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Alterations in the salivary proteome associated with periodontitis

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Abstract

Aim: To identify changes in the salivary proteome associated with active periodontitis.

Materials and Methods: Quantitative proteomics (two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis) was used to investigate whole saliva from individuals with severe periodontitis and their proteomic profiles before and after periodontal treatment were compared.

Results: A comparison of 128 proteins across all saliva samples identified 15 protein spots with altered abundance. The predominant alteration observed was an increase in the abundance of the S100 proteins S100A8/A9/A6. Of the remaining proteins with altered abundance, haptoglobin, prolactin inducible protein and parotid secretory protein have previously been associated with host defence.

Conclusion: These results highlight the predominant involvement of S100 proteins in the host response during periodontitis, identify host defence components that have not been linked previously to this disease and suggest new potential biomarkers for monitoring disease activity in periodontitis.

Key words: periodontitis; proteomic; saliva

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Periodontitis is a common disease characterized by bacterially induced inflammatory destruction of the support tissues of the teeth. The aetiology of periodontal inflammation is complex and most likely varies from individual to individual, but in general the disease can be considered to result from an inappropriate balance between pro-inflammatory and anti-inflammatory signals. This balance is affected by factors such as smoking, the accumulation of bacterial load and an individual's propensity to develop and resolve inflammation (Kornman 2008). If this inflammation remains unresolved, then tissue destruction occurs as a result of the ongoing secretion of proteases and other inflammatory components. This tissue-destructive activity may occur in an episodic manner over a long period of time (Socransky et al. 1984).

There is a considerable interest in identifying biomarkers that are associated with periodontal disease activity. Such markers have the potential to assist in the development of new periodontal therapies and in the detection and monitoring of disease (Ebersole 2003). Gingival crevice fluid (GCF) and saliva have been extensively examined as these fluids are known to reflect oral disease status (Kaufman & Lamster 2002, Taba et al. 2005, Lamster & Ahlo 2007). GCF is primarily composed of serum and inflammatory exudate from the gingiva. In contrast, whole saliva is composed of fluids and particles from a variety of oral and airway sources, but has the advantage of being more easily collected. A range of candidate host- and pathogen-derived markers has been investigated in these fluids but to date, no single marker has been found which clearly identifies the presence of periodontitis (Kaufman & Lamster 2000, Ozmeric 2004).

The inability to identify a single marker for periodontal disease suggests that an effective clinical side test may involve a combination of biomarkers. As such, there is an ongoing interest in identifying new molecules that indicate disease. These markers have traditionally been selected for investigation on the basis of their known role in inflammation. An alternate approach is to use techniques such as genomics and proteomics to identify changes in genes, proteins or cellular pathways. These approaches do not require the selection

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of candidate proteins and can simultaneously survey large numbers of potential markers. Although genomic techniques, such as the microarray analysis of mRNA, have been used in models of periodontal disease, proteomics has only been applied to the host response in periodontitis in two previous studies, this being a comparison of GCF and serum from periodontitis patients (Kojima et al. 2000) and more recently, a comparison of whole unstimulated saliva from generalized aggressive periodontitis patients with control individuals (Wu et al. 2009). In this study, we used quantitative proteomics (Two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, 2D SDS-PAGE) to investigate whole saliva from individuals with periodontitis, comparing their salivary profiles before and after periodontal treatment in a pairwise manner. This study design allowed us to compare the salivary proteome in the presence or absence of active periodontitis and to eliminate the effect of the high inter-individual variability known to exist in saliva samples. This variability may make consistent, significant changes in biomarker abundance over the course of the disease difficult to detect in a direct disease *versus* healthy control group comparison, even if the same trend is observed in most or all individuals. A pairwise comparison within individuals removes this source of variation. The proteomic approach also enables alternate post-translational isoforms of proteins to be differentiated and quantified, information which is not necessarily provided by an enzyme-linked immunosorbent assay or western blot techniques. As such, this investigation applies a novel approach to the identification of salivary biomarkers in periodontitis.

Materials and methods

Sample collection

Nine participants (seven male and two female, ages ranging between 35 and 66 years, the average age being 54.9 years) were recruited for the study following determination of their eligibility from a clinical examination and health questionnaire. Criteria for inclusion in the study were at least two pocket probing depths of 5 mm or more, generalized periodontitis (at least 50% of the teeth exhibiting pocket probe depths of 3 mm or more and at least 10% exhibiting bleeding upon probing), general good health, non-smoker, non-diabetic and no intake of antibiotics in the last 6 months. Participants provided an initial saliva sample immediately before their first appointment for periodontal therapy and a second sample at the completion of their treatment, once they met the criteria for inactive disease. The criteria for inactive periodontitis was a minimum 50% average reduction in abnormal pocket probe depth across all probe sites (2 mm was considered to be normal pocket depth) and an 80% reduction in bleeding upon probing. All patients received the same treatment regime, which consisted of routine root planing and cleaning. Saliva samples were collected between 08:00 and 10:00 hours following overnight fasting. Participants were provided with a parafilm bolus to chew and provided 5 ml of saliva by expectoration. Samples were mixed with a Complete protease inhibitor (Roche, Mannheim, Germany) and placed on ice before centrifugation at 10,000 *g* for 10 min. The pellet was discarded and the sample was stored at -80°C before analysis.

Sample and image analysis

The concentration of protein in each sample was measured using a Coomassie protein assay kit (BioRad, Hercules, CA, USA). Duplicate analyses were performed with 300 μg of protein from each sample. Samples were lypholized and then solubilized, focused and separated by 2D SDS-PAGE, as described previously (Smolenski et al. 2007). No desalting was performed. Samples were focused on 18 cm *p*I 3–10 IPG strips (Amersham, Uppsala, Sweden), separated in the second dimension using 13% (w/v) (SDS-PAGE) midi gels and then stained with colloidal Coomassie G-250. Gels were imaged on a GS-800-calibrated densitometer and image analysis was performed using the PDQuest v8.0 software package (BioRad). Spots that could be clearly identified on at least 50% of samples were included in the analysis. Gels were also assessed for the presence of spots which were unique to either the post- or pre-treatment sample set. Protein spot intensities for each gel were normalized to the sum of matched spot intensities on the gel, excluding the two most abundant proteins (serum albumin and salivary α-amylase). These two proteins were excluded to prevent the normalization from being skewed by the abundance of any one abundant protein spot. Normalized spot volumes were then averaged for the duplicate gels. For the spots that could not be accurately quantified on one of the sample duplicates, the spot volume was assigned from the remaining gel. For each protein spot, paired *t*-tests were performed on the log value of protein spot volumes (before and after treatment for each individual) using a two-tailed
t-test assuming equal variance.

Protein identification

Proteins that were found to be significantly different in abundance ($p < 0.05$) were excised from the gels for in-gel tryptic digestion and mass spectroscopy (MS) analysis. Digestion, MS and protein identification was performed as described previously (Smolenski et al. 2007) using either matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS or liquid chromatography (LC)-MS/MS. MALDI-TOF was performed using a Bruker Autoflex II (Billerica, MA, USA) and LC-MS/MS using a ThermoFinnigan LCQ Deca (Waltham, MA, USA) fitted with a nanospray interface. MALDI-TOF peptide mass fingerprint searches were performed with Mascot (Matrix Science, London, UK) against the human, archaea and eubacteria Swiss-Prot databases using a 100 ppm error, fixed carbamidomethylation and variable methionine oxidation (Perkins et al. 1999). A MOWSE score >55 was considered to be a positive identification. LC-MS/MS protein identification was performed with TurboSEQUEST (ThermoFinnigan) software searching against the human RefSeq database. Identification of at least three peptides (Xcorr greater than 1.65 for a +1 ion, 2.2 for a +2 ion or 3.7 for a +3 ion) were considered as positive identifications (Eng et al. 1994). The ion masses for each protein are included in the supporting information (Table S1).

Ethical considerations

This study was performed under approval from the Northern Y Health and Disability Ethics Committee, New Zealand (NTY/05/05/029). Signed informed consent was obtained from all participants before the donation of samples for this study.

Results

Whole saliva samples were collected from patients before and after treatment for periodontitis. The protein concentration ranged between 0.45 and 0.91 mg/ml (average 0.74 mg/ml). The time interval between these two samples ranged from 69 to 512 days, with a median of 187 days. Following treatment, the average gingival crevice probe depth was reduced from 3.17 to 2.25 mm and the percentage of sites exhibiting bleeding upon probing was reduced from 30.0 to 3.0% (data not shown). The saliva samples were analysed by 2D SDS-PAGE and the relative abundance of 126 protein spots was determined. Paired *t*-tests were performed on the quantified proteins and 15 of them were found to be statistically different in abundance ($p < 0.05$) between pre- and post-periodontal treatment saliva samples. No protein spots were identified which were unique to either the pre-treatment sample or the post-treatment sample. A previous power analysis experiment, which measured the variation in repeated samples from individuals over time, following analysis by duplicate 2D SDS-PAGE gels, had estimated that this study design had 80% power to detect 2.5-fold differences at $p < 0.05$ with a sample size of nine participants (data not shown). Figure 1 is a 2D SDS-PAGE gel of whole saliva and indicates the proteins with altered abundance. Eleven protein spots were more abundant in the saliva from individuals with active disease while four were observed to be decreased on average (Table 1). MS was successful in identifying 10 of these protein spots and all were human in origin (Table 1, supporting information). The remaining proteins could not be confidently identified. Figure 2 indicates the fold change of each protein for each study participant.

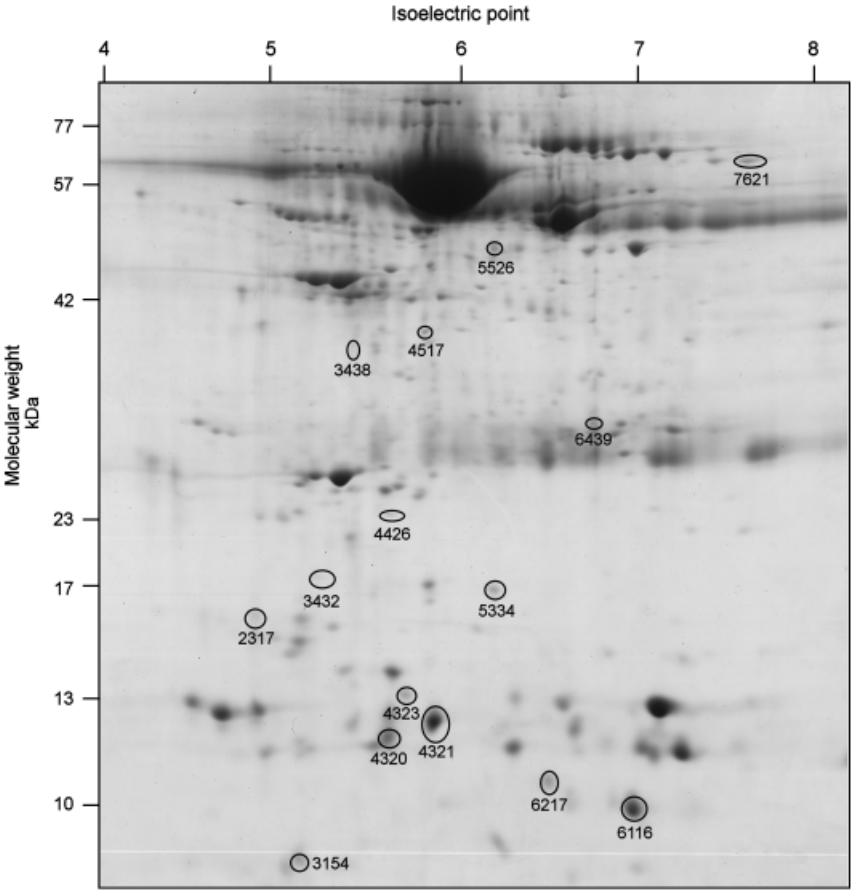

Fig. 1. Saliva sample from an individual before periodontal treatment, focused over the *p*I range of 3–10 and separated on a 13% sodium dodecyl sulphate polyacrylamide gel. Circles indicate position and spot number of proteins with a significant change in abundance between pre- and post-treatment samples.

Table 1. Identification of protein spots with a significant change in abundance ($p < 0.05$, paired *t*-test) between pre- and post-treatment samples

Spot number	Swiss-Prot accession number	Protein name	Fold change
2317	P12273	Prolactin-inducible protein	− 1.36
3154	P06703	S100A6	1.64
3432	Unidentified		− 1.61
3438	Q96DR5	Parotid secretory protein	− 1.50
4320	P06702	S100A9	1.45
4321	P06702	S100A9	1.99
4323	Unidentified		− 1.49
4426	Unidentified		2.61
4517	P37837	Transaldolase 1	1.65
5334	P00738	Haptoglobin α-chain subunit	3.64
5526	P50395	Rab GDP-dissociation inhibitor	2.17
6116	P05109	S100A8	2.31
6217	Unidentified		2.50
6439	Unidentified		2.30
7621	P29401	Transketolase	5.37

Fold change indicates abundance in pre-treatment sample (active periodontitis) over post-treatment sample (inactive disease) and is the average of the values for each individual.

Discussion

This investigation was designed to identify changes in the salivary proteome in the presence or absence of active periodontitis. To achieve this, individuals were recruited using selection criteria that is consistent with studies which have examined advanced or severe periodontitis (Savage et al. 2009). A second sample was taken following periodontal therapy, when evidence of both tissue repair (reduced pocket probe depths) and reduced inflammation (reduced bleeding) could be observed. The reduction in these parameters can only be achieved through a decrease in the periodontal disease activity.

By comparing these samples using proteomic analysis, 15 proteins with altered abundance were identified. These changes likely indicate proteins that are induced or repressed by the host

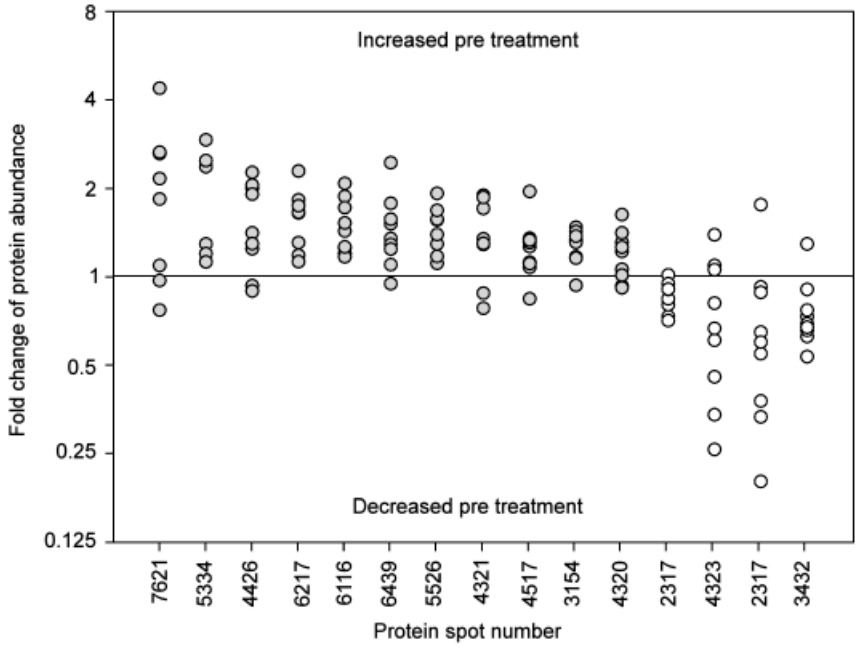

Fig. 2. Fold change in abundance for each protein across all study participants. Fold change indicates abundance in the pre-treatment sample over the post-treatment sample. Protein spots with an average fold change >1 (increased in disease) are indicated by grey symbols and <1 (decreased in disease) by open symbols.

inflammatory response but may also include proteins that are altered during the post-treatment repair process. As the analysed samples were whole saliva, these proteins may have originated from salivary secretions, GCF, mucosal exudates, exfoliated cells or the host microbiota. The changes identified are predominantly an increase in the abundance of certain protein spots during active disease, as would be expected during inflammation when a range of host defence factors are upregulated, released or produced by the infiltrating immune cells.

The predominant change observed in the salivary proteome was the increased abundance of S100 proteins in disease, which accounted for four of the 15 protein spots with altered abundance. The S100 proteins are a group of calcium-binding proteins with a variety of regulatory functions, including the regulation of inflammation (Donato 2001). S100A8 and S100A9 proteins form a heterodimer (calprotectin), which is expressed by neutrophils, activated monocytes, macrophages and squamous epithelia (including oral keratinocytes) and which constitutes a large proportion of the soluble cytosolic component of granulocytes (Gebhardt et al. 2006). As well as neutrophil and macrophage chemotactic activity, the S100A8/A9 molecules have been shown to inhibit macrophage H_2O_2 production, inhibit immunoglobulin synthesis, modulate neutrophil adhesion and exhibit antimicrobial activity (Yui et al. 2003). Levels of S100A8/A9 in GCF and saliva have been shown previously to correlate with periodontitis and are likely to result from active secretion by infiltrating neutrophils and gingival keratinocytes, both of which are known to secrete S100A8/A9 in the presence of bacterial lipopolysaccharides (Kido et al. 1999, 2004, Kojima et al. 2000, Ross & Herzberg 2001, Ramseier et al. 2009). In contrast to the S100A8/A9 proteins, there are few reports linking S100A6 to inflammation. Upregulation of S100A6 has been observed in a mouse model of asthma and following TNF-α stimulation and NF-κB activation (Joo et al. 2003, Calvo et al. 2009). S100A6 is found in a range of cell types, including epithelial cells and fibroblasts, and is upregulated in a variety of tumours. It has been shown to interact with the RAGE receptor, which is expressed on the cell surface of a range of cells, including monocytes and macrophages, where it may stimulate apoptosis (Donato 2001, Leclerc et al. 2007). Although the function of this protein is unclear, our results suggest that S100A6 protein is increased in the oral cavity during periodontal inflammation, potentially as the result of cytokine- or pathogen-dependent NF-κB activation.

Prolactin-inducible protein (PIP) is a small glycoprotein which is found in a variety of secretory fluids and mucosal tissues and is a marker for breast carcinomas with apocrine features (Mazoujian et al. 1983). PIP binds to a variety of oral bacteria, suggesting it has a role in protecting the oral mucosa through the inhibition of colonization or bacterial growth (Schenkels et al. 1997). PIP has been observed previously with decreased abundance in two inflammatory conditions: nasal lavage fluid from individuals with allergic rhinitis and tears from blepharitis patients (Bryborn et al. 2005, Koo et al. 2005). PIP has also been reported to belong to a group of salivary proteins whose abundance is reduced in the presence of oral bleeding (Huang 2004). In the present study, participants had elevated levels of bleeding gingiva at the time of active disease sample collection and the reduced levels of PIP we found may reflect this.

An acute phase protein, haptoglobin, was also identified. Haptoglobin levels have previously been shown to be increased in the serum of individuals with periodontitis and the level correlates with the disease severity (Ebersole et al. 1997). Although haptoglobin is primarily produced by the liver, it can also be produced locally by keratinocytes, where its expression is increased in certain inflammatory conditions, and in neutrophils, where it is released during neutrophil activation (Li et al. 2005, Theilgaard-Monch et al. 2006). The increased levels of haptoglobin observed in this study likely result from local inflammation rather than from serum-derived protein, as no other serum acute-phase proteins were identified.

Parotid secretory protein (PSP or SPLUNC2) is a member of the BPI-like family of proteins (Bingle & Craven 2002). The BPI-like family members have been proposed to have a role in the host defence of the oral cavity and recombinant PSP exhibits bacteriostatic activity towards *Pseudomonas aeruginosa* (Geetha et al. 2003, LeClair 2003). PSP is predominantly expressed by the salivary glands and multiple differentially glycosylated isoforms are observed in saliva (Geetha et al. 2003, Bingle et al. 2009). PSP is also detected in gingival keratinocytes, where it is upregulated in response to exposure to

Porphyromonas gingivalis and TNF-α (Geetha et al. 2003, Shiba et al. 2005). Our data identified a decrease in an isoform of PSP in the saliva from individuals with periodontitis. This agrees with the results of another salivary proteomics study (Wu et al. 2009) and suggests that although PSP may be locally upregulated in the gingival tissue during inflammation, the overall level of PSP in the oral cavity may be decreased. As PSP appears to be a substrate for matrix metalloproteinase-9, decreased levels of PSP protein may result from the increased levels of this protease, which are known to occur in the GCF of individuals with severe periodontitis (Teng et al. 1992, Geetha et al. 2005).

Three common intra-cellular proteins were also identified: transketolase, transaldolase and GDP dissociation inhibitor β. None of these proteins have a direct role in the immune response and all have been previously observed in saliva (Denny et al. 2008). Transketolase and transaldolase are components of the pentose phosphate pathway. Increased abundance of transketolase has been reported in the proteomic analysis of monocytes stimulated with lipopolysaccharide (Gadgil et al. 2003). The pentose phosphate pathway generates NADPH, which can be used to reduce glutathione or generate hydrogen peroxide, two pathways that may be under increased load in an environment where levels of reactive oxygen species are increased or phagocytic cells are concentrated, such as might occur in the inflamed gingiva. The GDP dissociating inhibitor (GDI) β is a member of a family of proteins involved in the regulation of GTPase proteins. These ubiquitous proteins are involved in a variety of cell signalling pathways, including the parotid gland secretory cycle (Benhar et al. 1997).

Of the nine different proteins identified here, only S100A8, S100A9 and PSP have been identified previously as altered in saliva during periodontitis. The remaining proteins are novel findings, although S100A6, haptoglobin and PIP have been associated with inflammation in other conditions. These proteins may serve as new biomarkers for periodontitis. A number of changes in other salivary proteins have been reported by other investigators, but these were not observed here. This may be due to the technical limitations of the proteomics approach used here. Although 2D SDS-PAGE is capable of accurately quantifying a large number of proteins in the same sample, proteins below 1 μg/ml in concentration, smaller than 10 kDa in size or of high hydrophobicity, are either poorly resolved or not detected. Proteins such as cytokines and matrix metalloproteinases, which have been associated with periodontal disease status in a number of previous studies, are only present in ng/ml concentrations in saliva and are thus not quantified using this technique. The use of a more sensitive fluorescent stain, instead of Coomassie blue, may have increased our ability to detect these lower abundance proteins. A comparison of saliva proteins identified using 2D SDS-PAGE (Ghafouri et al. 2003, Huang 2004, Vitorino et al. 2004, Walz et al. 2006) with studies that have measured changes in any of these components in whole saliva in periodontal disease identifies 12 proteins that have previously been linked to disease and may have been observable (α-amylase, carbonic anhydrase VI, zinc α-2 glycoprotein chain B, cystatin C/D/SA, lactoferrin, lysozyme, PSP, S100A8 and S100A9) (Henskens et al. 1996, Kojima et al. 2000, Fine et al. 2002, Jentsch et al. 2004, Ito et al. 2008, Wu et al. 2009). Our results are in agreement with the data on S100A8, S100A9 and PSP. Of the remaining nine proteins, only changes in lactoferrin and amylase have been observed in more than one study, and none have been observed here. Further investigations of larger groups of affected individuals, ideally stratified for specific forms of periodontal disease, would be required to confirm the changes observed in this study and other studies that have identified putative markers in whole saliva.

Figure 2 indicates the fold change of each protein for each study participant. Only four proteins are upregulated in disease across the entire sample set and all proteins have fold changes that are close to neutral for some of the individuals. This suggests that none of these proteins will by themselves provide a marker with sufficient power to confidently predict the disease state, but, taken together, may improve the diagnostic capability of existing markers as part of a diagnostic array. This appears to be the most likely approach to developing an effective rapid test for periodontitis, given that no previously characterized test, based on a single biomarker, has been demonstrated to be entirely predictive (Kaufman & Lamster 2000, Ozmeric 2004). An array of biomarkers which indicate alterations in the range of cellular pathways that may be perturbed in this complex disease may also differentiate the alternate disease processes. For example, the levels of S100A6 might indicate gingival inflammation, S100A8/A9 neutrophil influx and activation, PSP protease activity and PIP bleeding gingiva. Such a biomarker array may have considerable value in differentiating the forms of periodontitis and tailoring therapy to the specific needs of the individual.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Mass spectrophotometry data for identified proteins.

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Clinical Relevance

Scientific rationale for study: Biomarkers for periodontitis may assist in the detection and monitoring of disease. In this study, we used a novel approach, proteomics, to screen for new potential biomarkers in whole saliva.

Principal findings: The predominant alteration involved an increase in the abundance of a group of immune-related proteins, namely the S100 proteins, in the saliva of patients with active severe periodontitis. Other host defence proteins were also observed to be altered in abundance when pre- and post-treatment samples were compared.

Practical implications: We have identified a novel set of proteins for evaluation as salivary biomarkers for periodontitis activity.