cortex of post-mortem brains and cerebrospinal fluid of heavy alcohol users, and some of the same metabolites were seen to be altered in the central nervous system. This is an important study that will form the basis for hypothesis generation around diet-microbe-host interactions in alcohol use disorder. The work is done in a highly rigorous manner, and the rigorously collected human samples are a clear strength of this work. Overall, many new insights may be gained by this work, and it is poised to have a high impact on the field.

**Strengths:**

- (1) The rigorously collected patient-derived samples.
- (2) There is high rigor in the metabolomics investigation.
- (3) Statistical analyses are well-described and strong.
- (4) An evident strength is the careful control of taking blood samples at the same time of the day to avoid alterations in meal- and circadian-related fluctuations in metabolites.

**Weaknesses:**

- (1) Some validation in animal models of ethanol exposure compared to pair-fed controls would help strengthen causal relationships between metabolites and alterations in the CNS.
- (2) The classification of "heavy alcohol users" based on autopsy reports may not be that accurate.
- (3) The fact that most people with alcohol use disorder choose to drink over eating food, there needs to be some more discussion around how dietary intake (secondary to heavy drinking) most likely has a significant impact on the metabolome.

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**Reviewer #2 (Public Review):**

The authors carried out the current studies with the justification that the biochemical mechanisms that lead to alcohol addiction are incompletely understood. The topic and question addressed here are impactful and indeed deserve further research. To this end, a metabolomics approach toward investigating the metabolic effects of alcohol use disorder and the effect of alcohol withdrawal in AUD subjects is valuable. However, it is primarily descriptive in nature, and these data alone do not meet the stated goal of investigating biochemical mechanisms of alcohol addiction. The current work's most significant limitation is the cross-sectional study design, though inadequate description and citation of the underlying methodological approaches also hampers interest.

Most of the data are cross-sectional in the study design, i.e., alcohol use disorder vs controls. However, it is well established that there is a high degree of interpersonal variation with metabolism, and further, there is somewhat high intra-personal variation in metabolism over time. This means that the relatively small cohort of subjects is unlikely to reflect the broader condition of interest (AUD/withdrawal). The authors report a comparison of a later time-point after alcohol withdrawal (T2) vs. the AUD condition. However, without replicative time points from the control subjects it is difficult to assess how much of these changes are due to withdrawal vs the intra-personal variation described above. Overall, there is not enough experimental context to interpret these findings into a biological understanding. For example, while several metabolites are linked with AUD and associated with microbiome or host metabolism based on existing literature, it's unclear from the current study what

function these changes have concerning AUD, if any. The authors also argue that alcohol withdrawal shifts the AUD plasma metabolic fingerprint towards healthy controls (line 153). However, this is hard to assess based on the plots provided since the change in the direction of the orange data subset is considers AUD T2 vs T1. In contrast, AUD T2 vs Control would represent the claimed shift. To support these claims, the authors would better support their argument by showing this comparison as well as showing all experimental groups (including control subjects) in their multi-dimensional model (e.g., PCA). The authors attempt to extend the significance of their findings by assessing post-mortem brain tissues from AUD subjects; however, the finding that many of the metabolites changed in T2/T1 are also present in AUD brain tissues is interesting; however, not strongly supporting of the authors' claims that these metabolites are markers of AUD (line 173). Concerning the plasma cohort itself, it is unclear how the authors assessed for compliance with alcohol withdrawal or whether the subjects' blood-alcohol levels were independently verified.

The second area of concern is the need for more description of the analytical methodology, the lack of metabolite identification validation evidence, and related statistical questions. The authors cite reference #59 regarding the general methodology. However, this reference from their group is a tutorial/review/protocol-focused resource paper, and it is needs to be clarified how specific critical steps were actually applied to the current plasma study samples given the range of descriptions provided in the citations. The authors report a variety of interesting metabolites, including their primary fragment intensities, which are appreciated (Supplementary Table 3), but no MS2 matching scores are provided for level 2 or 3 hits. Further, level 1 hits under their definition are validated by an in-house standard, but no supporting data are provided besides this categorization. Finally, a common risk in such descriptive studies is finding spurious associations, especially considering many factors described in the current work. These include AUD, depression, anxiety, craving, withdrawal, etc. The authors describe the use of BH correction for multiple-hypothesis testing. However, this approach only accounts for the many possible metabolite association tests within each comparison (such as metabolites vs depression). It does not account for the multi-variate comparisons to the many behavior/clinical factors described above. The authors should employ one of several common strategies, such as linear mixed effects models, for these types of multi-variate assessments.

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