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ORIGINAL ARTICLE

Asymmetric and symmetric dimethylarginine gingival crevicular fluid levels in periodontitis

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Abstract

Objective: This study aimed to evaluate the level of ADMA (asymmetric dimethylarginine), SDMA (symmetric dimethylarginine), and IL-1β (Interleukin-1β) in gingival crevicular fluid (GCF) from periodontitis patients and control subjects.

Background: ADMA and SDMA are potentially hazardous non-proteinogenic amino acids that limit nitric oxide (NO) synthesis and have many functions in various human disorders. ADMA causes a structural change in nitric oxide synthase, while SDMA blocks arginine cell uptake. Increased plasma ADMA has been widely recognized as a "trigger" initiating impaired NO bioavailability and vascular dysfunction, which ultimately leads to oxidative stress.

Methods: Twenty-five patients with periodontitis (P) (Stage III, Grade C, $n = 25$) and 20 control (C) subjects were included in the study. The IL-1β level of GCF was measured by enzyme immunoassay (ELISA) and ADMA and SDMA by liquid chromatography-mass spectrometry (LC–MS/MS).

Results: Periodontitis patients had higher clinical parameters than controls ($p < .001$). Levels of IL-1β, ADMA and SDMA GCF were statistically significantly higher in group P than in group C (respectively; $p = .003$, $p < .0001$, $p < .0001$). There was no difference in the ADMA/SDMA ratio ($p = .312$) between the groups. There were significant positive correlations between clinical periodontal parameters and IL-1β, ADMA, and SDMA levels ($p < .05$). ADMA and SDMA levels were significantly correlated with IL-1β ($p < .05$).

Conclusions: These findings suggest that ADMA and SDMA may be involved in the pathogenesis of the periodontal disease.

KEYWORDS
gingival crevicular fluid, methylated arginines, periodontitis

1 | INTRODUCTION

Periodontitis is a chronic, progressive inflammatory disease with damage of the periodontium that can lead to tooth loss.[1] Disease features involve the activation of inflammatory cells such as PMNLs, macrophages, T and B cells[2] and, therefore, the expression of mediators such as cytokines,[2] prostaglandins,[3] acute phase reactants,[4] and nitric oxide (NO).[2]

Nitric oxide, synthesized from L-arginine by nitric oxide synthase (NOS), is a free radical that is involved in a multitude of physiological

functions. Some of the functions are modulation of vascular tone and integrity,[4] regulation of platelet aggregation,[5] neurotransmission,[6] vasodilatation,[7] and potent oxidative activity contributing to the part that destroys microorganisms.[8] Besides these beneficial effects, NO has dose-dependent harmful effects such as cytotoxicity and increasing neutrophil collagenase activity.[9] The protective and destructive effects of NO are modulated by inhibitors of NO production such as methylarginines, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA).[4]

Asymmetric dimethylarginine is a competitive inhibitor of NOS, while SDMA reduces NO primarily by inhibiting cellular uptake of L-arginine.[10] Due to its inhibitory effect on NOS, ADMA may reduce the bioavailability of NO, which is an important vasodilator.[11,12] Thus, the increased ADMA expression may induce NO deficiency.[13]

The importance of ADMA was first described in end-stage renal disease patients[14] and following studies have shown that it plays an essential role in the pathogenesis of various systemic disorders.[15–18] Periodontal disease, an inflammatory disease, can be considered ADMA and SDMA may play a role in the pathogenesis of periodontitis. Studies report that serum and salivary ADMA concentrations are increased in patients with periodontitis and coronary artery disease[16] and that periodontal treatment reduces ADMA expression with periodontal and renal disease.[19] In our previous study, we evaluated salivary and serum methylarginine levels in patients with Stage III Grade B periodontitis.[20] We concluded that salivary ADMA was increased in periodontitis and could be an important marker of periodontal inflammation. GCF is a plasma exudate or transudate depending on the degree of inflammation of the periodontal tissues, proving a unique window for analysis of periodontal conditions.[21,22] Although saliva can serve as a diagnostic fluid,[23] it is a hypotonic solution of gingival crevicular fluid (GCF), serum, salivary acini, oral mucosal secretions, and microorganisms.[24] Therefore, it is important to assess the host response directly at the site of destruction. In this study, we planned with the hypothesis that methylarginines may play a role in the pathogenesis of periodontitis, we aimed to evaluate the levels of ADMA, SDMA, and IL1-β in GCF of periodontitis patients and determination of their relationship with periodontal clinical parameters.

2 | MATERIALS AND METHODS

2.1 | Study design

Twenty-five patients with periodontitis (Stage III, Grade C, $n = 25$; 15 females, 10 males; mean age: 33.0 ± 9.4 years, P group) and 20 periodontally healthy control subjects ($n = 20$; 12 females, 8 males, mean age: 32.8 ± 9.2 years, C group), who applied to Ankara University Faculty of Dentistry, Department of Periodontology between 2018 and 2019, were included in the study. The Ankara University Human Research Ethics Committee authorized the design and execution of this study. The Declaration of Helsinki, as modified in 2013, was followed when we conducted our research (no. 36290600/52, 26.05.2017). A formal informed consent form was signed by everyone who consented to take part in the study.

The diagnosis was made based on the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.[25] Participants must have a minimum of 16 natural teeth, excluding third molars. Patients were diagnosed with periodontitis if they had interdental radiographic bone loss ≥2 non-adjacent teeth and PD >3 mm at least 2 teeth. The following conditions were used for Stage III, Grade C: (1) In the radiographic examination, alveolar bone loss in the middle or apical third of the root. (2) Tooth loss ≤4. (3) Effected teeth ≥%30. (4) Interdental CAL ≥5 mm. (5) % bone loss/age >1.

Controls were the individuals with no periodontal disease history or symptoms with clinically healthy periodontium (BOP < 10%, PD ≤ 3 mm) and lacked clinical signs of gingival inflammation with well-maintained oral hygiene. All measurements were recorded using a periodontal probe ('O' Probe with Williams, The University of Michigan) from six sides of each tooth by an expert periodontist (ZG). Pregnant and lactating mothers, tobacco users, those who had systemic diseases, such as diabetes mellitus, rheumatoid arthritis, obesity, and cancer, or those who used antibiotics, anti-inflammatory medications, immunosuppressive drugs, or contraceptives, or those who have received periodontal treatment in the past 3 months were excluded.

2.2 | GCF sampling

Gingival crevicular fluid samples were obtained from three single-rooted teeth per individual. We chose single-rooted teeth to accessibility to the area and the relatively low probability of plaque and saliva contamination. In addition, in a study performed in healthy subjects, there was a significant correlation between the GCF volume of single-rooted teeth and GI and BOP, whereas no correlation was observed for multi-rooted teeth.[26] The mesiobuccal and distobuccal sampling sites were selected based on disease severity (probing depth ≥ 5 mm) in the periodontitis groups. In the C group, single-rooted teeth were randomly selected. Three samples were collected using standardized paper strips (Periopaper, ProFlow) from each site, by consecutively placing a strip for 30 s and pooling for analysis. The area was isolated, the supragingival plaque was removed with sterile curettes, and tooth surfaces were gently air-dried. GCF samples were obtained using the extra sulcular technique[27] and blood-contaminated samples were discarded. The amount of GCF was measured using a calibrated instrument (Periotron 8000, Oraflow Inc.). Instrument readings were converted to volume (microliter) by reference to a standard curve.[28] Samples were stored at −80°C until further analysis.

2.3 | Analysis of IL-1β

Samples were eluted from the pooled strips as detailed previously.[27] Eluted samples were analyzed for IL-1β using a commercial ELISA

kit (Elabscience Biotechnology Co.) according to the manufacturer's instructions. Concentrations were determined based on the respective assay standard curve. The intra- and inter-assay accuracy was <10% and <12%. The assay detection range was 7.81–500 pg/ml for IL-1β. All samples were analyzed in duplicate, and the average was used in subsequent calculations. The obtained IL-1β values were corrected for the initial GCF volume, and the results are expressed in μg per site.

2.4 | Analysis of methylated arginine metabolites

The GCF levels of ADMA and SDMA were evaluated by liquid chromatography-mass spectrometry (LC–MS/MS, ESI Source, Thermo Scientific Accessmax) which was performed by modifying the method of Di Gangi et al.[29] Since a different brand of LC–MS/MS device was used, modifications were made in column type, optimization, MRM, CE, and capillary heat values. The sample preparation procedure was carried out as follows. One hundred microliters of GCF sample, 100 μl of ADMA-D7 internal standard (1 μM), and 1 ml of methanol were mixed in a 2 ml Eppendorf tube. After vortexing this mixture for 1 min, it was centrifuged at 9500 *g* for 10 min. The supernatant was transferred to a new tube and evaporated under nitrogen conditions and 200 μl of derivatization reagent (19:1 butanol: acetyl chloride) was added. After that samples were incubated for 30 min at 65°C and evaporated under nitrogen conditions at 65°C. After evaporation, samples were dissolved with 200 μl of dissolution reagent (10% methanol, 0.1% formic acid).

2.5 | Statistical analysis

Sample size analysis was performed in advance, using specific software. Using a large effect size (1.0) for two groups of ADMA level analysis, an α-error of 0.05, and a power of 80%, the total sample size was 34. Considering the possibility of case losses, a total of 50 individuals were included in the study.

All analyses were performed using statistics software (SPSS v.22, IBM SPSS Inc.). Data normality was tested by Shapiro–Wilk before further analysis. Intergroup comparisons of biochemical and clinical parameters were assessed using the Mann–Whitney *U* test. Correlation between IL-1β, ADMA, SDMA, and periodontal clinical parameters was performed using Spearman's correlation analysis. All tests were performed at a significance level of $\alpha = 0.05$.

3 | RESULTS

3.1 | Clinical parameters

Clinical periodontal parameters and GCF volume are reported in Table 1. Periodontitis group had significantly higher clinical periodontal parameters than the control group ($p < .001$). There was no significant difference between the groups in terms of age and sex (respectively; $p = .918$, $p = .731$).

TABLE 1 Comparison of clinical periodontal parameters between periodontitis and healthy groups

Clinical parameters	Periodontitis (P; *n* = 25)	Control (C; *n* = 20)
Age (year)	33.0 ± 9.4 32 (25–41)	32.8 ± 9.2 31 (25–37.6)
PI	1.47 ± 0.60* 1.26 (1.12–1.85)	0.44 ± 0.32 0.48 (0.16–0.65)
GI	0.90 ± 0.44* 0.93 (0.65–1.12)	0.13 ± 0.23 0.04 (0.03–0.08)
PD (mm)	3.83 ± 0.82* 3.60 (3.24–4.41)	1.46 ± 0.33 1.41 (1.16–1.69)
BOP (%)	74.66 ± 13.33* 72 (63–89)	7.96 ± 4.73 7.14 (5.66–12.50)
CAL (mm)	4.90 ± 1.39* 4.97 (3.64–5.62)	1.50 ± 0.33 1.5 (1.25–1.70)
GCFV (μl)	2.52 ± 0.73* 2.52 (2.11–3.07)	0.47 ± 0.20 0.45 (0.34–0.53)

Note: Data are expressed as the median/mean (min-max). Mann–Whitney *U* test.

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment lost; GCVF, gingival crevicular fluid volume; GI, gingival index; PD, probing depth; PI, plaque index.

*Statistically significant difference from control group ($p < .001$).

3.2 | Biochemical parameters

Figure 1 and Table 2 present the total amount of GCF IL-β, ADMA, and SDMA. In the periodontitis group, GCF IL-1β, ADMA, and SDMA levels were statistically significantly higher than in the control group (respectively; $p = .003$; $p < .0001$; $p < .0001$). There was no statistically significant difference between the groups in ADMA/SDMA ratio ($p = .312$).

3.3 | Correlation between periodontal and biochemical parameters

Table 3 shows correlation coefficients between the periodontal clinical parameters and GCF content of biochemical markers. IL-1β, ADMA, and SDMA levels were significantly positively correlated with clinical periodontal parameters ($p < .05$). ADMA and SDMA levels were significantly positively correlated with IL-1β level ($p < .05$). ADMA level was significantly correlated with SDMA ($p < .001$).

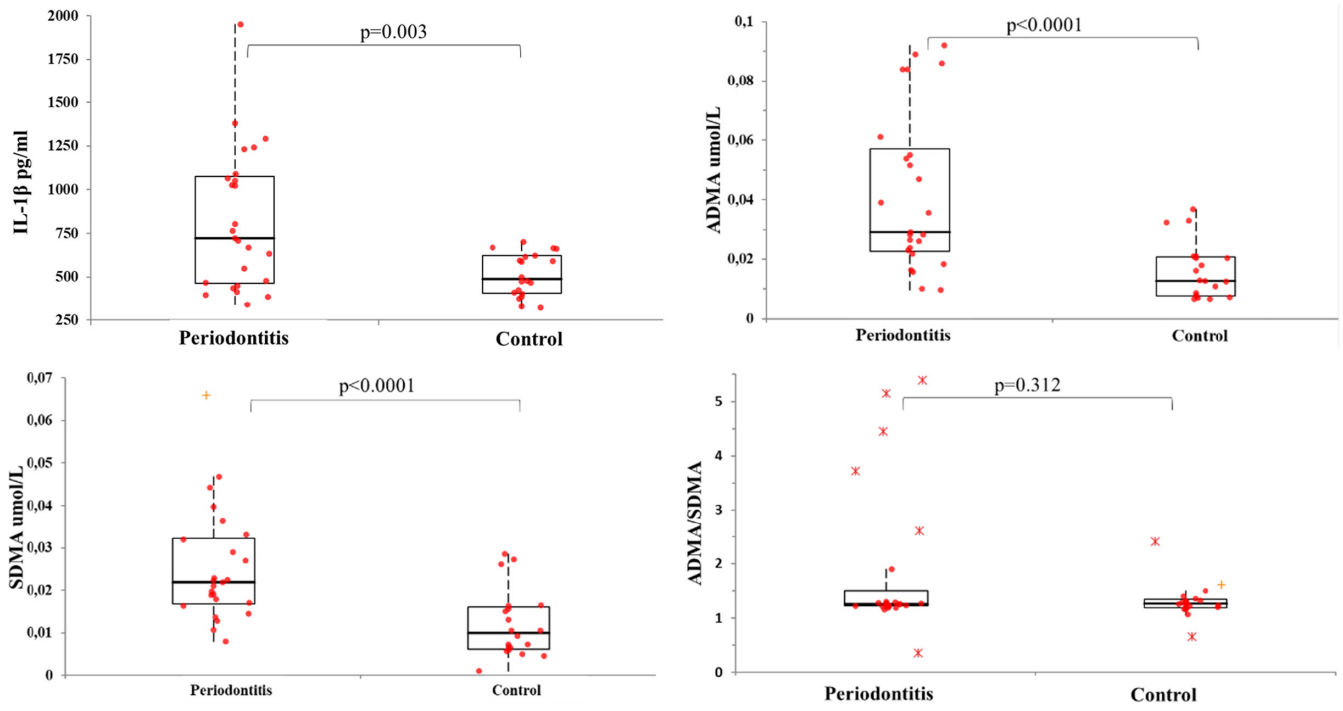

FIGURE 1 IL-1β, ADMA, and SDMA levels in control (C) and periodontitis (P) groups. Box-and-whisker plots with the median (horizontal line), interquartile range (box) and outlier (circles) values are shown. Significantly different ($p < .05$)

TABLE 2 IL-1β, ADMA, and SDMA levels in periodontitis and control groups

Biochemical parameters	Periodontitis (P; $n = 25$)	Control (C; $n = 20$)	p
IL-1β (pg/ml)	720 (468–1066)	488 (408–618)	**.003**
ADMA (μmoL)	0.03 (0.02–0.06)	0.01 (0.01–0.02)	**<.0001**
SDMA (μmoL)	0.02 (0.02–0.03)	0,01 (0.01–0.02)	**<.0001**
ADMA/SDMA ratio	1.26 (1.19–1.33)	0.80 (0.33–1.41)	.312

Note: Data are expressed as the median/mean (min-max). Mann–Whitney *U* test.

Abbreviations: ADMA, asymmetric dimethylarginine; IL-1β, interleukin-1β; SDMA, symmetric dimethylarginine.

Bold value indicates statistically significant difference between the groups $p < .05$.

4 | DISCUSSION

In the current study, the aim was to evaluate the GCF levels of ADMA and SDMA in Stage III, Grade C periodontitis. ADMA, SDMA, and IL-1β levels were higher in the periodontitis group than the control group and significantly positively correlated with clinical periodontal parameters, consistent with our hypothesis that the methylated arginine metabolites might be associated with periodontal inflammation.

Asymmetric dimethylarginine and SDMA are endogenous inhibitors of NO production and are responsible for regulating NO expression in tissue.[4] NO is mainly produced by endothelial and immune system cells, which influences the inflammatory process of several systemic diseases and a role in periodontal disease, depending on its amount.[30] ADMA and SDMA can reduce the negative effects of inflammation on the endothelium at physiological levels, but elevated ADMA levels are known predictive of endothelial dysfunction[31] and have pro-inflammatory effects.[32,33]

Asymmetric dimethylarginine inhibits NO production in macrophages[34] and regulates LPS-induced NO synthesis while increasing superoxide production via NOS uncoupling.[35,36] ADMA activity is dose-dependent, and there is an inverse relationship between the decrease in ADMA-induced NO levels and the increase in ADMA-induced ROS (reactive oxygen species) expression. Increased ROS levels decrease the activity of DDAH which is the catabolic enzyme of ADMA and increases the serum levels of ADMA.[37] Furthermore, in the presence of high ADMA concentrations, NOS produces ROS instead of NO, and decreased DDAH levels lead to increased ADMA levels.[38] Increased ADMA levels could be explained by this mechanism in the current study. In the previous study of our group, it was shown that salivary and serum ADMA levels were increased in patients with Stage III Grade B periodontitis compared to the control group.[20] Almeida et al. also showed that serum ADMA levels were increased in chronic kidney patients with periodontitis and decreased following periodontal therapy.[19] Our observations were consistent with these reports.

TABLE 3 Correlation of biomarkers between clinical periodontal parameters

Variables	IL-1β	ADMA	SDMA	ADMA/SDMA
IL-1β	–	**.315***	**.316***	–.019
ADMA	–	–	**.744****	**.481***
SDMA	–	–	–	.000
PI	**.404****	**.464****	**.460****	.035
GI	**.310***	**.476****	**.466****	.076
PD	**.403****	**.590****	**.559****	.185
BOP	**.416****	**.587****	**.566****	.122
CAL	**.435****	**.619****	**.556****	.203
GCF	.278	**.688****	**.621****	.185

Abbreviations: ADMA, asymmetric dimethylarginine; BOP, bleeding on probing; CAL, clinical attachment lost; GCV, gingival crevicular fluid; GI, gingival index; IL-1β, interleukin-1β; PD, probing depth; PI, plaque index; SDMA, symmetric dimethylarginine.

Values in bold are different from 0 with a significance level alpha $<.05$ (Spearmen correlation test $^*p<.05$, $^{**}p<.001$), $r=.20–.40$ positive and low correlation, $r=.40–.60$ positive and mild correlation, $r=.60–.80$ positive and high correlation.

Increased ROS production due to periodontal inflammation causes tissue damage by stimulating the expression of proinflammatory cytokines such as IL-1β.[39] In experimental models, exogenous applications of ADMA have been observed to increase the expression of IL-1β and TNF-α.[40,41] Also in a human study, Chen et al. suggested that ADMA may be a strong proinflammatory mediator. Consistent with these findings, we observed increased ADMA and IL-1β levels in the periodontitis group (respectively; $p<.0001$, $p=.003$). However, the low positive correlation between ADMA and IL-1β indicates that this increase may be related not only to increased ADMA expression but also to other mechanisms of the host response.

As a result of current study, it was shown that the GCF levels of SDMA were significantly higher in patients with periodontitis ($p<.001$). Although ADMA and SDMA inhibit NO production through different mechanisms, they similarly increase reactive oxygen species and show a pro-inflammatory effect. Proinflammatory cytokines may increase the arginine concentration.[41,42] The increased amount of SDMA may have developed to prevent arginine uptake into cells due to arginine concentration. The only study evaluating the SDMA level in periodontitis patients belongs to our research group, and it was found that salivary SDMA levels were higher in the periodontitis group, although there was no statistically significant difference.[20] In current study, we evaluated the SDMA levels in GCF. GCF is a fluid originating directly from periodontal tissues and includes all parameters of destruction.[21,22] Saliva is derived from serum, GCF, mucosal and acinar secretions, and therefore, it is not surprising that SDMA is found in lower concentrations in saliva than GCF from the degradation site. It was also observed that the serum SDMA level was decreased in periodontitis patients in our previous study.[20] Even though there are studies in the literature that found high SDMA levels in serum[43–46] contradicting with our studies, the possibility cannot be excluded that, SDMA concentrations might be higher in the microcirculation than plasma concentration and could be effectively transported into the cell, affecting NO synthesis locally.[47] Although the serum level is low, its increased level in the oral tissues may be related to this mechanism.

According to our results, it can be concluded that ADMA and SDMA may be an inflammatory marker in periodontal disease. However, further studies are needed to evaluate the relationship between oxidative stress and methylated arginine metabolites in periodontal disease.

5 | CONCLUSION

As a result of the current study, methylated arginine metabolites (ADMA and SDMA) are involved in the pathogenesis of periodontal disease and can be evaluated as a marker of periodontal inflammation. Although physiological levels of ADMA and SDMA have a regulatory effect on endothelial dysfunction, aberrant levels due to the periodontal inflammation may indirectly contribute to tissue damage.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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