

# TMAO is associated with higher age, cardiometabolic risk factors and degenerative alterations of the brain in healthy elderly adults

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## ABSTRACT

Trimethylamine N-oxide (TMAO) is a microbiome-based metabolite known to increase with age, cardiovascular and other diseases, with average concentrations mostly higher in males. It was recently found to be associated with neurodegenerative diseases. In this context we aimed to investigate associations between TMAO, cardiometabolic risk factors, cognitive function and brain atrophy in older individuals ( $67.81 \pm 8.86$  years of age) with normal neurological status.

TMAO was measured by liquid-chromatography tandem mass spectrometry in serum samples from 487 individuals. All participants underwent cognitive testing capturing executive function, motor and memory abilities. In 180 of these individuals, brain MRI was performed to investigate vascular and neurodegenerative changes.

In the total cohort higher TMAO concentrations were positively associated with advanced age ( $p=0.01$ ), BMI ( $p<0.01$ ), fasting blood sugar ( $p<0.01$ ), HbA1c ( $p<0.01$ ) and diabetes ( $p=0.02$ ). In contrast, we observed negative associations with total cholesterol ( $p=0.01$ ), LDL ( $p=0.04$ ) and HDL cholesterol ( $p=0.04$ ). No significant differences in TMAO concentrations were found between females and males. MRI revealed higher TMAO levels to be associated with lower total grey matter, frontal lobar and hippocampal volume ( $p=0.01$ ).

Summarized, TMAO is associated with higher age, cardiometabolic risk factors and brain atrophy, while there was no difference between the two sexes of this community-dwelling elderly cohort. These findings support that TMAO is mainly a marker of aging processes but also associated with neurodegeneration, even though associations with cognitive functioning were not observed.

## INTRODUCTION

Trimethylamine oxide (TMAO) is a metabolite whose precursor Trimethylamine (TMA) is generated by the gut microbiome from dietary compounds (mainly L-carnitine and choline), which are found in greater

amounts in red meat or eggs [1, 2]. Both compounds are metabolized to TMA by TMA (high) producing gut microbiota [3]. Subsequently, TMA enters the circulation and is transported to the liver, where it is oxidized to TMAO by flavin monooxygenase 3 (FMO3) [2]. Consequently, the intestinal microbiome

and the amount of available L-carnitine, choline and other TMA precursors are potential targets for beneficial lifestyle interventions that modify serum TMAO concentrations [4].

High circulating blood TMAO concentrations seem to be associated with a wide range of diseases, including inflammation, oxidative stress, cardiovascular disease (CVD) and cardiovascular mortality [5]. Additional relationships have also been reported for liver steatosis, type 2 diabetes mellitus, stroke, hypertension, frailty, and importantly renal failure [2, 6]. Despite such promising data, existing literature is conflicting. Some studies even found beneficial effects of TMAO. In one study for example, TMAO appeared to promote protein stability and to protect cells from osmotic and hydrostatic stress [7]. Another study suggested that TMAO may ameliorate hepatic damage [8]. In view of the progressive increase of TMAO with declining renal function [9], it is important to emphasize that most associations between TMAO and non-renal diseases seem to attenuate after correction for renal function [10], which suggests reversed causality [11]. Those controversies have fuelled an ongoing debate whether TMAO plays an active role in the aforementioned diseases or simply represents a bystander [7, 12].

Recently, TMAO has also sparked attention in the field of neurodegenerative disorders. Del Rio et. al. were able to detect and quantitate TMAO in human cerebrospinal fluid (CSF), but concentrations did not differ between distinct neurological diseases [13]. In another clinical cohort study, Vogt et. al. found a positive association of TMAO levels in CSF with cognitive impairment and Alzheimer's disease (AD) dementia [14]. These findings nurtured the gut-brain axis theory [15]. More recently, a study in elderly individuals with a family history of AD-like dementia showed early alterations in the blood concentrations of TMAO, L-carnitine,  $\gamma$ -butyrobetaine, a metabolite of L-carnitine, and other acylcarnitines [16]. First preclinical studies further support a role of TMAO in neurodegeneration. In a mouse model of accelerated senescence, exogenous TMAO increased the number of senescent cells in the brain, enhanced mitochondrial impairment, stimulated superoxide production and increased synaptic damage [17]. In a rat model of vascular dementia, TMAO was found to increase oxidative stress-induced neuro-inflammation, apoptosis and to exacerbate the development of vascular dementia [18]. However, a study combining two clinical cohorts from the Rotterdam Study and the Leiden Longevity Study observed no associations between TMAO and cognition, incident dementia, or neuroimaging markers, although an increased dementia risk was reported among individuals with impaired renal function [19].

Taken together, (high) TMAO concentrations were found to be associated with age-related cognitive dysfunction and also to induce mitochondrial dysfunction, oxidative stress, neuronal senescence and synaptic brain damage [20] but results in previous studies were inconsistent.

In a previous study, we could show that vitamin D deficiency occurred in individuals from a cohort of well-characterized, community dwelling older individuals, with a modest reduction of memory function without structural brain atrophy [21]. A clinical cross-sectional observational population study found high TMAO concentrations to be associated with vitamin D deficiency and non-alcoholic fatty liver disease [22]. Together this prompted us to investigate associations between TMAO, cardiometabolic risk, cognitive function and brain atrophy in this cohort of elderly adults with normal neurological status.

## MATERIALS AND METHODS

### Study population

The study population comprised participants from the Austrian Stroke Prevention Study (ASPS) and the Austrian Stroke Prevention Family Study (ASPS-Fam) [21]. ASPS is a single-centre, prospective study that was designed to investigate the effects of vascular risk factors on brain function and structure in an elderly population [23]. ASPS participants were randomly selected from the community register of Graz, Austria, and were enrolled between 1991 and 2004. Inclusion criteria were a normal neurologic examination, and absence of dementia and stroke. ASPS-Fam was designed as an extension of ASPS with a comparable study protocol including both original ASPS participants and their first-degree relatives [24]. Brain MRI with 3D T1 and FLAIR sequences were available for ASPS-Fam participants, permitting automated evaluation of brain atrophy in this subgroup. All research procedures were conducted in accordance with the Declaration of Helsinki. All study protocols were approved by the ethics committee of the Medical University of Graz, Austria ([23] and ethics protocol number 17-088 ex 05/05), and written informed consent was obtained from all participants.

### Cardiometabolic risk factors

Established cardiometabolic risk factors for impaired cognitive function and dementia were assessed and recorded as follows. Hypertension was defined as history of hypertension or systolic blood pressure > 140 mmHg or a diastolic blood pressure > 90 mmHg and current use of antihypertensive agents. Subjects were

classified as diabetic on the basis of a documented history of diabetes, use of anti-diabetics or a fasting blood glucose level > 126 mg/dL (7.0 mmol/L) at the time of examination [21]. Hypercholesterolemia was defined as history of hypercholesterolemia, current use of cholesterol lowering drugs, or total cholesterol > 200 mg/dL or LDL cholesterol > 130 mg/dL [25]. Hypertriglyceridemia was defined as history of hypertriglyceridemia, current use of triglyceride lowering drugs, or triglyceride levels > 200 mg/dL [26]. The presence of atrial fibrillation was confirmed by an electrocardiogram obtained during the study visit [21]. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation from 2021 which includes creatinine, age and sex [27]. Smoking status was dichotomized into “current or former smoking” versus “never smoking”. Alcohol consumption was defined as current regular alcohol intake. Neurofilament light chain (NfL), a biomarker of neuronal injury associated with various neurological disorders, was quantitated in serum samples from ASPS-Fam participants using a Single Molecule Array (Simoa) platform at the University Hospital Basel, Switzerland [28].

### Serum TMAO measurements

After blood collection, separation and aliquotting, serum samples were stored at -80° C until analysis. TMAO concentrations were measured by a validated in-house liquid chromatography-tandem mass spectrometry (LC-MS/MS) method previously published by Enko et al. [29]. Analyses were performed on a SCIEX QTRAP 6500 triple quadrupole instrument (Applied Biosystems, Framingham, MA, USA). Within-day imprecision (coefficients of variation; CVs) of this method ranges from 5.5% at 2.8 µmol/L to 2.2% at 12.8 µmol/L. Between-day CVs were 9.9% at 2.8 µmol/L and 7.6% at 12.6 µmol/L. Stability testing with high and low quality controls over 7 years showed no deviation from the specified target value ranges. The 487 participant samples available for analysis were processed in multiple batches over several weeks, including quality controls in every batch.

### Neuropsychological testing

Cognitive function was evaluated using a comprehensive panel of neuropsychological tests covering the domains of memory, executive function and motor abilities. Detailed descriptions of the individual tests have been published previously [23]. Individual tests were grouped into composite measures representing the cognitive domains of memory, executive function and motor skills. The composite scores were derived by converting individual test results

into z-scores based on the mean and standard deviation of the combined ASPS and ASPS-Fam sample, followed by averaging the z-scores within each domain [21]. In addition, principal components analysis was used to calculate a measure of global cognitive ability (g-factor) combining the results from individual tests into a single summary metric [30].

### Magnetic resonance imaging (MRI)

ASPS-Fam participants underwent MRI scanning on a 3T whole-body MR system (TimTrio; Siemens Healthcare, Erlangen, Germany). The MRI protocol included a high-resolution T1-weighted 3D MPRAGE sequence covering the whole brain and an axial T2-weighted-FLAIR sequence [21]. The methods used for calculating structural and microstructural brain metrics have been described previously [31]. Structural brain measures were derived from the T1-weighted MPRAGE images using FreeSurfer 5.3 for segmentations [32]. From these segmentations, total gray matter, cortical and subcortical gray matter volumes, hippocampal volume, and lobar measures of cortical volume were calculated. Regional values were summed or averaged, as appropriate, to obtain lobar-level metrics. All FreeSurfer-derived volumes were normalized to total intracranial volume to account for individual differences in head size. White matter hyperintensities (WMH) were assessed on FLAIR images by a blinded expert. WMH were outlined using a custom written IDL program (Exelis Visual Information Solutions, USA). Lesion areas were segmented by combined region growing and local thresholding following manual selection, as described earlier [31]. The total lesion volume in cubic millimeter was calculated using the program FSLMATHS by multiplying the lesion area with the slice thickness and was normalized for head size. Due to a skewed distribution, the lesion load was logarithmically transformed [31]. To assess microstructural changes, we used the peak width of the skeletonized mean diffusivity (PSMD). PSMD is a robust, fully automated and easy-to-implement marker for cerebral small vessel disease based on diffusion tensor imaging, white matter tract skeletonization (as implemented in FSL-TBSS) and histogram analysis [31, 33].

### Statistical analysis

Statistical analyses were conducted using R software, version 4.4.3 (Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>). The distribution of continuous variables was assessed by visual inspection and the Shapiro-Wilk test. Normally distributed variables are presented as mean ± standard deviation (std) and non-normally distributed variables

are summarized as median and interquartile range (IQR). Group comparisons were performed using the Chi-Square test for categorical variables and either the t-test or Mann-Whitney-U test for continuous variables, depending on their distribution. Given the right-skewed distribution of TMAO levels, log10-transformed TMAO levels were used in all association analyses to better approximate normality and thereby satisfy the assumptions underlying Pearson correlation analyses and linear mixed-effects models. Univariate relationships between log10-transformed TMAO and age as well as log10-transformed Nfl levels were evaluated using Pearson correlation coefficients. To assess associations requiring covariate adjustment, we applied linear mixed-effects models incorporating the family structure of the ASPS-Fam cohort as a random effect. Associations between TMAO and metabolic risk factors were examined using separate linear mixed-effects model for each risk factor, with log10-transformed TMAO as the dependent variable, the respective risk factor as the independent variable, and age, sex and eGFR as covariates. The association between TMAO and cognition was determined using a linear mixed-effects model for each cognitive domain, with the cognitive domain as the dependent variable, log10-transformed TMAO as the independent variable and age, sex, education, hypertension, diabetes, atrial fibrillation, hypercholesterolemia, hypertriglyceridemia and eGFR as covariates. Associations between TMAO and brain atrophy markers were analysed using linear mixed-effects model for each MRI marker, with the MRI marker as the dependent variable, log10-transformed TMAO as the independent variable, and age, sex, hypertension, diabetes, atrial fibrillation, hypercholesterolemia, hypertriglyceridemia and eGFR as covariates. The linear mixed effects models were implemented using the `lme4` function from the `coxme` R package version 2.2-22 (Mixed Effects Cox Models, <https://CRAN.R-project.org/package=coxme>) with a kinship matrix calculated using the `kinship2` package (Pedigree Functions, R package version 1.9.6.1, <https://cran.r-project.org/package=kinship2>) to account for relatedness between ASPS-Fam participants. When data from ASPS and ASPS-Fam were pooled, study origin was additionally included as a covariate to account for potential study-specific differences. The results of linear mixed-effects model analyses are reported as regression coefficients (beta), 95% confidence intervals (95% CI) and p-values. For p-values within each age stratum in Table 1, false discovery rate (FDR) correction was applied to adjust for multiple testing. Visualizations were generated using the `ggplot2` R package for the scatterplot and the `forestploter` R package for the forest plot. Prior to analysis, continuous risk and lifestyle factors depicted

in the forest plot were z-transformed to facilitate comparability.

## RESULTS

### Study characteristics

Demographics, serum TMAO concentrations and risk factors of 487 neurologically normal community-dwelling individuals are presented in Table 2. The cohort had a mean age of  $67.8 \pm 8.9$  years and median TMAO levels were  $3.73 \mu\text{mol/L}$  ranging from  $0.5 \mu\text{mol/L}$  to  $80.2 \mu\text{mol/L}$ . Men had slightly higher TMAO levels than women (median [IQR]:  $3.74 (2.63 - 5.26)$  versus  $3.72 [2.54 - 4.97] \mu\text{mol/L}$ ) although this difference did not reach statistical significance.

As previous studies showed that associations between TMAO and other diseases fade after correction for renal function [10, 11, 34] further analyses were adjusted/covared for estimated glomerular filtration rate (eGFR).

### Associations of TMAO, age and cardiometabolic risk factors

We found a positive correlation between TMAO levels and age ( $r_{\text{Pearson}}=0.13$ ,  $p=0.004$ ; Figure 1) but this association was no longer significant after adjustment for eGFR (beta=0.003, 95% CI:  $-0.0004 - 0.005$ ,  $p=0.09$ ). However, participants aged  $\geq 70$  years ( $N=231$ ) had significantly higher TMAO concentrations (median [IQR]:  $4.06 [2.89 - 5.47] \mu\text{mol/L}$ ) than those  $< 70$  years ( $N = 256$ , median [IQR]:  $3.24 [2.36 - 4.64] \mu\text{mol/L}$ ,  $p_{\text{Mann-Whitney-U test}} = 0.00003$ ). This difference remained significant after adjustment for eGFR (beta=0.06, 95% CI= $0.01 - 0.11$ ,  $p=0.01$ ).

Higher TMAO levels were associated with higher BMI (beta=0.04, 95% CI= $0.02 - 0.07$ ,  $p=0.0002$ ), higher fasting blood sugar (beta=0.03, 95% CI= $0.01 - 0.05$ ,  $p=0.008$ ), higher HbA1c (beta=0.04, 95% CI= $0.01 - 0.06$ ,  $p=0.002$ ) and diabetes (beta=0.08, 95% CI= $0.01 - 0.15$ ,  $p=0.02$ ) after adjustment for age, sex and eGFR (Figure 2). Higher TMAO concentrations were also associated with lower total (beta= $-0.03$ , 95% CI= $-0.05 - -0.01$ ,  $p=0.01$ ), lower LDL (beta= $-0.03$ , 95% CI= $-0.05 - -0.001$ ,  $p=0.04$ ) and lower HDL cholesterol (beta= $-0.03$ , 95% CI= $-0.05 - -0.001$ ,  $p=0.04$ ).

### TMAO concentrations and cognition

Cognitive test data were available for up to 444 study participants. Serum TMAO levels showed no association with executive function, motor skills, or memory after adjustment for demographic and clinical

**Table 1. Association between TMAO levels and cognition.**

|                    | N   | beta   | se   | p    |
|--------------------|-----|--------|------|------|
| executive function | 444 | -0.005 | 0.10 | 0.96 |
| motor skills       | 442 | -0.199 | 0.14 | 0.15 |
| memory             | 443 | -0.091 | 0.14 | 0.52 |
| g-factor           | 441 | -0.238 | 0.14 | 0.09 |

Analyses are adjusted for age, sex, education, hypertension, diabetes, atrial fibrillation, hypercholesterolemia, hypertriglyceridemia, eGFR, family structure and cohort.

**Table 2. Demographics, TMAO levels and risk factors.**

|                                                    | Total              | Females              | Males              | p-value* |
|----------------------------------------------------|--------------------|----------------------|--------------------|----------|
| N                                                  | 487                | 306 (62.8%)          | 181 (37.2%)        |          |
| age [y], mean $\pm$ std                            | 67.81 $\pm$ 8.86   | 67.97 $\pm$ 8.56     | 67.54 $\pm$ 9.35   | 0.59     |
| TMAO [ $\mu$ mol/L], median [IQR]                  | 3.73 (2.60 – 5.00] | 3.72 (2.54 – 4.97]   | 3.74 (2.63 – 5.26) | 0.32     |
| eGFR [ml/min/1.73 m <sup>2</sup> ], mean $\pm$ std | 71.23 $\pm$ 14.27  | 69.44 $\pm$ 14.02    | 74.25 $\pm$ 14.22  | 0.0003   |
| BMI [kg/m <sup>2</sup> ], mean $\pm$ std           | 26.89 $\pm$ 4.40   | 26.74 $\pm$ 4.87     | 27.14 $\pm$ 3.46   | 0.29     |
| diabetes, N (%)                                    | 63 (12.7%)         | 39 (12.7%)           | 23 (12.7%)         | 0.99     |
| fasting blood sugar [mg/dL], median [IQR]          | 93 [86 – 103]      | 92 [86 – 102]        | 94 [87 – 104]      | 0.07     |
| HbA1c [%], median [IQR]                            | 5.6 [5.4 – 5.9]    | 5.6 [5.4 – 5.9]      | 5.6 [5.3 – 5.9]    | 0.29     |
| hypercholesterolemia, N (%)                        | 394 (80.9%)        | 257 (84.0%)          | 137 (75.7%)        | 0.02     |
| total cholesterol [mg/dL], mean $\pm$ std          | 218.20 $\pm$ 39.93 | 225.55 $\pm$ 40.08   | 205.79 $\pm$ 36.56 | 9E-8     |
| LDL [mg/dL], mean $\pm$ std                        | 130.11 $\pm$ 35.96 | 133.31 $\pm$ 37.19   | 124.66 $\pm$ 33.15 | 0.01     |
| HDL [mg/dL], median [IQR]                          | 60 [50 – 74]       | 68.56 $\pm$ 20.51    | 55.29 $\pm$ 14.39  | 2.9E-14  |
| hypertriglyceridemia, N (%)                        | 92 (18.9%)         | 48 (15.7%)           | 44 (24.3%)         | 0.02     |
| triglycerides [mg/dL], median [IQR]                | 107 [75 – 152]     | 101 [74.75 – 145.00] | 113 [83.5 – 167.5] | 0.01     |
| hypertension, N (%)                                | 345 (70.8%)        | 213 (69.6%)          | 132 (72.9%)        | 0.44     |
| systolic blood pressure [mmHg], mean $\pm$ std     | 143.30 $\pm$ 23.09 | 143.81 $\pm$ 24.07   | 142.42 $\pm$ 21.36 | 0.51     |
| diastolic blood pressure [mmHg], mean $\pm$ std    | 87.71 $\pm$ 10.01  | 87.74 $\pm$ 10.21    | 87.66 $\pm$ 9.70   | 0.94     |
| atrial fibrillation, N (%)                         | 31 (6.4%)          | 21 (6.9%)            | 10 (5.5%)          | 0.56     |
| current or former smoker, N (%)                    | 201 (41.3%)        | 89 (29.4%)           | 112 (62.9%)        | 7.2E-13  |
| alcohol consumption, N (%)                         | 83 (17%)           | 21 (6.9%)            | 62 (34.3%)         | 7.9E-15  |

\*variables were compared between females and males using either t-test, Mann-Whitney-U test or Chi<sup>2</sup> test depending on variable type and distribution.

Std, standard deviation; IQR, interquartile range; eGFR, estimated glomerular filtration rate; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

factors including renal function (Table 1). Similarly, no association was observed with overall cognitive performance as captured by the g-factor.

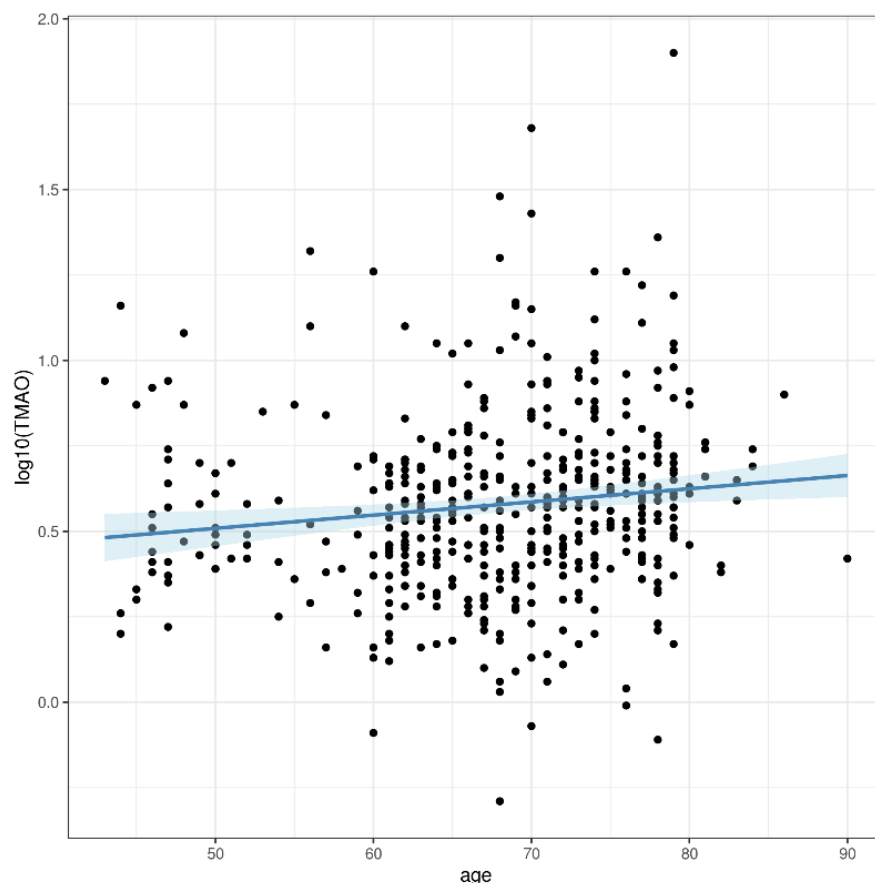
### TMAO concentrations and brain atrophy

MRI was performed in 180 individuals from the ASPS-Fam cohort. Demographics, TMAO concentrations and risk factors of this subset are presented in Supplementary Table 1.

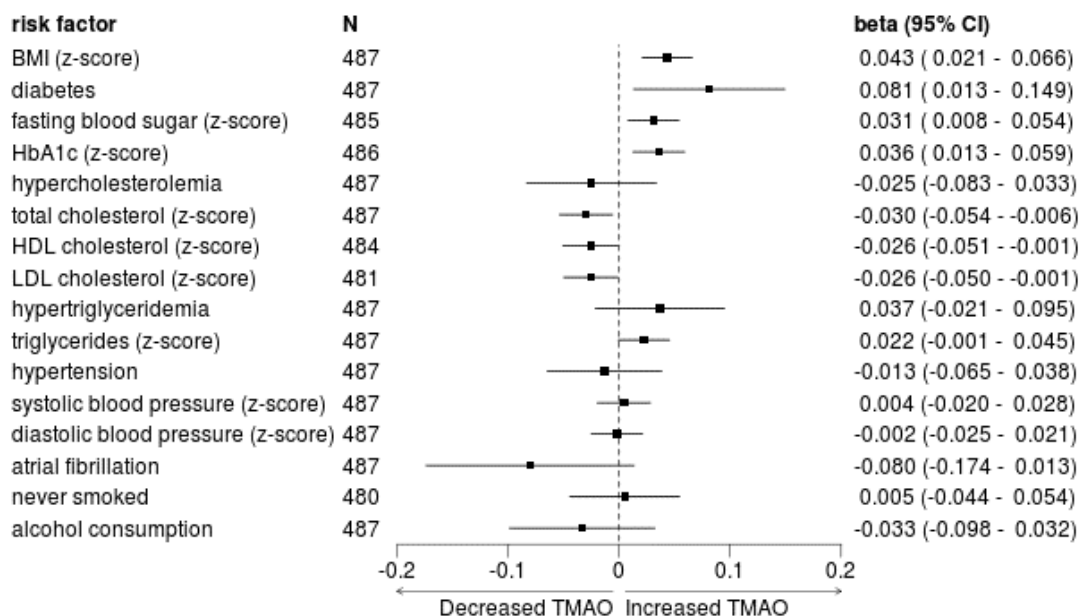
Higher TMAO concentrations were associated with lower total gray matter volume (beta= -0.015, se=0.01,

pFDR=0.03), lower hippocampus volume (beta= -0.0002, se=0.0001, pFDR=0.03) and lower frontal lobe volume (beta= -0.005, se=0.002, pFDR=0.03) after adjustment for age, sex, hypertension, diabetes, atrial fibrillation, hypercholesterolemia, hypertriglyceridemia and eGFR (Supplementary Table 2). Stratifying for age, the association with hippocampus volume remained significant in individuals older than 65 years (beta= -0.005, se=0.002, pFDR=0.03), while there were no associations in individuals < 65 years of age.

Investigating a serum/plasma marker for various neurological diseases in the ASPS Fam cohort, we



**Figure 1. Association between age and log10 transformed TMAO levels.**



**Figure 2. Associations of risk and lifestyle factors with TMAO.** Forest plot showing the results of individual models with log10(TMAO) as the dependent variable and risk/lifestyle factors as the independent variable covaried for age, sex and eGFR. eGFR: estimated glomerular filtration rate; BMI, body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

observed a positive correlation between TMAO and NfL ( $r_{\text{Pearson}}=0.21$ ,  $p=0.01$ ,  $N=167$ ). However, this association was no longer significant after adjustment for age ( $\beta=0.003$ , 95% CI=-0.0004 – 0.006,  $p=0.09$ ).

## DISCUSSION

The present study found independent associations of serum TMAO with age and cardiometabolic risk factors, such as BMI, fasting blood glucose, HbA1c and diabetes. Inverse relationships were observed with total, LDL and HDL cholesterol. In the entire ASPS cohort, no associations between TMAO and cognition were found; however, MRI brain parameters from 180 individuals in the ASPS Fam subcohort showed an association between higher TMAO concentrations and structural indices of brain health.

The age-related increase of circulating TMAO is in line with a previous study that measured TMAO longitudinally in healthy juvenile individuals at 11, 13, 15, 17, 19 and 26 years of age [35]. The rather young age of this juvenile cohort suggests the age-related rise in TMAO to be independent of kidney function. Furthermore, another study with mice and healthy humans suggests the age-related increase in TMAO to be unrelated to the dietary intake of TMAO precursors [36]. These observations nurture speculations that age-dependent changes in the gut microbiome composition and alterations in liver metabolism might promote the conversion of TMA into TMAO. This concept is supported by existing data showing variations of the gut microbiome composition from childhood through puberty and adolescence [37]. Common age-related changes in nutritional habits could prompt alterations in the bacterial colonization of the gut resulting in a progressive abundance of TMAO high producing bacteria.

Surprisingly, TMAO measurements in the participants of ASPS and ASPS Fam showed no sex-difference, which is in contrast to existing data in younger individuals where males had higher serum TMAO concentrations than females [35, 38, 39]. Although the impact of estrogen on TMAO synthesis is not fully clarified [40], lower estrogen levels in post-menopausal female participants of ASPS and ASPS Fam could at least partially contribute to this discrepancy. Estrogen decreases hepatic FMO3 expression, the principal enzyme involved in the conversion of TMA to TMAO [40]. As most participants of ASPS and ASPS Fam were above 50 years of age, almost all females were either in peri- or postmenopause with markedly lower estrogen levels than in younger, premenopausal cohorts. It can be speculated that the menopausal decline in estrogen production could result in higher FMO3 expression and a higher TMAO synthesis.

In addition to estrogen, dietary habits and constitutional differences in gut microbiome composition between males and females could also promote sex differences in circulating TMAO. In males, a relative enrichment of *Firmicutes* over *Bacteroidetes* was found to stimulate TMAO production [41]. In summary, more research is needed to clarify the impact of age and sex on microbiome composition and TMAO metabolism in aging humans.

In line with previous studies [2], the present results confirm positive associations of TMAO with widely used indices of metabolic health, such as BMI, fasting blood sugar, HbA1c and diabetes. Moreover, we identified negative associations with total cholesterol and the subfractions HDL and LDL cholesterol. While the negative correlation between TMAO and HDL cholesterol reflects TMAO as potential disease marker, the other found correlations remain controversial [42] and unexplained after extensive literature search, adding to the body of inconsistent data. Additionally, especially the findings of residual confounding of renal function [43], which simply suggest that TMAO accumulates in the circulation when the renal filtration is impaired, challenges TMAO's potential as a surrogate marker for an unhealthy diet and reduced metabolic health [7, 11].

Some researchers suggest to focus on TMAO precursors and metabolites rather than TMAO itself. A recent observational study in overweight individuals and normal weight controls found no associations of TMAO and its precursor choline with incident type 2 diabetes [44]. In the same study, plasma betaine was consistently associated with a lower risk of hyperglycemia, insulinemia, and type 2 diabetes. Consumed TMAO can also be a source for TMA as it is reduced to TMA in the gut. However, preclinical data from mice with intraperitoneal or oral administration of TMAO for 8 weeks suggest that adverse effects on glucose and lipid metabolism are mediated by gut microbiota derived TMA and not from injected TMAO [45].

The present study found independent associations of serum TMAO with structural indices of neuro-degeneration, but no relationship with cognitive function or biochemical markers of neuronal damage. After adjustment for potential confounders, higher TMAO concentrations were associated with lower total gray matter volume, hippocampus volume and frontal lobe volume, which was primarily observed in elderly subjects. In this cohort of community dwelling older individuals with normal neurological status, the aforementioned associations were neither paralleled by cognitive function nor by serum NfL, a biochemical surrogate marker of neuronal damage.

Our results add to the inconclusive data of existing studies. For example, Xu et al. identified TMAO as an independent risk factor for mild cognitive impairment (MCI) in type 2 diabetics [46]. Along this line, also Bai et al. found TMAO, as well as its dietary precursors betaine and carnitine, to be associated with MCI [47]. Another recent study in MCI patients and controls demonstrated inverse association between higher TMAO and hippocampal-dependent learning, memory abilities, and synaptic plasticity [48]. Preclinical data from the same study further suggests that TMAO regulates GSK-3 $\beta$  activity, an enzyme involved in learning and memory. In contrast, Yaqub et al. combined TMAO measurements from the large scale Rotterdam Study and the Leiden Longevity Study, and found no association of TMAO with cognitive function, MRI-based indices of neurodegeneration or incident dementia. Only the TMAO precursor choline was inversely linked to cognitive decline, brain atrophy, and markers of cerebral small vessel disease [19]. In the prospective Cardiovascular Health Study (CHS), which included adults aged 65 years and older,  $\gamma$ -butyrobetaine, crotonobetaine, but not TMAO, carnitine, choline or betaine, were associated with cognitive impairment and dementia [49]. The present results from the ASPS Fam subcohort showed inverse associations of serum TMAO with total gray matter volume, lower hippocampus volume and lower frontal lobe volume, whereas cognitive function was unrelated to serum TMAO. Overall, the observed associations with structural brain changes but not with cognitive performance are particularly noteworthy, as they provide important nuance to the current literature.

The conflicting results of existing studies may reflect the heterogeneity of the cohorts investigated. The ASPS and ASPS Fam included neurologically normal elderly individuals, thereby enabling the investigation of possible associations in this specific population. Observed relationships with structural markers of neurodegeneration were rather weak, which suggests a limited impact of TMAO in neurodegenerative processes that is probably insufficient to cause cognitive impairment. This hypothesis is supported by the measurement of circulating NfL, which was unrelated to structural and functional markers of neurodegeneration. NfL is considered a promising biomarker for neurological disorders [50]. Bai et al. speculated that there could exist an optimal concentration range for serum levels of TMAO and related biomarkers that could mitigate MCI risk [47], a hypothesis that merits further investigation.

When interpreting the present results, the following study limitations should be taken into account. Serum TMAO measurements were obtained at a single point,

which impedes a judgment on intraindividual variability of TMAO. Furthermore, stool samples for microbiome analysis were not collected from this cohort, and TMAO precursors (i.e., choline, betaine, L-carnitine, TMA) could not be measured which hampers the estimation of substrate availability. As brain MRI with 3D T1 and FLAIR sequences were not available at the time when ASPS participants were recruited, respective MRI data is only available in ASPS-FAM.

## CONCLUSION

In this cohort of neurologically normal elderly individuals serum TMAO was found to be linked to advanced age and several cardiometabolic risk factors, while differences in concentrations between females and males seem to disappear with age. As far as the neurological status is concerned, serum TMAO was found to be associated with lower hippocampus volume, whereas cognitive function and NfL showed no relationship. Overall the observed associations are modest and may reflect TMAO as a marker of systemic aging processes rather than a direct mediator of neurodegeneration.

In light of these observations, there are several aspects that future studies should focus on. First of all, the interaction between TMAO, and its precursors and down-stream metabolites need to be investigated more in depth. Second, future studies should focus on cohorts with particular dietary habits that impact substrate availability and microbiome composition in order to shed further light on the role of TMAO metabolism. Third, the investigation of potential gender-differences in general and in relation to the TMAO metabolism and age should get more attention.

## AUTHOR CONTRIBUTIONS

The authors have accepted responsibility for the entire content of this manuscript and approved its submission. Gunter Almer, manuscript writing (lead). Edith Hofer, statistical analysis. Reinhold Schmidt, study initiation, clinical data acquisition and supervision. Andreas Meinitzer, laboratory analysis. Markus Herrmann, analytical resources and manuscript writing. Dietmar Enko, supervision and manuscript writing.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## ETHICAL STATEMENT AND CONSENT

The study was approved by the local Ethics Committee of the Medical University of Graz

(registered at the Office for Human Research Protections of the US Department of Health and Human Services (IRB00002556 Institutional Review Board Registry Number)). Local registration number 17-088 ex 05/05. All study participants gave their written consent.

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## SUPPLEMENTARY MATERIALS

### Supplementary Tables

Please browse Full Text version to see the data of Supplementary Table 2.

**Supplementary Table 1. Demographics, TMAO levels and risk factors of the ASPS-Fam MRI subgroup.**

|                                                    | Total                | Females               | Males                  | p-value  |
|----------------------------------------------------|----------------------|-----------------------|------------------------|----------|
| N                                                  | 180                  | 112 (62.2%)           | 68 (37.8%)             |          |
| age [y], mean $\pm$ std                            | 64.93 $\pm$ 10.73    | 64.84 $\pm$ 10.22     | 65.09 $\pm$ 11.60      | 0.88     |
| TMAO [ $\mu$ mol/L], median [IQR]                  | 3.72 [2.65 – 5.29]   | 3.73 [2.66 – 5.15]    | 3.58 [2.61 – 5.99]     | 0.75     |
| eGFR [ml/min/1.73 m <sup>2</sup> ], mean $\pm$ std | 73.34 $\pm$ 15.39    | 73.21 $\pm$ 14.68     | 73.56 $\pm$ 16.60      | 0.88     |
| BMI [kg/m <sup>2</sup> ], mean $\pm$ std           | 26.61 $\pm$ 4.52     | 26.46 $\pm$ 5.00      | 26.85 $\pm$ 3.60       | 0.55     |
| diabetes, N (%)                                    | 21 (11.7%)           | 13 (11.6%)            | 8 (11.8%)              | 0.98     |
| fasting blood sugar [mg/dL], median [IQR]          | 95 [88.5 – 104]      | 95 [88 – 104]         | 97 [89 – 105.5]        | 0.63     |
| HbA1c [%], median [IQR]                            | 5.6 [5.4 – 5.9]      | 5.6 [5.4 – 5.9]       | 5.5 [5.4 – 5.7]        | 0.15     |
| hypercholesterolemia, N (%)                        | 134 (74.4)           | 87 (77.7%)            | 47 (69.1%)             | 0.20     |
| total cholesterol [mg/dL], mean $\pm$ std          | 209.45 $\pm$ 39.10   | 216.27 $\pm$ 38.00    | 196.37 $\pm$ 39.14     | 0.0009   |
| LDL [mg/dL], mean $\pm$ std                        | 117.23 $\pm$ 32.85   | 118.22 $\pm$ 34.28    | 115.58 $\pm$ 30.48     | 0.61     |
| HDL [mg/dL], median [IQR]                          | 65 [54 – 81]         | 77 [60 – 89]          | 55.5 [47.75 – 65.00]   | 5.2E-9   |
| hypertriglyceridemia, N (%)                        | 35 (19.4%)           | 14 (12.5%)            | 21 (30.9%)             | 0.003    |
| triglycerides [mg/dL], median [IQR]                | 98 [69 – 147.5]      | 94 [68 – 140]         | 107.5 [71.75 – 163.75] | 0.10     |
| hypertension, N (%)                                | 115 (63.9%)          | 67 (59.8%)            | 48 (70.6%)             | 0.15     |
| systolic blood pressure [mmHg], mean $\pm$ std     | 136.77 $\pm$ 21.31   | 136.66 $\pm$ 22.35    | 137.71 $\pm$ 19.62     | 0.75     |
| diastolic blood pressure [mmHg], mean $\pm$ std    | 85.27 $\pm$ 9.02     | 84.92 $\pm$ 9.38      | 85.65 $\pm$ 8.48       | 0.60     |
| atrial fibrillation, N (%)                         | 9 (5%)               | 8 (7.1%)              | 1 (1.5%)               | 0.16     |
| current or former smoker, N (%)                    | 84 (46.7%)           | 43 (39.4%)            | 41 (63.1%)             | 0.003    |
| alcohol consumption, N (%)                         | 47 (26.1%)           | 16 (14.3%)            | 31 (45.6%)             | 0.000004 |
| Serum NfL [pg/mL], median [IQR]                    | 32.6 [23.15 – 44.60] | 33.10 [22.43 – 44.08] | 32.3 [24.5 – 46.1]     | 0.90     |

\*variables were compared between females and males using either t-test, Mann-Whitney-U test or Chi<sup>2</sup> test depending on variable type and distribution.

Std, standard deviation; IQR, interquartile range; eGFR, estimated glomerular filtration rate; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NfL, neurofilament light chain.

**Supplementary Table 2. Association between TMAO concentrations and MRI.**