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RESEARCH ARTICLE

DIET-DERIVED TRIMETHYLAMINE N-OXIDE (TMAO) PATHWAY DISRUPTION IN PATIENTS WITH CONCURRENT ALCOHOL USE DISORDER AND SMOKERS PERSISTS AFTER ALCOHOL DETOXIFICATION

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ABSTRACT

Purpose: Trimethylamine N-oxide (TMAO), a gut-microbiota-derived metabolite, is associated with cardiometabolic diseases, and therapeutic strategies targeting TMAO reduction are gaining interest for disease management. This study aimed to compare serum TMAO levels in patients with alcohol use disorder (AUD) and smokers versus healthy controls, and to assess changes following medically supervised alcohol detoxification.
Material and Methods: Demographic data, biochemical parameters, and serum TMAO levels were analyzed. These parameters were quantified in healthy controls and patients with AUD and smokers before and after alcohol detoxification.
Results: The study included thirty-four healthy controls (six females and 28 males) and thirty-three patients (five females and 28 males) with AUD and smokers. At baseline, patients demonstrated significantly lower TMAO levels but higher levels of ferritin, CRP, AST, ALT, GGT, and MCV compared to healthy controls. Following detoxification, patients exhibited a further decrease in TMAO levels (p=0.024), alongside significant improvements in hepatic enzymes (AST and GGT) and ferritin levels (p<0.001).
Conclusion: These findings provide novel evidence that AUD combined with smoking is associated with markedly reduced TMAO levels, and that detoxification treatment leads to a further decline. This suppression likely reflects underlying gut dysbiosis, impaired hepatic function, and malnutrition, and persists even after detoxification.

Keywords: Alcoholism, dysbiosis, inflammation, smoking, trimethylamine N-oxide

INTRODUCTION

Smoking and alcohol consumption are established contributing factors to the various health complications, including cardiovascular and cerebrovascular diseases, gastric ulcers, and cancers (1). Smoking is highly prevalent among individuals with alcohol use disorder (AUD), and the concurrent use of both substances can exacerbate these health risks (2). Furthermore, alcohol consumption may potentiate the harmful effects of tobacco smoking (3). The pathophysiology of alcohol-related diseases may involve changes in the gut microbiota composition (4). An increasing amount of research indicates that gut microbiota may play a role in the development of alcohol-related diseases (5-9). AUD may lead to disruption of the gut microbial balance, dysbiosis, resulting in increased intestinal barrier permeability (10), and altered brain and liver function (11). In a study, AUD led to increased intestinal permeability, facilitating the entry of gut microbiota metabolites into the portal venous bloodstream (12). Increased intestinal permeability due to AUD may result in alterations in the production and systemic release of gut microbiota metabolites, which can contribute to the development of the disease.

Trimethylamine N-oxide (TMAO) is a gut microbiota-derived metabolite that is produced in the liver (13). The TMAO production is catalysed by trimethylamine (TMA) lyase, an enzyme of the gut microbiota, and hepatic flavin monooxygenase-3 (FMO3) enzyme (13). TMAO has been linked to the pathogenesis of cardiometabolic diseases, cancer, inflammatory conditions, and neurological disorders (13-15). It has also been proposed as a potential risk factor for non-alcoholic fatty liver disease (16), with the potential to trigger the onset of this condition due to metabolic dysfunction (17). Excessive alcohol consumption and smoking lead to dysbiosis (18,19), which may result in a decrease in the number of TMA-producing bacteria, and TMAO production may be affected in individuals who smoke. Therefore, both alcohol and smoking can modulate TMAO production by altering gut microbiota composition. Recent studies have investigated the TMAO in alcohol-related disorders, however, the findings of these studies vary (10,20). In a study, serum TMAO levels were found to be slightly elevated at the onset of alcohol withdrawal (10), while another study in patients with alcohol-related hepatitis found levels to be low (20). Despite the high prevalence of smoking among individuals diagnosed with AUD, research evaluating TMAO levels in patients who consume both alcohol and cigarettes is scarce. To the best of our knowledge, no study has been published on the effect of co-consumption of alcohol and cigarettes and detoxification treatment on circulating TMAO levels. Therefore, this study aimed to evaluate serum TMAO levels in individuals with concurrent AUD and smoking before and after alcohol detoxification, to determine whether detoxification reverses TMAO metabolic disruption.

MATERIAL AND METHODS

This retrospective study was approved by the Izmir Katip Çelebi University Non-Interventional Clinical Research Ethics Committee (Decision No: 0397) and was conducted in accordance with the principles of the Declaration of Helsinki. Blood samples previously collected for another study before detoxification (considered as baseline) and on day 14 ± 3 of the detoxification periods were used in this study. These samples were stored at -80°C for three months prior to the day of study.

Study Design and Population

In the previous study, patients aged between 18 and 65 who were admitted to the Alcohol-Drug Addiction Research, Treatment and Training Center of Izmir Atatürk Training and Research Hospital consumed more than four drinks/day for at least ten years and were diagnosed with AUD according to DSM-V criteria, were included. Patients who had smoked in the previous 6 months were selected in our study. Pregnant patients, patients under 18 years old, and patients with liver disease, kidney disease or AIDS were excluded. Serum samples were also obtained from healthy controls who reported not consuming alcohol and cigarettes over the past six months. Informed consent was obtained from patients and healthy controls for inclusion in that study. The age, sex, and smoking status were documented for each individual. In the present study, the before and after detoxification treatment results of the same AUD patients were compared to reduce the impact of individual differences. For this reason, an AUD group that did not receive detoxification treatment was not included.

Alcohol Detoxification Protocol

Patients with AUD experience alcohol withdrawal symptoms in response to the abrupt reduction or cessation of alcohol consumption. These symptoms

were managed using an alcohol detoxification protocol, and benzodiazepines are used as the gold standard (21). Alcohol withdrawal severity was assessed using the Clinical Institute Withdrawal Assessment for Alcohol Scale, Revised (CIWA-Ar) (22). Patients with moderate or severe withdrawal symptoms received a tapering course of diazepam, initiated at 30–50 mg/day.

The dosage and duration of the treatment were determined by the clinical evaluation based on the CIWA-Ar scores. Following withdrawal symptoms stabilized (typically within 2–3 days), the diazepam dose was gradually reduced and discontinued over a period of 7–10 days. Circulating folate and vitamin B12 levels were measured, and deficiencies were corrected with vitamin B complex supplements.

Measurement of Routine Biochemical Parameters

The results of the routine serum biochemical tests [hemoglobin, albumin, and C reactive protein (CRP)] were quantified utilizing the spectrophotometric method (AU5200, Beckman, USA). Ferritin levels were determined using a chemiluminescence immunoassay (DxI 800, Beckman, USA). Indirect and nonspecific AUD biomarkers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transferase (GGT) (23,24) were measured spectrophotometrically (Beckman Coulter 5800, USA) both before and after the detoxification period.

Mean corpuscular volume (MCV), an indirect and nonspecific biomarker of AUD, was measured using the sheath-flow DC detection method before detoxification (SYSMEX XN-1000, Japan). Post-detoxification MCV levels were not measured as these levels do not change over the life of the erythrocytes (120 days). All of the aforementioned parameters were also obtained from healthy controls.

Determination of Serum TMAO Levels

Serum TMAO levels were determined by the ELISA method (human TMAO ELISA kit-E4733Hu, Bioassay Technology Lab, China) before and after detoxification in AUD patients and healthy controls. The modified ELISA kit protocol was used in this study (25). Briefly, standards were added to the standard wells, and serum samples and biotinylated antibodies were added to the sample wells. After an incubation period of 90 minutes at 37°C and three washes, streptavidin-horseradish peroxidase was added, and the mixture was incubated at 37°C for a further 30 minutes. Then, five washes were performed, and the substrate solutions were added. The solutions were then incubated at 37°C for 20 minutes. Immediately after adding the stopping solution, the absorbance was measured at 450 nm using BioTek ELX800 (USA).

Statistical Analysis

The required sample size was calculated using the G*Power 3.1® package program, with the study conducted by Coulbault et al. (10) used as a reference. A minimum of 24 participants were included in the study, with 12 in each group. This was in accordance with the criteria of 90% power and 5% type 1 error rate.

The Shapiro-Wilk test was used to evaluate the normal distribution of the data, and it was determined that the data did not fit the normal distribution model. Descriptive statistics were presented as percentages and the median [25th-75th percentile (Q1-Q3)]. The chi-square test was used for categorical variables, while the Mann-Whitney U and Wilcoxon signed-rank tests were used for continuous variables. GraphPad Prism® software (USA) and IBM SPSS® (USA) were used for statistical analysis and for generating corresponding graphs. *p* values less than 0.05 were considered statistically significant.

RESULTS

Characteristics of healthy controls and patients with alcohol use disorder and smokers

This study included 34 healthy controls and 33 patients. The healthy controls and patients were selected based on their age and sex distribution (p=0.665 and p=0.783, respectively). The median age of the control group was 48.50 [33.75-54.00], whereas in the patient group it was 45.00 [38.00-56.00]. The healthy control group included six females (17.65%), while the patient group consisted of five (15.15%). Regarding the socio-demographic characteristics of the participants in our study, the patient and control groups have similar age and sex distributions, so the effect of these variables on the findings has not been considered.

Routine serum biochemical parameters before and after alcohol detoxification treatment

The hemoglobin, albumin, ferritin, and CRP levels are presented in Table 1. Before the detoxification period, patients exhibited statistically lower levels of hemoglobin and albumin than the healthy controls.

Table 1. Serum biochemical parameters in healthy controls, and before and after alcohol detoxification treatment in patients with AUD and smokers.

	Healthy controls	Before detoxification in patients	After detoxification in patients	*p*-value
Hemoglobin (g/dl)	14.15 [13.48-15.10]	13.25 [12.60-14.58] *	ND	0.016 (Healthy controls vs. before in patients)
Albumin (g/L)	44.70 [42.00-47.13]	40.50 [38.25-43.00] ***	40.00 [36.00-42.10] ***	*p*<0.001 (Healthy controls vs. before in patients) *p*<0.001 (Healthy controls vs. after in patients) 0.387 (Before vs. after in patients)
Ferritin (µg/L)	34.82 [17.31-65.17]	131.00 [67.50-327.5] ***	51.05 [24.19-82.25] ###	*p*<0.001 (Healthy controls vs before in patients) 0.127 (Healthy controls vs. after in patients) *p*<0.001 (Before vs. after in patients)
CRP (mg/L)	2.13 [0.69-3.72]	7.30 [1.96-14.19] ***	5.34 [2.33-11.07] ***	0.0004 (Healthy controls vs. before in patients) 0.0007 (Healthy controls vs. after in patients) 0.064 (Before vs. after in patients)
AST (U/L)	19.00 [17.00-23.50]	52.00 [28.50-94.00] ***	32.50 [20.75-52.00] ***, ##	*p*<0.001 (Healthy controls vs. before in patients) *p*<0.001 (Healthy controls vs. after in patients) *p*=0.002 (Before vs. after in patients)
ALT (U/L)	19.50 [13.75-29.25]	34.00 [24.50-50.00] ***	18.00 [12.50-45.00]	*p*<0.001 (Healthy controls vs. before in patients) 0.448 (Healthy controls vs. after in patients) 0.179 (Before vs. after in patients)
GGT (U/L)	23.50 [12.75-33.25]	83.00 [42.50-381.05] ***	71.00 [28.00-194.50] ***, ###	*p*<0.001 (Healthy controls vs. before in patients) *p*<0.001 (Healthy controls vs. after in patients) *p*<0.001 (Before vs. after in patients)
MCV (fL)	86.70 [83.40-89.85]	95.70 [89.95-101.30] ***	ND	*p*<0.001 (Healthy controls vs before in patients)
TMAO (ng/mL)	23.19 [12.76-78.62]	8.43 [6.51-10.76] ***	7.74 [5.29-10.11] ***, #	*p*<0.001 (Healthy controls vs. before in patients) *p*<0.001 (Healthy controls vs. after in patients) 0.024 (Before vs. after in patients)

Abbreviations: AUD, alcohol use disorder; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C reactive protein; GGT, γ-glutamyl transferase; MCV, mean corpuscular volume; ND, Not done; TMAO, trimethylamine N-oxide. The Mann Whitney U test was used for statistical analysis in comparison to healthy controls and patients and the Wilcoxon Signed Rank test was used for the statistical analysis in comparison to patients before and after alcohol detoxification treatment. Values were given as median [Q1-Q3]. $^{*}p < 0.05$ and $^{***}p<0.001$ for healthy controls vs. before or after detoxification in patients and $^{\#}p < 0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ for before vs. after detoxification in patients.

Ferritin and CRP levels were significantly higher in the AUD group than in the healthy controls before detoxification (Figure 1). A statistically significant decrease was observed in ferritin levels (Figure 1a) after the detoxification treatment. Conversely, there was no statistically significant change in albumin and CRP levels following the detoxification treatment among AUD patients (Figure 1b).

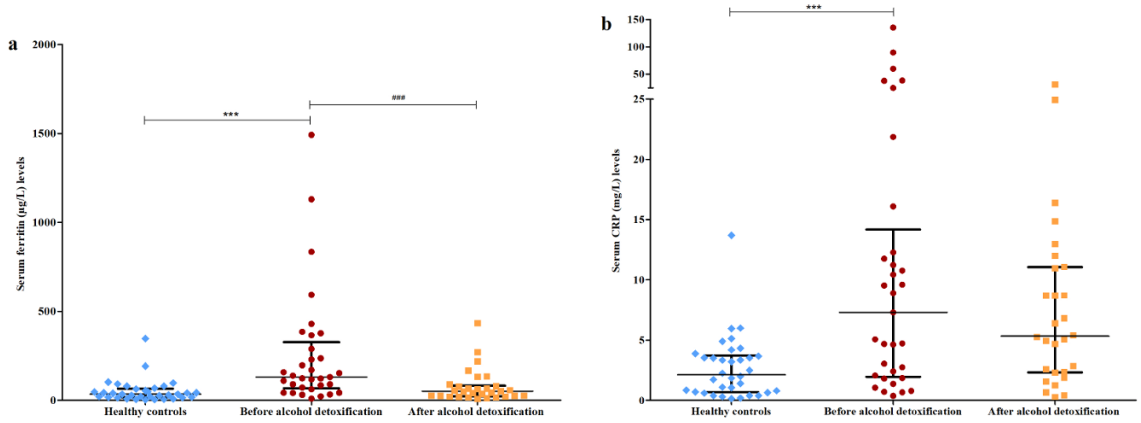


Figure 1. The serum levels of ferritin (a) and CRP (b) in the healthy controls and in patients with AUD and smokers. Data were shown as median [IQR]. ***p<0.001 for healthy controls vs. before alcohol detoxification. ###p<0.001 for before vs. after alcohol detoxification. Abbreviations AUD, alcohol use disorder; CRP, C reactive protein; IQR, interquartile range.

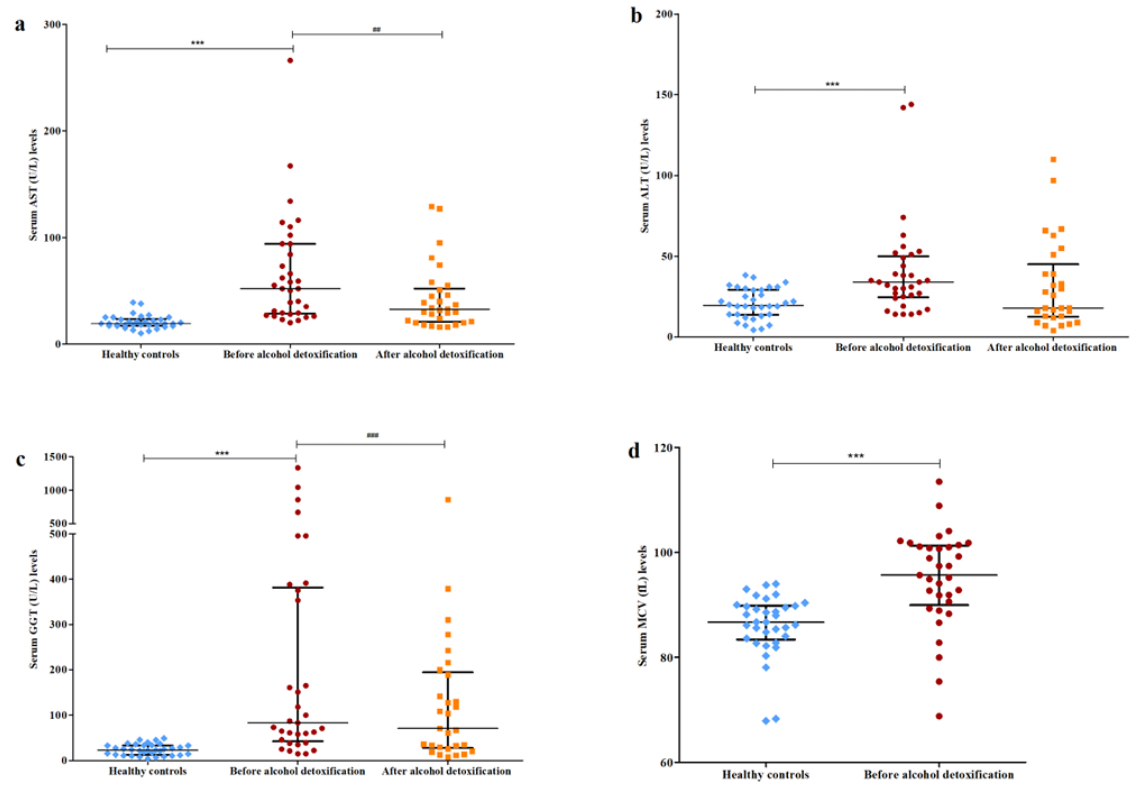


Figure 2. The serum levels of AST (a), ALT (b), GGT (c), and MCV (d) in the healthy controls and in patients with AUD and smokers. Data were shown as median [IQR]. ***p<0.001 for healthy controls vs. before alcohol detoxification. ##p<0.01 and ###p<0.001 for before vs. after alcohol detoxification. Abbreviations AUD, alcohol use disorder; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; IQR, interquartile range; MCV, mean corpuscular volume.

Serum levels of alcohol use disorder biomarkers before and after alcohol detoxification treatment

The median [Q1-Q3] values of AUD markers, including serum AST, ALT, GGT, and MCV levels, are presented in Table 1. The patients exhibited elevated AST, ALT, GGT, and MCV levels compared to the healthy controls (Figure 2a-d). Following detoxification, a statistically significant reduction in AST and GGT levels was observed (Figure 2a-c). On the other hand, the decrease in serum ALT levels after detoxification treatment was not statistically significant.

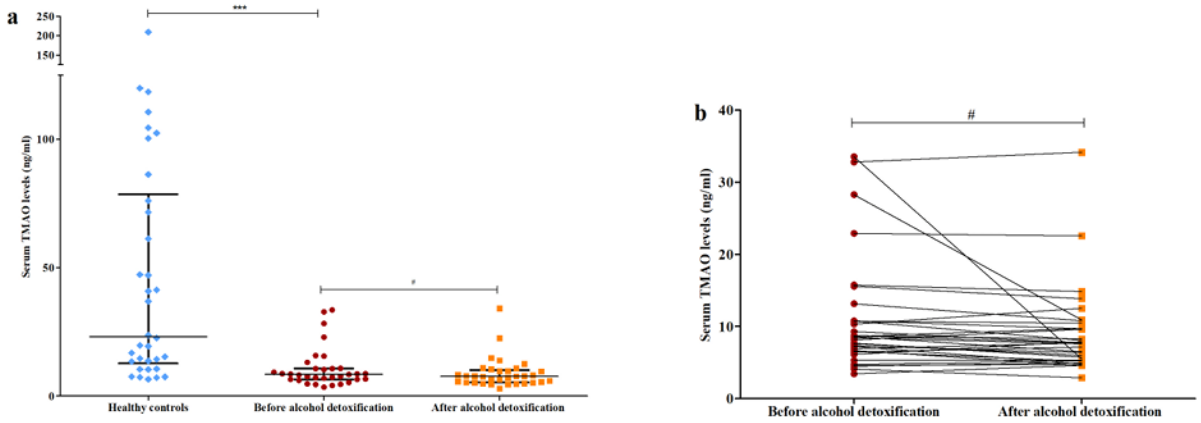


Figure 3. Comparison of serum TMAO levels in healthy controls and patients with AUD and smokers before and after alcohol detoxification. Data were shown as median [IQR]. ***p<0.001 for healthy controls vs. before alcohol detoxification. $^{\#}p$<0.05 for before vs. after detoxification in AUD group. Abbreviations AUD, alcohol use disorder; IQR, interquartile range; TMAO, trimethylamine N-oxide.

Serum TMAO levels before and after alcohol detoxification treatment

Serum TMAO levels were found to be statistically significantly higher in the healthy controls than in the AUD group both before and after detoxification treatment (Table 1 and Figure 3). Following detoxification treatment, statistically significant reductions in serum TMAO levels were observed in the AUD group (Figure 3).

DISCUSSION

Key findings of this study are (a) Serum TMAO levels were lower in the patients with AUD and smokers than healthy controls, (b) After alcohol detoxification, a considerable reduction in serum TMAO levels were observed in patients with AUD and smokers, (c) Before alcohol detoxification, patients had lower serum hemoglobin and albumin levels and higher serum ferritin, CRP, AST, ALT, GGT, and MCV levels, (d) Following alcohol detoxification, a substantial decrease was observed in ferritin, AST, and GGT levels.

In a study examining serum TMAO levels at the onset of alcohol withdrawal, mean TMAO levels were found to be slightly elevated in patients with AUD than in the control group (10). However, considerable interindividual variability in serum TMAO concentrations was observed, with the detection of both very low and very high TMAO concentrations. This study revealed that serum TMAO concentrations were markedly low in most AUD patients and that a greater number of biological abnormalities were observed in these subjects. On the other hand, high serum TMAO levels have been suggested to be associated with the severity of alcoholic liver disease in another study (26). In addition, another study was conducted on patients with alcohol-related hepatitis; during this study, choline metabolites were determined (20). While TMA levels were found to be elevated in these patients, TMAO levels were low (20). Consistent with this finding, our study observed that patients with AUD-smoking also exhibited lower serum TMAO levels compared to healthy controls. Dietary deficiencies in TMAO precursors such as choline, betaine, and carnitine (27) and malnutrition, as indicated by low albumin levels (28), could potentially contribute to the observed low TMAO levels in these patients. FMO3 activity suppression in patients has been hypothesised as a potential cause of low TMAO levels, and further research is required to validate this hypothesis. This suppression would result in inhibition of the conversion of TMA to TMAO. The findings showing decreased FMO3 mRNA levels in the liver of patients with alcohol-related hepatitis (20), and findings suggesting a competitive role of the FMO3 enzyme in nicotine metabolism in smokers (29,30), suggest that this suppression may be due to these factors. The preclinical phase of the previous study proposed that the inhibition of TMA production by the administration of non-lethal bacterial choline TMA lyase inhibitors in mice resulted in the prevention of ethanol-induced liver damage (20). As a result, TMA has been identified as a contributing factor in alcohol-related hepatitis, and therapeutic approaches targeting TMA production have been proposed that may be beneficial for these patients.

Another study indicated that patients with alcoholic hepatitis exhibited elevated breath TMA levels, with a moderate correlation between TMA levels and the severity of alcoholic hepatitis (31). However, this study excluded patients who smoked, which limits the generalizability of these findings (31).

The gut-liver axis is suggested to exert an influence on the inflammation, intestinal immune response, and intestinal barrier function and is implicated in the pathogenesis of alcohol-related liver disease (32). AUD and smoking have been associated with increased levels of inflammatory markers, including CRP and ferritin (33-36). Consistent with these findings, the present study also observed elevated serum CRP and ferritin levels in patients compared to healthy controls, indicating the presence of systemic inflammation. TMAO has been proposed to be involved in the inflammation pathogenesis through the induction of the release of pro-inflammatory cytokines and the triggering of oxidative stress (37). However, interestingly, healthy individuals have higher TMAO levels compared to patients with AUD and smokers in our study. Following alcohol detoxification treatment with diazepam, a significant decrease was observed in serum TMAO and ferritin levels; however, a decrease in serum CRP levels was not statistically significant. While these findings may be partly attributable to the potential anti-inflammatory properties associated with benzodiazepines (38-40), the elevation in ferritin levels has also been shown to be reversible within approximately 15 days of alcohol cessation (41). Hence, abstaining from alcohol during this period likely played a crucial role in mitigating the inflammatory response, particularly by reducing ferritin levels.

The AUD is known to cause an elevation in the levels of AST, ALT, GGT, and MCV. These elevated levels are observed to return to within the normal range a few weeks after alcohol consumption has ceased. While the typical range for AST and ALT is 2-4 weeks, and for GGT, 2-6 weeks, the mean time for the MCV level to return to normal is 8-16 weeks (42). In our study, serum levels of AST, ALT, and GGT were quantified before and following a nearly two-week of detoxification period in patients. Elevated serum AST levels have been observed to normalize in patients following detoxification treatment. Additionally, a substantial decrease of approximately 50% in Q3 values was observed in the GGT levels. Notably, serum ALT levels were within the normal range in patients. The serum MCV level was found to be elevated in patients. However, since MCV reflects approximately 120 days of erythropoiesis, the measurement was not repeated due to the expectation of no change during the two-week detoxification period.

Limitations

This is a retrospective study, with limitations such as potential selection bias and unmeasured confounding factors to be taken into account when interpreting the findings. The single-center setting and modest sample size underscore the need for validation in larger cohorts. In addition, the male-dominated sex distribution of participants may limit the generalizability of the findings, particularly regarding TMAO metabolism. The absence of a control group of patients with AUD not receiving detoxification treatment restricts the causal interpretation of these findings. Confounding variables could not be fully assessed due to the data constraints. These include dietary composition, particularly the intake of TMAO precursors such as choline and carnitine, as well as renal function, a key determinant of TMAO clearance. Despite the smoking status being documented, the absence of data on the number of cigarettes smoked per day and the duration of smoking limits a comprehensive understanding of the effect of cigarette exposure on TMAO metabolism. Decreased hepatic FMO3 activity in alcoholic liver disease is attributed to changes in TMAO metabolism; however, the present study was limited in directly assessing this mechanism due to the absence of serum TMA measurements or FMO3 expression data. Finally, variability in dosage and duration in personalised detoxification based on CIWA-Ar scores limited the ability to conduct a comprehensive analysis of treatment effects within the scope of this study.

CONCLUSION

AUD and smoking are preventable risk factors for non-communicable diseases, and the prevalence of cigarette consumption is notably higher among patients diagnosed with AUD (43). The present study is, to the best of our knowledge, the first to evaluate TMAO levels in patients with AUD and smokers before and after alcohol detoxification. Significantly lower TMAO levels were found in these patients compared to the healthy control group. Notably, alcohol withdrawal management with a benzodiazepine-based detoxification protocol was

associated with a further reduction in serum TMAO levels alongside improvements in inflammatory parameters. These findings suggest a potential interaction between detoxification treatment and TMAO levels; however, the retrospective design limits the establishment of causal relationships. Nevertheless, the results of this study contribute to this field, where research has been limited, and serve as a guide for future studies. Further studies with larger sample sizes, standardized protocols, and direct measurement of TMA, precursor of TMAO, and FMO3 activity are required to elucidate the underlying mechanisms and to assess the potential of the TMAO pathway as a therapeutic target in patients with AUD and smokers.

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Conflict of interests: The authors declare no conflict of interest.
Ethical approval: The Izmir Katip Celebi University Non-Interventional Clinical Research Ethics Committee granted approval for this study (Date: 21.09.2023, Decision No: 0397).
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