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1 Article

2 **Trimethylamine N-oxide (TMAO) and indoxyl sulfate concen-**
3 **trations in patients with Alcohol Use Disorder**4 **Laurent Coulbault^{1,2,*}, Alice Laniepce³, Shailendra Segobin¹, Céline Boudehent⁴, Nicolas Cabé^{1,4} and Anne Lise**
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11 * Correspondence: laurent.coulbault@unicaen.fr; Tel.: +33 23106324212 **Abstract: Background:** Trimethylamine N-oxide (TMAO) and indoxyl sulfate (IS) are produced by
13 the microbiota and the liver, and can contribute to brain aging and impaired cognitive function.
14 This study aims to examine serum TMAO and IS concentrations in patients with alcohol-use dis-
15 order (AUD) at the entry for alcohol withdrawal, and the relationships with several biological,
16 neuropsychological, and clinical parameters. **Methods:** TMAO and IS were quantified in thirty
17 AUD inpatients and fifteen healthy controls (HC). The severities of AUD and alcohol withdrawal
18 syndrome (AWS), and general cognitive abilities were assessed in AUD patients. **Results:** TMAO
19 concentrations did not differ between HC and AUD patients. Several biomarkers assessing nutri-
20 tional status and liver function were significantly different in AUD patients with the lowest TMAO
21 concentrations compared to other AUD patients. IS concentration was significantly lower in AUD
22 patients and a significant positive predictor of serum prealbumin variation during the acute phase
23 of alcohol withdrawal. No relationship was observed between the concentrations of these metabo-
24 lites and the severities of alcohol dependence, AWS, or cognitive deficits. **Conclusions:** Our data
25 suggest that AUD patients with low concentrations of TMAO or IS should probably benefit from a
26 personalized refeeding program during the acute phase of alcohol withdrawal.27 **Citation:** Coulbault, L.; Laniepce, A.;
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(<https://creativecommons.org/licenses/by/4.0/>).**1. Introduction**

Chronic and heavy alcohol consumption in patients with alcohol-use disorder (AUD) induces biological perturbations which contribute to liver disease, cancer and brain abnormalities [1,2]. Ethanol has its own effects on the body but is also metabolized in acetaldehyde in tissues, which is a reactive compound producing deleterious effects particularly on proteins and DNA [1,2].

AUD promotes an alteration of the gut barrier and increases intestinal permeability, contributing to modify the absorption of micronutrients. It contributes also to the transfer of various metabolic compounds produced by the gut microbiota in the portal venous bloodstream, such as lipopolysaccharide (LPS) or indol compounds [3], and to bacterial translocation. AUD can also promote gut dysbiosis characterized by an alteration of the microbiota composition [3]. All together, these mechanisms facilitate the development of a low grade inflammation, liver disease and probably play a role in brain neuroinflammation in AUD patients [4].

44 Trimethylamine (TMA) is a compound produced by the gut microbiota and come
45 from the metabolism of choline, betaine and L-carnitine, three compounds found in food,
46 mainly from animal origin [5]. TMA is absorbed in the gut and is rapidly metabolized in
47 the liver by flavin containing monooxygenase 1 and 3 (FMO1 and FMO3) to produce
48 trimethylamine-N-oxide (TMAO). Recent studies showed that this compound may play a
49 role in various diseases. TMAO is found, for example, at high levels in renal failure, and
50 composition of diet seems to affect its blood concentrations [6–9]. However, the effect of
51 diet on TMAO plasma concentration is still a matter of debate. Although TMAO concen-
52 tration can vary in a large extent after carnitine supplementation [8], a recent study
53 showed that TMAO plasma concentrations are not affected by consumption of eggs (a
54 food rich in choline) in healthy volunteers [7,10–12]. Another study in a population of
55 older women showed that a high protein diet can increase serum levels of carnitine and
56 indoxyl sulfate (IS), but not TMAO serum concentrations [13].

57 In experimental models and human studies, high TMAO concentrations induce
58 synthesis of proinflammatory cytokines, oxidative stress, endothelial dysfunction, vas-
59 cular inflammation and perturbation of lipid metabolism (see Janeiro *et al.* [14] for re-
60 view). These mechanisms contribute probably to insulin resistance and atherosclerosis,
61 then promoting the development of cardiovascular diseases [14].

62 Many authors proposed that high concentrations of TMAO could play a role in the
63 development of neurological disorders. TMAO is found in the cerebrospinal fluid, and
64 could promote blood brain barrier disruption, aggregation of A β protein and tau-tubulin
65 assembly [14]. TMAO could also be involved in the perturbation of beta-amyloid and tau
66 homeostasis and thus may play a role in the development of cognitive deficits in AUD
67 patients [15–17]. Lastly, another recent experimental work showed that TMAO can con-
68 tribute to brain aging [18]. Therefore, we can hypothesize that high TMAO concentra-
69 tions contribute to cognitive deficits observed in AUD patients.

70 IS is another compound coming from the metabolic transformation of dietary tryptophan
71 by the gut microbiota and the liver. Tryptophan, which is an essential amino acid,
72 is metabolized in the gut to form various indole compounds, which go across the gut
73 barrier and are then sulfoconjugated by the liver, and finally eliminated as IS by kidney.
74 IS is considered like an uremic toxin as blood levels of this compound greatly increase
75 during chronic renal failure and contribute to the development of cardiovascular diseases
76 [19]. Recent experimental works also showed that IS can induce blood brain barrier
77 permeability, neuroinflammation [20,21], anxiety and depression-like behaviour in mice
78 treated with IS [22]. In addition, IS administration in mice can alter cognitive perfor-
79 mance [22–24].

80 There is a growing interest for TMAO and IS in cardiovascular and neurological
81 diseases, and to our knowledge, these metabolites have not been studied yet in AUD pa-
82 tients. The objective of the present study was therefore to quantify serum TMAO and IS
83 concentrations in a sample of AUD inpatients at treatment entry for alcohol withdrawal,
84 and to examine their relationships with several biological markers, the severity of AUD,
85 the severity of alcohol withdrawal syndrome (AWS), and general cognitive abilities
86 evaluated at the end of the withdrawal.

87 2. Materials and Methods

88 2.1. AUD Patients and Healthy Controls

89 This study was approved by the local ethics committee (Comité de Protection des
90 Personnes CPP Nord-ouest III; ALCOBRAIN study, NCT01455207). Thirty AUD patients
91 were recruited during their alcohol withdrawal in the Addiction department of Caen
92 University Hospital. They met the “alcohol dependence” criteria according to the
93 DSM-IV-TR (American Psychiatric Association, 2000) or “severe AUD” criteria according
94 to the DSM-5. To be included, participants had to have French as their native language, to
95 be between 18 and 70-year-old. Comorbid conditions such as psychiatric disorders, a

96 history of serious chronic disease, or neurological pathologies were exclusion criteria. All
97 participants did not fulfill the criteria for substance use disorder (other than alcohol for
98 AUD patients) except tobacco.

99 Fifteen healthy controls (HC) were also recruited to match the demographics (age,
100 sex, education) of the AUD patients. All patients and HC had given a written and in-
101 formed consent for blood sampling and analysis.

102 Fasting blood sampling was performed at treatment entry in the addiction depart-
103 ment. Most AUD patients (n=27) underwent a second blood sampling to measure nutri-
104 tional markers such as serum albumin and prealbumin at the end of the acute alcohol
105 withdrawal phase. Fasting blood sampling was performed at the inclusion for HC. Serum
106 samples were stored at -80°C up to analysis.

107 2.2. Alcohol screening tool and assessment of alcohol withdrawal syndrome (AWS)

108 The Alcohol Used Disorders Identification Test (AUDIT) was performed at the in-
109 clusion to identify the severity of alcohol harmful drinking and dependence in AUD pa-
110 tients [25]. HC were also interviewed with AUDIT [25] to ensure that they did not meet
111 the criteria for DSM-IV-TR criteria for alcohol abuse (AUDIT < 7 for men and < 6 for
112 women).

113 In AUD patients, the severity of AWS was assessed using the Cushman's maximum
114 score [26]. This score takes the following clinical variables into account: hearth rate, sys-
115 tolic blood pressure, respiratory rate, tremor, sweating, agitation, and sensorial disor-
116 ders. Each variable is scored from 0 to 3 according to the severity of the symptom. The
117 Cushman score refers to the sum of these subscores.

118 2.3. Neuropsychological examination

119 AUD patients underwent the Montreal Cognitive Assessment (MoCA) and Brief
120 Evaluation of Alcohol-Related Neuropsychological Impairments (BEARNI). MoCA and
121 BEARNI are two screening tools that have been validated in AUD patients to early detect
122 neuropsychological impairments [27,28]. These tests were performed at the end of the
123 acute withdrawal phase for AUD patients, and at inclusion in the study for HC.

124 2.4. Biological analyzes

125 Biochemical and haematological analyzes were performed in the Biochemistry and
126 Haematology departments of Caen University hospital. To explore a potential liver dis-
127 ease, the index of fibrosis Hepascore was determined using age, sex and serum concen-
128 trations of hyaluronic acid, α 2-macroglobulin, bilirubin, and gamma
129 glutamintranspeptidase (γ GT), as previously described [29].

130 2.5. TMAO and IS analysis

131 TMAO and IS quantitative analysis were performed using liquid chromatography
132 coupled with tandem mass spectrometry (LC-MS/MS). Briefly, serum samples were
133 mixed with deuterated internal standard (TMAO-d₉ or IS-d₄, Sigma-Aldrich, Saint-Louis,
134 USA) and proteins were precipitated using cold methanol/acetonitrile (50/50 v/v). After
135 centrifugation and dilution, supernatants were injected in an UFLC chromatographic
136 system (Shimadzu, Kyoto, Japan) connected to a SCIEX QTRAP® 5500 mass spectrometer
137 (SCIEX, Toronto, Canada). Chromatographic separation was performed using a Pur-
138 suit®pentafluorophenyl (PFP) column (Agilent technologies, Santa Clara, USA) in gra-
139 dient conditions, and using electrospray source in positive (TMAO) or negative (IS)
140 mode. Multiple Reaction Monitoring (MRM) transitions were m/z 76→58 and m/z 85→66
141 for TMAO and TMAO-d₉ respectively, and m/z 212→132 and m/z 216→136 for IS and
142 IS-d₄, respectively. All coefficients of variation (CV) were lower than 5% in validation
143 assays. TMAO and IS determination was performed in all AUD patients and HCat the

inclusion in the study, and only in 12 AUD patients at the end of alcohol withdrawal due to low sample quantity.

2.6. Statistical analysis

This study was retrospective, as an ancillary investigation of large research protocol, and biological variability of IS or TMAO was not known in our population at the start of the study. It was then not possible to perform power calculation and to determine the number of subjects a priori.

All data were expressed as mean±standard deviation. Statistical analysis was performed using Statistica software (Tibco software Inc, Palo Alto, USA) and figures were made using GraphPad Prism software (Graphpad, La Jolla, USA). Normality was checked using Kolmogorov-Smirnov tests. When data were not normally distributed, the variables were Log transformed to approach a normal distribution. Comparisons between AUD patients and HC were conducted using Student t test or ANOVA for comparison between tertiles of AUD patients (supplemental data). A paired sample t test was used to compare TMAO and IS variations of concentration during alcohol withdrawal (supplemental figure). Differences were considered statistically significant at $p<0.05$. Demographics, clinical neuropsychological and biological data are summarized in Table 1.

Table 1. Demographic, clinical, neuropsychological and biological data observed in AUD patients and HC. Data are expressed as the mean±standard deviation. AUD: Alcohol Use Disorder. HC: Healthy Controls. AUDIT: Alcohol Use Disorders Identification Test. MoCA: Montreal Cognitive Assessment. BEARNI: Brief Evaluation of Alcohol-Related Neuropsychological Impairments. Significantly different at $p<0.05$. NA: Not applicable. ND: Not done.

	AUD Patients (n=30)	Healthy Controls (n=15)	p
Demographic and Clinical variables			
Age (y)	47.5±10.1	46.2±5.1	NS
Sexe (M/F)	24/6	12/3	NS
Body Mass Index (kg/m ²)	24.1±4.3	24.3±4.3	NS
AUDIT	28.6±7.8	2.6±1.4	$p<0.05$
Cushman's score	4.9±2.2	NA	ND
Benzodiazepin prescription (days)	6.7±5.3	NA	ND
MoCA score (/30)	24.5±5.0	27.4±1.4	$p<0.05$
BEARNI score (/30)	13.5±6.1	19.1±2.9	$p<0.05$
Length of hospital stay (days)	20.2±5.6	NA	ND
Biological measures			
Glycemia (mmol/L)	5.3±0.9	5.3±0.6	NS
Glycated haemoglobin (%)	5.4±0.6	5.4±0.3	NS
Total serum protein (g/L)	66.4±6.9	70.3±3.5	$p=0.05$
Albumin (g/L)	37.2±4.7	43.2±2.8	$p<0.05$
Prealbumin (g/L)	0.27±0.08	0.31±0.04	$p<0.05$
γ -glutamyl-transpeptidase (U/L)	442.2±812.2	16.9±10.5	$p<0.05$
Ammonium (μ mol/L)	42.8±23.3	28.1±7.2	$p<0.05$
ASAT (U/L)	69.3±60.3	19.6±7.8	$p<0.05$
ALAT (U/L)	50.3±71.7	21.1±12.5	$p<0.05$
ASAT/ALAT ratio	2.2±2.5	1.0±0.3	$p<0.05$
Bilirubin (μ mol/L)	25.1±17.4	17.9±10.5	$p<0.05$
α 2 macroglobulin (g/L)	2.1±0.6	1.7±0.5	$p<0.05$
Hyaluronic acid (μ g/L)	121.4±320.7	15.2±5.5	$p<0.05$
Score of fibrosis HEPASCOPE	0.46±0.31	0.22±0.13	$p<0.05$
Serum creatinine (μ mol/L)	61.9±12.7	78.7±12.8	$p<0.05$
Urea (mmoles/L)	2.7±1.0	4.4±0.8	$p<0.05$

Haemoglobin (g/dL)	14.1±1.4	14.8±0.7	p<0.05
Mean Cellular Volume (µm ³)	98.0±6.0	87.7±4.3	p<0.05
Platelets (G/L)	205±82	280±61	p<0.05
Prothrombin time (%)	96.3±8.4	96.9±5.2	NS
Trimethylamine N-oxide TMAO (µmol/L)	12.1±19.2	4.6±2.5	NS
Indoxyl sulfate IS (µmol/L)	2.1±1.0	3.9±1.5	p<0.05

In AUD patients, we also performed simple Pearson correlations between TMAO or IS concentration on the one hand, and demographic, biological or clinical parameters on the other hand. Backward multiple regressions were then used to identify 1) the best predictors of TMAO or IS levels, and 2) whether TMAO or IS levels could be good predictors of different clinical, biological or neuropsychological parameters. Only the variables presenting a statistically significant correlation were entered in the regression model, and only predictors of the final solution are presented.

3. Results

3.1. Serum TMAO concentrations in AUD patients and HC

TMAO concentrations were not significantly different between AUD patients and HC (12.1±19.2 µmol/L vs 4.6±2.5 µmol/L, NS; Table 1 and Figure 1.A). However, an important inter-individual variability was observed for serum TMAO concentrations in AUD patients at treatment entry (Figure 1.A). Some AUD patients had very low TMAO serum concentrations (<2 µmol/L) compared to HC, while others presented very high concentrations (>20 µmol/L, Figure 1.A). Such very high or very low concentrations of serum TMAO were not observed in HC. We then explored whether TMAO concentrations could be related to biological, neuropsychological or clinical characteristics in AUD patients. In a first attempt, the biological and clinical profiles of AUD patients were studied according to their distribution profile into tertiles of serum TMAO concentrations (tertile 1, [TMAO]<2.55 µmol/L; tertile 2, 2.55 µmol/L≤[TMAO]≤6.76 µmol/L; tertile 3, [TMAO]>6.76 µmol/L; see Supplemental Table S1). Interestingly, AUD patients with the lowest TMAO values presented several significant biological alterations (see Supplemental Table S1). AUD Patients with higher TMAO concentrations presented the weakest biological perturbations compared to other AUD patients (see Supplemental Table S1).

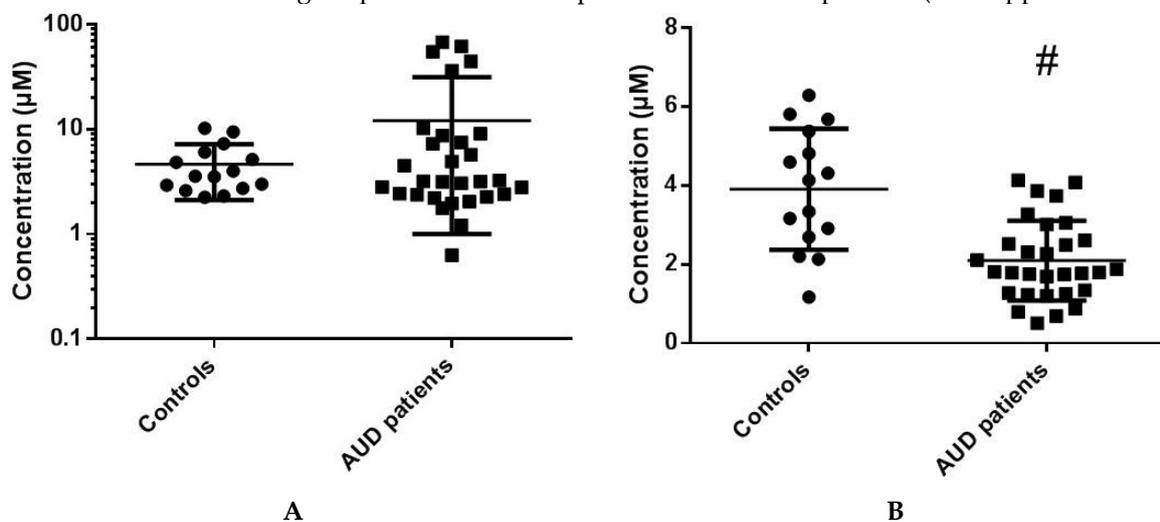


Figure 1. (A) TMAO and (B) IS concentrations in serum of AUD patients at the treatment entry and in HC. #: p<0.05 when compared to controls. TMAO: Trimethylamine N-oxide. IS: Indoxyl Sulfate. AUD: Alcohol Use Disorder. HC: Healthy Controls.

3.2. Serum IS concentrations in AUD patients and HC

Serum IS concentrations were significantly lower in AUD patients when compared to HC ($2.1 \pm 1.0 \mu\text{mol/L}$ vs $3.9 \pm 1.5 \mu\text{mol/L}$, $p < 0.05$; Table 1 and Figure 1.B). The inter-individual variability in AUD patients and in HC was lower by comparison to TMAO data.

The biological and clinical profiles of AUD patients has also been studied according to their distribution profile into tertiles of serum IS concentrations (tertile 1, $[\text{IS}] < 1.7 \mu\text{mol/L}$; tertile 2, $1.7 \mu\text{mol/L} \leq [\text{IS}] \leq 2.4 \mu\text{mol/L}$; tertile 3, $[\text{IS}] > 2.4 \mu\text{mol/L}$; see Supplemental Table S2).

Conversely to TMAO, no difference was seen when we compared tertiles of IS concentrations in AUD patients. However, the ASAT/ALAT ratio reached significance for the tertile 1 of AUD patients with the lower IS concentrations (see Supplemental Table S2). No correlation was found between TMAO and IS serum concentrations neither in AUD patients nor in HC (data not shown).

3.3. Evolution of serum TMAO and IS concentrations in AUD patients during the acute alcohol withdrawal phase

TMAO and IS determination was performed only in 12 AUD patients at the end of the acute phase of alcohol withdrawal. Concerning TMAO concentrations, no significant difference was observed in these 12 AUD patients during before and after alcohol withdrawal. However, a high intra-individual variability was observed in these patients. Indeed, some AUD patients presented very high serum TMAO concentrations at treatment entry which typically decreased and returned at the end of alcohol withdrawal to serum levels usually observed in HC. However, it was not observed for all AUD patients, and in some cases, TMAO concentrations were quite similar at the end of alcohol withdrawal when compared to the concentrations at treatment entry (see Supplemental Figure S1).

Concerning IS, serum concentrations significantly increased during the acute phase of alcohol withdrawal by applying a paired sample t test ($p = 0.037$). In addition, we observed a lower intra-individual variability when compared to TMAO concentrations (see Supplemental Figure S2).

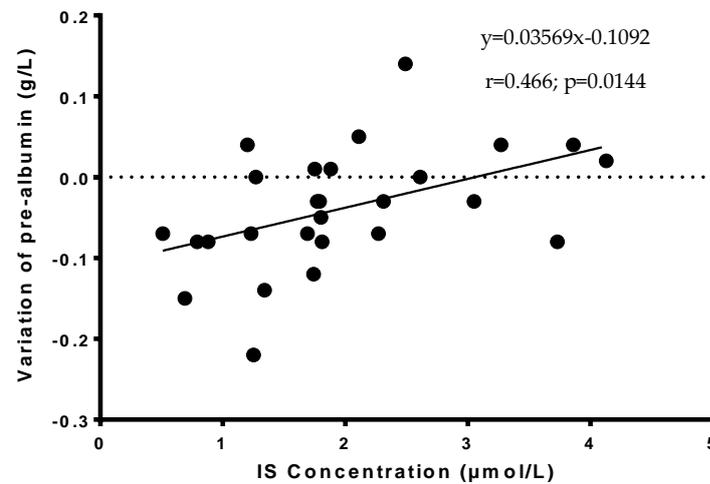
3.4. Correlations of serum TMAO or IS concentrations with biological and clinical markers in AUD patients

Simple correlations were performed in AUD patients between TMAO or IS concentrations at treatment entry on the one hand, and several biological and clinical parameters on the other hand. Interestingly, TMAO (Log transformed) correlated with various parameters such as $\alpha 2$ macroglobulin ($p = 0.0007$), glycated haemoglobin ($p = 0.0008$), total serum protein ($p = 0.01$), sodium ($p = 0.009$), ASAT/ALAT ratio ($p = 0.031$), glycemia ($p = 0.037$), Hepascore ($p = 0.037$), and length of hospital stay ($p = 0.0067$; see Supplemental Table S3.A and S6.A). In addition, IS concentrations at treatment entry correlated with the AUDIT total score ($p = 0.0032$), platelets levels ($p = 0.038$), ASAT/ALAT ratio ($p = 0.0465$) and the mean number of self-reported meals per day before hospitalization ($p = 0.0082$, see Supplemental Table S4).

3.5. Correlations of serum TMAO or IS concentrations in AUD patients with the variation of prealbumin or albumin concentrations during the acute phase of alcohol withdrawal

Serum albumin and prealbumin, two biological markers of nutritional status, were also measured at the end of the acute phase of alcohol withdrawal for 27 AUD patients, and it is of interest to note that variations of serum prealbumin during the acute phase of alcohol withdrawal correlated with IS concentration at the entry (Figure 2). In addition, variations of serum prealbumin correlated with variations of albumin during alcohol withdrawal (data not shown). Typically, prealbumin seems to decrease during alcohol

243 withdrawal in most AUD patients, but that seems to be less important when AUD pa-
 244 tients present medium to high levels of IS at treatment entry (Figure 2).



245
 246 **Figure 2.** Correlation between serum IS concentrations of AUD patients at the treatment entry and
 247 variations of serum prealbumin concentrations during alcohol withdrawal. Statistically significant
 248 at $p < 0.05$.

249 3.6. Predictors of TMAO and IS concentrations in AUD patients

250 We attempted to identify significant predictors of serum TMAO and IS concentra-
 251 tions at the entry by applying a backward stepwise multiple regression analyse. $\alpha 2$
 252 macroglobulin ($\beta = -0.497$, $p = 0.00069$), ASAT/ALAT ratio ($\beta = -0.454$, $p = 0.0013$) and
 253 natremia ($\beta = 0.423$, $p = 0.0025$) were identified as significant predictors of serum TMAO
 254 concentrations (adjusted $r^2 = 0.7059$, $p = 0.0000127$ for whole model; see Supplemental Table
 255 S3.B. The AUDIT score was the only predictor for IS concentrations ($\beta = -0.517$, $p = 0.004058$;
 256 see Supplemental Table S4.B).

257 3.7. Predictors of clinical, biological and neuropsychological parameters in AUD patients

258 Finally, we also attempted to identify predictors for different clinical and neuro-
 259 psychological parameters. Indicating the severity of AWS, the Cushman's maximum
 260 score was best predicted by alkaline phosphatase (ALP) and carbohydrate deficient
 261 transferrin (CDT) (data not shown). No relationship was also shown between TMAO or
 262 IS concentrations and duration of benzodiazepin prescription, which is also an indicator
 263 of the severity of AWS (data not shown).

264 Concerning the neuropsychological assessment of AUD patients, age and glycemia
 265 were only the best predictors for BEARNI total score, which detect neuropsychological
 266 impairments in AUD patients. In this study, we failed to find significant predictors for
 267 MoCA (data not shown). When studying serum prealbumin variation during alcohol
 268 withdrawal, it is interesting to note that IS concentration was the best positive predictor
 269 ($\beta = 0.408$, $p = 0.002363$), and Albuminemia ($\beta = -0.527$, $p = 0.00016$) and Hyaluronic acid
 270 ($\beta = -0.487$, $p = 0.000516$) were the best negative predictor (adjusted $r^2 = 0.668$, $p = 0.00000533$
 271 for whole model; see Supplemental Table S5).

272 Finally, TMAO concentrations (Log transformed) at the entry was the only predictor
 273 of length of hospital stay in our population of AUD patients ($\beta = -0.622$, $p = 0.001996$; see
 274 Supplemental Table S6).

275 4. Discussion

276 Chronic alcohol consumption is often associated with gut and nutritional disorders,
 277 and AUD patients can develop brain damages leading to cognitive deficits. TMAO and IS

278 are two metabolites coming from transformation of various dietary compounds by
279 microbiota and then liver. Whereas there is growing interest for TMAO and IS in cardi-
280 ovascular and neurological diseases, at our knowledge, no study assessed TMAO and IS
281 concentrations in AUD patients. We thus aimed at measuring TMAO and IS concentra-
282 tions in a group of AUD patients and at examining, in these patients, the relationships
283 between serum levels on the one hand and biological, clinical or neuropsychological
284 characteristics on the other hand.

285 In this study, we showed that serum TMAO concentrations in AUD patients pre-
286 sented an important interindividual variability when compared to HC. It is in agreement
287 with literature data as the concentrations observed in our population were comparable to
288 those reported in other studies [8]. Very low or very high TMAO concentrations were not
289 observed in HC, potentially because the number of HC was limited in our study. As
290 TMAO concentration may vary with dietary intake, liver function or dysbiosis, it would
291 be then interesting to quantify these parameters in a larger cohort to assess the biological
292 variability in nonpathological conditions.

293 We showed that many AUD patients had very high levels of serum TMAO, which
294 may be deleterious. Indeed, deleterious effects of high plasma concentrations of TMAO
295 and IS in the development of cardiovascular and/or neurological pathologies have been
296 previously reported [20,24,30–32]. Regarding AUD patients, they have a higher risk to
297 develop cardiovascular disease [33] such as hypertension, myocardial infarction or is-
298 chemic stroke. We can therefore hypothesize that TMAO or IS may play a role in the de-
299 velopment of cardiovascular disease in AUD patients, but this hypothesis presupposes
300 that high concentrations may be found in these patients, and that it could be deleterious.
301 However, surprisingly, AUD patients with very high serum TMAO concentrations pre-
302 sented the weakest biological perturbations compared to other AUD patients. It is intri-
303 guing as high levels of TMAO are often presented in literature data as promoting deleter-
304 ious effects contributing to diseases. One hypothesis is that the diet in this subgroup of
305 patients may be close to a balanced western diet (in quantity and quality), which would
306 perhaps allow to have sufficient nutritional intakes of micronutrients (trace elements and
307 vitamins) to fight against the harmful metabolic effects of alcohol (acetaldehyde produc-
308 tion and oxidative stress). Another explanation could be the consumption of red meat in
309 these patients the day before blood sampling during refeeding in the hospital setting.

310 Interestingly, many AUD patients had very low serum TMAO concentrations, and
311 these patients presented unexpectedly more biological perturbations for different bi-
312 omarkers assessing nutritional status and liver function. These effects are of interest but
313 must be confirmed in a larger cohort of AUD patients.

314 We also report that AUD patients exhibit significant lower serum IS concentrations
315 compared to HC. It should be noted that very high IS concentrations were not found in
316 our group of AUD patients.

317 All these data were unexpected and different explanations can be proposed. Several
318 factors probably affect the production of TMAO and IS: First, low concentrations of
319 TMAO and IS could be related to a lower choline, betaine, carnitine or tryptophane die-
320 tary intake, which is dependent from the diversity, the quality and the quantity of food
321 intake in a population of AUD patients which is often underprivileged. For different
322 reasons, AUD patients can have reduced aminoacid and micronutrients intake, which
323 directly affects TMA and indolic compounds production. A reduced food intake can
324 further induce chronic denutrition and may promote a clinical state with a low-grade in-
325 flammation. In the present study, IS concentration at treatment entry, but not TMAO,
326 was correlated with the number of meals per day in AUD patients, which reflects proba-
327 bly a lower tryptophan intake. Regarding TMAO, several studies showed an effect of diet
328 but others also failed to find such an effect [8,13,34–36]. In the present study, AUD pa-
329 tients and HC have a western diet and we cannot exclude an effect of such diet. Unfor-
330 tunately, the nutritional intakes of choline, carnitine and betaine have not been investi-

331 gated, and these intakes could not also be controlled in AUD patients during the days
332 before blood sampling.

333 If we consider diet to be the main factor that affects TMAO or IS production, then
334 our data suggest that medium or high serum levels of TMAO or IS could reflect adequate
335 food intake and probably a good nutritional status in AUD patients at treatment entry,
336 and this could be an indicator of a good prognosis in these patients.

337 Second, hepatic function is another factor that can explain low concentrations of
338 TMAO or IS observed in some AUD patients. TMA and indolic compounds go through
339 the gut and follow the venous portal system up to the liver, and then these metabolites
340 are transformed by hepatic enzymes such as FMO1 and FMO3 [11,35]. We can hypothe-
341 size that a reduced metabolic function of the liver affects the metabolism of TMA and
342 indolic compounds, and reduces the levels of TMAO and IS concentrations in the blood
343 of these patients. Then low concentrations of TMAO or IS could reflect liver dysfunction,
344 in agreement with the levels of different biomarkers assessing hepatic cytolysis
345 (ASAT/ALAT ratio), cholestase and oxidative stress in the liver (γ GT, α 2 macroglobulin),
346 or fibrosis (Hepascore).

347 Third, we can also hypothesize that alcohol consumption could modify composition
348 of microbiota and alter the quantity of bacteria in the gut, as described in previous stud-
349 ies [37,38]. For example, it could modify TMA production in the gut and then TMAO
350 concentrations in blood after hepatic metabolism. Alcohol withdrawal and renutrition
351 can then promote changes of microbiota composition and contribute to variations of se-
352 rum TMAO observed during withdrawal [38].

353 Finally, we did not show any link between serum concentrations of TMAO or IS,
354 and the severity of AUD, the severity of AWS and cognitive deficits at the end of the
355 acute phase of alcohol withdrawal. These findings suggest that the level of TMAO or IS
356 does not affect directly neurological state of these AUD patients. Although we did not
357 observe any effect in this study, we cannot exclude that an exposure to high concentra-
358 tions of TMAO during several weeks or months is required to observe deleterious effects.
359 In addition, although IS seems to be the main compound issued from tryptophan me-
360 tabolism by microbiota, it should be noted that other indole derivatives, such as
361 indol-3-acetic acid, can be produced and that they could also play a role in the develop-
362 ment of liver, brain or cardiovascular diseases [3,39]. However, these compounds were
363 not quantified in this study.

364 Considering the nutritional status, we showed in this study that IS level at treatment
365 entry was a significant positive predictor of variation of serum prealbumin during alco-
366 hol withdrawal. Prealbumin is considered as a biomarker assessing denutrition and the
367 efficiency of renutrition: an increase is observed in patients when renutrition is appro-
368 priated. In our study, serum prealbumin seems to decline during alcohol withdrawal in
369 most AUD patients, probably reflecting metabolic changes during this period. However,
370 the lower IS concentration at treatment entry, the more severe the decline of serum
371 prealbumin concentration. These data encourage the identification of AUD patients with
372 the lowest IS concentrations at treatment entry. They would require additional and spe-
373 cific nutritionist intervention associated with a personalized refeeding program and fol-
374 low-up examinations during alcohol withdrawal.

375 Interestingly, we also showed that TMAO concentrations (Log transformed) is a
376 significant predictor of the length of hospital stay. It is important to note that all AUD
377 patients benefited from a personalized therapeutic course during their hospitalization.
378 Therefore, the management of care may be heterogeneous between patients, some bene-
379 fitting from specific cares such as education workshop, thus increasing the length of stay.
380 It can induce bias in the interpretation of data and this relationship needs to be studied in
381 a larger cohort.

382 Then it could be of interest to further investigate a potential utility to quantify serum
383 TMAO and IS in a larger cohort of AUD patients. It could be of importance to detect these
384 patients early in the course of the treatment program in order to improve clinical practice,

385 to improve nutritional status, and perhaps to reduce the length of hospital stay and the
386 cost of hospitalization in this population of patients.

387 5. Strengths and limitations of this study

388 Several strengths could be mentioned in this work. First, the different inclusion cri-
389 teria in this study allowed to have a homogeneous population of AUD patients, limiting
390 potential biases. In addition, the use of modern analytical technologies allows to quantify
391 more easily and specifically many metabolic compounds coming from microbiota and
392 liver metabolism.

393 Nevertheless, this work has several limitations. The main limitation is the small
394 sample size. Moreover, although AUD patients were strictly selected and our population
395 can be considered as homogeneous, this population can also be considered as partially
396 representative of AUD patients hospitalized in an addictology department. From a sta-
397 tistical point of view, we did not perform corrections for multiple comparisons on the
398 correlations before entering in the regression model as this remains an exploratory anal-
399 ysis and we did not want to over-correct and take the risk of discarding true positives
400 [40]. In addition, dietary intakes were not registered before the entry in the addiction care
401 unit, and only few declarative data were available. The quantity and quality of the food
402 ingested were not registered, and we cannot exclude that high concentrations of plasma
403 TMAO observed in some AUD patients could result from the consumption of food from
404 animal origin the day before blood sampling. At last, only IS was quantified in our pa-
405 tients, but not other metabolites from tryptophan. Thus, we cannot exclude that these
406 other metabolites can have an effect on the severity of AWS or cognitive function during
407 and after the hospitalization. In addition, metabolomic studies using Nuclear Magnetic
408 Resonance could not be performed because these technologies were not available locally.
409 This would have made it possible to identify and quantify many other metabolites in
410 these samples and to better understand the mechanisms explaining low TMAO or IS
411 concentrations observed in many AUD patients. Finally, neuropsychological tests were
412 performed at the end of the acute alcohol withdrawal phase, and the effects of long-term
413 exposure to high concentrations of TMAO or IS on cognitive function were not assessed.
414 Therefore, we cannot exclude that long-term exposure to high levels of TMAO or IS is
415 finally deleterious for AUD patients; it would require multiple blood sampling to assess
416 evolution of these metabolites.

417 6. Conclusions

418 The present study is the first to explore TMAO and IS serum concentrations in AUD
419 patients. Our data show first a relationship between low TMAO concentrations and
420 higher biological perturbations, and second between low IS concentrations and the vari-
421 ations of serum prealbumin during the acute phase of alcohol withdrawal. In this popu-
422 lation, AUD patients with high serum TMAO concentrations presented the weakest bio-
423 logical perturbations. Although we did not see any relationship between low TMAO or IS
424 concentrations and the severity of AWS or cognitive deficits, our data suggest that AUD
425 patients with low concentrations of TMAO or IS at treatment entry could be related to a
426 poor nutritional status, and should probably benefit from a personalized refeeding pro-
427 gram. Biological quantification of these metabolites in larger cohorts could be of interest
428 in AUD patients.

429 **Supplementary Materials:** The following supporting information can be downloaded at:
430 www.mdpi.com/xxx/s1, Supplemental Table S1: Clinical and biological data in AUD patients ac-
431 cording to their distribution profile into tertiles of serum TMAO concentrations. Supplemental Ta-
432 ble S2: Clinical and biological data in AUD patients according to their distribution profile into
433 tertiles of serum IS concentrations. Supplemental Table S3: (A) Pearson correlations of TMAO
434 concentrations with biological and clinical parameters at the entry and (B) predictors of TMAO
435 concentration from stepwise multiple linear regression analyses. Supplemental Table S4: (A)

Pearson correlations of IS concentrations with biological and clinical parameters at the entry and (B) predictors of IS concentration from stepwise multiple linear regression analyses. Supplemental Table S5: (A) Pearson correlations of variations of serum prealbumin during alcohol withdrawal with others continuous variables, and (B) predictors of variation of serum prealbumin during alcohol withdrawal from stepwise multiple linear regression analyses. Supplemental Table S6: (A) Pearson correlations of length of hospital stay with others continuous variables and (B) predictors of length of hospital stay from stepwise multiple linear regression analyses. Supplemental Figure S1: Evolution of TMAO concentrations in AUD patients (n=12) during alcohol withdrawal. Supplemental Figure S2: Evolution of IS concentrations in AUD patients (n=12) during alcohol withdrawal. #: p<0.05.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the local ethics committee (Comité de Protection des Personnes CPP Nord-ouest III; ALCOBRAIN study, NCT01455207).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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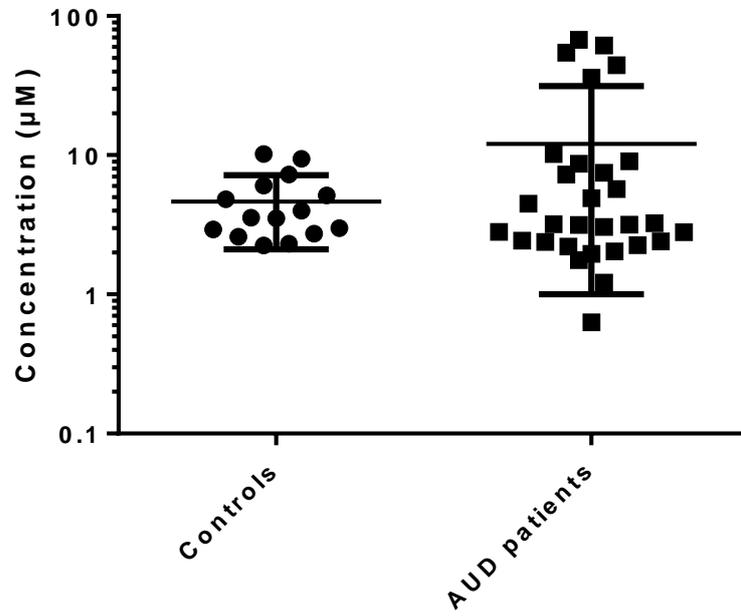
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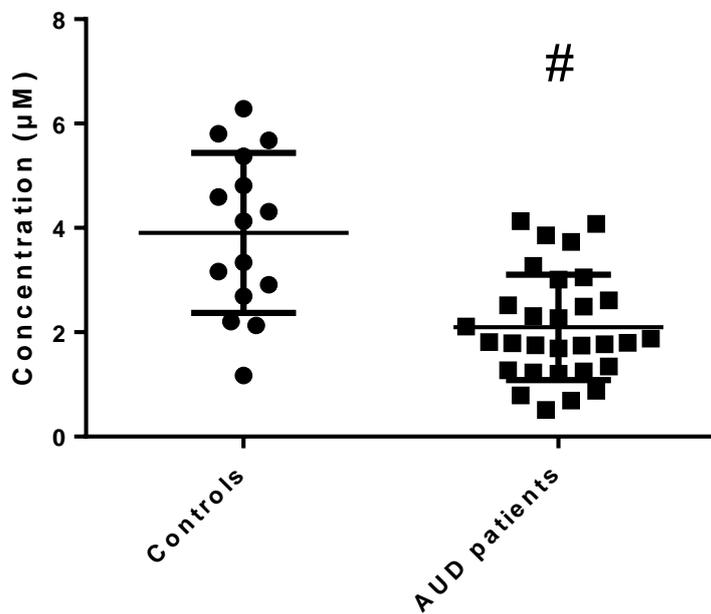
Fig. 1A



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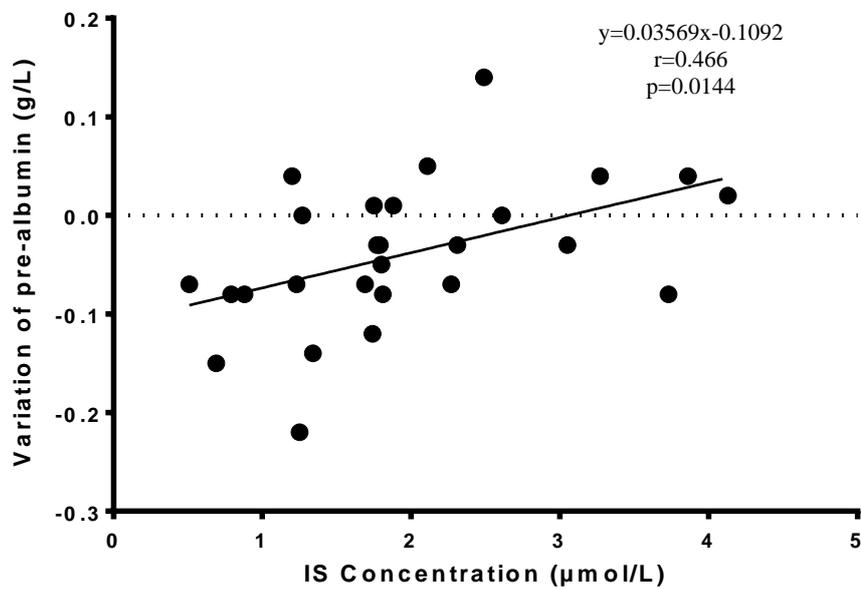
Fig. 1B



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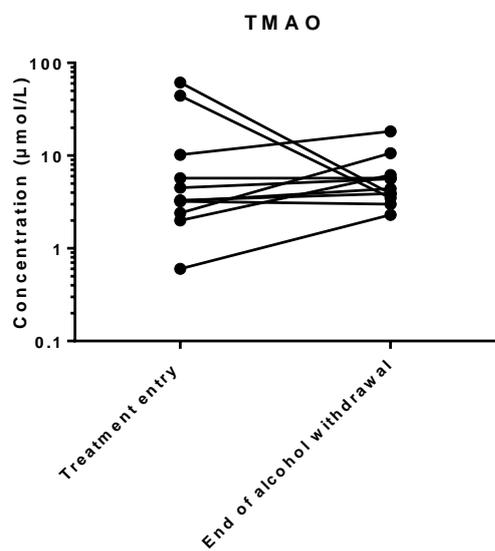
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Fig. 2

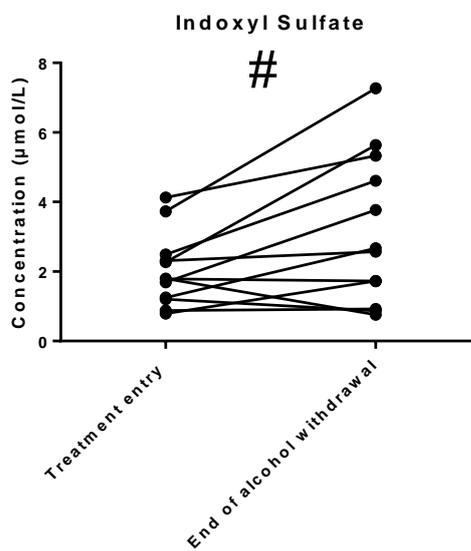


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Supplemental Figure S1



Supplemental Figure S2



Supplemental data – Table S1

Clinical and biological data in AUD patients according to their distribution profile into tertiles of serum TMAO concentrations (tertile 1, [TMAO]<2.55 µM; tertile 2, 2.55 µM≤[TMAO]≤6.76 µM; tertile 3, [TMAO]>6.76 µM). Statistically significant at p<0.05.

	TMAO concentration			
	Tertile 1 (n=10) [TMAO]<2.55µmol/L	Tertile 2 (n=10) 2.55≤[TMAO]≤6.76µmol/L	Tertile 3 (n=10) [TMAO]>6.76µmol/L	
AUDIT	30.2±4.7	26.5±12.7	26.4±8.7	NS
BMI (kg/m ²)	22.9±3.6	26.1±5.9	23.2±1.9	NS
Number of meal per day	1.9±1.1	2.1±0.9	2.1±0.9	NS
Cushman's score	5.0±1.8	5.4±2.4	5.2±2.3	NS
Benzodiazepin prescription (days)	6.3±6.2	7.1±5.9	6.8±3.7	NS
BEARNI (/30)	14.0±6.0	12.9±6.0	13.8±6.7	NS
MoCA score (/30)	23.9±4.4	25.0±3.9	24.7±6.8	NS
Length of hospital stay (days)	23.5±7.5	19.9±2.8	17.3±3.6	NS
Total protein (g/L)	67.1±8.3	69.2±6.4	63.0±4.8	NS
Albumin (g/L)	35.9±6.7	38.1±3.8	37.7±3.1	NS
Prealbumin (g/L)	0.25±0.09	0.26±0.09	0.29±0.07	NS
Variation of prealbumin concentration during alcohol withdrawal (g/L)	-0.06±0.09	-0.03±0.08	-0.03±0.05	NS
Variation of albumin concentration during alcohol withdrawal (g/L)	-0.4±7.0	-0.6±4.2	1.8±1.9	NS
γ-glutamyl-transpeptidase (U/L)	806.9±988.3	390.7±933.9	128.9±130.3	NS
Ammonium (µmol/L)	54.9±25.3	42.4±25.3	31.0±13.1	NS
ASAT (U/L)	97.0±72.8	63.0±56.9	49.0±49.6	NS
ALAT (U/L)	44.6±35.6	42.6±42.1	67.7±114.1	NS
ASAT/ALAT ratio	2.5±1.3 \$	1.7±0.8	1.3±0.8	p<0.05
Bilirubin (µmol/L)	34.1±21.1	23.8±18.0	17.4±7.0	NS
α2 macroglobulin (g/L)	2.4±0.5 \$	2.2±0.5	1.6±0.3	p<0.05
Hyaluronic acid (µg/L)	270.6±536.6	59.6±75.0	34.1±18.4	NS
Score of fibrosis (HEPASCORE)	0.58±0.37	0.49±0.33	0.31±0.17	NS
Serum creatinin (µmoles/L)	51.7±5.3 #	69.9±12.6	64.1±11.8	p<0.05
Urea (mmoles/L)	2.3±1.0	2.9±0.7	2.8±1.1	NS
Prothrombin time (%)	92.9±12.5	97.2±6.2	98.8±3.4	NS
Haemoglobin (g/dL)	13.0±1.4	14.6±1.3	14.6±0.9	NS
Mean Cellular Volume (µm ³)	99.3±7.5	94.9±5.0	99.9±4.3	NS

Platelets (G/L)	149.7±85.8	236.6±66.5	227.7±70.4	NS
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#: p<0.05 when compared to tertile 2.
\$: p<0.05 when compared to tertile 3.

Supplemental data – Table S2

Clinical and biological data in AUD patients according to their distribution profile into tertiles of serum IS concentrations (tertile 1, [IS]<1,7 µM; tertile 2, 1.7 µM ≤ [IS] ≤2.4 µM; tertile 3, [IS]>2.4µM). Statistically significant at p<0.05.

Indoxyl sulfate concentration

	Tertile 1 (n=10) [IS]<1.7µmol/L	Tertile 2 (n=10) 1.7≤[IS]≤2.4µmol/L	Tertile 3 (n=10) [IS]>2.4µmol/L	p
AUDIT	32.0±4.5	26.9±5.1	24.2±13.8	NS
BMI (kg/m ²)	23.4±4.1	25.7±5.2	23.2±3.3	NS
Number of meal per day	1.4±1.0	2.4±0.7	2.3±0.8	p<0.05
Cushman's score	5.9±1.8	4.9±2.4	4.8±2.1	NS
Benzodiazepin prescription (days)	7.9±4.6	5.0±4.9	7.3±6.3	NS
BEARNI (/30)	13.0±4.2	15.2±5.2	12.4±8.3	NS
MoCA score (/30)	25.4±4.4	24.9±6.0	23.3±4.8	NS
Length of hospital stay (days)	23.6±7.2	18.0±4.5	19.1±2.8	NS
Total protein (g/L)	67.0±8.6	66.4±7.7	65.9±4.5	NS
Albumin (g/L)	36.0±6.9	38.3±3.5	37.4±3.0	NS
Prealbumin (g/L)	0.27±0.10	0.29±0.07	0.24±0.06	NS
Variation of prealbumin concentration during alcohol withdrawal (g/L)	-0.08±0.07 \$	-0.03±0.05	+0.02±0.07	p<0.05
Variation of albumin concentration during alcohol withdrawal (g/L)	-0.8±6.0	+1.4±2.1	+1.6±5.6	NS
γ-glutamyl-transpeptidase (U/L)	859.8±1261.2	287.1±362.6	179.6±326.2	NS
Ammonium (µmol/L)	45.9±30.1	45.3±26.3	37.3±10.5	NS
ASAT (U/L)	76.2±60.2	69.4±54.0	61.3±73.6	NS
ALAT (U/L)	30.7±17.6	53.9±46.9	68.9±114.4	NS
ASAT/ALAT ratio	2.6±1.3	1.6±0.8	1.4±0.8	p=0.052 Tert.1 vs Tert.3
Bilirubin (µmol/L)	34.6±23.2	21.6±13.7	19.1±9.7	NS
α2 macroglobulin (g/L)	2.2±0.5	1.8±0.6	2.2±0.6	NS
Hyaluronic acid (µg/L)	239.1±521.4	86.3±184.9	38.9±30.7	NS
Score of fibrosis (HEPASCORE)	0.59±0.34	0.34±0.29	0.44±0.29	NS
Serum creatinin (µmoles/L)	56.7±8.8	65.0±15.1	64.0±12.9	NS
Urea (mmoles/L)	2.5±0.9	2.8±1.1	2.6±0.9	NS
Prothrombin time (%)	96.3±8.2	96.5±10.7	96.1±6.6	NS
Haemoglobin (g/dL)	13.5±1.3	14.9±1.4	13.8±1.3	NS
Mean Cellular Volume (µm ³)	99.1±6.3	101.1±5.0	94.8±5.8	NS

Platelets (G/L)	167.4±68.7	195.4±66.8	251.2±92.8	p=0.07 Tert.1 vs Tert.3
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§: p<0.05 when compared to tertile 3.

Supplemental data - Table S3.A and S3.B

A. Pearson correlations of TMAO concentrations with biological and clinical parameters at the entry.

Variable	r	p
Alpha 2 macroglobulin	-0.5831	0.0007
Glycated haemoglobin	0.6477	0.0008
Natremia	0.4669	0.009
Total protein	-0.4597	0.01
ASAT/ALAT ratio	-0.3992	0.031
Glycemia	0.383	0.037
Hepascore	-0.382	0.037

B. Predictors of TMAO concentration from stepwise multiple linear regression analyses.

All variables with a statistically significant correlation were entered in the regression model, and only predictors of the final solution are presented in the table.

Variable entered	β	(95%CI)	p
Alpha 2 macroglobulin	-0.497	(-0.741;-0.253)	0.000697
ASAT/ALAT ratio	-0.454	(-0.694;-0.214)	0.001353
Natremia	0.423	(0.181;0.665)	0.002502
	adjusted r^2		p
Whole model	0.7059		0.0000127

Supplemental data - Table S4.A and S4.B

A. Pearson correlations of IS concentrations with biological and clinical parameters at the entry.

Variable	r	p
AUDIT	-0.5202	0.0032
Number of meal/day	0.4734	0.0082
Platelets	0.3806	0.038
ASAT/ALAT ratio	-0.3727	0.0465

B. Predictors of IS concentration from stepwise multiple linear regression analyses.

All variables with a statistically significant correlation were entered in the regression model, and only the predictor of the final solution is presented in the table.

Variable entered	β	(95%CI)	p
AUDIT	-0.517	(-0.847;-0.187)	0.004058
	adjusted r ²		p
Whole model	0.2404		0.004058

Supplemental data - Table S5.A and S5.B

A. Pearson correlations of variations of serum prealbumin during alcohol withdrawal with others continuous variables.

Variable	r	p
Platelets	0.5878	0.001
Total protein	-0.5843	0.001
GGT (Log)	-0.5028	0.007
ASAT	-0.5024	0.012
Indoxyl sulfate	0.4655	0.014
Potassium	0.4646	0.014
ASAT/ALAT ratio	-0.5024	0.015
Total bilirubin	-0.4566	0.017
Albuminemia	-0.4498	0.018
Hyaluronic acid (Log)	-0.4488	0.019
Cholesterol	-0.4303	0.025
Hepascore	-0.4259	0.027

B. Predictors of variation of serum prealbumin during alcohol withdrawal from stepwise multiple linear regression analyses.

All variables with a statistically significant correlation were entered in the regression model, and only predictors of the final solution are presented in the table.

Variable entered	β	(95%CI)	p
Albuminemia	-0.527	(-0.759;-0.295)	0.00016
Hyaluronic acid (Log)	-0.487	(-0.727;-0.247)	0.000516
Indoxyl sulfate	0.408	(0.170;0.646)	0.002363
	adjusted r^2		p
Whole model	0.668		0.00000433

Supplemental data - Table S6.A and S6.B

A. Pearson correlations of length of hospital stay with others continuous variables.

Variable	r	p
Albuminemia	-0.496	0.005
Glycated haemoglobin	-0.5602	0.0054
TMAO (Log)	-0.4841	0.0067
Prealbuminemia	-0.4331	0.019
Natremia	-0.4226	0.02
Hepascore	0.4163	0.022
Alkaline phosphatase	0.3775	0.0397

B. Predictors of length of hospital stay from stepwise multiple linear regression analyses.

All variables with a statistically significant correlation were entered in the regression model, and only the predictor of the final solution is presented in the table.

Variable entered	β	(95%CI)	p
TMAO (Log)	-0.622	(-0.972;-0.272)	0.001996
	adjusted r ²		p
Whole model	0.3562		0.001996