Abstract

Background: Salivary factors modulate the balanced dynamic mineralization process of the dental enamel. Salivary proteins such as statherin and acidic proline-rich proteins (PRPs) protect oral surfaces by regulating oral calcium homeostasis and remineralization of enamel. Thus, they possibly play vital roles in dental caries. Aim: The present study aims to find the association of salivary statherin, proline-rich protein, and calcium levels with dental caries. Methods: A descriptive, cross-sectional study was conducted among 188 healthy participants (age between 18 and 50 years), from dental clinic of MAHSA University, Malaysia. Dental caries was measured using standard WHO criteria. Stimulated whole mouth saliva was collected, and salivary statherin, acidic PRP (aPRP), and calcium levels were estimated using ELISA Kit and calorimetric assay kit, respectively. Data were analyzed using Spearman’s rho and Pearson’s correlation coefficient (SPSS statistical package-version 25.0) to find correlation of salivary statherin, calcium, and proline-rich protein levels with dental caries. Results: A statistically significant ($P < 0.001$) moderate negative correlation ($r = −0.500$) was found between salivary statherin and proline-rich protein levels. There was no statistically significant association of dental caries with salivary statherin, calcium, and aPRP levels. Conclusion: Salivary statherin and aPRP levels appear to perform mutually complementing functions and thus may have potential role in the maintenance of tooth integrity.

Keywords: Calcium, dental caries, proline-rich protein, statherin

Introduction

Dental caries is one of the most prevalent chronic disease as well as a severe oral health burden across the globe.[1-3] Reports have shown large individual differences in caries development despite similar exposures to sugars.[4] Cariogenicity may result from the interplay of the biofilm composition, diet, and the genetically determined host environment within each individual’s oral cavity.[5]

Dental enamel happens to be the toughest acellular tissue among the other mineralized human tissues such as the bones, cementum, and dentin. The enamel contains about 96% carbonated hydroxyapatite (HA) mineral that imparts exceptional hardness to the same.[6] In healthy condition, the enamel remains stable with a maintained dynamic equilibrium between demineralization and remineralization processes at the interfaces between the tooth and salivary pellicle as well as between saliva and plaque.[7,8]

Any disruption in the balanced dynamic mineralization process leads to increased susceptibility of the enamel to dental caries.[9] Saliva represents a dilute and viscous solution containing many electrolytes and proteins that participate in the regulation of the oral microbiota, prevention of enamel from dissolving, and other protections of the oral tissues. The salivary proteins play an important role in protecting oral surfaces and as precursors of acquired enamel pellicle (AEP).[10] Therefore, they can modulate and influence the enamel demineralization-remineralization process and dental caries formation.[10]

Statherin, an asymmetric 43-residue tyrosine-rich dephosphorylated acidic salivary peptide (MW 5380D), is secreted by the acinar cells of the parotid and submandibular salivary glands. Its concentration in human saliva is about 10–40 μM.[11] The key function mediated by the prime amino acid sequences is HA adsorption, thereby aiding in situ enamel remineralization processes at the interfaces and between saliva and plaque.

How to cite this article: Pateel DG, Gunjal S, Dutta S. Association of salivary statherin, calcium, and proline-rich proteins: A potential predictive marker of dental caries. Contemp Clin Dent 2022;13:84-9.
biomimetic remineralization.[12] Thus, statherin finds relevance in regulating the mineralization of enamel and needs proper research to find whether it can be considered as potent salivary marker for dental caries.

Among the innumerable salivary proteins, the acidic and basic proline-rich proteins (PRPs), with a high amount of proline residues, find particular significance in modulating dental caries.[5] The role of various PRPs in dental caries susceptibility relies on the properties of their subclasses.[13] The acidic PRPs can attach firmly to the dental surfaces, while the basic PRPs to the cell walls and cell membranes of oral streptococci, thereby protecting the dental enamel from the microbial adherence and neutralizing the acids produced by the microbes.[5,13]

There is a growing cluster of research works directed to find potential salivary markers of dental caries.[10,14] The presence or absence of the inherited salivary markers will be of great value for evaluating dental caries risk. It may help to identify the risk factors and aid dental screening along with personalized dental treatment. Thus, the present study aims to find the association of salivary statherin, PRPs, and calcium with dental caries. The following research hypothesis was tested: there is an association of dental caries with the salivary statherin, acidic PRP (aPRP), and calcium.

Methods

Ethical approval

The research protocol was reviewed and approved by Joint Research Review and Ethics Committee MAHSA University (RMC/AL03/2017). The study was conducted according to the guidelines of the Helsinki Declaration. Voluntary written informed consent was obtained by all the participants.

Study design and participants

An analytical cross-sectional study was conducted to correlate levels of the salivary PRP, statherin, and calcium levels in participants with varying levels of dental caries [Figure 1]. The present study comprised participants aged between 18 and 40 years having various scores of the decayed-missing-filled-Teeth (DMFT) index recruited from September 2017 to March 2018 from outpatients of Faculty of Dentistry, MAHSA University, Malaysia. Exclusion criteria included participants with known systemic diseases such as diabetes, under medication with B-adrenergic drugs, salivary gland pathologies, precancerous and cancerous conditions, pathologies of thyroid and parathyroid glands, altered Vitamin D metabolism, and hormonal disturbances affecting calcium metabolism, who does not practice brushing daily, and who has received fluoride treatment within 6 months of time.

Consecutive sampling technique was employed to recruit a total of 188 participants meeting the inclusion and exclusion criteria. An information sheet was given to all participants.

A brief history, oral examination, and demographic data were recorded.

Clinical examination

Dental caries was assessed using DMFT index using standard WHO criteria.[15] A single trained examiner performed caries examination and recorded the DMFT index, wherein the number of decayed teeth, missing and filled teeth because of caries are scored and added to get the DMFT value for each patient, followed by calculation of mean DMFT for all the study participants.

Reproducibility

Before the start of the study, the examiner underwent training by the assessment of clinical photographs and clinical examinations. Reproducibility was assessed on 5% of the total samples, and the time interval between the examinations was 1 week. The kappa value was 0.92.

Saliva collection

Stimulated whole mouth saliva was collected using saliva collection kit (Fitzgerald, USA) (includes saliva transfer tube and Safe Seal microtube 2 ml PP) and paraffin wax (Kemdent College Wax, UK) between 10:00 am and 1:00 pm. Saliva collection procedure was performed after making sure that the participants did not to eat or drink from the past 2 h. The participants rinsed their mouth with distilled water to remove food debris for 5 s, followed by chewing of paraffin wax to stimulate salivary flow. The stimulated whole saliva collected in the mouth was transferred into the saliva collection vials using saliva transfer tubes provided in the kit, and the procedure was repeated until 2 ml of saliva was collected in the vials. The saliva samples were then stored under −70°C until further analysis. Salivary samples were centrifuged at 1000 rpm for 20 min using centrifuge. Each sample was assessed for statherin, aPRP, and calcium concentration.

Quantification of salivary statherin, acidic proline rich protein, and calcium

Salivary statherin

Salivary statherin was estimated using ELISA Kit (Elabscience, USA). All the reagents were of analytical grade. Assays were conducted according to manufacturer’s protocol. Standard curve was prepared, standard statherin was centrifuged at 10,000 ×g for 1 min, serial dilution was performed to obtain standard solution of 1000, 500, 250, 125, 62.5, 31.25, and 15.63 ng/mL. Saliva sample (100 μl) was added to 96-well micro ELISA plate (dismountable). After incubation at 37°C, 90 min, the liquid was removed from each well, and 100 μl of biotinylated detection antibody was added immediately. The ELISA plate was sealed, mixed gently, and followed by 1 h incubation at 37°C. Then, the plate was rinsed with washing buffer for three times. Next, 100 μl horseradish peroxidase conjugate was added into the wells and mixed gently. After incubation for 30 min, the plate was rinsed with washing
buffer for five times. A volume of 90 μl of substrate reagent was added to each well, and the plate was sealed immediately and incubated for 15 min. To stop the reaction, 50 μl of stop solution was added to each well. The absorbance value of each well was measured at 450 nm.

**Salivary acidic proline-rich protein**

Salivary aPRP level was measured using ELISA Kit (Abbexa, United Kingdom). For standard, 100 μl of the diluted standard was pipetted into the standard wells, whereas for control, 100 μl of standard diluent buffer was aliquoted to the control well. In sample wells, 100 μl of centrifuged saliva was added, and the plate was shaken to mix thoroughly. After sealing with cover, the plate was incubated for 1 h at 37°C. After incubation, the liquid was discarded, and 100 μl of detection reagent A working solution was added into each well, and the plate was incubated for 1 h. Next, the plate was washed for three times with wash buffer after the liquid was discarded. The steps were repeated after adding detection reagent B working solution. Then, 90 μl of 3,3’,5,5’-tetramethylbenzidine substrate (TMB Substrate) was added into each well and incubated for 20 min at 37°C. The reaction was stopped by adding 50 μl of stop solution. The plate was then mixed gently, and the absorbance of aPRP was measured at 450 nm immediately.

**Salivary calcium**

Calcium level was assessed using calcium calorimetric assay kit (BioVision, Milpitas, CA, USA). A volume of 25 μl saliva was added into a 96-well plate and topped up with distilled water until 50 μl. For standards, controls, and samples, 90 μl of chromogenic reagent was added. The plate was mixed gently. Then, 60 μl of the calcium assay buffer was added to each well and mixed gently. The plate was then incubated for 10 min in the dark at room temperature. The absorbance of sample was measured at 575 nm, and the concentration of calcium was calculated using equation: \( C = \frac{Sa}{Sv} \) (µg/µl or mg/ml); Sa, calcium sample amount (in µg) from standard curve; and Sv, is the sample volume (µl) added into the sample well.

**Statistical analyses**

All assays were performed in triplicate and independently repeated three times. The data were then analyzed and tabulated using SPSS statistical package (version 25.0 SPSS Inc., Chicago, IL, USA). Descriptive statistics were performed to evaluate the distribution and normality of the data. Based on the Kolmogorov–Smirnov test, normality distribution assumption was checked for quantitative variables. Mean and standard deviation was reported for normal distributed data and median (interquartile range [IQR]) for skewed distributed data. Spearman’s and Pearson’s correlation coefficient was calculated to evaluate the association between salivary statherin, calcium, aPRP, and dental caries. Power analysis was performed with \( r = 0.5 \), the significant correlation between salivary statherin and aPRP. The total sample size of 188 in the present study could be considered adequate since power of study calculated was >80% at level of significance of 0.05.
Results

The present cross-sectional study comprised 188 participants, with mean age of 29 ± 9 years, having 87 (46.27%) males and 101 (53.73%) females. Majority of the participants 117 (62.24%) were in the age group of 18–25 years. The DMFT ranged from 0 to 24 with a median and IQR of 4 and 6, respectively. Majority of the participants 98 (52.12%) had DMFT ≥4, followed by 61 (32.44%) having DMFT “1–3” and 29 (15.44%) having “0” DMFT. The median and IQR of salivary statherin in the study participants was 33.35 (61.67) ng/ml. The mean salivary calcium concentration and aPRP were 2.86 ± 1.04 mg/ml and 91.52 ± 39.03 ng/ml, respectively [Table 1].

Spearman’s rho test showed moderate negative correlation between salivary statherin and salivary PRP levels with statistically significant difference (r = −0.500, P = 0.001). However, there was no statistically significant correlation of salivary statherin (r = −0.006, P = 0.930), calcium (r = 0.015, P = 0.841), and aPRP (r = 0.025, P = 0.733) with dental caries [Table 2].

Discussion

The etiopathology of dental caries is orchestrated by three major sets of factors, namely, the host factors, oral microorganisms, and dietary substrates. When their interactions cause an imbalance in tooth mineralization process, demineralization of enamel overwhelms the remineralization events, leading to dental caries formation. AEP, a noncellular glycoprotein film on tooth surface, is formed through physical bonds between salivary proteins and surface substrates. This pellicle shields the tooth enamel against virulence factors of the oral microbes and influences the mineralization process by binding to calcium and phosphates.

The dental enamel is continually exposed to saliva, which has multivariate components to modulate mineralization processes. Thus, an array of salivary proteins plays important role to regulate the pathogenesis of dental caries. The present study explores the possible role of two of the most important salivary proteins, namely, statherin and aPRPs, in maintenance of tooth integrity. The salivary aPRP levels in the present study showed no statistically significant correlation with dental caries (r = 0.025; P = 0.733). However, a recent study revealed that aPRP levels were significantly increased in severe caries group when compared to minimum caries group.

The aPRPs accounts for 20%–30% of all human salivary proteins. After secretion, the aPRPs rapidly adhere on to the tooth surfaces, get degraded into potential innate-immunity peptides by dental plaque proteolysis, and also may exhibit buffering capacity, antibacterial property, and lubrication. These peptides have been shown to desorb bound bacteria and decrease dental plaque pH in vitro. Moreover, another PRP variant, with a 21-amino-acid internal tandem repeat, is associated with altered saliva adhesion of bacteria and caries susceptibility. The authors believe that the association between aPRP and dental caries could be statistically significant if the study limitations concerning a wider age range and convenience sampling were nullified. This probable increase in salivary aPRP level may be suggested to be a protective response against enamel disruption [Figure 2].

On the contrary, salivary statherin showed a moderate negative correlation with aPRP levels (r = −0.500, P = 0.001). Moreover, statherin levels in saliva showed poor correlation of salivary statherin (r = −0.500) with dental caries [Table 2].

| Variables | Participants (%) | Mean±SD |
|-----------|------------------|---------|
| Age (years) |                   |         |
| 18-25     | 117 (62.24)      | 28.67±9.3 |
| 26-35     | 22 (11.70)       |         |
| 36-35     | 25 (13.29)       |         |
| ≥4        | 98 (52.12)       |         |
| Gender    |                   |         |
| Males     | 87 (46.27)       |         |
| Females   | 101 (53.73)      |         |
| DMFT      |                   |         |
| 0         | 29 (15.44)       | 4 (6)   |
| 1-3       | 61 (32.44)       |         |
| ≥4        | 98 (52.12)       |         |
| Salivary statherin (ng/ml) | 188 (100) | 33.35 (61.67) |
| Salivary aPRP (ng/ml) | 188 (100) | 2.86±1.04 |
| Salivary calcium (mg/ml) | 188 (100) | 91.52±39.03 |

*Median (IQR). DMFT: Decayed Missing and Filled Teeth; SD: Standard deviation; aPRP: Acidic proline-rich proteins; IQR: Interquartile range.

Table 2: Distribution of demographic and clinical (Decayed, Missing, and Filled Teeth, salivary acidic proline-rich proteins, calcium, and statherin levels) characteristics

| Variables | Participants (%) | Mean±SD |
|-----------|------------------|---------|
| DMFT      |                  |         |
| r         | 1.000            | −0.006+ | 0.015+ | 0.025+ |
| P         | 0.930            | 0.841   | 0.733  |
| Statherin*|                  |         |
| r         | 1.000            | −0.104+ | −0.500+ |
| P         | 0.154            | <0.001+ |
| Calcium*  |                  |         |
| r         | 1.000            | −0.037+ |         |
| P         | 0.611            |         |
| aPRP*     |                  |         |
| r         | 1.000            |         |         |

*a/ng/ml; *mg/ml; * Spearman’s rho; * Karl pearson’s correlation; *Statistically significant. DMFT: Decayed Missing and Filled Teeth; aPRP: Acidic proline-rich proteins.
declining trend with increase in levels of dental caries, while the decline is not statistically significant \((r = -0.006, P = 0.930)\). However, the observation that the salivary statherin levels tend to decrease with increase in dental caries levels may be explained by the fact that statherin is essential for enamel remineralization, thereby its decline may lead to increase in the levels of dental caries. The salivary statherin proteins are potential precursors of the AEP because it has a strong affinity to HAP.\(^{[23]}\) These proteins also maintain supersaturation of saliva with calcium phosphate salts which enhances enamel remineralization.\(^{[24]}\) Statherin thereby plays a vital role in inhibiting caries progression [Figure 2]. The reduction in statherin level thereby leads to inadequate remineralization of enamel, thereby increasing the risk of dental caries.\(^{[10]}\) Thus, the result of the present study showing a slight decline in salivary statherin level with increase in levels of dental caries, though not statistically significant, may support the crucial role of statherin in maintaining tooth integrity, and further studies are encouraged to find whether salivary statherin levels can serve as an indicator of dental caries formation. The statistical analysis was not performed based on DMFT severity (caries free, low caries, and high caries) as there were unequal participants in different DMFT groups as shown in Table 1.

There have been some potential studies supporting the hypothesis of the present study. The study by Vitorino et al. has reported a strong correlation between large amounts of phosphoproteins, including statherin and histatin 1, and the absence of dental caries.\(^{[25]}\) Xiao et al. have also studied the functional domains of statherin and similar peptides in the acquired enamel and suggested the presence of a covalently linked phosphate group (at residues 2 and 3) in statherin peptides that modulates the effect of HA growth inhibition.\(^{[26]}\) Wang et al. have reported that DE-11, a peptide derived from salivary statherin, has the potential to be a highly promising as a restorative biomaterial for enamel remineralization in the anticaries applications.\(^{[27,28]}\) Shimotoyodome et al. through their experiments found that statherin and histatin 1 could inhibit Streptococcus mutans adhesion onto HA surfaces.\(^{[29]}\) Our earlier reports on the role of salivary proteins in dental caries had also put forth the importance of these salivary phosphoproteins in the maintenance of tooth integrity and indicate that the development of biologically stable statherin peptide could have therapeutic role against dental caries and/or periodontal disease.\(^{[24]}\)

Enamel mineralization status immensely depends on the level of calcium and phosphate. The present study showed no statistically significant correlation between salivary calcium levels and dental caries \((r = -0.015, P = 0.841)\). Salivary calcium precipitation may serve as niches for accumulation of more dental plaque, leading to the formation of dental caries. The decrease in calcium levels results in a reduction of enamel crystallinity, increasing one’s retentive surface and decreasing overall resistance. A study conducted to compare the salivary calcium levels between 23 caries affected versus 32 caries-free individuals and found that calcium levels in caries-free group were far higher than caries group.\(^{[30]}\) Our observation of weak negative correlation between salivary statherin and calcium levels \((r = -0.104, P = 0.154)\) may support the concept that statherin inhibits the precipitation of calcium phosphate salts from saliva [Figure 2].

The present study had some limitations. (1) The selection of participants was not equally distributed among different DMFT groups and could not be compared with salivary proteins. (2) Wider age range could have affected the saliva composition and in turn salivary proteins and calcium levels. (3) The role of other salivary proteins, salivary flow, and pH were not assessed as they might have played a role in dental caries.

The present study has demonstrated an inverse relationship between salivary statherin and aPRP levels. It can be assumed that the increase in the salivary aPRP level is the result of increased caries activity as a protective response against enamel disruption, together with hint of reduced salivary statherin levels which may be responsible for inhibition in remineralization of enamel. The additional functions of statherin and increased proteolytic activity

---

**Figure 2:** Conceptual diagram of regulation of mineralization and remineralization of dental enamel by salivary statherin, aPRP, and calcium salts. AEP: Acquired enamel pellicle; aPRP: Acidic proline rich peptide; Ca\(^{2+}\): Calcium ions; PO\(_4\)\(^{3-}\): Phosphate ions
in the oral cavity may also have influenced the salivary statherin levels in the present study. Along with these factors, other key factors that can influence the salivary protein concentrations such as the salivary flow rates, the total protein content, and narrow age range should be considered in the future studies. However, the conclusions obtained through this study provide a platform for further investigations in this direction to reach to a consensus on the suggested probable association among the salivary statherin, aPRP, calcium, and dental caries.

**Conclusion**

The study explores the possible roles played by the salivary proteins like statherin acidic proline-rich proteins (aPRP) in dental caries formation. The present study reveals an inverse relationship between salivary statherin and aPRP levels and may suggest having mutually complementary functions.

**Acknowledgments**

We would like to thank our undergraduate dental students Lam Jiunn Liang, Rosie AS Gubat, Eliie Tan Swin Yee, and Lim Ka Ye, for helping in the data collection.

**Financial support and sponsorship**

The research was funded by MAHSA University research grant (RP105-10/16).

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Hujoel PP, Hujoel ML, Kotsakis GA. Personal oral hygiene and dental caries: A systematic review of randomised controlled trials. Gerodontology 2018;35:282-9.
2. Ramamurthy P, Rath A, Sidhu P, Fernades B, Nettem S, Mitalib K, et al. Sealants for preventing dental caries in primary teeth. Cochrane Database Syst Rev 2018;2018:CD012981.
3. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet 2007;369:51-9.
4. Jonasson A, Eriksson C, Jenkinson HF, Källestål C, Johansson I, Strömberg N. Innate immunity glycoprotein gp‑340 variants may modulate human susceptibility to dental caries. BMC Infect Dis 2007;7:57.
5. Levine M. Susceptibility to dental caries and the salivary proline‑rich proteins. J Proteomics 2016;134:47‑56.
6. Ekström J, Khosravani N, Castagnola M, Messana I, Saliva and the control of its secretion. In: Dysphagia. Springer, 2017. p. 21‑57.
7. Li T, Pratt P, Jonsson AP, Ryberg M, Johansson I, Griffiths WJ, et al. Possible release of an ArgGlyArgProGln pentapeptide with innate immunity properties from acidic proline‑rich proteins by proteolytic activity in commensal streptococcus and actinomyces species. Infect Immun 2000;68:5425‑9.
8. Bennick A. Salivary proline‑rich proteins. Mol Cell Biochem 1982;45:83‑99.
9. Manconi B, Castagnola M, Cabras T, Olianas A, Vitali A, Desiderio C, et al. The intriguing heterogeneity of human salivary proline-rich proteins: Short title: Salivary proline-rich protein species. J Proteomics 2016;134:47‑56.
10. Pateel DG, Gunjal S, Math SY, Murugeshappa DG, Nair SM. Statherin‑derived peptide promotes hydroxyapatite crystallization and in situ remineralization of artificial enamel caries. RSC Adv 2018;8:1647‑55.