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ORIGINAL ARTICLE

Determination of Reference Interval of Serum Trimethylamine-N-Oxide In Humans By LC-MS/MS Method: Retrospective Study

İnsanlarda Serum Trimetilamin-N-Oksit Referans Aralığının LC-MS/MS Yöntemiyle Belirlenmesi: Retrospektif Çalışma

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ABSTRACT

Aim: The aim of our study is to determine the reference range of serum TMAO levels in individuals aged 18-65 using the LC-MS/MS method.
Methods: TMAO levels in serum samples from 185 healthy individuals aged 18-65 were determined using the LC-MS/MS method. Statistical analysis classified the data by gender and age. The lower and upper limits with a 90% confidence interval were estimated using the robust method. Outliers were identified and excluded from the log-transformed data using the Tukey criterion. Skewness and kurtosis estimates were based on log-transformed data.
Results: TMAO levels were similar between men and women, with median values of 180 ng/mL and 200 ng/mL, respectively (Z = −0.476, p=0.633). Additionally, no significant difference was observed in TMAO levels between women under 30 years of age and those aged 30 and above (median: 178 ng/mL vs. 186 ng/mL; Z = −0.739, p = 0.460). However, men aged 30 and above had significantly higher TMAO levels compared to men under 30 (median: 215 ng/mL vs. 191 ng/mL; Z = −2.225, p = 0.026). The reference interval for serum TMAO was determined as follows: 27–974 ng/mL in men under 30 years, 52–942 ng/mL in men aged 30 and above, 45–655 ng/mL in women under 30 years, and 33–539 ng/mL in women aged 30 and above.
Conclusions: This study determined age- and gender-specific reference intervals for serum TMAO levels using the LC-MS/MS method. To our knowledge, this is the first study to establish such reference intervals using this analytical technique. The findings are expected to provide valuable data for future clinical and epidemiological studies involving TMAO as a biomarker.

Keywords: TMAO, LCMS/MS, Reference Interval

ÖZ

Amaç: Bu çalışmanın amacı, 18-65 yaş aralığındaki bireylerde serum TMAO düzeylerinin referans aralığını LC-MS/MS yöntemi kullanarak belirlemektir.
Gereç ve Yöntemler: 18-65 yaş aralığında sağlıklı 185 bireyden elde edilen serum örneklerindeki TMAO düzeyleri LC-MS/MS yöntemiyle ölçüldü. İstatistiksel analizde veriler cinsiyet ve yaş gruplarına göre sınıflandırıldı. Alt ve üst referans aralık sınırları, %90 güven aralığı ile birlikte "robust" yöntem kullanılarak tahmin edildi. Uç değerler (aykırı değerler), logaritmik dönüşüm uygulanan verilerde Tukey kriterine göre belirlendi ve analizden çıkarıldı. Çarpıklık ve basıklık (skewness ve kurtosis) değerleri logaritmik dönüşümlü verilere dayanarak hesaplandı.
Bulgular: Erkekler ve kadınlar arasında TMAO düzeyleri benzer olup, sırasıyla medyan değerleri 180 ng/mL ve 200 ng/mL olarak belirlendi (Z = −0.476, p=0.633). Ayrıca, 30 yaş altı kadınlar ile 30 yaş ve üzeri kadınlar arasında TMAO düzeyleri açısından anlamlı bir fark saptanmadı (medyan değerler sırasıyla 178 ng/mL ve 186 ng/mL; Z = −0.739, p=0.460). Bununla birlikte, 30 yaş ve üzeri erkeklerde TMAO düzeyleri 30 yaş altı erkeklere göre istatistiksel olarak anlamlı derecede daha yüksek bulundu (medyan değerler sırasıyla 215 ng/mL ve 191 ng/mL; Z = −2.225, p = 0.026). Serum TMAO referans aralıkları şu şekilde belirlendi: 30 yaş altı erkekler için 27–974 ng/mL, 30 yaş ve üzeri erkekler için 52–942 ng/mL, 30 yaş altı kadınlar için 45–655 ng/mL ve 30 yaş ve üzeri kadınlar için 33–539 ng/mL.
Sonuçlar: Bu çalışma, LC-MS/MS yöntemi kullanarak serum TMAO düzeylerine ait yaş ve cinsiyet temelli referans aralıklarını belirlemiştir. Bildiğimiz kadarıyla, bu analitik yöntemle referans aralıklarını ilk kez ortaya koyan çalışmadır. Bulguların, gelecekte TMAO'nun bir biyobelirteç olarak değerlendirileceği klinik ve epidemiyolojik çalışmalara değerli veriler sunması beklenmektedir.

Anahtar Kelimeler: TMAO, LC-MS/MS, Referans Aralığı

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Introduction

Trimethylamine N-oxide (TMAO), a member of the amine oxide class with the chemical formula $(CH_3)_3NO$, is an important gut microbiota-derived metabolite that has attracted significant scientific interest in recent years due to its potential health implications in humans. TMAO is primarily produced through the hepatic oxidation of trimethylamine (TMA), which is generated by gut microbiota from dietary precursors such as choline, phosphatidylcholine, carnitine, betaine, dimethylglycine, and ergothioneine. After intestinal absorption, TMA enters the circulation and is converted to TMAO by hepatic flavin-containing monooxygenase enzymes (FMO1 and especially FMO3). Although TMAO can also be absorbed directly from certain marine foods, such as saltwater fish, endogenous production is considered the major contributor to circulating TMAO levels.

Foods rich in phosphatidylcholine and choline—such as eggs, liver, dairy products, red and white meat, and fish—are the primary dietary sources of TMAO. Conversely, plant-based diets rich in vegetables, whole grains, and dietary fiber have been associated with lower circulating TMAO levels. TMAO concentrations are influenced by several factors, including age, sex, dietary habits, gut microbiota composition, hepatic FMO activity, and most notably, renal function. In fact, TMAO is predominantly eliminated from the body via the kidneys, and renal clearance capacity plays a critical role in regulating its plasma levels [1].

Numerous studies have reported positive associations between elevated plasma TMAO concentrations and the development of various cardio-renal and metabolic disorders, including cardiovascular diseases, atherosclerosis [2], hypertension, ischemic stroke [3,4], atrial fibrillation [5], heart failure [6], acute myocardial infarction [7], and chronic kidney disease [8], as well as diabetes mellitus [9], metabolic syndrome, colorectal and gastric cancers [10–12], and certain neurodegenerative diseases [13]. While experimental evidence has shown that TMAO may promote the formation of atherosclerotic plaques [14,15], enhance platelet activation [16,17], and contribute to renal fibrosis [18], some studies have also proposed potential protective roles of TMAO in the cardiovascular system under specific conditions [19].

To enable the clinical use of TMAO as a novel biomarker, it is essential to establish reliable reference intervals (RIs) based on healthy individuals. RIs are typically derived from the distribution that encompasses approximately 95% of a reference population and play a central role in the biochemical characterization of that population [20]. Recognizing this need, organizations such as the Clinical and Laboratory Standards Institute (CLSI) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have published guidelines outlining the recommended pre-analytical and analytical procedures for determining reference intervals. This process involves the selection of appropriate reference individuals based on specific criteria, collection of biological samples, statistical analysis of measured values, and final calculation of reference limits [21].

Although some studies have reported reference values for TMAO levels in healthy individuals using the nuclear magnetic resonance (NMR) technique, inconsistencies in analytical methods and pre-analytical conditions have been noted in the literature. Additionally, the influence of ethnicity and dietary variation highlights the necessity of establishing population-specific reference intervals. Among the currently available methodologies, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the gold standard for the accurate quantification of TMAO due to its high sensitivity and specificity. Nevertheless, pre-analytical variables -such as blood collection tube type, sample storage conditions, and handling- must be carefully controlled to ensure measurement reliability.

In this context, the aim of the present study was to determine the reference interval for serum TMAO levels in healthy individuals using the LC-MS/MS method. The establishment of reliable reference values is expected to contribute significantly to the clinical utility of TMAO as a biomarker in disease risk assessment.

Materials and Methods

Study Sample

This study is a retrospective analysis conducted using the leftover blood samples from hospital personnel who had their blood drawn during a general screening. Information regarding whether the individuals had any chronic diseases was accessed through the hospital information system. Initially, 240 individuals were included in the study. However, those with chronic diseases and those whose age information could not be obtained were excluded. The study ultimately included 185 healthy individuals, comprising 86 men and 99 women, aged 18-65, who did not have chronic diseases or a COVID-19 diagnosis. The study was conducted using the leftover blood samples from the general screening of staff at Selcuk University Faculty of Medicine Hospital. Ethical approval for the study was obtained from the Selcuk University Local Ethics Committee with the approval number 2023/160.

Blood samples from the 185 participants were collected in yellow-capped gel biochemistry tubes and centrifuged. The resulting serum samples were transferred to 1.5 mL Eppendorf tubes and stored at -80°C until the day of analysis.

Measurement of Serum TMAO Levels

Deuterated Trimethylamine-N-oxide (d9-TMAO) was obtained from Cambridge Isotope Laboratories (Catalog: DLM-4779-1). All other reagents were purchased from Sigma-Aldrich. This study was conducted by modifying the methodology from the work of Liam M. Heaney and colleagues. In the study, the Q1 and Q3 values for TMAO were set at 59-76 atomic mass units, and for d9-TMAO, at 68-85 (6).

The analyses were performed using the ABSCIEX API

3200 High-Performance Liquid Chromatography-Mass Spectrometry (LC-MS/MS) system in the Biochemistry Laboratory of Selcuk University Faculty of Medicine, after thawing the pre-prepared serum samples at room temperature. A Phenomenex Luna (50 mm x 4.6 mm, 5 µm) C18 HPLC column and Turbo Ion Spray Electrospray (ESI) in positive mode were utilized. Two different mobile phases were employed during the analysis: HPLC-grade water containing 0.1% formic acid in pump A and methanol containing 0.1% formic acid in pump B, achieving a gradient elution.

Blood samples were centrifuged to obtain serum, which was then transferred to Eppendorf tubes. Each tube was spiked with the TMAO isotope. Methanol was added as a precipitant. The tubes were carefully vortexed for 30 seconds, followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was then transferred to clean tubes and evaporated under nitrogen gas at 28°C. The residues were reconstituted with HPLC-grade water. The tubes were centrifuged again at 4,500 rpm for 10 minutes, and the supernatants were transferred to vials compatible with the LC-MS/MS system. The TMAO concentrations in the samples were determined against the TMAO standard using the LC-MS/MS system with two mobile phases. The results were statistically analyzed.

Statistical Analysis

All statistical analysis was performed using R version 3.6.0 (The R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org). The data distribution was evaluated by density plots, box-plots, Q-Q plots and Shapiro-Wilk's normality test. Outliers were detected on log-transformed data overall or within strata of gender and age using Tukey's criterion, and excluded before the reference range estimation. The reference interval was estimated using the robust method on log-transformed data, and back transformed to the original scale of biomarkers. If the skewness and kurtosis values of the transformed data for normality were in range of ± 1.96, it was accepted that the data was suitable for normal distribution. 90% confidence interval estimates for the lower and the upper limit of the estimated reference were calculated using 5000 bootstrap replicates of the data. To test for differences between age and gender groups, we used a Mann-Whitney U test. A two-tailed p-value less than 5% was considered to be statistically significant.

Results

The median TMAO levels between men and women were comparable (180 [IQR, 123.25 – 267.25] vs. 200.5 [IQR, 118 – 283]; Z = −0.476, p = .633). Similarly, median TMAO levels in women under 30 years of age and those aged 30 years and older were also comparable (178 [IQR, 95.6 – 246] vs. 186 [IQR, 126.75 – 271.5]; Z = −0.739, p = .460). However, men aged 30 years and older had significantly higher median TMAO levels compared to those under 30 (215.5 [IQR, 141 – 327] vs. 191 [IQR, 84.75 – 227.5]; Z = −2.225, p = .026) (Table 1).

According to the results obtained, the TMAO reference range was determined as 27-974 ng/mL in men <30 years of age and 52-942 ng/mL in men ≥30 years of age. The TMAO reference range was determined as 45-655 ng/mL in women <30 years of age and 33-539 ng/mL in women ≥30 years of age.

Table 1. 95% Reference intervals of TMAO in healthy volunteers

				95% reference intervals			
Biomarker	**Age (years)**	**Gender**	***n***	**Lower limit (90% CIs)**	**Upper limit (90% CIs)**	**Skewness**	**Kurtosis**
TMAO							
	<30	Female	30	44.580 (31.138 – 66.813)	655.089 (475.007 – 822.574)	−0.370	−0.455
		Male	32	26.933 (17.422 – 52.334)	973.886 (559.900 – 1405.393)	−0.837	0.119
	≥30	Female	69	32.851 (20.761 – 48.861)	538.768 (454.164 – 621.896)	0.003	0.074
		Male	54	52.446 (38.254 – 72.730)	941.912 (671.913 – 1293.479)	−0.701	1.351

Stratified according to gender and age. The lower and upper limits with 90% confidence intervals (CIs) were estimated using the robust method. Outliers were detected on log-transformed data using test Tukey's criterion and excluded. Skewness and kurtosis estimates are based on the log-transformed data.

Discussion

The findings of this study demonstrate that age and gender have a significant impact on serum TMAO levels. The difference between the TMAO reference interval observed in men under 30 years of age (27–974 ng/mL) and those aged 30 and above (52–942 ng/mL) may initially suggest a decreasing trend in TMAO levels with advancing age. However, this interpretation should not be based solely on the upper reference limit. In fact, the increase in the lower limit in older men indicates a narrowing of the distribution range and reduced variation, rather than a direct age-related decrease in TMAO levels. This may reflect changes in biological regulation mechanisms associated with aging. Similarly, in women, the reference interval was 45–655 ng/mL in those under 30, and 33–539 ng/mL in those aged 30 and above, further supporting the influence of age on TMAO concentrations.

As a biologically active metabolite, TMAO is subject to changes during the aging process. Age-related alterations in the composition of gut microbiota and decreased hepatic flavin-containing monooxygenase (FMO) enzyme activity may affect TMAO production and circulating levels. Moreover, the decline in renal function commonly observed with aging may slow the renal excretion of TMAO, potentially resulting in elevated plasma levels.

When considering gender, higher TMAO levels were observed in men compared to women. This may be attributed to higher consumption of animal-based foods rich in carnitine and phosphatidylcholine, such as red meat, commonly seen in men. Lower

TMAO levels in women may be related to hormonal differences, dietary preferences, or variations in hepatic enzyme activity.

Chemically, TMAO is a compound of the amine oxide class containing three methyl groups, with the molecular formula $(CH_3)_3NO$. The molecule includes three methyl (CH_3) groups bonded to a nitrogen atom, which in turn is oxidized to form an N→O bond. Some studies have suggested potential ethnic variations in methylation pathways and related enzyme activity across populations such as Turks, Japanese, and Europeans. The presence and activity of methyl groups are significant in physiological processes including DNA and protein methylation, and estrogen metabolism. In females, especially during phases influenced by hormonal fluctuations (e.g., pregnancy, menstruation), methylation processes may be more prominent. Nevertheless, in the current study, the TMAO reference intervals in females were lower than in males, suggesting that methylation alone may not be the primary determinant of TMAO levels, or that other physiological factors may override its effect.

The use of TMAO as a biomarker for cardiovascular risk has gained increasing attention in recent years. Numerous studies have shown that elevated TMAO levels are associated with an increased risk of cardiovascular disease and atherosclerosis. Therefore, determining age- and gender-specific reference intervals is essential to enhance the clinical utility of TMAO. The data presented in this study clearly highlight the importance of considering demographic characteristics when interpreting TMAO levels in clinical practice.

Conclusion

In conclusion, this study demonstrates that serum TMAO levels are significantly influenced by both age and gender, emphasizing the need to account for demographic factors when establishing reference ranges and interpreting TMAO as a biomarker in clinical practice. Specifically, the elevated lower limit observed in older age groups suggests a narrowing of the distribution and potential age-related changes in biological regulation rather than a direct decrease in TMAO levels. Higher TMAO levels in men may be attributed to dietary habits, hormonal differences, or other physiological factors.

These findings also underscore TMAO's emerging role as a cardiovascular risk indicator, underscoring the importance of defining age- and gender-specific reference intervals for more accurate clinical assessment. However, limitations such as the retrospective design, lack of comprehensive dietary and lifestyle data, single-center sample, and the absence of genetic and gut microbiota information highlight the need for caution in generalizing the results. Future large-scale, multicenter, prospective studies that address these limitations and include more detailed confounding variables would be instrumental in refining our understanding of TMAO metabolism and its potential clinical relevance.

Conflict of interest

The authors declare no conflict of interest.

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