Gingival crevicular fluid biomarkers analysed by mass spectrometry can predict periodontal disease progression

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**Gingival crevicular fluid biomarkers analysed by mass spectrometry can predict
periodontal disease progression**

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Running Title: Biomarkers in gingival crevicular fluid

Key Words: gingival crevicular fluid; mass spectrometry; biomarkers; periodontal disease
progression

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Abstract

Background: Gingival crevicular fluid has been suggested as a possible source of biomarkers for periodontal disease progression. This paper describes a technique for the analysis of gingival crevicular fluid from individual sites using mass spectrometry. It explores the novel use of mass spectrometry to examine the relationship between the relative amounts of proteins and peptides in gingival crevicular fluid and their relationship with clinical indices and periodontal attachment loss in periodontal maintenance patients. The aim of this paper was to assess whether the mass spectrometric analysis of GCF may allow for the site-specific prediction of periodontal disease progression.

Material and Methods: Forty-one periodontal maintenance subjects were followed over 12 months, with clinical measurements taken at baseline and every 3 months thereafter. Gingival crevicular fluid was collected from subjects at each visit and analysed using MALDI-TOF mass spectrometry. Samples were classified based upon pocket depth (PD), modified gingival index (MGI), plaque index and attachment loss, and analysed within these groups. A genetic algorithm was used to create a model based on pattern analysis to predict sites undergoing attachment loss.

Results: Three-hundred and eighty-five GCF samples were analysed. Twenty-five sites under observation in 14 patients exhibited attachment loss of > 2 mm over the 12 month period. The clinical indices of PD, MGI, plaque levels and bleeding served as poor discriminators of GCF mass spectra. Models generated from the GCF mass spectra could predict attachment loss at a site with a high specificity (97% recognition capability and 67% cross validation).

Conclusions: GCF mass spectra could be used to predict attachment loss sites. The use of algorithm generated models based on GCF mass spectra may provide utility in the diagnosis of periodontal disease.

Introduction

Chronic and aggressive periodontitis are inflammatory diseases of the periodontium which result in the destruction of the supporting structures of the teeth, and, which if untreated, eventually lead to tooth loss. Bacteria, in the form of dental plaque, are the primary aetiological factor for the initiation and progression of periodontitis, though most of the destruction of periodontal tissues is believed to be host mediated.

The management of periodontal disease has traditionally involved a cause related phase of therapy, consisting of non-surgical and surgical tooth debridement, followed by a regular maintenance treatment. The progression of periodontal disease in maintenance patients has been assessed on a site basis (as loss of attachment), and on a subject basis (as tooth loss). The maintenance phase is initiated once the clinician feels that the disease has stabilized, and for subjects with moderate to advanced periodontitis, generally involves recall visits of between 3-12 months.

Periodontal disease can generally be successfully treated with both non-surgical and surgical treatment, and prevented with effective long-term maintenance care (1). However, even in well maintained populations, disease progression may still occur as measured by tooth loss (2, 3) or increases in clinical attachment loss (4). More aggressive therapy is generally associated with more attachment loss in the short term, particularly in shallow sites where such treatment may not be required.

The diagnosis of attachment loss is currently only retrospective in nature, and no reliable diagnostic tests are available. Inflammatory mediators are generally short lived, and have not demonstrated utility as predictors of disease activity (5). Enzymes and tissue breakdown products, while more actively reflecting current disease status, are still fairly non specific, correlating more closely with gingival inflammation than clinical attachment loss

(5). Clinical measures of gingival inflammation, plaque levels, bleeding on probing, pocket depth and previous attachment loss are all poor predictors of future attachment loss (6, 7).

Periodontal disease progression in maintenance patients generally occurs on a site basis, and so it makes sense that locally produced products may be of diagnostic value. Gingival crevicular fluid is an inflammatory exudate of the periodontium, and as such has been shown to contain serum proteins (8), as well as inflammatory proteins and peptides and tissue breakdown products (9).

Mass spectrometry (MS) is an analytical tool which can provide mass spectra of proteins and peptides within a sample. These spectra can be used for the identification of these proteins and peptides, or it can be used to create models for disease diagnosis. MS has served as an adjunct to gel electrophoresis (10) and liquid chromatography (11) in identification of certain proteins and peptides in GCF. Eight peptides in GCF from healthy sites have been identified using LC coupled with electro-spray ionisation-MS (12). MS has been used in conjunction with gel electrophoresis and LC-MS/MS to identify 33 peptides and 64 proteins in GCF (8). In more recent MS studies 432 (13) and 327 (14) proteins in human GCF samples were identified and differences were detected in some proteins from healthy subjects compared with those with chronic periodontitis.

MS can detect proteins and peptides in the femtomole range and the detection of a peptide or protein (which may be of diagnostic value) can be made without knowing its identity. Models of disease progression may be constructed from mass spectra, which may then be applied to future samples to classify sites sampled as stable or progressing.

The development of a mass spectral model of periodontal disease progression may enable the prediction of active (progressing) sites, which would facilitate a more targeted management of periodontal disease. To date, a method for the analysis of raw GCF mass spectra for the purpose of predicting disease progression has not been developed. The precise

peptide / protein profile of GCF in health and varying degrees of inflammation is lacking. This paper describes a reproducible technique for the analysis of protein and peptide profiles of GCF using mass spectrometry and examines the relationship between the relative amounts of proteins and peptides in GCF with clinical indices in periodontal maintenance patients. The aim of the study was to determine whether the mass spectrometric analysis of GCF was capable of predicting sites at risk of disease progression.

Materials and Methods

Patient recruitment

Forty-one patients in a periodontal disease maintenance program aged between 30 and 75 were recruited for the study from the periodontics clinic at the Royal Dental Hospital of Melbourne. Subjects had been diagnosed with moderate to severe chronic periodontitis in the past and were treated surgically or non-surgically. GCF and clinical measurements were obtained at baseline and every three months for the period of a year. Periodontal maintenance consisting of supra- and subgingival scaling and root planing, where required, continued during this time.

Ethics approval was obtained from the University of Melbourne Human Research and Ethics Committee, and the Royal Dental Hospital of Melbourne in accordance with the World Medical Association Declaration of Helsinki. Inclusion criteria included greater than 18 years of age, previously diagnosed with and treated for chronic periodontitis, and the presence of at least twenty teeth. Exclusion criteria included the use of antibiotics in the past 3 months, pregnancy or lactation, systemic conditions which may affect the progression of periodontitis, any condition requiring premedication prior to treatment, and long term therapy with non-steroidal anti-inflammatory drugs. Informed written consent was obtained from each patient.

Clinical measures

Two calibrated examiners (LHN and AGL) were responsible for clinical assessment and GCF collection, as well as the periodontal maintenance received by each subject. Throughout the study duration, subjects were seen by the same examiner. Clinical assessments were performed at baseline and every 3 months thereafter over a period of 12 months. The following measurements were made:

1. Gingival inflammation at the test sites were assessed using the modified gingival index (MGI) (15). Sites were scored from 0-4, with 0 being clinically healthy and 4 grossly inflamed.
2. Plaque levels at test sites were assessed using the plaque index (16). Plaque was scored from 0-4, with a score of 0 for an absence of plaque and 4 for abundant plaque deposits.
3. Probing depth. Periodontal pocket depths (PD) were measured at a constant force of 20 grams using an electronic Florida Probe[®] pocket probe (FP, Florida Probe[®] Company, Gainesville, FL, USA) with a tip diameter of 0.35 mm. PD values were recorded to the nearest 0.2 mm.
4. Relative attachment level. The relative attachment level (RAL) was recorded from a fixed point on the occlusal surface on the tooth at the point of PD measurement. RAL were measured at a constant force of 20 grams using an electronic Florida Probe[®] disc probe (FP, Florida Probe[®] Company, Gainesville, FL, USA). Values were recorded to the nearest 0.2 mm
5. Bleeding on probing. Bleeding from the base of the periodontal pocket which occurred up to 30 seconds after periodontal probing was recorded as either present or absent.

6. Suppuration on probing. Suppuration from the periodontal pocket after probing was recorded as either present or absent.

For PD and RAL measurements, a double pass technique was used. Each site was probed twice and the average of the two values taken. Where the second measurement differed from the first by >0.2 mm, a third measurement was taken and an average of the two closest values taken. The five deepest sites in each patient were chosen based upon previous periodontal charts, and were used as test sites. Molar sites (except the mesial surface) were excluded. Subjects received oral hygiene instruction and routine maintenance care throughout the study duration. At each visit, clinical indices were recorded for each test site, followed by GCF collection, subgingival plaque removal with a sterile curette. At the end of each visit, supportive periodontal therapy was provided for the remaining sites in the mouth.

GCF collection

GCF was collected using sterile glass microcapillary tubes (Microcaps, Drummond Scientific, Brookmall, PA, USA) following the method previously described (17). The site where GCF was to be collected was first isolated, if necessary, by cotton rolls and gently air dried to remove any saliva present. Any supragingival plaque was removed with a sterile curette. A sterile glass capillary tube was then placed at the entrance to the periodontal pocket and left for 30 sec. Crevicular fluid (0.2-1.5 μ l) within the pocket was drawn into the glass tube through capillary action. Individual samples were placed into microcentrifuge tubes and placed on ice, until final storage at -70°C . Any samples visually contaminated with blood were discarded.

GCF was dispensed from the microcapillary tube by means of gentle air pressure from one end of the tube, via a bulb. Trifluoroacetic acid (TFA) (2.5%) (4 μ l) was then drawn into each tube and dispensed with the rest of the sample.

Chemicals

All chemicals used, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used was MilliQ grade (Billerica, MA, USA). Acetone was purchased from EMD Chemicals (Darmstadt, Germany), acetonitrile from Riedel-de Haën (Seelze, Germany), and sequencing grade TFA from Agilent Technologies (Palo Alto, CA, USA).

Mass spectrometry

GCF samples were analysed using an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). Preparation of GCF for analysis involved a desalting step via the use of ZipTips (Milipore, Billerica, MA, USA). TFA (0.1% aqueous) was used for washing, before direct elution with 50% acetonitrile and 0.1% aqueous TFA onto 600 μ m anchors on an AnchorChip target plate (Bruker Daltonics). Matrix solution comprising 10 mg α -cyano-4-hydroxycinnamic acid / 1 ml of 50% acetonitrile in 0.1% aqueous TFA was prepared and 1.5 μ l added to the sample and the spot left to dry before analysis. GCF samples from each site were analysed individually.

Samples were analysed with the mass spectrometer in linear mode. Each GCF sample was spotted onto a single target spot on the plate. Each mass spectrum was obtained from 400 laser shots (50 shot bursts) onto the sample. The sample was analysed manually over its entire area in order to obtain the best quality spectrum for each spot.

Processing of mass spectra included smoothing (Savitzky Golay algorithm) (18) with a width of 0.2 m/z and for 1 cycle and baseline subtraction (median algorithm with a flatness of 0.8) (FlexAnalysis; Bruker Daltonics, Bremen, Germany). All spectra were normalized to their total ion count (TIC) using ClinProTools 2.1 software (Bruker Daltonics). For each spectrum the TIC is determined by the sum of all intensities of the spectrum. All spectra were

recalibrated to reduce mass shifts. A total average spectrum was calculated from the recalibrated data. The spectra were weighted with the reciprocal size of the classes to get an equal representation of classes with a very different number of spectra. Peak detection was then performed on the total average spectrum, followed by peak calculation on individual spectra (ClinProTools). Normalization of peak areas was performed for statistical analysis with the Genetic Algorithm (GA), as this allowed for small peaks to be given equal importance to larger peaks during analysis. The GA used in this study for spectral pattern analysis has been described in detail previously (19-21).

Samples for analysis

Samples were classified into two groups according to MGI at the site at the time of collection: low (MGI 0-1, 100 samples) and high (MGI 2-4, 90 samples). Samples were also classified into two groups based upon their PI: low PI (112 samples, PI 0-1) and high PI (81 samples, PI 2-4) groups. For PD values, the shallowest and deepest sites in all subjects (lowest and highest 25th percentile) were used to make two groups for statistical analysis. Samples from the lowest percentile had a pocket depth in the range of 1.2-2.4 mm (mean 2.0 mm), whilst samples from the highest percentile had a pocket depth in the range of 4.2-10.8 mm (mean 5.7 mm).

Attachment loss was defined as an increase in clinical attachment of ≥ 1.2 mm. Calibration of examiners demonstrated a standard deviation between examiners of 0.6 mm (data not shown). Test samples were obtained from the site at the appointment prior to the increase in RAL being clinically detected. Control samples were matched paired samples (based on PD, PI and GI) which did not have attachment loss, and were obtained from the same patient and collected on the same day.

Samples from different patients were analysed to assess the inter-patient variation in GCF profiles. Differences between patients may explain difficulties in the development of diagnostic tests, while similarities may highlight possible proteins which are similarly regulated in different patients.

Statistical Analysis

Peak areas were the unit of analysis. Dependent variables included MGI, PD, PI, smoking status (non-smokers versus smokers) and GCF from sites collected before attachment loss, during attachment loss, after attachment loss and control samples. Mean values and standard deviations were calculated for individual peaks. P values at less than 0.05 were regarded as being statistically significant.

The GA was used to create a “model spectrum” which could be used to differentiate between groups of samples (19-21). The results of the GA were validated by a Cross Validation procedure, which is a measure of the reliability of a calculated model (determines the predicted amount of false negatives). Recognition capability using the GA, and cross validation values were calculated using ClinProTools 2.2 software (Bruker Daltonics). This software was also used for the statistical analysis (T-test/ANOVA) of peak areas.

Prior to statistical analysis and model generation, where possible GCF mass spectra from each category were randomly divided into *training* and *test* groups. The *training* group of mass spectra was used by the GA to generate models for that class. *Test* group spectra were used to validate the model spectra, and provide sensitivity and specificity values.

Results

Forty-four subjects were initially enrolled in the study. Two subjects did not return after the initial screening exam, and 1 subject took antibiotics after the baseline exam, and so data for

41 subjects were included in the analysis. Twelve month data were available for 31 patients, with 9 month data for 2 patients, 6 month for 6 patients and 3 month data for 2 patients. Of the subjects with partial data, 3 subjects took antibiotics during the course of the study, and so only data before this was included. Five subjects did not wish to continue with the study or failed to return during maintenance, 1 subject did not wish to continue with maintenance due to ill health, and 1 patient moved interstate. Patient details are listed in Table 1.

Mass spectrometry

MALDI-MS analysis of GCF provided spectra in the mass range of 0.5-20 kDa. Following processing (smoothing), over 160 well defined peaks (with a signal to noise ratio of greater than 10) were seen in the mass spectra (Figure 1). A total of 385 mass spectra were obtained from GCF samples. Mass spectra were sorted into groups or classes based upon gingival inflammation (MGI), pocket depth values (low PD 1.2-2.4 mm and high PD 4.2-10.8 mm), and plaque levels (PI 0-1 and PI 2-4).

Patient variation in GCF profiles

Differences in GCF profiles from two different patients were also examined (54 and 65 year old female subjects). Although only a limited number of samples were available (25 per subject), high RC and CV (100% and 86% respectively) were seen with the generated model. When this model was validated, high sensitivity and specificity values (75% and 88%) were found (Table 2).

Mass spectra and gingival inflammation

Samples were classified into two groups according to MGI at the site at the time of collection: low (MGI 0-1, 100 samples) and high (MGI 2-4, 90 samples) (Table 3). Using the

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2
3 MGI as the discriminating parameter, from the *training* set data an overall recognition
4 capability (RC) of 86% was calculated (see Table 2). However, the large range in individual
5 peak intensities from individual spectra meant that calculated cross validation values (CV)
6 were low (Table 2), ranging from 54-57%. This low CV reflects the low sensitivity (63%)
7 and specificity (50%) values when the model was tested with *test* data (169 samples, see
8 Table 2). For sites with MGI 2-4, the majority of samples were incorrectly classified by the
9 model as belonging to the low MGI group.
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20 21 **Mass spectra and pocket depth**

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23 Samples were classified into two groups according to PD at the site at the time of collection.
24 Samples from the lowest percentile had a pocket depth in the range of 1.2-2.4 mm (mean 2.0
25 mm), while samples from the highest percentile had a pocket depth in the range of 4.2-10.8
26 mm (mean 5.7 mm) (Figure 2). Following division into *training* and *test* groups, there were
27 67 samples in the low PD, and 69 samples in the high PD groups (Table 2). Statistical
28 analysis using the GA demonstrated that the spectra from the low and high PD group were
29 distinctly different, with an overall RC of 91% and a CV of 65%. When samples were
30 divided into pocket depth ranges (0-3; 3.1-4.9; and 5+ mm) and compared against one
31 another, both RC (68.2%) and CV (33.9%) values were lower (data not shown).
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45 46 **Mass spectra and plaque levels**

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48 Samples were classified into two groups based upon their PI: low PI (112 samples, PI 0-1)
49 and high PI (81 samples, PI 2-4) groups (Table 2). When these two groups were compared
50 and analysed, overall RC and CV values were 88% and 61% respectively. When the model
51 was validated with *test* set data, although a good sensitivity value of 72% was seen, the
52 specificity value was 44%.
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Mass spectra in non-smokers versus smokers

To examine the potential influence of smoking on GCF protein profiles, GCF samples from non-smokers (202 samples) and smokers (64 samples) were compared. The generated model had an overall RC of 98%, and CV of 73% (94% for non-smokers and 52% for smokers). Using a *test* set of 115 samples, a sensitivity of 91% and specificity of 59% was seen.

Attachment loss

Twenty-five out of 205 sites (12%) in 14 subjects (out of 41 subjects) exhibited attachment loss of ≥ 1.2 mm over the 12 month observation period. Some sites exhibited attachment loss over 2 consecutive recall periods, such that the total number of test samples was 34. A matching number of control samples were chosen. Clinical data for test and control samples are presented in **Table 4**.

A link was found between smoking and attachment loss, with 3 of 5 smokers in the study population losing attachment in at least one site over the 12 month period. This trend between attachment loss and smoking supports previously published reports from studies illustrating smoking as a risk factor for periodontal disease (22). One of the smokers who did not lose attachment was a light smoker (1-2 cigarettes per day). Sixty percent of smokers lost attachment compared with 34% of the total population.

Analysis of mass spectra from these sites allowed for average spectra for test and control groups to be constructed (**Figure 3**). One spectrum from each group could not be calibrated and were excluded from the analysis. A total of 33 disease samples and 34 control samples were included in the averaged spectra and analysis. Due to the small number of sites exhibiting attachment loss, data could not be divided into training and test sample sets. Hence

cross validation values generated from the analysis were used to test the strength of the generated MS model.

A GA was used to calculate an MS model (10 peaks) for diseased sites and for healthy sites. Selected peak areas for the disease model are shown in Table 5. Recognition capabilities were calculated (the ability of the MS model to identify spectra which belongs to that group) for each group, as well as cross validation values (refer to Table 6). When comparing stable sites (control) and sites exhibiting attachment loss (test), a high overall recognition capability of 87.9% was obtained, with the recognition capability for test sites 97% and for control sites 78.8%. The higher recognition capability obtained for test sites versus control sites suggests that there is a high variability of crevicular fluid contents from a non-progressing site. A cross validation value of 64.2% was found.

As smoking was associated with attachment loss, an analysis of GCF obtained from smokers who had attachment loss versus non-smokers who had attachment loss was performed (Table 7). A demonstrable difference between GCF obtained from smokers and non-smokers was seen, with overall recognition capabilities of 93.9% and a cross-validation value of 77.6%. The higher cross validation value with smokers suggests that smoking may play a role in the variability of GCF constituents.

When GCF from smokers (3 patients) with attachment loss were examined, a recognition value of 100% for both attachment loss sites and control sites was found. No cross validation values were able to be calculated due to the small number of samples.

Comparison of Peaks used for Model Generation

Table 8 lists the various peaks identified by the GA as being discriminatory and used for model generation. No similarity can be seen between any of the categories.

Discussion

Mass spectral analysis of GCF samples in this study has demonstrated that differences exist between GCF obtained from stable and progressing sites. It was also clear from the MS analysis of GCF, that there was a large variation in individual mass spectra. The peptide and protein peaks observed in the mass spectra obtained from the analysis of GCF in this study represent a small fraction of the peptidome/proteome of GCF.

Increases in gingival inflammation have been correlated with a corresponding increase in GCF volume (23, 24). GCF volume rather than content may reflect the clinical indices of MGI and PI, which are at least partially subjective in nature. Although recognition values are high, the low cross validation values reflect the great variation in mass spectra. Low cross validation values are reflected in the low sensitivity and specificity values obtained when validating the models. The view that all GCF is an inflammatory exudate contrasts with Alfano (25) who described that in health GCF is a transudate of the tissues, whilst with inflammation it becomes an exudate. It is well accepted that an increase in gingival inflammation results in a corresponding increase in GCF flow. Histopathological studies on both human (26) and animals (24, 27) have noted that histologically (as opposed to clinically) healthy sites do not have any measurable GCF exudate. The difference in peak patterns between individual samples evident from this study reflects the change in protein and peptide constituents of individual GCF sites, which may be a result of specific inflammatory processes happening at a site level. Periodontal disease is site specific in nature, and within individuals, the subgingival flora has been shown to differ between sites.

Early studies looking at GCF using gel electrophoresis to visualize proteins (10-100kDa mass range), demonstrated a difference in the quality of GCF (28) from inflamed versus healthy sites, as well as from sites with progressive destructive disease. This is supported by the results of the current study, which has demonstrated that the mass spectra of

GCF samples (0.5-20kDa) show some variation with gingival inflammation. In a cross sectional study on untreated patients, a correlation was reported between cathepsin B/L-, elastase-, tryptase-, trypsin- and dipeptidyl peptidase IV-like activities in GCF and clinical parameters (29). Studies have also demonstrated links between GCF cytokine levels and pocket depth values. Increased levels of TGF- α , IL-1 β , β_2 -microglobulin and total protein content have been significantly associated with increased pocket depth in subjects with varying severity of periodontal disease (30). In untreated periodontitis patients, higher levels of IL-1 β were associated with deeper pocket depths, particularly in subjects with more severe periodontal disease (31). Increased IL-1 β levels as well as CRP were also associated with clinical parameters (pocket depth, PI, GI and CAL) in an adult population (32).

Similarly, a greater statistical significance was seen in this study when mass spectra from sites with low and high pocket depths were compared. Sensitivity and specificity values obtained were greater when examining pocket depth groups when compared with MGI or PI groups. Sites with an increased pocket depth have demonstrated increased numbers of pathogenic bacterial species (33) many of which are proteolytic. Greater pocket depths in association with higher plaque levels may therefore result in increased levels of proteolytic enzymes expressed on the cell surface or secreted into the periodontal pocket, and consequently an increase in protein breakdown products within the periodontal pocket may be observed. The increased mass of bacteria (as pocket depths increase) may also result in an increase in extracellular bacterial proteins in GCF.

Samples from the same patient displayed the highest sensitivity and specificity. GCF mass spectra from the same patient showed the greatest recognition capability (RC) cross validation (CV) and specificity values, when comparing with clinical indices (Table 3). This may be expected given the obvious difference between patients, in relation to the quality and quantity of plaque, inflammatory response, and acquired risk factors (such as smoking).

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3 Distinct variations in GCF profile on a patient basis may be important in the interpretation of
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5 any GCF based test. These differences did not appear to be related to age, gender, or degree
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7 of gingival inflammation, and may reflect individual variations in host inflammatory response
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9 or subgingival bacterial profiles. In this study, the statistical algorithm demonstrated that
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11 there was more similarity in GCF profile between patients, than between sites with differing
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13 levels of inflammation. The differences in GCF profiles between patients may help to explain
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15 the difficulty so far in the development of diagnostic tests for periodontal disease progression.
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17 The use of bioinformatics in this study demonstrates that even with these differences, GCF
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19 mass-spectra profiles may be used to predict attachment loss.
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23 The use of models generated from GCF mass spectra in this study allowed for
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25 attachment loss sites to be predicted (97% recognition capability and 67% cross validation).
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27 Note that the prediction of attachment loss as described in this study differs significantly from
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29 cross-sectional studies which claim to identify potential biomarkers for periodontal disease
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31 but have not related the biomarkers to periods of clinical attachment loss. Cross validation
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33 values would be expected to improve with increased sample sizes, or with the addition of
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35 more samples to the previously generated algorithmic model. The high recognition capability
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37 reflects the ability of the model to correctly diagnose disease sites and compares favorably
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39 with previous studies which have used other methods to diagnose periodontal disease
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41 progression. In a 3-month longitudinal study on 25 untreated periodontal patients (366 sites)
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43 it was reported that total crevicular fluid alkaline phosphatase (ALP) levels may serve as a
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45 predictor of future or current disease activity (34). The difference between ALP levels in
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47 active sites versus control sites was significant with a paired t-test $p < 0.003$. Calculated
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49 sensitivity and specificity values for ALP levels were 68% and 64% for current or recent
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51 disease activity. Bacterial protease [gingipain and bacterial dipeptidyl peptidase (DPP)] levels
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53 (35), and cathepsin B total enzyme and enzyme concentration (35) have been studied as
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possible markers of periodontal disease activity. The results from these studies showed a high correlation between levels of gingipain and DPP ($p < 0.0001$) and cathepsin B ($p < 0.0001$) and attachment loss when comparing active sites with matched control sites from the same patient. It was concluded from these studies that DPP, gingipain and cathepsin B in GCF could all serve as predictors of periodontal attachment loss. Unfortunately no sensitivity or specificity values were given for the use of these factors in predicting future attachment loss in these or follow-up studies.

As with the current study, it seems that the combination of more than one factor improves the ability to predict sites of attachment loss. Total amounts of prostaglandin E2, collagenase, ALP, alpha-2 macroglobulin, osteocalcin and antigenic elastase in GCF have shown significant diagnostic values (sensitivity: 80%, specificity: 91%) in identifying sites of future attachment loss (36). This previous study quantified six factors in GCF using six individual assays, and while the results seem promising, the study involved only 8 subjects and the results have not been validated. As well, the population studied by Nakashima (36) was untreated periodontal patients, and so the results are not directly comparable with this current study. The use of multiple assays for periodontal diagnosis may not be practical due to cost and time constraints. While the factors examined by Nakashima (36) are known inflammatory mediators and tissue turnover / breakdown products, no attempt in this current study has been made to identify the proteins which are used in the algorithmic disease model.

Smoking was seen in this current study to have an influence on biomarkers in GCF. When GCF from smokers and non-smokers who exhibited attachment loss were compared, a notable difference was seen, with high recognition values. This supports reports which have demonstrated that smoking affects the levels of various inflammatory cytokines in the periodontal tissues (37).

Although statistically significant RC, CV, sensitivity and specificity values in this current study were demonstrated, the standard deviations for all peaks were very large. The use of the genetic algorithm allows one to develop models which can look at peak proportions rather than absolute peak intensities. Peak intensities and spectra quality may be affected by sample collection, sample preparation and analysis variation (38). For mass spectrometry where many peptide and protein peaks are present in individual spectra, the relationships of a few peaks may be more important than the absolute heights of the peaks themselves.

An advantage of using the genetic algorithm to calculate a disease model, is that as more samples are added, the accuracy of the calculated model in recognizing healthy and diseased sites increases (the possibility of sharing the ‘model’ will only improve the diagnostic accuracy of using this technology). The use and acceptance of these algorithm-generated models, necessitates a paradigm shift in periodontal diagnostics. Diagnostic tests have traditionally focused on the detection of individual or multiple factors which are known to be either involved in the inflammatory process, or are breakdown products of connective tissue or bone destruction. Mass spectrometry combined with the use of bioinformatics negates the need for the identification of factors which can be used in disease diagnosis.

No similarity was found between the peaks used for model generation between the different categories studied. This reinforces the fact that no single “biomarker” for periodontal disease has yet been identified, and highlights the potential benefit of the use algorithms to identify multiple discriminatory disease markers. The identification of the peaks identified by the GA as being possible disease markers is still under investigation.

All patients examined in this study were diagnosed with moderate to severe chronic periodontitis, and were in maintenance care. The examination of healthy subjects, as well as those with more aggressive forms of the disease, may be required to ascertain whether subject

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3 based differences in GCF profile may enable the detection of “at-risk” individuals for
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5 periodontal disease. The analysis of GCF samples as undertaken in this study has the
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7 potential for automation and high-throughput, and may make large scale screening for oral
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9 diseases economically feasible. At this stage however the high cost of sample analysis as well
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11 as the difficulty in obtaining GCF samples will limit the clinical application. The collection
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13 of GCF from individual sites and analysed on an individual basis is ideal for the detection of
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15 at-risk sites but may not be ideal for screening and detection of at-risk subjects, where
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17 salivary testing may be more convenient. The use of algorithmic models of disease means
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19 that the diagnosis of disease can be independent of the identification of individual biomarkers
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21 and their absolute amounts. In the future, the knowledge of disease biomarkers may facilitate
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23 the development of more rapid screening tests for periodontal disease progression based on a
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25 chairside multiplex assay.
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30 Our results have demonstrated that with mass spectrometry and bioinformatics, GCF
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32 may be used to predict periodontal attachment loss at a site level with a high positive
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34 predictive value before it can be measured clinically. This can be achieved with a single test
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36 on individual GCF samples.
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41
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Table 1. Patient demographics. This table lists basic demographic data for the study population, as well as subjects who suffered attachment loss at one or more sites. (Smokers denote current smokers)

	Total Population			Patients with RAL		
	Male	Female	All	Male	Female	All
Number	12	29	41	2	12	14
Average age	54.4	62.7	60.3	45.0	64.2	61.5
(SD) years	(9.6)	(11.0)	(11.2)	(4.2)	(9.3)	(11.1)
Smokers	2	3	5	1	2	3

Table 2. Model generation for GCF samples based clinical and subject criteria. Recognition capability (RC). Cross validation (CV)

Class	Training set			Test set		
	Samples	RC %	CV %	Samples	Correct	Incorrect
MGI 0-1	100	86	54	169	59	38
MGI 2-4	90	86	57		34	38
Low PD	67	92	61	35	13	5
High PD	69	90	68		12	4
PI 0-1	112	88	76	169	71	27
PI 2-4	81	87	47		31	40
Non smoker	202	99	94	115	96	9
Smoker	64	97	52		16	11
Subject 1	17	100	94	16	6	2
Subject 2	17	100	79		7	1

Table 3. MGI and PI clinical data. Mean PD values (mm) are shown for the MGI and PI subgroups examined

Class	mean PD
MGI 0-1	3.25
MGI 2-4	3.87
PI 0-1	3.46
PI 2-4	3.62

Table 4. Clinical data for attachment loss samples and control samples. Test samples were samples obtained immediately before attachment loss was diagnosed clinically. Control samples were matched samples from a different site in the same patient. The range of PD, MGI and PI values can be seen.

	Number	mean PD (range)	MGI				PI			
			1	2	3	4	0	1	2	3
Test	34	4.2 (1.8-9.8)	16	9	6	2	3	15	12	3
Control	34	2.8 (1.4-5.0)	22	5	6	0	3	18	10	2

Table 5. Mass spectra peaks used for model generation. This table lists the mass peaks used for the algorithm generated disease ‘model’. The difference in peak area between test and control samples, as well as the standard deviation, is shown. PTTA: P-value T-Test/ANOVA

Mass	Weight	Average Peak Area		Standard deviation		PTTA
		Test	Control	Test	Control	
2023.5	0.213	1.56	2.15	1.11	2.57	0.907
4042.8	0.238	3.13	3.72	1.57	1.93	0.907
4490.3	0.117	2.55	2.28	2.06	1.13	0.907
4525.3	0.177	2.78	2.43	1.59	1.15	0.907
5232.3	0.199	10.62	7.3	15.26	6.81	0.907
5502.2	0.377	2.98	3.93	1.45	2.09	0.558
6890.1	0.432	1.81	2.39	0.89	1.01	0.449
10939	0.125	3.17	3.4	1.34	1.3	0.907
12833	0.251	1.53	1.82	0.81	0.81	0.907
14008	0.502	0.96	1.3	0.4	0.55	0.449

Table 6. Recognition values and validation of model mass spectra obtained using the genetic algorithm. Cross validation utilizes an internal calibration system and is reflective of the specificity of the model. The recognition capability of the model reflects the sensitivity of the model in correctly assigning all test sites to the disease model.

Sites	Cross validation	Recognition capability
Test sites	68.3%	97.0%
Control sites	60.0%	78.8%
Total	64.2%	87.9%

Table 7. Recognition values and validation of model mass spectra obtained with genetic algorithm for all sites from smokers versus non-smokers who had attachment loss. Cross validation utilizes an internal calibration system and is reflective of the specificity of the model. The recognition capability of the model reflects the sensitivity of the model in correctly assigning all test sites to the disease model.

Sites	Cross validation	Recognition capability
Smokers	60.8%	89.8%
Non-smokers	94.4%	98.0%
Total	77.6%	93.9%

Table 8. Comparison of peaks used in model generation. This table lists the various peaks identified by the genetic algorithm as being discriminatory for each parameter. No mass peak is shared between any of the categories.

AL vs Stable sites	Smoker vs Non-Sokers	AL in smokers	Low and High PD	Gingival Index
2023.5	448.88	540.88	1059.66	558.79
4042.8	505.45	705.44	3463.34	4525.11
4490.3	516.78	3797.44	5465.87	6888.79
4525.3	558.96	4133.51	10230.58	10981.8
5232.3	1436.92	4148.54	13537.89	12001.98
5502.2	3877.92	5695.27		
6890.1	4610.86	6952.8		
10939	5174.19	9050.99		
12833	6708.17	11004.63		
14008	11080.09	13537.12		

Figure Legends

Figure 1: Mass spectrum of a GCF sample from a single site following desalting and analysis on a MALDI mass spectrometer with CHCA as a matrix. The x-axis depicts the m/z value of the peptide/protein, while its relative intensity is shown on the y-axis. Major peaks have been labeled. The m/z range of 500-20000 was used for MALDI analysis of GCF samples.

Figure 2: Averaged mass spectra chromatogram for low PD and high PD classes. The averaged mass spectrum for low PD (average PD 2.0 mm) and high PD (average PD 5.7 mm) can be seen. The mass spectra have been magnified to identify peak m/z 6385, which was identified by the algorithm as one of the discriminating peaks used in the model to differentiate low and high PD groups.

Figure 3: Total averaged spectra for test and control groups. The averaged mass spectrum for Test (attachment loss - 33 samples) samples (red line) and Control samples (green line - 34 samples). The y-axis depicts the intensity of the peak, while the x-axis depicts the m/z value. Peak areas from these averaged spectra were used by the genetic algorithm for model generation.

Figure 1

Figure 2

Figure 3