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 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 alcohol addiction 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 AUD patients, including analysis of central nervous system (CNS) tissues(10). Furthermore,
74 the methodology could differ across studies, some using nuclear magnetic resonance (NMR)
75 while others using more sensitive mass spectrometry (MS) coupled with liquid or gas
76 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 severe AUD on the
81 blood 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 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

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

143 metabolites belonging to the class of bilirubins, the levels of identified metabolites that were
144 significantly changed upon alcohol withdrawal (the major ones being indoles, acylcarnitines,
145 glycerophospholipids and xanthines) came back towards to the levels observed in controls
146 (Table S3). In the course of alcohol abstinence, we noted a significant decrease in 16-chain
147 acylcarnitines, LPCs with 16- or 18-chain fatty acid tails excluding LPCs with ether bonds,
148 retinol, tryptophyl-phenylalanine dipeptide and 3-hydroxyvaleric acid (Fig. 2c). On the
149 contrary, LPCs with odd-chain fatty acid tails or ether bonds show a significant increase along
150 with tryptophan derivatives 3-indoleacetic acid and 3-indolepropanoic acid and metabolites of
151 the xanthine family such as theophylline, paraxanthine and trigonelline during alcohol
152 abstinence. Moreover, Fig. 2c demonstrates that alcohol withdrawal shifted the AUD plasma
153 metabolic fingerprint towards healthy controls.
154
155 **Correlations between blood metabolites and psychological symptoms**
156 Correlation analysis shows that, at T1, 96 annotated features were significantly ($p<0.05$)
157 correlated with psychological scores of anxiety, depression and alcohol craving (with sub-
158 scores of obsession and compulsion) (Fig 3). Annotated bile acids, drugs,
159 lysophosphoethanolamines (LPE), fatty acids, LPCs apart from LPCs with an ether-bond or
160 15:0 or 17:0 fatty acid tail were consistently positively correlated with psychological symptoms,
161 and more particularly with the compulsive component of alcohol craving. Members of the
162 xanthine family, pyrocatechol sulfate, a pentose sugar (mannose/fructose), hippuric acid, 1-
163 methyl-pyridone-carboxamide, acylcarnitines with maximum of 10 carbons, creatinine and
164 kynurenine were negatively correlated with psychological symptoms. Within the acylcarnitine
165 metabolite class, an interesting pattern was observed, as the shorter chain-length acylcarnitines
166 were consistently negatively correlated with the psychological parameters, and the long-chain
167 ones demonstrated positive correlation.

168

169 **Alterations in the brain metabolome of AUD patients**

170 Based on the annotated significantly altered plasma metabolites, we conducted a targeted search
171 in a metabolomics dataset consisting of CSF and frontal cortex samples collected from deceased
172 individuals with a history of heavy alcohol use and control individuals. 79 and 74 of the
173 annotated plasma metabolites were identified from the CSF and frontal cortex, respectively
174 (Fig. 4a, Table S3). We looked specifically at metabolites significantly correlated with at least
175 one psychological symptom. 3-Hydroxyvaleric acid, cotinine, theobromine and paraxanthine
176 were indeed present in the CNS and significantly (Welch *t*-test $p<0.05$) different between heavy
177 alcohol use and control groups in both frontal cortex (Fig 4b) and CSF (Fig 4c). Additional
178 significantly altered metabolites found only in the frontal cortex were LPE 20:3, nordiazepam,
179 PC 16:0_18:1 and urea (Fig. 4b). In the CSF, the independent differential metabolites were FA
180 16:1 (palmitoleic acid), hippuric acid, LPCs 16:0 and 18:1, LPEs 16:0, 18:1 and 20:4 and
181 pyrocatechol sulfate (Fig. 4c).

182

183

184 **DISCUSSION**

185

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

192

193 **Impact of chronic alcohol abuse on the blood metabolome**

194 In 2019, a review summarized the results obtained from 23 studies that have used a
195 metabolomics approach for measuring changes in metabolite profiles in relation to alcohol use
196 (1). Changes in lipids have been highlighted as the most consistent changes across studies.
197 Lipids are an integral part of cell membranes and signaling molecules in the body. PCs and
198 LPCs have been suggested to form a new class of biomarkers for alcohol consumption (2). For
199 instance, in our study palmitoleic acid (FA 16:1) was largely increased in AUD patients
200 compared to controls, and in other studies, this metabolite has likewise been significantly
201 associated with alcohol consumption (17,18). Another clear observation in our study was the
202 lower level of odd-chain lipids in AUD patients. Since the lipids containing FA 15:0 and FA
203 17:0 have been suggested to be products from bacterial metabolism (19,20), the existence of
204 gut dysbiosis in AUD patients could explain the lower abundance of LPC 17:0 and LPC 15:0
205 (14).

206 We found that some bile acids, sulphated steroids and 3-hydroxyvaleric acid were positively
207 associated with the amount of alcohol consumed. Sulphated steroids and hydroxyvalerate have
208 previously been associated with alcohol intake (21,22). Metabolites belonging to the xanthine

209 family (theobromine, theophylline, paraxanthine) and microbial metabolites (hippuric acid,
210 indole-3-propionic acid, p-cresol sulfate, pyrocatechol sulfate) were negatively correlated with
211 alcohol consumption. Altogether, these results suggest that these metabolites are sensitive to
212 alcohol exposure. Interestingly, these metabolites were also correlated with the severity of the
213 psychological symptoms suggesting that they could play a role in the symptomatology of
214 alcohol addiction.
215
216 **Effect of short-term alcohol abstinence on the blood metabolome**
217 Since alcohol consumption is known to influence lipid metabolism, it was expected that a short-
218 term alcohol abstinence could reverse or ameliorate lipidomic alterations. Indeed, we found that
219 some phospholipids that were increased in AUD patients at baseline, such as PC 16:0_18:1,
220 PC18:1_14:0 and 16:0_16:1 as well as LPC 16:1, were downregulated during alcohol
221 withdrawal to reach the levels of healthy controls after detox. On the other hand, LPC 15:0 and
222 LPC 17:0 that were decreased in AUD patients at baseline, increased during alcohol
223 withdrawal, but did not reach the levels of controls at the end of detox.
224 The metabolite that contributed the most to the differences observed with alcohol detoxification
225 was bilirubin. While bilirubin was not statistically higher in AUD patients at baseline vs
226 controls, we observed a significant reduction of this metabolite after a 3-week alcohol
227 withdrawal. In a previous study, serum bilirubin was found to be associated with alcohol
228 consumption, cigarette smoking and coffee consumption (23). Interestingly, the caffeine
229 metabolites belonging to the xanthine family such as paraxanthine, theophylline and
230 theobromine that were decreased at baseline in AUD patients compared to controls, increased
231 significantly during alcohol withdrawal to reach the levels of healthy controls. Changes in
232 dietary intake of coffee, tea and chocolate during alcohol withdrawal could explain these results
233 (data not shown). Also, the bacterial metabolites indole derivatives such as 3-indolepropionic

234 acid and 3-indoleacetic acid increased during alcohol withdrawal to reach the levels of healthy
235 controls. Intriguingly, 3-hydroxyvaleric acid significantly decreased during alcohol withdrawal
236 and was found to be lower than healthy controls at the end of detoxification period.
237 Metabolites that remained significantly higher in AUD patients at the end of detoxification
238 compared to controls are stress hormone cortisol, palmitoleic acid (FA 16:1), some bile acids,
239 some drugs (diazepam, trazodone), vitB6 metabolite (4-pyridoxic acid, which is likely due to
240 the fact that patients received vitamin B supplement during their hospital stay) and cotinine
241 (nicotine metabolite that reveals the higher proportion of smokers in AUD patients compared
242 to controls).
243
244 **Identification of blood metabolites with potential neuroactive properties**
245 The metabolites belonging to the xanthine family (theobromine, paraxanthine and theophylline)
246 are metabolites of caffeine produced upon cytochrome P450 dependent oxidation in the liver.
247 They were all decreased in the blood of AUD patients at baseline and were negatively correlated
248 with alcohol intake, alcohol craving, depression and anxiety. The decrease in caffeine
249 metabolites has previously been described in the urine of AUD patients, that is linked to the
250 increasing severity of alcoholic liver disease (24).
251 Theobromine is the principle alkaloid found in cocoa beans and is responsible for the bitter taste
252 of chocolate. It is known for its mood improving effect (25). Like caffeine, theobromine is an
253 inhibitor of brain adenosine receptors and phosphodiesterase. A study in rats showed that the
254 antagonist of A2a adenosine receptor produced a reduction of ethanol reinforcement (26),
255 suggesting adenosine receptor as a potential target for the treatment of alcohol abuse. In a
256 randomized, double-blind, placebo-controlled trial, the phosphodiesterase inhibitor
257 pentoxifylline associated with escitalopram showed greater reduction of depression scores
258 compared to patients receiving escitalopram alone (27). In another study, Apremilast which is

259 also a phosphodiesterase inhibitor, suppressed excessive alcohol drinking in AUD patients (28).
260 Paraxanthine has a psychostimulant effect and can modulate dopamine release in the striatum
261 (29). Interestingly, in 2017 a systematic review indicated that consumption of coffee, tea and
262 cocoa could have protective effects against depression (30).
263 Lipids, and mostly LPCs (except ether LPC derivatives) and LPEs were significantly and
264 positively correlated with the compulsive component of alcohol craving. LPCs are secreted by
265 the liver and are actively transported via the blood-brain barrier (BBB) and have been associated
266 with pro-inflammatory events (31). LPCs are also precursors of brain lysophosphatidic acid
267 (LPA), which regulates glutamatergic transmission and cortical excitability within the CNS.
268 Recently, LPA has been shown to induce hyperphagia following food restriction and this effect
269 was dependent on hypothalamic agouti-related peptide (AgRP) neurons (32). AgRP neurons
270 have also been implicated in circuitry controlling non-feeding behavior, including those
271 associated with reward, anxiety and compulsive disorders, more particularly in anorexia
272 nervosa (33). Therefore, we hypothesize that the positive correlation between peripheral LPC
273 and compulsion for alcohol drinking found in AUD patients who have just been deprived of
274 alcohol could be mediated by the effect of LPA on AgRP neurons. Consistent with that,
275 postmortem brain tissues from patients consuming a high intake of alcohol showed increased
276 levels of many LPCs (10).
277 Circulating bile acids can reach the brain by crossing the BBB, either by simple diffusion or
278 active transport. Some bile acids show neuroprotective effects (34) while others are rather
279 neurotoxic (35). In Alzheimer disease patients, the levels of glycochenodeoxycholic acid was
280 associated with worse cognition (36). In our study, both primary (glycochenodeoxycholic acid
281 and glycocholic acid) and secondary (glycohyodeoxycholic acid, tauroursodeoxycholic acid)
282 bile acids were positively correlated with depression and anxiety in AUD patients.

283 3-Hydroxyvaleric acid, also called β-hydroxypentanoate, was significantly and positively
284 correlated with anxiety and alcohol craving. This metabolite is formed from odd carbon fatty
285 acids in the liver and can reach the brain. 3-hydroxyvaleric acid is a C5-ketone body and is a
286 precursor of propionyl-CoA that refills intermediates of citric acid cycle and is useful for
287 alternative energy fuel in the brain (37,38).
288 Other co-metabolites, i.e. produced by the gut microbiota and then processed by the liver, were
289 negatively correlated with all psychological symptoms. Pyrocatechol sulfate is a phenolic
290 compound derived from the gut microbiota, present in the CSF of mice, and implicated in
291 synapse formation and fear extinction learning (39). In Parkinson disease patients, the plasma
292 level of pyrocatechol sulfate is decreased compared to controls (40). In our study, blood
293 pyrocatechol sulfate was significantly and negatively correlated with all psychological
294 symptoms of AUD patients (i.e. anxiety, depression and craving) suggesting a neuroprotective
295 role of this metabolite. Interestingly, 4-ethylphenylsulfate, another gut-derived metabolite
296 linked with neurodevelopment abnormalities, autism and anxiety behavior in mice (41–43)
297 showed negative correlations with depression and the compulsive component of alcohol
298 craving.
299 Hippuric acid, the glycine conjugate of benzoic acid has long been associated with the microbial
300 degradation of specific dietary components, including polyphenolic compounds (like
301 chlorogenic acid and catechin) found in fruits, vegetables, coffee and tea (44). Hippuric acid is
302 indeed a host-microbe cometabolite (45). It is synthesized in the liver and in the renal cortex
303 from the microbial metabolite benzoate. The plasma concentration of hippuric acid has been
304 shown to be 17-fold higher in conventional mice compared with their germ-free counterparts
305 suggesting a substantial contribution of the gut microbiota in its production (46). We showed
306 that blood hippurate levels, that correlated negatively with anxiety, depression and craving,
307 were decreased in AUD patients, as shown in ethanol-treated mice (6) and humans

308 characterized by high alcohol intake and those with major depression (47,48). Urinary hippurate
309 excretion is also decreased in depression, schizophrenia and autism spectrum disorders patients
310 (44). A recent Mendelian randomization study including > 13,000 individuals from five
311 European cohorts characterized for depression suggested that low hippuric acid levels in the
312 circulation is part of the causal pathway leading to depression (49), which was consistent with
313 a significant decrease of the dietary sources of hippuric acid including fresh fruits and
314 vegetables in depressed patients (49).
315 Another way to support the neuroactive effects of the blood metabolites that are correlated with
316 one or several psychological factors is to demonstrate their presence in the brain. We therefore
317 conducted a targeted search in a database of post-mortem frontal cortex and CSF metabolomics
318 analysis (10) and found that 3-hydrovaleric acid, caffeine metabolites (theobromine,
319 paraxanthine and theophylline) and microbial metabolites (hippuric acid and pyrocatechol
320 sulfate) that were correlated with anxiety, depression and alcohol craving in our plasma cohort
321 were also present in the brain and in CSF, and the direction of their changes in the plasma
322 (increase or decrease) mimicked changes in the central nervous system.
323
324 **Advantages and limitations of the study**
325 Most of the studies assessing the impact of alcohol consumption on the blood metabolome were
326 cross-sectional, and only included male participants (47). Here, we reported longitudinal data
327 to assess the impact of a short-term alcohol abstinence on the blood metabolome, both in male
328 and female AUD patients. In the study of Zhu et al (9), the AUD patients recruited were alcohol
329 abstinent, but for various periods of abstinence. To avoid the bias of abstinence duration, our
330 patients were enrolled in a rigorous and standardized manner, within 24 hours after the last
331 drink. Furthermore, alcohol consumption was carefully evaluated with the time line follow back
332 method, which allows precise calculation of the amount (and type) of alcohol consumed (50).

333 This study also presents some limitations. First, the metabolomics analysis was conducted with
334 LC-MS while some important molecules, like lipoproteins, could have been measured with
335 NMR-based methods. Combining NMR and MS-based methods could have covered a wider
336 spectrum of metabolites. However, the non-targeted metabolic profiling with two different
337 chromatographic methods and ionization polarities covers a wide range of metabolites ideal for
338 our discovery-based approach. Large studies are usually required in metabolomics to observe
339 small and medium size changes. Here, we included only 96 AUD patients, but they were all
340 well characterized and received standardized therapies (for instance, vitB supplementation)
341 during alcohol withdrawal.
342 The selection of the control group is always challenging in alcohol research. Here, the healthy
343 subjects were matched for sex, age and BMI but not for smoking status or nutritional intake.
344 Alcohol addiction is a major cause of malnutrition in developed countries and tobacco smoking
345 is more prevalent in alcohol users compared to healthy subjects. These two main confounding
346 factors, although being an integral part of the alcoholic pathology, are known to influence the
347 blood metabolome (51–53).
348
349 **Conclusion**
350
351 LC-MS metabolomics plasma analysis allowed for the identification of metabolites that were
352 clearly linked to alcohol consumption, and reflected changes in metabolism, alterations of
353 nutritional status, and gut microbial dysbiosis associated with alcohol intake. In particular the
354 changes in lipid class involving odd-chain fatty acids and ether-bond lipids as well as
355 compounds produced by gut microbiota seem to be the most prominent indicators of metabolic
356 malfunction related to alcohol use disorder, and thus warrant further studies and targeted
357 intervention. Also, the discovery of metabolites associated with behavioral and psychiatric traits

358 related to AUD were of importance, and could be considered potential new therapeutic targets
359 in the management of AUD, namely as adjuvants in the period of alcohol abstinence. The
360 novelty of our work was to characterize the impact of chronic alcohol abuse on the blood
361 metabolome, and the impact of a short-term alcohol abstinence in the same individuals, within
362 a cohort that included both male and female patients. Intervention studies are needed in order
363 to bring the proof of concept that nutritional interventions – namely the addition of specific
364 lipids, or of nutrients modulating the gut microbiome - for example prebiotic dietary fibers -
365 may be essential and so far underestimated components of alcohol withdrawal efficacy.
366
367 **SUBJECTS AND METHODS**
368
369 **Study design and participants**
370 A total of 96 AUD patients hospitalized for a 3-week detoxification program in the alcohol
371 withdrawal unit at Cliniques Universitaires Saint-Luc, Brussels, Belgium were recruited. These
372 patients belong to two different cohorts, namely ALCOHOLBIS (patients recruited in 2015 and
373 2019) and GUT2BRAIN (patients recruited in 2018-2019) (Table 1). The severity of AUD was
374 evaluated by a psychiatrist using the Diagnostic and Statistical Manual of Mental Disorders
375 (DSM) criteria, fourth edition (DSM-IV) (ALCOHOLBIS cohort) or fifth edition (DSM-5)
376 (GUT2BRAIN cohort). Patients were eligible if they had been drinking until the day of
377 admission to the detoxification unit or the day before, and if they also did not suffer from
378 inflammatory bowel disease, other chronic inflammatory diseases (such as rheumatoid arthritis)
379 or cancer, nor from metabolic disorders such as obesity (BMI > 30 kg/m2), diabetes and
380 bariatric surgery, or severe cognitive impairment (MMSE < 24). We also excluded subjects who
381 had taken antibiotics, probiotics, or prebiotics in the 2 months prior to enrolment and those who
382 were taking non-steroidal anti-inflammatory drugs or glucocorticoids within 1 month of

383 inclusion. Patients with known cirrhosis or significant liver fibrosis (≥F2) detected by Fibroscan
384 (>7.6 kPa) on the day of admission were also excluded from the study. No other psychiatric
385 diseases, and no other addiction (except tobacco) have been diagnosed in these patients.
386 AUD patients were tested twice, on the day following their admission (T1) and on days 18–19
387 (T2) corresponding to the last days of the detoxification program. The patients of the
388 GUT2BRAIN cohort were initially enrolled in a randomized, double-blind, placebo-controlled
389 study assessing the impact of prebiotic fiber supplementation on the gut-liver-brain axis (54,55).
390 For this reason, only biological and psychological data obtained at admission (T1), and before
391 the beginning of the prebiotic/placebo treatment, were considered. The patients of the
392 ALCOHOLBIS cohort did not take part in any other clinical study during the 3-week
393 hospitalization stay. Therefore, for this cohort, data obtained at both times of alcohol
394 withdrawal (T1 and T2) were considered. Thirty-two healthy controls (13 from the
395 GUT2BRAIN cohort and 19 from the ALCOHOLBIS cohort) matched for age, gender and BMI
396 with no AUD (Alcohol use disorders test [AUDIT] score <8 in males and <7 in females) were
397 also recruited using flyers posted in Brussel's public setting (Table S1). The inclusion/exclusion
398 criteria were the same as for AUD patients except for the alcohol related items. Healthy controls
399 and AUD patients were not matched for smoking status.
400 The study was approved by the "Comité d'éthique Hospitalo-facultaire Saint-Luc UCLouvain"
401 (2017/04JUL/354 and 2014/31dec/614, identification number NCT03803709 at
402 ClinicalTrials.gov). The study has been carried out in accordance with The Code of Ethics of
403 the World Medical Association and followed the ethical guidelines set out in the Declaration of
404 Helsinki. All participants provided written informed consent in compliance with the European
405 law 2001/20/CE guidelines.
406 For investigating presence of the potentially neuroactive metabolites in the CNS, we used
407 metabolomics data from frontal cortex (Broadman area 9) and CSF samples from the Tampere

408 Sudden Death Study (TSDS) cohort, which have been described in detail elsewhere (10). TSDS
409 was collected from forensic autopsies done in the area of the Pirkanmaa Hospital District during
410 2010-2015, a total of 700 subjects. Out of these we identified 97 heavy alcohol users based on
411 autopsy reports and medical records (diagnosis of alcohol-related diseases: ICD-10 codes
412 F10.X, G31.2, G62.1, G72.1, I42.6, K70.0-K70.4, K70.9, and K86.0, or signs of heavy alcohol
413 use in the clinical or laboratory findings, e.g., increased levels of gamma-glutamyl transferase,
414 mean corpuscular volume, carbohydrate-deficient transferrin). Lack of these findings was
415 inclusion criteria for the control group (n = 100), most of whom had died due to cardiovascular
416 diseases (Table S2). Samples were stored at -80 °C until use.
417
418 **Assessment of psychological symptoms**
419 AUD patients were tested for depression, anxiety and alcohol craving with self-reported
420 questionnaires: the Beck Depression Inventory [BDI] (56), the State-Trait Anxiety Inventory
421 (STAI form YA)(57), and the Obsessive-Compulsive Drinking Scale [OCDS](58) (see
422 supplemental material for details)
423
424 **Biological sampling**
425 To avoid variation due to fasting state and circadian rhythm, blood samples were collected in
426 all participants in the morning between 8:00 and 8:30 am after an overnight fasting, at T1 and
427 T2. Blood was drawn in tubes containing EDTA as an anticoagulant. The samples were centrifuged
428 at 1000 g for 15 min at 4°C and the plasma was frozen at −80°C until analysis.
429
430 **Nontargeted metabolomics analysis**
431 Plasma sample preparation and LC-MS measurement were performed as previously detailed
432 in Klåvus et al (59) (see supplemental material for details).

433

434 **Statistical analysis**

435 R software version 4.0.3. was used for statistical analyses. Multivariate analyses, namely PCA
436 for dimension reduction and sPLS-DA for group discrimination, were conducted by
437 'mixOmics' R package v. 6.14.1 (60). For the sPLS-DA model we used a cross-validation (CV)
438 procedure of 10-fold CV repeated 50 times. Univariate analyses were conducted by 'notame'
439 R package v. 0.2.1(59). Significant features were shortlisted using Welch's and paired t-tests.
440 All *p*-values were corrected using the Benjamini-Hochberg false discovery rate (FDR) to
441 calculate the *q*-value. For all tests, *p* and *q* values < 0.05 were considered statistically
442 significant. Visualizations were created by the previously mentioned R packages and GraphPad
443 Prism v. 8.4.2. Correlation analyses were performed at T1 using R software version 3.6.1.
444 Spearman coefficient was calculated and p-value < 0.05 was considered statistically significant.

445

446

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632
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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.

663

664 **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*

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

667

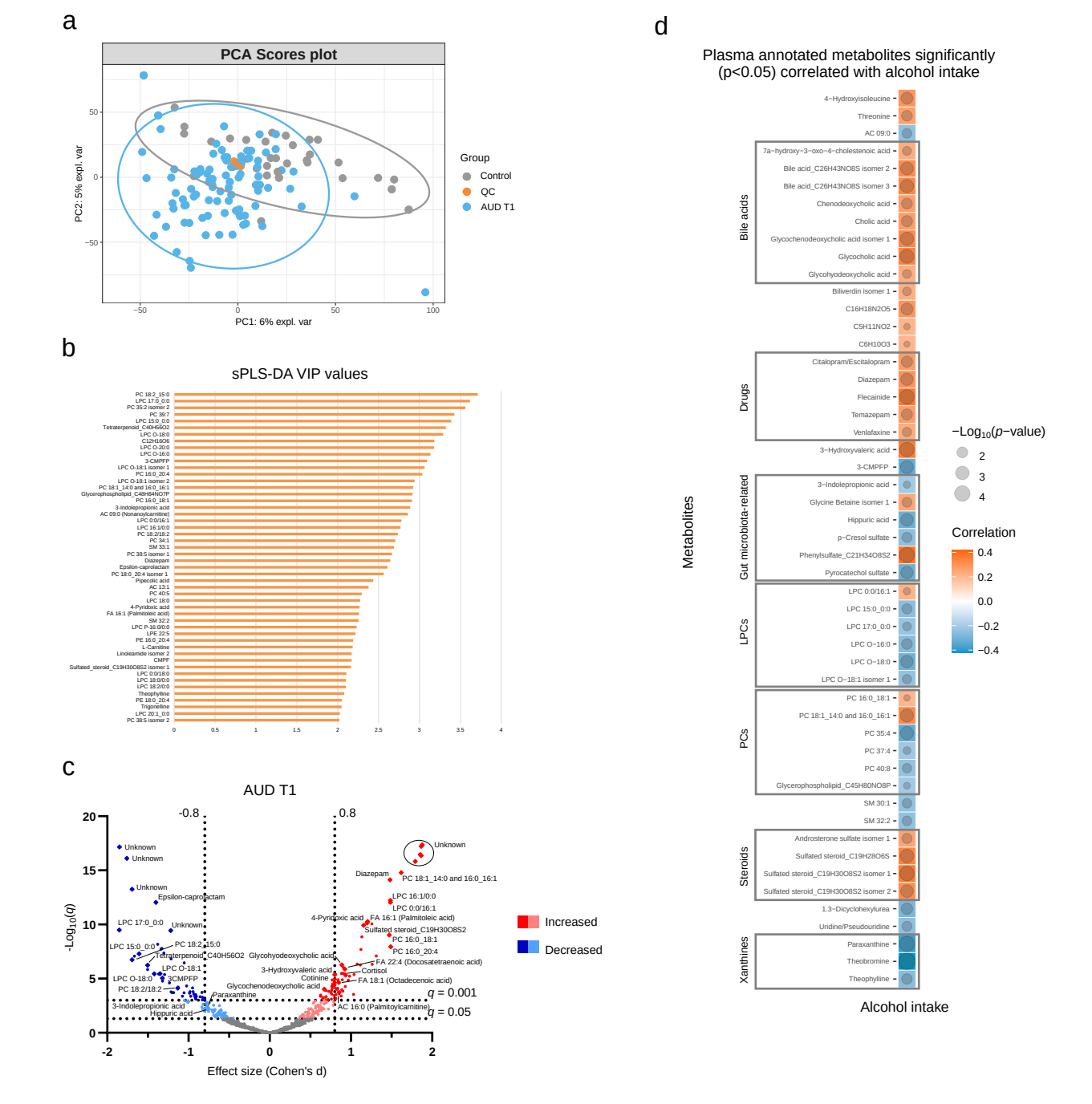
Figure legends

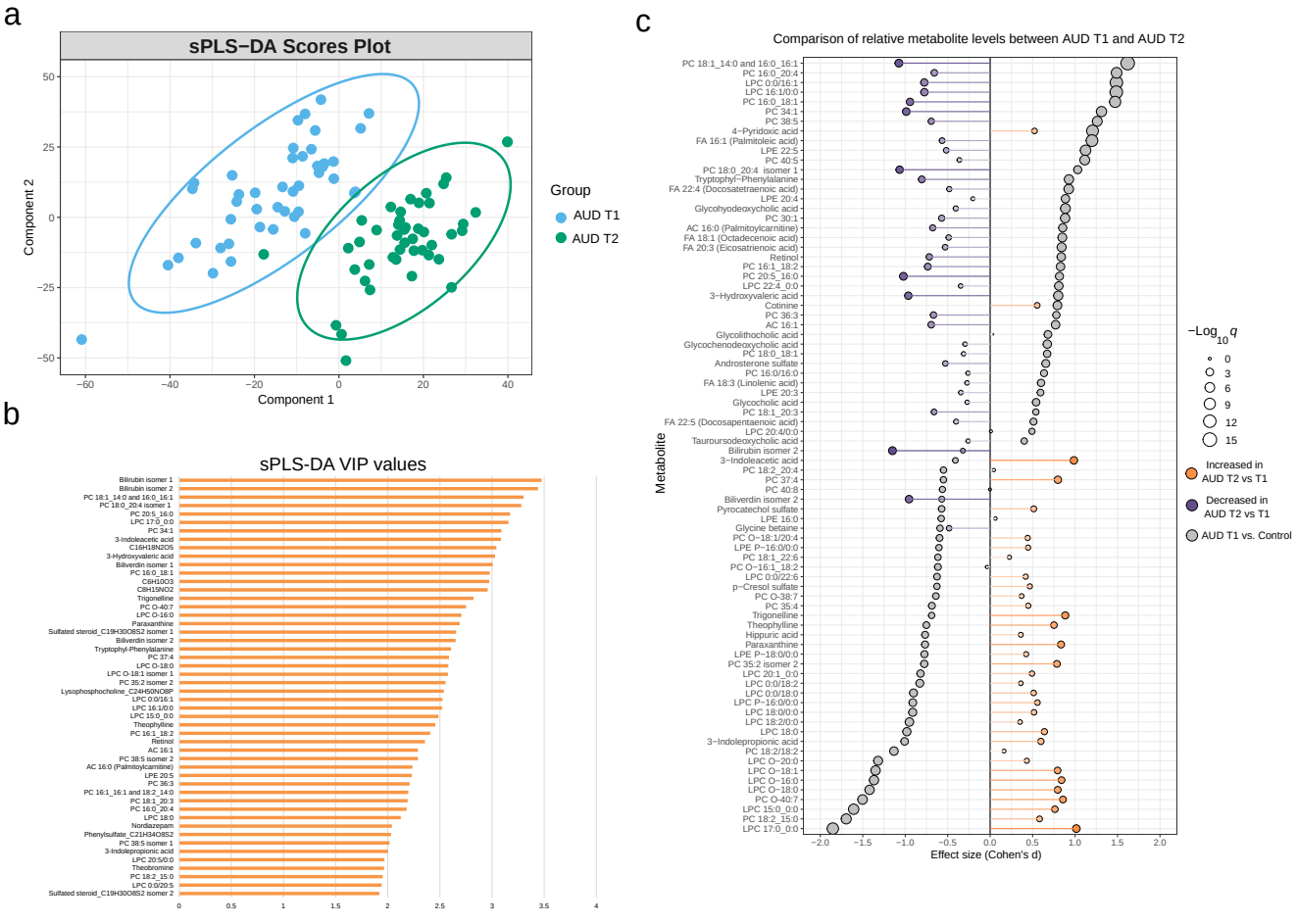
Figure 1. Chronic alcohol intake alters the plasma metabolome compared to healthy controls. **a** Principal component analysis score plot of the plasma metabolomic features between healthy controls and persons with AUD at T1. QC samples are colored in orange. **b** Top 50 annotated metabolites ranked 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.

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 AUD. **b** Top 50 annotated metabolites having a variable importance in projection (VIP) score of > 1.5 in the sPLS-DA model. **c** Lollipop plot of the effect size (Cohen's D) and -Log_{10} transformed *q* values of the altered annotated metabolites between AUD T1 and healthy controls as well as AUD T1 and AUD T2. Circle size refers to the level of significance, grey color to the comparison between controls and AUD 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.

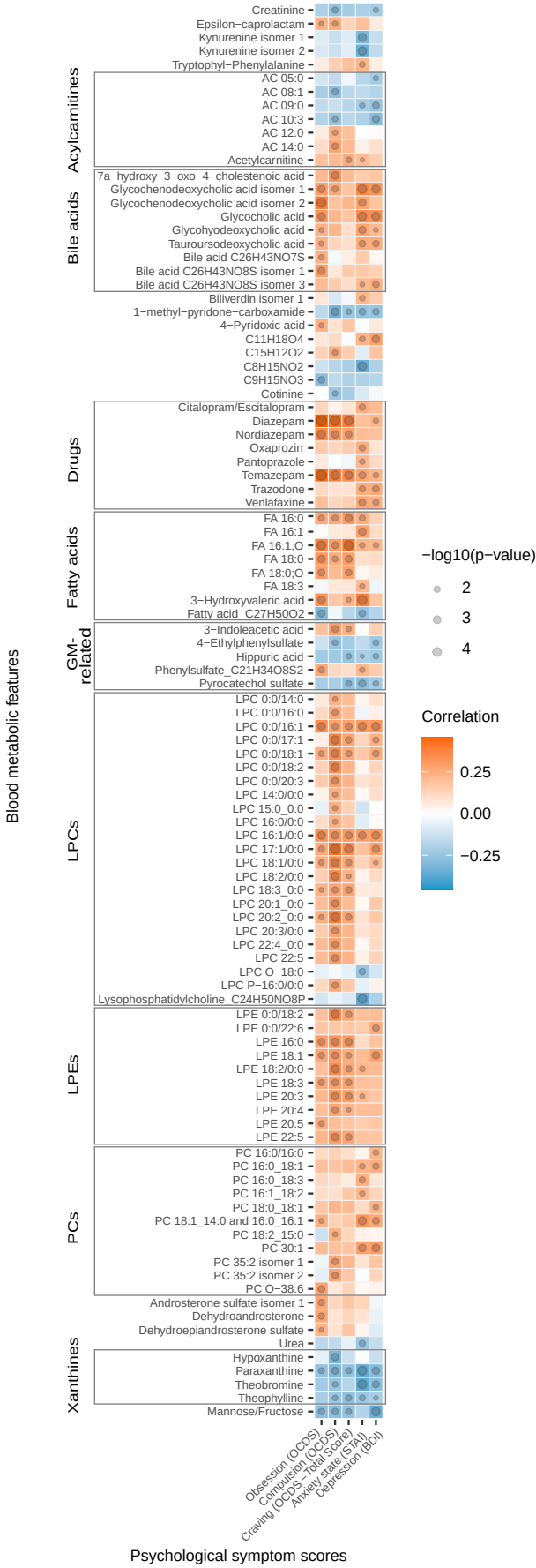
Figure 3. Plasma metabolites associated with psychological symptom scores. Heatmap of the annotated metabolites having significant ($p<0.05$) Spearman correlation with one or more psychological symptom score of obsession, compulsion, alcohol craving, anxiety state or depression. Circle size refers to 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.

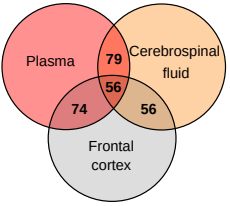

a
PCA Scores plot
PC2: 5% expl. var
PC1: 6% expl. var
Group
Control
QC
AUD T1
b
sPLS-DA VIP values
PC 18:2_15:0
LPC 17:0_0:0
PC 35:2 isomer 2
PC 39:7
LPC 15:0_0:0
Tetraterpenoid_C40H56O2
LPC O-18:0
C12H16O6
LPC O-20:0
LPC O-16:0
3-CMPFP
LPC O-18:1 isomer 1
PC 16:0_20:4
LPC O-18:1 isomer 2
PC 18:1_14:0 and 16:0_16:1
Glycerophospholipid_C48H94NO7P
PC 16:0_18:1
3-Indolepropionic acid
AC 09:0 (Nonanoylcarnitine)
LPC 0:0/16:1
LPC 16:1/0:0
PC 18:2/18:2
PC 34:1
SM 33:1
PC 38:5 isomer 1
Diazepam
Epsilon-caprolactam
PC 18:0_20:4 isomer 1
Pipecolic acid
AC 13:1
PC 40:5
LPC 18:0
4-Pyridoxic acid
FA 16:1 (Palmitoleic acid)
SM 32:2
LPC P-16:0/0:0
LPE 22:5
PE 16:0_20:4
L-Carnitine
Linoleamide isomer 2
CMPF
Sulfated_steroid_C19H30O8S2 isomer 1
LPC 0:0/18:0
LPC 18:0/0:0
LPC 18:2/0:0
Theophylline
PE 18:0_20:4
Trigonelline
LPC 20:1_0:0
PC 38:5 isomer 2
c
AUD T1
-0.8
0.8
Unknown
Unknown
Unknown
Epsilon-caprolactam
LPC 17:0_0:0
Unknown
PC 18:2_15:0
LPC 15:0_0:0
Tetraterpenoid_C40H56O2
LPC O-18:1
LPC O-18:0
3-CMPFP
PC 18:2/18:2
3-Indolepropionic acid
Hippuric acid
Glycohyodeoxycholic acid
3-Hydroxyvaleric acid
Cotinine
Glycochenodeoxycholic acid
Paraxanthine
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
PC 16:0_20:4
FA 22:4 (Docosatetraenoic acid)
Cortisol
FA 18:1 (Octadecenoic acid)
AC 16:0 (Palmitoylcarnitine)
q = 0.001
q = 0.05
Increased
Decreased
-Log10(q)
Effect size (Cohen's d)
d
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)
Correlation


a
sPLS−DA Scores Plot
Component 2
Component 1
Group
AUD T1
AUD T2
b
sPLS-DA VIP values
Bilirubin isomer 1
Bilirubin isomer 2
PC 18:1_14:0 and 16:0_16:1
PC 18:0_20:4 isomer 1
PC 20:5_16:0
LPC 17:0_0:0
PC 34:1
3-Indoleacetic acid
C16H18N2O5
3-Hydroxyvaleric acid
Biliverdin isomer 1
PC 16:0_18:1
C6H10O3
C8H15NO2
Trigonelline
PC O-40:7
LPC O-16:0
Paraxanthine
Sulfated steroid_C19H30O8S2 isomer 1
Biliverdin isomer 2
Tryptophyl-Phenylalanine
PC 37:4
LPC O-18:0
LPC O-18:1 isomer 1
PC 35:2 isomer 2
Lysophosphocholine_C24H50NO8P
LPC 0:0/16:1
LPC 16:1/0:0
LPC 15:0_0:0
Theophylline
PC 16:1_18:2
Retinol
AC 16:1
PC 38:5 isomer 2
AC 16:0 (Palmitoylcarnitine)
LPE 20:5
PC 36:3
PC 16:1_16:1 and 18:2_14:0
PC 18:1_20:3
PC 16:0_20:4
LPC 18:0
Nordiazepam
Phenylsulfate_C21H24O8S2
PC 38:5 isomer 1
3-Indolepropionic acid
LPC 20:5/0:0
Theobromine
PC 18:2_15:0
LPC 0:0/20:5
Sulfated steroid_C19H30O8S2 isomer 2
c
Comparison of relative metabolite levels between AUD T1 and AUD T2
Metabolite
PC 18:1_14:0 and 16:0_16:1
PC 16:0_20:4
LPC 0:0/16:1
LPC 16:1/0:0
PC 16:0_18:1
PC 34:1
PC 38:5
4−Pyridoxic acid
FA 16:1 (Palmitoleic acid)
LPE 22:5
PC 40:5
PC 18:0_20:4 isomer 1
Tryptophyl−Phenylalanine
FA 22:4 (Docosatetraenoic acid)
LPE 20:4
Glycohyodeoxycholic acid
PC 30:1
AC 16:0 (Palmitoylcarnitine)
FA 18:1 (Octadecenoic acid)
FA 20:3 (Eicosatrienoic acid)
Retinol
PC 16:1_18:2
PC 20:5_16:0
LPC 22:4_0:0
3−Hydroxyvaleric acid
Cotinine
PC 36:3
AC 16:1
Glycolithocholic acid
Glycochenodeoxycholic acid
PC 18:0_18:1
Androsterone sulfate
PC 16:0/16:0
FA 18:3 (Linolenic acid)
LPE 20:3
Glycocholic acid
PC 18:1_20:3
FA 22:5 (Docosapentaenoic acid)
LPC 20:4/0:0
Tauroursodeoxycholic acid
Bilirubin isomer 2
3−Indoleacetic acid
PC 18:2_20:4
PC 37:4
PC 40:8
Biliverdin isomer 2
Pyrocatechol sulfate
LPE 16:0
Glycine betaine
PC O−18:1/20:4
LPE P−16:0/0:0
PC 18:1_22:6
PC O−16:1_18:2
LPC 0:0/22:6
p−Cresol sulfate
PC O-38:7
PC 35:4
Trigonelline
Theophylline
Hippuric acid
Paraxanthine
LPE P−18:0/0:0
PC 35:2 isomer 2
LPC 20:1_0:0
LPC 0:0/18:2
LPC 0:0/18:0
LPC P−16:0/0:0
LPC 18:0/0:0
LPC 18:2/0:0
LPC 18:0
3−Indolepropionic acid
PC 18:2/18:2
LPC O−20:0
LPC O−18:1
LPC O−16:0
LPC O−18:0
PC O-40:7
LPC 15:0_0:0
PC 18:2_15:0
LPC 17:0_0:0
Effect size (Cohen's d)
−Log10 q
0
3
6
9
12
15
Increased in AUD T2 vs T1
Decreased in AUD T2 vs T1
AUD T1 vs. Control

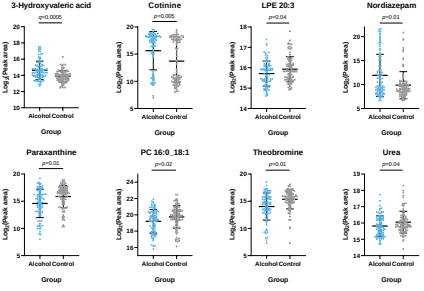
Plasma annotated metabolites significantly (p<0.05) correlated with psychological symptom scores



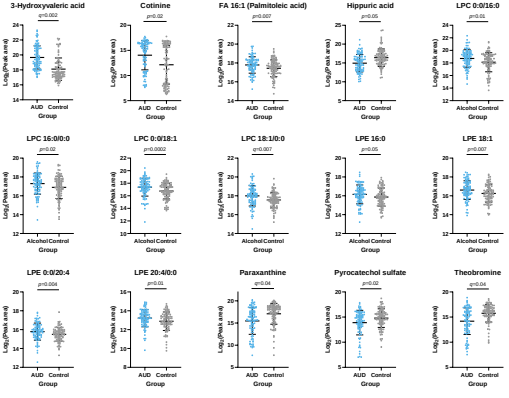
a


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