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ORIGINAL ARTICLE

Trimethylamine N-oxide, A Gut Microbiota-dependent Metabolite in Chronic Hepatitis B

Kronik Hepatit B'de Bağırsak Mikrobiyota Bağımlı Bir Metabolit Olan Trimetilamin-N-oksit

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ABSTRACT

Background: Trimethylamine N-oxide (TMAO), a gut microbiota metabolite is produced in the liver from dietary precursors such as choline, betaine, and L-carnitine. TMAO has been linked to inflammatory processes and oxidative stress, both of which are critical factors in the progression of hepatitis. This article aims to examine the impact of TMAO on chronic hepatitis B (CHB).
Materials and Methods: The study included 41 treatment-naïve CHB patients with HBV DNA levels above 2000 IU/mL, as well as 46 age and gender-matched controls. Serum TMAO levels were measured using Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS). All statistical analysis was performed with R version 4.2.1.
Results: Patients with CHB have a more significant increase in serum level of TMAO than healthy controls (1860 [IQR, 808 – 2720] vs. 552.5 [IQR, 252 – 876.5], p<0.001). Serum ALT and AST were higher in patients with CHB (p<0.001 and p<0.001). TMAO levels were positively correlated with ALT and AST levels (r=0.466, p<0.001; r=0.376, p<0.001) and had predictive power for CHB with an area under the curve of 0.808.
Conclusions: Our results indicate that there is a link between TMAO, a gut microbiota-dependent metabolite, and CHB disease. Since TMAO is synthesized mainly in the liver, its raised levels may be associated with liver-related diseases.

Keywords: HBV, TMAO, Microbiota, LC/MS/MS

ÖZ

Giriş: Bağırsak mikrobiyota metaboliti olan Trimetilamin-N-oksit (TMAO), kolin, betain ve L-karnitin gibi besin kaynaklarından karaciğerde üretilir. TMAO, hepatit ilerlemesinde kritik faktörler olan inflamatuar süreçler ve oksidatif stres ile ilişkilendirilmiştir. Bu çalışma, TMAO'nun Kronik Hepatit B (KHB) üzerindeki etkisini incelemeyi amaçlamaktadır.
Gereç ve Yöntemler: Çalışmaya, HBV DNA düzeyleri 2000 IU/mL'nin üzerinde olan 41 tedavi-naif KHB hastası ve yaş ve cinsiyet açısından eşleştirilmiş 46 kontrol grubu dahil edilmiştir. Serum TMAO seviyeleri, Likit Kromatografi-Tandem Kütle Spektrometrisi (LC/MS/MS) kullanılarak ölçülmüştür. Tüm istatistiksel analizler R versiyon 4.2.1 ile gerçekleştirilmiştir.
Bulgular: KHB hastalarında serum TMAO düzeyleri, sağlıklı kontrollere göre anlamlı derecede daha yüksekti (1860 [IQR, 808 – 2720] vs. 552.5 [IQR, 252 – 876.5], p<0.001). Serum ALT ve AST düzeyleri KHB hastalarında daha yüksekti (p<0.001 ve p<0.001). TMAO düzeyleri, ALT ve AST seviyeleri ile pozitif korelasyon göstermiştir (r=0.466, p<0.001; r=0.376, p<0.001) ve KHB tanısı için eğri altındaki alan 0.808'di.
Tartışma: Sonuçlarımız, bağırsak mikrobiyota bağımlı bir metabolit olan TMAO ile KHB hastalığı arasında bir bağlantı olduğunu göstermektedir. TMAO esas olarak karaciğerde sentezlendiğinden, artmış seviyeleri karaciğer hastalıklarıyla ilişkili olabilir.

Anahtar Kelimeler: TMAO, Mikrobiyota, HBV, LC/MS/MS

Introduction

Trimethylamine N-oxide (TMAO), an amine oxide compound with the chemical formula (CH3)3NO, is a dietary component that is derived from trimethylamine (TMA) through oxidation (1). TMAO, a gut microbiota metabolite produced in the liver from dietary precursors such as choline, L-carnitine, and betaine by hepatic enzyme flavin-containing monooxygenase-3 (2), has garnered significant attention in recent years for its role in various health conditions such as cardiovascular diseases (3, 4), diabetes (5, 6), chronic kidney disease (7), colorectal cancer (8), and even all-cause mortality (9).

In both in vitro and in vivo studies, TMAO has been shown to exert prooxidative, proinflammatory, and profibrotic effects through the activation of key inflammatory pathways, thereby contributing to various pathological conditions (10-12). TMAO also serves as a piezolyte, providing stability to proteins and nucleic acids (10).

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Evidence from experimental studies suggests that TMAO directly may trigger liver inflammation by interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-a) (13). Oxidative stress arises when the production of reactive oxygen species (ROS) exceeds the body's ability to counteract them with its antioxidant defenses (14). This state of heightened oxidative activity leads to cellular and molecular damage, contributing to inflammation, tissue dysfunction, and disease progression (15). Elevated levels of TMAO have been linked to increased production of ROS, exacerbating oxidative stress, and promoting inflammation (16, 17). Several studies have proven that increased TMAO concentrations can result in endothelial dysfunction in cultured endothelial cells via oxidative stress (18, 19). Thus, TMAO undeniably plays a role in the pathogenesis of chronic inflammation, acting as a pivotal mediator that triggers and sustains inflammatory pathways.

Chronic hepatitis B (CHB) is an infection of the liver caused by the hepatitis B virus (HBV) for more than six months, affecting millions of people worldwide. Despite significant advances in vaccination and antiviral therapies, CHB remains a major global health concern due to its potential to lead to severe liver complications, including cirrhosis and hepatocellular carcinoma. The virus is primarily transmitted through sexual, parenteral, and vertical routes, making it highly contagious.

An increasing amount of evidence highlights the crucial role of interactions between microbiota and host in maintaining overall health, affecting not only gut homeostasis but also playing a key role in various human diseases by generating biologically active substances (20). Recently, the potential impact of the gut microbiota on disease risk has led to the hypothesis that changes in the metabolome profile, specifically TMAO, could serve as a novel biomarker for assessing human health conditions associated with intestinal microbiota (2, 21).

TMAO has been linked to inflammatory processes and oxidative stress, both of which are critical factors in the progression of hepatitis. Since TMAO is synthesized mainly in the liver, its local levels may be associated with liver-related diseases. This article aims to examine the impact of TMAO on CHB. By investigating the relationship of TMAO with CHB disease, we also aim to shed light on new research avenues and potential therapeutic interventions in the fight against Hepatitis B.

Materials and Methods

Patients and Study Design

This prospective study included 41 CHB patients with HBV DNA levels above 2000 IU/mL who presented to the Infectious Diseases Department of XXX University Hospital, along with 46 age- and gender-matched controls. Participants who had received antiviral therapy or immunosuppressive treatments were excluded from the study. Moreover, individuals co-infected with hepatitis C virus (HCV) and hepatitis delta virus (HDV) were excluded. Individuals with other chronic diseases, active infections, a history of malignancy, pregnant women, and those with incomplete data were also excluded from the study. The research received approval from the Ethics Committee at XXX University Faculty of Medicine (2024/176).

Laboratory Analysis

Venous blood samples were collected into BD Vacutainer SST II Advance serum gel separator tubes (Becton Dickinson, NJ, USA) from all participants following a minimum fasting period of 8 hours. The collected blood samples were then subjected to centrifugation at 2000xg for 10 minutes. After centrifugation, serum samples were carefully separated and transferred into Eppendorf tubes. To ensure the stability of the serum samples, they were stored at -80°C until analysis.

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBsAg, HBeAg, anti-HBs, and anti-HBe were measured using the Abbott Architect ci16200 and Abbott Architect i2000sr (Abbott Laboratories Ltd, Abbott Park, Illinois, US) auto-analyzers.

TMAO levels in serum samples, which were brought to room temperature, were analyzed using a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system. Briefly, A 250 µL aliquot of serum was taken from each sample, to which 100 µL of d9-TMAO, the isotope of TMAO, was added as an internal standard. Following this, 100% methanol was added as a precipitating agent. The tubes were vortexed for 30 followed by centrifugation at 14,000 rpm for 10 minutes. Then, the supernatant transferred to clean tubes was evaporated under nitrogen gas at 28°C. Subsequently, after adding 250 µL of High-Performance

Liquid Chromatography (HPLC) grade water as a solvent, the samples were then vortexed. After another round of centrifugation at 4,500 rpm for 10 minutes, the supernatant was collected and transferred into vials. Analytical separation was achieved using a Shimadzu HPLC system (Kyoto, Japan) coupled with a Phenomenex C18 column (50 mm x 4.6 mm, 5 µm, 100 Å). TMAO concentration was quantified using an ABSciex API 3200 tandem mass spectrometer (Applied Biosystems/MDS Sciex).

Statistical analysis

Statistical analysis was conducted using R version 4.2.1. Software (The R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org). The normality of the data was checked via Shapiro-Wilk's normality test and the homogeneity of the variance was assessed via Levene's test. Numerical variables were summarized as mean ± standard deviation (SD) or median with quartiles [1st quartile – 3rd quartile], as appropriate. Categorical variables were described as count (n) and percentage (%). A Mann-Whitney U test (unpaired Wilcoxon test) or student's t-test was conducted to assess whether there was a statistically significant difference in demographical and laboratory parameters between groups. In addition, the Chi-square test with Yates continuity correction was used to evaluate the relationship between sex distribution and study groups. For further analysis, the receiver operating characteristic (ROC) curve analysis to identify the diagnostics performance of serum TMAO level to predict the CHB was performed. Calculation of the area under the curve (AUC) was performed, and the optimal cut-off point was derived based on the Youden index criteria. The sensitivity, specificity, negative (NPV), and positive predictive value (PPV) were calculated for the determined optimal cut-off point. Next, Spearman's rho correlation analysis was applied to assess the relationship between serum TMAO level and age, ALT and AST levels, and HBV-DNA level both in all cohorts and each study arm. A two-tailed p-value less than 0.05 was deemed to demonstrate statistical significance.

Results

A total of 87 participants, among whom 54% (n=47) were females, including 41 CHB patients and 46 healthy controls who met eligibility criteria, were enrolled in this study. Table 1 provides a summary of the demographic and laboratory characteristics of the study groups. Patients were broadly comparable in terms of age (38.89 ± 10.16 vs. 38.83 ± 12.60, p=0.980) and sex (47.8% vs. 61% for females, p=0.311) distribution between the study groups. Higher serum levels of ALT and AST were found in patients with CHB than those in healthy controls. The mean HBV viral load in log10 was 4.44 ± 1.18 U/mL. 4 (9.8%) out of 41 patients with CHB were HBeAg-positive. Mann-Whitney U test revealed that CHB patients had a more significant increase in serum level of TMAO than healthy controls (1860 [IQR, 808 – 2720] vs. 552.5 [IQR, 252 – 876.5], p<0.001, Figure 1-A).

Table 1. Demographical and clinical characteristics, and laboratory findings of the study groups

Characteristics	**Controls (*n*=46)**	**CHB (*n*=41)**	***p*-value**
Age (years)	38.89 ± 10.16	38.83±12.60	0.980[1]
Sex (female/ male)	22 (47.8)/ 24 (52.2)	25 (61)/16 (39)	0.311[2]
ALT (U/L)	18 [14.25 – 26.25]	31 [24-47]	<0.001[3]
AST (U/L)	19 [14.25 – 21.75]	28 [23-37]	<0.001[3]
HBV-DNA (log_{10} U/mL)		4.44±1.18	
HBeAg-positive		4 (9.8)	
Anti-Hbe		37 (90.2)	
TMAO (ng/mL)	552.5 [252-876.5]	1860 [808-2720]	<0.001[3]

1Student's t-test; 2Chi-square test with Yates continuity correction; 3Mann-Whitney U test (unpaired Wilcoxon test). Data were presented as mean±standard deviation or median with quartiles [1st quartile-3rd quartile] for numerical variables, and data were described as number (n) and percentage (%) for categorical variables. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, CHB: Chronic hepatitis B, HBV: Hepatitis B virus, TMAO: Trimethylamine N-oxide,

The area under the curve (AUC) of serum TMAO level to distinguish CHB from healthy controls was 0.808, and ROC analysis identified that a TMAO level of 1560 ng/mL was able to predict the CHB with a sensitivity of 58.54%, a specificity of 100%, a PPV of 100% and an NPV of 73.02% (Figure 1-B).

As shown in Figure 1, Spearman's rho correlation analysis indicated that both serum ALT (Spearman's rho=0.466, p<0.001, Figure 1-C) and AST (Spearman's

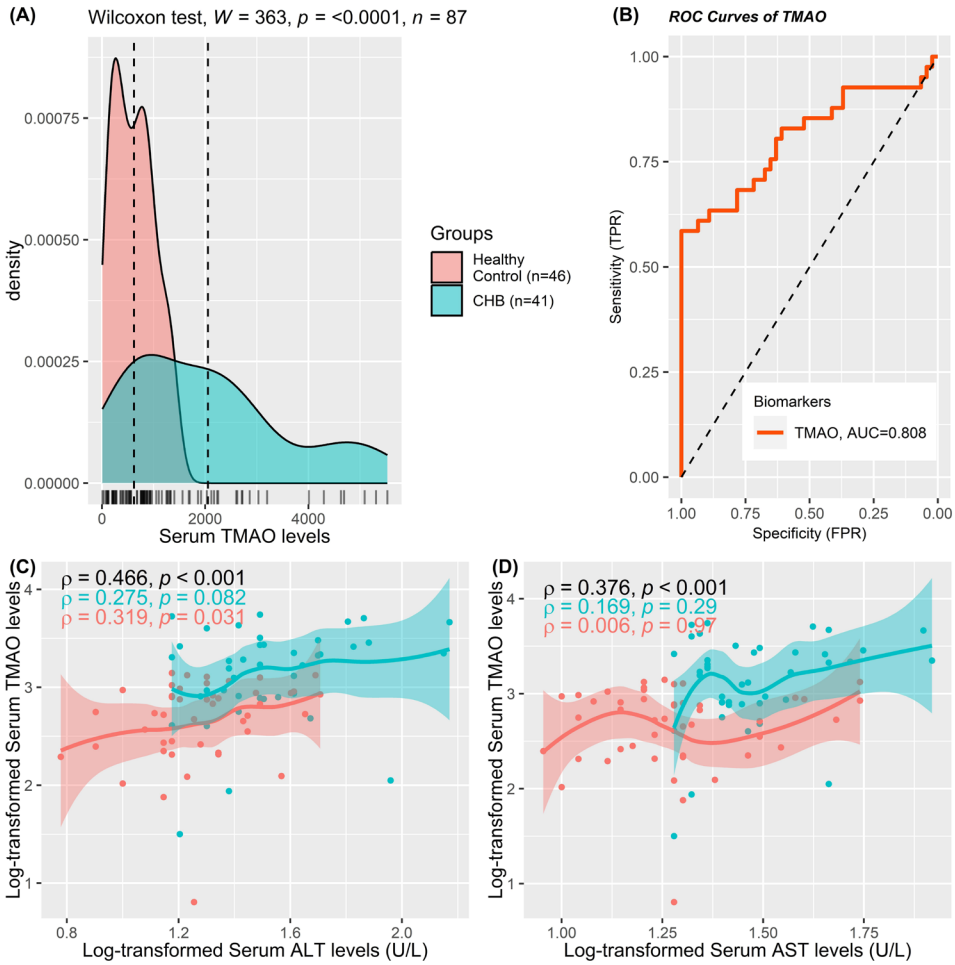

Figure 1. (A) The serum level of TMAO in patients with CHB and healthy controls. (B) ROC curve of serum TMAO level to predict the CHB. (C) The scatter plot between serum level of TMAO and serum ALT level in all cohorts (colored black) and each study arm. (D) The scatter plot between serum level of TMAO and serum AST level in all cohorts (colored black) and each study arm. TMAO: Trimethylamine N-oxide, CHB: Chronic hepatitis B, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase.

rho=0.376, p<0.001, Figure 1-D) levels positively correlated with serum TMAO level in all patients.

Discussion

In our study, we compared serum TMAO levels between patients with CHB and healthy controls and found that TMAO levels were significantly higher in CHB patients. Additionally, TMAO levels were positively correlated with ALT and AST levels and had predictive power for CHB with an AUC of 0.808.

Adequate choline intake is vital for maintaining liver health and its deficiency can exacerbate hepatic steatosis and contribute to the progression of liver inflammation and hepatic damage (22, 23). The results of a case-control study involving 297 incident cases and 631 matched controls suggest a close relationship between serum choline levels and hepatocellular carcinoma (24). The connection between TMAO and choline is crucial for comprehending their metabolic pathways. Choline, along with betaine and L-carnitine, is metabolized by gut microbiota to form TMA. This TMA is then transported to the liver, where it is oxidized to form TMAO. Recent studies have highlighted a significant connection between elevated levels of TMAO and an increased risk of colorectal cancer (8, 25, 26). Despite the limitation of direct evidence implicating TMAO in liver diseases, TMAO is linked to metabolic conditions such as diabetes mellitus (27), and dyslipidemia (28), as well as inflammation (29), all of which can potentially contribute to metabolic liver disease. Due to the close relationship between the liver and gut in TMAO formation, it has been hypothesized that TMAO could also be associated with liver diseases. Indeed, increased TMAO levels have recently been associated with non-alcoholic fatty liver disease (30, 31). A case-control study involving 671 primary liver cancer cases and 671 controls found a significant association between elevated TMAO levels and primary liver cancer (32).

The gut microbiota is essential in guiding the maturation of the immune system and modulating the functional diversity of immune cells. Changes in the gut microbiota have been observed in many liver diseases, including CHB (33). Analyzing urine samples from 42 Bangladeshi patients with Hepatocellular Carcinoma, 47 with cirrhosis, 46 with CHB, and seven healthy controls using nuclear magnetic resonance spectroscopy, they found that urine TMAO levels were statistically significantly lower in Hepatocellular Carcinoma patients and elevated, though not statistically significant, in patients with CHB compared to healthy controls (34). Shi et al. demonstrated that during a hepatotoxicity assessment with Bay41-4109, a newly recognized strong inhibitor of HBV replication, increased TMAO levels were detected in the cohort of rats with liver damage caused by Bay41-4109 (35). However, while it is evident that TMAO influences various pathways in hepatitis B through different mechanisms, the exact mechanism remains unclear.

Recent research highlights TMAO's multifaceted impact on liver inflammation through diverse molecular pathways. Beyond its association with metabolic dysregulation, TMAO is implicated in promoting inflammatory responses within hepatic tissues via distinct signaling cascades. Liu et al. suggest that TMAO induces hepatocytes to release Exos that exacerbate inflammation and impair endothelial function through miRNA-mediated mechanisms and NF-κB activation, thus stimulating the upregulation of inflammatory markers and induction of cell apoptosis and hindering cell migration (13). The intake of high carnitine, a significant precursor of TMAO, led to liver injury by increasing inflammatory hepatic cytokines, reducing the antioxidant capacity, and altering gut microbiota composition in mice (36). Sun et al. reported that TMAO has the potential to trigger oxidative stress and initiate the ROS-TXNIP-NLRP3 inflammasome signaling cascade, leading to the production of inflammatory cytokines and endothelial dysfunction in human umbilical vein endothelial cells (37). It has been found by Chen et al. that TMAO can directly bind to hepatic protein kinase R-like endoplasmic reticulum kinase, which in turn activates the unfolded protein response and promotes metabolic dysfunction (38).

The liver plays a crucial role in detoxifying endogenous and xenobiotic metabolism products, which makes it more susceptible to damage from harmful substances like ROS. Initially, hepatic inflammation induces tissue repair and the restoration of normal cellular function, however, prolonged inflammation due to persistent stressors can result in the loss of hepatocytes and the development of fibrosis (10). Oxidative stress damage has been implicated in the pathogenesis of chronic liver metabolic and inflammatory diseases through mechanisms involving alterations in lipid and glucose metabolism as well as modifications of the inflammatory response (39-41). In a three-month study by Florea et al., oral administration of TMAO in varying doses led to dose-dependent increases in liver oxidative stress and inflammation via iNOS and COX-2 activation, along with vascular alterations, but did not result in significant biochemical or histologic liver fibrosis or inflammation associated with IL-1a and TNF-a within the study period in mice (10). Their study also demonstrated that TMAO administration leads to a dose-dependent decrease in reduced glutathione levels along with a decrease in catalase and an increase in oxidized glutathione, indicating oxidative stress.

Long-term inflammation and liver toxin exposure can cause fibrogenesis by activating hepatic stellate cells to transform into myofibroblasts, leading to the production of extracellular matrix (ECM). Whereas collagen constitutes the majority of this ECM tissue, fibrosis is traditionally diagnosed through biopsy due to the lack of potential biomarkers for non-invasive diagnosis of liver fibrosis. Recently, it has been shown that TMAO induces cardiac fibrosis (42), aortic valve fibrosis (43), kidney fibrosis (44), and liver fibrosis (45). In their study, Yang et al. proved that mice supplemented with TMAO and choline demonstrated increased myocardial fibrosis, which was subsequently reversed by 3,3 Dimethyl-1-butanol, an inhibitor of microbial TMA formation (46). Florea et al., on the other hand, demonstrated in mice administered TMAO for 3 months that there was no increase in fibrosis, which could be attributed to dose or duration effects (10). It remains unclear whether TMAO contributes to fibrosis in chronic hepatitis, highlighting the need for further studies in this area.

Considering the limitations of our study, several aspects need to be addressed. Firstly, we did not simultaneously evaluate oxidative stress parameters, inflammatory markers, and liver fibrosis, which are interconnected pathways. Additionally, factors such as diet may have influenced the results. Moreover, our study was performed at a singular center, potentially limiting the generalizability of our findings to broader

patient populations. Future research should involve longitudinal follow-up of larger patient groups to evaluate whether individuals with elevated TMAO levels are more prone to developing conditions such as cirrhosis and malignancy over time.

Conclusion

This study demonstrates a significant increase in serum TMAO levels among patients with CHB compared to healthy controls. The positive correlation of TMAO with ALT and AST levels further supports its role in liver disease progression. Our study is, to the best of our knowledge, the first to investigate the relationship between CHB and TMAO. The link between TMAO and liver diseases is a relatively new area of research, and our study serves as a pioneering effort to understand this connection in the context of gut microbiota-dependent metabolite TMAO and CHB. We believe that our findings will pave the way for more comprehensive studies involving larger patient cohorts and examining other parameters of this pathway. This could ultimately lead to a deeper understanding and novel therapeutic approaches in Hepatitis B management. Whether TMAO is a causative factor or merely a consequence remains unclear, necessitating further research to elucidate its precise role in Hepatitis B.

Conflict of Interest

The authors have stated that they have no conflicts of interest.

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Author Contributions

All authors contributed equally to this study.

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