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ORIGINAL ARTICLE

Evaluation of salivary and serum methylated arginine metabolites and nitric oxide synthase in advanced periodontitis patients

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Abstract

Objectives Methylated arginine metabolites and nitric oxide synthase (NOS) play a critical role in regulating endothelial function. The aim of this study was to determine levels of NOS, and methylated arginine metabolites (ADMA, SDMA, homoarginine, arginine, and L-NMMA) and IL-6 in serum and saliva in patients with advanced periodontal diseases and identify their association with clinical parameters.

Materials and methods The study consisted of two groups: healthy individuals (control: $n=24$), and generalized Stage III Grade B periodontitis (P: $n=21$). Clinical periodontal parameters (probing pocket depth, bleeding on probing, clinical attachment level) were recorded. IL 6 and NOS levels in saliva and serum were analyzed by enzyme-linked immunosorbent assay (ELISA). ADMA, SDMA, homoArg, arginine, and L-NMMA in saliva and serum were analyzed by liquid chromatography–mass spectrometry (LC MS/MS).

Results Clinical parameters were significantly higher in the periodontitis group ($p<0.001$). In periodontitis group, NOS, ADMA, and arginine levels in saliva were statistically significantly higher than control group ($p<0.05$). Serum levels of SDMA were statistically significantly lower, and IL-6 was statistically significantly higher in P group than C group ($p<0.05$). ADMA, NOS, and arginine levels were significantly positive correlated with all clinical periodontal parameters ($p<0.05$).

Conclusions These findings suggest that there is a relationship between severity of periodontal disease and endothelial dysfunction by means of ADMA. Salivary ADMA may be related with periodontal inflammation.

Clinical relevance ADMA levels in periodontal inflammation are associated with endothelial dysfunction. According to the results of our study, periodontal inflammation is effective on both local and systemic methylated arginine metabolites and nitric oxide synthase levels. This may shed light on the relationship between periodontal disease and systemic status.

Keywords (MeSH): Asymmetric dimethylarginine (ADMA) · Periodontitis · Nitric oxide synthase (NOS)

Introduction

Nitric oxide (NO), which is found in small amounts in the human body [1–3], is synthesized from arginine by nitric oxide synthase (NOS) [4]. Nitric oxide is an important regulator of the physiological processes of the immune system and circulatory system [5, 6] due to its functions such as regulation of endothelial vascular tone [7], prevention of platelet aggregation [8, 9], inhibition of expression and activation of chemokines and adhesion molecules [10, 11]. While low level production of NO is seen in normal biological activities. its overproduction is implicated in cell death (such as apoptosis) and tissue damage [12, 13].

Asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) and monomethyl arginine (L-NMMA), which are analogs of methylarginine, act together as nitric oxide synthase inhibitors [14, 15]. While L-NMMA and ADMA are potent endogenous inhibitors of nitric oxide synthase (NOS), SDMA indirectly inhibits NO production by blocking the uptake of L-arginine [15]. NO and NOS, which is responsible for its expression, and ADMA, which is an inhibitor of NOS, are important factors in the pathogenesis of inflammatory disease [16–19]. NO

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is formed by the oxidation of the amino acid L-arginine by the NOS enzyme. There are 3 isoforms of the NOS enzyme in mammalian tissues: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). While nNOS and eNOS are constitutively expressed in the body, iNOS is expressed only in response to the inflammatory stimulus [20].

The relationship of these markers with inflammatory systemic diseases such as cardiovascular disease and rheumatoid arthritis has been demonstrated by several studies [13–15]. Although in two of these studies ADMA levels were studied on the relationship between systemic disease and periodontitis [17, 21], these studies focused only on ADMA levels. In one of these studies, Almeida et al. [21] reported that active periodontal therapy reduces serum ADMA levels in individuals with periodontitis with chronic kidney disease. Isola et al. [17] showed that individuals with chronic periodontitis and coronary artery disease have higher serum and salivary ADMA levels compared to chronic periodontitis and healthy individuals. In our study, methylated arginine metabolites in detail (ADMA, SDMA, L-NMMA, L-arginine, arginine, homoarginine) and their relationship with NOS and periodontal clinical parameters were evaluated in periodontitis patients was revealed.

Thus, we hypothesized that methylated arginine metabolites might have roles in the pathogenesis of periodontitis, and its levels were associated with periodontal inflammation. Therefore, our aim was to analyze the expression of methylated arginine metabolites in saliva and serum samples of patients with Stage III Grade B periodontitis and identify its association with NOS, IL-6, and clinical periodontal parameters.

Materials and methods

Study population

Twenty-one patients with Stage III Grade B generalized periodontitis and 24 periodontally healthy individuals were enrolled for this cross-sectional study, which was conducted at the Department of Periodontology, School of Dentistry, Ankara University, Turkey. The present study was approved by the human subject's ethics board of Ankara University (No: 09/01, on 24.07.2019) for use and access of human subjects in research and was performed according to the Helsinki Declaration. All participants have given written consent before the study.

The clinical diagnosis of periodontitis and periodontally healthy subjects was based on clinical and radiographic criteria, as detailed previously [22]. The presence of PPD ≤ 3 mm and no signs of inflammation were defined as healthy periodontium. Patients who had interdental radiographic bone loss of ≥ 2 mm in nonadjacent, distinct teeth or buccal or oral radiographic bone loss up to 15% with a PPD of > 3 mm for ≥ 2 teeth were diagnosed as (Stage III) periodontitis. Periodontitis patients were graded according to the bone loss/age index as Grade B (index score between 0.25 and 1.00).

The exclusion criteria were as follows: use of antibiotics, anti-inflammatory or immunosuppressive drugs, and contraceptives within 3 months preceding the study; use of drugs that may determine gingival hyperplasia (nifedipine, cyclosporin A, hydantoin, etc.); periodontal treatment (previous 3 mo.); having at least 16 natural teeth excluding third molar; status of pregnancy or lactation.

All participants included in this study were systemically healthy (e.g., cardiovascular disease, adiposity, metabolic syndrome, prediabetes, kidney disease, rheumatoid arthritis). All participants were non-smokers, i.e., never smokers or had reportedly quit for a minimum period of 2 years before enrollment [23].

Periodontal examination

All clinical periodontal measurements were performed by a single, calibrated examiner (V.Ş.). Clinical periodontal parameters (probing pocket depth (PPD), bleeding on probing (BOP), clinical attachment level (CAL)) were recorded at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, distolingual) by a periodontal probe.[1]

Saliva and serum sampling

Unstimulated saliva samples were collected from each patient during early hours of the day (9:00 am to 11:00 am) after the collection protocol was explained to each patient. The patients were initially asked to rinse their mouth thoroughly using distilled water and instructed to sit comfortably then spit into the plastic tubes for 5 min. The samples were then centrifuged at 2800 × g for 10 min, transferred into Eppendorf tubes [21].

Venous blood samples were collected by standard venipuncture, and samples were kept at room temperature for 30 min, to allow clotting. After centrifugation at 4000 × g for 10 min, serum was separated from blood samples. All samples were preserved at − 80 °C until the day of the analysis [21].

[1] William's probe, Hu-Friedy, Chicago, IL.

NOS and IL-6 analysis

NOS and IL-6 levels were determined in both serum and saliva samples by commercially available ELISA kits.[2] All assays were performed according to manufacturers' instructions, with colorimetric assessment performed using a microplate reader at 450 nm.[3] Concentrations were determined based on the respective assay standard curve. Intra- and inter-assay precision was < 10% and < 12%. Assay detection range were 7.8–500 pg/ml for IL-6 and 0.156–10 ng/ml for NOS. All samples were analyzed in duplicate, and the average used in subsequent calculations.

Methylated arginine metabolites analysis

Salivary and serum levels of ADMA, SDMA, homoArg, Arginine, and L-NMMA were assessed by use of liquid chromatography–mass spectrometry (LC MS/MS)[4] measurements, carried out by modifying the method of Di Gangi et al. [24]. Two hundred-microliter aliquot of serum sample, 100 µl of ADMA-D7 (1 µM) internal standard, and 1 ml of methanol were mixed in an Eppendorf tube. After this mixture is vortexed for 1 min, it is centrifuged at 13,000 rpm for 10 min. Then, the supernatant was transferred to a new tube and evaporated under nitrogen. Later, 200 µl of derivatization reagent (19:1 butanol:acetyl chloride) was added and incubated again for 30 min at 65 °C. The samples were evaporated once more under nitrogen at 65 °C. Finally, after the evaporation process, it was dissolved with 200 µl of dissolving reagent (10% methanol, 0.1% formic acid). The injection volume was 20 µl.

Statistical analyses

Sample size analysis was performed a priori, using specific software.[5] Using a large effect size (0.8) for two groups analysis of ADMA levels, a 0.05 α-error, and an 80% power, the resulting total sample size was 42. However, considering the possibility of confounders and incomplete data, the study was designed to include 50 patients.

All analyses were performed using commercially available statistical software.[6] Shapiro–Wilk test was applied to determine data normality. For intergroup comparisons, where the samples were normally distributed between disease categories as between healthy and periodontitis, the Mann–Whitney *U* test was used to compare the differences between the parameters recorded, while Student's *t* test was used for non-normally distributed samples. Correlations between biochemical and periodontal clinical parameters were performed using Spearman and Pearson correlation analysis according to the distribution data. All tests were performed at a significance level of $\alpha = 0.05$.

Table 1 Comparison of demographic and clinical periodontal parameters between the groups

Clinical parameters	Periodontitis ($n = 21$)	Control ($n = 24$)	p
Age (year)*	40.1 ± 2.20	34.6 ± 2.06	0.077
Gender (n, f/m)	12 / 9	16 / 8	0.552
PI*	1.84 ± 0.52	0.77 ± 0.31	< 0.001
PD (mm)*	4.24 ± 1.05	1.61 ± 0.20	< 0.001
BOP (%) **	60 (35.75–84.75)	5 (2–8)	< 0.001
CAL (mm)**	4.48 (3.87–5.57)	0.01 (0.00–0.13)	< 0.001

* Data as mean ± standard deviation. Student *t* test

** Data as median-interquartile range. Mann–Whitney *U* test

Results

Study population and periodontal clinical parameters

Twenty-one periodontitis patients (P group; 12 females, 9 males and mean age: 40.1 ± 11.5 years) and twenty-four periodontally healthy control individuals (Control (C) group 16 females, 8 males and mean age: 34.6 ± 8.7) were recruited and completed all study procedures.

Demographic and periodontal clinical parameter data are presented in Table 1. Gender and age distribution showed no significant difference between the groups ($p > 0.05$). Clinical periodontal parameters were found to be significantly higher in the P group than the C group ($p < 0.001$).

Biochemical parameters

Figure 1 presented salivary and serum IL-6, NOS, ADMA, SDMA, homoarginine, and arginine levels. In patients with periodontitis, salivary NOS, ADMA, and arginine levels were statistically significantly higher than the healthy controls (respectively; $p = 0.001$, $p = 0.01$, and $p < 0.001$). Serum NOS and ADMA were found to be statistically significantly higher in P group than in C group (respectively; $p = 0.042$ and $p = 0.040$). Serum SDMA level was found

[2] Human IL-6 ELISA Kit, Uscn, Cloud-Clone Corp, USA and Human NOS1 ELISA Kit, Uscn, Cloud-Clone Corp, USA.

[3] Synergy HT Microplate Reader, Bio-Tek Instruments, Winooski, WT, USA.

[4] TSQ Quantum Access MAX Triple Stage Quadrupole Mass, Thermo Scientific, USA.

[5] 3.1.9.2 G*Power; https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-undarbeitspsychologie/gpower.html.

[6] SPSS for Windows v.26, IBM SPSS Inc., New York, NY, USA.

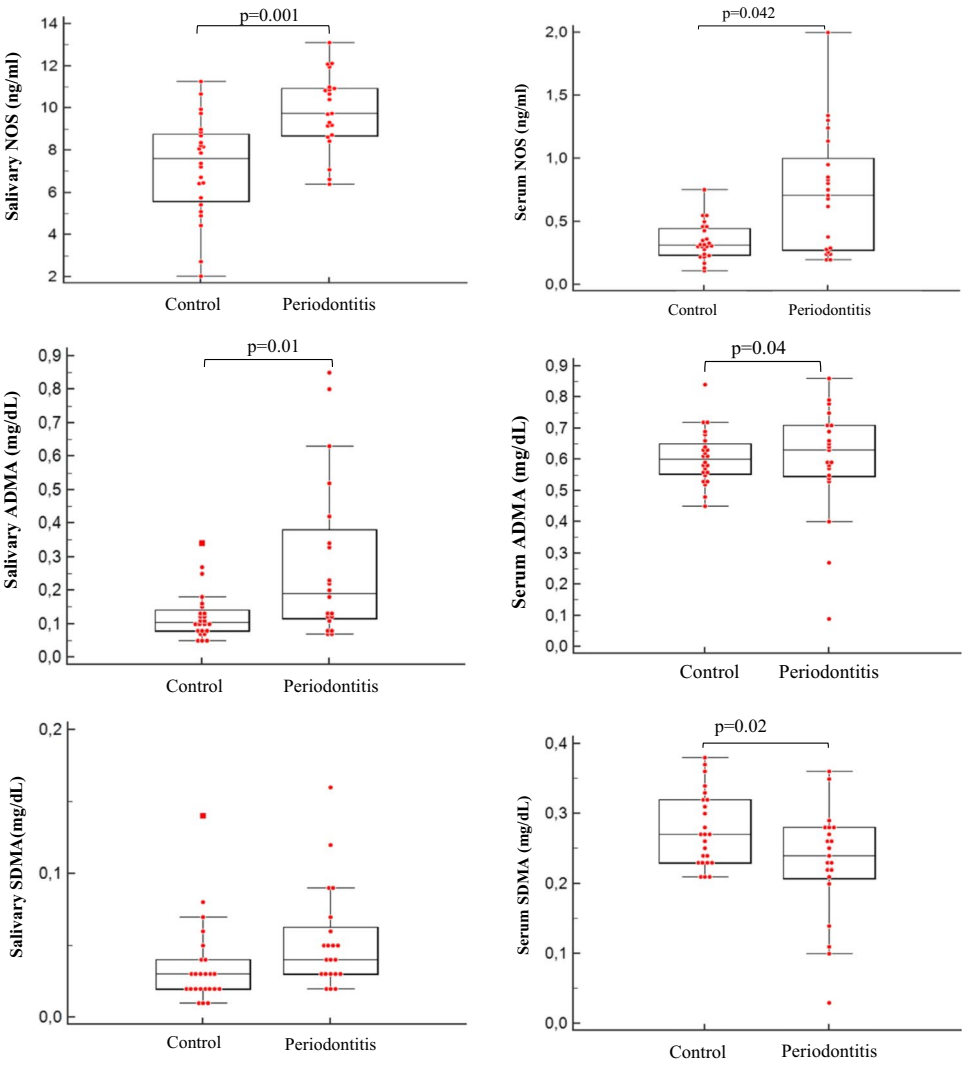


Fig. 1 Saliva and serum levels of ADMA, SDMA, L-NMMA, L-arginine, and L-homoarginine levels in control (C) and periodontitis (P) groups. Box-and-whisker plots with the median (horizontal line), interquartile range (box), and outlier (circles) values shown. Significantly different ($p < 0.05$)

to be statistically significantly lower in P group than in C group ($p = 0.02$). Although IL-6 serum and saliva levels increased in periodontitis group, these differences were not statistically significant (Fig. 2).

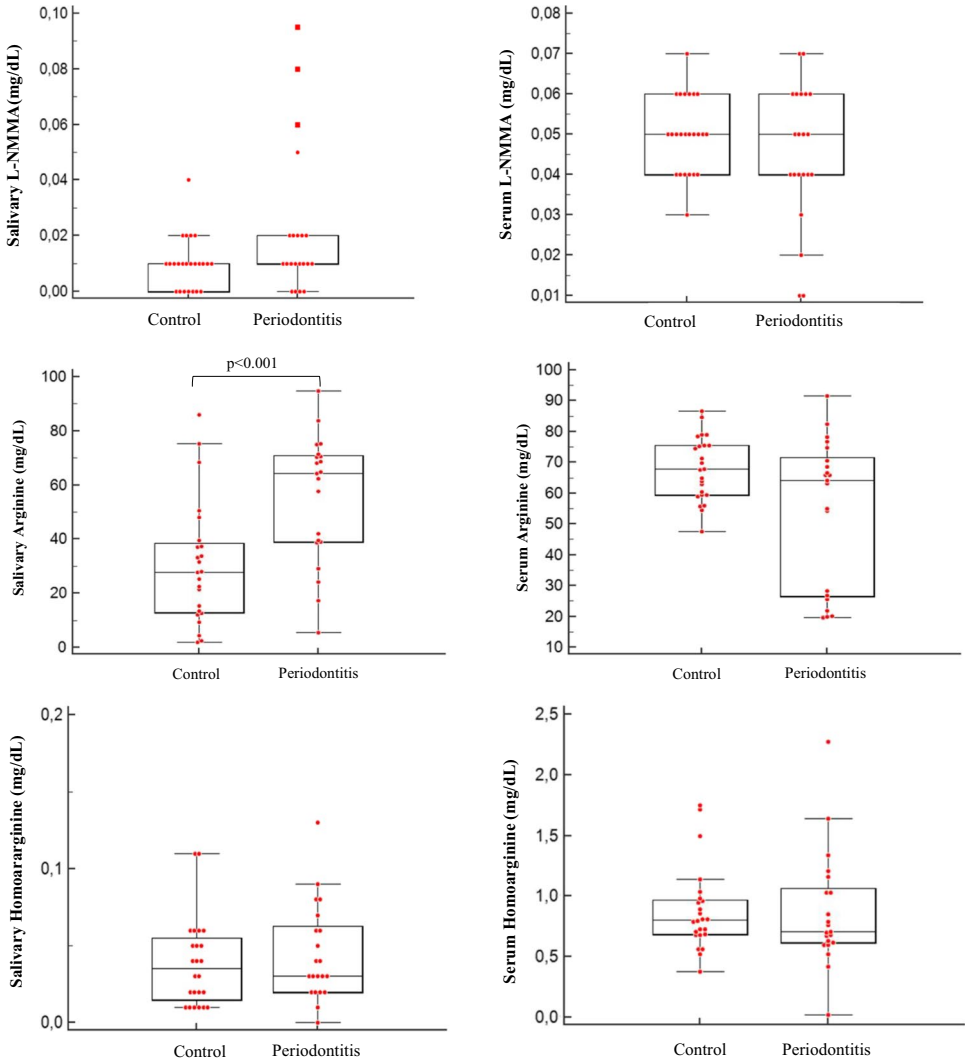
Correlations between biochemical and clinical periodontal parameters

These analyses, reported in Table 2, were based on data from all 45 patients. PPD, BOP, and CAL had significantly positive correlations with salivary NOS, ADMA, and arginine. Serum NOS levels were statistically positive correlated with PI, PPD, and CAL. Statistically significant positive correlation was observed between serum NOS and serum IL-6 (Table 2).

Discussion

In this study, saliva and serum methylated arginine metabolites and NOS levels were evaluated in Stage III Grade B periodontitis patients and periodontally healthy individuals, as well as possible relationships between periodontal clinical measurements, and these biomarkers were tried to be explained. Salivary and serum NOS and ADMA levels were found to be higher in periodontitis patients compared to controls. Salivary arginine levels were significantly higher in individuals with periodontitis compared to controls ($p < 0.001$). The results also show that PPD, BOP, and CAL values, which are clinical markers of periodontal inflammation and tissue destruction, are positively associated with both saliva and serum NOS and salivary ADMA and arginine levels. These results confirm our hypothesis.

Fig. 1 (continued)



IL-6 is an important pro-inflammatory cytokine involved in bone resorption in the presence of inflammation [25] and is among the initiators of endothelial dysfunction and plays a key role in the maintenance of inflammation in chronic injuries [26]. While this cytokine induces iNOS[28] and inhibits eNOS expression [27, 28], it contributes to the accumulation of ADMA [29, 30]. It is known that with the increase of IL-6 level, the arginine/ADMA ratio and accordingly the NO level decrease [31]. As a result of our study, there was no statistically significant difference between the two groups in terms of IL-6 levels, but it was observed that IL-6 levels were higher in the periodontitis group in both serum and saliva samples. For this reason, it is possible to emphasize that IL-6 level may act in relation to NOS, especially with

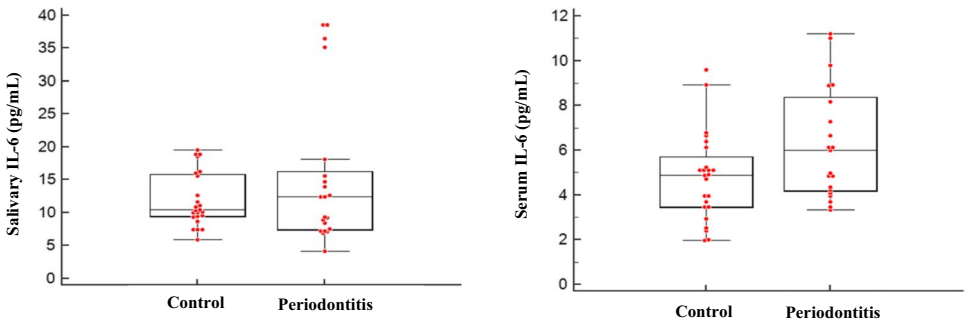


Fig. 2 Saliva and serum levels of IL-6 levels in control (C) and periodontitis (P) groups. Box-and-whisker plots with the median (horizontal line), interquartile range (box), and outlier (circles) values shown. Significantly different ($p < 0.05$)

Table 2 Correlations between biomarkers and periodontal clinical parameters (Spearman correlation coefficients, *r*)

Parameters	Salivary IL-6	Serum IL-6	Salivary NOS	Serum NOS	Salivary ADMA	Serum ADMA	Salivary arginine	Serum arginine	Salivary homoarginine	Serum homoarginine	Salivary SDMA	Serum SDMA	Salivary L-NMMA
Serum IL-6	−0.147	–	-	-	-	-	-	-	-	-	-	-	-
Salivary NOS	0.239	−0.006	-	-	-	-	-	-	-	-	-	-	-
Serum NOS	−0.041	**0.314***	0.230	-	-	-	-	-	-	-	-	-	-
Salivary ADMA	−0.126	−0.049	0.272	0.138	-	-	-	-	-	-	-	-	-
Serum ADMA	0.140	−0.005	0.024	0.069	0.142	-	-	-	-	-	-	-	-
Salivary arginine	0.034	−0.040	**0.442***	0.127	0.165	−0.111	-	-	-	-	-	-	-
Serum arginine	0.267	−0.234	0.071	0.059	−0.069	**0.442***	−0.168	-	-	-	-	-	-
Salivary homoarginine	0.146	−0.017	0.264	0.225	**0.476***	−0.042	0.019	0.022	-	-	-	-	-
Serum homoarginine	−0.041	−0.223	0.043	−0.232	−0.016	0.239	0.069	**0.298***	−0.021	-	-	-	-
Salivary SDMA	−0.217	0.049	0.066	0.181	**0.768****	−0.012	−0.018	−0.149	**0.656****	−0.112	-	-	-
Serum SDMA	0.021	0.038	−0.192	−0.132	−0.175	0.232	−0.172	0.158	0.039	**0.340***	−0.172	-	-
Salivary L-NMMA	−0.084	0.015	**0.317***	0.003	**0.800****	0.185	0.058	0.076	**0.471***	0.009	**0.660****	−0.211	-
Serum L-NMMA	0.038	−0.021	−0.035	−0.184	0.131	**0.651****	−0.210	**0.385***	−0.010	0.196	0.030	0.129	0.136
PI	−0.060	**0.380***	**0.439***	**0.430***	0.142	0.165	**0.408***	0.026	0.015	−0.013	0.106	−0.127	0.059
PPD (mm)	−0.137	0.105	**0.565****	**0.341***	**0.411***	0.066	**0.528****	−0.125	0.041	−0.117	0.289	−0.232	0.229
CAL (mm)	−0.034	0.100	**0.580****	**0.374***	**0.436***	−0.016	**0.559****	−0.280	0.072	−0.197	0.295	−0.290	0.209
BOP (%)	−0.221	0.077	**0.511****	0.256	**0.398***	0.165	**0.475***	−0.078	−0.090	−0.018	0.283	−0.246	0.225

the iNOS type, and may contribute to higher ADMA levels in the disease group.

As a result of our study, both saliva and serum ADMA levels were found to be statistically significantly higher in group P than in group C. ADMA is an endogenous inhibitor of NOS and is used to evaluate endothelial function [15]. Like our findings, Hendek et al., in their study examining the effect of initial periodontal treatment on cardiovascular disease-related inflammatory markers in individuals with severe chronic periodontitis, found serum ADMA concentrations higher in chronic periodontitis patients than in periodontally healthy individuals. Also, they observed a significant increase in eNOS level as a result of the decrease in ADMA concentration with periodontal treatment [32]. Unlike this, Isola et al. found no difference for saliva and serum ADMA levels in individuals with periodontitis without systemic disease but found a significant difference between periodontitis and control groups in individuals with cardiovascular disease.

Based on the information in the literature that ADMA inhibits NOS, it was expected that NOS level would decrease while ADMA increased, but our results showed high NOS levels together with high ADMA levels. This may be due to the difference in NOS type. In addition to iNOS during the normal inflammation process, it is a natural process that the increases in the eNOS levels outside the physiological limits due to edema, and this may contribute to the increase in the total amount of NOS. Also, due to the high level of ADMA in the disease group, the high level of NOS to reduce or stop the existing endothelial dysfunction may be considered due to the ability of the tissue to protect itself. On the other hand, it is possible for ADMA to act as a regulator that tries to reduce inflammation in response to the increased amount of NOS.

The formation of NO from L-arginine by the catalysis of NOS is inhibited by ADMA, L-NMMA, and SDMA [33], and when these methylated arginines are in high concentration, they compete with both arginine and each other [34]. In our results, salivary concentrations of NOS, its substrate arginine, and ADMA were found significantly higher in the disease group. It can explain that ADMA competes with arginine and cannot use arginine as a substrate despite high NOS concentration, resulting in excess arginine in the saliva. The excess arginine in the environment seems likely to be used by iNOS and play a role in the continuation of the inflammatory process because methylarginine groups act as competitive inhibitors of arginine and inhibit the use of L-arginine by NOS [35].

Homoarginine is a homolog of L-arginine and is synthesized enzymatically from L-arginine by arginine:glycine amidino transferase [36]. Like L-arginine, homoarginine may be involved in the regulation of the expression of NO. The homoarginine level is known to be unchanged in many physiological and pathological conditions and is used as an internal standard in studies evaluating the role of ADMA in pathological conditions [37]. According to the findings of our study, no statistically significant difference was found between the control and disease groups in terms of homoarginine values. Our findings are compatible with the literature and reveal that the main evaluation should be made over arginine.

The effect of oxidative stress in the chronic phase of periodontitis has been demonstrated through free oxygen radicals [38, 39]. Oxidative stress reduces the activity of DDAH which is the enzyme responsible for ADMA catabolism. Thus, the level of ADMA in the blood increases due to the decrease of its DDAH degradation [40].

DDAH, which is responsible for the metabolism of a large part of the ADMA, is not involved in SDMA metabolism. DDAH expression is inhibited in the presence of potent oxidants such as peroxynitrite and excessive NO production due to eNOS. This has been demonstrated especially in the pathobiology of atherosclerosis. In the presence of high concentrations of ADMA due to reduced DDAH activity, eNOS produces superoxide instead of NO. This situation causes more DDAH oxidation and more ADMA accumulation [41]. Increased ADMA levels cause endothelial dysfunction due to increased vascular superoxide levels and decreased vascular NO presence [42]. Cai and Harrison showed that increased reactive oxygen species can cause endothelial dysfunction [43]. Antoniades et al. reported that oxidative stress and pro-inflammatory cytokines increase ADMA levels and therefore cause endothelial dysfunction [44]. Based on this literature, the known presence of increased reactive oxygen species in periodontitis seems to be compatible with the increased ADMA level in the disease group. Because increasing oxidative stress in the disease group inhibits the activity of the DDAH enzyme responsible for ADMA catabolism and increases the ADMA level. In addition, the increase in eNOS level and the production of NO beyond physiological limits can also inhibit the activity of DDAH. This information supports the positive correlation between the clinical parameters of tissue destruction such as increased CAL and PPD and the ADMA level.

Apart from ADMA, SDMA and L-NMMA are also synthesized endogenously. L-NMMA is as potent as ADMA in inhibiting NOS activity, but its concentration in plasma is 10 times lower [45]. Although the circulating concentration of SDMA is comparable to ADMA, it has no effect on NOS [46]. Although some studies have identified SDMA as a more important cardiovascular and renal risk factor than ADMA and L-NMMA, circulating SDMA has recently been identified only as a cardiovascular risk factor [44]. Consistent with the literature in our study, although the concentration of SDMA and L-NMMA was not found to be significantly higher in the periodontitis group, it suggests that particularly ADMA may play an important role to

demonstrate the local and systemically effect of periodontal inflammation.

The cross-sectional nature of this study is its main limitation; due to study design, randomization in the selection of patients was not possible, which may lead to selection bias. Although the sample size of the study was equal or greater than similar studies, sample size is a relative limitation. Therefore, further studies are needed in larger populations.

Conclusions

It is known that proinflammatory cytokines and oxidative stress are involved in the pathogenesis of periodontitis; it has also been reported that they increase ADMA levels and therefore cause endothelial dysfunction. As a result of our study, the significantly higher salivary ADMA levels in the periodontitis group compared to the control group and the positive correlation of ADMA levels with PPD, CAL, and BOP suggest that ADMA may be a biomarker in the course of periodontal disease.

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Declarations

Ethics approval The present study was approved by the human subject's ethics board of Ankara University (No: 09/01, on 24.07.2019) for use and access of human subjects in research and was performed according to the Helsinki Declaration.

Consent to participate All participants have given written consent before the study.

Conflict of interest The authors declare no competing interests.

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