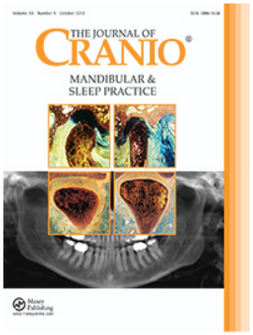

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Salivary biomarkers for caries susceptibility and mental stress in individuals with facial pain

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ABSTRACT

Objective: To assess salivary biomarkers for dental caries susceptibility and mental stress in young adults with perceived facial pain.

Methods: Males and females who reported facial pain and pain-free controls participated in this study. Facial pain was investigated using the RDC/TMD. Unstimulated saliva was then collected for the evaluation of salivary flow rate (SFR), pH, *Streptococcus mutans* counts, morning cortisol, and S-IgA.

Results: Women with facial pain had significantly lower SFR values, and the facial pain group showed different correlations among biomarkers for caries susceptibility and cortisol levels when compared to controls. Notably, higher SFR values were associated with a lower likelihood of having facial pain.

Conclusion: Differences in SFR values, particularly in women, and markedly distinct interactions among the salivary biomarkers analyzed were observed between individuals with facial pain and pain-free controls. Hence, a connection between the dynamics of saliva, stress response, and facial pain perception might exist.

KEYWORDS

Dental caries susceptibility; facial pain; gender; immunoglobulin A; psychological stress; saliva

Introduction

Facial pain affects the region above the neck, in front of the ears, and/or below the orbitomeatal line. It shows high prevalence, especially in patients with temporomandibular disorders (TMD), and evolves into chronic pain in 11% of cases [1]. The mechanisms involved in the development and progression of facial pain have been increasingly understood and related to changes in peripheral and centralized pain processing, as well as psychosocial comorbidities [2]. Additionally, individuals with facial pain are more likely to experience distress-related comorbidities due to neuroendocrine responses to mental stress [3–5].

Indeed, levels of secretory immunoglobulin A (S-IgA), the major antibody isotype of the mucosal immune system, is thought to be decreased in individuals undergoing stress [6,7], while females with TMD showed increased levels of cortisol, a hormonal stress biomarker [3,8]. Nonetheless, myofascial pain therapy was not found to increase S-IgA or cortisol levels in a clinical sample [9], thus, representing a controversial association between stress, facial pain, and these so-called salivary stress biomarkers.

Interestingly, associations between stress, altered cortisol or S-IgA levels, and augmented predisposition to oral infections have been proposed [7,10]. Increased mental stress is believed to modify salivary flow rate (SFR) and composition, affecting the oral microbiome and driving higher accumulation of dental biofilm [6,7,10]. Saliva and its biological components play a role in dental caries pathogenesis, and certain salivary parameters have been utilized as biomarkers for caries susceptibility, including the SFR, pH, levels of *Streptococcus mutans*, and S-IgA [11–15].

Dental caries is a highly prevalent disease associated with tooth decay [16]; however, while the worldwide prevalence of tooth loss has decreased over the past decades, the disability linked to caries has increased [17]. Prior reports have demonstrated higher levels of stress biomarkers, including salivary cortisol, in children with carious lesions, when compared to healthy controls [18,19].

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Therefore, it is clinically relevant to investigate whether stress-related disorders can affect the susceptibility to dental caries before its occurrence in systemically healthy individuals with good oral health.

Notably, alterations in salivary pH have been linked to the stimuli of an acute environmental stressor in males [20], whereas limited data have suggested a relationship between facial pain and reduced SFR [21]. Nevertheless, possible associations between facial pain and salivary biomarkers for caries susceptibility and mental stress have not yet been evaluated. Still, considering the need for reliable biomarkers for facial pain, several molecules have been tested as potential complementary diagnostic tools, but fluids and tissues utilized usually include either blood or muscle samples [22,23]. These are considered invasive when compared to saliva, thereby justifying the search for salivary biomarkers for facial pain.

Therefore, it was hypothesized that individuals with facial pain would be more suitable to changes in stress biomarkers accompanied by a modified oral environment. Hence, the purpose of this study is to investigate: (1) salivary biomarkers associated with the host susceptibility to dental caries in men and women with perceived facial pain; (2) salivary biomarkers linked to mental stress response in individuals with perceived facial pain; and (3) whether the parameters evaluated could be useful to detect the presence of facial pain.

Materials and methods

Participants

The study was reviewed and approved by the local Research Ethics Committee (process #633/11). Eighty individuals among undergraduate students attending a private university in Brazil were recruited. The sample was homogenous, as all volunteers were dental students, whose skin color was self-declared as white by the majority. Regarding the family income, all participants reported to receive at least twice the minimum wage. The participation in this study was voluntary, and the participants were, thereby, not monetarily compensated for their participation nor did they receive compensation of any nature, including academic benefits.

The present study excluded individuals with any endocrine, immunosuppressive, mental, musculoskeletal, neurological, or metabolic disease; those taking any type of medication/supplementation (including hormonal contraceptives) or under treatment for TMD; individuals with history of headache, dental or neuropathic pain in the previous 12 months; individuals who presented with extensive tooth loss, untreated caries or history of moderate/severe periodontitis; women who reported symptoms of premenstrual syndrome or those who were experiencing the menstrual phase of the menstrual cycle; pregnant women; and students undergoing university-related stressful events (e.g., exams, seminars). Each participant signed an informed consent.

Self-reported facial pain

The participants were evaluated in terms of the presence or absence of perceived facial pain by answering questions on Axis II of the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) [24]. This allowed the authors to determine each patient's score of the Graded Chronic Pain Scale (GCPS). GCPS scores ranged from 0 to 4, indicating the absence of facial pain symptomatology and highest severity, respectively.

Saliva collection

The participants of both groups then underwent saliva collection for evaluation of salivary microbial and non-microbial parameters. Unstimulated saliva was collected up to two hours after awakening using the split method, as previously described [25,26], in order to obtain a cortisol awakening pulse [27]. Samples that were contaminated with blood were excluded from this study [28]. For the immunoenzymatic tests, saliva samples were centrifuged (1,900 rpm, 10 min), the protease inhibitor phenylmethylsulfonyl fluoride (Sigma-Aldrich) at final concentration of 1 mM was added to the supernatant, and the samples were stored at −20ºC for later use. All experiments were conducted in a double-blind fashion, in which the person who collected data from questionnaires and saliva samples (C. M.A.) was different from those who performed the subsequent analysis of flow rate, pH, and *S. mutans* counting (J.F. F.O, L.V.G-M.).

Salivary flow and pH evaluations

The participants were instructed to allow the saliva to accumulate in the floor of the mouth and to expectorate it into graduated test tubes. Unstimulated SFR was determined by reading the total saliva obtained from the patient in 10 min, being the result expressed in milliliters of saliva produced per minute (mL/min) [26]. The salivary pH of the samples was determined by placing pH indicator test strips 0–14 (Merck, Kenilworth, NJ, USA) in contact with the saliva and comparing the color on the test strips with the manufacturer's color scale [25].

Culture of S. mutans

Saliva samples collected for this evaluation were homogenized and diluted (10^{-1} to 10^{-4}) in a sterile saline solution. Bacterial cultivation was conducted through the inoculation of 20 µL pure saliva in duplicate dilution cultures (Petri dishes with *Mitis Salivarius* Agar medium, containing 200 U/L bacitracin, 15% sucrose and 1% potassium tellurite). The material was read 48 hours after incubation (37° C, 10% CO_2) by counting the total colony-forming units per milliliter of saliva (CFU/mL).

Isolation, DNA extraction, and identification of S. mutans

Polymerase Chain Reaction (PCR) was performed as previously reported [25]. Briefly, the isolates were cultivated in 3 mL Brain Heart Infusion (37°C, 10% CO_2, 24 hours), followed by DNA extraction through boiling. PCR testing was conducted with a final volume of 25 µL that contained 12.5 µL PCR Master Mix-Promega, plus 10 picomoles of each primer (sense: 5′ACTACACTTTCGGGTGGCTTGG3′; antisense: 5′CAGTATAAGCGCCAGTTTCATC3′). The amplified product underwent electrophoresis in a 2% agarose gel, and subsequently, in Tris-borate-EDTA buffer. The gels were then dyed in an ethidium bromide solution (1:1000) and observed in an ultraviolet transilluminator.

Salivary levels of cortisol and s-IgA

Salivary concentrations of cortisol and S-IgA were determined with the Enzyme Linked Immunosorbent Assay (ELISA) using specific commercially available kits (Cortisol Parameter Kit, R&D Systems, Minneapolis, MN, USA; and Human IgA ELISA kit, Bethyl Laboratories, Montgomery, TX, USA). The immunoassays were conducted in duplicate.

Statistical analysis

The Shapiro-Wilk test was used to determine the normality of numerical variables, followed by the Student's *t*-test or by the non-parametric Mann-Whitney test. Spearman's correlation investigated correlations among salivary parameters. A backward conditional logistic regression was then conducted, using the presence of facial pain (GCPS > 0) as dependent variable. Multiple linear regressions were developed using cortisol level as dependent variable in the facial pain and in the control group. The level of significance considered for all analysis was 5%. Data were analyzed using the Statistical Package for Social Sciences – IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Out of the 80 volunteers initially evaluated, 7 were excluded from both the facial pain group (2 females and 1 male) and the control group (3 females and 1 male), due to insufficient amounts of saliva to conduct all experiments or due to contamination with blood. The final sample consisted of 73 age- and sex-matched individuals (46 females and 27 males). They were divided into two groups: facial pain (n = 40; mean age: 23.4 y), which included participants with perceived facial pain (GCPS scores ranging from 1 to 4); and controls (n = 33; mean age: 21.5 y), including those with no episode of facial pain in the past 6 months (GCPS 0).

As demonstrated in Table 1, the facial pain group had a significantly lower median of SFR ($p < .05$) when both males and females were considered. Even though male participants with facial pain presented with reduced SFR, it was not significantly different from controls ($p > .05$), while female participants with facial pain showed a lower median of SFR that reached statistical significance when compared to controls ($p < .05$). Similarly, an increased SFR was also associated with a lower likelihood of having facial pain (odds ratio [OR] = 0.05, 95% confidence interval [CI]: 0.005–0.54, $p < .05$; Table 2), and this association was maintained when gender was included as a co-variable in the model. Salivary pH and *S. mutans* counts were similar in both facial pain and the control group, independently of the gender ($p > .05$; Table 1).

Although both male and female individuals from the facial pain and the control group showed similar levels of cortisol and S-IgA ($p > .05$, Table 1), cortisol levels were discreetly associated with a lower likelihood of having facial pain (OR = 0.99, 95% CI: 0.97–0.99, $p < .05$; Table 2), independent of the gender. In participants with facial pain, none of the biomarkers for caries susceptibility predicted salivary cortisol levels ($p > .05$; data shown). In the control group, salivary pH and *S. mutans* counts predicted cortisol levels (R square: 0.46, $p < .05$; Figure 1). A negative correlation was found between SFR and cortisol (R = −0.367, $p < .05$), and a positive correlation between pH and cortisol was shown (R = 0.531, $p < .01$). Lastly, a negative correlation between SFR and S-IgA was observed in both facial pain (R = −0.421, $p < .01$) and the control group (R = −0.465, $p < .01$).

Table 1. Comparison of salivary biomarkers for caries susceptibility and mental stress among male and female participants from the facial pain and control groups.

Parameter	Group	n	Mean (SD)	Median	*p*-value	Male (n)	Mean (SD)	Median	*p*-value	Female (n)	Mean (SD)	Median	*p*-value
Salivary flow (mL/min)	Facial pain	40	0.49 (0.27)	0.5	0.02[a,*]	10	0.55 (0.34)	0.55	0.46[a]	23	0.43 (0.16)	0.40	0.01[a,*]
	Control	33	0.67 (0.40)	0.6		11	0.78 (0.59)	0.60		18	0.63 (0.27)	0.60	
Salivary pH	Facial pain	40	7.26 (0.87)	7.0	0.59[a]	10	7.75 (1.18)	7.75	0.36[b]	23	6.89 (0.60)	7.0	0.43[a]
	Control	33	7.17 (0.55)	7.5		11	7.36 (0.55)	7.50		18	7.02 (0.55)	7.0	
S. mutans (CFU/mL)	Facial pain	40	$6.2x10^4$ ($1.1x10^5$)	$1.4x10^4$	0.21[a]	10	$3.4x10^4$ ($5.3x10^4$)	$2x10^4$	0.75[a]	23	$6x10^4$ ($8.3x10^4$)	$1.2x10^4$	0.72[a]
	Control	33	$3.7x10^4$ ($5.5x10^4$)	$1.4x10^4$		11	$1.8x10^4$ ($1.8x10^4$)	$9x10^3$		18	$3.2x10^4$ ($5.1x10^4$)	$1.3x10^4$	
Cortisol (ng/mL)	Facial pain	40	844.71 (401.77)	798.80	0.13[b]	10	801.83 (415.65)	854.15	0.46[b]	23	863.36 (403.63)	798.80	0.15[b]
	Control	33	1008.28 (446.73)	953.20		11	946.93 (462.01)	847.20		18	1058.56 (454.59)	1109.90	
S-IgA (µg/mL)	Facial pain	40	21.35 (5.27)	22.30	0.29[a]	10	20.25 (7.81)	22.37	0.80[a]	23	21.31 (4.19)	22.11	0.58[b]
	Control	33	20.65 (3.72)	21.11		11	21.0 (4.61)	20.45		18	20.65 (3.26)	21.49	

n: number of subjects; CFU: colony forming units; SD: standard deviation; S-IgA: secretory immunoglobulin A; *$p < 0.05$, [a]according to Mann-Whitney test; [b]according to the Student's *t*-test.

Table 2. Binary logistic regression model showing predictors for the presence or absence of facial pain. The presence/absence of facial pain was used as the dependent variable.

Parameter[a]	OR (95% CI)	*p*-value	Parameter[b]	OR (95% CI)	*p*-value
Salivary flow	0.009 (0.00–0.28)	0.007*	Salivary flow	0.05 (0.005–0.54)	0.01*
Salivary pH	1.51 (0.62–3.65)	0.35	-	-	-
Cortisol	0.99 (0.98–0.99)	0.04*	Cortisol	0.99 (0.97–0.99)	0.03*
S-IgA	0.86 (0.71–1.04)	0.12	-	-	-

Variables included in the model: gender, salivary flow, salivary pH, *S. mutans*, cortisol and S-IgA;
[a]Variables excluded in the 4th step of regression: gender, *S. mutans*; [b]Variables excluded in the 6th step of regression: gender, Salivary pH, *S. mutans* and S-IgA; CI: confidence interval; GCPS: Graded Chronic Pain Scale; OR: odds ratio; S-IgA: secretory immunoglobulin A; *$p < 0.05$.

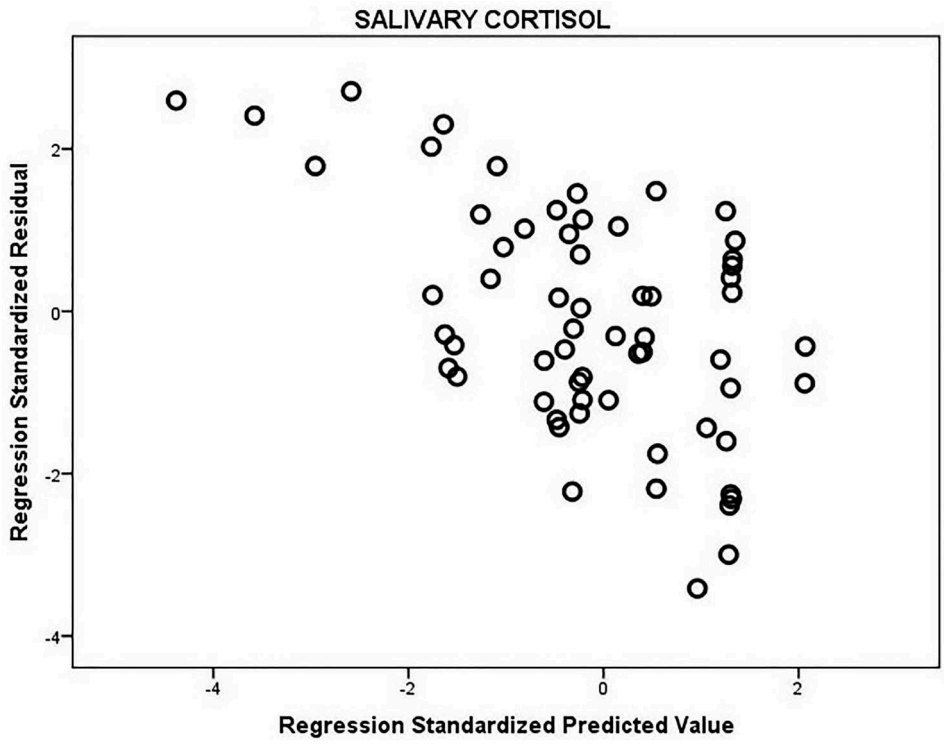

Figure 1. Multilevel backward multiple linear regression model testing candidate salivary biomarkers for cortisol levels in the control group. Dependent variable: salivary cortisol; predictors: salivary pH, $p < .001$; *S. mutans*, $p = .02$ (R square: 0.46).

Discussion

Saliva consists of a complex mixture of substances originated from microbial and host components, playing an important role in early detection of oral infections and reflecting the physiological state of the body [7,14,15]. Some distinctions in salivary biomarkers for caries susceptibility and cortisol levels in a group of young adults reporting facial pain were demonstrated in the current study. These results included lower SFR, more specifically in women, and a different pattern of correlations among these parameters in individuals with perceived facial pain. SFR and cortisol values also predicted the presence of facial pain. To the best of the authors' knowledge, this is the first time the association between facial pain, stress response, and salivary dynamics linked to caries susceptibility has been scrutinized.

Facial pain related to TMD has recently been clustered according to the characteristics of pain and comorbidity, being characterized by low experimental pain sensitivity and low mental distress, by higher pain sensitivity, or by higher pain sensitivity and mental distress [29]. Here, it was initially considered that stress might influence saliva flow and composition, which could induce a higher susceptibility to oral infections [7,15,20,30]. Because dental caries is the most prevalent oral disease, salivary biomarkers for caries-risk assessment were analyzed [17]. Therefore, one major hypothesis was that facial pain could affect the patients'salivary pattern related to caries risk. The salivary parameters analyzed in the current study are linked to the functional properties of saliva, mucosal immunity and microbial profile, and are considered to be biomarkers for caries susceptibility [15].

The lower SFR values observed in women with facial pain should be emphasized, as this can lead to higher caries susceptibility [12,14,15]. Reduced SFR has been related to high incidence of caries, due to the lubricating and antimicrobial action of saliva [14,16]. Notably, the current study group had previously demonstrated similar levels of SFR in a group of healthy individuals either male or female [25], suggesting that the occurrence of facial pain may play a role in salivary secretion in a gender-dependent manner, thereby leading to SFR alterations. SFR changes in patients with perceived facial pain may be explained, among other factors, by increased sympathetic activity in a stressed body, leading to a reduction in the secretory activity [5].

A significant reduction in SFR was previously reported in male and female individuals with orofacial pain; however, this study included a higher frequency of women in the group with orofacial pain and patients who were

taking several medications [21]. Thus, it could be implied that such greater SFR reduction could be linked to the drugs' side effects [30]. Importantly, considering that most participants with facial pain included in the present study showed mild/moderate GCPS scores and reduced flow rate without using any medication, the connection between facial pain and SFR then sounds reasonable.

In addition, even though SFR is one of the determinants of volume and composition of the dental biofilm, most parameters included here should be analyzed together towards assessing caries susceptibility [12–15]. The present analysis showed that pH was negatively correlated with cortisol levels in the controls, but not in patients with facial pain. In a recent study [20], salivary pH was increased in men exposed to mental stress when compared to controls. Some evidence suggests that hydrocortisone affects renal acid-based transporters, promoting changes in bicarbonate secretion [31]. Furthermore, increased cortisol levels were linked to decreased blood pH during intense exercise [32]. Nevertheless, physiological mechanisms for explaining the relationship between cortisol response and salivary pH regulation are yet to be understood.

The levels of *S. mutans* in saliva have been significantly correlated with its proportion in biofilm, thereby legitimating the salivary measure as an alternative for caries-risk assessment [15]. Shankland et al. [33] evaluated the oral microbiota of patients with craniofacial pain and found *Streptococcus* species to be the most common group of bacteria present in intra-bony cultures. Conversely, the current findings demonstrated similar *S. mutans* counts between individuals with and without facial pain, and *S. mutans* counts were found to predict cortisol levels only in the control group, suggesting that any microbial change might occur in a facial pain-independent manner.

In regard to the salivary stress biomarkers evaluated, salivary cortisol levels predicted the presence/absence of facial pain. Conversely, a recent report observed no significant difference in salivary cortisol levels between women with orofacial pain/distress and controls [34]. It is worth mentioning that individuals of both genders were recruited in the present study. Additionally, although prior studies have suggested S-IgA levels to be decreased in stressed individuals [6,7], no significant S-IgA reductions in individuals with perceived facial pain were shown in the present study. Likewise, no difference in S-IgA levels between distressed patients and distress-free ones had been previously reported [35]. Interestingly, the salivary level of S-IgA has been evaluated as a potential biomarker for chronic pain [36]. Moreover, correlations among SFR, pH, and cortisol were observed only in the control group. In linear regression analysis, pH and *S. mutans* predicted cortisol levels only in the control group, as well.

Limitations of the present study comprise the higher number of women recruited as compared to men, the exclusion of some participants due to low-quality samples of saliva, and the fact that females were not clustered according to each phase of the menstrual cycle. On the other hand, the strengths of this study include a comparison according to the gender and a combination of versatile salivary biomarkers for both caries susceptibility and mental stress. Overall, these findings raise the hypothesis that the oral environment of individuals with facial pain includes distinguished interactions among components of saliva; however, the reasons for such distinct patterns are unclear and deserve to be further explored.

Conclusion

In summary, women with facial pain presented with reduced SFR. Additionally, individuals with facial pain showed a different pattern of interactions between salivary biomarkers for caries susceptibility and cortisol; however, caries risk needs to be further evaluated in this population, as current findings are inconclusive. Notably, higher SFR values apparently indicate a lower likelihood of having facial pain. Hence, salivary flow should be investigated through longitudinal studies as a potential candidate biomarker for detecting facial pain.

Conflict of Interest

The authors declare no conflict of interest.

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