Open access • Journal Article • DOI:10.1021/PR900941Z

Application of label-free absolute quantitative proteomics in human gingival crevicular fluid by LC/MS E (gingival exudatome) — Source link

N Bostanci, Wendy E. Heywood, Kevin Mills, Mohamed Parkar ...+2 more authors

Institutions: UCL Eastman Dental Institute, UCL Institute of Child Health

Published on: 07 May 2010 - Journal of Proteome Research (American Chemical Society)

Topics: Periodontal pathogen and Aggressive periodontitis

Application of label-free absolute quantitative proteomics in human gingival crevicular fluid by LC/MS E (gingival exudatome)

Bostanci, N; Heywood, W; Mills, K; Parkar, M; Nibali, L; Donos, N

Application of label-free absolute quantitative proteomics in human gingival crevicular fluid by LC/MS E (gingival exudatome)

Abstract

Periodontal disease is perhaps the most common infectious disease in humans. Gingival crevicular fluid (GCF) is a local inflammatory exudate of the periodontal tissues. Its composition greatly varies between health and periodontal disease. GCF collection is rapid and noninvasive, but previous approaches aiming to analyze its composition have mainly involved single protein biomarkers. The aim of this study was to perform analysis of the GCF exudatome from healthy and periodontally diseased sites by LC/MS(E), a label-free mass spectrometry method that enables simultaneous protein identification and absolute quantification in biological fluids. In total, 154 proteins of human, bacterial, and viral origin were identified in the 40 GCF samples obtained from the 10 subjects (five healthy and five generalized aggressive periodontitis). The proportion of bacterial, viral, and yeast protein was increased in disease, compared to health. The presence of host defense-related proteins, such as Cystatin-B and defensins, was confirmed to be present only in health. Among the newly identified GCF proteins were L-plastin detected only in disease (15.6 +/- 12.1 fmol) and Annexin-1 detected in 5-fold higher levels in health. Nevertheless, pro-inflammatory cytokines or periodontal pathogen proteins were rarely detected. Conclusively, the LC/MS(E) technology may facilitate characterization of GCF proteome in periodontal health and disease, thus conferring prognostic and diagnostic value. Larger cohort studies are required to characterize the complete GCF proteome in health and disease.

Application of label free-absolute quantitative proteomics in human gingival crevicular fluid by LC/MS^{E} *(Gingival exudatome)*

N. Bostanci [†§*], W. Heywood[‡], K. Mills[‡], M. Parkar[#], L. Nibali[†], N. Donos[†*]

[†] Periodontology Unit, Division of Clinical Research, UCL Eastman Dental Institute, London, UK

[§] Institute of Oral Biology, Center for Dental and Oral Medicine and Cranio-Maxillofacial Surgery, Faculty of Medicine, University of Zürich, Zürich, Switzerland

[#] Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, London, UK

[‡] Biochemistry Group, Clinical and Molecular Genetics Unit, UCL Institute of Child Health, London, UK

[*]Corresponding Authors:

Prof. Nikolaos Donos. Unit of Periodontology, Department of Clinical Research, UCL Eastman Dental Institute, 256 Grays Inn Road, London WC1X 8LD.
E-mail: n.donos@eastman.ucl.ac.uk. Tel: +44 20 7915 1075; Fax: +44 20 7915 1137

Dr. Nagihan Bostanci. Institute of Oral Biology, Center for Dental and Oral Medicine and Cranio-Maxillofacial Surgery, Faculty of Medicine, University of Zürich, Plattenstrasse 11, 8032, Zürich, Switzerland
E-mail: nagihan.bostanci@zzm.uzh.ch. Tel: +41 44 634 57 53
Fax: +41 44 634 43 10

Key words: Gingival crevicular fluid, periodontal disease, aggressive periodontitis, proteomics, biomarkers, diagnosis, LC/MS^{E}, qTOF MS

Abstract

Periodontal disease is perhaps the most common infectious disease in humans. Gingival crevicular fluid (GCF) is a local inflammatory exudate of the periodontal tissues. Its composition greatly varies between health and periodontal disease. GCF collection is rapid and non-invasive, but previous approaches aiming to analyse its composition have mainly involved single protein biomarkers. The aim of this study was to perform analysis of the GCF exudatome from healthy and periodontally diseased sites by LC/MS^E, a label-free mass spectrometry method that enables simultaneous protein identification and absolute quantification in biological fluids. In total, 154 proteins of human, bacterial and viral origin were identified in the 40 GCF samples obtained from the 10 subjects (five healthy and five generalized aggressive periodontitis). The proportion of bacterial, viral and yeast protein was increased in disease, compared to health. The presence of host defence-related proteins, such as Cystatin-B and defensins was confirmed to be present only in health. Among the newly identified GCF proteins were L- plastin detected only in disease (15.6 ± 12.1 fmol) and Annexin-1 detected in 5-fold higher levels in health. Nevertheless, pro-inflammatory cytokines or periodontal pathogen proteins were rarely detected. Conclusively, the LC/MS^E technology may facilitate characterisation of GCF proteome in periodontal health and disease, thus conferring prognostic and diagnostic value. Larger cohort studies are required to characterise the complete GCF proteome in health and disease.

Introduction

Periodontal disease is perhaps the most common infectious disease in humans caused by bacteria present in the oral cavity, which attach on the teeth and cause inflammation to the surrounding (periodontal) tissues. Longitudinal epidemiological studies show that whilst 90% of the population will develop some signs of the early disease namely gingivitis, only 7-13% appear to be at high risk of developing severe destructive periodontitis (Brown and Loe, 1993). Two main forms of destructive periodontal disease are currently recognized, chronic and aggressive periodontitis. Aggressive periodontitis is often characterized by a rapid and severe periodontal destruction in mainly younger individuals and a tendency for cases to aggregate in families (Albandar and Rams, 2002). In its severe forms, periodontitis may lead to loosening and drifting of the teeth and ultimately tooth loss, resulting in a significant impact on masticatory function and quality of life. Moreover, recent evidence has indicated that periodontal disease could contribute to the development of life-threating conditions namely cardiovascular disease (CVD) or stroke (Danesh et al., 1997). The growing global burden of periodontitis creates a need to identify biomarkers for this disease, establish novel diagnostic tests, or develop new therapies directed at the attenuation of the action of these markers.

Gingival crevicular fluid (GCF) is a serum transudate or an inflammatory exudate that results from the interaction between the bacterial biofilm adherent to the tooth surfaces and the cells of the periodontal tissues (Alfano, 1974). GCF plays a special part in maintaining the structure of junctional epithelium and in the antimicrobial defence of periodontium and acts as a medium for the transport of bacterial products into and host derived products out of the periodontal environment (Ebersole, 2003;

Krasse, 1996). The collection of GCF is a non-invasive, safe procedure, and perhaps the most applicable aid for analysis for development of diagnostic markers(Lamster et al., 1988), since it is closely approximated to the tissues where periodontal disease begins and consequently opens to the whole body (Champagne et al., 2003). Because GCF composition reflects the nature and amplitude of the host response to the microbial plaque challenge, determination of GCF constituent levels may help identify subjects undergoing a transition from health to disease (Bostanci et al., 2007). The circulating GCF proteome holds great promise as a reservoir of information that will be applicable for diagnosis of periodontal disease.

The identification of the composition of GCF as opposed to that of serum or saliva has been more challenging due to its small sample volume. Initial protein analysis of GCF has been elucidated using techniques such as crossed immunoelectrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis coupled with mass spectrometry (MS) and ELISA (Estreicher et al., 1996; Golub and Kleinberg, 1976; Pisano et al., 2005). Using these approaches, more than 60 individual components of GCF have been assessed as candidate markers of disease progression (Lamster and Ahlo, 2007; Ozmeric, 2004; Uitto, 2003). Specifically, plasma proteins, bacterial and host enzymes, various inflammatory mediators have been among the myriad components studied. Although these strategies have provided some insights, it is acknowledged that studying individually these markers is of limited value. To date, however, there is a lack of information regarding proteomic approaches and sample processing in order to analyse the expression of various proteins present in GCF. We anticipate that integrated analysis of the proteome of GCF is required to dissect fully the molecular

mechanisms responsible for periodontal diseases and consequently linked systemic diseases such as CVD and diabetes mellitus (DM).

New developments in proteomic analysis technology could allow for successful analysis of the full complement of proteins in GCF. Recent reports employing label-free quantitative liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS) have demonstrated that protein profiles of oral samples such as saliva may be useful for diagnosis of DM(Rao et al., 2009) **.** More recently, a new variant of label-free quantification known as LC/MS^{E} was introduced for quadrupole time-of-flight (Q-Tof) mass spectrometers. For this method, alternating scans of low collision energy and elevated collision energy during LC/ MS analysis are used to obtain both protein quantification and protein identification data in a single sample (Silva et al., 2006; Silva et al., 2005). This technology offers unique advantages including reduced sample consumption, improved detection sensitivity and enhanced data quality for proteomic studies. The aim of this study was to perform a comprehensive analysis of the GCF exudatome from healthy and periodontally diseased sites by LC/MS^{E}. The results of the present study suggest that the current technology, which requires only a small volume of fluid, is an appropriate platform for analysis of GCF proteome and allowed us to uncover a number of proteins that were not previously known to be present in GCF.

Research Design and Methods

Subjects. From a case-control study, 10 subjects were chosen for GCF proteome analysis. The study had been reviewed and approved by the Joint UCL/UCLH Committees on the Ethics of Human Research (REF:05/Q0502/84). Subjects with

chronic systemic or oral infections, pregnant or lactating, receiving systemic medications meeting, or current smokers were excluded. The selection of the subjects was made according to the criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions(Armitage, 1999). Patients fulfilling the inclusion criteria were provided with an written informed consent. 40 GCF samples were obtained from healthy subjects (n=5) and patients with generalized aggressive periodontitis (G-AgP) (n=5). Clinical periodontal parameters including probing pocket depth (PPD), clinical recession level (REC), full-mouth bleeding scores (FMBS) and plaque scores (FMPS) were assessed by two calibrated examiners at six sites/tooth with a UNC-15 periodontal probe with a force of 0.3N. Presence or absence of bleeding on probing (BOP) and plaque accumulation was recorded in a dichotomous way. If bleeding occurred within 15 sec after retrieval of the probe, the site is recorded as BOP-positive (Muhlemann and Son, 1971). Presence of visible plaque after running the probe along the gingival margin was recorded as positive. The mean FMPS and FMPS are given as percentage (Table 1). The healthy group consisted of 3 females and 2 males ranging in age from 27 to 38 years with a mean age of 32.4 ± 3.9 years. They exhibited PPD < 3 mm, and no radiographic evidence of alveolar bone loss. The G-AgP group consisted of 3 females and 2 males between the ages of 25 and 44 years (mean of 34.6 ± 6.9 years) with a generalized pattern of severe periodontal tissue destruction and loss of periodontal support inconsistent with age and plaque levels (Armitage, 2004). They exhibited clinical attachment loss ≥ 5 mm on eight or more teeth; at least three of those were other than central incisors or first molars. Statistical analysis for clinical and demographic data included normality testing using the Kolmogorov-Smirnov test and Student *t* tests or Mann-Whitney tests as appropriate. A *P* value of less than 0.05 was considered

statistically significant. All data analyses were performed using the SPSS 12.0 software.

Sample Collection and Processing. The GCF samples were collected from the four deepest periodontal pockets (one site per quadrant) in G-AgP patients. In the healthy group, the GCF samples were collected from four standard sites (one first molars and one central incisors in each jaw) exhibiting PPD up to 3 mm without bleeding on probing or radiographic evidence of bone loss. The selected sites were isolated from salivary contamination. A sterile Periopaper strip (ProFlow Inc. Amityville, NY) was then gently inserted into the periodontal pocket and left in place for 30 seconds (Lamster, 1997). Mechanical irritation avoided and the strips contaminated with blood were discarded.

The GCF sample volume was measured with a calibrated Periotron 8000 (Periotron 8000, Proflow, Inc., Amityville, NY, USA) and then the readings were converted to an actual volume (µl) by reference to the standard curve. Four samples from each subject were pooled in an Eppendorf tube prior to storing at -80°C prior to analysis. On the day of the analysis, the GCF samples were re-eluted in 100µl PBS, then centrifuged at 13000 x g for 15 min.

MSe Label Free Quantitation. 10 µl of 100mM Tris, pH 7.8, containing 6M urea was added to 10µl of eluted GCF sample protein and was left shaking at room temperature for 1hr. Disulphide bridges were reduced by the addition of 3 µl of 100mM Tris-HCL, pH 7.8 containing 5M DTE and incubation at room temp for 60 min. Free thiol groups were carboamidomethylated followed by incubation with 6 µl

of 100mM Tris-HCL, pH 7.8 containing 5M iodoacetamide. The solution was then diluted with 155 µl H_2O, vortexed and 2 µg of sequence grade trypsin (Sigma Aldrich, UK) added to the solution. Samples were incubated overnight at 37 ^{0}C in a water bath. After incubation, digested peptides were extracted using self made C8 resin columns. Peptides in aqueous solution were loaded onto the columns washed with 0.1% TFA and eluted in 50% ACN 0.1%TFA. Eluted peptides were then dried down using a Jouan R10.22 Speeedvac. Dried GCF peptides were then re-suspended in 3% ACN with 50fmol of MassPrep^{TM} yeast enolase digestion standard (Waters, Milford, USA) prior to LC/MS^E analysis. Each sample was analyzed in two independent experimental runs. The LC/MS^E analysis was performed using 10 µl of the final tryptic digest. A standard yeast enolase digest was run before and after the samples to monitor sensitivity.

GCF proteins were identified and quantitated by direct analysis of the reaction mixture described above. All analyses were performed using a nanoAcquity HPLC and QTOF Premier mass spectrometer (Waters Corporation, Manchester, UK). Peptides were trapped and desalted prior to reverse phase separation using a Symmetry C18 5µm, 5mm X 300µm pre-column. Peptides were then separated prior to mass spectral analysis using a 15cm X 75 µm C18 reverse phase analytical column. Peptides were loaded onto the pre-column at a flow rate of 4µl / min in 0.1% formic acid for a total time of 4 mins. Peptides were eluted off the pre-column and separated on the analytical column using a gradient of 3-40% acetonitrile [0.1% formic acid] over a period of 90 min and at a flow rate of 300 nl/min. The column was washed and re-generated at 300 nl/min for 10 min using a 99% acetonitrile [0.1%] rinse. After all non-polar and non peptide material was removed the column was re-equilibrated at

the initial starting conditions for 20 min. All column temp were maintained at 35 ^{0}C. Mass accuracy was maintained during the run using a lock spray of the peptide [glu1]-fibrinopeptide B delivered through the auxiliary pump of the nanoAcquity at a concentration of 300 fmol/l and at a flow rate of 300 nl/min.

Peptides were analysed in positive ion mode using a Q-Tof Premier mass spectrometer (Waters Corp., Manchester, UK) and was operated in v-mode with a typical resolving power of 10,000 FWHM. Prior to analyses, the TOF analyser was calibrated using [glu1]-fibrinopeptide B fragments obtained using collision energy of 25 eV and over the mass range 50-2000 m/z. Post calibration of data files were corrected using the doubly charged precursor ion of [glu1]-fibrinopeptide B (785.8426 m/z) with a sampling frequency of 30 sec. Accurate mass LC-MS data were colleted in a data independent and alternating, low and high collision energy mode. Each low/high acquisition was 1.5s with 0.1 sec interscan delay. Low energy data collections were performed at constant collision energy of 4 eV, high collision energy acquistions were performed using a 15-40eV ramp over a 1.5 sec time period and a complete low/high energy acquisition achieved every 3.2 sec.

Data Processing and Database Searching.

ProteinLynx GlobalServer version 2.3.5 was used to process all data acquired. Protein identifications were obtained by searching UniProt/Swiss-Prot databases to which the sequence of yeast enolase was added manually. Protein identification from the low/high collision spectra for each sample was processed using a hierarchical approach where more than three fragment ions per peptide, seven fragment ions per protein and more than two peptides per protein had to be matched. Since single-

peptide protein identifications are more likely to represent false-positive data points, all proteins with greater than two peptides identified with high confidence were considered for protein quantification. Carboamidomethylation was set as a fixed modification and oxidation was set as variable modification. The average intensity value of the top three ionizing peptides to yeast enolase was used to convert the average intensity of the top three ionizing peptides for proteins to the corresponding absolute quantity of protein loaded on column. The lowest detectable concentration was 0.1 fmol. Quantitative data in fmol was analysed in Microsoft Excel.

Enzyme-linked immunosorbent assay (ELISA)

To validate the LC/MS^E findings, neutrophil defensin (HNP) was selected as marker. HNP levels were measured in the same GCF samples by a commercially available ELISA kit, according to the manufacturer's instructions (HyCult Biotechnology, Uden, The Netherlands). The absorbance was measured at 450 nm with a wavelength correction set at 570nm, using a microplate reader (Tecan Infinite 200, Tecan Group Ltd, Switzerland). A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel). The values of the samples were assigned according to the generated standard curve. The sensitivity of the assay was 0.41 pg/ml.

Results and Discussion

Identification and Quantification of GCF Proteins.

In the present study we used label-free quantitative LC/MS^E analysis approach to characterise multiple proteins simultaneously in the GCF samples collected from healthy subjects and patients with a generalized severe form of periodontal disease.

There are several reports in the literature that have investigated single potential biomarkers by employing immunoassays using this biological material (Uitto, 2003). To our knowledge, this is the first report to make use of the label-free LC/MS^E approach for full identification and quantification of proteins in the GCF, unveiling a large number of proteins that were not previously known to be present in GCF.

The demographic and clinical data are shown in Table 1 and Table 2. There were no significant differences between the groups in terms of gender and age (Table 1). Gender-mixing in a small sample size would have been a limitation if we sought to verify disease markers in such a small sample. Since the aim of the study was to evaluate if this particular method is an appropriate platform for detection of proteins in gingival crevicular fluid, a mixed gender sample in both groups is potentially more suitable in this context.

When full mouth clinical parameters of the studied subjects were compared, the G-AgP group had significantly higher mean PPD, REC and FMBS ($p<0.05$) (Table 1). G-AgP groups had also significantly higher mean PPD, REC and BS scores of sampling sites than the healthy group ($P<0.05$) (Table 2). In contrast, no significant differences were detected between the two groups regarding the plaque levels ($P>0.05$).

A total of 154 proteins with at least two unique peptide hits, validated from two different runs, were identified and quantified in the two subject groups (Table 3). When the identified proteins are categorized based on their origin, the disease group exhibited an increase in the proportion of bacterial, yeast, and viral protein, at the expense of human-derived proteins (Figure 1 A-B). This is rationale, as the

microorganism load is expected to be higher in disease. The pie chart in Figure 2 A-B also shows the functional classification of the GCF human-origin proteome based on their annotations in the SwissProt database. The annotation of the biological process revealed no quantitative changes in the proportions of human proteins involved in immune response, transportation, metabolism, signal transduction and enzyme regulation. However, the percentage of proteins involved in cell differentiation was decreased in disease, while that of cell organization was increased. The plausible explanation is that in the disease status, the cells undergo morphological alteration in response to the microorganism challenge, while the cell differentiation mechanisms are diminished, denoting a impeded tissue formation. Although the percentage of immune response regulated proteins was similar in health and disease, qualitative differences exist, as elaborated further.

Greater number of proteins were identified in diseased samples (n=115) than healthy ones (n=88). Interestingly, a few markers were found exclusively in either group, whereas a greater number of them were found present in both groups, albeit at different relative levels. The putative roles of these proteins in relation to periodontal health/disease are discussed below. There was also great variability of GCF analytes between the subjects, even within the same group. The mean +/- SD value derives from five individual samples in each group. The fact that some SDs are 0.0 is due to the protein was detected only in one single sample in that group. This suggests that the protein composition of GCF is rather individual specific, and pooling of the samples from the same clinical presentation based on diagnosis may result in loss of important information. Since the sample size was small with the large SD, it was not possible to perform further statistical analysis.

Among the large number of proteins that were found in both diseased and healthy subjects, the most abundant proteins were human albumin, followed by immunoglobulins and various keratins. Previously, around 70 individual components of GCF have been assessed as potential markers for the disease (Lamster and Ahlo, 2007). These have been mostly proteolytic enzymes and pro-inflammatory cytokines. However, in the present study we rarely identified cytokines. This could potentially be due to their low concentrations or low molecular weight being masked by the presence of albumin or immunoglobulins (Jacobs et al., 2005). While many studies employing proteomic technologies apply techniques to deplete serum and plasma of high-abundance proteins prior to analysis, recent studies have shown that these practices may concomitantly remove potentially important diagnostic information. For instance, human albumin is a protein vehicle within the blood stream that binds to hormones, cytokines, and lipoproteins (Dea et al., 2002). In the present study, albumin concentration was the highest among all detected proteins which was at the level of 220.4 ± 230.9 fmol in health and 241.8 ± 240.4 fmol in periodontitis. On the contrary to cytokines, we did identify a number of enzymes associated with polymorphonuclear neutrophils (PMNs), such as matrix metalloproteinase-8 (MMP-8), cathepsin G and myeloperoxidase, all of which were previously identified and proposed as candidate diagnostic markers for periodontal disease (Sorsa et al., 1999; Tervahartiala et al., 1996; Yamalik et al., 2000). Interestingly, MMP-8 and cathepsin G were not detected in GCF samples from healthy sites, but were present in periodontitis. MMPs are a family of zinc-dependent endopeptidases that are expressed in many inflammatory conditions including periodontitis and contribute to connective tissue breakdown. The fact that some other MMPs were not detected by this method,

despite that they have been detected by conventional protein detection assays suggests that the current method has its limitations. In the case of relatively low abundant proteins, such as MMPs and potentially cytokines, a drop of peptide signal is still a limiting factor for detection. Selective enrichment or depletion of the highly abundant GCF proteins prior to mass spectrometric analysis may help overcome this problem (Freije et al., 2008). Although the current approach was quite successful, clearly there is more room for improvement identifications of whole GCF proteins.

Myeloperoxidase was detected only in one out of five healthy samples, but in all periodontitis samples, which was on average 2-fold greater. Other proteins in the diseased group were most notably heat shock protein 70 kDa (HSP70). HSP70 has been shown to be an activator of the innate immune system (Kiang and Tsokos, 1998) and its release can be induced by periodontal pathogens (Saba et al., 2007).

Among the proteins not previously identified in GCF, the present data indicates that L-plastin (Plastin-2), also known as lymphocyte cytosolic protein 1, is of special interest. This protein was not detected in any of the five healthy samples, whereas it was present in 4 out of 5 diseased samples, with a mean concentration of 15.6 ± 12.1 fmol. L-plastin belongs to the actin-binding protein family, which is found in cells of the hematopoetic origin such as leukocytes. A role for L-plastin in the regulation of leukocyte adhesion and PMN signal transduction has been suggested (Jones et al., 1998). However, there is no known literature suggesting a role for L-plastin in periodontal disease. Higher concentrations of this protein in GCF may facilitate recruitment of PMNs at sites of inflammation. This may be relevant in view of the

potential constitutive PMN hyper-reactivity in periodontitis patients (Matthews et al., 2007).

In contrast, cystatin B and neutrophil defensins were not detected in any of the periodontitis group samples. The average concentrations in health were 11.7 ± 10.4 fmol for cystatin B, and 14.8 ± 3.4 fmol for neutrophil defensin. A commercially available ELISA for human neutrophil defensin was also employed to validate the findings from the proteomic analysis. Accordingly, human neutrophil defensin levels were under detection limit in the periodontal disease samples, whereas the mean concentration of the healthy samples was 125.13 ± 129.04 ng/ml. These findings are consistent with the LC/MS^E data, as well as previously published data (Lundy et al., 2005). The fact that neutrophil defensin, which has an antimicrobial properties was absent in the diseased samples supports the idea that the progression of periodontal disease could be linked to a decrease in the defensin barrier (Lundy et al., 2005). To this extent, defensins are suggested to have an important protective role for the host immune response to infection by periodontal pathogens (Brogden et al., 2003). Cystatin B, a neutral cysteine proteinase inhibitor, belonging to the cystatin family that minimizes proteolysis-associated tissue damage in inflammation (Chen et al., 1998; Dickinson, 2002). Proteins belong to the cystatin family such cystatin A, but not cystatin B, C or S, have previously been detected in GCF by immunoblotting or ELISA (Blankenvoorde et al., 1997). Cystatin B in particular could have been derived from serum, or from local cellular secretions such as monocytes, epithelial cells and PMNs (Jarvinen et al., 1987).

Members of the calcium binding protein family including Calgranulin B, S100-P and Annexins were also differentially present in GCF. The S100 family proteins are thought to modulate biological activity via calcium binding and they expressed by macrophages and by epithelial cells in acutely inflamed tissues (Marenholz et al., 2004). Annexins are a group of Ca^{2+}-binding proteins that are associated with inflammatory and defense responses (Perretti and D'Acquisto, 2009). In the present study, annexin-1 appears to be more associated with a healthy status, since it was more frequently detected in health (four out of five samples in health vs two out of five in disease) and at 5-fold higher levels compared to disease. Likewise, Annexin-2 was not detected in any of the disease samples, but was found in two out of five healthy samples, at a mean concentration of 3.5 ± 1.7 fmol. Annexin-1 has long been suggested to be a critical endogenous negative regulator of pro-inflammatory mediators including IL-1, IL-6 and cyclooxygenase-2 (Parente and Solito, 2004). In several disease models, such as experimental animal models of endotoxemia or arthritis, absence of Annexin-1 has been associated with increased levels of cytokines and exacerbation of acute inflammation (Damazo et al., 2005; Yang et al., 1999). Therefore, it is tempting to postulate that the higher levels of annexins in GCF may be associated with a healthy periodontal status, or that reduced levels or absence may have a potential to explain many aspects of inflammatory status in periodontal disease. These proteins, which were mainly found in health, may have an important protective role and their function in periodontal disease needs to be addressed in a larger cohort of patient samples.

Other proteins of the immune system, such as α_1-antitrypsin, α_1-1-antichymotrypsin, α-1-acid glycoprotein, immunoglobulins (Ig) were also identified. α_1-antitrypsin was

detected in two out of five healthy samples (30.0 ± 15.2 fmol), but was found in only one disease sample (2.4 ± 0.0 fmol). Presence of α-1-antitrypsin in GCF has been previously reported (Cox et al., 2006). Deficiencies in α-1-antitrypsin are known to predispose individuals to destructive inflammatory disease, including periodontitis (Peterson and Marsh, 1979; Yang et al., 2000). The balance between proteases and protease inhibitors is critical for maintenance of healthy tissue, and imbalances may lead to tissue destruction. The α-1-acid glycoprotein is one of the acute phase proteins, which is increased in response bacterial infection. In addition, its serum concentrations remain stable under physiological conditions (Fournier et al., 2000; Hochepied et al., 2003).A comparison of the healthy samples versus the diseased ones showed that α-1-acid glycoprotein 1 levels were up-regulated by 2-fold in periodontitis. Higher levels of this molecule have been previously reported in the sera of subjects with periodontitis (Glurich et al., 2002).

Confirming previous reports (Eley and Cox, 2003; Mooney and Kinane, 1997; Reinhardt et al., 1989; Wilton et al., 1993), GCF was enriched with immunoglobulins including four IgG subclass proteins and IgA. IgG concentrations were lower in GCF from patients with periodontitis than healthy ones. IgG1 protein was detected in both health and disease and was at highest concentration in healthy samples (86.2 ± 61.5 fmol). IgG3 was not present in any of the disease samples, whereas its mean concentration in health was 11.6 ± 0.0 fmol. IgA was present only in health (in 2 samples out of 5) with a mean concentration of 4.7 ± 2.5 fmol. Immunoglobulins arrive at the periodontal lesion site from both systemic and local tissue sources. The relative abundance of immunoglobulins in GCF may play a crucial role in the local defence mechanisms.

Notably, twenty-five different keratins were identified and quantified in GCF. However, this is not surprising since a great complexity of cytokeratins have been shown to be expressed in the gingiva or periodontal pockets (Mackenzie and Gao, 1993; Mackenzie et al., 1991; Ouhayoun et al., 1985). Although it should not be excluded that keratins are usual contaminants from the air, skin or hair, in the present study this was controlled for by simultaneous digestion of all samples. The present results indicated different and distinctive patterns of keratin protein expression in healthy and diseased GCF. For instance, cytokeratins 12 and 23 were only present in GCF from healthy sites, whereas cytokeratin 1 was consistently found in all samples. Other cytokeratins were detected at variable levels, but clearly their concentrations were lower in periodontal disease compared to health. This may be expected since the normal turnover rate of healthy sulcular epithelium is known to be one of the most rapid of all the epithelial tissues (Rowat and Squier, 1986). Therefore, it would be expected that healthy GCF contains large number of cytokeratins resulting from this vigorous turnover. In periodontal disease, where the periodontal pocket epithelium is ulcerated, the presence of cytokeratins in GCF may be attributed to passive release due to epithelial cell detachment or lysis (Ouhayoun et al., 1990).

There were also proteins from bacteria, viruses and yeast present in the GCF, since protein identification searches were not only limited to human protein sequence database. Evidently, greater proportions of these organisms are present in the GCF samples from periodontal disease than healthy ones. The higher presence of viral proteins, such as herpes virus protein 2, in the diseased samples corroborates previous reports that support the involvement of viral infection in the pathogenesis of

periodontal disease (Slots, 2007). Interestingly, no proteins from putative periodontal pathogens were detected in any of the diseased samples, with one exception, the methylmalonyl-CoA mutase of *Porphyromonas gingivalis*, which is one of the major pathogens implicated in periodontitis (Curtis et al., 2001). Although previous studies have shown that *P. gingivalis* proteolytic enzymes can be detectable in GCF (Aas et al., 2007), the present approach may not be optimal for the detection of periodontal pathogens. Notably, *E. coli* proteins were detected in GCF of both healthy and periodontally diseased subjects. Since the oral cavity is not a natural habitat for *E. coli*, the presence of its proteins in GCF detected in the present study may reflect their transfer from non-oral sites via the serum route.

Conclusions

The findings of the present study demonstrate that label-free quantitative LC/MS^E based mass spectrometry technology is an appropriate platform for the analysis of GCF proteome. The protein content of GCF could potentially be used as a diagnostic aid in periodontal disease, since the characterisation of the GCF proteome will greatly increase the clinical importance of this biological exudate. The comprehensive analysis of GCF by the suggested approach may provide better understanding of the pathological processes involved in periodontal disease, and facilitate the development of accurate diagnostic and prognostic markers. Due to the interindividual variations of protein biomarkers in GCF, larger cohort studies are required to characterise the complete GCF proteome in health and disease, before its diagnostic and prognostic value reaches optimal full potential.

Abbreviations:

CVD, cardiovascular disease; DM, diabetes mellitus; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; G-AgP: generalized aggressive periodontitis; GCF, gingival crevicular fluid; GO, gene ontology; IL, interleukin; LC, liquid chromatography; LC-MS/MS, liquid chromatography/tandem mass spectrometry; PD, periodontal disease; PPD, probing pocket depth; REC, recession level; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Q-Tof, quadrupole time-of-flight.

Acknowledgement

This study was partially supported by the research account of the Periodontology Unit and UCLH/UCL Comprehensive Biomedicine Centre. Dr. NB was employed as Walport Clinical Academic Lecturer at Eastman Dental Institute, funded by the NIHR.

References

Aas, J. A., Barbuto, S. M., Alpagot, T., Olsen, I., Dewhirst, F. E., and Paster, B. J. (2007). Subgingival plaque microbiota in HIV positive patients. J Clin Periodontol *34*, 189-195.

Albandar, J. M., and Rams, T. E. (2002). Risk factors for periodontitis in children and young persons. Periodontol 2000 *29*, 207-222.

Alfano, M. C. (1974). The origin of gingival fluid. J Theor Biol *47*, 127-136.

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. Ann Periodontol *4*, 1-6.

Armitage, G. C. (2004). Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000 *34*, 9-21.

Blankenvoorde, M. F., Henskens, Y. M., van der Weijden, G. A., van den Keijbus, P. A., Veerman, E. C., and Nieuw Amerongen, A. V. (1997). Cystatin A in gingival crevicular fluid of periodontal patients. J Periodontal Res *32*, 583-588.

Bostanci, N., Ilgenli, T., Emingil, G., Afacan, B., Han, B., Toz, H., Atilla, G., Hughes, F. J., and Belibasakis, G. N. (2007). Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. J Clin Periodontol *34*, 370-376.

Brogden, K. A., Heidari, M., Sacco, R. E., Palmquist, D., Guthmiller, J. M., Johnson, G. K., Jia, H. P., Tack, B. F., and McCray, P. B. (2003). Defensin-induced adaptive immunity in mice and its potential in preventing periodontal disease. Oral Microbiol Immunol *18*, 95-99.

Brown, L. J., and Loe, H. (1993). Prevalence, extent, severity and progression of periodontal disease. Periodontol 2000 *2*, 57-71.

Champagne, C. M., Buchanan, W., Reddy, M. S., Preisser, J. S., Beck, J. D., and Offenbacher, S. (2003). Potential for gingival crevice fluid measures as predictors of risk for periodontal diseases. Periodontol 2000 *31*, 167-180.

Chen, H. Y., Cox, S. W., and Eley, B. M. (1998). Cathepsin B, alpha2-macroglobulin and cystatin levels in gingival crevicular fluid from chronic periodontitis patients. J Clin Periodontol *25*, 34-41.

Cox, S. W., Rodriguez-Gonzalez, E. M., Booth, V., and Eley, B. M. (2006). Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis. J Periodontal Res *41*, 477-485.

Curtis, M. A., Aduse-Opoku, J., and Rangarajan, M. (2001). Cysteine proteases of Porphyromonas gingivalis. Crit Rev Oral Biol Med *12*, 192-216.

Damazo, A. S., Yona, S., D'Acquisto, F., Flower, R. J., Oliani, S. M., and Perretti, M. (2005). Critical protective role for annexin 1 gene expression in the endotoxemic murine microcirculation. Am J Pathol *166*, 1607-1617.

Danesh, J., Collins, R., and Peto, R. (1997). Chronic infections and coronary heart disease: is there a link? Lancet *350*, 430-436.

Dea, M. K., Hamilton-Wessler, M., Ader, M., Moore, D., Schaffer, L., Loftager, M., Volund, A., and Bergman, R. N. (2002). Albumin binding of acylated insulin (NN304) does not deter action to stimulate glucose uptake. Diabetes *51*, 762-769.

Dickinson, D. P. (2002). Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. Crit Rev Oral Biol Med *13*, 238-275.

Ebersole, J. L. (2003). Humoral immune responses in gingival crevice fluid: local and systemic implications. Periodontol 2000 *31*, 135-166.
Eley, B. M., and Cox, S. W. (2003). Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. Periodontol 2000 *31*, 105-124.
Estreicher, A., Broggiato, A., Duroux, P., Andersen, E., and Cimasoni, G. (1996). Low molecular-weight proteins in human gingival crevicular fluid. Arch Oral Biol *41*, 733-738.
Fournier, T., Medjoubi, N. N., and Porquet, D. (2000). Alpha-1-acid glycoprotein. Biochim Biophys Acta *1482*, 157-171.
Freije, R., Klein, T., Ooms, B., Kauffman, H. F., and Bischoff, R. (2008). An integrated high-performance liquid chromatography-mass spectrometry system for the activity-dependent analysis of matrix metalloproteases. J Chromatogr A *1189*, 417-425.
Glurich, I., Grossi, S., Albini, B., Ho, A., Shah, R., Zeid, M., Baumann, H., Genco, R. J., and De Nardin, E. (2002). Systemic inflammation in cardiovascular and periodontal disease: comparative study. Clin Diagn Lab Immunol *9*, 425-432.
Golub, L. M., and Kleinberg, I. (1976). Gingival crevicular fluid: a new diagnostic aid in managing the periodontal patient. Oral Sci Rev, 49-61.
Hochepied, T., Berger, F. G., Baumann, H., and Libert, C. (2003). Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. Cytokine Growth Factor Rev *14*, 25-34.
Jacobs, J. M., Adkins, J. N., Qian, W. J., Liu, T., Shen, Y., Camp, D. G., 2nd, and Smith, R. D. (2005). Utilizing human blood plasma for proteomic biomarker discovery. J Proteome Res *4*, 1073-1085.
Jarvinen, M., Rinne, A., and Hopsu-Havu, V. K. (1987). Human cystatins in normal and diseased tissues--a review. Acta Histochem *82*, 5-18.
Jones, S. L., Wang, J., Turck, C. W., and Brown, E. J. (1998). A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function. Proc Natl Acad Sci U S A *95*, 9331-9336.
Kiang, J. G., and Tsokos, G. C. (1998). Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther *80*, 183-201.
Krasse, B. (1996). Serendipity or luck: stumbling on gingival crevicular fluid. J Dent Res *75*, 1627-1630.
Lamster, I. B. (1997). Evaluation of components of gingival crevicular fluid as diagnostic tests. Ann Periodontol *2*, 123-137.
Lamster, I. B., and Ahlo, J. K. (2007). Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. Ann N Y Acad Sci *1098*, 216-229.
Lamster, I. B., Oshrain, R. L., Fiorello, L. A., Celenti, R. S., and Gordon, J. M. (1988). A comparison of 4 methods of data presentation for lysosomal enzyme activity in gingival crevicular fluid. J Clin Periodontol *15*, 347-352.
Lundy, F. T., Orr, D. F., Shaw, C., Lamey, P. J., and Linden, G. J. (2005). Detection of individual human neutrophil alpha-defensins (human neutrophil peptides 1, 2 and 3) in unfractionated gingival crevicular fluid--a MALDI-MS approach. Mol Immunol *42*, 575-579.
Mackenzie, I. C., and Gao, Z. (1993). Patterns of cytokeratin expression in the epithelia of inflamed human gingiva and periodontal pockets. J Periodontal Res *28*, 49-59.
Mackenzie, I. C., Rittman, G., Gao, Z., Leigh, I., and Lane, E. B. (1991). Patterns of cytokeratin expression in human gingival epithelia. J Periodontal Res *26*, 468-478.

Marenholz, I., Heizmann, C. W., and Fritz, G. (2004). S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun *322*, 1111-1122.
Matthews, J. B., Wright, H. J., Roberts, A., Ling-Mountford, N., Cooper, P. R., and Chapple, I. L. (2007). Neutrophil hyper-responsiveness in periodontitis. J Dent Res *86*, 718-722.
Mooney, J., and Kinane, D. F. (1997). Levels of specific immunoglobulin G to Porphyromonas gingivalis in gingival crevicular fluid are related to site disease status. Oral Microbiol Immunol *12*, 112-116.
Muhlemann, H. R., and Son, S. (1971). Gingival sulcus bleeding--a leading symptom in initial gingivitis. Helv Odontol Acta *15*, 107-113.
Ouhayoun, J. P., Goffaux, J. C., Sawaf, M. H., Shabana, A. H., Collin, C., and Forest, N. (1990). Changes in cytokeratin expression in gingiva during inflammation. J Periodontal Res *25*, 283-292.
Ouhayoun, J. P., Gosselin, F., Forest, N., Winter, S., and Franke, W. W. (1985). Cytokeratin patterns of human oral epithelia: differences in cytokeratin synthesis in gingival epithelium and the adjacent alveolar mucosa. Differentiation *30*, 123-129.
Ozmeric, N. (2004). Advances in periodontal disease markers. Clin Chim Acta *343*, 1-16.
Parente, L., and Solito, E. (2004). Annexin 1: more than an anti-phospholipase protein. Inflamm Res *53*, 125-132.
Perretti, M., and D'Acquisto, F. (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol *9*, 62-70.
Peterson, R. J., and Marsh, C. L. (1979). The relationship of alpha1-antitrypsin to inflammatory periodontal disease. J Periodontol *50*, 31-35.
Pisano, E., Cabras, T., Montaldo, C., Piras, V., Inzitari, R., Olmi, C., Castagnola, M., and Messana, I. (2005). Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. Eur J Oral Sci *113*, 462-468.
Rao, P. V., Reddy, A. P., Lu, X., Dasari, S., Krishnaprasad, A., Biggs, E., Roberts, C. T., and Nagalla, S. R. (2009). Proteomic identification of salivary biomarkers of type-2 diabetes. J Proteome Res *8*, 239-245.
Reinhardt, R. A., McDonald, T. L., Bolton, R. W., DuBois, L. M., and Kaldahl, W. B. (1989). IgG subclasses in gingival crevicular fluid from active versus stable periodontal sites. J Periodontol *60*, 44-50.
Rowat, J. S., and Squier, C. A. (1986). Rates of epithelial cell proliferation in the oral mucosa and skin of the tamarin monkey (Saguinus fuscicollis). J Dent Res *65*, 1326-1331.
Saba, J. A., McComb, M. E., Potts, D. L., Costello, C. E., and Amar, S. (2007). Proteomic mapping of stimulus-specific signaling pathways involved in THP-1 cells exposed to Porphyromonas gingivalis or its purified components. J Proteome Res *6*, 2211-2221.
Silva, J. C., Denny, R., Dorschel, C., Gorenstein, M. V., Li, G. Z., Richardson, K., Wall, D., and Geromanos, S. J. (2006). Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome: a sweet tale. Mol Cell Proteomics *5*, 589-607.
Silva, J. C., Denny, R., Dorschel, C. A., Gorenstein, M., Kass, I. J., Li, G. Z., McKenna, T., Nold, M. J., Richardson, K., Young, P., and Geromanos, S. (2005). Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem *77*, 2187-2200.

Slots, J. (2007). Herpesviral-bacterial synergy in the pathogenesis of human periodontitis. Curr Opin Infect Dis *20*, 278-283.
Sorsa, T., Mantyla, P., Ronka, H., Kallio, P., Kallis, G. B., Lundqvist, C., Kinane, D. F., Salo, T., Golub, L. M., Teronen, O., and Tikanoja, S. (1999). Scientific basis of a matrix metalloproteinase-8 specific chair-side test for monitoring periodontal and peri-implant health and disease. Ann N Y Acad Sci *878*, 130-140.
Tervahartiala, T., Konttinen, Y. T., T, I. N., Hayrinen-Immonen, R., Ding, Y., and Sorsa, T. (1996). Cathepsin G in gingival tissue and crevicular fluid in adult periodontitis. J Clin Periodontol *23*, 68-75.
Uitto, V. J. (2003). Gingival crevice fluid--an introduction. Periodontol 2000 *31*, 9-11.
Wilton, J. M., Bampton, J. L., Hurst, T. J., Caves, J., and Powell, J. R. (1993). Interleukin-1 beta and IgG subclass concentrations in gingival crevicular fluid from patients with adult periodontitis. Arch Oral Biol *38*, 55-60.
Yamalik, N., Caglayan, F., Kilinc, K., Kilinc, A., and Tumer, C. (2000). The importance of data presentation regarding gingival crevicular fluid myeloperoxidase and elastase-like activity in periodontal disease and health status. J Periodontol *71*, 460-467.
Yang, P., Tremaine, W. J., Meyer, R. L., and Prakash, U. B. (2000). Alpha1-antitrypsin deficiency and inflammatory bowel diseases. Mayo Clin Proc *75*, 450-455.
Yang, Y., Hutchinson, P., and Morand, E. F. (1999). Inhibitory effect of annexin I on synovial inflammation in rat adjuvant arthritis. Arthritis Rheum *42*, 1538-1544.

Figure Legends

Figure 1

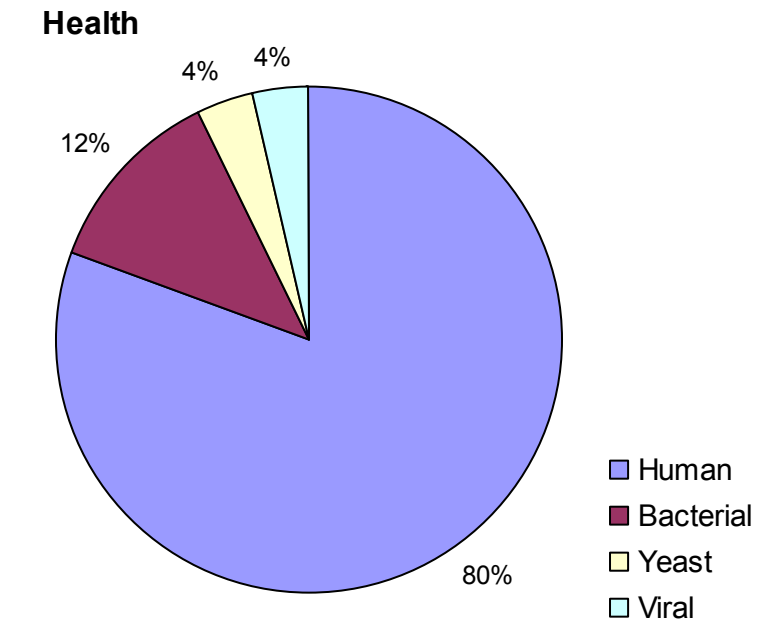
The pie-charts show the distribution of the proportion of gingival crevicular fluid (GCF) proteins according to their source of origin, in health (A) and disease (B). The proportions are expressed as percentage (%) of the total protein in each group. Four soureces of origin were identified, namely human, bacetrial, yeast and viral. An increase in the proportion of bacterial, viral and yeast origin proteins was detected in disease, compared to health.

Figure 2

The pie-charts show the distribution of the proportion of gingival crevicular fluid (GCF) proteins according to their biological function, in health (A) and disease (B). The proportions are expressed as percentage (%) of the total protein in each group. The sorting of protein function was based on the UniProt/Swiss-Prot database annotations. A functional classification was assigned to each unique protein, and the total number and percentage of proteins in each class were determined.

Figure 1

A)


Health
4%
4%
12%
80%
Human
Bacterial
Yeast
Viral

B)

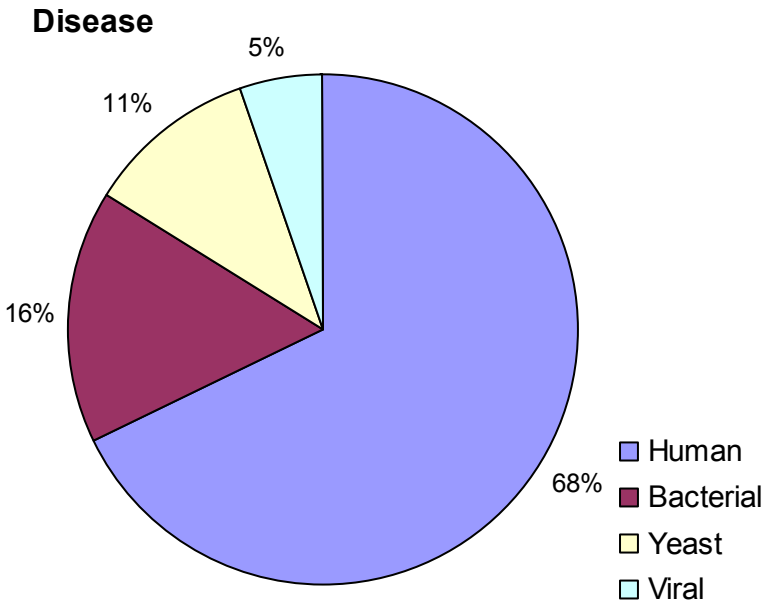
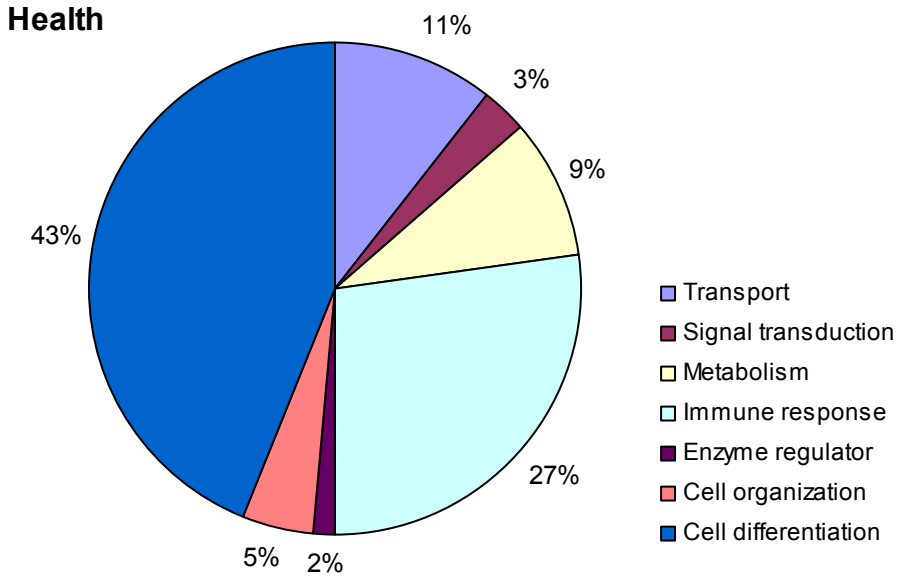

Disease
5%
11%
16%
68%
Human
Bacterial
Yeast
Viral

Figure 2

A)


Health
11%
3%
9%
43%
27%
5%
2%
Transport
Signal transduction
Metabolism
Immune response
Enzyme regulator
Cell organization
Cell differentiation

B)

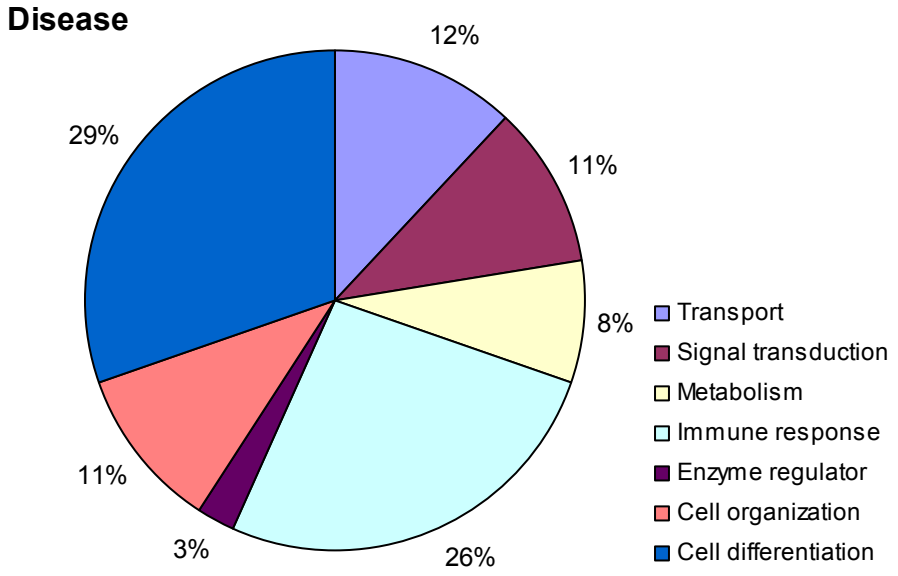

Disease
12%
11%
29%
8%
11%
3%
26%
Transport
Signal transduction
Metabolism
Immune response
Enzyme regulator
Cell organization
Cell differentiation

Table 1: Demographic and full mouth clinical parameters of the studied subjects (five healthy and five patients with generalized aggressive periodontitis (G-AgP) (mean ± SD)

	Healthy (n=5)	**G-AgP (n=5)**
Gender F:M	3:2	3:2
Age	32.4 ± 3.9	34.6 ± 6.9
PPD (mm)	1.7 ± 0.6	4.6 ± 2.3*
REC (mm)	0.009 ± 0.1	0.48 ± 1.17*
FMPS(%)	43.8 ± 49.6	57.3 ± 31.5
FMBS (%)	9.05 ± 28.7	71.9 ± 23.01*

To determine the clinical periodontal status, all subjects had a clinical periodontal examination including the measurement of probing pocket depth (PPD), clinical recession level (REC), full-mouth bleeding (FMBS) and full-mouth plaque scores (FMPS) at six sites around each tooth with a manual probe as detailed in Materials and Methods. The asterisk (*) indicates significant difference from the healthy group (Mann–Whitney U-test, $p<0.05$).

Table 2: Clinical parameters of the sampling areas in the study groups (mean ± SD)

	Healthy	**G-AgP**
PPD sites (mm)	1.85 ± 0.36	6.65 ± 2.70*
REC sites (mm)	0	0.55 ± 1.3*
PS (%)	60 ± 45.4	65 ± 41.8
BS (%)	0	85 ± 13.6*
GCF (μl)	0.16 ± 0.09	0.58 ± 0.30*

The gingival crevicular fluid (GCF) samples were collected from the four deepest periodontal pockets (one site per quadrant) in G-AgP patients. In the healthy group, the GCF samples were collected from four standard sites (one first molars and one central incisors in each jaw) exhibiting PPD up to 3 mm without bleeding on probing and radiographic evidence of alveolar bone loss. The GCF sample volume was measured with a calibrated Periotron 8000. PPD, probing pocket depth; REC, clinical recession level; BS, bleeding score; PS, plaque score. The asterisk (*) indicates significant difference from the healthy group (Mann–Whitney U-test, $p<0.05$).

Table 3

Gingival crevicular fluid Proteins Identified Using LC/MS^{E}

GCF proteins were identified and quantitated in healthy subjects (n=5) and patients with generalized aggressive periodontitis (n=5) by LC/MS^{E}. Proteins having at least two peptide identifications found in human GCF are listed by alphabetical order with their UniProt/Swiss-Prot entry name, accession number and description. Proteins are grouped according to their origin. The absolute quantity of each protein is expressed as fmol. The arrows indicate the relative up-regulation or down-regulation of any given protein in disease, compared to health. The mean and +/- SD value is derived from five individual samples in each group.

Table 3. Gingival crevicular fluid Proteins Identified Using LC/MS[E]

Accession	SwissProt Entry	Protein description	Function	Health	Disease	Regulation
Human						
P02763	A1AG_HUMAN	Alpha-1-acid glycoprotein 1	Immune response	3.1±2.3	6.0±4.4	↑
P19652	A1AH_HUMAN	Alpha-1-acid glycoprotein 2	Signal transduction	2.3±0.0	6.3±4.1	↑
P01009	A1AT_HUMAN	Alpha-1-antitrypsin	Immune response	30.0±15.2	2.4±0.0	↓
P01011	AACT_HUMAN	Alpha-1-antichymotrypsin	Immune response	14.5±0.0	ND	↓
P35250	AC14_HUMAN	Replication factor C	Cell organization	ND	4.4±0.0	↑
P02768	ALBU_HUMAN	Serum albumin	Transport	220.4±230.9	241.8±240.4	↑
P01876	ALC1_HUMAN	Ig alpha-1	Immune response	4.7±2.5	ND	↓
P04083	ANX1_HUMAN	Annexin A1	Immune response	10.5±6.8	2.4±1.4	↓
P07355	ANX2_HUMAN	Annexin A2	Cell differentiation	3.5±1.7	ND	↓
P12429	ANX3_HUMAN	Annexin A3	Enzyme regulator	ND	3.7±0.0	↑
O95236	APL3_HUMAN	Apolipoprotein L3	Transport	1.5±0	ND	↓
P08311	CATG_HUMAN	Cathepsin G	Immune response	ND	37.9±0.0	↑
P01024	CO3_HUMAN	Complement C3	Immune response	4.5±0.0	3.2±0.0	↓
P35321	CORA_HUMAN	Cornifin-A	Cell differentiation	4.5±3.3	ND	↓
Q9UBC9	CORC_HUMAN	Small proline-rich protein 3	Cell differentiation	4.0±1.4	1.6±0.0	↓
Q08828	CYA1_HUMAN	Adenylate cyclase type 1	Signal transduction	ND	1.1±0.0	↑
P04080	CYTB_HUMAN	Cystatin-B	Enzyme regulator	11.7±10.4	ND	↓
P81605	DCD_HUMAN	Dermcidin	Immune response	2.3±0.0	ND	↓
P59665	DEFN_HUMAN	Neutrophil defensin 1-3	Immune response	14.8±3.4	ND	↓
Q01469	FABE_HUMAN	Fatty acid-binding protein	Metabolism	2.2±0.0	ND	↓

Q7Z6J6	FRMD5_HUMAN	FERM domain protein 5	Signal transduction	ND	0.5±0.0	↑
P04406	G3P2_HUMAN	GAPDH 3	Metabolism	8.3±6.5	ND	↓
P01857	GC1_HUMAN	Ig gamma-1 chain C	Immune response	86.2±61.5	53.8±45.2	↓
P01859	GC2_HUMAN	Ig gamma-2 chain C	Immune response	19.9±10.6	25.2±15.3	↑
P01860	GC3_HUMAN	Ig gamma-3 chain C	Immune response	11.6±0.0	ND	↓
P01861	GC4_HUMAN	Ig gamma-4 chain C	Immune response	26.4±17.9	20.8±10.9	↓
P52565	GDIR_HUMAN	Rho GDP-dissociation inhibitor 1	Signal transduction	ND	4.6±3.7	↑
P14136	GFAP_HUMAN	Glial fibrillary acidic protein	Signal transduction	ND	4.7±0.0	↑
P62807	H2BA_HUMAN	Histone H2B type 1	Immune response	3.3±0.0	71.2±0.0	↑
P57053	H2BS_HUMAN	Histone H2B type F-S	Immune response	ND	71.2±0.0	↑
P62805	H4_HUMAN	Histone H4	Cell organization	ND	1.2±0.0	↑
P69905	HBA_HUMAN	Hemoglobin subunit alpha	Transport	0.3±0.0	59.3±60.5	↑
P68871	HBB_HUMAN	Hemoglobin subunit beta	Transport	ND	22.5±0.0	↑
P02042	HBD_HUMAN	Hemoglobin subunit delta	Transport	ND	158.2±196.7	↑
P00738	HPT2_HUMAN	Haptoglobin	Metabolism	10.9±9.1	12.0±4.0	↑
P11142	HSP7C_HUMAN	Heat shock 71 kDa protein	Immune response	ND	3.1±0.0	↑
P54652	HSP72_HUMAN	Heat shock-related 70 kDa	Immune response	ND	1.2±0.0	↑
P01777	HV3P_HUMAN	Ig heavy chain V-III region	Immune response	ND	2.8±0.0	↑
P35527	K1CI_HUMAN	Keratin, type I cytoskeletal 9	Cell differentiation	10.0±9.2	6.6±0.0	↓
P13645	K1CJ_HUMAN	Keratin, type I cytoskeletal 10	Cell differentiation	22.2±13.6	8.7±0.0	↓
Q99456	K1CL_HUMAN	Keratin, type I cytoskeletal 12	Cell differentiation	26.6±9.4	ND	↓
P13646	K1CM_HUMAN	Keratin, type I cytoskeletal 13	Cell differentiation	32.2±23.0	10.4±7.1	↓
P02533	K1CN_HUMAN	Keratin, type I cytoskeletal 14	Cell differentiation	16.5±17.5	7.5±3.5	↓
P19012	K1CO_HUMAN	Keratin, type I cytoskeletal 15	Cell differentiation	21.7±16.6	8.2±4.6	↓
P30654	K1CP_HUMAN	Keratin, type I cytoskeletal 16	Cell differentiation	27.7±16.0	10.3±0.0	↓
Q04695	K1CQ_HUMAN	Keratin type I cytoskeletal 17	Cell differentiation	45.5±0.0	6.6±2.7	↓
P08727	K1CS_HUMAN	Keratin, type I cytoskeletal 19	Cell differentiation	43.8±0.0	4.5±3.5	↓

P35900	K1CT_HUMAN	Keratin, type I cytoskeletal 20	Cell differentiation	13.7±6.3	1.4±0.0	↓
Q9C075	K1CW_HUMAN	Keratin, type I cytoskeletal 23	Cell differentiation	3.9±0.0	ND	↓
P35908	K22E_HUMAN	Keratin, type II cytoskeletal 2	Cell differentiation	73.3±97.6	10.4±5.8	↓
Q01546	K22O_HUMAN	Keratin, type II cytoskeletal 2 oral	Cell differentiation	11.0±10.4	4.6±0.6	↓
P04264	K2C1_HUMAN	Keratin, type II cytoskeletal 1	Cell differentiation	60.1±51.8	15.8±9.6	↓
P12035	K2C3_HUMAN	Keratin, type II cytoskeletal 3	Cell differentiation	9.1±7.3	ND	↓
P19013	K2C4_HUMAN	Keratin, type II cytoskeletal 4	Cell differentiation	8.4±7.5	1.2±0.0	↓
P13647	K2C5_HUMAN	Keratin, type II cytoskeletal 5	Cell differentiation	85.6±114.4	7.5±1.7	↓
P08729	K2C7_HUMAN	Keratin, type II cytoskeletal 7	Cell differentiation	9.2±0.0	ND	↓
P05787	K2C8_HUMAN	Keratin, type II cytoskeletal 8	Cell differentiation	8.7±7.6	4.5±0.0	↓
P02538	K2CA_HUMAN	Keratin, type II cytoskeletal 6A	Cell differentiation	66.0±61.4	11.5±2.7	↓
P04259	K2CB_HUMAN	Keratin, type II cytoskeletal 6B	Cell differentiation	168.5±0.0	12.4±3.8	↓
P48668	K2CC_HUMAN	Keratin, type II cytoskeletal 6C	Cell differentiation	203.9±262.4	11.5±2.7	↓
P48667	K2CD_HUMAN	Keratin, type II cytoskeletal 6D	Cell differentiation	119.6±178.2	6.9±8.0	↓
P48668	K2CE_HUMAN	Keratin, type II cytoskeletal 6E	Cell differentiation	151.2±195.9	10.5±2.9	↓
P48669	K2CF_HUMAN	Keratin, type II cytoskeletal 6F	Cell differentiation	62.7±55.4	17.8±0.0	↓
P01834	KAC_HUMAN	Ig kappa chain C	Immune response	15.5±13.5	6.8±2.6	↓
P78368	KC12_HUMAN	Casein kinase I isoform gamma-2	Signal transduction	ND	0.5±0.0	↑
Q9C0H6	KHL4_HUMAN	Kelch-like protein 4	Cell organization	1.8±0.0	ND	↓
P14618	KPY2_HUMAN	Cytosolic thyroid hormone protein	Metabolism	0.6±0.0	ND	↓
P30613	KPYR_HUMAN	Pyruvate kinase 1	Metabolism	ND	1.4±0.0	↑
P04206	KV3G_HUMAN	Rheumatoid factor	Immune response	ND	2.7±0.3	↑
P01842	LAC_HUMAN	Ig lambda chain C	Immune response	12.8±18.4	4.6±1.1	↓
P00695	LYC_HUMAN	Lysozyme C	Metabolism	12.1±14.7	3.0±1.4	↓
P49736	MCM2_HUMAN	DNA replication licensing factor	Cell organization	ND	1.2±0.0	↑
P55081	MFAP1_HUMAN	Microfibrillar-associated protein 1	Metabolism	ND	1.1±0.0	↑
P22894	MMP8_HUMAN	Matrix metalloproteinase 8	Immune response	ND	1.0±0.0	↑

P22712	MPB1_HUMAN	C myc promoter binding protein	Cell differentiation	1.5±0.0	ND	↓
P35579	MYH9_HUMAN	Myosin-9	Immune response	ND	4.2±0.0	↑
P80188	NGAL_HUMAN	Neutrophil gelatinase-lipocalin	Transport	ND	18.9±11.2	↑
P11678	PERE_HUMAN	Eosinophil peroxidase	Transport	1.2±0.0	ND	↓
P05164	PERM_HUMAN	Myeloperoxidase	Immune response	3.1±0.0	6.8±4.5	↑
P13796	PLSL_HUMAN	Lymphocyte cytosolic protein 1	Transport	ND	15.6±12.1	↑
P13797	PLST_HUMAN	Plastin-3	Cell organization	ND	9.8±7.9	↑
P07737	PRO1_HUMAN	Profilin-1	Cell organization	9.4±0.0	13.9±0.0	↑
Q596K9	Q596K9_HUMAN	Migration-inducing protein 12	Cell organization	ND	24.5±0.0	↑
Q6ICQ8	Q6ICQ8_HUMAN	ARHG protein	Signal transduction	ND	2.1±0.0	↑
Q6NSB4	Q6NSB4_HUMAN	HP protein	Enzyme regulator	ND	5.4±0.0	↑
Q01201	RELB_HUMAN	Transcription factor RelB	Cell organization	55.8±0.0	ND	↓
P02753	RETB_HUMAN	Retinol-binding protein 4	Transport	3.3±0.0	ND	↓
Q13671	RIN1_HUMAN	Ras interaction/interference protein 1	Signal transduction	ND	0.2±0.0	↑
P06702	S109_HUMAN	Calgranulin B	Immune response	10.3±0.3	11.0±0.2	↑
P25815	S10E_HUMAN	Protein S100-P	Transport	ND	1.3±0.0	↑
O15056	SYJ2_HUMAN	Synaptojanin-2	Signal transduction	0.6±0.0	ND	↓
P17542	TAL1_HUMAN	T-cell acute lymphocytic leukemia 1	Cell differentiation	ND	0.9±0.0	↑
P10599	THIO_HUMAN	Thioredoxin	Metabolism	ND	2.5±0.0	↑
P29401	TKT_HUMAN	Transketolase	Metabolism	0.8±0.0	ND	↓
Q03169	TNAP2_HUMAN	TNFalpha-induced protein 2	Immune response	0.6±0.0	ND	↓
P02787	TRFE_HUMAN	Serotransferrin	Transport	16.8±12.06	6.4±4.7	↓
P02788	TRFL_HUMAN	Lactotransferrin	Transport	ND	2.9±0.0	↑
P62328	TYB4_HUMAN	Thymosin beta-4	Cell organization	ND	1.1±0.0	↑
P49427	UBC3_HUMAN	Ubiquitin-conjugating enzyme E2 R1	Cell organization	ND	1.4±0.0	↑
P62988	UBIQ_HUMAN	Ubiquitin	Metabolism	ND	1.3±0.0	↑
P02774	VTDB_HUMAN	Vitamin D-binding protein	Transport	14.6±0.0	ND	↓

Bacterial

P57448	AMPA_BUCAI	Cytosol aminopeptidase	Bacterial	ND	1.0±0.0	↑
Q9ZL64	ACO2_HELPJ	*H. pylori* aconitate hydratase	Bacterial	ND	0.4±0.0	↑
P0A1B5	AROF_SALTY	*S.typhimurium* DAHP synthetase	Bacterial	ND	1.6±0.0	↑
P0A1Y2	ASRC_SALTY	*S. typhimurium* sulfite reductase	Bacterial	ND	2.6±0.0	↑
P12999	BIOC_ECOLI	*E. coli* biotin synthesis protein	Bacterial	0.9±0.0	ND	↓
P0A518	CH61_MYCTU	*M.tuberculosis* chaperonin	Bacterial	17.9±0.0	ND	↓
Q02929	CPS_CLOTM	Putative sensory transducer	Bacterial	ND	2.1±0.0	↑
P0A9F3	CYSB_ECOLI	*E. coli* cysB	Bacterial	ND	0.7±0.0	↑
P31680	DJLA_ECOLI	*E. coli* DnaJ-like protein	Bacterial	2.9±0.0	ND	↓
P66004	DLDH_MYCTU	*M. tuberculosis* dihydrolipoyl DH	Bacterial	20.8±0.0	ND	↓
Q9PHE3	DNAA_XYLFA	*Xylella fastidiosa* dnaA	Bacterial	ND	2.9±0.0	↑
O25242	DP3B_HELPY	*H. pylori* DNA polymerase	Bacterial	ND	8.8±0.0	↑
Q9ZML6	DXR_HELPJ	*H. pylori* reductoisomerase	Bacterial	ND	15.7±0.0	↑
P06176	FLIC_SALCH	*S. choleraesuis* flagellin	Bacterial	ND	2.2±0.0	↑
Q05755	GLTB_AZOBR	A. brasilense glutamate synthase	Bacterial	13.9±0.0	ND	↓
P07018	MCP4_ECOLI	*E. coli* MCP-IV	Bacterial	ND	2.4±0.0	↑
Q57714	PORB_METJA	Pyruvate synthase porB	Bacterial	46.6±0.0	6.2±0.0	↓
Q59677	MUTB_PORGI	*P. gingivalis* Methylmalonyl-CoA	Bacterial	ND	0.6±0.0	↑
P11278	PELP_YERPS	*Yersinia pseudotuberculosis* pelY	Bacterial	12.7±0.0	ND	↓
P16682	PHND_ECOLI	*E. coli* phosphonates-binding protein	Bacterial	17.7±0.0	ND	↓
O07497	SECA_BORBU	Protein translocase	Bacterial	ND	1.7±0.0	↑
Q9Z7P1	SYH_CHLPN	*C. pneumoniae* h-tRNA synthetase	Bacterial	ND	1.0±0.0	↑
P16966	TTR_PSESY	*P. syringae* acetyltransferase	Bacterial	1.9±0.0	ND	↓
P76349	YEEL_ECOLI	*E. coli* protein yeeL	Bacterial	ND	8.5±0.0	↑
P75287	YF00_MYCPN	*M. pneumoniae* adhesin P1	Bacterial	ND	0.8±0.0	↑
P76469	YFAU_ECOLI	*E. coli* KDR aldolase	Bacterial	ND	1.2±0.0	↑

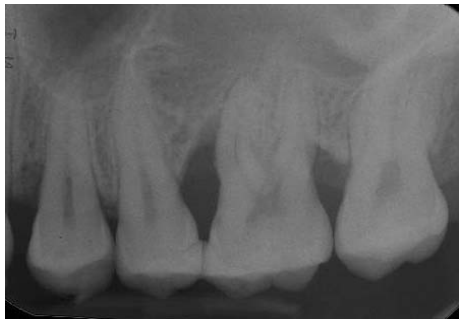
P54390	YPIB_BACSU	*B. subtilis* protein ypiB	Bacterial	6.2±0.0	ND	↓
Yeast						
O94049	ACSA_CANAL	C.albicans acetyl coenzyme A	Yeast	ND	0.2±0.0	↑
O93803	CET1_CANAL	C. albicans mRNA-capping	Yeast	ND	1.9±0.0	↑
P87131	CYSK_SCHPO	S. pombe cysteine synthase	Yeast	ND	1.4±0.0	↑
P14020	DPM1_YEAST	S. cerevisiae DPM synthase	Yeast	ND	3.2±0.0	↑
Q02207	FOX2_YEAST	S. cerevisiae β-oxidation protein	Yeast	0.1±0.0	ND	↓
P22147	KEM1_YEAST	S. cerevisiae exoribonuclease 1	Yeast	9.9±0.0	ND	↓
P40957	MAD1_YEAST	S.cerevisia MAD1	Yeast	ND	2.0±0.0	↑
P33755	NPL4_YEAST	S. cerevisiae NPL4	Yeast	ND	10.7±0.0	↑
P46673	NU85_YEAST	S. cerevisiae NUP85	Yeast	ND	2.1±0.0	↑
P07922	PYRF_KLULA	C. sphaerica OMP decarboxylase	Yeast	6.1±0.0	21.3±8.9	↑
P33332	SEC3_YEAST	Protein PSL1	Yeast	ND	0.5±0.0	↑
P26637	SYLC_YEAST	S. cerevisiae l-tRNA synthetase	Yeast	ND	3.9±0.0	↑
P34252	YKK8_YEAST	SLD2	Yeast	ND	1.3±0.0	↑
P36113	YKZ7_YEAST	RING finger protein	Yeast	ND	3.5±0.0	↑
Viral						
P00544	FGR_FSVGR	Sarcoma virus tyrosine- kinase	Viral	4.2±0.0	11.3±9.2	↑
P36848	FIBP_ADE31	Human adenovirus fiber protein	Viral	10.2±0.0	ND	↓
P13121	RPC1_BPP1	Enterobacteria phage C1	Virus	ND	0.4±0.0	↑
P69202	RPC2_BPP22	Enterobacteria phage P22 C2	Virus	ND	2.1±0.0	↑
P26109	RRP3_IARUD	Polymerase basic protein 2	Virus	ND	0.81±0.0	↑
Q08099	US02_HSVBS	Herpes virus Protein US2	Virus	ND	4.4±0.0	↑
P21044	VC13_VACCC	Vaccinia virus Protein C13	Virus	1.9±0.0	ND	↓
P25243	VGA_BPAL3	Enterobacteria phage A protein	Virus	ND	2.2±0.0	↑

Table of Contents Synopsis

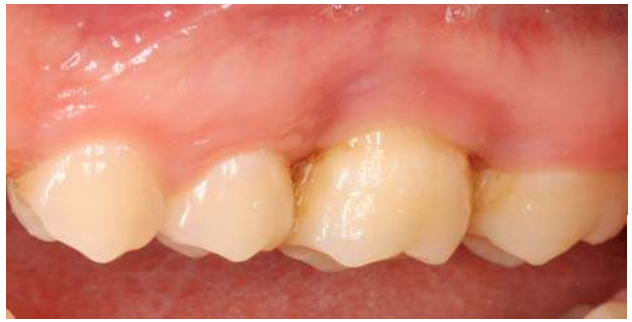
GCF is a local inflammatory exudate of the diseased periodontal tissues that can be used to study potential disease markers. As opposed to previous approaches studying single biomarkers, the present study used label-free LC/MS^E and identified 154 proteins of human, bacterial and viral origin, in health and periodontitis. This technology may facilitate characterisation of GCF proteome in periodontal health and disease, aiding the development of accurate diagnostic and prognostic markers.

Periodontal disease

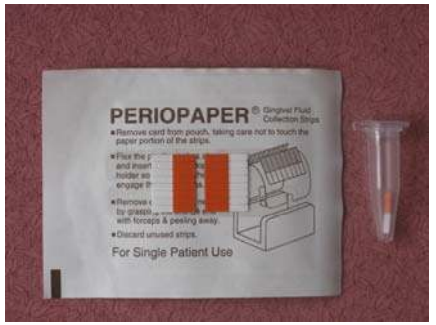
Radiographic



Clinical

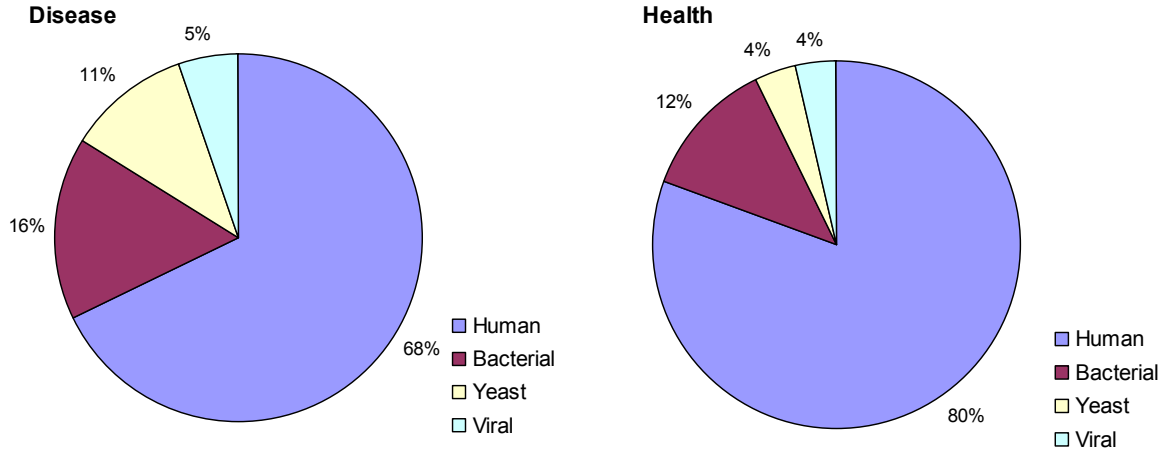


GCF



***Gingival exudatome* analysis by LC/MS^{E}**

Data Processing and Database Searching



Protein origin in health and disease