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Psychological Stress and its relationship to Periodontal flora and salivary Nitrite/Nitrate

Elisa Ponzio [a], Jacopo Dolcini [a]*, Scilla Sparabombe [b], Giorgio Firmani [a], Marcello M. D'Errico [a], Pamela Barbadoro [a]

[a] *Department of Biomedical Science and Public Health, Unit of Hygiene, Preventive Medicine and Public Health, Università Politecnica delle Marche, Ancona, Italy*
[b] *Department of Clinical Sciences and Stomatology (DISCO), Università Politecnica delle Marche, Ancona 60126, Italy*

ABSTRACT

Objective: Psychological stress can be a common risk factor for the development of oral and systemic disease; therefore, analysis of a pathophysiologic mechanisms that may explain this association may be significant in planning preventive strategies. The aim of this study was to investigate the association amongst academic stress, periodontal health, and salivary cortisol and nitrite and nitrate levels in a sample of university students.
Methods: Participants (N = 14) were classified into 2 groups according to their exposure to academic stress due to periods of university exams (n = 6 and n = 8, respectively). All participants were subjected evlauted for their behavioural, psychological, and anthropometric parameters, as well as an oral health examination. A real-time polymerase chain reaction analysis in samples of saliva and plaque was used to detect *Prevotella intermedia* and *Veillonella dispar* as well as the total bacterial count. Nitrite/nitrate ratio (NR ratio) and cortisol in saliva were evaluated by enzyme-linked immunosorbent assay.
Results: Full Mouth Bleeding Score, Full Mouth Plaque Score, and Gingival Index were significantly higher in the group exposed to academic stress. Nitrite was directly related to the presence of *V dispar* (coefficient, 0.13; P = .00; CI, 0.07 to 0.19) and inversely related to total bacterial count (coefficient, −0.07; P = .012; CI, −0.13 to 0.02). NR ratio was directly related to *V dispar* (coefficient, 4.35; P = .010; 95% CI, 1.35 to 7.36) and inversely related to total bacterial count (coefficient, −4.05; P = .018; 95% CI, −7.32 to 0.86).
Conclusions: These results confirm the importance of stress on periodontal health and salivary nitrite concentration and highlight a potential differential role of specific bacteria on nitrite concentration in saliva.

Introduction

Psychological stress has been related to different conditions, including gum tissue inflammation and periodontal disease.[1−3] The results of a recent review show that periodontal diseases are associated with different systemic pathologies, including rheumatoid arthritis, cardiovascular pathologies, and neurodegenerative pathologies.[4] In this context, the specific relation of oral microbiology and serum reactive oxygen metabolite levels, such as nitric oxide (NO), has been studied.[5-7] Previous research has shown the role of stress hormones on growth of selected periodontitis-related bacteria,[6] and evidence shows that NO deficiency at the endothelial level may be associated with the development of hypertension and other forms of cardiovascular disease.[7] Moreover, periodontal and cardiovascular diseases have been linked,[8−11] which preempts a hypothesis of a linkage amongst stress, periodontal disease, oral microbiota, and systemic homeostasis. According to different authors, academic stress is an important psychosocial challenge for both students and undergraduates,[12] and the role of academic stress has already been studied with regards to oral NO levels.[5,13] Moreover, the observation of salivary nitrite/nitrate modifications in

healthy participants exposed to acute stress may be a useful model to study the linkage amongst stress, periodontal health, and nitrite/nitrate levels. Since the definition of a possible pathophysiologic mechanism linking periodontal disease and stress may be significant in planning preventive strategies targeting different diseases, this relationship has been an intensive area of research.

The aim of this study was to evaluate the correlation between academic stress, periodontal health, gum inflammation (measured via Full Mouth Bleeding Score [FMBS], Full Mouth Plaque Score [FMPS], and Gingival Index [GI]), nutritional habits, salivary cortisol, and nitrite/nitrate levels in a sample of university students. Periodontal health status was evaluated by investigating the load of *Veillonella dispar*, which is associated with oral health, and *Prevotella interemedia*, implicated in gingivitis and periodontitis, as according to some studies these species are linked to the concentration of NO.[14]

Materials and methods

Study population and methodology

A cross-sectional study was carried out in 2016 amongst students attending their third year of different degree programmes in the Faculty of Medicine and Surgery at the Università Politecnica delle Marche, Ancona, Italy. A total of 14 participants, all aged 22 years, were recruited. Participants were classified into 2 groups according to exposure to academic stress: one group was about to face an exam session and the other group was not. Students with diabetes or mental health conditions, pregnant students, and those taking selected drugs (calcium channel blockers; anticonvulsant, anti-inflammatory, immunosuppressive, and antihypertensive agents; narcotic substances; and antibiotics during the 6 weeks before enrollment) were excluded from the study.[15] Moreover, other exclusion criteria included having tooth decay or periodontal probing ≥4 mm; having had periodontal treatment during the 6 months before the study; and having had an oral hygiene session performed in the last 3 months.

All participants were evaluated for distribution of sociodemographic, behavioural, psychological, and anthropometric parameters; oral health examination; and assessment of selected components of the oral microbiota, salivary cortisol, salivary nitrate/nitrite levels, and pH.

Behavioural, psychological, and anthropometric parameters included sex, body mass index (BMI; classified as underweight, BMI < 18.4 kg/m^2; normal weight, BMI = 18.5−24.9 kg/m^2; and overweight, BMI > 25 kg/m^2), smoking habits, alcohol consumption as evaluated by the 4-item Alcohol Use Disorders Identification Test,[16] physical activity as evaluated by the International Physical Activity Questionnaire (IPAQ) 27-item questionnaire.[17] Self-perceived stress was evaluated with the Perceived Stress Scale Questionnaire (PSS-10), a 10-item instrument[18] measuring the degree to which one's life situations are appraised as stressful using a 5-point Likert scale, and a Visual Analogue Scale (VAS; ranging from 0 to 10).

Nutritional habits were examined using a food frequency questionnaire, including the evaluation of consumption of coffee (never, rarely, several times a week, every day, several times a day), magnesium-rich foods (nuts and seeds, dark chocolate, bananas, avocado, soybeans), green leafy vegetables, and fish as well as pro- and anti-inflammatory foods such as ginger, garlic, and curcuma.[19] Food frequency consumption options ranged from several times a day to daily, weekly, and monthly. Students underwent a clinical examination of the oral cavity with evaluation of the dental formula and plaque index (FMPS) and the dental gingival bleeding index (FMBS). Presence of any dental caries and the state of the restorations were evaluated by means of an oral and physical examination an assisted interview. Periodontal probing was performed to evaluate the presence of periodontal pockets ≥4 mm (see exclusion criteria). GI was assessed evaluating 6 sites in each student. GI ranged from 0 to 3 (0 = normal gum; 1 = medium inflammation, slight change of colour, no bleeding on probing; 2 = moderate inflammation, redness and oedema, bleeding on probing; 3 = severe inflammation, marked redness and oedema, ulceration with a tendency for spontaneous bleeding), and an accurate anamnesis was carried out.

This study was approved by Ethics Committee of the Polytechnic University of Marche (Prot.n. 0000197). All enrolled participants provided written informed consent. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Laboratory evaluation

Sample collection

Plaque samples was collected during a dental visit, and salivary samples were collected on awakening (between 7:00 and 8:30 AM, t1) and then after 15 (t2) and 30 minutes (t3). Participants refrained from eating, drinking, smoking, and brushing teeth from awakening upto the saliva and plaque collection time point. At least 1 mL of saliva was collected in a Salivette device (Sarsted Aktiengesellschaft & Co.), and the procedures were conducted as previously described.[20] Saliva samples were centrifuged at 1000 rpm for 2 minutes, and the supernatant was collected and stored at −20 °C. Supragingival and subgingival plaque sampling was performed using sterile paper points (ISO 30, Dentsply, GmbH). Supragingival plaque samples from the mesial-buccal surface of the first molar (second molar if the first molar was missing) in each quadrant were collected. The pooled plaque from each participant was placed in a microcentrifuge tube containing 1.5 mL sterile 1X Tris-EDTA buffer. Subgingival plaque was sampled from the site of the deepest pocket of the same tooth where the supragingival plaque was collected. Collections were stored at −20 °C until real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis.

Oral microbiology evaluation

Genomic DNA extraction from the specimens was performed using a PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions with some modifications. DNA concentrations were determined

spectrophotometrically using Qubit 2.0 Fluorometer (Invitrogen).

Bacteria-specific TaqMan probe and primer sets were designed from the species-specific region on the 16S rRNA and were used to identify *Prevotella intermedia* and *Veillonella dispar*.[21-23] In addition, a universal primer was used to quantify the total amount of bacterial species in the oral plaque.[20] Real-time polymerase chain reaction (PCR) was carried out using a Rotor-Gene 3000 (Corbett-Research); all samples were run in duplicate. Each PCR was performed in a total volume of 20 μL containing 4 μL of 5X Takara Buffer (Takara Bio Inc), 0.2 μL each of forward and reverse primers (final concentration, 500 nM each), an appropriate dose of TaqMan Probe (final concentration, 200 nM), an appropriate amount of MgCl$_2$ solution (final concentration, 1.5–6 mM) and dNTP mixture (final concentration, 0.2 mM), 1 U Taq DNA Polymerase, 2 μL of template DNA solution, and an appropriate dose of sterilised UltraPure DNase/RNase-Free Distilled Water (Invitrogen). Amplification conditions are shown in Table 3. The bacterial DNA level was quantified by qRT-PCR and transformed to theoretical cell numbers as previously described.[22] In the present study, the total bacterial load in the specimens was calculated with the assumption that the 16s rRNA gene copy numbers of the eubacterial species were not significantly different from each other.[21]

Salivary cortisol detection

Procedures were conducted as previously described.[24] At least 1 mL of saliva was collected in Salivette devices (Sarsted Aktiengesellschaft & Co.). Saliva samples were centrifuged at 1000 rpm for 2 minutes, and supernatant was collected and stored at −20 °C. A commercial enzyme immunoassay kit was used to determine salivary cortisol (DiaMetra) according to the manufacturer's instructions. Cortisol concentration was expressed as nmol/L. The lower limit of detection for the assay was 0.5 nmol/L, and the upper limit of the standard curve was 1750 nmol/L. To investigate the circadian pattern of basal cortisol secretion, 3 salivary samples were collected: at wake-up (between 7:00 and 8:30 AM, t1) and then after 15 (t2) and 30 minutes (t3).

NO determination

An enzyme immunoassay kit (R&D Systems, Inc.) to determine NO$_2^-$ and NO$_3^-$ in saliva was used according to the manufacturer's instruction. The assay determines nitric oxide concentration based on the enzymatic conversion of nitrate to nitrite through the activity of nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the 2-step diazotisation reaction in which acidified NO$_2^-$ produces a nitrosating agent, which reacts with sulfonic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm and is then read by the enzyme-linked immunosorbent assay reader. For the determination of NO, the saliva sample upon awakening (between 7:00 and 8:30 AM, t1) was used.

Salivary pH and flow measurement

For measurement of unstimulated flow, patients were asked not to swallow for 1 minute, and at the end of that time the salivary sample was collected using a 2.5-Cl syringe in a glass and measured. Then, salivation was stimulated by an acid liquid, such as lemon juice, inserted into the oral cavity of the patient and removed after 10 seconds; then, stimulated salivary flow was measured. Salivary pH was measured using unstimulated saliva.

Statistical analysis

Normal distribution for raw cortisol values, NO$_2^-$ and NO$_3^-$ concentration, and bacterial count was checked with Shapiro−Wilk test; if the test rejected normality, data were log-transformed. NR ratio was calculated as follows: 100 * [nitrite] / ([nitrate] + [nitrite]). Bivariate analyses were performed to analyse the association of stress and periodontal health variables in the 2 groups of students, using Chi-square and Student t test as appropriate. Multiple linear regression models were developed to evaluate factors independently associated with salivary nitrite, nitrate, and NR capacity levels. FMPS was categorised into 2 levels: 0% to 20% and 50% to 100% considering the percentage of sites harbouring plaque <20% as an accepted standard and as a tolerable level of oral hygiene amongst the general population, and FMBS was categorised into 2 levels: 0% to 20% and 50% to 100% according to the cut-offs for localised and generalised gingival inflammation. The significance level for variables to enter the multiple logistic regression models was set at ≤0.2, and for removing them from the model at ≤0.4. Analyses were performed with STATA, version 15 (Stata Corp.). The level of significance was set at 0.05.

The comparison of cortisol levels between the 2 groups was performed using the ggstatsplot v.0.8.0 package.[25] The analysis was carried out in RStudio v. 4.2.1.[26]

To better understand the complexity and interrelationships amongst the variables, a principal component analysis (PCA) was performed and the exposed and the unexposed groups were analysed using the ggplot2 package. The analysis was carried out in RStudio v. 4.2.1.[26]

Results

Fourteen students were enrolled in this study; 71.4% (n = 10) were female (Table 1). Bivariate analysis revealed no statistical differences between groups regarding sociodemographic variables and dietary and habits sych as smoking alcohol consumption and physical activity. No differences were detected in the PSS-10 score, but the VAS score was significantly higher in the group exposed to academic stress (P = .04; Table 1). Selected nutritional habits did not show any significant effect in the present sample.

FMBS, FMPS, and GI were significantly higher in the group exposed to academic stress, and basal levels of salivary cortisol on awakening (t1) and after 30 minutes (t3; Table 2) were also significantly higher.

The linear regression model showed that nitrate concentration was inversely related to female sex (coefficient, −1.02;

Table 1 – Distribution of sociodemographic, behavioural, psychological, and anthropometric parameters in participants exposed and not exposed to academic stress.

	Academic stress		
	Not exposed	Exposed	P value
Sex	No. (%)	No. (%)	
Female	7 (87.50)	3 (50.00)	.12*
Male	1 (12.50)	3 (50.00)	
Smoking			
Yes	3 (37.50)	5 (83.33)	.39*
No	5 (62.50)	1 (16.67)	
BMI			
Underweight	1 (12.50)	0 (0.00)	.42*
Normal	6 (75.00)	6 (100.00)	
Overweight	1 (12.50)	0 (0.00)	
Physical excercise			
Yes	4 (50.00)	5 (83.33)	.21*
No	4 (50.00)	1 (16.67)	
Alcohol consumption			
Never	4 (50.00)	4 (66.67)	.53*
Rarely	4 (50.00)	2 (33.33)	
Fish consumption			
Rarely	4 (50.00)	3 (50.00)	1.00*
Several times a week	4 (50.00)	3 (50.00)	
Fruit and vegetable consumption			
Every day	5 (62.50)	2 (33.33)	.28*
Several times a day	3 (37.50)	4 (66.67)	
Spinach consumption			
Never/rarely	6 (75.00)	3 (50.00)	.33*
Several times a week	2 (25.00)	3 (50.00)	
Coffee consumption			
Never	1 (12.50)	0 (0.00)	.10*
Rarely	0 (0.00)	1 (16.67)	
Several times a week	3 (37.50)	1 (16.67)	
Every day	1 (12.50)	2 (33.33)	
Several times a day	3 (37.50)	0 (0.00)	
Consumption of high-magnesium food			.63*
Rarely	4 (50.00)	2 (33.33)	
Several times a week	3 (37.50)	2 (33.33)	
Every day	1 (12.50)	2 (33.33)	
Turmeric			.72*
Yes	1 (12.50)	1 (12.50)	
No	7 (87.50)	4 (66.67)	
Saffron			1.00*
Yes	4 (50.00)	3 (50.00)	
No	4 (50.00)	3 (50.00)	
Ginger			
Yes	7 (87.50)	5 (83.33)	.825*
No	1 (12.50)	1 (16.67)	
FMPS			
0%−20%	8 (100.00)	1 (16.67)	.001*
50%−100%	0 (0.00)	5 (83.33)	
FMBS			
0%−20%	8 (100.00)	1 (16.67)	.001*
50%−100%	0 (0.00)	5 (83.33)	
GI			
Normal	8 (100.00)	2 (33.33)	.024*
Mild inflammation	0 (0.00)	3 (21.43)	
Moderate inflammation	0 (0.00)	1 (16.67)	
	Mean (SEM)	Mean (SEM)	
PSS-10 score	21.50 (1.09)	21.83 (1.49)	.86†
VAS	3.50 (0.78)	6.00 (0.68)	.04†
Salivary cortisol			
At awakening	27.31 (14.16)	68.61 (9.09)	.02†
15 min after awakening	52.92 (17.39)	87.59 (21.78)	.12†
30 min after awakening	31.02 (10.84)	113.29 (38.17)	.04†
Bacterial count			
Total bacterial count	1.66E+04 (1.20E+04)	1.67E+08 (1.67E+08)	.76†
Prevotella intermedia	287.50 (156.34)	1700.00 (1660.12)	.83†
Veilonella dispar	165.14 (120.42)	1.67E+05 (1.67E+05)	.35†
NO (μmol/L)	61.41 (17.35)	57.07 (16.57)	.86†
pH	6.38 (0.18)	6.50 (0.22)	.67†

* Chi-square test.
† Student *t* test. BMI, body mass index; FMBS, Full Mouth Bleeding Score; FMPS, Full Mouth Plaque Score; GI, gingival index; NO, nitric oxide; PSS-10, Perceived Stress Scale Questionnaire; VAS, Visual Analogue Scale

Table 2 – Results of multiple linear regression modeling for factors related to NO$_3$ salivary levels.

Nitrate	Coefficient	P value	95% CI
Nitrite	0.27	.43	−0.46 to 1.01
Salivary cortisol t3	−0.3	.008	−0.50 to 0.92
Sex	−1.02	.012	−1.76 to −0.27

Table 3 – Results of multiple linear regression modeling for factors related to NO$_2$ salivary levels.

Organism	Coefficient	P value	95% CI
Veilonella dispar	0.13	<.001	0.07 to 0.19
Total bacterial count	−0.07	.012	−0.12 to 0.02

Table 4 – Results of multiple linear regression modeling for factors related to nitrite/nitrate ratio (NR).

NR	Coefficient	P value	95% CI
Veilonella dispar	4.35	.010	1.35 to 7.36
Total bacterial count	−4.05	.018	−7.23 to 0.86
FMBS	8.71	.051	−0.03 to 17.44
Alcohol intake	−3.58	.075	−7.61 to 0.44
PSS-10 score	−1.19	.081	−4.15 to 0.29
Smoking habit	−9.91	.127	−23.23 to 3.41

FMBS, Full Mouth Bleeding Score; PSS-10, PSS-10, Perceived Stress Scale Questionnaire.

P = .012; 95% CI, −1.76 to 0.27) and to concentration of salivary cortisol at t3 (coefficient, −0.3; P = .008; 95% CI, −0.50 to 0.92; Table 2). Nitrite was directly related to the presence of *V dispar* (coefficient, 0.13; P = 0.00; 95% CI, 0.07 to 0.19) and inversely related to total bacterial count (coefficient, −0.07; P = .012; 95% CI, −0.12 to 0.02; Table 3). Moreover, despite being significant (P = .0267), the only factors independently related to NR ratio were the count of *V dispar* (coefficient, 4.35; P = .010; 95% CI, 1.35 to 7.36) and the total bacterial count (−4.05; P = .018; 95% CI, −7.32 to 0.86). However, the significance of the model explaining the NR ratio variability was improved by the inclusion of increasing FMBS (coefficient, 8.71; P = .051; 95% CI, −0.03 to 17.44), reduction of alcohol intake (coefficient, −3.58; P = .075; 95% CI, −7.61 to 0.44), reduction of PSS-10 scores (coefficient, −1.19; P = .081; 95% CI, −4.15 to 0.29), and smoking habit (coefficient, −9.91; P = .127; 95% CI, −23.23 to 3.41; see Table 4).

The ggstatsplot showed that in these 2 groups the cortisol levels measured upon awakening (t$_{\text{Welch}}$ = 3.11; P = .02) and after 30 minutes (t$_{\text{Welch}}$ = 2.62; P = .02) were higher in exposed participants than in those not exposed to stress (Figures 1 and 2).

Figure 3 shows the relationships amongst 3 variables simultaneously (total bacterial count, cortisol t3, and *V dispar*). These 3 variables were selected after calculating the correlation coefficient through the cor() function. Variables that share more than 80% of the covariance were selected.
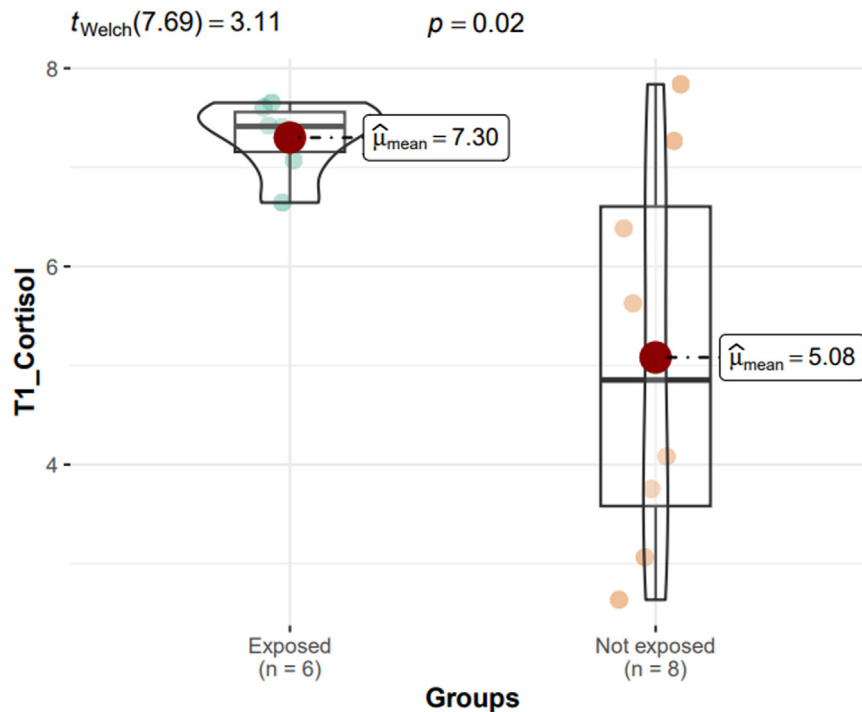
Fig. 1 – ggstatsplot showing that cortisol levels upon awakening (t1) are higher in participants exposed to stress than in those not exposed to stress.
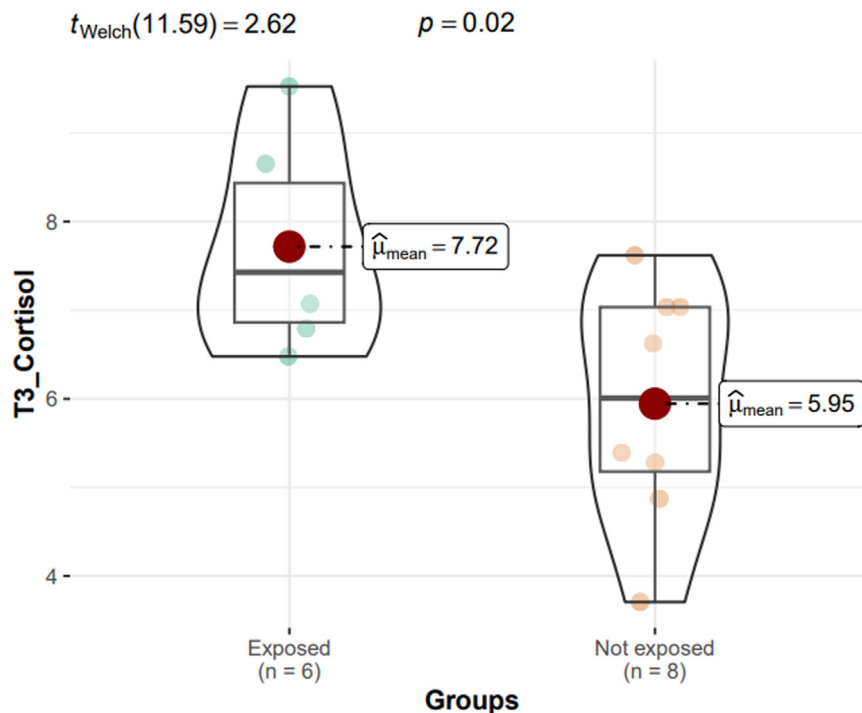

Fig. 2 – ggstatsplot showing that cortisol levels after 30 minutes (t3) are higher in participants exposed to stress than in those not exposed to stress.

Discussion

According to other studies evaluating the association of stress with increased plaque index, our work has highlighted significant periodontal modifications in participants undergoing stress. In fact, our results agree with those of Deinzer et al,[3] since 83.3% of participants exposed to academic stress had an FMPS level between 50% and 100%, which was significantly higher than controls. Moreover, the proportion of participants with bleeding gums was as high as 83.3%, significantly greater
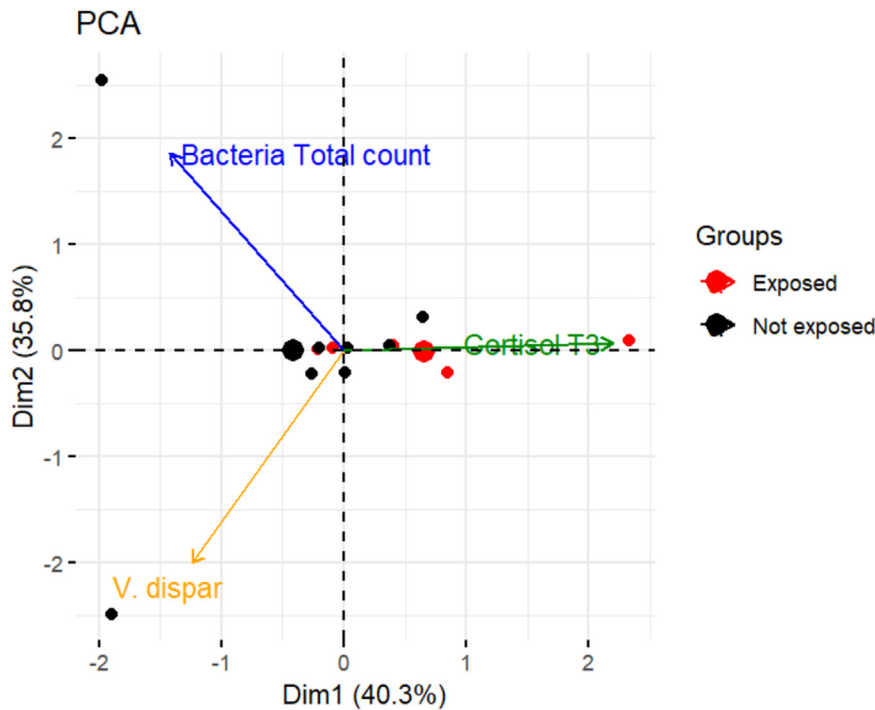
Fig. 3 – Principal component analysis score relates the variables bacterial total count, cortisol t3, and *Veillonella dispar* in participants exposed and not exposed to stress.

than that of students not exposed to academic stressors ($P < .01$).

In the context of this association, at awakening (t1) and after 30 minutes (t3), the academic stress group had a greater concentration of salivary cortisol than the control group, which is in agreement with observations by Johannsen et al[27] in 2010, which revealed that the increase of bacterial plaque in students experiencing academic stress was related to elevated levels of salivary cortisol.

PCA showed that variables that provide similar information are grouped together, showing a possible relationship (Figure 3). Total bacterial count and V *dispar* are examples of 2 positively related variables. When the numerical value of one variable increases or decreases, the numerical value of the other variable tends to change in the same way. This is because total bacterial count comprises both bacteria associated with good (ie, V *dispar*) and poor oral hygiene. When variables are negatively ("inversely") correlated, they are placed on opposite sides of the origin of the chart. For example, the variables cortisol t3 and V *dispar* are inversely related, which means that when cortisol levels increase, V *dispar* decreases. In addition, the distance from the origin of the axes also communicates information. The farther a variable is from the origin of the diagram, the stronger the impact is that the variable has on the model. Figure 3 shows how the impact of the variable cortisol t3 is stronger in participants exposed to stress (red dots) than in those not exposed (black dots). Again, for example, there were higher levels of V *dispar* in participants not exposed to stress than those exposed to stress. Multiple linear regression modelling showed a reduced NO_3^- concentration in female participants and increased salivary

cortisol at t3. Reduced levels of NO_3^- could be explained by the interaction of many factors; it is known that increases in the adrenal−cortisol axis may decrease the production of NO.[28] Moreover, despite the role of estrogens in female participants in increasing NO levels,[29] this upregulation could be attenuated by their cortisol-induced downregulation in those exposed to chronic stress. Similar results have been recently reported in participants of the National Health and Nutrition Examination Survey where exhaled NO was greater in men and obese individuals.[30] In our sample, BMI was not related to NO; however, the adjustment for weight was significant in the whole model.

Following the results by Ozer et al,[31] the amount of salivary NO in students exposed to academic stress was lower than that in individuals not exposed, suggesting that the production of NO may be suppressed in individuals with periodontitis. However, contrasting findings were reported by Reher et al[32] and Parwani et al.[33]

On the other hand, despite our small sample size, a significant variation in clinical data testifies to a clinically important correlation amongst stress and oral microbiota (ie, V *dispar*) and oral NO_2^-, confirming previous results showing that the effects of nitrate reduction in the oral cavity was linked to the presence of commensal bacteria[34,35] and specifying the potentially different role of total bacterial count (reducing nitrite levels) with respect to other species (ie, V *dispar*). Moreover, the toll of *Veillonella spp.* as a nitrate reducer is confirmed in the present study, in agreement with results of previous works.[36]

Our study has some important limitations: We had a small sample size in terms of recruited participants, and we have been able to study only 2 bacterial species whilst a deepened analysis between oral and general health status should

include searching of more bacteria species and their relative prevalence. For these reasons, our results are extremely preliminary in the context of a pilot study and future work is needed to better investigate this scenario.

Conclusions

This study demonstrated that academic stress was associated with gum inflammation and periodontal health risk in a cohort of healthy young participants. From a clinical point of view, patients should be advised on stress as a risk factor for the homeostais of periodontal health. Professional help should be provided for patients who are unable to maintain appropriate oral hygiene to obtain a more intensive follow-up during psychological stress periods. Despite the lack of statistical significance as an independent variable regarding periodontal levels, results suggested the importance of oral microbiota in cardiovascular disease and blood pressure control.[37–39]

These results point out the necessity to plan appropriate strategies to improve health education, promoting good oral health habits particularly in university students exposed to high academic stress. Moreover, awareness must be increased about stress as an important risk factor for gingival inflammation and periodontal disease.

Conflict of interest

None disclosed.

Acknowledgements

Author contributions

PB and EP conceived the study and contributed to data acquisition; PB and EP prepared the original draft; SS provided assistance with study design; and JD provided assistance in preparing the manuscript. Laboratory analysis was done by EP and GF. Statistical analyses were done by PB, and MMD supervised the project. All authors read and agreed to the published version of the manuscript.

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