Quantitative Proteomic Analysis of Gingival Crevicular Fluid in Different Periodontal Conditions

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Abstract

Aim: To quantify the proteome composition of the GCF in periodontal health (HH) and in sites with different clinical conditions in chronic periodontitis (CP) subjects.

Material and Methods: 5 subjects with HH and 5 with CP were submitted to full-mouth periodontal examination, and GCF sampling. Sites in the CP group were classified and sampled as periodontitis (P, probing depth, PD>4 mm), gingivitis (G, PD≤3mm with bleeding on probing, BOP), and healthy sites (H, PD≤3mm without BOP). GCF proteins were subjected to liquid chromatography electrospray ionization mass spectrometry for identification, characterization and quantification.

Results: 230 proteins were identified; 145 proteins were detected in HH, 214 in P, 154 in G, and 133 in H. Four proteins were exclusively detected at HH, 43 proteins at P, 7 proteins at G, and 1 protein at H. Compared to HH group, 35 and 6 proteins were more abundant in P and G (p<0.001), respectively; and 4, 15 and 37 proteins were less abundant in P, G and H (p≤0.01), respectively.

Conclusions: There are marked differences in the GCF proteome according to disease profile. Comprehension of the role of the identified proteins in the etiopathogenesis of periodontal disease may lead to biomarkers definition.

Introduction

Clinical periodontal conditions are described as periodontal health (PH), gingivitis (G) and periodontitis (P) [1]. Gingivitis induced by dental biofilm is the presence of gingival inflammation without clinical attachment loss (CAL) and with no radiographic evidence of alveolar bone loss. Periodontitis is the presence of gingival inflammation with CAL, and re-absorption of the alveolar bone. In most patients, the increased probing depth (PD) or the formation of periodontal pockets follows the development of the periodontitis [1,2]. Chronic periodontitis (CP) is the most common destructive periodontal disease in adults [3].

The etiological agent of periodontitis is the high levels and proportions of periodontopathic bacteria in the subgingival biofilm [4,5]. The interplay between the pathogenic biofilm and the periodontal tissues in susceptible subjects leads to immunological responses that can be detected in the tissues and in the inflammatory exudates of the gingival crevicular fluid (GCF) [6,7]. In the GCF, pro-inflammatory cytokines and a vast array of other proteins can be found, especially in diseased subjects [8]. Recent reports have shown that, even in healthy periodontal microenvironment, GCF contains local proteins derived from extracellular matrix components and degradation products, as well as serum-derived proteins [7,9,10].

As GCF is an oral cavity-specific fluid, it has been studied in order to determine which constituents could be used as biomarkers for periodontal diagnosis or prognostic for the progression of periodontitis [6,8]. In the recent years, it has been recognized that the multivariate model is more promising than...
a single biomarker for risk assessment of disease [11]. The ability to screen and discover multiple biomarkers simultaneously may provide a more valid clinical diagnosis and may be more useful for recognizing molecular patterns predictive for disease development. This multi-biomarker approach has progressed by recent advances in clinical proteomics [12–14]. Many studies have been performed in GCF analyzing its proteome profile through mass spectrometry (MS) technology [7,9,10,15–19]. Those studies differed in relation to the clinical condition, including studies of experimental gingivitis [15,16], aggressive periodontitis subjects [7], CP subjects [9,18,19], and healthy subjects [10].

Different findings are reported in those studies, not only because of differences inherent to periodontal condition but also because of the MS technique employed. On the other hand, to the moment, proteomics of GCF is in its beginning and there is a vast array of possible biomarkers [8,14]. Thus, comprehensive studies of GCF in different clinical conditions would contribute to a better understanding of the diagnostic potential of the GCF, improving the ability to early detection of disease [6,8,14].

Based on it, we hypothesized that the GCF proteome of PH subjects qualitatively and quantitatively differs from the proteome of CP subjects. This working hypothesis was explored by using quantitative proteomics approach based on label-free LC-MS on GCF of subjects with PH and CP; in CP subjects, sites with different clinical conditions as periodontal health, gingivitis and periodontitis were assessed.

Materials and Methods

Subject Population

Ten subjects who sought dental treatment in December of 2011 at the Dental School of the Federal University of Rio de Janeiro were enrolled in the present study. All participants were informed about the nature of the study and a signed consent form was obtained from each individual prior to entering into the study. The study protocol (#148/11) was reviewed and approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital of the Federal University of Rio De Janeiro.

In order to participate of the study, subjects had to present at least 14 teeth, ≥ 18 years of age, and clinical diagnosis of CP or PH. Exclusion criteria included smoking, pregnancy, nursing, periodontal therapy in the last year, and use of antibiotics in the previous six months, as well as any immunological condition that could affect the progression of periodontitis. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. Furthermore, PH subjects should present clinical attachment level (CAL) characteristics as described bellow in order to avoid potential incipient destructive periodontal disease.

Clinical evaluation

A calibrated examiner performed all clinical examinations. The intra-class correlation coefficient for CAL at the site was 0.90, and for probing depth (PD), 0.92. Full-mouth measurements including PD, CAL, presence or absence of supragingival biofilm (SB) and bleeding on probing (BOP) were recorded at six sites per tooth in all teeth, but third molars. Clinical diagnosis of periodontal status was established for all subjects based on the following criteria: periodontal health (PH), ≤ 10% of sites with BOP, no PD or CAL > 3 mm, although PD or CAL = 4 mm in up to 5% of the sites without BOP was allowed; and chronic periodontitis (CP), > 10% of teeth with PD and/or CAL ≥ 5 mm and BOP [20]. CP subjects had to have at least 5 sites with gingivitis (PD ≤ 3 mm with BOP) and 4 sites with clinical periodontal health (PD ≤ 3 mm without BOP).

GCF sampling

Fourteen GCF samples from non-adjacent sites were collected from each participant. In CP subjects, different categories of sites were selected: 5 deep (PD >4 mm) sites (P), 5 shallow sites with BOP as gingivitis (G), and 4 shallow sites without BOP as health (H). In PH subjects, 14 buccal sites from the upper jaw were sampled (HH samples).

After removal of supragingival biofilm, the teeth were isolated with cotton rolls and a sterile Periopaper strip (ProFlow Inc., Amityville, NY, USA) was gently inserted into the selected subgingival sites and left there for 30s. Then, the volume of the GCF was measured using a Periotron 8000 (Oraflow Inc., Smithtown, NY, USA). The strips were stored in microcentrifuge tubes at -80°C.

Sample preparation

For each CP subject, the paper strips were pooled according to the clinical categories (H, G and P) in microcentrifuge tubes. For PH subjects, two pools were made, dividing the 14 strips in two tubes. Each tube was incubated with 150 μL of a solution containing 80% acetonitrile, 19.9% distilled water and 0.1% trifluoroacetic acid and sonicated for 1 min. This procedure was repeated three times, in order to elute all proteins from the paper strips. Eluted proteins from sites of the same clinical category were pooled. In the end, there were three pools for the 5 CP subjects as P, G and H, and there was one pool for the 5 PH subjects. The pools were concentrated by a rotary evaporator. The total protein concentration was assessed by Micro Bicinchoninic acid (Micro BCA™) Assay (Thermo Scientific, Rockford, USA). Equal protein amount (10 μg) from sites categories was dried by a rotary evaporator, denatured and reduced for 2 h by the addition of 200 μL of 4M urea, 10 mM dithiothreitol (DTT), 50 mM NH4HCO3, pH 7.8. After four-fold dilution with 50 mM NH4HCO3, pH 7.8, trypsin digestion was carried out for 18 h at 37°C, after the addition of 2% (w/w) sequencing-grade trypsin (Promega, Madison, WI, USA) [21].

Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

Equal amounts of all samples were dried by rotary evaporator and re-suspended in 20 μL of 97.5% H2O/2.4% acetonitrile/0.1% formic acid and then subjected to reversed-phase LC-ESI-MS/MS. Peptide separation and mass spectrometric analyses were carried out with a nano-HPLC Proxeon (Thermo Scientific, San Jose, CA, USA) which allows in-line LC with the capillary column, 75 μm x 10 cm (PicoTip™ Emitter, New Objective, Woburn, MA, USA) packed in-house.
using Magic C18 resin of 5 µm diameter and 200 Å pores size (Michrom BioResources, Auburn, CA, USA) linked to mass spectrometer (LTQ Velos, Thermo Scientific, San Jose, CA, USA) using an electrospray ionization in a survey scan in the range of m/z values 390–2000 tandem MS/MS. The nano-flow reversed-phase HPLC was performed with linear 100 min gradient ranging from 5% to 55% of solvent B in 65 min (97.5% acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min with a maximum pressure of 280 bar. Electro spray voltage and the temperature of the ion transfer capillary were 1.8 kV and 250°C, respectively.

**Enzyme-linked immunosorbent assay (ELISA)**

To validate the differential protein level identified by quantitative mass spectrometry approach, ELISA was carried out on lysozyme, a protein with well-known characteristics and functions in the oral cavity. ELISA microtiter plate (96-wells) was coated with 100 µl of GCF protein material from each group (10 µg/ml) at 37°C for 1 hour. The plate was then washed three times with 250 µl Tris Buffered Saline (TBS) per well and 200 µl TBST containing 3% BSA added to each well to block uncoated sites, and incubated overnight at 4°C. Primary anti lysozyme antibody (50 µl; 1:1000 dilution, Abcam, ab97950, MA, USA) in TBST containing 1% BSA was added to each well and incubated at 37°C for 1.5 h, followed by washing three times, and incubation with horse radish peroxidase (HRP) linked Anti- MOUSE IgG (H&L) (GOAT) Antibody Peroxidase Conjugated (100µl; 1:5000 dilution, ROCKLAND, PA, USA) in TBST containing 1% BSA. After incubation in the dark for 1 h at room temperature OPD (o-phenylenediamine dihydrochloride, Sigma-Aldrich, MO, USA) was added and product was analyzed spectrophotometrically at 490 nm. The experiment was performed in triplicate.

**Data analysis**

A statistical program (SPSS Statistics 19, IBM Brazil, São Paulo, SP, Brazil) was used for clinical analysis. Full-mouth clinical data were averaged in each patient and within groups. Clinical parameters for the 14 sampled sites were also computed for each patient and averaged within groups. Significant differences in demographic and clinical parameters among groups were determined by Kruskal-Wallis, Mann-Whitney and χ² tests. For MS data, each survey scan (MS) was followed by automated sequential selection of seven peptides for a standard collision-induced (CID) method, with dynamic exclusion of the previously selected ions. The obtained MS/MS spectra were searched against human protein databases (Swiss Prot and TrExEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA), using at least two peptides. Search results were filtered for a False Discovery rate of 1% employing a decoy search strategy utilizing a reverse database [21]. The proteins identified were grouped into 9 different categories based on their known biological functions.

For quantitative proteome analysis, three MS raw files from each pooled clinical categories were analyzed using SIEVE software (Version 2.0 Thermo Scientific, San Jose, CA, USA). Signal processing was performed in a total of 12 MS raw files. The SIEVE experimental workflow was defined as “Control Compare Trend Analysis” where one class of samples are compared to one or more other classes of samples. In the present study, the HH group was compared to each of the other group (H, G and P). For the alignment step, a single MS raw file belonging to the HH group was selected as the reference file and all of the other files were adjusted to generate the best correlation to this reference file. After alignment, the feature detection and integration (or framing) process was performed using the MS level data with a feature called “Frames From MS2 Scans” only. When using this type of framing only MS mass-to-charge ratio (m/z) values that were associated with MS2 scan are used. Any m/z measurements that do not have MS2 were ignored. The parameters used consisted of a frame m/z width of 1500 ppm and a retention time width of 1.75 min. A total of 216,096 MS2 scans were present in all of the 12 RAW files that resulted in a total of 20,158 frames. Then peak integration was performed for each frame and these values were used for statistical analysis. Next, peptide sequences obtained from the database search using SEQUEST algorithm in Proteome Discoverer 1.3 were imported into SIEVE. A filter was applied to the peptide sequences during the import that eliminated all sequences with a Percolator q-value greater than 1% (false discovery rate). Peptides were grouped into proteins and a protein ratio and p-value were calculated, using a weighted average of the peptide intensities for the protein calculation. Only proteins observed in all four groups were quantified. HH group was used as the default group and all other three groups were compared with HH group. Relative abundance of an individual protein from HH group was considered significantly different protein level when the values observed were <0.75 for decreased abundance or >1.25 for increased abundance, and a p-value <0.05 as described previously [22,23].

For ELISA results, mean (± standard-deviation) values were calculated for each group. Afterwards, Analysis of Variance and Student-Newman-Keuls test for pairwise comparisons was carried out to identify significant differences among groups at a 5% level.

**Results**

**Demographic and clinical findings**

Table 1 shows the demographic and clinical data of the sample population. CP subjects presented significantly higher mean age than HH subjects (p < 0.01, Mann-Whitney test). Full-mouth clinical data show that CP had significantly higher mean PD and CAL (p < 0.01), and mean % of sites with BOP and SB than HH subjects. In fact, HH subjects presented no sites with BOP or SB. Regarding the clinical data of the sampled sites, significantly differences among sites from diseased subjects and HH subjects were detected (p < 0.01, Kruskal-Wallis test). Sites with periodontitis (P) presented the highest means for PD and CAL than the other categories; and all sites with gingivitis (G) presented BOP. The volume of GCF samples differed significantly among groups (p = 0.016). The
GCF mean volume obtained from P sites of the CP group (0.3 µL ± 0.06) was significantly higher than samples from the HH group (0.1 µL ± 0.03, p = 0.016, Mann-Whitney test), H sites (0.06 µL ± 0.02, p = 0.009), and G sites (0.1 ± 0.04, p = 0.028). However, there was no significant difference between the HH group and G or H sites of CP.

### Table 1. Demographic and clinical data (full-mouth and sampled sites; mean ± SEM) of the study population.

| Variables                  | Groups          | CP (n = 5) | PH (n = 5) |
|----------------------------|-----------------|------------|------------|
| Age (years)                |                 | 46.20 ± 4.84 | 22.20 ± 0.66* |
| Gender - Females (%)       |                 | 100        | 100        |
| Full mouth                 |                 |            |            |
| PD (mm)                    |                 | 2.67 ± 0.13 | 1.16 ± 0.03* |
| CAL (mm)                   |                 | 2.78 ± 0.12 | 1.16 ± 0.03* |
| BOP (%)                    |                 | 51.51 ± 6.39 | 0*         |
| Supragingival biofilm (%)  |                 | 39.25 ± 7.24 | 0*         |
| Sampled sites              |                 |            |            |
| PD (mm)                    | P               | 5.16 ± 0.27 | 1.89 ± 0.19 |
| CAL (mm)                   | G               | 5.24 ± 0.25 | 2.01 ± 0.16 |
| BOP (%)                    | G               | 75.0 ± 14.66 | 100 ± 0.0   |
| Supragingival biofilm (%)  | H               | 40.66 ± 12.93 | 41.57 ± 13.82 |
| GCF volume (µL)            | HH              | 0.3 ± 0.06 | 0.1 ± 0.04 |
|                            |                 | 0.06 ± 0.02 | 0.1 ± 0.03|  

* p < 0.01, †p < 0.05, Mann-Whitney test; ‡p < 0.01, §p = 0.016, Kruskal-Wallis test; CP: chronic periodontitis; PH: periodontal health; P: periodontitis; G: gingivitis; H: health in periodontitis; HH: healthy sites in healthy subjects; PD: Probing depth; CAL: Clinical attachment level; Bleeding on probing; GCF: gingival crevicular fluid.

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Figure 1. Examples of base-peak chromatograms of the clinical groups. Peptide separation was achieved using a nano-flow reverse-phase HPLC column, with gradient elution ranging from 5 to 55% solvent B in 100 min. P: sites with probing depth >4 mm; G: sites with probing depth ≤3 mm and bleeding on probing; H: sites with probing depth ≤3 mm without bleeding on probing in periodontitis subjects; HH: sites with probing depth ≤3 mm without bleeding on probing in healthy subjects.

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GCF mean volume obtained from P sites of the CP group (0.3 µL ± 0.06) was significantly higher than samples from the HH group (0.1 µL ± 0.03, p = 0.016, Mann-Whitney test), H sites (0.06 µL ± 0.02, p = 0.009), and G sites (0.1 ± 0.04, p = 0.028). However, there was no significant difference between the HH group and G or H sites of CP.

### Protein identification

After protein elution from the paper strip and trypsinization, equal amounts of peptides were subjected to nanoscale LC-ESI-MS/MS. A total of 3 runs per group were carried out. The base-peak chromatogram for reversed-phase chromatography monitored by the mass spectrometer represents the intensity of all peptide ions in the sample in a single scan. GCF proteome from all four different groups showed a consistent elution of protein/peptides range from 20 to 60 min (Figure 1).
A total of 230 different proteins were identified in GCF of all four groups (Table 2). Seventy proteins were related to cell differentiation, 60 to cell organization, 3 to coagulation, 1 to enzyme regulation, 36 to immune response, 23 to metabolism, 11 to signal transduction, 16 to transport, and 11 had unknown function. The HH group presented 145 proteins, while in the CP group P sites showed 214 proteins, G 154 proteins, and H 133 proteins. One hundred and five proteins were identified in all four groups, indicating a high overlap in GCF proteins (Figure 2). Four proteins were detected exclusively in the HH group, including keratin type II cytoskeletal 7, neuroblast differentiation-associated protein AHNAK, and 2 gial fibrillary acidic proteins. On the other hand, only one protein (nucleoprotein TPR) was detected in H sites. Moreover, 7 proteins were found exclusively in G sites: histones H1.1, H1.5 and H1t, fibrinogen alpha and beta chain, cathelicidin antimicrobial peptide, and myosin-9. Forty-three proteins were only detected in P sites and were distributed as follows: 10 were related to cell differentiation, 3 to cell organization, 1 to coagulation, 8 to immune response, 9 to metabolism, 8 to signal transduction, 2 to transport, and 2 had non-specified function (Figure 1 and Table 2).

Relative quantitative abundance ratios of proteins in GCF

The relative abundance ratios of the detected proteins in GCF samples from the studied groups are displayed in Table 3. Thirty-five proteins were found to be significantly high abundant in P sites of the CP compared to the HH group (p < 0.001). Many of them (12 proteins) were related to immune response, followed by metabolism (8 proteins), transport (6 proteins), cell differentiation (3 proteins), cell organization (3 proteins), signal transduction (2 proteins), and enzyme regulation (1 protein). Only 4 proteins were significantly low abundant in P sites compared to the HH group: 2 proteins with cell organization function (histone H2A, serum amyloid A 4 protein), and 2 proteins related to immune response (apolipoprotein AIV and myosin-9) (p < 0.001). Conversely, the G and H site categories had most proteins significantly low abundant compared to the HH group. In G sites, 37 proteins were significantly low abundant, mainly proteins related to cell differentiation (21 proteins), followed by immune response function (6 proteins) (p ≤ 0.01); and only 6 proteins were significantly high abundant (p ≤ 0.01). In H sites, 15 proteins were significantly low abundant (p ≤ 0.01), which included 5 proteins with cell organization function and 4 proteins with immune response function.

ELISA

A significant reduced protein level was observed by MS at H and HH concentration compared to control G and P groups (Table 3). By ELISA Lysozyme contents were demonstrated to decrease from 0.805 ± 0.021 μg/10 μg GCF total protein in the P group, to 0.15 ± 0.05, 0.05 ± 0.02 and 0.113 ± 0.04 in the G, H, HH groups, respectively (Figure 3).

Discussion

The aim of the present case-control study was to explore the human proteins present in GCF from healthy subjects as well as in subjects with CP. Additionally, analysis of GCF in different clinical conditions in CP subjects was possible because of the sampling strategy. Although, case-control studies may present limited level of evidence, they are often used to identify factors that may contribute to the disease development. The design experiment and the outcome data belong to the primary phase of five consecutive phases. Phase one, as preclinical exploratory studies, is the first (initial) step for the search of biomarkers [24].

The current study identified 230 proteins. In previous study [19], a similar amount of 231 proteins was identified; 168 proteins from those were identified in GCF samples from a healthy site, and 167 proteins were detected in periodontitis sites. Sixty-four proteins were contained only in the healthy samples, and 63 proteins were detected only in the periodontitis samples. In the current analysis, 4 proteins were detected exclusively in the HH group, and 43 proteins were only detected in P sites. The different amount of proteins detected in healthy subjects between studies could be explained by the number of subjects sampled, where in the previous study only one healthy subject was analyzed [19]. It has been demonstrated that when GCF is pooled from multiple sites, the site-specific variability in GCF constituents is reduced [10,17]. Perhaps, by increasing the number of subjects sampled, it is possible that the range of proteins detected increases. Other studies that evaluate the GCF from healthy or diseased subjects have reported a lower number of detected proteins [7,10,15–17], while others have identified a higher amount [9,18]. In the study of Baliban et al. [9], 432 human proteins were found. From those, 79 were exclusively found in healthy subjects and 123 in periodontitis subjects. Another study have identified 327 human proteins in healthy subjects [18]. The difference in the amount of identified proteins can also be explained by the MS technique employed as well as the number of peptides used to identify the proteins. Studies that have used only one peptide to identify proteins may have increased the sensibility [9,18], however it may have reduced the specificity.

The most frequently detected proteins in the current study were actins, keratins, histones, annexins, protein S100-A9, apolipoprotein A-I, ALB protein, albumin and serum albumin. These findings are in accordance with a prior proteome study [9], in which the proteins more often detected in both healthy and CP samples were serum albumin, serotransferrin and α-2-macroglobulin. In that study, the authors have also found a wide variety of Type I and II keratins. The high frequency of these proteins observed in the current and other investigations may reflect the high turnover and rate of differentiation of oral epithelia [7,9]. In contrast, cytokines were not detected, as reported also by previous studies applying proteomic based mass spectrometric [7,9,16]. Possible explanations for this finding may be their low concentrations or low molecular weight, for which a drop of peptide signal is still a limiting factor.
Table 2. Gingival crevicular fluid proteome from periodontal health (HH, 145 proteins), chronic periodontitis in three categories P (deep probing depth sites, 214 proteins), G (shallow probing depth sites with bleeding on probing, 154 proteins) and H (shallow sites without bleeding on probing, 133 proteins), and number of hits of each protein achieved during three mass spectrometric runs.

| Accession/ function | Protein name | Number of hits during three mass spectrometry runs |
|---------------------|--------------|---------------------------------------------------|
|                     |              | HH | P | G | H |
| Cell differentiation | Q5T8M7       | 5  | 24 | 11 | 4 |
|                     | Q5T8M8       | 5  | 27 | 11 | 2 |
|                     | Q5T9N7       | 4  | 15 | 11 | 2 |
|                     | A6NL76       | 5  | 27 | 11 | 7 |
|                     | E7EQV5       | 11 | 22 | 11 | 2 |
|                     | P69133       | 3  | 30 | 11 | 4 |
|                     | P62736       | 3  | 27 | 11 | 4 |
|                     | P60709       | 7  | 34 | 13 | 6 |
|                     | CSUTX5       | 12 | 12 | 11 | 2 |
|                     | CSUML1       | 2  | 15 | 11 | 2 |
|                     | CSUJR7       | 12 | 15 | 11 | 2 |
|                     | E7EVS6       | 6  | 17 | 11 | 2 |
|                     | F5GYT4       | 0  | 22 | 0  | 0 |
|                     | P63261       | 7  | 34 | 13 | 6 |
|                     | F5H0N0       | 16 | 33 | 11 | 6 |
|                     | B8ZZJ2       | 4  | 12 | 11 | 2 |
|                     | C9JFL5       | 27 | 15 | 13 | 2 |
|                     | E9PG30       | 0  | 17 | 8  | 4 |
|                     | P63267       | 3  | 27 | 11 | 4 |
|                     | P13645       | 61 | 46 | 41 | 62 |
|                     | C9JAJ77      | 3  | 20 | 18 | 25 |
|                     | P13646       | 44 | 23 | 25 | 39 |
|                     | P02533       | 36 | 23 | 26 | 33 |
|                     | A8MT21       | 2  | 5  | 0  | 8 |
|                     | B3KRA2       | 8  | 5  | 0  | 13 |
|                     | P19012       | 24 | 16 | 15 | 25 |
|                     | P06779       | 30 | 14 | 16 | 21 |
|                     | Q04695       | 12 | 8  | 6  | 14 |
|                     | C5JM50       | 2  | 7  | 29 | 10 |
|                     | P06727       | 16 | 13 | 13 | 16 |
|                     | P35900       | 7  | 0  | 0  | 7 |
|                     | Q7Z3Z0       | 4  | 4  | 7  | 2 |
|                     | Q7Z3Y8       | 4  | 4  | 7  | 2 |
|                     | Q7Z3Y7       | 4  | 4  | 7  | 2 |
|                     | P35527       | 28 | 53 | 11 | 49 |
|                     | Q9NSB2       | 2  | 2  | 0  | 0 |
|                     | P04264       | 86 | 71 | 40 | 68 |
|                     | Q7SZ94       | 2  | 2  | 0  | 0 |
|                     | P35906       | 22 | 24 | 19 | 30 |
|                     | Q01546       | 4  | 5  | 7  | 7 |
|                     | P12035       | 4  | 3  | 3  | 2 |
|                     | F5H8K9       | 0  | 13 | 4  | 9 |
|                     | P19013       | 8  | 13 | 4  | 9 |
|                     | E7EU87       | 0  | 16 | 14 | 28 |
|                     | P13647       | 25 | 20 | 17 | 30 |
|                     | E7EU88       | 0  | 22 | 23 | 39 |
|                     | P02536       | 30 | 22 | 26 | 42 |
|                     | F5H6G5       | 0  | 13 | 14 | 34 |
|                     | P04259       | 30 | 18 | 19 | 40 |
| Accession/ function | Protein name                  | Number of hits during three mass spectrometry runs |
|---------------------|-------------------------------|-----------------------------------------------|
|                     |                               | HH  | P  | G  | H  |
| E7EQ7               | Keratin, type II cytoskeletal 6C | 17  | 21 | 22 | 39 |
| P49668              | Keratin, type II cytoskeletal 6C | 30  | 21 | 22 | 39 |
| E7ES34              | Keratin, type II cytoskeletal 7 | 0   | 8  | 0  | 0  |
| F5GZD1              | Keratin, type II cytoskeletal 7 | 2   | 0  | 0  | 0  |
| P08729              | Keratin, type II cytoskeletal 7 | 3   | 8  | 15 | 0  |
| Q3SY94              | Keratin, type II cytoskeletal 71 | 0   | 2  | 0  | 0  |
| Q14CN4              | Keratin, type II cytoskeletal 72 | 0   | 6  | 0  | 7  |
| Q86Y46              | Keratin, type II cytoskeletal 73 | 4   | 7  | 3  | 4  |
| Q7RTS7              | Keratin, type II cytoskeletal 74 | 0   | 2  | 0  | 2  |
| Q96E76              | Keratin, type II cytoskeletal 75 | 0   | 10 | 7  | 13 |
| Q5XE5               | Keratin, type II cytoskeletal 79 | 5   | 7  | 7  | 8  |
| P05787              | Keratin, type II cytoskeletal 8 | 6   | 8  | 0  | 2  |
| Q9H552              | Keratin-B-like protein 1       | 0   | 2  | 0  | 0  |
| Q09666              | Neuroblast differentiation-associated protein AHNAK | 2   | 0  | 0  | 0  |
| Q0PS4               | TMSB4X protein (fragment)      | 2   | 2  | 2  | 0  |
| Q5U58               | Tropomyosin 3                  | 0   | 2  | 0  | 0  |
| Q5U66               | Tropomyosin 3                  | 0   | 2  | 0  | 0  |
| Q5U61               | Tropomyosin 3                  | 0   | 2  | 0  | 0  |
| Q5U72               | Tropomyosin 3                  | 0   | 2  | 0  | 0  |
| P06753              | Tropomyosin alpha-3 chain      | 0   | 2  | 0  | 0  |
| P67936              | Tropomyosin alpha-4 chain      | 0   | 2  | 0  | 0  |
| Cell organization   |                               |     |    |    |    |
| P02654              | Apolipoprotein C-I             | 0   | 13 | 2  | 0  |
| P02655              | Apolipoprotein C-II            | 0   | 2  | 2  | 0  |
| P02656              | Apolipoprotein C-III           | 0   | 10 | 2  | 6  |
| B0YIW2              | Apolipoprotein C-III variant 1 | 2   | 10 | 8  | 2  |
| P17661              | Desmin                        | 0   | 9  | 0  | 0  |
| Q02539              | Histone H1.1                  | 0   | 0  | 2  | 0  |
| P16403              | Histone H1.2                  | 10  | 8  | 11 | 2  |
| P16402              | Histone H1.3                  | 10  | 8  | 11 | 2  |
| P16412              | Histone H1.4                  | 10  | 8  | 11 | 2  |
| P16401              | Histone H1.5                  | 0   | 0  | 2  | 0  |
| P22492              | Histone H1t                   | 0   | 0  | 2  | 0  |
| C9J386              | Histone H2A                   | 5   | 6  | 0  | 4  |
| A6NF38              | Histone H2A                   | 0   | 6  | 0  | 4  |
| A6NKY0              | Histone H2A                   | 0   | 6  | 0  | 6  |
| A6NN01              | Histone H2A                   | 11  | 10 | 3  | 8  |
| C9J0D1              | Histone H2A                   | 4   | 10 | 3  | 10 |
| C9JE32              | Histone H2A                   | 22  | 11 | 13 | 9  |
| P0C058              | Histone H2A type 1            | 12  | 20 | 17 | 15 |
| Q96QV6              | Histone H2A type 1-A          | 2   | 12 | 13 | 9  |
| P04908              | Histone H2A type 1-B/E        | 6   | 18 | 17 | 14 |
| Q93077              | Histone H2A type 1-C          | 6   | 18 | 17 | 14 |
| P20671              | Histone H2A type 1-D          | 12  | 20 | 19 | 15 |
| Q96K5               | Histone H2A type 1-H          | 12  | 20 | 19 | 15 |
| Q98786              | Histone H2A type 1-J          | 12  | 20 | 19 | 15 |
| Q9F113              | Histone H2A type 2-A          | 12  | 21 | 19 | 15 |
| Q8UE6               | Histone H2A type 2-B          | 3   | 11 | 10 | 3  |
| Q16777              | Histone H2A type 2-C          | 12  | 21 | 19 | 15 |
| Q7L7L0              | Histone H2A type 3            | 6   | 18 | 17 | 14 |
| Q9B1M1              | Histone H2A.J                 | 12  | 20 | 19 | 15 |
| Q71U9               | Histone H2A.V                 | 0   | 10 | 6  | 10 |
| P16104              | Histone H2A.x                 | 2   | 12 | 13 | 9  |
Table 2 (continued).

| Accession/ function | Protein name       | Number of hits during three mass spectrometry runs |
|----------------------|--------------------|-------------------------------------------------|
|                      |                    | HH    | P    | G    | H    |
| P0C055               | Histone H2A.Z      | 0     | 10   | 3    | 10   |
| B4DR52               | Histone H2B        | 4     | 17   | 6    | 4    |
| Q96A08               | Histone H2B type 1-A | 2    | 3    | 0    | 0    |
| P33778               | Histone H2B type 1-B | 7    | 14   | 8    | 4    |
| P62807               | Histone H2B type 1-C/E/F/G/I | 11  | 17   | 8    | 4    |
| P58876               | Histone H2B type 1-D | 11  | 17   | 8    | 4    |
| Q93079               | Histone H2B type 1-H | 7    | 17   | 8    | 4    |
| P06899               | Histone H2B type 1-J | 7    | 14   | 8    | 4    |
| O60814               | Histone H2B type 1-K | 3    | 17   | 4    | 4    |
| Q98880               | Histone H2B type 1-L | 7    | 17   | 8    | 4    |
| Q99879               | Histone H2B type 1-M | 7    | 17   | 8    | 4    |
| Q98877               | Histone H2B type 1-N | 7    | 17   | 8    | 4    |
| P23527               | Histone H2B type 1-O | 19  | 14   | 20   | 23   |
| Q16778               | Histone H2B type 2-E | 7    | 14   | 8    | 4    |
| Q5ONW8               | Histone H2B type 2-F | 7    | 17   | 8    | 4    |
| Q8N257               | Histone H2B type 3-B | 6    | 9    | 8    | 3    |
| P57053               | Histone H2B type F-S | 11  | 17   | 8    | 4    |
| B4DEB1               | Histone H3         | 4     | 0    | 4    | 0    |
| P69431               | Histone H3.1       | 2     | 2    | 4    | 0    |
| Q16695               | Histone H3.1t      | 2     | 0    | 4    | 0    |
| Q71D13               | Histone H3.2       | 2     | 4    | 4    | 0    |
| P84243               | Histone H3.3       | 2     | 0    | 4    | 0    |
| P62805               | Histone H4         | 26    | 30   | 29   | 23   |
| P41219               | Peripherin        | 0     | 4    | 0    | 0    |
| P13796               | Plastin-2          | 0     | 2    | 0    | 0    |
| P07737               | Profilin-1        | 0     | 4    | 2    | 0    |
| P35542               | Serum amyloid A-4 protein | 0 | 2    | 11   | 0    |
| P62328               | Thymosin beta-4    | 2     | 2    | 2    | 0    |
| A3MW06               | Thymosin beta-4-like protein 3 | 6    | 2    | 2    | 0    |
| **Coagulation**      |                    |       |      |      |      |
| P02671               | Fibrinogen alpha chain | 0 | 0    | 3    | 0    |
| P02675               | Fibrinogen beta chain | 0   | 0    | 2    | 0    |
| Q9BYX7               | Putative beta-actin-like protein 3 | 0 | 10   | 0    | 0    |
| **Enzyme regulator** |                    |       |      |      |      |
| P04080               | Cystatin-B        | 3     | 2    | 0    | 0    |
| **Immune response**  |                    |       |      |      |      |
| P01009               | Alpha-1-antitrypsin | 3    | 10   | 10   | 3    |
| D6RA62               | Annexin           | 2     | 3    | 0    | 0    |
| D6RA28               | Annexin           | 5     | 2    | 0    | 0    |
| D6RCA8               | Annexin           | 26    | 2    | 0    | 0    |
| D6RFG5               | Annexin           | 7     | 30   | 0    | 0    |
| P04683               | Annexin A1        | 17    | 39   | 18   | 10   |
| Q5T3N0               | Annexin A1 (fragment) | 2 | 19   | 8    | 3    |
| Q5T3N1               | Annexin A1 (fragment) | 10   | 28   | 13   | 9    |
| P06727               | Apolipoprotein A-V | 0     | 20   | 5    | 0    |
| P49913               | Cathelicidin antimicrobial peptide | 0    | 0    | 2    | 0    |
| P08311               | Cathepsin G       | 7     | 19   | 17   | 4    |
| P31146               | Coronin-1A        | 2     | 6    | 0    | 0    |
| P81605               | Dermcidin         | 2     | 2    | 6    | 6    |
| A5JHP3               | Dermcidin isofrom 2 | 2    | 2    | 6    | 26   |
| B4DL87               | Heat shock protein beta-1 | 32  | 7    | 0    | 0    |
| C9J3N8               | Heat shock protein beta-1 | 30  | 3    | 0    | 0    |
| P04792               | Heat shock protein beta-1 | 0    | 8    | 0    | 0    |
| Accession/function | Protein name                  | Number of hits during three mass spectrometry runs |
|--------------------|-------------------------------|----------------------------------------------------|
|                    |                               | HH | P | G | H |
| P01857             | Ig gamma-1 chain C region     | 0  | 2 | 0 | 0 |
| P0CG05             | Ig lambda-2 chain C regions   | 0  | 3 | 12| 0 |
| P0CG06             | Ig lambda-3 chain C regions   | 0  | 3 | 0 | 0 |
| F5GWP8             | Junction plakoglobin          | 0  | 7 | 9 | 14|
| B7ZAX2             | Lactoferrin-C                 | 4  | 2 | 0 | 0 |
| C9J8S5             | Lactoferrin-C                 | 23 | 2 | 0 | 0 |
| C9JCF5             | Lactoferrin-C                 | 28 | 2 | 0 | 0 |
| E7EQB2             | Lactoferrin-C                 | 0  | 2 | 0 | 0 |
| E7ER44             | Lactoferrin-C                 | 0  | 2 | 0 | 0 |
| E7ERT3             | Lactoferrin-C                 | 0  | 2 | 0 | 0 |
| P02788             | Lactotransferrin              | 0  | 2 | 0 | 0 |
| P30740             | Leukocyte elastase inhibitor  | 0  | 3 | 0 | 0 |
| P05164             | Myeloperoxidase               | 8  | 31| 2 | 0 |
| P33579             | Myosin-9                      | 0  | 0 | 3 | 0 |
| P59665             | Neutrophil defensin 1         | 8  | 10| 5 | 5 |
| P59666             | Neutrophil defensin 3         | 8  | 10| 5 | 5 |
| P12270             | Nucleoprotein TPR             | 0  | 0 | 0 | 2 |
| P05109             | Protein S100-A8               | 8  | 17| 7 | 2 |
| P06702             | Protein S100-A9               | 66 | 81| 64| 46|
| **Metabolism**     |                               |    |    |    |    |
| P31946             | 14-3-3 protein beta/alpha     | 0  | 3 | 0 | 0 |
| P31947             | 14-3-3 protein sigma          | 0  | 3 | 0 | 0 |
| P27348             | 14-3-3 protein theta          | 0  | 3 | 0 | 0 |
| F5H1C1             | Actin, alpha cardiac muscle 1 | 11 | 24| 14| 4 |
| P68032             | Actin, alpha cardiac muscle 1 | 3  | 30| 11| 4 |
| P02647             | Apolipoprotein A-I            | 14 | 95| 66| 44|
| P02652             | Apolipoprotein A-II           | 2  | 23| 15| 9 |
| P02649             | Apolipoprotein E              | 0  | 8 | 0 | 0 |
| E9PK25             | Cofilin-1                     | 0  | 4 | 0 | 0 |
| E9PLJ3             | Cofilin-1                     | 0  | 2 | 0 | 0 |
| E9PP50             | Cofilin-1                     | 0  | 2 | 0 | 0 |
| E9PQ87             | Cofilin-1                     | 0  | 2 | 0 | 0 |
| P23528             | Cofilin-1                     | 0  | 4 | 0 | 0 |
| P61626             | Lysozyme C                    | 3  | 13| 7 | 2 |
| P31949             | Protein S100-A11              | 3  | 6 | 4 | 2 |
| E7EW61             | Transferrin                   | 0  | 20| 11| 0 |
| F5H566             | Transferrin                   | 0  | 19| 5 | 0 |
| P02766             | Transferrin                   | 0  | 23| 9 | 2 |
| F5H288             | Vimentin                      | 0  | 37| 12| 0 |
| P08670             | Vimentin                      | 9  | 37| 10| 0 |
| Q5JV88             | Vimentin (fragment)           | 0  | 15| 2 | 0 |
| B0YJC4             | Vimentin variant 3            | 7  | 37| 10| 0 |
| B0YJC5             | Vimentin variant 4            | 8  | 22| 3 | 0 |
| **Signal transduction** |                            |    |    |    |    |
| Q04917             | 14-3-3 protein eta            | 0  | 3 | 0 | 0 |
| P61986             | 14-3-3 protein gamma          | 0  | 3 | 0 | 0 |
| Q8UK7              | ALB protein                   | 21 | 55| 57| 51|
| P12429             | Annexin A3                   | 0  | 5 | 0 | 0 |
| E7EMB3             | Calmodulin                    | 0  | 2 | 0 | 0 |
| E7ETZ0             | Calmodulin                    | 0  | 2 | 0 | 0 |
| P62158             | Calmodulin                    | 0  | 2 | 0 | 0 |
| P80511             | Protein S100-A12              | 0  | 2 | 0 | 0 |
| P11151             | Protein S100-A7               | 0  | 2 | 0 | 0 |
for detection or being masked by the presence of other proteins as albumin [7,9,16,25].

An efficient periodontal biomarker should be able to predict future attachment loss in a susceptible subject. Therefore, the analysis of gingivitis sites in at risk subjects might help in the identification of which factors are already present and what might indicate future breakdown. Having that in mind, some proteins only found in G sites of CP subjects when two peptides were used should be highlighted as histones H1.1, H1.5 and cathelicidin antimicrobial peptide [16]. Among those proteins, only cathelicidin antimicrobial peptide has been described as involved in periodontal disease [26]. Most recently, histones have been shown to play an important role in an extracellular defense mechanism, neutrophil extracellular traps (NETs) [27]. Moreover, in 2010 it was the first time that a histone protein was reported in GCF [17]. Low concentrations of histone H2A-histone H2B-DNA complexes may present antibacterial property against the species *Shigella flexneri*, *Salmonella typhimurium*, and *Staphylococcus aureus* [27]. Moreover, NETs contain other proteins such as neutrophil

| Accession/ function | Protein name | Number of hits during three mass spectrometry runs |
|---------------------|--------------|---------------------------------------------------|
| C9JKR2 | Albumin, isoform CRA-k | 4 78 49 55 |
| P68905 | Hemoglobin subunit alpha | 0 31 16 3 |
| P68871 | Hemoglobin subunit beta | 0 50 24 20 |
| E9PEW8 | Hemoglobin subunit delta | 0 23 22 0 |
| E9PTF6 | Hemoglobin subunit delta | 0 23 8 0 |
| P02042 | Hemoglobin subunit delta | 0 26 11 6 |
| P25815 | Protein S100-P | 0 2 0 2 |
| P02787 | Serum transferrin | 0 2 0 0 |
| B7WNR0 | Serum albumin | 9 97 85 68 |
| D6RHD5 | Serum albumin | 10 63 77 64 |
| E7ESU5 | Serum albumin | 23 113 94 81 |
| P02768 | Serum albumin | 28 113 94 81 |
| D6RAK8 | Vitamin D-binding protein | 8 12 0 0 |
| D6RF35 | Vitamin D-binding protein | 8 12 0 0 |
| P02774 | Vitamin D-binding protein | 0 9 0 0 |

The proteins identified were grouped into 9 different categories based on their known biological functions.
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for detection or being masked by the presence of other proteins as albumin [7,9,16,25].

An efficient periodontal biomarker should be able to predict future attachment loss in a susceptible subject. Therefore, the analysis of gingivitis sites in at risk subjects might help in the identification of which factors are already present and what might indicate future breakdown. Having that in mind, some proteins only found in G sites of CP subjects when two peptides were used should be highlighted as histones H1.1, H1.5 and H1t, fibrinogen alpha and beta chains, cathelicidin antimicrobial peptide, and myosin-9. Additionally, some other proteins that were relative more abundant in G compared to the HH group such as histone H2A.J, and glial fibrillary acidic protein might also be important (p <0.005). Proteins of the intermediate filament family as glial fibrillary acidic protein, peripherin, and desmin bind together to form intermediate filaments, providing support and strength to cells, and determining the placement of the nucleus and other specialized structures within the cell. Kido et al. [19] have described high levels of actin and myosin related proteins in GCF samples mainly from periodontitis subjects. Those proteins may be related to periodontal tissue degradation and inflammation [19]. Furthermore, myosin-9 can stimulate leukocyte migration and monocyte differentiation [10]. Myosin-9 and fibrinogen alpha chain were detected only in the resolution phase of experimental gingivitis, while histone H1.5 was detected in relative high frequency in both phases (induction and resolution) [15]. In another experimental gingivitis study, the authors also found myosin-9, fibrinogen alpha and beta chains, histone H1.5 and cathelicidin antimicrobial peptide [16]. Among those proteins, only cathelicidin antimicrobial peptide has been described as involved in periodontal disease [26]. Most recently, histones have been shown to play an important role in an extracellular defense mechanism, neutrophil extracellular traps (NETs) [27]. Moreover, in 2010 it was the first time that a histone protein was reported in GCF [17]. Low concentrations of histone H2A-histone H2B-DNA complexes may present antibacterial property against the species *Shigella flexneri*, *Salmonella typhimurium*, and *Staphylococcus aureus* [27]. Moreover, NETs contain other proteins such as neutrophil
elastase, cathepsin G, myeloperoxidase, lactoferrin, and gelatinase [27]. Additionally, the outflow of GCF associate to NETs may be an important mechanism of clearance of the gingival sulcus or periodontal pocket [28].

From the 214 proteins detected in P sites, 43 were exclusively detected in this category. It is known that greater pocket depths associated to clinical signs of inflammation (i.e. bleeding) may result in increased levels of protein-breakdown products expressed in GCF [17]. Those 43 proteins detected included actin cytoplasmatic, tropomyosins, plastin-2, putative beta-actin-like protein 3, peripherin, desmin, Ig gamma-1 and -3 chain C regions, lactoferrin-C, lactotransferrin, leukocyte elastase inhibitor, 14-3-3 protein beta/alpha, 14-3-3 protein sigma, 14-3-3 protein theta, 14-3-3 protein epsilon 3, protein S100-A12, protein S100-A7, serotransferrin, and vitamin D-binding protein. In another analysis, many proteins showed significant higher relative abundance in P sites when compared to the HH group as alpha 1 antitrypsin, annexin, apolipoprotein AIV, cathelicidin antimicrobial peptide, cathepsin G, coronin-1A, dermcidin isoform 2, heat shock protein beta-1, myeloperoxidase, neutrophil defensin 3, S100 A8, and S100 A9 were present in samples from deep pockets and/or have elevated relative abundance compared to samples from healthy sites (Table 3). For instance, dermcidin isoform 2 displays antimicrobial activity and is highly effective against Escherichia coli, Enterococcus faecalis, S. aureus and Candida albicans [29]. Surprisingly, two proteins of the immune system, Annexin A1 and myosin 9, showed significantly decreased relative abundance in P sites compared to HH group (Table 3, p < 0.001). Conversely, other studies have shown that myosin 9 was found only in disease [7] or in higher frequency in GCF of periodontitis subjects [9]. In a study of periodontally healthy and generalized aggressive periodontitis subjects, a total of 101 human proteins was found in GCF, 35 from those were exclusively detected in aggressive periodontitis [7]. In accordance with the current findings, the authors found that annexin A3, cathepsin G, and S100 P were identified only in diseased subjects, and that myeloperoxidase and profilin 1 were up-regulated in disease. In contrast, the proteins serotransferrin and alpha 1 antitrypsin were low abundant in disease, while neutrophil defensin 3 was detected just in healthy subjects. However, comparisons between those studies should be interpreted carefully, given that aggressive periodontitis subjects may present a more severe condition which is modulated by a genetic pre-disposition and different factors compared to chronic periodontitis subjects [1,2,30].

Findings from the HH group showed that a total of 145 proteins were detected, and only 4 proteins were exclusively found in that group. In the analysis of abundance ratios, it was

Figure 2. Venn diagram summarizing the absolute number of proteins detected in gingival crevicular fluid samples from periodontally healthy (145 proteins), and chronic periodontitis subjects in three categories P (deep probing depth sites, 214 proteins), G (shallow probing depth sites with bleeding on probing, 154 proteins) and H (shallow sites without bleeding on probing, 133 proteins).

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**Figure 2.** Venn diagram summarizing the absolute number of proteins detected in gingival crevicular fluid samples from periodontally healthy (145 proteins), and chronic periodontitis subjects in three categories P (deep probing depth sites, 214 proteins), G (shallow probing depth sites with bleeding on probing, 154 proteins) and H (shallow sites without bleeding on probing, 133 proteins).
Table 3. Abundance* ratios of the detected proteins in gingival crevicular fluid samples from periodontally health (HH) and chronic periodontitis subjects (P: deep probing depth sites; G: shallow probing depth with bleeding on probing sites; and H: shallow probing depth without bleeding on probing sites).

| Accession/ function | Protein name | Ratio HH/HH | Ratio P/HH | p-value | Ratio G/HH | p-value | Ratio H/HH | p-value |
|---------------------|--------------|-------------|------------|---------|------------|---------|------------|---------|
| Cell differentiation | Actin, alpha skeletal muscle | 1 | 2.19 | < 0.001 | - | - | - | - |
| | Actin, cytoplasmic 1, N-terminally processed | 1 | 3.24 | < 0.001 | - | - | - | - |
| | Actin, gamma-enteric smooth muscle | 1 | 2.09 | < 0.001 | 0.53 | < 0.001 | 0.35 | < 0.001 |
| | Keratin, type I cytoskeletal 9 | 1 | - | - | 0.71 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 10 | 1 | - | - | 0.65 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 13 | 1 | - | - | 0.66 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 13 | 1 | - | - | 0.38 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 14 | 1 | - | - | 0.66 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 15 | 1 | - | - | - | 0.48 | < 0.001 | - |
| | Keratin, type I cytoskeletal 16 | 1 | - | - | 0.57 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 19 | 1 | - | - | 0.56 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 25 | 1 | - | - | 0.47 | < 0.001 | - | - |
| | Keratin, type I cytoskeletal 28 | 1 | - | - | 0.55 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 1 | 1 | - | - | 0.69 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 2 epidermal | 1 | - | - | 0.73 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 2 oral | 1 | - | - | 0.56 | 0.008 | - | - |
| | Keratin, type II cytoskeletal 4 | 1 | - | - | 0.52 | 0.009 | - | - |
| | Keratin, type II cytoskeletal 5 | 1 | - | - | 0.62 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 5 | 1 | - | - | 0.65 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 6A | 1 | - | - | 0.50 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 6A | 1 | - | - | 0.60 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 6B | 1 | - | - | 0.51 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 6C | 1 | - | - | 0.56 | < 0.001 | - | - |
| | Keratin, type II cytoskeletal 7 | 1 | - | - | 0.52 | < 0.001 | - | - |
| Cell organization | Apolipoprotein CII variant 1 | 1 | 0.74 | < 0.001 | - | - | - | - |
| | Histone H1.4 | 1 | 2.11 | < 0.001 | 0.64 | < 0.001 | 0.60 | < 0.001 |
| | Histone H1.5 | 1 | - | - | 1.56 | < 0.001 | - | - |
| | Histone H1t | 1 | - | - | 0.43 | 0.016 | - | - |
| | Histone H2A | 1 | 0.70 | < 0.001 | 0.68 | < 0.001 | 0.44 | < 0.001 |
| | Histone H2A-J | 1 | - | - | 1.67 | < 0.001 | - | - |
| | Histone H2A.x | 1 | - | - | - | - | - | - |
| | Histone H2B | 1 | 1.84 | < 0.001 | - | - | 0.74 | 0.001 |
| | Histone H3 | 1 | - | - | 0.47 | < 0.001 | - | - |
| | Histone H4 | 1 | - | - | - | 0.42 | < 0.001 | - |
| | Profilin 1 | 1 | 2.83 | < 0.001 | - | - | - | - |
| | Serum amyloid A 4 protein | 1 | 0.11 | < 0.001 | - | - | - | - |
| Enzyme regulator | Cystatin B | 1 | 1.78 | < 0.001 | 0.61 | < 0.001 | 0.27 | < 0.001 |
| Immune response | Alpha 1 antitrypsin | 1 | 2.16 | < 0.001 | - | - | - | - |
| | Annexin | 1 | 1.59 | < 0.001 | - | - | - | - |
| | Annexin A1 | 1 | 0.74 | < 0.001 | 0.70 | < 0.001 | 0.69 | 0.003 |
| | Apolipoprotein AIV | 1 | 1.56 | < 0.001 | 0.51 | < 0.001 | - | - |
| | Cathelicidin antimicrobial peptide | 1 | 1.44 | < 0.001 | - | - | - | - |
| | Cathepsin G | 1 | 2.27 | < 0.001 | - | - | - | - |
| | Coronin-1A | 1 | 3.06 | < 0.001 | - | - | 0.66 | < 0.001 |
| | Dermcidin isofrom 2 | 1 | - | - | 0.5 | < 0.001 | 0.42 | < 0.001 |
| | Heat shock protein beta-1 | 1 | 1.56 | < 0.001 | - | - | - | - |
| | Heat shock protein beta-1 | 1 | 1.80 | < 0.001 | - | - | - | - |
| | Myeloperoxidase | 1 | 1.80 | < 0.001 | - | - | - | - |
Table 3 (continued).

| Accession/ function | Protein name            | Protein name | Ratio HH/HH | Ratio P/HH | p-value   | Ratio G/HH | p-value | Ratio H/HH | p-value |
|---------------------|-------------------------|--------------|-------------|------------|-----------|------------|---------|------------|---------|
| P35579              | Myosin 9                |              | 1           | 0.73       | < 0.001   | 0.62       | < 0.001 |            |         |
| P59666              | Neutrophil defensin 3   |              | 1           | 1.77       | < 0.001   | 0.48       | < 0.001 | 0.44       | < 0.001 |
| P05109              | Protein S100 A8         |              | 1           | 1.26       | < 0.001   | -          | -       | -          |         |
| P06702              | Protein S100 A9         |              | 1           | 1.46       | < 0.001   | 0.57       | < 0.001 | -          |         |
|                     | **Metabolism**          |              |             |            |           |            |         |            |         |
| P02647              | Apolipoprotein AI       |              | 1           | 1.36       | < 0.001   | -          | -       | 0.33       | < 0.001 |
| P02652              | Apolipoprotein All      |              | 1           | 3.05       | < 0.001   | -          | -       | -          |         |
| P02649              | Apolipoprotein E        |              | 1           | 2.97       | < 0.001   | -          | -       | -          |         |
| E9PLJ3              | Cofilin-1               |              | 1           | 2.81       | < 0.001   | -          | -       | -          |         |
| P61626              | Lysozyme C              |              | 1           | 2.13       | < 0.001   | 0.71       | < 0.001 | 0.29       | < 0.001 |
| P31949              | Protein S100 A11        |              | 1           | 2.77       | < 0.001   | 0.51       | < 0.001 | -          |         |
| F5H868              | Transthyretin            |              | 1           | 1.65       | < 0.001   | -          | -       | -          |         |
| B0YJC4              | Vimentin variant 3      |              | 1           | 2.20       | < 0.001   | -          | -       | -          |         |
|                     | **Signal transduction** |              |             |            |           |            |         |            |         |
| E7ETZ0              | Calmodulin              |              | 1           | 1.67       | < 0.001   | -          | -       | -          |         |
| E9PAX3              | Glial fibrillary acidic protein |          | 1           | -          | -         | 1.47       | < 0.001 | -          |         |
| P80511              | Protein S100 A12        |              | 1           | 1.99       | 0.002     | 0.50       | 0.020   | -          |         |
|                     | **Transport**           |              |             |            |           |            |         |            |         |
| P69905              | Hemoglobin subunit alpha|              | 1           | 2.35       | < 0.001   | 1.43       | < 0.001 | -          |         |
| E9PFT6              | Hemoglobin subunit delta|              | 1           | 1.95       | < 0.001   | -          | -       | -          |         |
| P02042              | Hemoglobin subunit delta|              | 1           | -          | 1.33      | < 0.001   | -       | -          |         |
| P25815              | Protein S100 P          |              | 1           | 2.07       | < 0.001   | 0.69       | < 0.001 | -          |         |
| P02787              | Serotransferrin         |              | 1           | 1.80       | < 0.001   | -          | -       | -          |         |
| B7WNR0              | Serum albumin           |              | 1           | 2.74       | < 0.001   | -          | -       | 0.68       | < 0.001 |
| D6RF35              | Vitamin D-binding protein|             | 1           | 2.06       | < 0.001   | 0.71       | < 0.001 | -          |         |
|                     | **Non specified**       |              |             |            |           |            |         |            |         |
| A6NBZ8              | Uncharacterized protein |              | 1           | -          | -         | 1.52       | < 0.001 | -          |         |

* Relative abundance of an individual protein from HH group was considered significant protein level when the values observed were < 0.75 for decreased abundance or > 1.25 for increased abundance, and a p-value < 0.05. The proteins identified were grouped into 9 different categories based on their known biological functions.

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Figure 3. ELISA experiment with 10 µg of GCF material for each group and anti-lysozyme antibody. Bars represent standard deviation of the mean, calculated from three independent experiments. Different lower case letters denote statistical difference according to Analysis of variance and Student-Newman-Keels’ test. P: sites with probing depth >4 mm; G: sites with probing depth ≤3 mm and bleeding on probing; H: sites with probing depth ≤3 mm without bleeding on probing in periodontitis subjects; HH: sites with probing depth ≤3 mm without bleeding on probing in healthy subjects.

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interesting to observe that all significant results showed that the detected proteins in H sites were low abundant compared to the HH group. Although, the sites sampled were clinically similar in both groups, with no attachment loss or BOP, metabolically they are completely different by the expression of the proteins actin, apolipoprotein, histones, cystatin B, annexin, coronin-1A, dermcidin isoform 2, neutrophil defensin 3, and lysozyme C. Many of those proteins are related to the immune system. Although there is a clinical similarity, in a susceptible diseased subject healthy sites may not be very effective in the immune response as in a healthy subject. Other authors have also found a great number of proteins in GCF from healthy subjects [7,9,10,18], where coiffin 1, profilin 1, plastin 1, lactotransferrin, myeloperoxidase, calmodulin and alpha 1 antitrypsin were more frequently detected in samples from health subjects compared to CP [9]. Surprisingly, they found leukocyte elastase inhibitor only in healthy subjects. In the current study, one type of heat shock protein beta-1 was detected in P sites, and higher frequency of detection of other two heat shock protein beta-1 was detected in HH patients. Likewise, other authors have identified heat shock protein beta-1 in healthy subjects [16]. Conversely, Bostani et al. [7] detected heat shock protein only in aggressive periodontitis subjects. The proteins S100A8 and S100A9, which are produced by neutrophils and macrophages, can be found in plasma, but their levels in GCF may differ significantly during periodontal disease owing to the local inflammatory response and recruitment of those white cells [10]. This may reinforce the idea that even though serum contributes to its composition, the GCF from healthy periodontal sites is neither pure serum nor are its proteins all serum derived [10].

Although our sample size is not large, it was enough to show significant differences among the clinical groups through MS analysis. Moreover, the differential protein levels identified among groups were confirmed by ELISA on the levels of Lysozyme. On the other hand, the studies previously cited in the current report were performed in relatively small sample size as well. For instance, Bostani et al. [7] have studied 5 healthy subjects and 5 aggressive periodontitis subjects; and Kido et al. [19] have studied 8 subjects with CP and 1 with periodontal health. Additionally, some studies have analyzed only 10 healthy subjects [10] or 12 CP subjects under maintenance therapy [17].

In general, an increase in gingival inflammation results in an increase in GCF flow, and differences exist between GCF obtained from stable and progressing sites [17]. The current results demonstrated that there are markedly differences in the human proteome of GCF according to disease profile. Therefore, more studies completing the phases described by Pepe et al. [24], including multicenter studies [31], are necessary to understand the role of this vast range of identified proteins in the etiopathogenesis of periodontal disease. Through that comprehension, the discrimination of biomarkers for diagnosis and prognosis of periodontal diseases might be possible.

**Author Contributions**

Conceived and designed the experiments: WLS CMSB APVC. Performed the experiments: WLS CMSB YX MHT CR. Analyzed the data: WLS CMSB. Contributed reagents/materials/analysis tools: WLS CMSB APVC. Wrote the manuscript: WLS CMSB APVC.

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