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Elevated serum trimethylamine N-oxide (TMAO) and trimethyllysine in patients with amyotrophic lateral sclerosis (ALS): An exploratory case–control study

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ABSTRACT

Trimethylamine N-oxide (TMAO) is a gut microbiota–derived metabolite implicated in protein homeostasis, inflammation, and chronic disease, but its relevance in amyotrophic lateral sclerosis (ALS) remains poorly characterized. In this exploratory pilot study, we quantified circulating TMAO and related trimethylammonium-containing compounds in a Sardinian ALS cohort using targeted LC–MS/MS. Serum samples were collected under fasting conditions from 12 ALS patients and 8 age- and sex-matched healthy controls. Median serum TMAO levels were markedly higher in ALS patients than in controls (27.9 vs. 4.0 μmol/L, $P < 0.05$), with substantial inter-individual variability in the ALS group (range 2.4–125.0 μmol/L). Trimethyllysine (TML) concentrations were also significantly elevated in ALS (0.43 vs. 0.34 μmol/L, $P < 0.05$), whereas choline, carnitine, betaine, ergothioneine, and γ-butyrobetaine levels did not differ between groups. Most ALS patients were receiving acetyl-L-carnitine (ALCAR) supplementation, suggesting that ALCAR intake may contribute to the observed metabolite profiles. Overall, these findings indicate alterations in trimethylammonium-containing compound metabolism in ALS and underscore the need for larger, well-controlled studies to determine whether such changes reflect disease-related mechanisms, treatment effects, or their interaction.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that typically manifests between 50 and 70 years of age (González-Sánchez et al., 2025; Ingre et al., 2015). It is characterized by degeneration of upper and lower motor neurons in the spinal cord, brainstem, and cortex (Khamaysa et al., 2025; Masrori and Van Damme, 2020). Clinical onset is often subtle, with symptoms such as cramps, fasciculations, weakness, and dysarthria, which gradually progress to loss of voluntary motor control and respiratory failure (Hu et al., 2019; Raymond et al., 2019; Keon et al., 2021; Zarei et al., 2015). Median survival is approximately three years after diagnosis, although clinical course is highly variable (Chiò et al., 2009; Vasta et al., 2025). ALS arises from a complex interplay of genetic and environmental factors. About 10 % of cases are familial and linked to mutations in genes such as C9orf72, SOD1, FUS, and TARDBP, while the remaining majority are sporadic (Vasta et al., 2022; Nijs and Van Damme, 2024; Huang et al., 2024). Pathologically, abnormal cytoplasmic aggregation of TDP-43 is observed in most cases, contributing to RNA processing dysfunction and neurodegeneration (Scotter et al., 2015; Suk and Rousseaux, 2020; Zhao

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and Souayah, 2025). Environmental exposures and lifestyle factors, together with emerging evidence on gut microbiota alterations, have also been implicated in disease risk and progression (Duan et al., 2023; Yu et al., 2014; D'Antona et al., 2021; Fontdevila et al., 2024). Microbial metabolism of dietary trimethylammonium-containing compounds, including choline (CHOL) and carnitine (CAR), produces trimethylamine (TMA), which is converted in the liver into trimethylamine-N-oxide (TMAO) by flavin-containing monooxygenase 3 (FMO3) (Zhen et al., 2023; Gatarek and Kaluzna-Czaplinska, 2021; Ilyas et al., 2022; Li et al., 2018; Robinson-Cohen et al., 2016). Elevated TMAO has been linked to cardiovascular, metabolic, and neurodegenerative conditions, partly through effects on inflammation, oxidative stress, and protein homeostasis (Jang et al., 2024; Cristian et al., 2024). However, little is known about TMAO metabolism in ALS. A hospital-based case–control study conducted in China involving 160 ALS patients reported significantly decreased levels of TMAO, CHOL, and gBB compared with age- and sex-matched healthy controls, while CAR and TMG concentrations were increased (Chen et al., 2020). Based on these considerations, we conducted an exploratory case–control study to assess serum TMAO and related precursors, including CHOL, CAR, trimethylglycine (TMG), Nε,Nε,Nε-trimethyllysine (TML), γ-butyrobetaine (gBB), and ergothioneine (ERT), in ALS patients compared with healthy controls. Unexpectedly, we observed markedly elevated TMAO concentrations in ALS patients. This prompted us to analyze possible explanatory factors, and we found that nearly all patients were receiving acetyl-L-carnitine (ALCAR), raising the possibility that supplementation contributed to the observed metabolite profiles.

Methods

Study population

This exploratory cross-sectional case–control study included 20 participants residing in the Sulcis-Iglesiente area, a historical subregion of southwestern Sardinia (Italy), enrolled between December 2023 and April 2024 at the tertiary referral center for ALS of the University of Cagliari. The study population comprised 12 ALS patients and 8 healthy controls. ALS patients (5 females, 7 males; age 48–83 years) were diagnosed according to the revised El Escorial criteria (Brooks et al., 2000) and classified as possible (n = 3), probable (n = 6), or definite (n = 3). Clinical phenotypes included spinal (n = 8) and bulbar onset (n = 4) (Chiò et al., 2011). Disease severity was assessed with King's staging system (Roche et al., 2012): 3 patients were at stage 1, 5 at stage 2, and 4 at stage 3. Cognitive and behavioral status was evaluated with the Edinburgh Cognitive and Behavioural ALS Screen (ECAS) (Poletti et al., 2016), excluding 2 patients who could not complete testing due to severe disease-related limitations. The mean ECAS score was 109.9 ± 10.3 (range 95–123). Genetic testing identified pathogenic variants in 7 of 12 patients: 5 carried TARDBP mutations and 2 a C9orf72 expansion. These mutations were found in both familial and sporadic cases. Most patients (n = 8) were treated with riluzole, administered either as tablets or liquid suspension, as part of standard therapy. Nearly all (n = 11) were receiving acetyl-L-carnitine (ALCAR) supplementation, and several were also taking additional nutritional or neuroprotective agents, including palmitoylethanolamide (sometimes combined with luteolin), alpha-lipoic acid, tauroursodeoxycholic acid (TUDCA), and essential amino acid mixtures. Additional medications were prescribed as required for comorbid conditions. Healthy controls (4 females, 4 males; age 19–71 years) had no neurological disorders and no current use of CAR-based supplements. Ethical approval was obtained from the Ethics Committee of Sardinia (code: 28, February 26, 2024). The study was conducted in accordance with the Declaration of Helsinki, and all participants provided written informed consent.

Sample collection and analyte measurement

Blood samples were collected in the morning after approximately 10 h of overnight fasting, using serum separator clot activator tubes without anticoagulants and containing a polymer gel separator. After allowing the blood to clot at room temperature for 10 min, the tubes were centrifuged for 10 min at 1900 × g at 4 °C. The upper serum phase was carefully transferred to a new tube and centrifuged again for 15 min at 3000 × g to remove any remaining cellular nucleic acids attached to cellular debris. The resulting serum was aliquoted into 250-μL aliquots and stored at −80 °C until analysis. For measurement, serum aliquots were thawed without previous freeze-thaw cycles and used to measure the analytes by a liquid-chromatography tandem mass spectrometry (LC-MS/MS) method (Sotgia, 2025). Briefly, 100 μL of serum were combined with 200 μL of acetonitrile containing the internal standard d9-TMAO (4 μmol/L). The mixture was vortexed and centrifuged at 17, 000 × g for 5 min. Following centrifugation, 120 μL of the resulting clear supernatant were collected and directly subjected to LC–MS/MS analysis. Instrumental analysis was performed using a Waters Acquity UPLC system coupled to a Waters TQD tandem quadrupole mass spectrometer (Waters Italia, Milan, Italy). Chromatographic separation was achieved on a Zorbax RR HILIC PLUS column (50 mm × 4.6 mm, 3.5 μm; Agilent Technologies) using an isocratic mobile phase consisting of methanol and 1 % aqueous formic acid (75:25, v/v) at a constant flow rate of 0.4 mL/min. A sample volume of 2 μL was injected, with a total run time of 7 min. Mass spectrometric detection was performed in positive electrospray ionization mode using multiple reaction monitoring (MRM). Transitions were optimized using MassLynx IntelliStart and included the following ion pairs: *m/z* 75.97 → 58.9 (TMAO), 162.11 → 102.9 (CAR), 104.02 → 58 (DMG), 104.02 → 60.1 (CHOL), 118.06 → 58.9 (TMG), 230.18 → 127.1 (ERT), 146.13 → 87 (gBB), 189.24 → 84.1 (TML), and 85.02 → 68.1 for the d9-TMAO internal standard. The method demonstrated excellent linearity across all target compounds ($R^2 > 0.995$). Analytical precision was confirmed by low intra- and inter-assay variability, with relative standard deviations (RSD%) averaging 2.88 % and 4.23 %, respectively. Recovery rates ranged from 95 % to 101 %, supporting the robustness and reproducibility of the assay. The method also achieved low limits of detection and quantification, enabling accurate measurement of analytes across physiological and pathophysiological concentration ranges.

Statistical analysis

Continuous data are reported as medians and interquartile ranges (IQR), with minimum and maximum values (range) provided to illustrate interindividual variability. Group comparisons for continuous variables between ALS patients and healthy controls were performed using the Mann–Whitney *U* test, selected for its robustness to non-normal distributions and suitability for small, independent samples. Categorical variables were compared using the chi-square test (χ^2) or Fisher's exact test when expected frequencies were low. A two-sided p-value < 0.05 was interpreted as indicative of statistical significance. Exact p-values from the Mann–Whitney *U* test are reported throughout the manuscript. All analyses were conducted using MedCalc Statistical Software version 23.1.3 (64-bit).

Results

As displayed in Table 1, the ALS cohort comprised 12 patients (5 females, 7 males; median age 59 years, IQR 53–66; disease duration 7–87 months, median 28). Seven (58 %) had comorbidities, most commonly hypertension, while others included nephrolithiasis, diabetes, diverticulosis, secondary epilepsy, glaucoma, benign prostatic hyperplasia, hypothyroidism, gastroesophageal reflux disease, and Hashimoto's thyroiditis. Controls (N = 8; 4 females, 4 males; median age 61 years, IQR 49–63) had comorbidities in 38 % (hypertension, celiac

Table 1
Demographic, clinical, and metabolic characteristics of the study participants. Data are presented as median (IQR) or mean ± standard deviation. Categorical data are presented as N (%). Statistically significant *P*-values ($P < 0.05$) are shown in bold. Abbreviations: NS, not significant.

	ALS Patients (N = 12)	Healthy Controls (N = 8)	P-value
Age (years)	59 [53–66]	61 [49–63]	NS
Sex (Female/Male), N (%)	5 (41.7) / 7 (58.3)	4 (50.0) / 4 (50.0)	NS
Current smoker, N (%)	4 (33.3)	1 (12.5)	NS
Ex-smoker, N (%)	3 (25.0)	3 (37.5)	
Never-smoker, N (%)	5 (41.7)	4 (50.0)	
Presence of comorbidities, N (%)	7 (58.3)	3 (37.5)	NS
Disease duration from onset (months)	28 [7–87]	-	
Phenotype (Spinal / Bulbar), N	8 / 4	-	
King's Stage (stage 1, 2, 3), N	3 / 5 / 4	-	
ECAS Score[1]	109.9 ± 10.3	-	
Genetic status (C9orf72 / TARDBP / Negative), N	2 / 5 / 5	-	
Riluzole treatment, N (%)	8 (66.7)	-	
TMAO, µmol/L	27.91 [8.29–41.38]	3.99 [2.99–6.90]	P < 0.05
TML, µmol/L	0.43 [0.37–0.49]	0.34 [0.31–0.36]	P < 0.05
TMG, µmol/L	39.70 [35.89–51.83]	33.96 [23.70–39.34]	NS
DMG, µmol/L	3.30 [2.98–4.64]	3.66 [3.47–4.64]	NS
CHOL, µmol/L	8.00 [6.90–8.69]	9.30 [8.10–12.85]	NS
gBB, µmol/L	0.62 [0.35–0.87]	0.38 [0.37–0.43]	NS
CAR, µmol/L	32.00 [28.97–38.83]	35.14 [32.53–43.82]	NS
ERT, µmol/L	1.34 [0.90–1.68]	1.27 [0.91–2.13]	NS

disease, osteoporosis). Groups did not differ significantly in sex (P = 1.00), age (P = 0.67), or smoking status (ALS: 33 % current, 25 % ex-, 42 % never; controls: 12.5 % current, 38 % ex-, 50 % never; P = 0.56). Eleven of 12 ALS patients received ALCAR, often with other agents (palmitoylethanolamide often combined with luteolin, alpha-lipoic acid, TUDCA, amino acid mixtures). Eight were also on riluzole (tablet or suspension) therapy. None of the controls received ALCAR or other CAR-based supplements, only medications for comorbidities. ALS patients had significantly higher TMAO than controls (median 27.91 vs 3.99 µmol/L; IQR 8.29–41.38 vs 2.99–6.90; U=15.00; P = 0.0096), with wider ranges (2.4–125.0 vs 1.1–20.3 µmol/L) (Fig. 1). The only patient not receiving ALCAR supplementation had 4.70 µmol/L, within the control range. TML was also significantly elevated in ALS (0.43 vs 0.34 µmol/L; IQR 0.37–0.49 vs 0.31–0.36; U=15.00; P = 0.0096), with greater variability (0.30–0.76 vs 0.27–0.41 µmol/L) (Fig. 2). No other metabolites differed significantly. ERT was 1.34 µmol/L (IQR 0.90–1.68, range 0.36–3.09) in patients vs 1.27 (IQR 0.91–2.13, range 0.58–5.82) in controls (U=47.00; P = 0.97). TMG was 39.70 (IQR 35.89–51.83, range 31.01–72.56) vs 33.96 (IQR 23.70–39.34, range 15.79–44.74) (U=24.00; P = 0.0691). gBB was 0.62 (IQR 0.35–0.87, range 0.25–1.19) vs 0.38 (IQR 0.37–0.43, range 0.36–0.59) (U=30.00; P = 0.1813). CAR was 32.00 (IQR 28.97–38.83, range 19.35–51.49) vs 35.14 (IQR 32.53–43.82, range 22.23–68.22) (U=34.00; P = 0.3054). DMG was 3.30 (IQR 2.98–4.64, range 2.35–6.00) vs 3.66 (IQR 3.47–4.64, range 3.11–6.63) (U=26.00; P = 0.0979). CHOL was 8.00 (IQR 6.90–8.69, range 4.83–17.25) vs 9.30 (IQR 8.10–12.85, range 7.61–13.54) (U=28.00; P = 0.1349). Patients showed broader ranges for most metabolites, though IQRs were generally similar or narrower. Restricting the analysis to the ALCAR-supplemented patient subgroup (n = 11) yielded results comparable to those obtained in the full ALS cohort when compared with controls (n = 8).

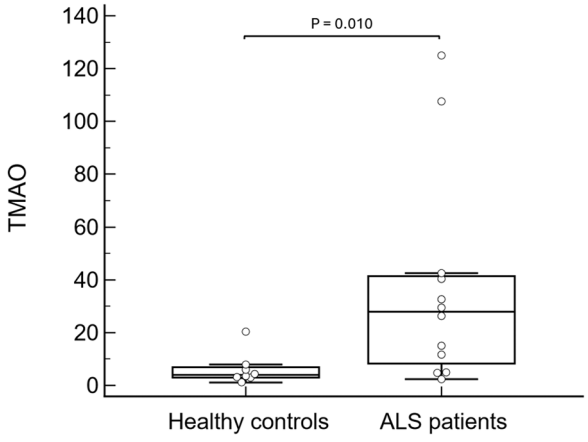

Fig. 1. Comparison of serum TMAO levels between healthy controls and ALS patients.

Discussion

The main finding of this exploratory study is that ALS patients exhibited markedly elevated serum TMAO compared with controls. This observation contrasts with a large Chinese case–control study (>150 patients), which reported reduced TMAO, together with lower CHOL and gBB, and higher CAR and TMG (Chen et al., 2020). To explore possible explanations for our findings, we examined potential contributing factors and found that nearly all patients in our cohort were receiving ALCAR supplementation. Since ALCAR is hydrolyzed to CAR, a known substrate for microbial TMA generation, it may plausibly contribute to elevated TMAO. The only patient not receiving ALCAR showed TMAO levels within the control range, although no definitive conclusions can be drawn from a single observation. Other potential contributors to elevated TMAO, such as impaired renal clearance, were considered. However, none of the patients showed clinical or biochemical evidence of renal dysfunction, renal comorbidities, or ongoing treatment for renal disease. The wide interindividual variability in TMAO levels among ALS patients is noteworthy and may reflect host-dependent differences in microbial TMA production and variability in hepatic FMO3 activity (Robinson-Cohen et al., 2016). Although potential indirect effects of concomitant therapies and comorbidities on TMAO metabolism cannot be entirely excluded, direct evidence for a specific modulation by riluzole or TUDCA remains limited. In contrast, the ALCAR–CAR–microbial TMA–TMAO pathway provides a biologically coherent and parsimonious explanation for the metabolic profile observed in our cohort. Owing to the exploratory nature of the study, dose–response analyses could not be reliably performed, as clinical heterogeneity and practical constraints in standardizing supplementation regimens and the timing of blood sampling prevented such

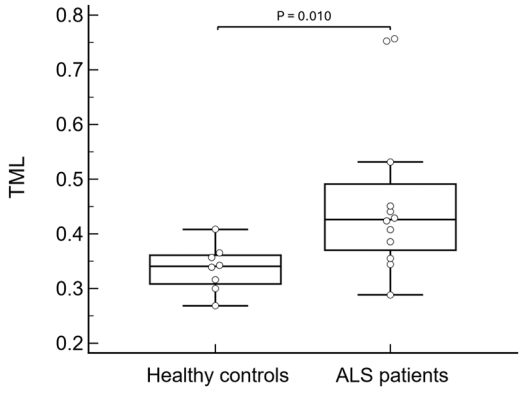

Fig. 2. Comparison of serum TML levels between healthy controls and ALS patients.

assessments. TML was also significantly elevated in ALS patients (median 0.43 μmol/L, IQR 0.37–0.49; range 0.30–0.76) compared with controls (0.34 μmol/L, IQR 0.31–0.36; range 0.27–0.41; P = 0.0096). TML is a methylated lysine derivative abundant in muscle proteins and the first intermediate in the biosynthetic CAR pathway (TML → gBB → CAR) (Maas et al., 2020; Strijbis et al., 2010). Its elevation may therefore reflect neurogenic muscle catabolism, a hallmark of ALS (Parvanovova et al., 2024). The absence of significant increases in downstream intermediates such as gBB and CAR could suggest limited enzymatic conversion or reduced demand for endogenous CAR in the context of exogenous ALCAR supplementation, potentially favoring TML accumulation. Although clinical progression was assessed using disease duration and King's clinical staging, the limited sample size and the ordinal nature of these measures did not allow for robust correlation analyses with circulating TMAO or TML levels. Similarly, no clear or consistent subgroup-specific patterns in TMAO or TML levels emerged when patients were explored according to genotype, clinical phenotype, or disease stage.

The marked elevation of TMAO in ALS raises concerns about its potential biological implications. TMAO is a well-established gut-derived uremic toxin that has been extensively studied for its detrimental effects on cardiovascular and renal function (Andreu-Sánchez et al., 2024; Zhang et al., 2024; Heinrich-Sanchez and Vital, 2025). Elevated levels have been associated with increased oxidative stress, endothelial dysfunction, and fibrosis in several pathological contexts (Caradonna et al., 2025). Accordingly, TMAO may aggravate an already severe disease by promoting comorbid cardiovascular and renal conditions and potentially exerting direct neurotoxic effects. In support of this, a recent meta-analysis of nine studies including 82,246 participants reported a significant association between high circulating TMAO and an increased risk of cognitive decline, with inflammatory signaling, oxidative stress, endoplasmic reticulum stress, and synaptic dysfunction proposed as potential mediators (Long et al., 2024). Notably, TMA, the precursor of TMAO, crosses the blood–brain barrier more efficiently than TMAO and can be partially converted into TMAO within the brain by monoamine oxidases (Zhang et al., 2021; Hoyles et al., 2021). Elevated TMAO has also been detected in the cerebrospinal fluid of both experimental models and humans with neurodegenerative conditions, including Alzheimer's disease and mild cognitive impairment (Vogt et al., 2018). Conversely, in the Chinese ALS cohort (Chen et al., 2020), although overall ALS patients exhibited lower plasma TMAO levels than controls, within the ALS group relatively higher TMAO concentrations were associated with less severe upper motor neuron involvement, suggesting that TMAO may not exert exclusively detrimental effects. Consistent with this notion, in vitro studies have demonstrated that TMAO can act as a chemical chaperone and osmolyte, stabilizing protein conformations and modulating phase behavior under stress conditions, including oxidative and hydrostatic stress (Ma et al., 2014; Pepelnjak et al., 2024; Hill et al., 2002). Importantly, TMAO has been reported to influence the liquid–liquid phase separation (LLPS) of intrinsically disordered proteins such as TDP-43, inhibiting their transition into insoluble fibrillar aggregates (Choi et al., 2018). This ability to modulate phase behavior may be particularly relevant in ALS, where cytoplasmic aggregation of misfolded, ubiquitinated, and phosphorylated TDP-43 disrupts RNA metabolism, axonal protein synthesis, and neuronal homeostasis, ultimately contributing to motor neuron degeneration (Choi et al., 2018; Ormeño et al., 2020).

Conclusion

In conclusion, this exploratory study provides preliminary evidence of altered trimethylamine-related metabolism in ALS, with unexpectedly high serum TMAO levels. Elevated TML was also observed, which may reflect increased muscle protein turnover characteristic of ALS. The widespread use of ALCAR supplementation in our cohort suggests a plausible contribution to these findings, although causality cannot be established. It is also unlikely that elevated TMAO levels were secondary to impaired renal function, as none of the patients had renal comorbidities and no concomitant increase was observed in other metabolites sensitive to renal clearance, such as TMG. Furthermore, considering that the large Chinese cohort study reported reduced TMAO levels in ALS patients compared with controls, the increase observed in our cohort is unlikely to be solely attributable to ALS pathology itself. However, given the exploratory design and the limited sample size, these findings should be interpreted as hypothesis-generating rather than as evidence derived from adequately powered statistical testing. Accordingly, larger longitudinal studies including ALS patients not receiving ALCAR, with detailed dietary and microbiome assessments, are required to clarify the significance of these findings and to evaluate the potential of TMAO and related metabolites as biomarkers in ALS.

CRediT authorship contribution statement

Salvatore Sotgia: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Angelo Zinellu:** Writing – review & editing, Investigation. **Stefano Zoroddu:** Writing – review & editing, Investigation. **Maria Ida Pateri:** Writing – review & editing, Resources. **Eleonora Loi:** Writing – review & editing, Resources. **Andrea Pisano:** Writing – review & editing, Resources. **Angela Sabalic:** Writing – review & editing, Resources. **Davide Tutedde:** Writing – review & editing, Resources. **Ana Florencia Vega Benedetti:** Writing – review & editing, Resources. **Francesca Floris:** Writing – review & editing, Resources. **Monica Puligheddu:** Writing – review & editing, Resources. **Paolo Valera:** Writing – review & editing, Resources. **Patrizia Zavattari:** Writing – review & editing, Resources. **Giuseppe Borghero:** Writing – review & editing, Resources. **Roberto Madeddu:** Writing – review & editing, Resources, Investigation.

Compliance with Ethical Standards

The study protocol was approved by the Ethics Committee of Sardinia (approval no. 28/2024) and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to inclusion in the study.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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