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Trimethylamine N-Oxide (TMAO) and TNF-α Levels in Periodontal Disease Associated With Smoking

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ABSTRACT

Aims: Trimethylamine N-oxide (TMAO) is a compound involved in the pathogenesis of various systemic inflammatory diseases, including cardiovascular conditions. The aim of this study was to determine differences in saliva and serum levels of TMAO between periodontitis and healthy patients according to smoking status.

Methods: The study included four systemically healthy groups: periodontally healthy non-smokers (NS-Control; $n = 25$), non-smokers with Stage-III-Grade-B periodontitis (NS-Periodontitis; $n = 25$), periodontally healthy smokers (S-Control; $n = 25$), and smokers with Stage-III Grade-C periodontitis (S-Periodontitis; $n = 25$). Periodontal parameters were recorded. TMAO levels were determined in saliva and serum samples using liquid chromatography-mass spectrometry (LC–MS/MS). TNF-α levels were measured by the ELISA method.

Results: Salivary TNF-α and TMAO levels were significantly elevated in the smoking periodontitis group compared to other groups ($p < 0.001$ and $p = 0.003$, respectively). Serum TMAO levels were also significantly higher in the smoking periodontitis group compared to non-smoking controls and non-smoking periodontitis. TMAO/SFR ratios were notably higher in the smoking periodontitis group compared to other groups, and a strong positive correlation was observed between salivary TMAO and TNF-α levels ($r = 0.892$, $p < 0.001$).

Conclusion: The data suggested that TMAO and TNF-α are associated with inflammatory mechanisms of periodontitis in cases where periodontitis coexists with smoking.

Trial Registration: NCT06580431

1 | Introduction

Approximately 8 million people a year worldwide have been come face to face with death by the use of tobacco products (Murray 2022). Smoking is correlated to increased levels of serum glucose, cortisol, free fatty acids, and adrenaline, as well as elevated blood pressure, heart rate, and consequently increased oxygen consumption by the heart (Abuse 2006). Inhalation of tobacco smoke or use of tobacco products leads to a significant increase in cardiovascular diseases, chronic obstructive pulmonary disease (COPD), Crohn's disease, and various types of cancers, tightly correlating with significant pathologies such as periodontitis; this underscores the potential harmful effects of smoking on human health (Chaffee et al. 2021). Reducing smoking is a critical step in preventing deaths and diseases. The complex effects of smoking on the vascular system, immune system,

and inflammatory processes have been extensively investigated (Dahdah et al. 2022). At a cellular level, smoking disrupts the regulated secretion of adhesion molecule components, triggering inappropriate leukocyte-endothelial binding and activation, leading to leukocyte accumulation in blood vessels and consequently exacerbating tissue damage associated with leukocytes (Ishida et al. 2024).

Periodontitis stands as one of the most widespread dental afflictions, stemming from an inflammatory cascade that impacts periodontal tissues, manifesting through inflammation of the periodontal soft tissues and the progressive deterioration of periodontal ligaments and alveolar (Yang et al. 2021). Smoking is also a significant risk factor in the pathogenesis and progression of periodontitis, suppressing clinical signs of the inflammatory response against infection (Johnson and Guthmiller 2007). Smoking reduces gingival vascularization, suppresses immune responses, impairs the morphological and functional healing of the periodontium, and alters biofilm complexity (Buduneli and Scott 2018). The effects of smoking on periodontal tissues have been associated with mechanisms such as decreased immunoglobulin G (IgG) production and increased prevalence of periodontopathogens. Furthermore, smoking may complicate the elimination of pathogens through mechanical therapy and decrease growth factor production (Adler et al. 2008). Periodontal clinical studies investigating the biochemical, clinical, and microbiological effects of smoking have revealed how pro-inflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-8, IL-6, and transforming growth factor-β (TGF-β)] alter the host response against bacterial biofilms (Sharma et al. 2011). Numerous animal studies have indicated that TNF-α plays a significant role in inflammatory processes such as alveolar bone loss and destruction of connective tissue in experimental periodontitis (Gomes et al. 2016). These findings support observations in human studies, emphasizing the critical role of TNF-α in the pathogenesis of periodontitis (Garlet 2010). Recently, in a clinical study evaluating the relationship between smoking and periodontitis in terms of serum TNF-α levels, it was reported that smokers with periodontitis exhibited significantly elevated TNF-α plasma levels (Knie et al. 2024).

Trimethylamine (TMA) is derived from dietary sources containing choline, carnitine, and betaine in humans, metabolized by gut microbiota. These metabolites are rapidly oxidized to TMA N-oxide (TMAO) by the liver enzyme flavin monooxygenase 3 (FMO3). TMAO is an amine oxide formed from the metabolism of choline-rich foods by intestinal bacteria, known for its pro-inflammatory effects contributing to vascular inflammation and endothelial dysfunction (Kumari et al. 2022; Janeiro et al. 2018). Elevated circulating TMAO levels are recognized as an independent predictor of adverse cardiovascular events. On the one hand, by activating nuclear factor kappa B (NF-kB), TMAO can increase the expression of inflammatory genes such as IL-1 and enhance oxidative stress, thereby augmenting the indicators of inflammatory markers associated with TMAO. Additionally, TMAO can contribute to inflammatory processes by enhancing macrophage chemotaxis and increasing the expression of inflammatory cytokines like TNF-α and IL-6 (Ma et al. 2017; Wang et al. 2023) Moreover, a broad literature indicates its association with various health issues including coronary artery disease, elevated blood lipids, diabetes, obesity, insulin resistance, metabolic syndrome, cancer, and kidney failure (Fletcher and Subramaniam 2018) (Janeiro et al. 2018). There is limited literature investigating the relationship between periodontal disease and TMAO. The clinical study indicates elevated circulating levels of TMAO in patients with Stage III-IV periodontitis, correlating with vascular endothelial dysfunction (Zhou et al. 2022). The animal study reports that *P. gingivalis* oral administration induced experimental periodontitis in mice, resulting in the increase of plasma TMAO and LPS, which was linked to aggravated atherosclerosis (AS) plaques (Xiao et al. 2022). Moreover, Wang et al. (2024) evaluated the effect of *P. gingivalis*, *Fusobacterium nucleatum*, and *Streptococcus mutans* oral administration on serum TMAO levels and TMAO-TMAO pathways in mice. Similar to previous animal study results, *Pg*, compared to the other selected bacteria, *caused elevation of* plasma TMAO levels and significantly influenced the TMA-TMAO pathway via lipid metabolism (Wang et al. 2024).

Although the relationship between TMAO and inflammation has been evaluated from a limited perspective, the role of TMAO on periodontal inflammation through smoking has not been fully elicited. To date, according to our knowledge, there is currently no data on TMAO and periodontal inflammation in smokers with periodontitis. The hypothesis of this study is that cigarette smoking may trigger periodontal inflammation by increasing TMAO levels both locally and systemically. Accordingly, our aim is to measure TMAO levels in serum and saliva samples from periodontitis patients who smoke and those who do not and to evaluate the relationship of these molecules with periodontal inflammation. The findings could contribute to understanding possible effects of periodontitis related to the pathogenesis of cardiovascular diseases under the influence of nicotine due to analyzing a unique marker.

2 | Materials and Methods

2.1 | Subjects and Study Design

Five hundred and twenty-seven patients who applied to Istanbul Medipol University Faculty of Dentistry Department of Periodontology were screened for study population. All participants included in this study were systemically healthy. The exclusion criteria for this study were (1) declined to participate and being under 18 and over 65 years of age; (2) having any systemic inflammatory diseases such as diabetes, rheumatoid arthritis, or systemic conditions, including immunodeficiency syndrome, cardiovascular disorders, or hepatic disorders; (3) the use of antibiotics and/or anti-inflammatory nonsteroidal anti-inflammatory drugs within 3 months preceding the study; (4) having nonsurgical periodontal treatment (previous 6 months); (5) having surgical periodontal treatment (previous 12 months); (6) having <20 natural teeth excluding the third molars. Smokers were classified as individuals smoking ≥ 10 cigarettes a day for at least 5 years, whereas non-smokers were individuals who had never smoked in their life (Yilmaz et al. 2024). The clinical diagnoses of periodontitis and a healthy periodontium were based on clinical and radiographic criteria, as detailed previously (Tonetti, Greenwell, and Kornman 2018). The healthy periodontium was defined to have a presence of a pocket probing depth (PPD) ≤ 3 mm and $<10\%$ bleeding (Chapple et al. 2018). Periodontitis was defined as individuals

with a detectable interdental clinical attachment level (CAL) at ≥2 non-adjacent teeth. For each tooth, interdental CAL at the side of the greatest loss was recorded, and CAL ≥5 mm was diagnosed as stage-III periodontitis (Tonetti, Greenwell, and Kornman 2018). The patients were graded according to the bone loss/age index as Grade B (index score between 0.25 and 1.00). However, the grade was upgraded to a higher grade as Grade C for smokers. Briefly, of 537 patients, 34 patients were excluded because of the remaining natural teeth number, 51 patients were excluded due to antibiotic usage during the last 3 months, and 15 patients declined to participate the study. Our data is presented in Figure 1 as a STROBE checklist (Figure 1).

The present study was approved by the human subject ethics board of Istanbul Medipol University (date: 21.12.2023; Number: 1061) for the use and access of human subjects in research and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The informed consent was signed by all included subjects.

2.1.1 | Clinical Parameters

The full-mouth periodontal indices of the plaque index (PI), PPD, CAL, and bleeding on probing (BOP) were recorded by a single calibrated examiner (I.B.) by using the William's markings periodontal probe (William's probe, Hu-Friedy, Chicago, IL) (Cekici et al. 2022). Mean scores for the full mouth of PPD and CAL in mm and the percentage of sites with BOP were calculated for each subject.

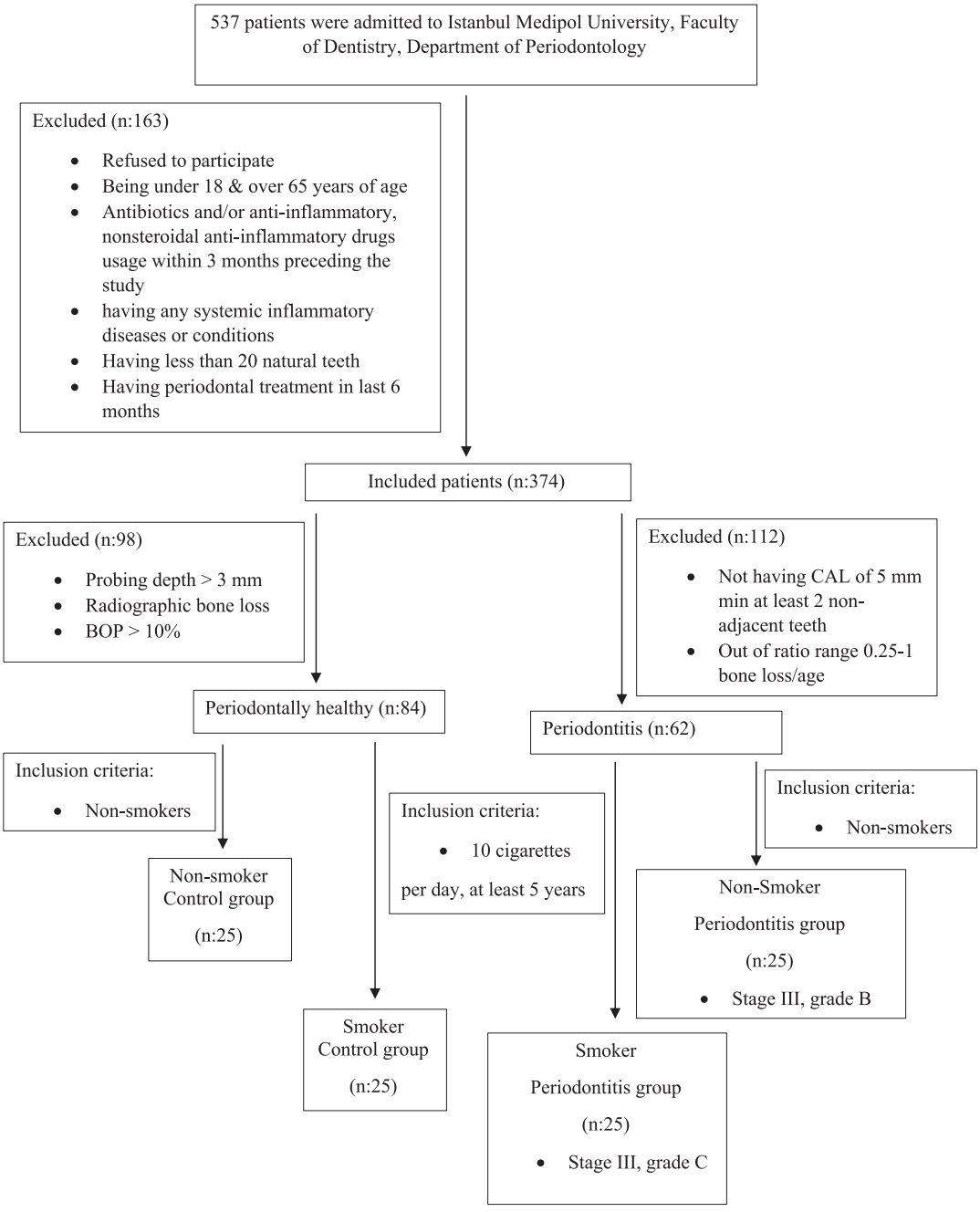

FIGURE 1 | STROBE checklist for healthy and periodontal disease participants due to smoking status.

2.1.2 | Sample Collection

To evaluate the selected markers in serum and saliva, samples were collected from each participant using standardized methods (Yilmaz et al. 2023). Unstimulated saliva samples were collected from each participant early in the morning when they were fasting. The patients were made to sit comfortably while giving a saliva sample and were asked to rinse their mouths with distilled water beforehand. After 10 min of spitting, the samples were centrifuged for 10 min at 2800 *g* and then transferred into Eppendorf tubes. Blood samples were collected immediately after saliva collection by qualified staff (NB) and centrifuged at 4000 *g* for 10 min to separate the serum. Saliva and serum were then stored at −80°C until analysis.

2.1.3 | Laboratory Analysis

2.1.3.1 | The TNF-α Analysis. TNF-α levels in collected saliva and serum samples were measured by commercial ELISA kits (ELISA Cloud Immunoassay, Cloud Clone Corp; cat #SEA079Hu) and analyzed according to manufacturers' instructions. The standard curve of the assay was used to determine the analyte concentrations in each sample. All samples were analyzed in duplicate, and the mean values were used in subsequent calculations.

2.1.3.2 | TMAO Analysis. TMAO levels in saliva and serum were determined by a liquid chromatography–mass spectrometry (LC MS/MS) method (LC–MS/MS, ESI Source, Thermo Scientific Accessmax), which was a modification of the method reported by Li et al. (2021) and similar to that reported in our previous study. To identify and quantify TMAO, samples were analyzed by an LC–MS/MS system equipped with a Thermo Dionex Ultimate 3000 UHPLC system and an TSQ Quantum Access Max quadrupole mass spectrometer (USA).

In addition to the determination of TMAO levels in saliva samples, the ratio of salivary TMAO to SFR was calculated for each group for evaluating the concentration of TMAO in a certain unit to uncover distinguishing characteristics among the study groups.

2.2 | Statistical Analysis

The sample size analysis was carried out a priori with special software (3.1.9.2 G*Power; https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower.html). For a large effect size of 0.40, power of 80%, and alpha value of 5%, the sample size was calculated to be at least 19 for each group, for a total of 76 people. To increase the power of the study, we decided to include 25 participants in each group (Yilmaz et al. 2024).

Statistical software (SPSS for Windows v.26, IBM SPSS Inc.) that is available commercially was used for all analyses. The Shapiro–Wilk test was used to evaluate the normality of the data. To compare variables among the four groups, the Bonferroni test was used as a "one-way analysis of variance (ANOVA)" multiple comparison test. The Kruskal–Wallis test was used for data that were not normally distributed. Correlations between biochemical and periodontal clinical data were analyzed by using the Spearman correlation test.

3 | Results

Table 1 represents demographic characteristics and clinical periodontal parameters of the study population (Table 1). A total of 100 systemically healthy subjects were involved in our study, comprising Group 1 = non-smokers with a healthy periodontium (NS-Control group; 12 females and 13 males; aged 37–46 years), Group 2 = non-smokers with Stage-III Grade B generalized periodontitis (NS-Periodontitis group; 13 females and 12 males; aged 40–50 years), Group 3 = smokers (≥10 cigarettes per day) with a healthy periodontium (S-Control group; 13 females and 12 males; aged; 38–48 years), and Group 4 = smokers (10 ≥ per day) with Stage-III Grade C generalized periodontitis (S-Periodontitis group; 11 females and 14 males; aged 42–46 years). There were no differences of age and gender among all groups. Saliva flow rate (SFR) was significantly lower in the S-periodontitis group compared to NS-Control and S-Control (respectively; $p = 0.001$, $p = 0.008$), whereas no differences were observed between periodontitis groups (NS-periodontitis and S-periodontitis) (Table 1). For S-control and S-periodontitis groups, the number of cigarettes per day was 10.64 ± 2.63 and 11.24 ± 2.89, respectively. The number of cigarettes for both smokers' groups was significantly higher compared to non-smokers groups, but there was no difference between S-periodontitis and S-control groups (Table 1). Briefly, all clinical periodontal parameters (PI, PPD, CAL, and BOP) were significantly higher in the non-smoking and smoking with periodontitis groups than in the NS-Control and S-Control groups ($p < 0.001$).

3.1 | Biochemical Results

The serum concentration of TNF-α was higher in the NS-periodontitis group than in the S-control group ($p < 0.05$), whereas saliva concentrations of TNF-α was higher in the S-Periodontitis group than in NS-control and NS-periodontitis, and the difference was statistically significant. Therewithal, saliva TNF-α concentrations in both NS and S periodontitis groups were significantly elevated compared to S-control (respectively; $p = 0.008$, $p < 0.001$) (Figure 2). Non-smokers and smokers with periodontitis demonstrated significantly higher TMAO ($p = 0.037$, $p = 0.003$) concentrations in saliva than non-smoking controls (Figure 2). In addition, serum concentration of TMAO was statistically significantly elevated in the S-Periodontitis group than in NS-control and NS-periodontitis ($p = 0.001$, $p = 0.023$; $p < 0.001$) (Figure 2).

Since SFR is affected by smoking, we also calculated the ratio between salivary TMAO and SFR for evaluating the concentration of TMAO in a certain unit with this way to determine the role of this molecule in the determined inflammatory pathway (Figure 3). Besides, SFR is affected by smoking, and TMAO values were proportional to SFR in our study. In the S-Periodontitis group, in saliva, TMAO/SFR ratios were significantly higher than those in the NS-Control, NS-Periodontitis, and S-Control groups (respectively; $p < 0.001$, $p = 0.023$, $p = 0.002$). Similarly, in

TABLE 1 | Demographic, clinical, and biochemical parameters of periodontitis and control groups.

Parameters	NS-Control, $n=25$	NS-Periodontitis, $n=25$	S-Control, $n=25$	S-Periodontitis, $n=25$	p
Age (year)	42 (37–46)	46 (40–50)	42 (38–48)	43 (42–46)	0.265
Gender F/M	12/13	13/12	13/12	11/14	0.932
PI[a]	**0.84 (0.46–0.97)**	**2.44 (2.20–2.74)**	**0.80 (0.65–0.91)**	**1.91 (1.62–2.20)**	**<0.001**
PPD (mm)[a]	**1.45 ± 0.24**	**3.74 ± 0.70**	**1.58 ± 0.26**	**3.67 ± 0.70**	**<0.001**
BOP (%)[a]	**4.92 ± 3.19**	**60.08 ± 23.86**	**6.50 ± 2.73**	**34.41 ± 12.43**	**<0.001**
CAL (mm)[a]	**0.00 (0.00–0.00)**	**3.99 (3.60–4.67)**	**0.00 (0.00–0.00)**	**4.14 (3.67–4.72)**	**<0.001**
SFR	0.40 (0.30–0.45)	0.30 (0.25–0.40)	0.40 (0.30–0.40)	**0.25 (0.20–0.35)[b]**	**0.002**
Number of cigarettes (per day)	0.00 ± 0.00	0.00 ± 0.00	**10.64 ± 2.63[c]**	**11.24 ± 2.89[c]**	**<0.001**
TNF-α serum (pg/mL)	209 (176–245)	**253 (233–270)**	**190 (164–199)**	206 (180–248)	**0.013**
TNF-α saliva (pg/mL)	**166 (155–189)**	**204 (148–339)**	**150 (126–167)**	**305 (239–368)**	**<0.001**
TMAO saliva (ng/mL)	5.70 (2.93–6.76)	**8.10 (4.06–14.0)**	5.20 (3.60–11.1)	**9.70 (6.03–14.2)**	**0.003**
TMAO serum (ng/mL)	169 (117–211)	185 (135–252)	167 (133–313)	**295 (217–335)**	**0.002**

Note: Data shown as mean ± standard deviation and median (IQR). Statistical difference with the control group $p<0.05$.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment lost; PI, plaque index; PPD, pocket probing depth; SFR, saliva flow rate; TMAO, trimethylamine N-oxide; TNF-α, tumor necrosis factor-alpha.
[a]Both periodontitis groups are different from both control groups, there is no difference between both periodontitis groups or control groups.
[b]S-Periodontitis is different from NS-Control and S-Control (respectively; $p=0.001$, $p=0.008$).
[c]Groups of smokers are different from groups of non-smokers, but there is no difference between groups of smokers.

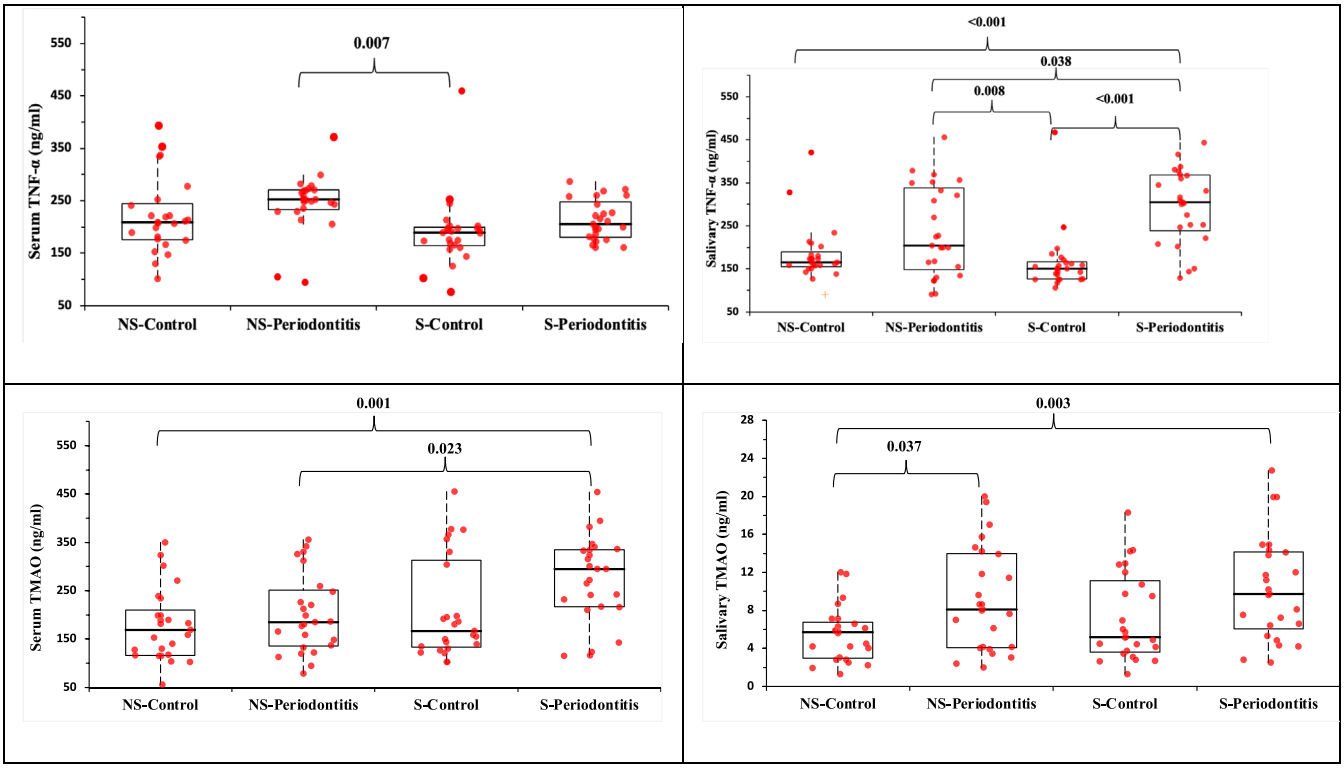

FIGURE 2 | Serum and salivary TNF-α and TMAO levels in periodontitis and control groups. Salivary and serum levels of TMAO and TNF-α levels in smoker and non-smoker control (S-C and NS-C) and periodontitis (S-P and NS-P) groups. Box-and-whisker plots with the median (horizontal line) and interquartile range (box) values are shown. Significantly different ($p<0.05$).

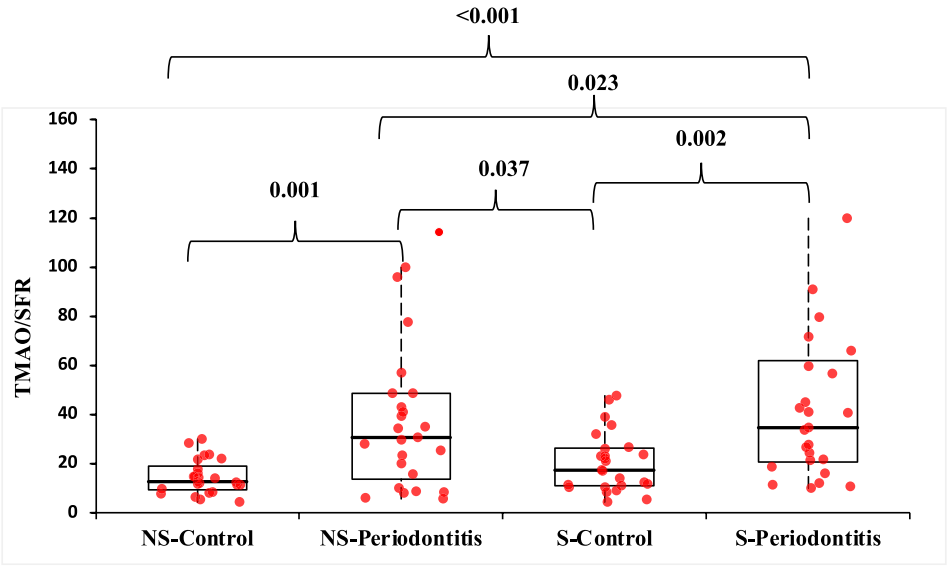

FIGURE 3 | Salivary TMAO/SFR ratio levels in periodontitis and control groups. Salivary TMAO/SFR ratio in smoker and non-smoker control (S-C and NS-C) and periodontitis (S-P and NS-P) groups. Box-and-whisker plots with the median (horizontal line) and interquartile range (box) values are shown. Significantly different ($p < 0.05$).

the NS-periodontitis group, ratios were different from those in the NS and S control groups, which was statistically significant ($p = 0.001$, $p = 0.037$; Figure 3).

3.2 | Correlation Analysis

Table 2 presents the correlation between clinical data and selected biochemical analytes. Saliva and serum levels of TNF-α were low degree, positively and significantly correlated with all the clinical periodontal parameters (r values 0.296 to 0.413; $p < 0.05$; Table 2). There was significant correlation between saliva TMAO and PI, CAL, PPD, and BOP ($r = 0.347$, $r = 0.302$, $r = 0.303$, $r = 0.208$; $p < 0.05$, $p < 0.001$, respectively). The number of cigarettes, PPD, and CAL measurements showed a positive and significant correlation with TMAO serum levels ($p < 0.05$, Table 2).

TMA/SFR was positively correlated with all clinical parameters but with a moderate degree of significant correlation ($p < 0.001$). The positive correlation was found between TMAO/SFR and TMAO levels in both saliva and serum; saliva TMAO was the strongest ($r = 0.892$, $p < 0.001$; Table 2).

4 | Discussion

In this study, the levels of TMAO and TNF-α in both saliva and serum were assessed in individuals with periodontitis as well as in those with healthy gingiva, with a focus on smoking

TABLE 2 | Correlations between biomarkers and periodontal clinical parameters (Spearman correlation coefficients, r values) ($n = 100$).

Variables	Serum TNF-α	Salivary TNF-α	Salivary TMAO	Serum TMAO	TMAO/SFR
Salivary TNF-α	0.135	—	—	—	—
Salivary TMAO	0.003	0.114	—	—	—
Serum TMAO	0.000	0.146	**0.555****	—	—
TMAO/SFR	−0.008	0.143	**0.892****	**0.533****	—
Age	0.075	0.124	0.181	−0.009	**0.201***
PPD	**0.334***	**0.393****	**0.303***	**0.231***	**0.405****
CAL	**0.308***	**0.413****	**0.302***	**0.248***	**0.428****
BOP	**0.327***	**0.296***	**0.208***	0.191	**0.351****
PI	**0.408****	**0.381****	**0.347****	0.159	**0.408****
SFR	−0.018	−0.126	−0.088	−0.175	**−0.481****
Number of cigarettes	**−0.304***	0.031	0.155	**0.230***	0.169

Note: Significantly different values are shown in boldface type, *$p < 0.05$; **$p < 0.001$.

status. The absence of significant differences in demographic variables, such as age and gender, indicates that the data distribution is appropriate and balanced. This research sought to determine whether smoking significantly influences these parameters in the context of periodontitis. The study groups were delineated as non-smoking controls, smoking controls, non-smoking periodontitis patients, and smoking periodontitis patients. In addition to comparing the serum and salivary levels of TMAO and TNF-α with clinical parameters, with the aim for elucidating the role of TMAO in periodontal inflammatory pathway, we computed the ratio of TMAO to SFR and analyzed the relative changes in the TMAO to SFR ratio to uncover distinguishing characteristics among the study cohorts. This approach can be considered as the main strength of the study. Figure 2 reveals that salivary TMAO levels were significantly higher in S-Periodontitis patients compared to the control group. Additionally, an increase in TMAO was observed in individuals with periodontitis, indicating that periodontitis contributes to elevated TMAO levels, thereby playing a role in the inflammatory process. However, the impact of smoking remains ambiguous. When comparing TMAO levels in smaller units, Figure 3 presents a more nuanced analysis by evaluating TMAO levels in relation to the SFR. This approach highlights a more meaningful result, demonstrating that TMAO levels increase with the presence of periodontitis, underscoring its role in inflammatory pathology. A significant difference in TMAO levels was found between S-Periodontitis patients and NS-Periodontitis patients, illustrating the cumulative effects of smoking and periodontitis on TMAO. The data suggest a notable change in TMAO levels per unit area. The principal distinction between Figures 2 and 3 is the overall increase in TMAO levels associated with periodontitis. The effect of smoking, however, remains a subject of discussion. Yet, when examining TMAO levels per unit area, the impact of periodontitis is clearly evident. The combination of smoking and periodontitis further elevates TMAO levels, indicating that this molecule is influenced by both factors. As a biomarker for cardiovascular disease, TMAO is considered a risk factor for both periodontitis and smoking independently. Therefore, TMAO emerges as a significant molecule when both smoking and periodontitis are present.

During the data processing phase, several limitations were encountered. One limitation was the lack of analyzing gingival crevicular fluid (GCF) of participants included. However, saliva is the most studied biofluid in the scientific literature and contains many enzymes and cytokines, which were indicators of periodontitis (Önder et al. 2023), and the content of the saliva is also influenced by various factors such as diabetes and AS and it harbors numerous microorganisms and their products which can cause challenge in detecting significant differences between the study groups (Li et al. 2022). In our study, for eliminating the factors that cause this complexity in saliva, only the systemically healthy participants were included, and the meticulous methodology was applied. Still, to understand the site-specific nature of the sample as a diagnostic marker, incorporating GCF analysis could have provided more valuable insights and would have strengthened the validity of the research. Another limitation encountered was the inability to classify the smoking groups based on the duration of smoking and not to include biochemical validation considering the serum nicotine levels. The association between the duration of smoking exposure and the prevalence as well as the extent of periodontitis has been thoroughly established in the literature (Costa et al. 2013). Nonetheless, investigating the length of smoking exposure and the confirmation of cigarette abstinence by systemic inspection in this research might have provided critical insights into the impact of the examined biomarkers on disease progression and this would also allow for stratification of the data by associating the specific dose–response relationship between smoking and TMAO levels. In the context of this study, including participants who had been smoking at least 10 cigarettes daily for a minimum of 5 years enhanced the transparency of our findings and improved the alignment with the study's objectives. Furthermore, the close similarity without significant differences in the daily cigarette consumption among the smoking groups (Table 1) supports the appropriateness of this criterion.

The investigation revealed that TMAO concentrations in saliva were significantly higher in both the NS-Periodontitis and S-Periodontitis groups compared to the non-smoking control group. Nevertheless, no significant difference was detected between the S-Periodontitis and S-Control groups. In contrast, serum TMAO levels were significantly elevated in the S-Periodontitis group relative to both the S-Control and NS-Periodontitis groups. However, no significant variation in serum TMAO levels was observed between the S-Periodontitis and S-Control groups, indicating that elevated serum TMAO may occur independently of periodontal disease and is influenced by smoking. This finding supports the mechanism proposed by Xiao et al. (2022), which suggests that alterations in the gut microbiome and the subsequent increase in TMAO levels are underlying factors contributing to this observation. This research with $ApoE^{-/-}$ mice has shown that experimental periodontitis leads to gut dysbiosis and increased TMAO levels. These results suggest that periodontitis may disrupt the gut microbiome and metabolic processes, thereby enhancing liver and gut inflammation and potentially accelerating AS (Xiao et al. 2022). Moreover, in patients with periodontitis, inflammatory cytokines are elevated in both serum and gingival tissues. This elevation may increase the risk of cardiovascular and metabolic diseases due to shared pathogenic mechanisms (Sanz et al. 2020). This finding underscores the systemic impact of smoking on TMAO levels. High serum TMAO is acknowledged as an independent marker for adverse cardiovascular events. In a related study using a different mouse model, chronic administration of TMAO induced early-stage AS in non-genetically modified mice. This condition was characterized by specific histopathological lesions, and these alterations were associated with elevated inflammatory marker levels in the serum (Florea et al. 2022). Another investigation observed that TMAO triggers excessive mitophagy in endothelial cells, thereby facilitating pyroptosis (Chen et al. 2024). The results comparing the S-Control and NS-Control groups indicate that elevated serum TMAO levels are associated with periodontal disease; however, smoking independently contributes to a significant increase in TMAO levels, regardless of periodontal disease. Furthermore, smoking appears to exacerbate the increase in serum TMAO levels associated with periodontal disease parameters. Comparisons between control groups reveal a positive correlation between TMAO levels in both serum and saliva.

Previous studies have observed elevated TNF-α plasma levels in smokers, with these levels being particularly increased in smokers with periodontitis (Nile et al. 2013). The variability in

TNF-α values in saliva across different groups is attributed to the influence of both systemic conditions and oral microbiota on salivary content. The presence of both smoking and periodontitis represents two distinct inflammatory processes and factors that alter host responses, leading to a significant increase in TNF-α levels. Although TNF-α levels are high in both the NS-Periodontitis and NS-Control groups, no significant difference is observed between them. A significant difference is noted between the S-Periodontitis and NS-Periodontitis groups. The variations in TNF-α levels between smokers and non-smokers suggest that TNF-α acts as a principal inflammatory mediator in periodontal disease for both smokers and non-smokers (Hao et al. 2023).

When evaluating the relationships between TMAO and TNF-α molecules with clinical parameters, the associations between age and periodontal parameters (PPD, CAL, BOP, and PI) are weak. Notably, salivary TMAO levels exhibit strong correlations with periodontal parameters. Additionally, while smoking may be associated with some biomarkers, its association with other biomarkers is not significant. Recent large-scale studies have demonstrated a strong association between both mild and severe periodontitis and cardiovascular diseases (CVDs), stroke, and endothelial dysfunction. In particular, it has been suggested that severe periodontitis may play a significant role in the pathophysiology of AS and CVDs by impairing endothelial function (Angjelova et al. 2024). The mechanisms explaining the relationship between periodontitis and these diseases involve periodontopathogens and inflammatory markers that affect the immune response. Although it is established that periodontal pathogens and their by-products enter the bloodstream, leading to endothelial dysfunction and atherosclerotic lesions, it remains unclear whether these microorganisms directly contribute to AS (Zardawi et al. 2021). Common periodontal pathogens identified in atherosclerotic plaques include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*. Epidemiological evidence links these pathogens to AS. The mechanisms by which these pathogens contribute to AS include the disruption of the endothelial barrier, activation of the immune system, facilitation of monocyte adhesion and aggregation, and promotion of foam cell formation. These mechanisms contribute to the progression and destabilization of atherosclerotic plaques (Huang et al. 2023). The elevated serum and salivary TMAO levels observed in our study support the mechanism proposed by a recent study from Guowu Gan et al. (2023), which highlights the association between *Porphyromonas gingivalis* infection, changes in gut microbiota, and the subsequent increase in TMAO levels. The study demonstrated that *Porphyromonas gingivalis* infection-induced chronic apical periodontitis significantly altered the composition and diversity of gut microbiota in mice (Gan et al. 2023). Additionally, elevated levels of TMA N-oxide (TMAO) and a positive correlation between TMAO levels and the relative abundance of *Lachnospiraceae* and *Porphyromonadaceae* were observed in ApoE$^{-/-}$ mice ($p < 0.05$). These findings suggest that gut microbiota may provide a critical link between oral infections and the pathogenesis of AS (Gan et al. 2023). According to our results, clinical indices BOP and PI, which show significant differences with salivary TMAO, exhibit positive correlations when analyzed (Table 1). Additionally, PI is corelated with the TMAO/SFR ratio (Table 2). The correlation of the inflammatory marker BOP index stands out more prominently in this study compared to the bacterial load marker PI index.

Saliva plays a crucial physiological role in normal oral functions such as swallowing, chewing, and taste perception (Vandenberghe-Descamps et al. 2016). The SFR is pivotal in maintaining oral homeostasis and protecting the oral mucosa from dryness. Normal SFR ranges from 0.3 to 0.65 mL/min under stimulation and 1.5 to 6 mL/min; thus, an SFR of < 0.2 mL/min indicates hyposalivation due to various external and internal factors such as smoking and glandular hypofunction (Grover et al. 2016). New smokers often report increased salivary flow due to continuous stimulation by nicotine, although protective components in saliva may be reduced. Some prospective studies suggest chronic smoking damages salivary glands, leading to a decrease in SFR. Interestingly, similar results have been observed in passive smokers, suggesting nicotine as a potent trigger. However, the effects of smoking are debated, as some studies show weak or no correlation between smoking and SFR (Qiu et al. 2017). Research has demonstrated that SFR initially increases significantly under nicotine stimulation, but prolonged exposure to nicotine and other toxic components can damage salivary gland tissues and cause oral dryness. This indicates that nicotine stimulation leads to periodic increases in SFR, which may help prevent dental caries by disrupting pathogenic activity and inhibiting tartar formation. Nevertheless, continuous stimulation of taste receptors can reduce SFR and minimize its protective effects on periodontal tissues (Nigar et al. 2022).

Smoking is a major addictive behavior that significantly contributes to the onset of systemic diseases such as cardiovascular issues, diabetes, and hypertension, all of which pose severe health risks. Furthermore, it influences the distribution and progression of periodontal conditions. The act of smoking alters the body's responses, elevates oxidative stress, promotes the formation of blood clots, decreases blood vessel formation, increases sympathetic nerve activity, and damages the endothelial lining (Kondo et al. 2019; Zee 2009). The resulting oxidative stress leads to cellular injury and is implicated in the development of several conditions, including atherosclerotic CVD (ASCVD). Recent studies have shown that TMAO activates Nod-like receptor pyrin domain-containing 3 (NLRP3) inflammasome in endothelial cells, involving the production of reactive oxygen species (ROS) through mitochondrial ROS signaling pathways (Al Samarraie, Pichette, and Rousseau 2023). Another study has demonstrated that cigarette smoke extract (CSE) increases oxidative stress, leading to the accumulation of ROS and mitochondrial damage. These effects of CSE are mitigated by TMAO. The findings indicate that oxidative stress and ROS accumulation play interconnected and interactive roles in the apoptosis induced by CSE (Chuangxin et al. 2015). ROS contribute to tissue damage through various mechanisms, including DNA damage, lipid peroxidation, protein damage (particularly affecting gingival hyaluronic acid and proteoglycans), and oxidation of antiproteases. Recent years have seen a growing interest in the role of oxidative stress in the pathogenesis of periodontal diseases. ROS play a critical role in the normal functioning of cellular metabolism and are continuously produced by cells (Wang, Andrukhov, and Rausch-Fan 2017). ROS are typically balanced by biochemical antioxidants in aerobic cells. Recent studies have highlighted the impact of increased oxidative stress and impaired antioxidant defenses on the development and progression

of complications in coronary artery disease. Neutrophils, as the predominant white blood cells in circulation, provide primary defense against bacterial infections; they migrate to periodontal tissues and gingival sulcus when activated by pathogenic biofilms and are considered major contributors to ROS production in periodontitis (Angjelova et al. 2024). Dysregulation of homeostasis is often associated with an increase in ROS or a decrease in antioxidant capacity, leading to oxidative stress. Oxidative stress is linked to various systemic diseases such as cardiovascular disorders, arthritis, and diabetes. Scientific literature indicates that oxidative stress may play a potential role in the development of periodontal diseases, as elevated ROS levels produced by inflammatory cells can contribute to tissue damage (da Silva et al. 2018). In a prospective randomized clinical study results showed that smoking cessation had minor effects on the gut microbiome and there was no significant changes in TMAO levels after an attempt at quitting smoking (Sublette et al. 2020). In a study conducted on a mice model, a significant increase in fecal choline and betaine levels, which are the precursor of TMAO, were observed after smoke exposure (Fluhr et al. 2021). In parallel with the findings of Fluhr et al. (2021), our study also observed similar TMAO elevations associated with smoking. Clarifying the relationship between smoking and TMAO production may provide beneficial information in understanding the factors that cause CVD.

Due to our results, a decrease in BOP values in smoking groups supports the notion that smoking might suppress the disease, likely due to its well-documented suppressive effects on vascular responses. Smoking influences the host's response by elevating levels of critical pro-inflammatory cytokines, such as TNF-α. TNF-α, which is produced by monocytes, exerts extensive pro-inflammatory and immune-regulatory effects on various cell types, including fibroblasts. Our research indicates that patients with periodontitis, particularly those who smoke, exhibit markedly higher levels of salivary TNF-α compared to individuals in the control group. These findings align with previous research. Additionally, the observed positive correlation between serum TNF-α levels and BOP underscores TNF-α's role as a significant biomarker for diagnosing periodontitis (Lang et al. 1986). The lack of similar studies limits comparisons with our findings; however, salivary TMAO and TNF-α levels are more distinctly detectable in relation to periodontitis and smoking. This suggests that TNF-α tends to increase due to negative conditions such as smoking and periodontitis and can be systemically detected when both conditions are present.

This study emphasizes the importance of TMAO and TNF-α as significant inflammatory markers in the context of periodontitis and smoking. The investigation reveals a notable increase in salivary TMAO and TNF-α levels in periodontitis patients who smoke compared to the control group, providing novel insights into the role of these biomarkers in the pathogenesis of periodontitis. The findings indicate that periodontitis is associated with a substantial increase in TMAO levels, highlighting its involvement in the inflammatory processes underlying the disease. In our study, no correlation was observed between TNF-α and TMAO in neither saliva nor serum. This data does not support previous studies which demonstrated that acute TMAO injection induces inflammatory gene expression, including TNF-α, in $LDLR^{-/-}$ mice and five-sixths nephrectomy-induced chronic kidney disease (CKD) rats (Lai et al. 2022; Seldin et al. 2016). As mentioned before, the presence of two distinct inflammatory processes and factors that alter host responses in our study may cause the results to be contradictory. Moreover, the cumulative effects of smoking and periodontitis on TMAO underscore TMAO as a central molecule influenced by both factors, reinforcing its relevance as a marker for CVD risk. It is also noteworthy that the TMAO/SFR ratio is significantly higher in smoking periodontitis patients compared to non-smoking periodontitis patients, demonstrating the combined impact of periodontitis, and smoking on TMAO levels. Owing to cues originating from the oral environment including not only salivary TMAO levels but also dental plaque accumulation quantity (PI representation) might be a significant differential risk factor for developing CVDs such as vascular endothelial dysfunction and myocardial infarction via eliminating the presence or absence of standard risk factors, which include hypercholesterolemia, diabetes, and hypertension. Understanding how TMAO works in periodontitis related to smoking could ultimately be subsidiary to monitor or even diagnose CVDs early in many people. Additionally, CVDs may become preventable with decreasing TMAO levels via treating the existing periodontitis.

Possible inflammatory molecules could have been evaluated together with TMAO in our study, but in this study, we preferred to evaluate TNF-α, whose relationship with TMAO has been evaluated many times in the context of systemic diseases, as a single guide molecule. Thus, it was thought that the TMAO–periodontitis–smoking interaction could be conveyed more clearly. However, a comprehensive and detailed analysis is needed to better understand the specific contributions of TMAO and related inflammatory molecules to periodontitis as modulated by smoking. This research makes a significant contribution to the literature by elucidating the effects of periodontitis and smoking on disease progression and clarifying the role of TMAO in these processes.

5 | Conclusion

This study is the first to investigate the relationship between TMAO and TNF-α in the context of smoking and periodontitis. Despite the study's constraints, the results demonstrate an increase in salivary levels of TMAO and TNF-α among individuals with periodontitis. Elevated TMAO and TNF-α levels in saliva may be linked to mechanisms involving heightened inflammation or could contribute to the disease's pathogenesis. When periodontitis is present alongside smoking, serum levels of TMAO and TNF-α may serve as potential indicators for the condition. Smoking is found to elevate the levels of these biomarkers in the serum. Notably, salivary TMAO and TNF-α levels are significantly higher in smokers with periodontitis compared to non-smokers with or without periodontitis.

Author Contributions

Ipek Bal: investigation, project administration, writing – original draft, methodology, writing – review and editing. **Nur Balci:** conceptualization, methodology, data curation, investigation, validation, project administration, writing – original draft, writing – review and editing, formal analysis. **Cem Sorguc:** data curation, software, validation, formal analysis, writing – review and editing. **Hilal Uslu Toygar:** methodology, supervision, writing – review and editing, validation, visualization. **Ceyhan C. Serdar:** conceptualization, visualization,

writing – original draft, writing – review and editing. **Sivge Kurgan:** conceptualization, methodology, data curation, formal analysis, supervision, writing – review and editing. **Muhittin A. Serdar:** methodology, software, data curation, supervision, formal analysis, writing – review and editing, resources.

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Ethics Statement

This study was approved by the Ethics Committee of Istanbul Medipol University (No: 1061), and it was carried out in accordance with the Helsinki Declaration.

Consent

At the start of the trial, all participants provided written permission.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

References

Adler, L., C. Modin, J. Friskopp, and L. Jansson. 2008. "Relationship Between Smoking and Periodontal Probing Pocket Depth Profile." *Swedish Dental Journal* 32: 157–163. https://tandlakarforbundet.se/app/uploads/2017/01/sdj-2008-4.pdf#page=3.

Al Samarraie, A., M. Pichette, and G. Rousseau. 2023. "Role of the Gut Microbiome in the Development of Atherosclerotic Cardiovascular Disease." *International Journal of Molecular Sciences* 24: 5420. https://doi.org/10.3390/ijms24065420.

Angjelova, A., E. Jovanova, A. Polizzi, et al. 2024. "Impact of Periodontitis on Endothelial Risk Dysfunction and Oxidative Stress Improvement in Patients With Cardiovascular Disease." *Journal of Clinical Medicine* 13: 3781. https://doi.org/10.3390/jcm13133781.

Buduneli, N., and D. A. Scott. 2018. "Tobacco-Induced Suppression of the Vascular Response to Dental Plaque." *Molecular Oral Microbiology* 33: 271–282. https://doi.org/10.1111/omi.12228.

Cekici, A., S. Sahinkaya, M. Fatih Donmez, E. Turkmen, N. Balci, and H. Toygar. 2022. "Serum and Saliva Sirtuin 6, Lipoxin A4, Caspase8 Levels in Correlation With Periodontal Status in Severe Periodontitis." https://doi.org/10.21203/rs.3.rs-1729628/v1.

Chaffee, B. W., E. T. Couch, M. V. Vora, and R. S. Holliday. 2021. "Oral and Periodontal Implications of Tobacco and Nicotine Products." *Periodontology 2000* 87: 241–253. https://doi.org/10.1111/prd.12395.

Chapple, I. L. C., B. L. Mealey, T. E. Van Dyke, et al. 2018. "Periodontal Health and Gingival Diseases and Conditions on an Intact and a Reduced Periodontium: Consensus Report of Workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions." *Journal of Periodontology* 89: S74–S84. https://doi.org/10.1002/JPER.17-0719.

Chen, Y., C. Yuan, W. Qin, B. Yu, D. Wei, and P. Wu. 2024. "TMAO Promotes Vascular Endothelial Cell Pyroptosis via the LPEAT-Mitophagy Pathway." *Biochemical and Biophysical Research Communications* 703: 149667. https://doi.org/10.1016/j.bbrc.2024.149667.

Chuangxin, H., J. J. Wang, J. H. Ma, J. Chenjin, Y. Qiang, and X. Z. Sarah. 2015. "Activation of the UPR Protects Against Cigarette Smokeinduced Induced RPE Apoptosis Through Up-Regulation of NrF2." *Journal of Biological Chemistry* 290, no. 9: 5367–5380. https://doi.org/10.1074/jbc.M114.603738.

Costa, F. O., L. O. M. Cota, E. J. P. Lages, et al. 2013. "Associations of Duration of Smoking Cessation and Cumulative Smoking Exposure With Periodontitis." *Journal of Oral Science* 55, no. 3: 245–253. https://doi.org/10.2334/josnusd.55.245.

da Silva, J. C., F. W. M. G. Muniz, H. J. R. Oballe, M. Andrades, C. K. Rösing, and J. Cavagni. 2018. "The Effect of Periodontal Therapy on Oxidative Stress Biomarkers: A Systematic Review." *Journal of Clinical Periodontology* 45, no. 10: 1222–1237. https://doi.org/10.1111/jcpe.12993.

Dahdah, A., R. M. Jaggers, G. Sreejit, et al. 2022. "Immunological Insights Into Cigarette Smoking-Induced Cardiovascular Disease Risk." *Cells* 11: 3190. https://doi.org/10.3390/cells11203190.

Fletcher, C., and S. Subramaniam. 2018. "Themed Section: Spotlight on Small Molecules in Cardiovascular Diseases Trimethylamine N-Oxide: Breathe New Life." *British Journal of Pharmacology* 175: 1344. https://doi.org/10.1111/bph.v175.8/issuetoc.

Florea, C. M., R. Rosu, G. Cismaru, et al. 2022. "Chronic Oral Trimethylamine-N-Oxide Administration Induces Experimental Incipient Atherosclerosis in Non-Genetically Modified Mice." *Journal of Physiology and Pharmacology* 73, no. 5: 633–643. https://doi.org/10.26402/jpp.2022.5.07.

Fluhr, L., U. Mor, A. A. Kolodziejczyk, et al. 2021. "Gut Microbiota Modulates Weight Gain in Mice After Discontinued Smoke Exposure." *Nature* 600, no. 7890: 713–719. https://doi.org/10.1038/s41586-021-04194-8.

Gan, G., R. Zhang, B. Lu, et al. 2023. "Gut Microbiota May Mediate the Impact of Chronic Apical Periodontitis on Atherosclerosis in Apolipoprotein E-Deficient Mice." *International Endodontic Journal* 56, no. 1: 53–68. https://doi.org/10.1111/iej.13845.

Garlet, G. P. 2010. "Critical Reviews in Oral Biology & Medicine: Destructive and Protective Roles of Cytokines in Periodontitis: A Re-Appraisal From Host Defense and Tissue Destruction Viewpoints." *Journal of Dental Research* 89: 1349–1363. https://doi.org/10.1177/0022034510376402.

Gomes, F. I. F., M. G. B. Aragão, F. C. B. Barbosa, M. M. Bezerra, V. De Paulo Teixeira Pinto, and H. V. Chaves. 2016. "Inflammatory Cytokines Interleukin-1β and Tumour Necrosis Factor-α—Novel Biomarkers for the Detection of Periodontal Diseases: A Literature Review." *Journal of Oral and Maxillofacial Research* 7, no. 2: e2. https://doi.org/10.5037/jomr.2016.7202.

Grover, N., J. Sharma, S. Sengupta, S. Singh, N. Singh, and H. Kaur. 2016. "Long-Term Effect of Tobacco on Unstimulated Salivary pH." *Journal of Oral and Maxillofacial Pathology* 20, no. 1: 16–19. https://doi.org/10.4103/0973-029X.180907.

Hao, C. P., N. J. Cao, Y. H. Zhu, and W. Wang. 2023. "The Impact of Smoking on Periodontitis Patients' GCF/Serum Cytokine Profile Both Before and After Periodontal Therapy: A Meta-Analysis." *BMC Oral Health* 23, no. 1: 60. https://doi.org/10.1186/s12903-023-02768-8.

Huang, X., M. Xie, X. Lu, et al. 2023. "The Roles of Periodontal Bacteria in Atherosclerosis." *International Journal of Molecular Sciences* 24: 12861. https://doi.org/10.3390/ijms241612861.

Ishida, M., C. Sakai, Y. Kobayashi, and T. Ishida. 2024. "Cigarette Smoking and Atherosclerotic Cardiovascular Disease." *Journal of Atherosclerosis and Thrombosis* 31: 189–200. https://doi.org/10.5551/jat.RV22015.

Janeiro, M. H., M. J. Ramírez, F. I. Milagro, J. A. Martínez, and M. Solas. 2018. "Implication of Trimethylamine n-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target." *Nutrients* 10: 1398. https://doi.org/10.3390/nu10101398.

Johnson, G. K., and J. M. Guthmiller. 2007. "The Impact of Cigarette Smoking on Periodontal Disease and Treatment." *Periodontology 2000* 44: 178–194.

Knie, L. V., K. N. Leknes, Y. Xue, S. A. Lie, and D. F. Bunæs. 2024. "Serum Biomarker Levels in Smokers and Non-Smokers Following Periodontal Therapy. A Prospective Cohort Study." *BMC Oral Health* 24, no. 1: 463. https://doi.org/10.1186/s12903-024-04196-8.

Kondo, T., Y. Nakano, S. Adachi, and T. Murohara. 2019. "Effects of Tobacco Smoking on Cardiovascular Disease." *Circulation Journal* 83: 1980–1985. https://doi.org/10.1253/circj.CJ-19-0323.

Kumari, K., K. S. Singh, K. Singh, R. Bakhshi, and L. R. Singh. 2022. "TMAO to the Rescue of Pathogenic Protein Variants." *Biochimica et Biophysica Acta – General Subjects* 1866: 130214. https://doi.org/10.1016/j.bbagen.2022.130214.

Lai, Y., H. Tang, X. Zhang, et al. 2022. "Trimethylamine-N-Oxide Aggravates Kidney Injury via Activation of p38/MAPK Signaling and Upregulation of HuR." *Kidney and Blood Pressure Research* 47, no. 1: 61–71. https://doi.org/10.1159/000519603.

Lang, N. P., A. Joss, T. Orsanic, F. A. Gusberti, and B. E. Siegrist. 1986. "Bleeding on Probing. A Predictor for the Progression of Periodontal Disease?" *Journal of Clinical Periodontology* 13: 590–596.

Li, Y., J. Kang, Y. Lee, J. Y. Chung, and J. Y. Cho. 2021. "A Validated Simple LC-MS/MS Method for Quantifying Trimethylamine N-Oxide (TMAO) Using a Surrogate Matrix and Its Clinical Application." *Translational and Clinical Pharmacology* 29, no. 4: 216–225. https://doi.org/10.12793/tcp.2021.29.e19.

Li, Y., M. Zhu, Y. Liu, et al. 2022. "The Oral Microbiota and Cardiometabolic Health: A Comprehensive Review and Emerging Insights." *Frontiers in Immunology* 13: 1010368. https://doi.org/10.3389/fimmu.2022.1010368.

Ma, G. H., B. Pan, Y. Chen, et al. 2017. "Trimethylamine N-Oxide in Atherogenesis: Impairing Endothelial Self-Repair Capacity and Enhancing Monocyte Adhesion." *Bioscience Reports* 37, no. 2: BSR20160244. https://doi.org/10.1042/BSR20160244.

Murray, C. J. L., 2022. "The Global Burden of Disease Study at 30 Years." *Nature Medicine* 28, no. 10: 2019–2026. https://doi.org/10.1038/s41591-022-01990-1.

Nigar, S., S. Hassan, A. Maqsood, et al. 2022. "An Assessment of Unstimulated Salivary Flow Rate, IgA and Clinical Oral Dryness Among Active and Passive Smokers." *International Journal of Occupational Medicine and Environmental Health* 35, no. 1: 39–51. https://doi.org/10.13075/IJOMEH.1896.01829.

Nile, C. J., S. Sherrabeh, G. Ramage, and D. F. Lappin. 2013. "Comparison of Circulating Tumour Necrosis Factor Superfamily Cytokines in Periodontitis Patients Undergoing Supportive Therapy: A Case-Controlled Cross-Sectional Study Comparing Smokers and Non-smokers in Health and Disease." *Journal of Clinical Periodontology* 40, no. 9: 875–882. https://doi.org/10.1111/jcpe.12134.

Önder, C., N. Akdoğan, Ş. Kurgan, et al. 2023. "Does Smoking Influence Tryptophan Metabolism in Periodontal Inflammation? A Cross-Sectional Study." *Journal of Periodontal Research* 58, no. 5: 1041–1051. https://doi.org/10.1111/jre.13166.

Qiu, F., C.-L. Liang, H. Liu, et al. 2017. "Impacts of Cigarette Smoking on Immune Responsiveness: Up and Down or Upside Down?" *Oncotarget* 8: 268–284. www.impactjournals.com/oncotarget/.

Sanz, M., A. Marco del Castillo, S. Jepsen, et al. 2020. "Periodontitis and Cardiovascular Diseases: Consensus Report." *Journal of Clinical Periodontology* 47, no. 3: 268–288. https://doi.org/10.1111/jcpe.13189.

Seldin, M. M., Y. Meng, H. Qi, et al. 2016. "Trimethylamine N-Oxide Promotes Vascular Inflammation Through Signaling of Mitogen-Activated Protein Kinase and Nuclear Factor-κB." *Journal of the American Heart Association* 5, no. 2: e002767. https://doi.org/10.1161/JAHA.115.002767.

Sharma, M., I. Bairy, K. Pai, et al. 2011. "Salivary IL-6 Levels in Oral Leukoplakia With Dysplasia and Its Clinical Relevance to Tobacco Habits and Periodontitis." *Clinical Oral Investigations* 15, no. 5: 705–714. https://doi.org/10.1007/s00784-010-0435-5.

Sublette, M. G., T. W. L. Cross, C. E. Korcarz, et al. 2020. "Effects of Smoking and Smoking Cessation on the Intestinal Microbiota." *Journal of Clinical Medicine* 9, no. 9: 2963. https://doi.org/10.3390/jcm9092963.

Substance Abuse and Mental Health Services Administration. 2006. "Results From the 2005 National Survey on Drug Use and Health: National Findings." https://books.google.com/books?hl=tr&lr=&id=UPpE_GTiRwYC&oi=fnd&pg=PR7&dq=(Abuse,+S.+(2006).+Results+from+the+2005+national+survey+on+drug+use+and+health:+national+findings.+http://www.+oas.+samhsa.+gov/nsduh/2k5nsduh/2k5Results.+pdf.)&ots=JU98ZeX9Cj&sig=-aThTsQoseK1c3mAtMgnEekT-_c.

Tonetti, M. S., H. Greenwell, and K. S. Kornman. 2018. "Staging and Grading of Periodontitis: Framework and Proposal of a New Classification and Case Definition." *Journal of Clinical Periodontology* 45: S149–S161. https://doi.org/10.1111/jcpe.12945.

Vandenberghe-Descamps, M., H. Labouré, A. Prot, et al. 2016. "Salivary Flow Decreases in Healthy Elderly People Independently of Dental Status and Drug Intake." *Journal of Texture Studies* 47, no. 4: 353–360. https://doi.org/10.1111/jtxs.12191.

Wang, Q., Y. Sun, T. Zhou, C. Jiang, A. Lan, and W. Xu. 2023. "Gut Microbiota-Dependent Trimethylamine n-Oxide Pathway Contributes to the Bidirectional Relationship Between Intestinal Inflammation and Periodontitis." *Frontiers in Cellular and Infection Microbiology* 12: 1125463. https://doi.org/10.3389/fcimb.2022.1125463.

Wang, X., L. Chen, Y. Teng, et al. 2024. "Effect of Three Oral Pathogens on the TMA-TMAO Metabolic Pathway." *Frontiers in Cellular and Infection Microbiology* 14: 1413787. https://doi.org/10.3389/fcimb.2024.1413787.

Wang, Y., O. Andrukhov, and X. Rausch-Fan. 2017. "Oxidative Stress and Antioxidant System in Periodontitis." *Frontiers in Physiology* 8: 910. https://doi.org/10.3389/fphys.2017.00910.

Xiao, L., L. Huang, X. Zhou, et al. 2022. "Experimental Periodontitis Deteriorated Atherosclerosis Associated With Trimethylamine N-Oxide Metabolism in Mice." *Frontiers in Cellular and Infection Microbiology* 11: 820535. https://doi.org/10.3389/fcimb.2021.820535.

Yang, B., X. Pang, Z. Li, Z. Chen, and Y. Wang. 2021. "Immunomodulation in the Treatment of Periodontitis: Progress and Perspectives." *Frontiers in Immunology* 12: 781378. https://doi.org/10.3389/fimmu.2021.781378.

Yilmaz, M., E. Yay, N. Atalay, et al. 2024. "Do Arginine Metabolites Have a Role in Periodontitis Due to Smoking? A New Perspective." *Oral Diseases* 30, no. 2: 743–753. https://doi.org/10.1111/odi.14448.

Yilmaz, M., E. Yay, N. Balci, et al. 2023. "Parkinson's Disease is Positively Associated With Periodontal Inflammation." *Journal of Periodontology* 94, no. 12: 1425–1435. https://doi.org/10.1002/JPER.23-0274.

Zardawi, F., S. Gul, A. Abdulkareem, A. Sha, and J. Yates. 2021. "Association Between Periodontal Disease and Atherosclerotic Cardiovascular Diseases: Revisited." *Frontiers in Cardiovascular Medicine* 7: 625579. https://doi.org/10.3389/fcvm.2020.625579.

Zee, K. 2009. "Smoking and Periodontal Disease." *Australian Dental Journal* 54, no. s1: S44–S50. https://doi.org/10.1111/j.1834-7819.2009.01142.x.

Zhou, J., S. Chen, J. Ren, et al. 2022. "Association of Enhanced Circulating Trimethylamine N-Oxide With Vascular Endothelial Dysfunction in Periodontitis Patients." *Journal of Periodontology* 93, no. 5: 770–779. https://doi.org/10.1002/JPER.21-0159.