



Free amino acid composition of saliva in patients with healthy periodontium and periodontitis

Nur Balci¹ · Şivge Kurgan² · Ali Çekici³ · Tülin Çakır³ · Muhittin A. Serdar⁴

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Abstract

Objectives To identify and compare the free amino acids in the saliva of periodontitis patients and healthy individuals and to assess their levels in different periodontal disease types.

Materials and methods There were three groups: healthy individuals (control (C); $n = 20$), Stage III Grade B generalized periodontitis (GP-B; $n = 20$), and Stage III Grade C generalized periodontitis (GP-C; $n = 20$). Clinical periodontal parameters were measured. Amino acid analysis of the saliva was accomplished by liquid chromatography–mass spectrometry (LC MS/MS), taking the mean concentration.

Results Citrulline and carnosine concentrations were significantly higher in patients with periodontitis than in the control group ($p < 0.017$). Methionine, glutamic acid, and arginine showed significantly higher concentrations in GP-C, whereas proline and tryptophan showed higher concentrations in the GP-B group ($p < 0.017$). There was a significant correlation between methionine, citrulline, arginine, and carnosine and clinical periodontal parameters.

Conclusions Our results demonstrate that periodontal status and disease type can result in variations in salivary amino acid (AA) content in correlation with clinical inflammatory signs. The significant correlation of methionine, citrulline, carnosine, and arginine with clinical parameters, regardless of systemic status, suggests that the levels of different salivary free AAs play roles in periodontitis.

Clinical relevance Salivary free AAs may be suggested as a potential diagnostic compound in patients with periodontitis.

Clinical trial registration [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04642716) NCT04642716

Keywords Free amino acids · Saliva · Periodontitis

Introduction

Periodontitis is a chronic inflammatory disease of tooth-supporting tissues that may result in tooth loss [1]. Dental biofilms play a primary role in the etiology of periodontitis, but an uncontrolled inflammatory host response triggers

increased levels of local and systemic proinflammatory mediators, which may lead to the destruction of periodontal tissues [2]. Amino acids (AAs) are organic molecules that consist of a basic amino group ($-NH_2$), an acidic carboxyl group ($-COOH$), and an organic R group (or side chain) that is unique to each AA [3]. Several studies have suggested an association between AAs and inflammatory conditions (such as intestinal inflammation) and innate immune diseases [4–6]. Current knowledge highlights the essential roles of AAs such as taurine, arginine, glutamine, and proline in the immune response by regulating T and B lymphocytes, natural killer cell and macrophage activation, lymphocyte proliferation, and the secretion of antibody cytokines and cytotoxic products [7, 8]. Metabolites of the essential AA tryptophan promote regulatory T-cell differentiation and suppress proinflammatory T-helper-cell (Th)1 and Th17 phenotypes. Suppression of the antigen-specific Th1 response that regulates interferon gamma ($IFN-\gamma$) and interleukin (IL)-17 secretion contributes to the

✉ Nur Balci
nbalci@medipol.edu.tr

¹ Department of Periodontology, Faculty of Dentistry, Istanbul Medipol University, Istanbul, Turkey

² Department of Periodontology, Faculty of Dentistry, Ankara University, Ankara, Turkey

³ Department of Periodontology, Faculty of Dentistry, Istanbul University, Istanbul, Turkey

⁴ Department of Medical Biochemistry, School of Medicine, Acibadem University, Ankara, Turkey

therapeutic effect of tryptophan in autoimmune disorders such as multiple sclerosis (MS) [9].

The findings of other studies suggest that several kinds of AA are implicated in the cell repair and protection process following inflammatory intestinal conditions such as inflammatory bowel diseases (IBDs), ulcerative colitis, and Crohn's disease [6, 10–13]. Glutamine has been experimentally confirmed to have an anti-inflammatory effect on the gastrointestinal tract, linked to a reduction in myeloperoxidase activity, the prevention of nuclear factor-kappa B (NF- κ B) activation, and inhibition of oxidative stress [11]. Moreover, similar to glutamine, L-cysteine, which is reported to have a protective function in intestinal inflammation, can regulate NF- κ B and nuclear erythroid-related factor 2 (Nrf2) signaling pathways and inhibit oxidative stress after lipopolysaccharide (LPS) challenge [10]. There is substantial evidence that AAs may have the ability to modulate the NF- κ B pathway and inhibit oxidative stress, both important pathways in the pathogenesis of periodontitis [14, 15]. Additionally, some existing studies suggest that AAs have anti-inflammatory effects on the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α) and IL-1B, which are well known to contribute to periodontal tissue destruction [16–18].

Saliva is a complex medium that contains electrolytes, bacteria, various enzymes, and inflammatory response products. It has a relatively stable amino acid concentration compared with plasma and urine [19]. Salivary amino acid components have been reported in patients with migraine conditions, phenylketonuria, and breast cancer [19–21]. Accordingly, the levels of AA in saliva have been proposed as biomarkers of Alzheimer's disease as well as breast and gastric cancers [21–23]. Given the importance of recognizing saliva as a contributor to health and diseases, there is a need to assess whether any AAs in the saliva might serve as an indicator of periodontal disease. In addition to researching AAs as an indicator of periodontal disease, this study was concerned with investigating promising therapeutic molecules that may specifically inhibit the mediators involved in the inflammatory cascade. Since AAs have been suggested as novel molecules for both identifying and treating inflammatory diseases [7, 19–22], previous studies have explored AAs and their levels in saliva in periodontitis patients [24–26]. These studies identified 16–21 different free AAs that showed elevated concentrations in stimulated whole saliva of patients with severe periodontitis, including glycine, proline, and taurine.

Even with today's advanced technologies, to the best of our knowledge, no study has identified the free AA content in whole saliva and assessed the AA levels in patients with periodontitis. Therefore, the aim of this study was to identify the free AAs present in the saliva of periodontitis patients relative to a group of healthy individuals using liquid chromatography–mass spectrometry (LC–MS/MS).

Materials and methods

Study population

Twenty systemically healthy patients with Stage III Grade B generalized periodontitis (age range: 43.3 ± 7.77 ; 9 females, 11 males), 20 systemically healthy patients with Stage III Grade C generalized periodontitis (age range: 28.8 ± 3.95 ; 8 females, 12 males), and 20 systemically and periodontally healthy individuals (age range: 36.6 ± 5.18 ; 12 females, 8 males) were enrolled in this study. This study was approved by the human subject's ethics board of İstanbul Medipol University's Faculty of Dentistry (date: 12.12.2014; Number: 10840098-327) for use and access of human subjects in research and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Clinical examination

The clinical diagnosis was made according to the “2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions” [27]. Good periodontal health (control [C] group) was defined based on a probing pocket depth (PPD) of ≤ 3 mm and bleeding on probing (BOP) of (+) $\leq 10\%$. 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 with periodontitis. The periodontitis groups consisted of patients with Stage III generalized periodontitis. These patients were graded according to the bone loss/age index (Grade B, 0.25–1.00 [GP-B] and Grade C, > 1.00 [GP-C]).

The clinical periodontal parameters of the plaque index (PI), PPD, gingival recession (GR), clinical attachment level (CAL), and BOP were recorded. Measurements were taken at four sites per tooth (mesio-buccal, mid-buccal, disto-buccal, and mid-lingual). Average scores for whole-mouth PPD, CAL, GR, and the percentage of sites with BOP were calculated for each subject.

The exclusion criteria were as follows: a history of regular use of systemic antibiotics, anti-inflammatory, or antioxidant drugs (previous 3 months); nonsurgical periodontal treatment (previous 6 months); surgical periodontal treatment (previous 12 months); presence of < 10 teeth; current medications affecting gingival health (calcium channel blockers, phenytoin, cyclosporine, or hormone replacement therapy); diabetes diagnosis; rheumatoid arthritis diagnosis; or pregnancy, lactating, or smoking. All participants were clinically examined at their first visit to the Department of Periodontology by two examiners with calibration training (NB, TC).

Saliva collection

The saliva collection protocol was explained to the patients in advance. Before collection, the patients performed oral hygiene and then rinsed their mouths thoroughly with distilled water. They were told to sit comfortably and to spit into the tubes 5 times per min for 10 min during the morning hours. The samples were transferred to Eppendorf tubes, centrifuged at 1000 ×g for 10 min and stored at –80 °C until the day of the analysis [28].

Amino acid analysis

Saliva samples were deproteinized with 6% sulfosalicylic acid at a ratio of 1:1, incubated at room temperature for 5 min, and centrifuged for 5 min at 15.000 rpm. The supernatants (10 L) were diluted with 800 L of 2 mM tridecafluoroheptanoic acid containing 0.375 M glucosaminic acid and internal standards and transferred to autosampler vials for analysis. Type calibration standards were prepared by mixing amino acid standards.

AA analysis of the saliva was performed by LC–MS/MS by using the Thermo Scientific TSQ Quantum Access MAX (Thermo Scientific, Schaumburg, IL, USA) according to a modified version of Le et al.’s [29] method.

Examiner calibration

Before beginning the study, two examiners were trained in a calibration process (NB, TC). A total of 10 volunteers were assessed by each examiner using a Williams probe (Hu-Friedy Co., Chicago, USA) once a week over a period of 1 month. The probing depth scores demonstrated good reproducibility, which was assessed by interexaminer analysis ($\kappa = 0.892$). In addition to the kappa agreement, the reproducibility assessment showed that for 90% of sites, the repeat probing mean measurements were within ±1 mm.

Statistical analysis

A statistical software package was used to conduct the data analysis (SPSS v.22, IBM SPSS Inc., Chicago, IL, USA).

Descriptive statistical methods (mean, standard deviation, frequency) were used to evaluate the study data. Data normality was examined using the Shapiro–Wilk test. A one-way ANOVA test was used for the comparison of quantitative data if there was a normal distribution. Kruskal–Wallis and Bonferroni-corrected tests were used for the comparison of nonnormally distributed parameters ($p < 0.017$). The chi-square test was used to compare the qualitative data, and Spearman’s rho correlation analysis was used to investigate the relationships between the parameters not conforming to a normal distribution. The overall significance of the study was evaluated at $p < 0.05$.

Results

Clinical parameters

The full-mouth clinical periodontal measurement results are reported in Table 1. All clinical periodontal measurements were significantly higher in the periodontitis groups than in the control group ($p < 0.001$).

AAs in saliva

The mean concentrations of the AAs and their metabolites in the saliva samples are listed in Table 2. A total of 31 AAs and their metabolites (19 essential AAs [except aspartic acid] and 12 metabolites) were found in each of the C, GP-B, and GP-C groups, but with considerable variations (Table 2).

The intergroup comparison revealed significantly different levels of specific AAs associated with periodontal disease type (Fig. 1). Proline and tryptophan were present in significantly higher concentrations in the GP-B group than in the C group, whereas the methionine, glutamic acid, and arginine levels in the GP-C group were significantly higher than those in the C group ($p < 0.017$) (Fig. 1). Ornithine showed a statistically higher concentration in the GP-B group than in the C and GP-C groups ($p < 0.017$) (Fig. 1). However, some AAs were observed at significantly higher concentrations in both

Table 1 Clinical periodontal measurements

Measurement	C (n=20)	GP-B (n=20)	GP-C (n=20)	p**
Plaque index	0.34 ± 0.12(0.18)	2.59 ± 0.14(2.67)	1.61 ± 0.51(1.84)	< 0.001
Probing depth (mm)	1.76 ± 0.24(1.78)	3.33 ± 0.67(3.34)	5.49 ± 0.97(5.67)	< 0.001
Bleeding on probing (%)	7.96 ± 22.57(2.00)	87.8 ± 21.45(96)	95.79 ± 10.57(100)	< 0.001
Clinical attachment level(mm)	1.86 ± 0.22(1.89)	4.3 ± 1.18(3.79)	5.71 ± 1.01(5.86)	< 0.001

Data are expressed as mean ± SD (median)

C group of periodontally and systemically healthy volunteers, GP-B group of systemically healthy patients with Stage III Grade B generalized periodontitis, GP-C group of systemically healthy patients with Stage III Grade C generalized periodontitis

**Significantly different from C group, both GP-B and GP-C groups ($p < 0.001$; Mann–Whitney U-test)

Table 2 Saliva AA concentrations of healthy (C), Stage III Grade B generalized periodontitis (GP-B), and Stage III Grade C generalized periodontitis (GP-C) patients (LC MS/MS results)

AAs	Saliva concentrations (ng/mL)		
	C	GP-B	GP-C
Tryptophan	0.22 ± 0.22 (0.11)	0.58 ± 0.57 (0.33)	0.47 ± 0.59 (0.2)
Phenylalanine	20.24 ± 24.63 (12.03)	28.98 ± 29.26 (24.62)	30.96 ± 23.28 (23.62)
Tyrosine	15.66 ± 16.44 (7.66)	26.51 ± 22.71 (16.08)	18.22 ± 15.1 (14)
Leucine	8.4 ± 10.59 (2.43)	14.36 ± 14.22 (9.66)	10.49 ± 8.53 (9.47)
Gamma-aminobutyric acid	2.1 ± 1.46 (1.87)	4.09 ± 3.76 (2.76)	1.76 ± 1.81 (1.27)
Isoleucine	4.37 ± 6.4 (1.45)	4.63 ± 7.06 (1.22)	1.52 ± 2.86 (0.73)
Methionine	0.03 ± 0.05 (0.01)	0.21 ± 0.31 (0.08)	0.3 ± 0.39 (0.13)
Valine	27.06 ± 23.63 (21.27)	30.66 ± 37.16 (13.97)	19.4 ± 14.24 (15.49)
Glutamic acid	7.43 ± 8.89 (3.23)	18.55 ± 27.09 (8.66)	24.46 ± 23.53 (18.86)
Amino adipic acid	0.62 ± 0.83 (0.33)	0.24 ± 0.31 (0.13)	0.25 ± 0.48 (0.06)
Ethanolamine	39.09 ± 36.83 (34.36)	48.9 ± 38.69 (56.5)	58.23 ± 38.06 (51.85)
Aminobutyric acid	24.34 ± 17.44 (19.78)	18.05 ± 16.52 (10.95)	19.92 ± 17.1 (16.19)
Threonine	5.11 ± 6.96 (1.57)	22.47 ± 37.88 (6.52)	17.54 ± 32.76 (3.62)
Alanine	57.67 ± 38.44 (58.14)	71.34 ± 79.67 (36.81)	52.26 ± 37.77 (59.29)
Beta-alanine	11.3 ± 8.83 (7.31)	10.47 ± 8.26 (9.62)	12.92 ± 7.94 (10.42)
Serine	15.33 ± 20.76 (2.34)	26.44 ± 33.4 (11.58)	16.84 ± 18.88 (10.99)
Proline	176.19 ± 175.24 (141.96)	349.15 ± 232.71 (273.86)	238.33 ± 188.99 (203.75)
Trans-4-hydroxy-L-proline	0.82 ± 1.44 (0.03)	0.44 ± 1.43 (0.04)	0.29 ± 0.78 (0.03)
Asparagine	0.33 ± 0.67 (0.07)	0.64 ± 0.84 (0.11)	0.39 ± 0.45 (0.25)
Glutamine	17.73 ± 23.75 (8.92)	20.83 ± 31.06 (7.67)	20.41 ± 16.28 (15.67)
Citrulline	11.19 ± 8.59 (8.08)	27.13 ± 21.89 (21.67)	27.38 ± 19.58 (25.7)
Homocysteine	0.02 ± 0.04 (0)	0.01 ± 0.03 (0)	0.02 ± 0.04 (0)
Cysteine	0.42 ± 0.58 (0.14)	0.55 ± 0.62 (0.33)	0.37 ± 0.43 (0.24)
Arginine	9.06 ± 6.33 (8.26)	16.49 ± 12.21 (16.94)	18.46 ± 11.89 (15.06)
Histidine	28.88 ± 26.35 (19.56)	29.02 ± 29.58 (21.21)	22.91 ± 11.45 (22.51)
Cystathionine	0 ± 0.01 (0)	0.01 ± 0.02 (0)	0.01 ± 0.01 (0)
Hydroxy-lysine	1.06 ± 2.57 (0.22)	0.84 ± 2.17 (0.11)	0.85 ± 3.03 (0.14)
Ornithine	16.19 ± 15.27 (10.75)	31.91 ± 24.75 (24.4)	14.22 ± 8.69 (10.74)
Lysine	36.71 ± 25.98 (31.71)	58.24 ± 51.99 (41.08)	41.16 ± 25.41 (40.7)
Carnosine	0.14 ± 0.17 (0.06)	1.04 ± 1.4 (0.47)	2.1 ± 3.44 (0.38)
1-methyl-L-histidine	2.35 ± 3.01 (1.21)	2.36 ± 2.86 (1.55)	2.62 ± 2.78 (2)

Data are expressed as mean ± SD (median)

Data were determined using Kruskal–Wallis test

C group of periodontally and systemically healthy volunteers, GP-B group of systemically healthy patients with Stage III Grade B generalized periodontitis, GP-C group of systemically healthy patients with Stage III Grade C generalized periodontitis

GP-B and GP-C than in the C group; these AAs included citrulline and carnosine ($p < 0.017$) (Fig. 1).

Correlation between the clinical measurements and the saliva AA concentrations

The correlations between the saliva AA concentrations and the clinical periodontal measurements are reported in Table 3. Tryptophan, tyrosine, and leucine were positively correlated with the clinical measurements ($r = 0.279$, $r = 0.359$, and $r = 0.282$,

respectively; $p < 0.05$), whereas aminobutyric acid was negatively correlated ($r = -0.275$, $p < 0.05$) only with BOP. PPD was negatively but not strongly correlated with hydroxy-lysine ($r = -0.275$, $p < 0.05$). Proline was positively correlated with only CAL ($r = 0.291$, $p < 0.05$), while phenylalanine and glutamic acid were both positively correlated ($r = 0.385$ and $r = 0.337$, and $r = 0.410$ and $r = 0.412$, respectively; $p < 0.05$) with both PPD and CAL. PI was positively correlated with several AA concentrations while the ornithine was the strongest correlated ($r = 0.447$, $p < 0.05$). Methionine, citrulline, arginine, and carnosine

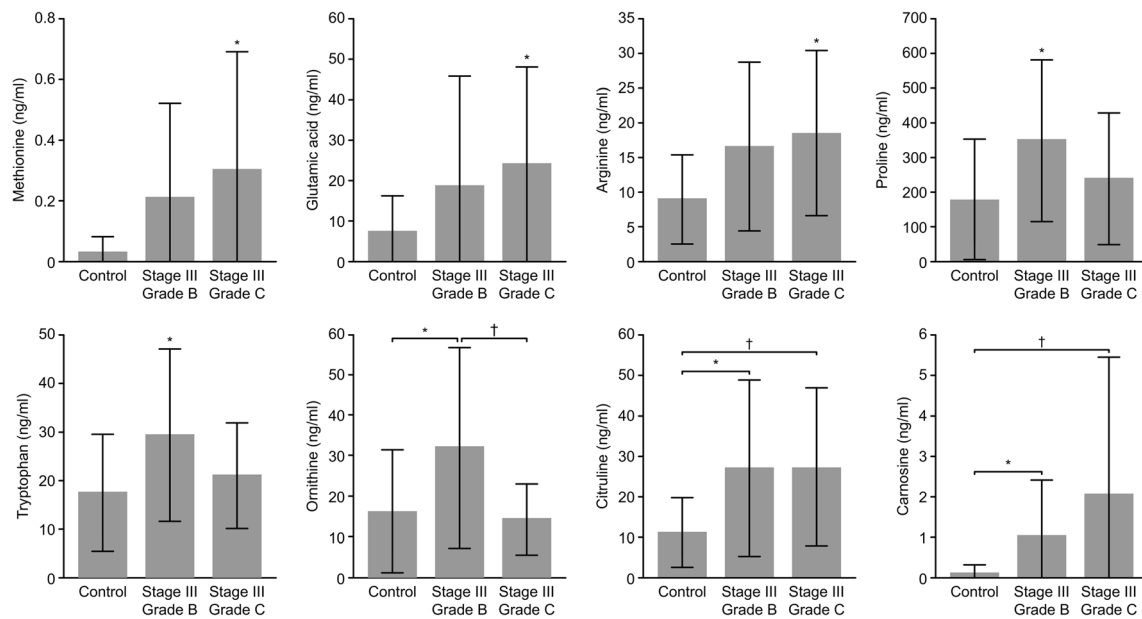


Fig. 1 Comparisons of saliva AA mean concentrations for patients with stage III grade B generalized periodontitis (GP-B), stage III grade C generalized periodontitis (GP-C), and periodontally healthy volunteers (C)

were strongly and positively correlated with all clinical parameters, with methionine showing the strongest correlation.

Discussion

Periodontitis is a chronic disease that involves a persistent inflammatory reaction with complement activation,

production of cytokines, and the release of other inflammatory cell products [1]. The purpose of the present study was to identify saliva-based AAs in different types of periodontal disease in comparison with healthy controls without the masking effects of any systemic diseases [30]. The results indicated that different specific free AAs in the saliva are more pronounced in different types of periodontal disease. In terms of the correlation between the clinical status and saliva-free AA levels, we found interesting results showing correlations (positive or negative) of various specific AAs with either one or more sets of clinical periodontal parameters.

Table 3 Correlations between clinical parameters and saliva AA concentrations (Spearman correlation coefficients)

Variables	PPD	BOP (%)	CAL	PI
Tryptophan	<i>r</i> 0.200	0.279*	0.222	0.379*
Phenylalanine	<i>r</i> 0.385*	0.249	0.337*	0.181
Tyrosine	<i>r</i> 0.195	0.359*	0.254	0.411*
Leucine	<i>r</i> 0.223	0.282*	0.202	0.316*
Methionine	<i>r</i> 0.514**	0.357	0.542**	0.313*
Glutamic acid	<i>r</i> 0.410*	0.221	0.412*	0.202
Aminobutyric acid	<i>r</i> -0.081	-0.275*	-0.050	-0.217
Proline	<i>r</i> 0.215	0.216	0.291*	0.357*
Citrulline	<i>r</i> 0.389*	0.390*	0.358*	0.324*
Arginine	<i>r</i> 0.421*	0.283*	0.411*	0.253
Ornithine	<i>r</i> 0.076	0.194	0.107	0.447**
Lysine	<i>r</i> 0.174	0.161	0.224	0.307*
Carnosine	<i>r</i> 0.449**	0.449**	0.431*	0.340*

PPD probing pocket depth, BOP bleeding on probing, CAL clinical attachment level, PI plaque index

p* < 0.05; *p* < 0.01

To our knowledge, this is the most up-to-date and comprehensive study aiming to identify free AA levels in saliva in different types of periodontal disease, and we think that it is important to determine the concentrations of amino acids to aid in understanding the pathophysiology of inflammatory diseases such as periodontitis.

There is a growing interest among researchers in investigating possible salivary biomarkers for the diagnosis of diseases, such as particular cancer types [21, 23] and Sjogren’s syndrome [31]. Using saliva is advantageous not only for researchers but also for patients due to the convenient, safe, and painless sample-collection process. In the present study, unstimulated whole saliva was used to analyze AA concentrations. The findings suggest that unstimulated whole saliva had a higher concentration of AAs than stimulated whole saliva; in other words, the parotid glands do not play a role in secreting AAs [32].

Earlier studies have listed between 16 and 24 different free AAs in human whole saliva [24–26, 33]. Our results indicate that the free AA content in whole saliva is in accordance with the findings of these studies [24–26, 33, 34]; however, in

addition to the AAs previously reported, we also found methionine, gamma-aminobutyric acid, amino adipic acid, ethanolamine, aminobutyric acid, beta-alanine, homocysteine, cystathionine, and 1-methyl-L-histidine in the saliva of periodontitis patients at various concentrations.

Amino acid analysis was performed with a sophisticated method (LC–MS/MS) in our study. Although this method is difficult to apply in routine dentistry because of its higher operational cost, it gives us an opportunity to observe the quantification of comprehensive AA profiling via good selective sensitivity. The metabolic profiling of amino acids in periodontitis differed from that in the control group, which indicates that certain amino acids could be potential biomarkers for the diagnosis of patients with periodontitis. For example, the concentrations of citrulline, carnosine, methionine, glutamic acid, arginine, proline, and tryptophan ($p < 0.017$) were significantly higher in periodontitis patients than in the control group, and proline had the highest concentration in all groups. Arginine is an essential amino acid, and it plays a critical role in intestinal inflammation through pathways that are known to be related to periodontal inflammation, such as the manipulation of immune responses and the oxidative system, the release of anabolic hormones, and the synthesis of nitric oxide (NO) [30, 35]. Likewise, arginine deficiencies are known or suspected in arteriosclerosis, pulmonary, and systemic hypertension, which suggests a mechanism involving NO production [36]. Citrulline has generated interest as a predictor in various diseases, such as rheumatoid arthritis, intestinal pathology, and arteriosclerosis [37]. Citrulline is also required in protein citrullination as a product of arginine by NO synthesis [36]. Wang et al. showed that periodontitis severity increases NO expression by inducing the action of certain cytokines [38]. NO mediates the pathological effects of lipopolysaccharide (LPS), TNF, and IL-1 and regulates leukocyte and epithelial cell adhesion, demonstrating that NO is likely related to periodontitis. Given the association of NO synthesis with the development of periodontal disease, it can be speculated that the citrulline-NO cycle may be involved in periodontitis pathogenesis by regulating the action of these and other cytokines. Tryptophan exerts a function in response to interferons and other cytokines that are released upon inflammation by tryptophan indoleamine 2,3-dioxygenase metabolism activation. Inflammation-related tryptophan indoleamine 2,3-dioxygenase activity is often considered in diseases characterized by excessive or chronic inflammation, including autoimmune disorders and cardiovascular disease, which can have reversible interactions with periodontitis [39]. Proline was detected at the highest concentrations in both the healthy and periodontitis groups. Proline originates from salivary proteins and is regarded as being responsible for much of the total AA residue of collagen as well as hydroxyproline and glycine [40]. In inflamed gingiva and periodontal destruction, the rates of collagen activity and degradation increase;

thus, proline is expected to be found in whole saliva, especially in periodontitis patients. On the other hand, we detected methionine and cystathionine at low concentrations. Syrjanen et al. [26] reported that methionine was not detected in human saliva, although it was readily observed in gingival fluid. Their findings were similar to ours, but they used wax-stimulated saliva in their study. This indicates that some AAs might degrade rapidly in saliva.

Another issue that needs to be evaluated to understand the role of amino acids in periodontal diseases is the bacteria-amino acid-periodontitis paradigm. The human oral microflora is colonized by >350 species of bacteria from the saliva to the gingival crevice; however, the distribution of their number, species, or metabolic activity is not uniform [41]. This complex bacterial community has a continuous interaction with host metabolism. There is a bidirectional balance between the inflammatory response and the microbiome in oral health and periodontitis. Microorganisms are responsive to metabolic and pathogen challenges and have the ability to produce AAs from nonspecific nitrogen sources [42]. Beyond periodontitis, similar observations have been made in obesity and other related diseases via the gut microbiota [43, 44]. Some amino acids (leucine, isoleucine, alanine, lysine, and methionine) levels were found to be correlated with specific bacteria in type 2 diabetes mellitus and obesity. It has been claimed that the gut bacteria can produce amino acids that act in metabolic product synthesis and have roles in the utilization of both exogenous and endogenous proteins [44, 45]. As the gut microbiota has effects on AAs in diabetic patients, the oral microbiota may also be responsible for similar interactions. When the linkage of diabetes and periodontitis is considered, bacteria could influence AA metabolism in periodontitis. It is therefore crucial to better understand and quantify the microbial biosynthesis of amino acids in periodontitis patients. Although the underlying mechanisms require further investigation, this study has provided a general framework for understanding the possible relationship between amino acids and microbiome via AAs and plaque index correlations in periodontitis (Table 3). However, the exact reason or molecular mechanism of these amino acids could not be extracted from the present findings, our study showed that several free saliva AAs are positively correlated with microbial intensity. One of the limitations of this study is that it does not support the understanding of other interactions between a wide variety of cytokines, chemokines, and bacterial effects that modulate the pathogenesis of periodontitis. Therefore, there is still much work to be done to explore the functions and signaling pathways of amino acids in humans and animals in periodontal inflammation.

The effects of free AAs on the pathology of diseases are significant not only for periodontal diseases but also for other conditions, such as IBDs, ulcerative colitis, breast cancer, and septic shock [6, 12, 21, 46]. Miyagi et al. [47] observed

significant increases in the concentrations of threonine proline, glycine, and alanine ($p < 0.001$), serine and ornithine ($p < 0.01$), and lysine ($p < 0.05$) in breast cancer patients. Cheng et al. [21] reported that the concentrations of 15 salivary-free AAs demonstrated significant differences ($p < 0.05$) between breast cancer patients at stages I–II and healthy controls. The results of these clinical studies suggest that different cancer types and degrees can alter free AA metabolism, mostly by increasing their concentrations in body fluids, including saliva. A pilot study investigated the relationship between plasma-based AA concentrations (arginine, glutamine, citrulline), inflammatory mediators (TNF- α , IL-10), and C-reactive protein (CRP) in patients with septic shock and reported that citrulline, glutamine, and arginine levels in plasma were negatively related to CRP, whereas there was no correlation between citrulline and TNF- α or IL-10 [46]. In the present study, we found a significant increase in eight specific salivary-free AA concentrations in the periodontitis groups, and this increase was associated with increased local inflammation. These results suggest that disease-specific salivary AAs might be more strongly modulated in the presence of periodontal inflammation. Although several studies have attempted to identify the mechanism for the abnormal AA profiles shown by patients with various inflammatory diseases, there is a lack of up-to-date similar studies on salivary-free AAs in patients with both chronic and aggressive forms of periodontal disease.

Periodontitis is a common inflammatory disease worldwide. Therefore, there has always been an interest in researching the etiopathogenesis of this disease to determine the diagnosis, prevention, and screening of periodontal patients. Although detecting sensitive biomarkers to improve the early detection of tissue destruction of the periodontium in dental clinics is a very desirable situation, in general, there are very few and limited practical and economical tests to be applied at the chair side. In this regard, the point of care (PoC) method has been used to detect biomarkers such as active MMP8 (matrix metalloproteinase 8) and IL-6 (interleukin 6) for the diagnosis of periodontitis at the chair side [48–53]. Researchers have shown that with PoC, it is easy, fast, and inexpensive to test oral fluids such as gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF), mouth rinse fluids, and saliva from patients with periodontitis, peri-implantitis, and other systemic diseases such as head cancer and diabetes. From the perspective of our study, further research should be planned to establish a chair-side testing method for AAs as a possible biomarker of periodontitis in clinical settings in the future.

When identifying AA profile changes in periodontal disease and evaluating the effects of inflammation on AA concentrations, it is also important to evaluate whether the clinical parameters are correlated. Positive correlations with clinical parameters were found for 11 of the 31 free salivary AAs observed in the current study. Additionally, the strong positive correlations between clinical parameters (PI, GI, BOP) and

methionine, citrulline, arginine, and carnosine are similar to the findings of studies focusing on AA concentration changes resulting from inflammatory systemic diseases [54, 55]. To the best of our knowledge, this is the first study demonstrating correlations of methionine, citrulline, arginine, and carnosine profiles in saliva with clinical parameters that indicate periodontitis severity. However, it cannot be concluded from these findings alone whether these amino acids play a role in the pathogenesis of periodontitis. It would be important and illustrative to evaluate the effects of these amino acids and their metabolism on periodontal conditions in further studies.

Detection and treatment of periodontitis require identifying the molecular interactions that occur during the activation and resolution of periodontal inflammation. In this context, detection of the salivary free AA profile may be expected to complement traditional diagnostic and treatment techniques for periodontal diseases. Nevertheless, the conclusions of this study have limitations owing to confounding factors such as pro/anti-inflammatory mediators not being analyzed. Additionally, if the selected AAs are to be taken into consideration for complementing the diagnosis of periodontitis, they need to be validated in studies of other body fluids (e.g., gingival crevicular fluid and blood serum) and with larger populations.

Conclusion

Within the limits of the present investigation, the results demonstrate that periodontal status and disease type can result in variations in salivary AA content. The significant correlation of methionine, citrulline, carnosine, and arginine with clinical periodontal parameters, regardless of systemic status, may suggest that the levels of different salivary free AAs have potential as diagnostic compounds of periodontitis. Large-scale clinical trials on patients with periodontitis are needed to establish the potential significance of the AA profile in understanding the relationship between periodontal inflammation and salivary free AAs.

Author contribution NB contributed to the design of the study, collected the samples, recorded clinical data, and wrote the manuscript with input from the other authors. SK contributed to the design of the study, analyzed the clinical data, helped interpret the results, and wrote the manuscript with input from the other authors. AC contributed to the design of the study, helped interpret the results, and wrote the manuscript with input from the other authors. TC helped to collect the samples and recorded clinical data. MS contributed to the design of the study, performed biochemical analysis and statistical analysis, and helped interpret the results. All authors reviewed and approved the submitted manuscript.

Declarations

Ethics approval All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of

the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent to participate Informed consent was obtained from all individual participants included in the study and/or their relatives or legal representatives.

Conflict of interest The authors declare no competing interests.

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