Gingival Crevicular Fluid Levels of Human Beta-defensin 2 and 3 in Healthy and Diseased Sites of Individuals with and without Periodontitis

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Abstract

Objectives: Human beta-defensins (hBDs) play an important role in the susceptibility to periodontitis. This study aimed to evaluate the levels of hBD-2 and hBD-3 in gingival crevicular fluid (GCF) of individuals with and without periodontitis.

Methods: Twenty periodontally healthy individuals (H) and 20 with periodontitis (P) were recruited. GCF samples were collected from healthy sites (Hh; n=20) from H individuals; and from healthy sites (Ph; n=20), sites with gingival inflammation but no attachment loss (Pg; n=20) and sites with inflammation and attachment loss (Pp; n=20) from P individuals. Levels of hBDs (pg/mL) were determined using ELISA. Comparisons between individuals and among sites were performed through hierarchical linear modelling.

Results: GCF levels [median(Q3–Q1)] of hBD-2 were: Hh=4.80 (0.00–30.69); Ph=33.29 (28.04–38.25); Pg=27.56 (23.14–35.50); and Pp=26.20 (23.20–42.54); and of hBD-3 were: Hh=0.00 (0.00–0.00); Ph=978.44 (760.48–1268.12); Pg=938.19 (806.75–1266.38); and Pp=613.63 (325.50–854.68). Periodontitis at the individual level was associated with higher levels of hBD-2 ($p=0.023$) and hBD-3 ($p<0.001$). No influence of site phenotype was observed on hBDs levels.

Conclusion: Individuals with periodontitis presented higher levels of hBD-2 and hBD-3 in the GCF. These levels seemed to be influenced by periodontitis at the individual level but not by periodontal site.

Keywords: Beta-Defensins, Elisa (Enzyme-Linked Immunosorbent Assay), Gingival Crevicular Fluid, Periodontitis.

Introduction

Periodontitis is a complex inflammatory disease resulting from the interaction between the dental biofilm and host immune responses. It affects the teeth supporting tissues leading to attachment loss and bone loss (Meyle and Chapple, 2015).

Human beta-defensins (hBDs), components of the innate immune response, may play a key role in the susceptibility to periodontitis (Joly *et al.*, 2005). hBDs are a class of peptides expressed by a variety of epithelial cells (Gursoy and Könönen 2012). They can also be found in saliva and gingival crevicular fluid (GCF) (Mathews *et al.* 1999, Dommisch and Jepsen 2015). hBDs are active against pathogenic microorganisms, being able to

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destroy gram-positive and -negative bacteria (Maisetta *et al*. 2005, Lee *et al*. 2013), yeasts (Feng *et al*. 2005), and viruses (Quiñones-Mateu *et al*. 2003). Moreover, they have a potential immunomodulatory effect, acting on cell signalling and on the communication between innate and adaptative immune responses (McCormick and Weinberg 2010, Gursoy and Könönen 2012).

hBDs may cause the maturation and activation of antigen-presenting cells, such as dendritic cells and monocytes (Biragyn *et al*. 2002, Funderburg *et al*. 2007), stimulate the migration of mast cells and act in the recruitment of macrophages (Soruri *et al*. 2007), induce mast cell degranulation, prostaglandin D2 production, histamine release and intracellular mobilization of Ca^{+2} (Niyonsaba *et al*. 2001), recruit T cells (Yang et al. 1999), inhibit tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Bian *et al*. 2016, Cui *et al*. 2017) and suppress nuclear factor-kappa B (NF-kB) signalling pathways (Cui *et al*. 2017, Park *et al*. 2017).

In the oral cavity, hBD-1, hBD-2, hBD-3 and hBD-4 have a basal level of expression, even in clinically healthy tissues. However, their levels can be altered by infectious and immunoinflammatory conditions (Vankeerberghen *et al*. 2005, Li *et al*. 2016). Recent studies have shown variations in tissues and GCF levels of hBDs in periodontitis (Bissel *et al*. 2004, Brancatisano *et al*. 2011, Yong *et al*. 2015, Costa *et al*. 2018). It is not clear whether these levels are increased or decreased and their role in the periodontal health-disease process is not yet fully understood (Dommisch *et al*. 2015). Nevertheless, it has been suggested that the sequential and differential expression of antimicrobial peptides during the development of periodontal inflammation play a central role as guardians of a healthy periodontium (Dommisch *et al*. 2019).

In a previous study (Costa *et al*. 2018), our research group demonstrated a reduced level of hBD-1 in individuals with periodontitis. Nevertheless, no differences were observed when healthy sites, sites with gingival inflammation but no attachment loss and sites with inflammation and attachment loss were compared within these individuals. Although some studies have compared the expression of hBD-2 and hBD-3 among individuals with and without periodontitis (Mathews *et al*. 1999, Dunsche *et al*. 2002, Bissell *et al*. 2004, Dommisch *et al*. 2005, Hosokawa *et al*. 2006, Vardar-Sengul *et al*. 2007, Brancatisano *et al*. 2011, Pereira *et al*. 2012, Pereira *et al*. 2013, Liu *et al*. 2014, Li *et al*.2016, Zorina *et al*. 2016), multilevel approaches considering sites and individuals are scarce (Lu *et al*. 2004).

Therefore, the aim of the present study was to evaluate and compare the levels of hBD-2 and hBD-3 in the GCF of healthy and diseased sites from individuals with and without periodontitis.

Materials and Methods

Study design and sampling strategy

This cross-sectional study comprised participants from a previous study (Costa *et al*. 2018), totalling 40 individuals selected from the Periodontology Clinic at the Federal University of Minas Gerais. Sample size was calculated using statistical software (GPower 3.1 Statistical Power Analysis for Windows, Düsseldorf, Germany) considering mean values from previous studies (Dommisch *et al*. 2005, Brancatisano *et al*. 2011, Ertugrul *et al*. 2013), an effect size of 0.30, a significance level of 0.05 and a study power of 0.80. Based on these parameters, a sample size of ~20 individuals per group was determined to be necessary.

Sampling strategy was as previously described (Costa *et al*. 2018). Briefly, during the period of data collection (August 2012 to December 2014), approximately 360 individuals sought dental care in the university, were determined to be eligible and invited to participate in the study. According to their availability and accessibility during the dental care routine, individuals who met the inclusion criteria were added to the study groups until the required sample size was reached. Individuals were excluded if they were pregnant or lactating women, smokers and former smokers or if they have any systemic diseases. Exclusion was also applied to individuals using drugs that could influence periodontal health, having received periodontal treatment within the last 6 months or having used antibiotics and anti- inflammatory drugs within the last 3 months.

Periodontal clinical examination

All participants underwent a full-mouth periodontal examination and the parameters of probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were recorded at 4 sites (buccal, distal, lingual, mesial) for all teeth present except any third molars. Intraoral radiographs were taken to detect bone loss (Costa *et al*. 2018). One single trained examiner performed all examinations with a manual periodontal probe (PCPUNC 15 Hu-Friedy®, Chicago, USA). Evaluations of PD and CAL parameters were performed and repeated within a 1-week interval for 10 individuals randomly selected during the recruitment of participants, in order to determine intra-examiner agreement. Analyses retrieved kappa values for PD and CAL greater than 90% and intra- class correlation coefficient greater than 89%. Those individuals were not included in the study.

Participants were divided into 2 groups (n = 20): periodontally healthy individuals (H) and individuals with periodontitis (P). Periodontally healthy individuals (H) should have a minimum of 20 natural teeth, PD ≤3 mm and no more than 20% of sites with BOP. It

is important to stress that CGF samples were collected in these individuals from sites with no BOP. Individuals with periodontitis should have a minimum of 15 natural teeth and at least 4 teeth with 1 or more sites with PD ≥4 mm and CAL ≥3 mm in the same site. The diagnosis of periodontitis was based on clinical and radiographic parameters as previously established (American Academy of Periodontology Task Force Report on the Update to the 1999 Classification of Periodontal Diseases and Conditions, 2015). Diagnosis and definition of periodontitis were updated according to the new classification scheme for periodontal diseases and conditions (Tonetti *et al.* 2018) and participants' periodontal condition was confirmed.

Gingival crevicular fluid sampling

GCF sampling was carried out 1 week after the periodontal examination among all participants. GCF fluid samples were obtained from healthy sites (Hh) of each periodontally healthy individual and from healthy sites, sites with gingival inflammation but no attachment loss (Pg) and sites with gingival inflammation and attachment loss (Pp) of each individual with periodontitis. For each sample category, 4 representative sites were selected in each participant and analyzed together (pool samples) to provide a mean value per category/individual (Hh [n = 20], Ph [n = 20], Pg [n = 20], Pp [n = 20]). A total of 320 sites were sampled (80 in H individuals and 240 in P individuals).

Healthy sites (Hh and Ph) for GCF collection showed PD and CAL ≤3 mm without BOP and without alveolar bone loss. In P individuals, sites with gingival inflammation but no attachment loss (Pg) for GCF collection showed PD and CAL ≤3 mm and BOP; sites with gingival inflammation and attachment loss (Pp) showed PD ≥4 mm, CAL ≥3 mm and BOP. The 4 Pp sites were selected according to the deepest PD at different non-adjacent teeth, preferably 1 site in each quadrant. Clinical parameters of sampling Pp sites were: mean PD 6.01±2.03 mm and mean CAL 6.40±2.31 mm). The 4 Hh and Ph sites (healthy sites), as well as the 4 Pg sites (sites with gingival inflammation but no attachment loss), were also collected at different non-adjacent teeth, preferably 1 site in each quadrant (Costa *et al.* 2018).

Before GCF sampling, all selected sites were isolated with cotton rolls to avoid contamination with saliva, supragingival plaque was removed and the tooth was gently air-dried. Paper strips (Periopaper, PerioCol Collection Strip, Oraflow, Plainview, NY, USA) were inserted into the gingival sulcus/pocket until mild resistance was felt and left in position for 30 seconds. Care was taken to avoid mechanical injury. Paper strips contaminated with blood were discarded (Griffiths 2003, Wassall and Preshaw 2016). The total GCF volume collected by each strip was measured with a calibrated device (Periotron 6000, IDE Interstate, Amityville, NY, USA).

Representative samples of the same site category (Hh, Ph, Pg, Pp) in the individual were placed into a dry Eppendorf tube (4 paper strips per tube) and frozen at −80°C until laboratory analysis.

Quantification of hBD-2 and hBD-3

GCF levels of hBD-2 and hBD-3 (pg/ml) were determined using the Enzyme-linked Immunosorbent Assay (ELISA) sandwich technique according to manufacturers' instructions (ELISA kits catalog #900-K172 and #900-K210, Peprotech, Rocky Hill, NJ, USA). Before the quantification of hBDs, the absorbed fluid was eluted from the paper strips by adding 600 µl (150 µl per strip) of phosphate- buffered saline (PBS; 10 mmol/L, pH 7.2) to each Eppendorf tube and shaking the tubes on an ELISA plate shaker for 30 minutes. Subsequently, the tubes were centrifuged at 13 000 g for 10 minutes at 4°C.

For the analysis, 96 wells were coated with 100 µl (0.25 mg/ml) of anti-hBD specific antibody for 12 hours. After 4 washes with PBS with 0.05% Tween 20 (PBST), wells were blocked for 1 hour at room temperature with 300 µl of blocking solution (1% bovine serum albumin in PBST). The plates were washed and 100 µl of sample or standard solution was added to the wells in duplicate; following this, the plates were incubated for 2 hours. After further washing, 100 µl of the detection antibody (0.5 µg/ml) was added to the wells and the plates were incubated for another 2 hours. After this period, the plates were washed and 100 µl of streptavidin-peroxidase (1:2000 in PBST) was added to the wells. Plates were incubated at room temperature for 30 minutes. Colorimetric reactions were produced with the use of o- phenylenediamine in the presence of 0.02% H_2O_2. After blocking the reaction with H_2SO_4 (2N), the absorbance was measured at 450 nm using an ELISA reader. The minimum and maximum detectable limits of hBD-2 were 16 - 2000 pg/ml and of hBD-3 was 63 - 4000 pg/mL. The levels of hBD-2 and hBD-3 in each sample were determined using the concentration values of standards included in the kit contents. The results were expressed in pg/ml.

Ethical considerations

The present study was approved by the Research Ethics Committee of the Federal University of Minas Gerais – COEP/UFMG (CAAE #0529.0.203.0001-11). All participants were informed of the aims of the study and they signed an informed consent form before inclusion in the study. Legal rights of the participants were preserved at all times.

Statistical analysis

Data normality was assessed using the Kolmogorov-Smirnov test. Descriptive analyses were presented for periodontal parameters and levels of hBDs. Descriptive and bivariate analyses, as well as the creation of 2 databases using site and individual values of hBD-2 and hBD-3, were performed using statistical software (Statistical Package for Social Sciences, IBM SPSS Statistics for Macintosh version 20, IBM Corp., Armonk, NY, USA). These databases were then used to perform the multilevel analyses (Hierarchical Linear and Nonlinear Modeling, HLM 6.06 Statistical Package, Skokie, IL, USA). The results were considered significant if $p < 0.05$.

For each defensin, data were hierarchically structured in 2 levels: periodontal sites (Level 1: healthy sites/sites with gingival inflammation but no attachment loss/sites with gingival inflammation and attachment loss) nested within individuals (Level 2: individuals with or without periodontitis). The multilevel structure of analyses included 80 mean site values (Level 1) from 40 individual values (Level 2). Multilevel linear modelling was used to assess the differences in hDB-2 and hBD-3 expression. During the first stage, a "null model" estimated the basic partition of data variability between the 2 levels before the inclusion of site and individual characteristics were taken into account. Level 1 variables were incorporated into the model. In the sequence, Level 2 variables were incorporated, with the calculation of p-values (Student's t test). Only variables with $p < 0.05$ were retained in the final model. The intraclass correlation coefficient, i.e., the proportion of total variance attributed to the cluster level (individual characteristics), was also calculated.

Results

The group of periodontally healthy individuals (H) was composed of 7 women and 13 men, mean age 39.6 ± 16.7 years (range 18 – 79). The group of subjects with periodontitis (P) was composed of 10 women and 10 men, mean age 44.4 ± 10.2 years (range 25 – 68). Full-mouth periodontal parameters of the study groups are shown in Table 1. Individuals with periodontitis presented a higher mean PD and CAL when compared to periodontally healthy individuals.

GCF levels of hBD-2 were [mean ± s.d.; median (Q3 – Q1)]: Hh = 18.03 ± 28.11 (4.80; 0.00 – 30.69); Ph = 33.43 ± 6.53 (33.29; 28.04 – 38.25); Pg = 29.60 ± 8.68 (27.56; 23.14 – 35.50); and Pp = 35.10 ± 20.04 (26.20; 23.20 – 42.54) (Figure 1). GCF levels of hBD-3 were [mean ± s.d.; median (Q3 – Q1)]: Hh = 41.12 ± 120.71 (0.00; 0.00 – 0.00); Ph = 1121.74 ± 565.87 (978.44; 760.48 – 1268.12); Pg = 1424.45 ± 1309.38 (938.19; 806.75 – 1266.38); and Pp = 852.76 ± 1121.98 (613.63; 325.50 – 854.68) (Figure 2).

Table 1. Periodontal status of study groups.

Periodontal clinical parameters	Study groups		p*
	Individuals without periodontitis (H) (n = 20)	Individuals with periodontitis (P) (n = 20)	
Mean PD (mm)	1.7 ± 0.2	4.2 ± 2.6	< 0.001
% of sites with PD			
4 mm	NA	5.7 ± 4.6	NA
5 – 6 mm	NA	7.6 ± 8.1	NA
≥7 mm	NA	2.1 ± 3.4	NA
Mean CAL (mm)	2.0 ± 0.5	3.1 ± 0.7	< 0.001
% of sites with CAL			
4 mm	NA	8.2 ± 4.6	NA
5 – 6 mm	NA	12.4 ± 11.1	NA
≥7 mm	NA	4.4 ± 6.1	NA
% of sites with BOP	NA	30.8 ± 20.7	NA

*Mann-Whitney test; PD = probing depth;
CAL = clinical attachment level;
BOP = bleeding on probing; NA = not applicable.

It is important to note that 17 hBD-3 samples (17/20) and 6 hBD-2 samples (6/20) in the control group were under the detection limit for the ELISA test. These samples represented 14,38% of the total samples. They were imputed a value of 0 (zero) for statistical analyses purposes.

In the hierarchical modelling, the analysis of the null model showed a significant variance in the values of hBD-2 ($p < 0.01$) and a marginally significant variance in the values of hBD-3 ($p = 0.096$) by the individual level (Level 2 groupings), indicating a need for running HLM analyses (Table 2).

Results from the multilevel model showed that periodontitis was associated with higher levels of hBD-2 (coefficient 14.68; $p = 0.023$) and hBD-3 (coefficient 1091.86; $p < 0.001$). There were no effects of site phenotype on the GCF levels of hBD-2 ($p = 0.266$ for Pg and $p = 0.625$ for Pp) (Table 3) and hBD-3 ($p = 0.302$ for Pg and $p = 0.359$ for Pp) (Table 4).

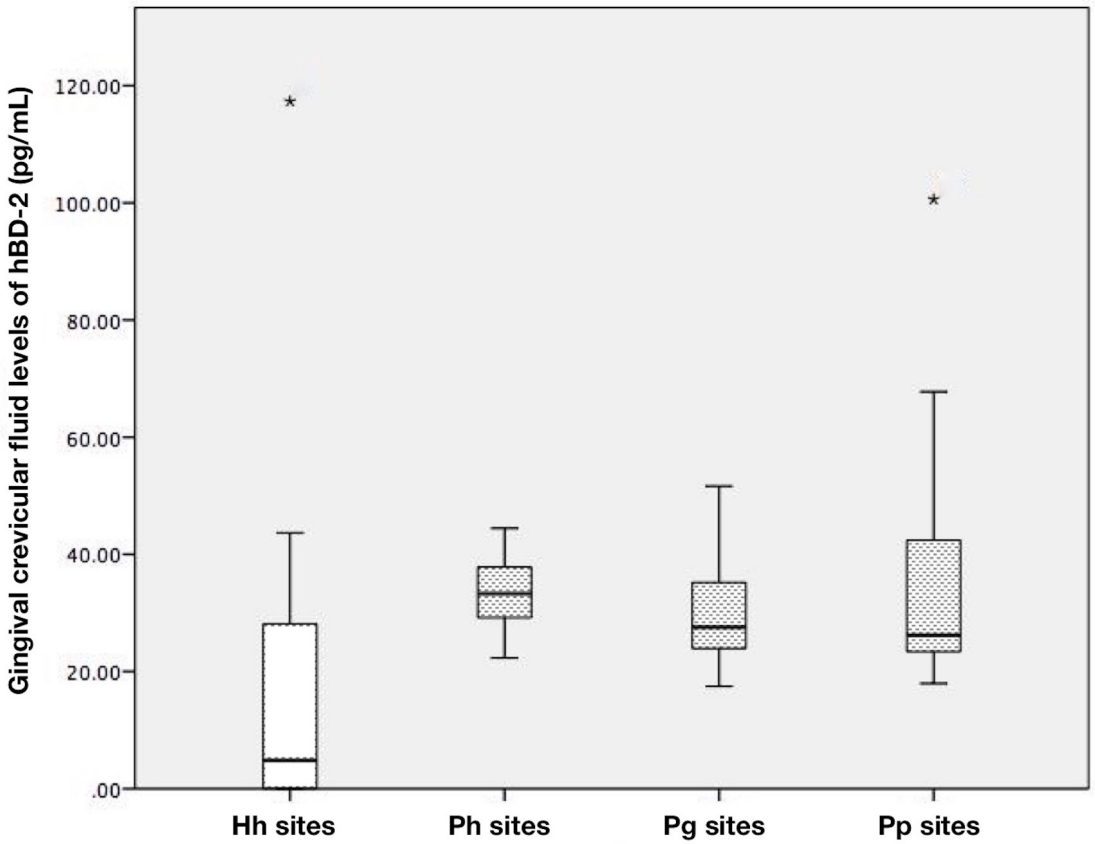

Figure 1. hBD-2 levels in gingival crevicular fluid from healthy sites (Hh) of periodontally healthy individuals, and from healthy sites (Ph), sites with gingivitis (Pg) and sites with periodontitis (Pp) of individuals with periodontitis. Hh < Ph = Pg = Pp (p = 0.023).

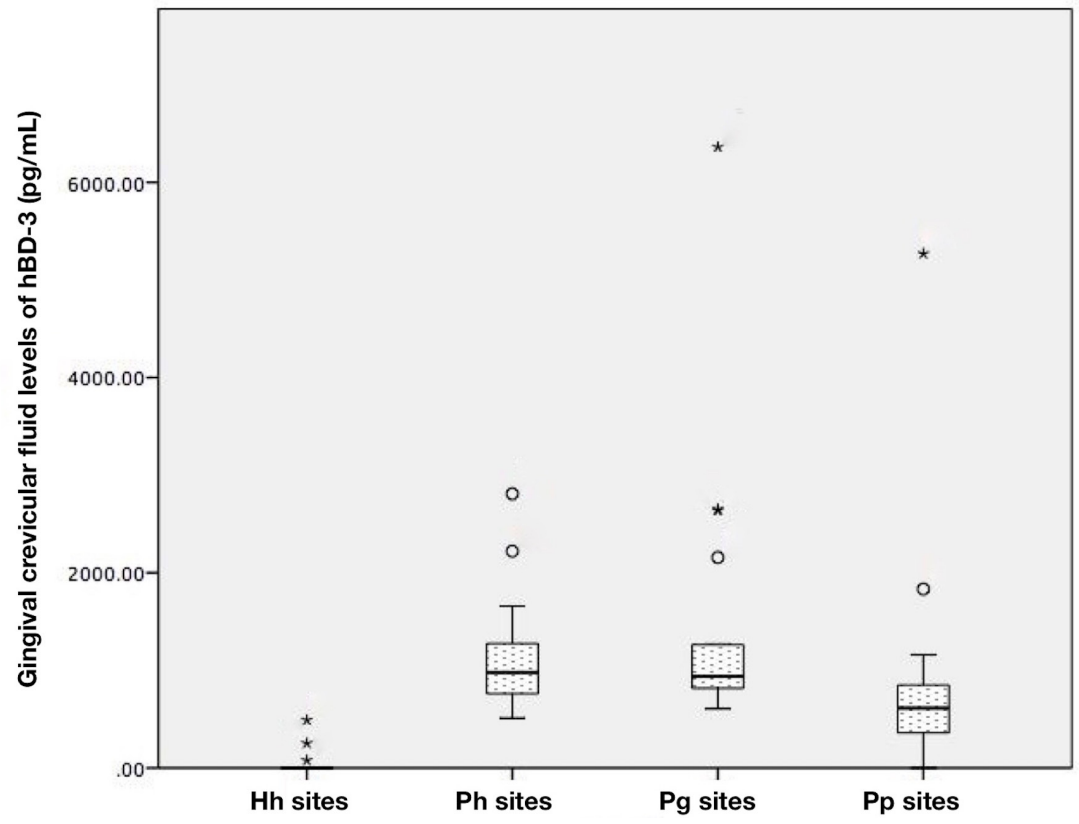

Figure 2. hBD-3 levels in gingival crevicular fluid from healthy sites (Hh) of periodontally healthy individuals, and from healthy sites (Ph), sites with gingivitis (Pg) and sites with periodontitis (Pp) of individuals with periodontitis. Hh < Ph = Pg = Pp (p < 0.001).

Table 2. Final estimation of variance components in the multilevel analysis ("null model").

	Random effect	SD	Variance component	*df*	Chi-square	p	ICC[a]
hBD-2	Intercept, U0	18.837	354.832	39	207.424	< 0.001	74.9%
hBD-3	Intercept, U0	427.408	182677.853	39	50.878	0.096	17.2%

[a]ICC = intraclass correlation coefficient, fraction of the total variance that is due to individuals (Level 2).

Table 3. Hierarchical linear models for gingival crevicular fluid levels of human beta- defensin-2.

Fixed effects	Coefficient	SE	T-ratio	*df* (aprox.)	*p*
Site level model (Level 1)					
Healthy sites (reference)	NA	NA	NA	NA	NA
Sites with gingival inflammation but no attachment loss	-3.833	3.414	-1.123	77	0.266
Sites gingival inflammation and attachment loss	1.675	3.414	0,491	77	0.625
Individual level model (Level 2)					
Periodontitis	14.677	6.178	2.376	38	0.023

NA = not applicable.

Table 4. Hierarchical linear models for gingival crevicular fluid levels of human beta- defensin-3.

Fixed effects	Coefficient	SE	T-ratio	*df* (aprox.)	*p*
Site level model (Level 1)					
Healthy sites (reference)	NA	NA	NA	NA	NA
Sites with gingival inflammation but no attachment loss	302.7132	128.051	1.039	77	0.302
Sites gingival inflammation and attachment loss	-268.984	291.290	-0.923	77	0.359
Individual level model (Level 2)					
Periodontitis	1091.861	242.869	4.496	38	< 0.001

NA = not applicable.

Discussion

A number of studies have evaluated the expression of hBD-2 and hBD-3 in periodontitis (Hosokawa *et al.* 2006, Vardar-Sengul *et al.* 2007, Pereira *et al.* 2013, Liu *et al.* 2014, Yong *et al.* 2015, Li *et al.* 2016, Zorina *et al.* 2016). Nevertheless, multilevel analysis evaluating periodontal sites and individuals are scarce. To best of our knowledge, this is the first study evaluating GCF levels of hBD-2 and hBD-3 through hierarchical modelling comparing healthy and diseased periodontal sites nested within individuals with and without periodontitis.

In the present study, when considering the individual level, hBD-2 and hBD-3 levels were higher in individuals with periodontitis when compared to individuals without periodontitis. This observation resembled the patterns of hBD expression in other tissues, where they are expressed mainly in the presence of infection (O'Neil *et al.* 1999, Liu *et al.* 1998, Lehmann *et al.* 2002). When the findings for hBD-2 and hBD-3 from the present study are considered together with findings of hBD-1 from our previous study (Costa *et al.* 2018), it can be observed that the GCF levels of each specific defensin manifests in a particular and different pattern.

These findings may be related to different biological, mechanisms of induction and regulation of hBDs in the different tissues. It has been noted that infection and inflammation influence the secretion of hBD-2 and hBD-3, while hBD-1 is secreted constitutively (Gursoy and Könönen 2012). Current evidence suggests that the expression and secretion of hBDs may be suppressed during periodontitis (Gursoy and Könönen 2012). Several hypotheses have been proposed to explain this find-

ing. Degradation of hBDs by bacteria- or host-derived proteolitic enzymes, may lead to suppressed expression (Brancatisano *et al.* 2011). Alternatively, the replacement of innate response with immune response during the periodontal disease process (from an initial gingival lesion towards the formation of an established lesion, antibacterial functions of hBDs are replaced by immune cells) (Dunsche *et al.* 2002). Even though no statistical differences were observed in hBD-3 levels among Ph, Pg and Pp sites in the present study, the decrease in hBD-3 levels with the formation of the disease was seen and may corroborate those hypotheses. Differential temporal expression and time-dependent regulation of antimicrobial peptides following inflammation has also been described previously (Dommisch et al. 2015).

Several bacteria have demonstrated an ability to induce the expression of hBD-2, such as *Aggregatibacter actinomycetemcomitans* (Noguchi *et al.* 2003), *Porphyromonas gingivalis* (Taguchi *et al.* 2006), and *Prevotella intermedia* (Hosokawa *et al.* 2006). Gursoy *et al.* (2012) found that *Fusobacterium nucleatum* was also able to stimulate the expression of hBD-2 and hBD-3 in a dento-epithelial organotypic model. Mathews *et al.* (1999) demonstrated that stimulation with interleukin-1β and *Escherichia coli* LPS could induce hBD-2 expression, but hBD-1 levels remained unchanged. Similarly, Krisanaprakornkit *et al.* (1998) found that expression of hBD-2 mRNA, but not hBD-1, was altered by a cell wall extract from *Fusobacterium nucleatum*, TNF-α or PMA (an epithelial cell activator).

Different patterns of hBDs expression arising from different stimuli have also been observed (Vankeerberghen *et al.*, 2005) wherby the expression of hBD-2 and hBD-3 increased but hBD-1 was not induced after stimuli with TNF-α, *Escherichia coli* or *Fusobacterium nucleatum*. Similar results have been noted after stimulation with PMA, where hBD-3 expression increased by more than 10,000-fold, hBD-2 by more than 100-fold, and hBD-1 by more than 10-fold. However, after stimulation with *Porphyromonas gingivalis*, all strains induced hBD-1, but the induction of hBD-3 was variable and no induction of hBD-2 was observed. On the other hand, after stimuli with *Aggregatibacter actinomycetemcomitans*, the expression of hBD-3 occurred, hBD-2 was variable according to the strain tested, and the expression of hBD-1 was small.

These different induction characteristics could explain why the levels of hBD-2 and hBD-3 were increased in individuals with periodontitis in the present study, contrasting from hBD-1 levels that were reduced in our previous study (Costa *et al.* 2018). High levels of these defensins could be the result of the organism's reaction to bacterial antigens and inflammatory mediators present in the tissues in an attempt to control periodontal infection, both by the antimicrobial capacity and by the immunomodulatory and anti-inflammatory capacities of these hBDs.

Comparing to studies that have used similar methodological approached, our results are consistent with Yong *et al.* (2015) who found elevated GCF levels of hBD-2 in individuals with gingivitis and periodontitis compared to healthy individuals. Conversely, they are divergent from Brancatisano *et al.* (2011) who verified extremely reduced GCF levels of hBD-3 in individuals with periodontitis. It should be taken into consideration, however, that Brancatisano *et al.* (2011) did not perform a differential analysis per site, so comparisons should be carefully taken.

Results of the present study are convergent to the few published longitudinal studies (Pereira *et al.* 2012, Zorina *et al.* 2016, Dommisch *et al.* 2015). Pereira et al. (2012) found high levels of hBD-2 in the saliva of individuals with periodontitis, and these levels were reduced after non-surgical treatment. Dommisch *et al.* (2015) verified an elevation on GCF levels of hBD-2 in individuals with experimental gingivitis. Zorina *et al.* (2016) found that hBD-3 mRNA levels in gingival epithelial cells were elevated in subjects with periodontitis, and that, after treatment, these levels decreased to values similar to those found in healthy individuals.

Different results have been reported in some studies where different methodological designs were used. Pereira *et al.* (2013) showed high levels of hBD-2 in the saliva of individuals with gingivitis and periodontitis, and Mathews *et al.* (1999) showed higher levels of hBD-2 mRNA in inflamed gingival tissues compared to non-inflamed tissues. Dommisch et al. (2005) and Li et al. (2016) demonstrated no significant differences in hBD-1, hBD-2 and hBD-3 mRNA expression in gingival tissue samples with different degrees of inflammation. On the contrary, Dunsche *et al.* (2002) and Liu *et al.* (2014) found that mRNA of hBD-1, hBD-2 and hBD-3 was reduced in inflamed tissues. Bissel *et al.* (2004) showed a significantly lower level of hBD-3 mRNA, but not of hBD-1 nor of hBD-2, in inflamed tissues. Moreover, Vardar-Sengul *et al.* (2007) demonstrated significantly higher expression of hBD-2 mRNA in individuals with aggressive periodontitis, but reduced in individuals with gingivitis and variable in individuals with chronic periodontitis

It is important to notet that comparisons between studies with different methods of hBDs evaluation should be interpreted with caution since different methods of evaluation could lead to different results (Hosokawa *et al.* 2006, Li *et al.* 2016). Hosokawa *et al.* (2006) observed that the expression of hBD-2 e hBD-3 mRNA was detected in a lower frequency in inflamed gingival. Nevertheless, levels of hBD-2, when analyzed through ELISA, were slightly higher in these inflamed tissues. Hence, there appears to be a complex and yet

unknown mechanism in the process of expression of hBDs in gingival tissue that makes the expression of the gene not necessarily proportional to the peptide concentration in the samples. Li *et al.* (2016) analyzed gingival tissue samples through immunohistochemistry and RT-PCR and also showed dissimilar results, suggesting that the fact that mRNA chains in eukaryotes have a short life span would be the probable cause for the differences in the expression of hBDs when evaluated by these different methods. Future studies comparing hBDs levels in gingival tissue samples and GCF through different methods are necessary to better understand the expression patterns, as well as the correlations between samples.

In the present study, when considering the site level, no significant differences in the levels of hBDs were observed. These findings suggest that, although periodontitis may exert an influence on the expression of hBD-2 and hBD-3 at the individual level, this influence does not appear to depend on the local inflammatory and infectious site condition, but rather on the condition of the individual as a whole.

Lu *et al.* (2004), when analyzing the expression of hBDs at the site level, found that the expression of hBD-2 in healthy individuals was higher than its expression in healthy or inflamed tissues from individuals with periodontitis. In this study it was further verified that the expression of hBD-2 in inflamed tissues of individuals with periodontitis is increased compared to non-inflamed tissues from the same group. However, it is important to note that the individuals with periodontitis evaluated in this study had previously undergone non-surgical periodontal therapy and the evaluated sites were those that did not show improvement in the inflammatory status after therapy. Nonetheless, it was previously demonstrated that periodontal treatment has the ability to reduce the levels of hBDs in the gingival tissues (Zorina *et al.* 2016). Therefore, the results of Lu *et al.* (2004) may not reflect pre-treatment levels, thus compromising possible comparisons with the present study.

It is also important to note that there are significant differential characteristics of hBD expression, induction, localization and degradation, together with other factors such as the high variation of hBDs among individuals of the same group (Bissell *et al.*2004, Dommisch *et al.* 2005, Vardar-Sengul *et al.* 2007) and their capacity to accumulate in the cytoplasm or cellular nucleus and not be secreted (Yilmaz *et al.* 2015) that may explain the enormous complexity involved in their mechanisms of action.

Overall, despite the differences between studies, the current consensus is that hBDs are important components in the health-disease process and maintenance of periodontal homeostasis (Vardar-Sengul *et al.* 2007, McCormick and Weinberg 2010, Brancatisano *et al.* 2011, Liu *et al.* 2014, Costa *et al.* 2018). This is corroborated by Ikuta *et al.* (2015) who have shown an association between hBD-1 genetic polymorphism (BD-1 gene genotype -44CC) and the susceptibility to periodontitis Furthermore it has been demonstyrated that there is an association between reduced numbers of genomic copies of hBD-2 with the severity of periodontitis (Jaradat *et al.*, 2013).

There are some limitations of the present study that must be recognized. There is considerable variation in the methods used for collecting, processing and analyzing GCF samples. In the present study protocol, paper strips were placed in microtubes with no buffer, immediately freezed, and elution was performed just before analysis with the addition of phosphate buffered saline. Since no enzyme inhibitors were used during sample collection, elution and analysis, one can speculate that the secreted peptides may have been degraded throughout these processes by host or bacterial factors.. Similar procedures have been previously used for GCF and saliva processing (Ishida *et al.* 2019, Costa *et al.* 2018, Yilmaz *et al.* 2018, Pereira *et a*l. 2013). The external validity of the present results should be done with caution and future studies with different larger samples should be performed to confirm the results. However, multilevel analyzes of healthy and diseased individuals and sites provided interesting information on the expression of hBD-2 and hBD-3. Studies investigating microbiological and genetic profiles may help to better clarify the regulation of the expression of these hBDs in periodontal tissues.

Conclusion

GCF levels of hBD-2 and hBD-3 appear to be influenced by periodontitis at the individual level, but not by site phenotype. Increased GCF levels of hBD-2 and hBD-3 in individuals with periodontitis may suggest either a normal response against periodontal infection or be considered a partially responsible factor for the exacerbated immunoinflammatory response commonly found in those individuals.

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References

American Academy of Periodontology Task Force Report on the Update to the 1999 Classification of Periodontal Diseases and Conditions. *Journal of Periodontology* 2015; **86**:835-838.

Bian T, Li L, Lyu J, Cui D, Lei L and Yan F. Human β-defensin 3 suppresses Porphyromonas gingivalis lipopolysaccharide-induced inflammation in RAW 264.7 cells and aortas of ApoE-deficient mice. *Peptides* 2016; **82**:92-100.

Biragyn A, Ruffini PA, Leifer CA, et al. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* 2002; **298**:1025-1029.

Bissell J, Joly S, Johnson GK, et al. Expression of beta-defensins in gingival health and in periodontal disease. *Journal of Oral Pathology & Medicine* 2004; **33**:278-285.

Brancatisano FL, Maisetta G, Barsotti F, et al. Reduced human beta defensin 3 in individuals with periodontal disease. *Journal of Dental Research* 2011; **90**:241-245.

Costa LCM, Soldati KR, Fonseca DC, et al. Gingival crevicular fluid levels of human beta-defensin 1 in individuals with and without chronic periodontitis. *Journal of Periodontal Research* 2018; **53**,736-742.

Cui D, Lyu J, Li H, et al. Human β-defensin 3 inhibits periodontitis development by suppressing inflammatory responses in macrophages. *Molecular Immunology* 2017; **91**:65-74.

Dommisch H and Jepsen S. Diverse functions of defensins and other antimicrobial peptides in periodontal tissues. *Periodontology 2000* 2015; **69**:96-110.

Dommisch H, Skora P, Hirschfeld J, Olk G, Hildebrandt L, Jepsen S. The guardians of the periodontium – sequential and differential expression of antimicrobial peptides during gingival inflammation. Results from in vivo and in vitro studies. *Journal of Clinical Periodontology* 2019; **46**:276-285.

Dommisch H, Açil Y, Dunsche A, Winter J and Jepsen S. Differential gene expression of human beta-defensins (hBD-1, -2, -3) in inflammatory gingival diseases. *Oral Microbiology and Immunology* 2005; **20:**186-190.

Dommisch H, Staufenbiel I, Schulze K, et al. Expression of antimicrobial peptides and interleukin-8 during early stages of inflammation: An experimental gingivitis study. *Journal of Periodontal Research* 2015; **50**:836-845.

Dunsche A, Açil Y, Dommisch H, Siebert R, Schröder JM and Jepsen S. The novel human beta-defensin-3 is widely expressed in oral tissues. *European Journal of Oral Sciences* 2002; **110**:121-124.

Ertugrul AS, Dikilitas A, Sahin H, Alpaslan N, Bozoglan A and Tekin Y. Gingival crevicular fluid levels of human beta-defensins 1 and 3 in subjects with periodontitis and/or type 2 diabetes mellitus: a cross-sectional study. *Journal of Periodontal Research* 2013; **48**:475-482.

Feng Z, Jiang B, Chandra J, Ghannoum M, Nelson S and Weinberg A. Human beta-defensins: differential activity against candidal species and regulation by Candida albicans. *Journal of Dental Research* 2005; **84**:445-450.

Funderburg N, Lederman MM, Feng Z, et al. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proceedings of the National Academy of Science of the United States of America* 2007; **104**:18631-18635.

Griffiths GS. Formation, collection and significance of gingival crevice fluid. *Periodontology 2000* 2003; **31**:32-42.

Gursoy UK and Könönen E. Understanding the roles of gingival beta-defensins. *Journal of Oral Microbiology* 2012; **4**:15127

Gursoy UK, Pöllänen M, Könönen E and Uitto V-J. A Novel Organotypic Dento-Epithelial Culture Model: Effect of *Fusobacterium nucleatum* Biofilm on B-Defensin-2, -3, and LL-37 Expression. *Journal of Periodontology* 2012; **83**:242-247.

Hosokawa I, Hosokawa Y, Komatsuzawa H, et al. Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clinical and Experimental Immunology* 2006; **146**:218-225.

Ikuta T, Inagaki Y, Tanaka K, et al. Gene polymorphism of β-defensin-1 is associated with susceptibility to periodontitis in Japanese. *Odontology* 2015; **103**:66-74.

Ishida A, Wada T, Ogami K, Ueda T, Sakurai K. Influence of chewing on expression level of human beta-defensin 2 and secretory immunoglobulin A in the epithelium. *Journal of Prosthodontic Research* 2019; 63:162-166.

Jaradat SW, Hoder-Przyrembel C, Cubillos S, et al. Beta-defensin-2 genomic copy number variation and chronic periodontitis. *Journal of Dental Research* 2013; **92**:1035-1040.

Joly S, Organ CC, Johnson GK, McCray PB and Guthmiller JM. Correlation between beta-defensin expression and induction profiles in gingival keratinocytes. *Molecular Immunology* 2005; **42**:1073-1084.

Krisanaprakornkit S, Weinberg A, Perez CN and Dale BA. Expression of the peptide antibiotic human β-defensin 1 in cultured gingival epithelial cells and gingival tissue. *Infection and Immunity* 1998; **66**:4222-4228.

Lee J-K, Chang SW, Perinpanayagam H, et al. Antibacterial efficacy of a human β-defensin-3 peptide on multispecies biofilms. *Journal of Endodontics* 2013; **39**:1625-1629.

Lehmann J, Retz M, Harder J, et al. Expression of human beta-defensins 1 and 2 in kidneys with chronic bacterial Infection. *BMC Infectious Diseases* 2002; **10**:1-10.

Li X, Duan D, Yang J, et al. The expression of human β-defensins (hBD-1, hBD-2, hBD-3, hBD-4) in gingival epithelia. *Archives of Oral Biology* 2016; **66**:15-21

Liu J, Chen J, Du X, Hu L, Chen L. The expression of hBDs in the gingival tissue and keratinocytes from healthy subjects and periodontitis patients. *Archives of Oral Biology* 2014; **59**:193-198.

Liu L, Wang L, Jia HP, et al. Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 1998; **222**:237-244.

Lu Q, Jin L, Darveau RP, Samaranayake LP. Expression of human beta-defensins-1 and -2 peptides in unresolved chronic periodontitis. *Journal of Periodontal Research* 2004; **39**:221-227.

Maisetta G, Batoni G, Esin S, et al. Susceptibility of Streptococcus mutans and Actinobacillus actinomycetemcomitans to bactericidal activity of human beta-defensin 3 in biological fluids. *Antimicrobial Agents and Chemotherapy* 2005; **49**:1245-1248.

Mathews M, Jia HP, Guthmiller JM, et al. Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infection and Immunity* 1999; **67**:2740-2745.

McCormick TS and Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. *Periodontology 2000* 2010; **54**:195-206.

Meyle J and Chapple I. Molecular aspects of the pathogenesis of periodontitis. *Periodontology 2000* 2015; **69**:7-17.

Niyonsaba F, Someya A, Hirata M, Ogawa H and Nagaoka I. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *European Journal of Immunology* 2001; **31**:1066-1075.

Noguchi T, Shiba H, Komatsuzawa H, et al. Syntheses of prostaglandin E2 and E-cadherin and gene expression of beta-defensin-2 by human gingival epithelial cells in response to Actinobacillus actinomycetemcomitans. *Inflammation* 2003; **27**:341-349.

O'Neil DA, Porter EM, Elewaut D, et al. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *Journal of Immunology* 1999; **163**:6718-6724.

Park O-J, Kim J, Ahn KB, et al. A 15-amino acid C-terminal peptide of beta-defensin-3 inhibits bone resorption by inhibiting the osteoclast differentiation and disrupting podosome belt formation. *Journal of Molecular Medicine (Berlin)* 2017; **95**:1315-1325.

Pereira AL, Franco GC, Cortelli SC, et al. Influence of periodontal status and periodontopathogens on levels of oral human β-defensin-2 in saliva. *Journal of Periodontology* 2013; **84**:1445-1453.

Pereira AL, Holzhausen M, Franco GCN, Cortelli SC and Cortelli JR. Human β-defensin 2 and protease activated receptor-2 expression in patients with chronic periodontitis. *Archives of Oral Biology* 2012; **57**:1609-1614

Quiñones-Mateu ME, Lederman MM, Feng Z, et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *AIDS* 2003; **17:**F39-48.

Soruri A, Grigat J, Forssmann U, Riggert J and Zwirner J. beta-Defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved. *European Journal of Immunology* 2007; **37**:2474-2486.

Taguchi Y and Imai H. Expression of β-defensin-2 in human gingival epithelial cells in response to challenge with Porphyromonas gingivalis in vitro. *Journal of Periodontal Research* 2006; **41**:334-339.

Tonetti MS, Greenwell H and Kornman KS. Staging and grading of periodontitis: framework and proposal of a new classification and case definition. *Journal of Clinical Periodontology* 2018; **45(suppl)**:S149-S161.

Vankeerberghen A, Nuytten H, Dierickx K, Quirynen M, Cassiman J-J and Cuppens H. Differential induction of human beta-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells. *Journal of Periodontology* 2005; **76**:1293-1303.

Vardar-Sengul S, Demirci T, Sen BH, Erkizan V, Kurulgan E and Baylas H. Human β defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. *Journal of Periodontal Research* 2007; **42**:429-437.

Yang D, Chertov O, Bykovskaia SN, et al. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999; **286**:525-528.

Yilmaz D, Caglayan F, Buber E, et al. Gingival crevicular fluid levels of human beta-defensin-1 in type 2 diabetes mellitus and periodontitis. *Clinical Oral Investigations* 2018; **22**:2135-2140.

Yilmaz D, Güncü GN, Könönen E, Barıs E, Caglayan F and Gursoy UK. Overexpressions of hBD-2, hBD-3, and hCAP18/LL-37 in gingiva of diabetics with periodontitis. *Immunobiology* 2015; **220**:1219-1226.

Yong X, Chen Y, Tao R, et al. Periodontopathogens and human β-defensin-2 expression in gingival crevicular fluid from patients with periodontal disease in Guangxi, China. *Journal of Periodontal Research* 2015; **50**:403-410.

Wassall RR, Preshaw PM. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontology 2000* 2016; **70**:65-79.

Zorina OA, Gankovskaya L V, Balykin RA and Svitich OA, Ivanyushko TP. The expression of TLR4 and HBD3 in epithelial cells of oral mucosa by surgical treatment of periodontitis. *Stomatologiia* 2016; **95**:13-15.