Blood metabolomic profiling reveals new targets in the management of psychological symptoms associated with severe 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 this disorder 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 severe AUD (sAUD), and of a three-week period of alcohol abstinence, on the blood metabolome (non-targeted LC-MS metabolomics analysis) in 96 sAUD patients hospitalized for alcohol withdrawal. 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 sAUD 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 exposure 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 sAUD.

The study was registered at clinicaltrial.gov under the identification number NCT03803709.

58 **Introduction**

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

65 Alcohol consumption clearly leads to alterations of the circulating metabolome (1–4). For
66 instance, changes in the levels of lipids (fatty acids, phosphatidylcholine, steroids) and amino
67 acids (glutamine, tyrosine, alanine, serotonin, asparagine) are commonly observed and,
68 interestingly, some changes in the blood metabolite profile precedes the emergence of alcohol
69 use related diseases, such as lower levels of serotonin and asparagine (5). Metabolomics studies
70 conducted in rodent models of alcohol exposure have mostly targeted the liver tissue or urine
71 (6–8). In human, plasma or serum metabolomics studies have tested the effects of
72 low/moderate/excessive alcohol intake (9) but are rarely performed in clinical populations of
73 severe AUD (sAUD) patients, including analysis of central nervous system (CNS) tissues(10).
74 Furthermore, the methodology could differ across studies, some using nuclear magnetic
75 resonance (NMR) while others using more sensitive mass spectrometry (MS) coupled with
76 liquid or gas chromatography (LC or GC), leading to the detection of different metabolites.

77 The human blood metabolome consists of 1) small molecules that directly represent the
78 functional changes in host metabolism, 2) metabolites produced by intestinal micro-organisms
79 and 3) metabolites originating from nutrition of other exogenous sources like drugs (1,11).

80 The aims of this study are multiple. First, we investigated the impact of sAUD on the blood
81 metabolome by non-targeted LC-MS metabolomics analysis. Second, we investigated the
82 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 sAUD 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 sAUD.

93 **RESULTS**

94

95 **Clinical characteristics of the study participants**

96 Two cohorts of sAUD patients (ALCOHOLBIS and GUT2BRAIN) were used in this study. All
97 patients were hospitalized for a 3-week detoxification program, and tested at two timepoints:
98 T1 which represents the first day of alcohol withdrawal, and T2 which represents the last day
99 of the detoxification program. Both groups of patients were similar in terms of age, gender,
100 smoking and drinking habits and presented with high scores of depression, anxiety and alcohol
101 craving at T1 (Table 1). These biological and psychological similarities allow us to combine
102 both cohorts (and consequently increase sample size) and compare them to a group of heathy
103 controls for metabolomics analysis".

104

105 **Alterations in the plasma metabolome of sAUD patients**

106 The metabolomics analysis allowed for sorting out a total of 11,651 features from the four
107 analytical modes of the plasma samples. An unsupervised principal component analysis (PCA)
108 model of the plasma metabolomic profiles between healthy controls and sAUD patients at the
109 beginning of the withdrawal (T1) is shown in Fig. 1a. In addition, the scores plot and the
110 performance of a supervised sparse partial least square discriminant analysis (sPLS-DA) model
111 are shown in Figs. S1 and S2, respectively. Between healthy controls and sAUD T1, the
112 annotated differential (Welch *t*-test $q<0.05$ and sPLS-DA variable importance in the projection
113 (VIP) score > 2.0) metabolites included compounds from several metabolite classes as indicated
114 in Fig. 1b and Table S3. Compared to healthy controls, the metabolic profiles of sAUD patients
115 were characterized by an increase in long-chain fatty acids, such as 16:1 (palmitoleic acid), 18:1
116 (octadecenoic acid) and 22:4 (docosatetraenoic acid) fatty acids and phospholipids holding
117 these fatty acids. In addition, several drugs (like diazepam, trazodone) and metabolites with

118 steroid backbone such glycinated bile acids (glycohyodeoxycholic acid and
119 glycochenodeoxycholic acid), steroid hormones and acylcarnitines were increased in the sAUD
120 group. We also observed a significant increase in vitamin B6 metabolite, 4-pyridoxic acid,
121 nicotine metabolite cotinine, a hydroxy fatty acid 3-hydroxyvaleric acid and stress hormone
122 cortisol. However, lysophosphatidylcholines (LPCs) holding a saturated odd-chain (e.g. LPC
123 15:0 and LPC 17:0), polyunsaturated 18-carbon fatty acid or an ether bond (O-) containing lipid
124 were consistently decreased in sAUD compared to controls (Fig. 1b). Further, we also showed
125 a decrease in furan fatty acids 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid (3-CMPFP)
126 and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), in a carotenoid compound
127 and in several metabolites belonging to the family of xanthine. In addition, several amino-acid
128 derived bacterial metabolites such as pipecolic acid, 3-indole propionic acid, p-cresol sulfate
129 and hippuric acid were significantly decreased in sAUD patients compared to controls. The top-
130 ranked metabolites in Fig. 1b remained unknown regardless of manual curation.
131 We then conducted a correlational analysis between blood metabolites and alcohol consumption
132 reported by the patients. Alcohol intake was positively correlated with annotated bile acids,
133 steroids and drugs while xanthines (paraxanthine, theobromine and theophylline), odd-chain or
134 ether-bond LPCs and bacterial metabolite hippuric acid, p-cresol sulfate, pyrocatechol sulfate,
135 and 3-indole propionic acid showed negative correlations (Fig. 1c).
136
137 **Alcohol withdrawal shapes the plasma metabolome**
138 The score plot of the sPLS-DA in Fig. 2a shows a clear discrimination in plasma metabolomic
139 profiles in the course of withdrawal in sAUD patients. The sPLS-DA model performance and
140 the unsupervised PCA model scores plot are shown in supplementary Figs. S3 and S4,
141 respectively. Annotated metabolites discriminating sAUD groups (paired *t*-test $q<0.05$, sPLS-
142 DA VIP score > 2.0) before (T1) and after (T2) the 3-week withdrawal period included

143 metabolites from a range of chemical classes (Fig. 2b, Table S3). Apart from the metabolites
144 belonging to the class of bilirubins, the levels of identified metabolites that were significantly
145 changed upon alcohol withdrawal (the major ones being indoles, acylcarnitines,
146 glycerophospholipids and xanthines) came back towards to the levels observed in controls
147 (Table S3). In the course of alcohol abstinence, we noted a significant decrease in 16-chain
148 acylcarnitines, LPCs with 16- or 18-chain fatty acid tails excluding LPCs with ether bonds,
149 retinol, tryptophyl-phenylalanine dipeptide and 3-hydroxyvaleric acid (Fig. 2b). On the
150 contrary, LPCs with odd-chain fatty acid tails or ether bonds show a significant increase along
151 with tryptophan derivatives 3-indoleacetic acid and 3-indolepropanoic acid and metabolites of
152 the xanthine family such as theophylline, paraxanthine, theobromine and trigonelline during
153 alcohol abstinence. The changes in metabolites belonging to the xanthine family during alcohol
154 withdrawal could be explained by the changes in dietary intake of coffee, tea and chocolate (see
155 Fig S5).
156 Overall, Fig. 2c demonstrates that a number of identified metabolites altered in sAUD patients
157 relative to control are affected by alcohol withdrawal. Apart from 4-pyridoxic acid, cotinine,
158 and heme metabolites bilirubin and biliverdin, the shifts observed in the selected metabolites
159 are generally in the opposite direction as compared to the baseline.
160

161 **Correlations between blood metabolites and psychological symptoms**

162 Correlation analysis shows that, at T1, 96 annotated features were significantly ($p<0.05$)
163 correlated with psychological scores of anxiety, depression and alcohol craving (with sub-
164 scores of obsession and compulsion) (Fig 3). Annotated bile acids, drugs,
165 lysophosphatidylethanolamines (LPE), fatty acids, LPCs apart from LPCs with an ether-bond
166 or 15:0 or 17:0 fatty acid tail were consistently positively correlated with psychological
167 symptoms, and more particularly with the compulsive component of alcohol craving. Members

168 of the xanthine family, pyrocatechol sulfate, a pentose sugar (mannose/fructose), hippuric acid,
169 1-methyl-pyridone-carboxamide, acylcarnitines with maximum of 10 carbons, creatinine and
170 kynurenine were negatively correlated with psychological symptoms. Within the acylcarnitine
171 metabolite class, an interesting pattern was observed, as the shorter chain-length acylcarnitines
172 were consistently negatively correlated with the psychological parameters, and the long-chain
173 ones demonstrated positive correlation.
174
175 **Heavy alcohol use-related alterations in the brain metabolome**
176 Based on the annotated significantly altered plasma metabolites, we conducted a targeted search
177 in a metabolomics dataset consisting of cerebrospinal fluid (CSF) and frontal cortex samples
178 collected from deceased individuals with a history of heavy alcohol use and control individuals.
179 79 and 74 of the annotated plasma metabolites were identified from the CSF and frontal cortex,
180 respectively (Fig. 4a, Table S3). We looked specifically at metabolites significantly correlated
181 with at least one psychological symptom. 3-Hydroxyvaleric acid, cotinine, theobromine and
182 paraxanthine were indeed present in the central nervous system (CNS) and significantly (Welch
183 *t*-test $p<0.05$) different between heavy alcohol use and control groups in both frontal cortex (Fig
184 4b) and CSF (Fig 4c). Additional significantly altered metabolites found only in the frontal
185 cortex were LPE 20:3, nordiazepam, PC 16:0_18:1 and urea (Fig. 4b). In the CSF, the
186 independent differential metabolites were FA 16:1 (palmitoleic acid), hippuric acid, LPCs 16:0
187 and 18:1, LPEs 16:0, 18:1 and 20:4 and pyrocatechol sulfate (Fig. 4c).
188
189

190 **DISCUSSION**

191

192 The circulating metabolome reflects the crosstalk between nutrition, microbiome and host
193 metabolism (12), with diet and microbiome being the strongest determinants of the human
194 blood metabolome (11,13). In this study, we showed the impact of sAUD, and the impact of a
195 short-term abstinence, on the blood metabolome. We analyzed the correlations between blood
196 metabolites and psychological symptoms, as well as the presence of identified metabolites in
197 the CNS of individuals considered as heavy alcohol drinkers.

198

199 **Impact of sAUD on the blood metabolome**

200 In 2019, a review summarized the results obtained from 23 studies that have used a
201 metabolomics approach for measuring changes in metabolite profiles in relation to alcohol use
202 (1). Changes in lipids have been highlighted as the most consistent changes across studies.
203 Lipids are an integral part of cell membranes and signaling molecules in the body. PCs and
204 LPCs have been suggested to form a new class of biomarkers for alcohol consumption (2). For
205 instance, in our study palmitoleic acid (FA 16:1) was largely increased in sAUD patients
206 compared to controls, and in other studies, this metabolite has likewise been significantly
207 associated with alcohol consumption (14,15). Another clear observation in our study was the
208 lower level of odd-chain lipids in sAUD patients. Since the lipids containing FA 15:0 and FA
209 17:0 have been suggested to be products from bacterial metabolism (16,17), the existence of
210 gut dysbiosis in sAUD patients could explain the lower abundance of LPC 17:0 and LPC 15:0
211 (18).

212 We found that some bile acids, sulphated steroids and 3-hydroxyvaleric acid were positively
213 associated with the amount of alcohol consumed. Sulphated steroids and hydroxyvalerate have
214 previously been associated with alcohol intake (19,20). 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 use disorder.

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 sAUD 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 sAUD 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 sAUD 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 (21). Interestingly, the caffeine metabolites belonging to the xanthine family such as paraxanthine, theophylline and theobromine that were decreased at baseline in sAUD 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

240 acid and 3-indoleacetic acid increased during alcohol withdrawal to reach the levels of healthy
241 controls. Intriguingly, 3-hydroxyvaleric acid significantly decreased during alcohol withdrawal
242 and was found to be lower than healthy controls at the end of detoxification period.
243 Metabolites that remained significantly higher in sAUD patients at the end of detoxification
244 compared to controls are stress hormone cortisol, palmitoleic acid (FA 16:1), some bile acids,
245 some drugs (diazepam, trazodone), vitB6 metabolite (4-pyridoxic acid, which is likely due to
246 the fact that patients received vitamin B supplement during their hospital stay) and cotinine
247 (nicotine metabolite that reveals the higher proportion of smokers in sAUD patients compared
248 to controls).
249
250 **Identification of blood metabolites with potential neuroactive properties**
251 The metabolites belonging to the xanthine family (theobromine, paraxanthine and theophylline)
252 are metabolites of caffeine produced upon cytochrome P450 dependent oxidation in the liver.
253 They were all decreased in the blood of sAUD patients at baseline and were negatively
254 correlated with alcohol intake, alcohol craving, depression and anxiety. The decrease in caffeine
255 metabolites has previously been described in the urine of AUD patients, that is linked to the
256 increasing severity of alcoholic liver disease (22).
257 Theobromine is the principle alkaloid found in cocoa beans and is responsible for the bitter taste
258 of chocolate. It is known for its mood improving effect (23). Like caffeine, theobromine is an
259 inhibitor of brain adenosine receptors and phosphodiesterase. A study in rats showed that the
260 antagonist of A2a adenosine receptor produced a reduction of ethanol reinforcement (24),
261 suggesting adenosine receptor as a potential target for the treatment of alcohol abuse. In a
262 randomized, double-blind, placebo-controlled trial, the phosphodiesterase inhibitor
263 pentoxifylline associated with escitalopram showed greater reduction of depression scores
264 compared to patients receiving escitalopram alone (25). In another study, Apremilast which is

265 also a phosphodiesterase inhibitor, suppressed excessive alcohol drinking in AUD patients (26).
266 Paraxanthine has a psychostimulant effect and can modulate dopamine release in the striatum
267 (27). Interestingly, in 2017 a systematic review indicated that consumption of coffee, tea and
268 cocoa could have protective effects against depression (28).
269 Lipids, and mostly LPCs (except ether LPC derivatives) and LPEs were significantly and
270 positively correlated with the compulsive component of alcohol craving. LPCs are secreted by
271 the liver and are actively transported via the blood-brain barrier (BBB) and have been associated
272 with pro-inflammatory events (29). LPCs are also precursors of brain lysophosphatidic acid
273 (LPA), which regulates glutamatergic transmission and cortical excitability within the CNS.
274 Recently, LPA has been shown to induce hyperphagia following food restriction and this effect
275 was dependent on hypothalamic agouti-related peptide (AgRP) neurons (30). AgRP neurons
276 have also been implicated in circuitry controlling non-feeding behavior, including those
277 associated with reward, anxiety and compulsive disorders, more particularly in anorexia
278 nervosa (31). Therefore, we hypothesize that the positive correlation between peripheral LPC
279 and compulsion for alcohol drinking found in sAUD patients who have just been deprived of
280 alcohol could be mediated by the effect of LPA on AgRP neurons. Consistent with that,
281 postmortem brain tissues from patients consuming a high intake of alcohol showed increased
282 levels of many LPCs (10).
283 Circulating bile acids can reach the brain by crossing the BBB, either by simple diffusion or
284 active transport. Some bile acids show neuroprotective effects (32) while others are rather
285 neurotoxic (33). In Alzheimer disease patients, the levels of glycochenodeoxycholic acid was
286 associated with worse cognition (34). In our study, both primary (glycochenodeoxycholic acid
287 and glycocholic acid) and secondary (glycohyodeoxycholic acid, tauroursodeoxycholic acid)
288 bile acids were positively correlated with depression and anxiety in sAUD patients.

289 3-Hydroxyvaleric acid, also called β-hydroxypentanoate, was significantly and positively
290 correlated with anxiety and alcohol craving. This metabolite is formed from odd carbon fatty
291 acids in the liver and can reach the brain. 3-hydroxyvaleric acid is a C5-ketone body and is a
292 precursor of propionyl-CoA that refills intermediates of citric acid cycle and is useful for
293 alternative energy fuel in the brain (35,36).
294 Other co-metabolites, i.e. produced by the gut microbiota and then processed by the liver, were
295 negatively correlated with all psychological symptoms. Pyrocatechol sulfate is a phenolic
296 compound derived from the gut microbiota, present in the CSF of mice, and implicated in
297 synapse formation and fear extinction learning (37). In Parkinson disease patients, the plasma
298 level of pyrocatechol sulfate is decreased compared to controls (38). In our study, blood
299 pyrocatechol sulfate was significantly and negatively correlated with all psychological
300 symptoms of sAUD patients (i.e. anxiety, depression and craving) suggesting a neuroprotective
301 role of this metabolite. Interestingly, 4-ethylphenylsulfate, another gut-derived metabolite
302 linked with neurodevelopment abnormalities, autism and anxiety behavior in mice (39–41)
303 showed negative correlations with depression and the compulsive component of alcohol
304 craving.
305 Hippuric acid, the glycine conjugate of benzoic acid has long been associated with the microbial
306 degradation of specific dietary components, including polyphenolic compounds (like
307 chlorogenic acid and catechin) found in fruits, vegetables, coffee and tea (42). Hippuric acid is
308 indeed a host-microbe cometabolite (43). It is synthesized in the liver and in the renal cortex
309 from the microbial metabolite benzoate. The plasma concentration of hippuric acid has been
310 shown to be 17-fold higher in conventional mice compared with their germ-free counterparts
311 suggesting a substantial contribution of the gut microbiota in its production (44). We showed
312 that blood hippurate levels, that correlated negatively with anxiety, depression and craving,
313 were decreased in sAUD patients, as shown in ethanol-treated mice (6) and humans

314 characterized by high alcohol intake and those with major depression (45,46). Urinary hippurate
315 excretion is also decreased in depression, schizophrenia and autism spectrum disorders patients
316 (42). A recent Mendelian randomization study including > 13,000 individuals from five
317 European cohorts characterized for depression suggested that low hippuric acid levels in the
318 circulation is part of the causal pathway leading to depression (47), which was consistent with
319 a significant decrease of the dietary sources of hippuric acid including fresh fruits and
320 vegetables in depressed patients (47).
321 Another way to support the neuroactive effects of the blood metabolites that are correlated with
322 one or several psychological factors is to demonstrate their presence in the brain. We therefore
323 conducted a targeted search in a database of post-mortem frontal cortex and CSF metabolomics
324 analysis (10) and found that 3-hydrovaleric acid, caffeine metabolites (theobromine,
325 paraxanthine and theophylline) and microbial metabolites (hippuric acid and pyrocatechol
326 sulfate) that were correlated with anxiety, depression and alcohol craving in our plasma cohort
327 were also present in the brain and in CSF, and the direction of their changes in the plasma
328 (increase or decrease) mimicked changes in the central nervous system.
329
330 **Advantages and limitations of the study**
331 Most of the studies assessing the impact of alcohol consumption on the blood metabolome were
332 cross-sectional, and only included male participants (45). Here, we reported longitudinal data
333 to assess the impact of a short-term alcohol abstinence on the blood metabolome, both in male
334 and female AUD patients. In the study of Zhu et al (9), the AUD patients recruited were alcohol
335 abstinent, but for various periods of abstinence. To avoid the bias of abstinence duration, our
336 patients were enrolled in a rigorous and standardized manner, within 24 hours after the last
337 drink. Furthermore, alcohol consumption was carefully evaluated with the time line follow back
338 method, which allows precise calculation of the amount (and type) of alcohol consumed (48).

339 This study also presents some limitations. First, the metabolomics analysis was conducted with
340 LC-MS while some important molecules, like lipoproteins, could have been measured with
341 NMR-based methods. Combining NMR and MS-based methods could have covered a wider
342 spectrum of metabolites. However, the non-targeted metabolic profiling with two different
343 chromatographic methods and ionization polarities covers a wide range of metabolites ideal for
344 our discovery-based approach. Large studies are usually required in metabolomics to observe
345 small and medium size changes. Here, we included only s96 AUD patients, but they were all
346 well characterized and received standardized therapies (for instance, vitB supplementation)
347 during alcohol withdrawal.
348 The selection of the control group is always challenging in alcohol research. Here, the healthy
349 subjects were matched for sex, age and BMI but not for smoking status or nutritional intake.
350 Alcohol addiction is a major cause of malnutrition in developed countries and tobacco smoking
351 is more prevalent in alcohol users compared to healthy subjects. These two main confounding
352 factors, although being an integral part of the alcoholic pathology, are known to influence the
353 blood metabolome (49–51). Furthermore, another limitation is that the control group was tested
354 only once, while the sAUD patients were tested twice (T1 and T2). This means that we do not
355 take into consideration the intra-personal variability of the metabolomics data when interpreting
356 the results of alcohol withdrawal effects.
357
358 **Conclusion**
359
360 LC-MS metabolomics plasma analysis allowed for the identification of metabolites that were
361 clearly linked to alcohol consumption, and reflected changes in metabolism, alterations of
362 nutritional status, and gut microbial dysbiosis associated with alcohol intake. In particular the
363 changes in lipid class involving odd-chain fatty acids and ether-bond lipids as well as

364 compounds produced by gut microbiota seem to be the most prominent indicators of metabolic
365 malfunction related to severe alcohol use disorder, and thus warrant further studies and targeted
366 intervention. Also, the discovery of metabolites associated with behavioral and psychiatric traits
367 related to sAUD were of importance, and could be considered potential new therapeutic targets
368 in the management of sAUD, namely as adjuvants in the period of alcohol abstinence. The
369 novelty of our work was to characterize the impact of sAUD on the blood metabolome, and the
370 impact of a short-term alcohol abstinence in the same individuals, within a cohort that included
371 both male and female patients. Intervention studies are needed in order to bring the proof of
372 concept that nutritional approaches – namely the addition of specific lipids, or of nutrients
373 modulating the gut microbiome - for example prebiotic dietary fibers - may be essential and so
374 far underestimated components of alcohol withdrawal efficacy.
375
376 **SUBJECTS AND METHODS**
377
378 **Study design and participants**
379 A total of 96 sAUD patients hospitalized for a 3-week detoxification program in the alcohol
380 withdrawal unit at Cliniques Universitaires Saint-Luc, Brussels, Belgium were recruited. These
381 patients belong to two different cohorts, namely ALCOHOLBIS (patients recruited in 2015 and
382 2019) and GUT2BRAIN (patients recruited in 2018-2019) (Table 1). The severity of AUD was
383 evaluated by a psychiatrist using the Diagnostic and Statistical Manual of Mental Disorders
384 (DSM) criteria, fourth edition (DSM-IV) (ALCOHOLBIS cohort) or fifth edition (DSM-5)
385 (GUT2BRAIN cohort). Patients evaluated with the DSM-IV received the diagnosis of "alcohol
386 dependence", while the patients evaluated with the DSM-5 received the diagnosis of "severe
387 alcohol use disorder" (6 or more criteria). To simplify, we used the term "sAUD" (for severe
388 alcohol use disorder) that includes both diagnosis (sAUD and alcohol dependence)". Patients

389 were eligible if they had been drinking until the day of admission to the detoxification unit or
390 the day before, and if they also did not suffer from inflammatory bowel disease, other chronic
391 inflammatory diseases (such as rheumatoid arthritis) or cancer, nor from metabolic disorders
392 such as obesity (BMI > 30 kg/m2), diabetes and bariatric surgery, or severe cognitive
393 impairment (MMSE < 24). We also excluded subjects who had taken antibiotics, probiotics, or
394 prebiotics in the 2 months prior to enrolment and those who were taking non-steroidal anti-
395 inflammatory drugs or glucocorticoids within 1 month of inclusion. Patients with known
396 cirrhosis or significant liver fibrosis (≥F2) detected by Fibroscan (>7.6 kPa) on the day of
397 admission were also excluded from the study. No other psychiatric diseases, and no other
398 addiction (except tobacco) have been diagnosed in these patients.

399 sAUD patients were tested twice, on the day following their admission (T1) and on days 18–19
400 (T2) corresponding to the last days of the detoxification program. The patients of the
401 GUT2BRAIN cohort were initially enrolled in a randomized, double-blind, placebo-controlled
402 study assessing the impact of prebiotic fiber supplementation on the gut-liver-brain axis (52,53).
403 For this reason, only biological and psychological data obtained at admission (T1), and before
404 the beginning of the prebiotic/placebo treatment, were considered. The patients of the
405 ALCOHOLBIS cohort did not take part in any other clinical study during the 3-week
406 hospitalization stay. Therefore, for this cohort, data obtained at both times of alcohol
407 withdrawal (T1 and T2) were considered. Thirty-two healthy controls (13 from the
408 GUT2BRAIN cohort and 19 from the ALCOHOLBIS cohort) matched for age, gender and BMI
409 with no AUD (Alcohol use disorders test [AUDIT] score <8 in males and <7 in females) were
410 also recruited using flyers posted in Brussel's public setting (Table S1). The inclusion/exclusion
411 criteria were the same as for sAUD patients except for the alcohol related items. Healthy
412 controls and sAUD patients were not matched for smoking status.

413 The study was approved by the "Comité d'éthique Hospitalo-facultaire Saint-Luc UCLouvain"
414 (2017/04JUL/354 and 2014/31dec/614, identification number NCT03803709 at
415 ClinicalTrials.gov). The study has been carried out in accordance with The Code of Ethics of
416 the World Medical Association and followed the ethical guidelines set out in the Declaration of
417 Helsinki. All participants provided written informed consent in compliance with the European
418 law 2001/20/CE guidelines.
419 For investigating presence of the potentially neuroactive metabolites in the CNS, we used
420 metabolomics data from frontal cortex (Broadman area 9) and CSF samples from the Tampere
421 Sudden Death Study (TSDS) cohort, which have been described in detail elsewhere (10). TSDS
422 was collected from forensic autopsies done in the area of the Pirkanmaa Hospital District during
423 2010-2015, a total of 700 subjects. Out of these we identified 97 heavy alcohol users based on
424 autopsy reports and medical records (diagnosis of alcohol-related diseases: ICD-10 codes
425 F10.X, G31.2, G62.1, G72.1, I42.6, K70.0-K70.4, K70.9, and K86.0, or signs of heavy alcohol
426 use in the clinical or laboratory findings, e.g., increased levels of gamma-glutamyl transferase,
427 mean corpuscular volume, carbohydrate-deficient transferrin). Lack of these findings was
428 inclusion criteria for the control group (n = 100), most of whom had died due to cardiovascular
429 diseases (Table S2). Samples were stored at -80 °C until use.
430
431 **Assessment of psychological symptoms**
432 sAUD patients were tested for depression, anxiety and alcohol craving with self-reported
433 questionnaires: the Beck Depression Inventory [BDI] (54), the State-Trait Anxiety Inventory
434 (STAI form YA)(55), and the Obsessive-Compulsive Drinking Scale [OCDS](56) (see
435 supplemental material for details)
436
437 **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 (57) (see supplemental material for methodological details of the LC-MS analysis, peak picking and data processing).

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 (58). 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(57). 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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640

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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.

671

672 **Table 1**. Clinical features of the study participants

	ALCOHOLBIS cohort	GUT2BRAIN cohort	All	p-value
Number of subjects	48	48	96	
Age	46 ± 10	48 ± 9	47 ± 10	0.24
Gender				
Men, n (%)	34 (71%)	30 (62.5%)	64 (67%)	0.39$
Women, n (%)	14 (29%)	18 (37.5%)	32 (33%)	
Smoking status				
Active smoker (%)	77	79	78	0.83$
Non-smoker (%)	23	21	22	
Alcohol history				
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		
Depression				
Score at T1	23 ± 11	26 ± 12	25 ± 12	0.26
Anxiety				
Score at T1	44 ± 11	46 ± 15	45 ± 13	0.27
Alcohol craving				
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*

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

675

Figure legends

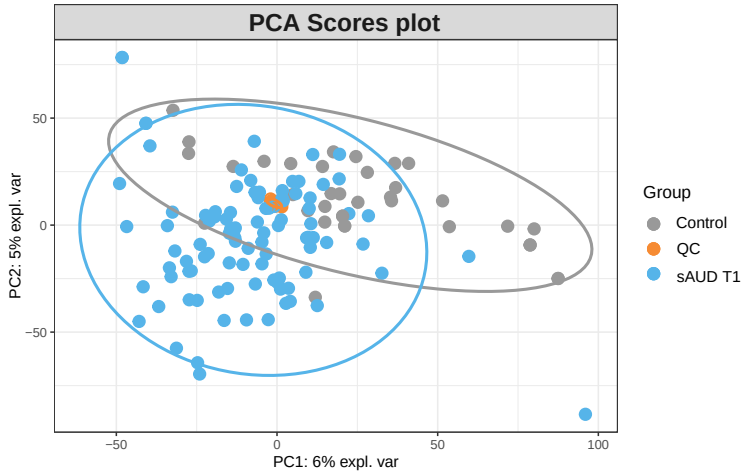
Figure 1. Chronic alcohol intake alters the plasma metabolome compared to healthy controls. **a** Principal component analysis score plot of the plasma metabolomic features between healthy controls and persons with sAUD at T1. QC samples are colored in orange. **b** Volcano plot depicting the effect 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 sAUD at T1. Circle size represent the variable importance in projections (VIP) scores derived from the sPLS-DA model for the plasma metabolomic features between persons with sAUD at T1 and healthy controls. **c** 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; FA fatty acid; LPC lysophosphatidylcholine; LPE lysophosphatidylethanolamine; PC phosphatidylcholine; PE phosphatidylethanolamine; SM sphingomyelin.

Figure 2. 3-week alcohol withdrawal shapes the plasma metabolome. **a** Scores plot of the sparse partial least square discriminant analysis (sPLS-DA) of the plasma metabolomes at the start (T1) and end (T2) of the alcohol withdrawal in persons with sAUD. **b** Volcano plot depicting the effect size (Cohen's D) and -Log_{10} transformed *q* values derived from paired *t*-test analysis of the metabolomic features different between in persons with sAUD at T1 and T2. Circle size represent the variable importance in projections (VIP) scores for the plasma metabolomic features in the sPLS-DA model of persons with sAUD at T1 and T2. **c** Lollipop plot of the effect size (Cohen's D) and -Log_{10} transformed *q* values of the altered annotated metabolites between sAUD T1 and healthy controls as well as sAUD T1 and sAUD T2. Circle size refers to the level of significance, grey color to the comparison between controls and sAUD T1, orange color to relative increase while violet to relative decrease towards the end of alcohol withdrawal (T2). AC acylcarnitine; FA fatty acid; LPC lysophosphatidylcholine; LPE lysophosphatidylethanolamine; PC phosphatidylcholine.

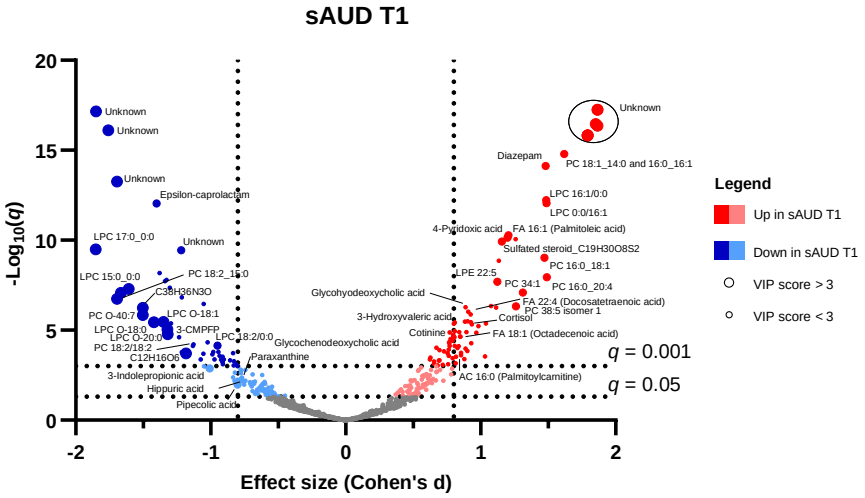
Figure 3. Plasma metabolites associated with psychological symptom scores. Heatmap of the annotated metabolites having significant ($p<0.05$) Spearman correlation with one or more psychological symptom score of obsession, compulsion, alcohol craving, anxiety state or depression. Circle size refers to 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 ± SD with individual values shown. Statistical values derived from Welch's *t*-test comparing metabolomic features between control and alcohol groups.

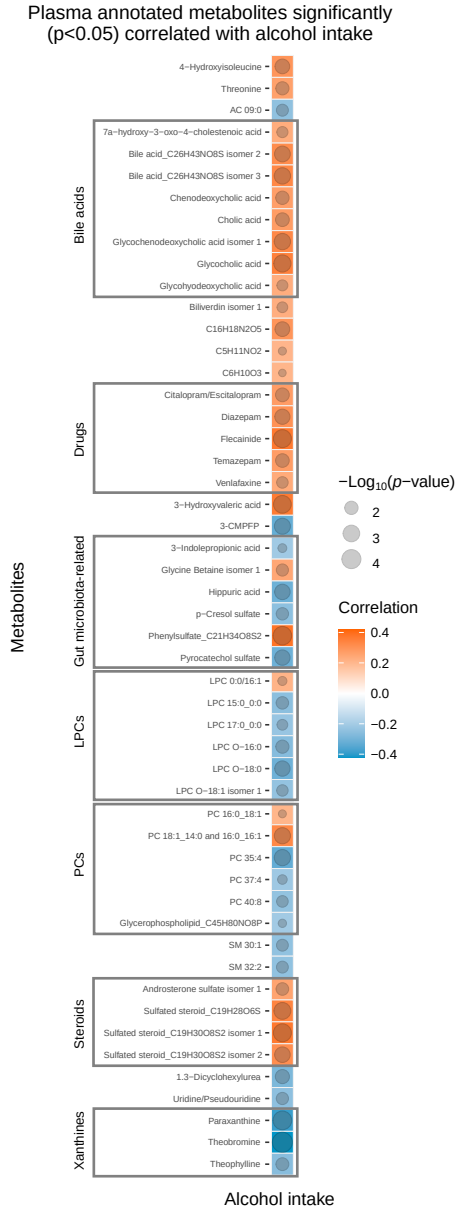
a

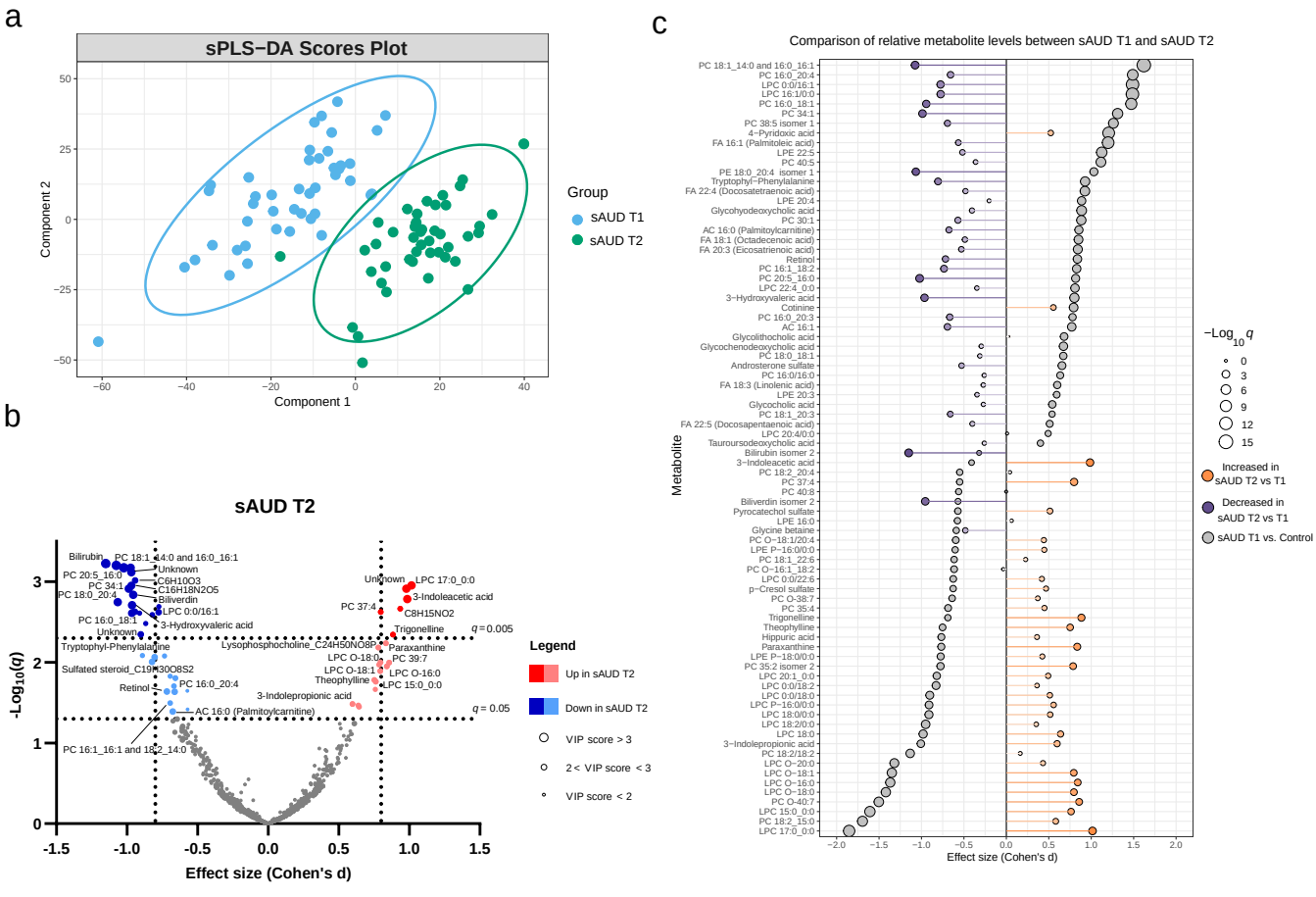

PCA Scores plot
PC2: 5% expl. var
PC1: 6% expl. var
50
0
−50
−50
0
50
100
Group
Control
QC
sAUD T1

b

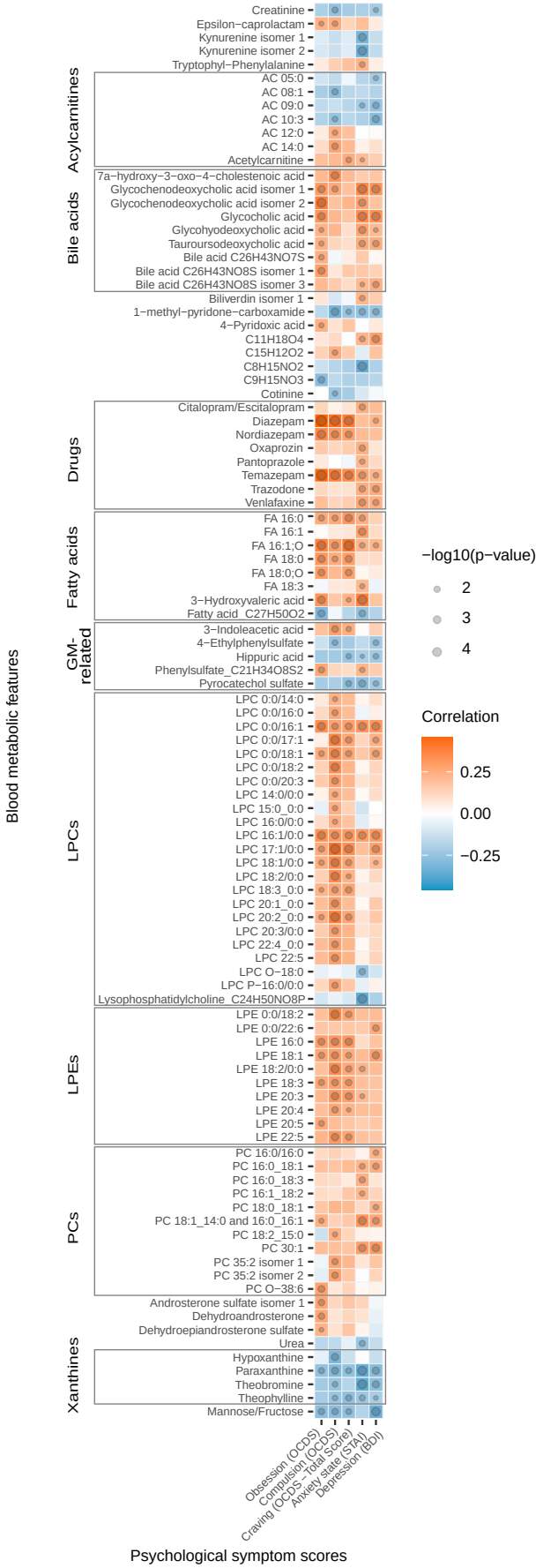

sAUD T1
-Log10(q)
Effect size (Cohen's d)
20
15
10
5
0
-2
-1
0
1
2
Unknown
Unknown
Unknown
Epsilon-caprolactam
LPC 17:0_0:0
Unknown
LPC 15:0_0:0
PC 18:2_15:0
C38H36N3O
PC O-40:7
LPC O-18:1
LPC O-18:0
3-CMPFP
LPC O-20:0
LPC 18:2/0:0
PC 18:2/18:2
C12H16O6
Glycochenodeoxycholic acid
Paraxanthine
3-Indolepropionic acid
Hippuric acid
Pipecolic acid
Unknown
Diazepam
PC 18:1_14:0 and 16:0_16:1
LPC 16:1/0:0
LPC 0:0/16:1
4-Pyridoxic acid
FA 16:1 (Palmitoleic acid)
Sulfated steroid_C19H30O8S2
PC 16:0_18:1
LPE 22:5
PC 34:1
PC 16:0_20:4
Glycohyodeoxycholic acid
FA 22:4 (Docosatetraenoic acid)
PC 38:5 isomer 1
3-Hydroxyvaleric acid
Cortisol
Cotinine
FA 18:1 (Octadecenoic acid)
AC 16:0 (Palmitoylcarnitine)
q = 0.001
q = 0.05
Legend
Up in sAUD T1
Down in sAUD T1
VIP score > 3
VIP score < 3

c

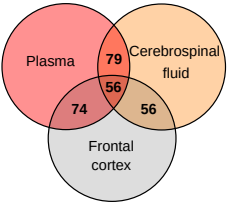

Plasma annotated metabolites significantly
(p<0.05) correlated with alcohol intake
Metabolites
Bile acids
4−Hydroxyisoleucine
Threonine
AC 09:0
7a−hydroxy−3−oxo−4−cholestenoic acid
Bile acid_C26H43NO8S isomer 2
Bile acid_C26H43NO8S isomer 3
Chenodeoxycholic acid
Cholic acid
Glycochenodeoxycholic acid isomer 1
Glycocholic acid
Glycohyodeoxycholic acid
Biliverdin isomer 1
C16H18N2O5
C5H11NO2
C6H10O3
Drugs
Citalopram/Escitalopram
Diazepam
Flecainide
Temazepam
Venlafaxine
Gut microbiota-related
3−Hydroxyvaleric acid
3-CMPFP
3−Indolepropionic acid
Glycine Betaine isomer 1
Hippuric acid
p−Cresol sulfate
Phenylsulfate_C21H34O8S2
Pyrocatechol sulfate
LPCs
LPC 0:0/16:1
LPC 15:0_0:0
LPC 17:0_0:0
LPC O−16:0
LPC O−18:0
LPC O−18:1 isomer 1
PCs
PC 16:0_18:1
PC 18:1_14:0 and 16:0_16:1
PC 35:4
PC 37:4
PC 40:8
Glycerophospholipid_C45H80NO8P
SM 30:1
SM 32:2
Steroids
Androsterone sulfate isomer 1
Sulfated steroid_C19H28O6S
Sulfated steroid_C19H30O8S2 isomer 1
Sulfated steroid_C19H30O8S2 isomer 2
1.3−Dicyclohexylurea
Uridine/Pseudouridine
Xanthines
Paraxanthine
Theobromine
Theophylline
Alcohol intake
−Log10(p−value)
2
3
4
Correlation
0.4
0.2
0.0
−0.2
−0.4


a
sPLS−DA Scores Plot
Component 2
Component 1
Group
sAUD T1
sAUD T2
b
sAUD T2
-Log10(q)
Effect size (Cohen's d)
Bilirubin
PC 18:1_14:0 and 16:0_16:1
Unknown
PC 20:5_16:0
PC 34:1
C6H10O3
C16H18N2O5
PC 18:0_20:4
Biliverdin
LPC 0:0/16:1
PC 16:0_18:1
3-Hydroxyvaleric acid
Unknown
Tryptophyl-Phenylalanine
Lysophosphocholine_C24H50NO8P
Sulfated steroid_C19H30O8S2
Retinol
PC 16:0_20:4
AC 16:0 (Palmitoylcarnitine)
PC 16:1_16:1 and 18:2_14:0
Unknown
LPC 17:0_0:0
3-Indoleacetic acid
PC 37:4
C8H15NO2
Trigonelline
Paraxanthine
LPC O-18:0
PC 39:7
LPC O-18:1
LPC O-16:0
Theophylline
LPC 15:0_0:0
3-Indolepropionic acid
q = 0.005
q = 0.05
Legend
Up in sAUD T2
Down in sAUD T2
VIP score > 3
2 < VIP score < 3
VIP score < 2
c
Comparison of relative metabolite levels between sAUD T1 and sAUD T2
Metabolite
Effect size (Cohen's d)
−Log10 q
Increased in sAUD T2 vs T1
Decreased in sAUD T2 vs T1
sAUD T1 vs. Control

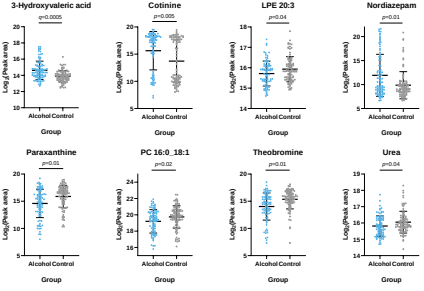
Plasma annotated metabolites significantly (p<0.05) correlated with psychological symptom scores



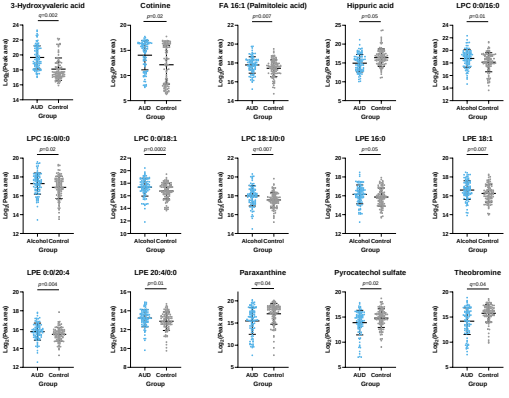
a


Plasma
Cerebrospinal fluid
Frontal cortex
79
56
74
56

b


3-Hydroxyvaleric acid
q=0.0005
Cotinine
p=0.005
LPE 20:3
p=0.04
Nordiazepam
p=0.01
Paraxanthine
p=0.01
PC 16:0_18:1
p=0.02
Theobromine
p=0.01
Urea
p=0.04
Log2(Peak area)
Alcohol Control
Group

c


3-Hydroxyvaleric acid
q=0.002
Cotinine
p=0.02
FA 16:1 (Palmitoleic acid)
p=0.007
Hippuric acid
p=0.05
LPC 0:0/16:0
p=0.03
LPC 16:0/0:0
p=0.02
LPC 0:0/18:1
q=0.0002
LPC 18:1/0:0
q=0.007
LPE 16:0
p=0.05
LPE 18:1
p=0.007
LPE 0:0/20:4
p=0.004
LPE 20:4/0:0
p=0.01
Paraxanthine
p=0.04
Pyrocatechol sulfate
p=0.02
Theobromine
q=0.04
Log2(Peak area)
AUD Control
Group