Relationship between gingival crevicular trappin-2 and gingipain levels in periodontal health and disease - An observational report

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Abstract

Background: Trappin-2 is a potent serine protease inhibitor produced locally by the epithelial and immune cells. It counters proteases like gingipain. This interaction in the crevicular environment is still unexplored. Thus, this novel study aims to investigate the gingival crevicular fluid (GCF) levels of gingipain and trappin-2 in periodontal health and disease.

Methods: GCF was collected from 60 systemically healthy non-smoking individuals. Periodontal parameters were recorded, and they were grouped as periodontally healthy group (n=20), gingivitis group (n=20), and periodontitis stage II grade A group (n=20). Gingipain activity was assessed by ultraviolet spectrophotometer and trappin-2 was analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: The GCF trappin-2 level is significantly higher in periodontal health, in comparison to gingivitis and periodontitis group ($P<0.01$). Conversely, the gingipain level is low in health and higher in the periodontitis group ($P<0.01$). Correlation analysis shows an inverse relationship between crevicular gingipain and trappin-2 levels in periodontitis individuals ($r = - 0.49$; $P<0.027$).

Conclusion: GCF Trappin-2 levels negatively correlates with gingipain in patients with periodontitis stage II grade A. The interplay between these could also help us to differentiate between gingival health, gingivitis, and early periodontitis.

Keywords: Trappin-2; Anti-proteases; Gingipain; Gingival Crevicular Fluid; Periodontitis; Inflammation.

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and is linked to several systemic diseases (Papapanou *et al*., 2018). The progression from periodontal health to disease involves complex interactions between the host and bacteria (Giannobile WV., 2008). As pro-inflammatory enzymes increase in the gingival crevicular fluid (GCF) the epithelial cells secrete protease inhibitors like elafin, trappin-2, and leukocyte protease inhibitors that protect the gingival tissue from excessive damage (Kretschmar S, *et al*., 2012). The epithelial expression of protease inhibitors is modulated by various oral bacteria (Laugisch O *et al*., 2012; Sallenave JM, 2010). Studies relate protease inhibitor levels, mainly elafin to various forms of periodontal disease (Yin L and Dale BA, 2007; Kantyka T *et al*., 2009). The results of elafin levels are quite contradictory, as some show increase in the aggressive form of periodontitis (Yin L and Dale BA, 2007), others show it to be decreased (Kantyka T *et al*., 2009). Trappin-2 is a precursor of elafin and is structurally related to secretory leukocyte protease inhibitor (Bingle CD and Vyakarnam A 2008). It possesses antibacterial properties (Laugisch O *et al*., 2012; Sallenave JM, 2010) and is mainly secreted by the epithelial cells (i.e. keratinocytes, endometrial cells, bronchial cells, and the epithelial cells of large intestine), alveolar macrophages, and gamma delta T cells (Verrier T *et al*., 2012).

There is enough evidence suggesting the contribution of proteolytic enzymes (gingipain) along with an array of other virulence factors such as

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lipopolysaccharides, capsular material, and fimbriae by red-complex microorganisms to pathogenicity of periodontal disease (Kantyka T *et al*., 2009; Kretschmar S *et al*., 2012; Drannik AG *et al*., 2011; Hamedi M *et al*., 2009). Collectively gingipain can impair neutrophil function, manipulate the complement pathway, interfere with coagulation and kinin cascades, cleave immunoglobulins, inactivate endogenous protease inhibitors, as well as degrade the extracellular matrix proteins and bioactive peptides (Zhang R *et al*., 2016; Kantyka T *et al*., 2009; Verrier T *et al*., 2012). Consequently it can be stated that gingipain play an important role in modulating the balance between host proteases and their inhibitors at the infection site (Imamura T *et al*., 2009). We hypothesize that trappin-2 level down regulation by gingipain is based on the severity of the periodontal disease. Therefore in this novel study, we aim to investigate the GCF levels of gingipain and trappin-2 in periodontally healthy, generalized gingivitis and periodontitis stage II grade A individuals.

Materials and methods

Source of data and ethics

Sixty participants who fulfilled the inclusion criteria were enrolled in the study, which was conducted from March – December 2019 at the Department of Periodontology; (Bangalore Institute of Dental Sciences and Post Graduate Research Center) Approval for the study was obtained from the ethics committee of the institute (No. 161117). A written informed consent was obtained from all the participants as per Helsinki's guidelines (2013) after the purpose, treatment procedures, and recall intervals were explained in detail. Medical and dental history was compiled and an oral examination was carried out by an experienced examiner (SRK). Inclusion criteria included systemically healthy non-smoking participants of both genders between 30-60 years of age. Exclusion criteria included systemic diseases such as diabetes mellitus, hypertension, coronary artery diseases, and pregnant or lactating women. None of the participants had received any form of periodontal therapy or gave a history of antibiotic or anti-inflammatory drug usage in the past 6 months. The number of cigarettes per day was recorded and only non-smokers (without history of cigarette smoking) were recruited for the study.

Clinical examination and grouping

Full mouth clinical examination was carried out by a single trained periodontist (SRK). Measurement of probing depth (PD), clinical attachment loss (CAL), gingival index (GI) (Loe H, 1967), plaque index (PI) (Turesky S *et al*., 1970), gingival bleeding index (GBI) (Ernest N, 1996) and periodontal disease index (PDI) (Sigurd P.Ramfjord, 1967) at six sites per tooth except third molars was done. A manual periodontal probe † was used to record clinical parameters. Orthopantomograph was taken for evaluating the radiographic bone loss. Participants were classified into three groups based on their periodontal status according to the criteria proposed by the 2017 World Workshop on the classification of Periodontal and Peri-implant diseases (Caton J *et al*., 2018). Periodontally healthy group (Group 1) included volunteers with an intact periodontium who had bleeding on probing (BOP) <10% and PD ≤ 3mm without CAL or radiographic signs of alveolar bone loss. The gingivitis group (Group 2) included volunteers with no probing attachment loss, PD ≤ 3mm, and BOP ≥ 30% with or without radiographic crestal bone loss. Periodontitis stage II (Group 3) included volunteers with inter-dental CAL (at site of greatest loss) 3-4mm, PD ≤ 5mm, and radiographic bone loss extending to the coronal third of the root (15-33%) and mostly horizontal. Since the patients included in the group 3 were non smokers and non diabetic the grade modifier was suggestive of grade A. The extent of the disease for group 2 and group 3 was generalized.

Collection of GCF samples

GCF samples were collected for all 60 individuals a day after the clinical parameters were recorded to avoid blood associated with the probing of inflamed sites. Sampling site selection was made by the same experienced examiner who did the clinical recordings (SRK) and the second examiner (DP) collected the samples. Two to three non-adjacent sites per individual were selected as sampling sites in the gingivitis group. Samples were taken from the mesiobuccal and distobuccal sites, which demonstrated a higher clinical sign of inflammation (GI scores of 2 and 3) whereas for the periodontitis group two sites from different quadrants (deepest periodontal site/pocket X quadrant) were chosen. Multiple sites (four to six sites) with an absence of clinical inflammation were selected for sampling in the periodontally healthy group to ensure the collection of an adequate amount of GCF. On the following day, the individuals were instructed to rinse their mouth and the area which was chosen for sample collection was air-dried gently, supra gingival plaque was removed without irritating the marginal gingiva using a manual universal scaler. Calibrated microcapillary pipette ǂ was placed at the entrance of the sulcus gently touching the gingival margin. A standardized volume of 2µL of crevicular fluid was collected. Correction for different volumes of GCF obtained from healthy sites if necessary was done using molecular-grade water or de-ionized water. The samples contaminated with blood or saliva was discarded. The collected GCF sample was immediately transferred into plastic vials and stored at – 80°C until further analysis. (Egelberg J and Attstrom R., 1973).

Analysis of trappin-2

The levels of trappin-2 in GCF were measured by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) using commercial kit § with the sensitivity of 0.10 pico-grams per millimeter (pg/mL). Standard dilutions of the collected GCF samples were done according to the manufacturer's instructions. The antigen-antibody reaction in the well plates was stopped using 50µl of stop solution in each well and the intensity of the color change from blue to yellow was measured at 450 nm. Results are presented as pg/mL of protein.

Analysis of gingipain activity

The activity of gingipain in the GCF was determined using chromogenic substrate N Benzoyl-L-Arginine-p-Nitroanilide (BAPNA). ‖ 10 µl of GCF samples were pre-incubated in 200 mM (milli mole) Tris hydrochloride (HCL),100 mM sodium chloride (NaCl), 5mM calcium chloride ($CaCl_2$,) pH 7.6, supplemented with 10 mM cysteine, for 5 minutes at 37°C and assayed for amidase activity with 0.5 mM substrate in the total volume of 200 µL. The ability to cleave the substrate was determined by measuring the p-nitroaniline levels indicated by the change in absorbance at 405 nm using an Ultraviolet (UV) spectrophotometer ¶ at 405 nm at intervals of 0 min and after 120 minutes. The difference between the initial absorbance and final absorbance (Optical density) was calculated to determine the activity of gingipain. (Eley BM., Cox SW, 1996) The rate of product development was computed by dividing the change in absorbance over time. Results are presented as nano mol per minute (nmol/min) of protein.

Statistical Analysis

Sample size estimation was done using G Power v. 3.1.9.2. # considering the effective size to be measured (f) at 40%, power of the study at 85% and the margin of the error at 10% the effective sample size was calculated to be around sixty individuals. To test the normality of the data Shapiro-Wilk test was applied and the non-parametric test was suggested. All the recorded clinical and biochemical parameters were statistically analyzed using SPSS software version 20 ** The median and Interquartile range were obtained for all the clinical and biochemical parameters and compared among the groups using the Kruskal Wallis test followed by post hoc Mann Whitney. Correlations between the biochemical and clinical parameters were done using Spearman's correlation statistics. The level of significance (P-Value) was set at $P<0.05$.

Results

Demographic data and clinical parameters among the three groups are summarized in Table 1. The mean age of the subjects included in Group 1, Group 2 and Group 3 was 36.50 ± 4.29, 37.05 ± 3.8 and 48.60 ± 5.8 respectively. The mean percentage of female and male subject in group 1 was 70% and 30%, Group 2 was 50% and 50% and in Group 3 was 35% and 65% respectively. The periodontitis group showed significantly higher PD, PI, GI, and CAL compared to gingivitis and healthy group. Intergroup comparison of clinical parameters (PD, CAL, GI, GBI, and PDI) using Kruskal-Wallis showed significant differences among the groups ($P < 0.01$).

Table 1. Demographics and full mouth parameters among study groups.

Patient characteristics	Periodontally healthy (N=20)	Gingivitis (N=20)	Periodontitis (N=20)	Kruskal wallis	P value
Age (years)	36.5	37.05	48	-	0.01*
Gender (F/M)	14/6	10/10	7/13	-	0.08
PD(mm)	1.55(0.43)	1.85(0.24)	4.1(0.85)	44.98	0.01*
CAL(mm)	0	0	4.17(1.6)		0.01*
PI	0.65(0.61)	2(0.29)	2.83 (0.42)	50.82	0.05*
GI	0.79(0.13)	1.99(0.42)	2.5(0.56)	47.69	0.05*
GBI	7.14(2.14)	70.08(16.96)	82.14(5.36)	51.93	0.01*
PDI	0.13(0.06)	0.72(0.33)	3.41(1.31)	52.50	0.05*

All data are given in terms of median (Inter quartile range); * statistically significant
PD-Probing depth; CAL- Clinical attachment loss; PI-plaque index; GI-gingival index; GBI- gingival bleeding index; PDI- periodontal disease index; F-Female; M- Male.

† University of North Carolina probe. Hu-Friedy, Chicago, IL.USA

‡ Micro pipettes,Sigma Aldrich,MO,USA.

§ RayBiotech, Inc, LA, USA

‖ BAPNA SIGMA, MO, USA

¶ TecanSPECTRA Flour Plus, Artisan technology group, IL, USA

Heinrich-Heine-Unversitat Dusseldorf, Germany.

** IBM® SPSS® Statistics, IBM Corporation, NY, USA.

Biochemical findings

GCF trappin-2 was detected in all samples. Median (Inter-quartile range) from ELISA readings were 6.1 (1.68) pg/mL in the healthy group; 3.79 (1.03) pg/mL in the gingivitis group; and 1.49 (0.68) pg/mL in the periodontitis group. The trappin-2 levels were significantly lower in the periodontitis group when compared to the healthy and gingivitis group (P< 0.01, Table 2). Inter-group post hoc analysis also revealed significant differences between the groups (P< 0.01; Table 3). GCF gingipain were detected in all samples. Median (Interquartile range) concentrations were 0.018 (0.01) nmol/min in the healthy group 0.048(0.02) nmol/min in the gingivitis group, 0.15 (0.06) nmol/min in the periodontitis group. The gingipain levels were significantly higher in the periodontitis group when compared to healthy and gingivitis group (P< 0.01, Table 2). Inter-group post hoc analysis also revealed significant differences between the groups (P< 0.01, Table 3).

Correlation analysis between trappin-2, gingipain, and clinical parameters

Spearman's correlation between clinical parameters (PI, GI, PDI and GBI) and GCF trappin-2 for group 1 showed weak negative insignificant results, while a weak positive correlation was found between trappin-2 and PD and CAL. In group 2 there was a weak to moderate negative correlation (PI, GI, PDI and CAL) with significance only with GI (P=0.037) and PDI (P=0.033). A weak to moderate positive insignificant correlation was found between trappin-2 and PD and GBI. In group 3 there was a weak moderate negative correlation between trappin-2 and PD, PDI, PI and CAL. The correlation was significant only for PD (P=0.008) and CAL (P=0.039) (Table 4a).

Spearman correlation analysis between levels of GCF Gingipain and clinical parameters in group 1 showed a moderate positive correlation with PI, GI, PDI and GBI but was insignificant. A moderate negative correlation was found for PD and CAL which was again insignificant. In group 2 there was a weak negative correlation to PI and GBI which was insignificant. A weak to moderate positive correlation was found between gingipains and PD, CAL (P=0.005) and GI. In group 3 there was a weak positive correlation with PI, PDI, PD and CAL, a very weak negative correlation between GI and GBI (Table 4b).

Correlation analysis between trappin-2 and gingipain

The Spearman's correlation between Trappin-2 and GCF-Gingipain are presented in (Figure 1). A weak negative correlation was observed for Trappin-2 and GCF-Gingipain in healthy and gingivitis groups but was not statistically significant. In the periodontitis group, a moderate negative correlation was observed (r= -0.49) and was statistically significant (P=0.027).

Table 2. Comparison of GCF trappin-2 and gingipain among groups using Kruskal-Wallis.

GCF Biomarkers	Periodontally healthy(n=20)	Gingivitis	Periodontitis (N=20)	Kruskal wallis	P value
Trappin-2 (pg/mL)	6.1 (1.68)	3.79 (1.03)	1.49 (0.68)	51.31	0.01*
Gingipain (nmol/min)	0.018 (0.01)	0.048(0.02)	0.15 (0.06)	47.38	0.01*

All data are given in terms of median (Inter quartile range); * statistically significant
pg/ml- picograms/milliliter; nmol/min- nanomol/ minute; GCF-gingival crevicular fluid.

Table 3. Post- hoc analysis using Mann Whitney.

Variables	Group 1 v/s Group 2		Group 1 v/s Group 3		Group 2 v/s Group 3	
	Mean diff	P value	Mean diff	P value	Mean diff	P value
PI	-1.47	0.01*	-2.21	0.01*	-0.733	0.01*
GI	-1.07	0.01*	-1.69	0.01*	-0.62	0.01*
PDI	-0.51	0.01*	-3.08	0.01*	-2.57	0.01*
PD	-0.29	0.01*	-2.78	0.01*	-2.49	0.01*
GBI	-63.22	0.01*	-74.88	0.01*	-11.65	0.01*
CAL	-0.12	0.15	-3.08	0.01*	-2.95	0.01*
TRAPPIN- 2	2.30	0.01*	4.65	0.01*	2.35	0.01*
GINGIPAIN	-0.029	0.01*	-0.123	0.01*	-0.094	0.01*

* statistically significant
PD-Probing depth; CAL- Clinical attachment loss; PI-plaque index; GI-gingival index; GBI- gingival bleeding index; PDI- periodontal disease index; Group1- healthy; Group2-Gingivitis; Group 3- Periodontitis

Table 4a. Spearman's correlation of clinical parameters with trappin-2 levels.

Clinical Parameters	Healthy		Gingivitis		Periodontitis	
	r value	P value	r value	P value	r value	P value
PI	-0.44	0.052	-0.23	0.31	-0.21	0.36
GI	-0.15	0.51	-0.46	0.037*	0.10	0.64
PDI	-0.38	0.09	-0.47	0.033*	-0.20	0.39
PD	0.25	0.28	0.054	0.82	-0.57	0.008*
GBI	-0.20	0.38	0.13	0.58	-0.093	0.69
CAL	0.36	0.11	-0.21	0.37	-0.46	0.039*

* statistically significant; PD-Probing depth; CAL- Clinical attachment loss; PI-plaque index; GI-gingival index; GBI- gingival bleeding index; PDI- periodontal disease index.

Table 4b. Spearman's correlation of clinical parameters with gingipain levels.

Clinical Parameters	Healthy		Gingivitis		Periodontitis	
	r value	P value	r value	P value	r value	P value
PI	0.15	0.51	-0.17	0.46	0.25	0.28
GI	0.14	0.53	0.15	0.52	-0.05	0.81
PDI	0.11	0.64	0.28	0.22	0.017	0.4
PD	-0.40	0.07	0.06	0.77	0.27	0.24
GBI	0.22	0.33	-0.07	0.76	-0.27	0.24
CAL	-0.43	0.05	0.59	0.005*	0.15	0.52

* statistically significant; PD-Probing depth; CAL- Clinical attachment loss; PI-plaque index; GI-gingival index; GBI- gingival bleeding index; PDI- periodontal disease index.

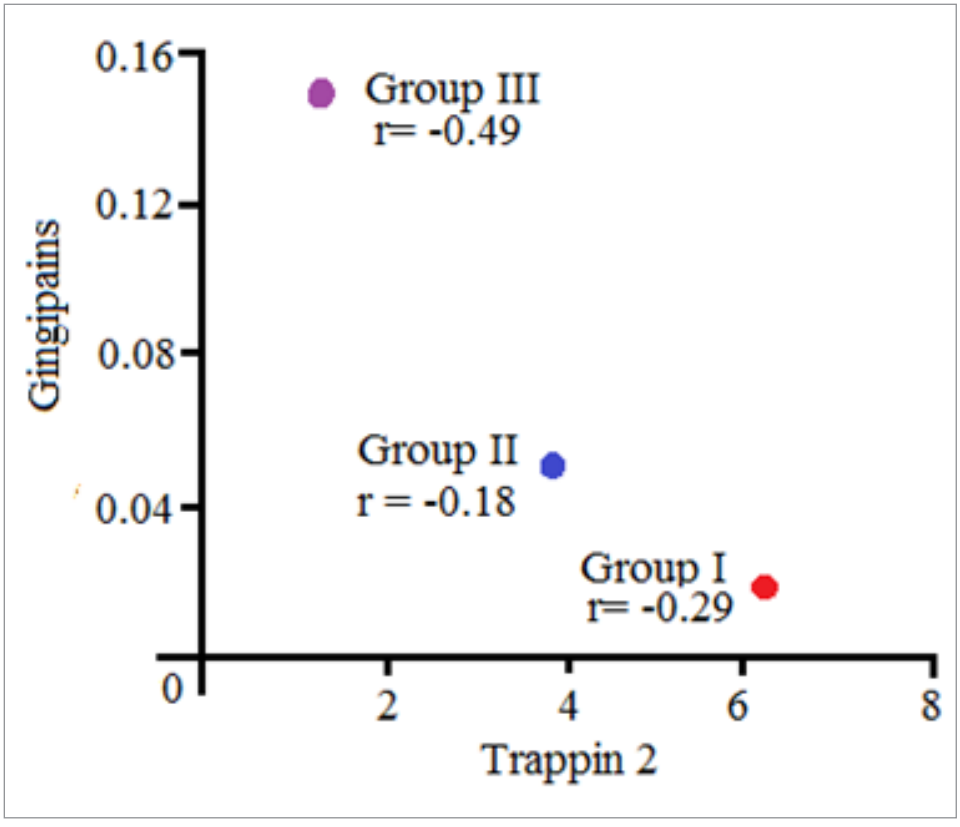

Figure 1. Correlation between trappin-2 and gingipain. Group I - Periodontally healthy; Group II- Gingivitis group; Group III- Periodontitis group.

Discussion

The results of this novel observational study, shows that GCF level of trappin-2 negatively correlates with the severity of periodontal disease, while gingipain show a positive correlation. It is of interest to note that the reduction of GCF trappin-2 levels from health to disease follows a unique predictive pattern and correlates strongly with increasing PD and CAL. We postulate that the thin line of difference between healthy and gingivitis to early stages of periodontitis is better predicted by measuring the GCF trappin-2 levels rather than going clinically. There is no literature evaluating GCF trappin-2 levels in periodontitis to allow comparison. However, the conclusion drawn is consistent with previous research which shows a negative correlation between salivary levels of trappin-2 to various systemic inflammatory diseases (Kretschmar S *et al*., 2012; Baranger K *et al*., 2008) and periodontitis (Afacan B *et al*., 2018) . Of late, innovative methods for gingipain inhibition in the periodontal pockets using vectors of bacterial, viral origin, plant-based, and also synthetic derivatives are being widely researched (Snipas SJ *et al*., 2001; Taiyoji M *et al*., 2013; Ingar Oslen and Jan Potempa, 2014). Therefore estimating levels of gingipain and trappin-2 will not only help us to understand the host-microbial interaction but also be of use in developing newer therapeutic strategies.

The microbial species of the red complex and orange complex predominate with increasing pocket depth (Papapanou PN *et al*., 2018; Socransky SS *et al*., 1998). In these host-microbial interactions, the dominance of P gingivalis in degrading protease inhibitors is of importance (Nakayama M and Ohara, 2017; Eley BM and Cox SW, 2003). Likewise, our results also show negligible gingipain levels in health and increased gingipain levels in the diseased. Correlation statistics in our study show a positive correlation between gingipain and periodontitis. This along with the inverse relation between gingipain and trappin-2 levels across the three groups in our study emphasizes the role of gingipain in the disease process; starting from adherence, colonization, nutrient acquisition, neutralization of host defenses, and manipulation of an inflammatory response. These may eventually lead to invasion, tissue destruction, and systemic dissemination (Jan P *et al*., 2003).

Trappin 2, also known as serine protease inhibitor belongs to the chelonianin family and is a precursor for elafin (Zani M-L *et al*., 2009). The previous study relates decreased salivary trappin-2 levels to periodontitis (Afacan B *et al*., 2018). Our study demonstrates trappin-2 levels in the GCF to be higher in the periodontally healthy group (6. 23 pg/ml) compared to gingivitis (3.92 pg/ml) and periodontitis groups (1.57 pg/ml). The influence of trappin-2 expression on the course of inflammatory diseases and periodontitis still remains inconclusive. Possible explanations for the reduction in levels could be the direct degradation of trappin-2 by bacteria proteases like gingipain or indirect degradation by pro-inflammatory cytokines like interleukin-1β and neutrophils elastases. The pleiotropic effects of trappin-2 play a pivotal role in the regulation of immune response (Drannik AG *et al*., 2012). Therapeutic agents are developed to target serine protease inhibitors in the medical field (Gibbons A *et al*., 2010; Korkmaz B *et al*., 2011). On the similar lines we feel the need to develop potential anti-proteinase molecules which help to restore the proteinase-antiproteinase balance, thereby limiting the exaggerated inflammation in diseased periodontal sites.

Correlating the gingipain and trappin-2 levels in our study confirms this interaction between bacterial enzymes and host proteinase inhibitors. This inverse relation is mainly because gingipain present as extracellular bacterial vesicles cleave elaffin and trappin-2. The degraded product obtained alters the peptide reserve pool and serves as nutrients for various other asaccharolytic pathogens, thereby changing the ecology from health to disease (Zhou J *et al*., 2017; Afacan B *et al*., 2018; Guyot N *et al*., 2005). However, we acknowledge that there may be additional factors other than gingipain, such as cathepsins and neutrophils elastase which may be involved to decrease the levels of protease inhibitors in gingival crevicular fluid (Takii R *et al*., 2005). However gingipain have shown to inhibit all of the above-said factors as well. Furthermore, gingipain may facilitate the bacterial invasion into the host tissues and activate the latent host tissue collagenases (Matrix Metalloproteinase 1 and Matrix Metalloproteinase -8), thus promoting host tissue enzyme-mediated tissue destruction, and thereby completely inactivating the proteins important in host defense (Bellemare A *et al*., 2010; Bartnicka D *et al*., 2019). Thus we would like to state that the site-specific interactions between gingipain derived from periodontopathic red-complex bacteria and trappin-2, can be more read into by the crevicular fluid analysis rather than saliva or serum and hold the key for further understanding of this complex host-microbiome interactions.

Absence of bacterial culture to prove these results could be an important limitation of this study. However additional interventional study involving a larger population across different age groups along with bacterial culture is needed to confirm these important findings of the present study.

Conclusion

The GCF gingipain and trappin-2 levels show a significant inverse relationship in periodontitis stage II grade A patients. Future researches using gingipain inhibitors and increasing levels of trappin-2 by various new

formulations could be of assistance for understanding the host bacterial interactions as well as rendering personalized periodontal care.

References

Afacan B, Ozturk VO, Emingil G, Kose T, Bostanci N. Alarm anti-protease trappin-2 negatively correlates with proinflammatory cytokines in patients with periodontitis. *J Periodontol* 2018;**89**:58-66.

Baranger K, Zani M-L, Chandenier J, Dallet-Choisy S, Moreau T. The antibacterial and antifungal properties of trappin-2 (pre-elafin) do not depend on its protease inhibitory function. *FEBS J*. 2008;**275**:2008-20.

Bartnicka D, Karkowska-Kuleta J, Zawrotniak M, Satala D, Michalik K, Zielinska G et al. Adhesive protein-mediated cross-talk between Candida albicans and Porphyromonas gingivalis in dual species biofilm protects the anaerobic bacterium in unfavorable oxic environment. *Sci Rep*. 2019;**9**:4376.

Bellemare A, Vernoux N, Morin S, Gagné SM, Bourbonnais Y. Structural and antimicrobial properties of human pre-elafin/trappin-2 and derived peptides against Pseudomonas aeruginosa. *BMC Microbiol*. 2010;**10**:253.

Bingle CD, Vyakarnam A. Novel innate immune functions of the whey acidic protein family. *Trends Immunol*. 2008;**29**: 444-453.

Caton J, Armitage G, Berglundh T, et al. A new classification scheme for periodontal and peri implant diseases and conditions— Introduction and key changes from the 1999 classification. *J Clin Periodontol* 2018;**45**(Suppl 20):S1–S8.

Drannik AG, Henrick BM, Rosenthal KL. War and peace between WAP and HIV: role of SLPI, trappin-2, elafin and ps20 in susceptibility to HIV infection. *Biochem Soc Trans*. 2011; **39**:1427-1432.

Drannik AG, Nag K, Yao XD, Henrick BM, Sallenave JM, Rosenthal KL. Trappin-2/elafin modulate innate immune responses of human endometrial epithelial cells to PolyI:C. *PLoS One*. 2012;7(4):24.

Egleberg J, Attstrom. Comprison between orifice and intracrevicular methods of sampling gingival fluid. *J Periodontal Res*.1973;**8**:384-388.

Eley BM, Cox SW. Correlation between gingivain/gingipain and bacterial dipeptidyl peptidase activity in gingival crevicular fluid and periodontal attachement loss in chronic periodontitis patients.A 2-year longitudunal study. *J Periodontol* 1996; **67**(7) 703-16.

Eley BM, Cox SW.Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. *Periodontol 2000*. 2003;**31**:105-24.

Ernest N. Indices to measure gingival bleeding. *J Periodontol* 1996; **67**:555-561.

Giannobile WV.Host-response therapeutics for periodontal diseases. *J Periodontol*. 2008;**79**:1592-1600.

Gibbons A, McElvaney NG, Cryan SA. A dry powder formulation of liposome-encapsulated recombinant secretory leukocyte protease inhibitor (rSLPI) for inhalation: preparation and characterization. *AAPS PharmSciTech* 2010; **11**: 1411–1421.

Guyot N, Zani ML, Maurel MC, Dallet-Choisy S, Moreau T. Elafin and its precursor trappin-2 still inhibit neutrophil serine proteinases when they are covalently bound to extracellular matrix proteins by tissue transglutaminase. *Biochemistry* 2005;**44**(47):15610-18.

Hamedi M, Belibasakis GN, Cruchley AT, Rangarajan M, Curtis MA, Bostanci N. Porphyromonas gingivalis culture supernatants differentially regulate interleukin-1beta and interleukin-18 in human monocytic cells. *Cytokine*. 2009; **45**:99-104.

Imamura T, Pike RN, Potempa J, Travis J. Pathogenesis of periodontitis: a major arginine-specific cysteine proteinase from Porphyromonas gingivalis induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. *J.Clin. Invest* 2009; **94**:361–367.

Ingar Oslen and Jan Potempa. Strategies for the inhibition of gingipain for the potential treatment of periodontitis and associated systemic diseases. *Journal of oral Microbiology* 2014; *6*:24800.

Jan P, Aneta S, Takahisa I, James T. Gingipain, the major cysteine proteinases and virulence factors of Porphyromonas gingivalis: structure, function and assembly of multidomain protein complexes. *Curr Protein Pept Sci*. 2003;**4**:397-407.

Kantyka T, Latendorf T, Wiedow O, et al. Elafin is specifically inactivated by RgpB from Porphyromonas gingivalis by distinct proteolytic cleavage. *Biol Chem*. 2009; **390**:1313-1320.

Korkmaz B, Horwitz MS, Jenne DE,Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol. Rev* 2011; **62**:726–759.

Kretschmar S, Yin L, Roberts F, et al.Protease inhibitor levels in periodontal health and disease. *J Periodontal Res* 2012;**47**(2):228-35.

Laugisch O, Schacht M, Guentsch A, et al.Periodontal pathogens affect the level of protease inhibitors in gingival crevicular fluid. *Mol Oral Microbiol*. 2012;**27**(1):45-56.

Loe H. The gingival index, the plaque index and the retention index systems. J Periodontol 1967; 38:610-6.

Nakayama M, Ohara N. Molecular mechanisms of Porphyromonas gingivalis-host cell interaction on periodontal diseases. *Jap Dent Sci Rev*. 2017;**53**:134-40.

Papapanou PN, Sanz M, Buduneli N et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and conditions. *J Clin Periodontol* 2018; **45**: 162-170.

Sallenave JM. Secretory leukocyte protease inhibitor and elafin/trappin-2: versatile mucosal antimicrobials and regulators of immunity. *Am J Respir Cell Mol Biol*. 2010; **42**:635-643.

Sigurd P.Ramfjord. Periodontal disease index *J Periodontol* 1967; **38** (Part II) 602-610.

Snipas SJ, Stennicke HR, Riedl S, Potempa J, Travis J.Inhibition of distant caspase homologues by natural caspase inhibitors. *Biochem J* 2001; **357**: 575-80.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent Jr RL. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998;**25**(2):134-44.

Taiyoji M, Yamanaka T, Tsuno T, Ohtsubo S. Potential value of a rice protein extract containing proteinaceous inhibitors against cysteine proteinases from Porphyromonas gingivalis, for managing periodontal diseases. *Biosci Biotechnol Biochem* 2013; 77: 80-6.

Takii R, Kadowaki T, Baba A, Tsukuba T, Yamamoto K. A functional virulence complex composed of gingipain, adhesins, and lipopolysaccharide shows high affinity to host cells and matrix proteins and escapes recognition by host immune systems. *InfectImmun*. 2005;**73**(2):883-93.

Turesky S, Gilmore ND and Glickman I. Reduced plaque formation by the chloromethyl analogue of victamine C. *J Periodontol* 1970; **41**:41-43.

Verrier T, Solhonne B, Sallenave JM, Garcia-Verdugo I. The WAP protein trappin-2/Elafin: a handyman in the regulation of inflammatory and immune responses. *Int J Biochem Cell Biol*. 2012;**44**(8):1377-80.

Yin L, Dale BA. Activation of protective responses in oral epithelial cells by Fusobacterium nucleatum and human beta-defensin-2. *J Med Microbiol*. 2007;**56**:976-987.

Zani M-L, Baranger K, Guyot N, Dallet-Choisy S, Moreau T. Protease inhibitors derived from elafin and SLPI and engineered to have enhanced specificity towards neutrophil serine proteases. *Protein Sci* 2009;**18**(3):579-94.

Zhang R, Yang J, Wu J, Sun WB, Liu Y. Effect of deletion of the rgpA gene on selected virulence of porphyromonas gingivalis. *J Dent Sci*. 2016;**11**(3):279-86.

Zhou J, Yao Y, Jiao K, et al. Relationship between gingival crevicular fluid microbiota and cytokine profile in periodontal host homeostasis. *Front Microbiol*. 2017;**8**:2144.