Journal of
PERIODONTAL RESEARCH

J Periodont Res 2013; 48: 331–341

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12012

Mass spectrometric analysis of gingival crevicular fluid biomarkers can predict periodontal disease progression

L. H. Ngo, I. B. Darby, P. D. Veith, A. G. Locke, E. C. Reynolds
Oral Health CRC, Melbourne Dental School and the Bio21 Institute, The University of Melbourne, Melbourne, Victoria, Australia

Ngo LH, Darby IB, Veith PD, Locke AG, Reynolds EC. Mass spectrometric analysis of gingival crevicular fluid biomarkers can predict periodontal disease progression. J Periodont Res 2013; 48: 331–341. © 2012 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objective: 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 gingival crevicular fluid may allow for the site-specific prediction of periodontal disease progression.

Material and Methods: Forty-one periodontal maintenance subjects were followed over 12 mo, with clinical measurements taken at baseline and every 3 mo thereafter. Gingival crevicular fluid was collected from subjects at each visit and was analysed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Samples were classified based upon pocket depth, modified gingival index (MGI), plaque index and attachment loss, and were 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 gingival crevicular fluid samples were analysed. Twenty-five sites under observation in 14 patients exhibited attachment loss of > 2 mm over the 12-mo period. The clinical indices pocket depth, MGI, plaque levels and bleeding on probing served as poor discriminators of gingival crevicular fluid mass spectra. Models generated from the gingival crevicular fluid mass spectra could predict attachment loss at a site with a high specificity (97% recognition capability and 67% cross-validation).

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

Eric C. Reynolds PhD, Oral Health CRC, Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Victoria, 3010, Australia
Tel: +61 3 9341 1547
Fax: +61 3 9341 1596
e-mail: e.reynolds@unimelb.edu.au

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

Accepted for publication August 21, 2012

Chronic periodontitis and aggressive periodontitis are inflammatory diseases of the periodontium that 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, although 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 nonsurgical and surgical tooth debridement, followed by 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 every 3 to 12 mo.

Periodontal disease can generally be successfully treated with both nonsurgical 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 a higher level of 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 nonspecific, correlating more closely with gingival inflammation than with 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 that can provide mass spectra of proteins and peptides within a sample. These spectra can be used to identify these proteins and peptides or to create models for disease diagnosis. MS has served as an adjunct to gel electrophoresis (10) and liquid chromatography (LC) (11) in the identification of certain proteins and peptides in gingival crevicular fluid. Eight peptides in the gingival crevicular fluid from healthy sites have been identified using LC coupled with electrospray ionization MS (12). MS has been used in conjunction with gel electrophoresis and LC–tandem MS (LC-MS/MS) to identify 33 peptides and 64 proteins in gingival crevicular fluid (8). In more recent MS studies, 432 (13) and 327 (14) proteins in human gingival crevicular fluid samples were identified and differences were detected in some proteins from healthy subjects compared with those from patients with chronic periodontitis.

MS can detect proteins and peptides in the femtomole range, and the detection of a peptide or a 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 gingival crevicular fluid mass spectra for the purpose of predicting disease progression has not been developed. The precise peptide/protein profile of gingival crevicular fluid in health and varying degrees of inflammation is lacking. This paper describes a reproducible technique for the analysis of protein and peptide profiles of gingival crevicular fluid using MS and examines the relationship between the relative amounts of proteins and peptides in gingival crevicular fluid with clinical indices in periodontal maintenance patients. The aim of the study was to determine whether the mass spectrometric analysis of gingival crevicular fluid was capable of predicting sites at risk of disease progression.

Material and methods

Patient recruitment

Forty-one patients (30–75 years of age) in a periodontal disease maintenance program 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 had received surgical or nonsurgical treatment. Gingival crevicular fluid and clinical measurements were obtained at baseline and every 3 mo for the period of a year. Periodontal maintenance, consisting of supragingival and subgingival scaling and root planing, where required, were continued during this time.

Ethics approval was obtained from the University of Melbourne Human Research and Ethics Committee and from the Royal Dental Hospital of Melbourne, in accordance with the World Medical Association Declaration of Helsinki. Inclusion criteria were: > 18 years of age; previously diagnosed with and treated for chronic periodontitis; and the presence of at least 20 teeth. Exclusion criteria were: the use of antibiotics in the past 3 mo; pregnancy or lactation; systemic conditions that may affect the progression of periodontitis; any condition requiring premedication before treatment; and long-term therapy with nonsteroidal anti-inflammatory drugs. Informed written consent was obtained from each patient.

Clinical measures

Two calibrated examiners (L.H.N. and A.G.L.) were responsible for clinical assessment and gingival crevicular fluid collection, as well as for the periodontal maintenance received by each subject. Throughout the duration of the study, subjects were seen by the same examiner. Clinical assessments were performed at baseline and every 3 mo thereafter over a period of 12 mo. The following measurements were made.

1 Gingival inflammation at the test sites was assessed using the modified gingival index (MGI) (15). Sites were scored on a scale of 0–4, with 0 being clinically healthy and 4 being grossly inflamed.
2 Plaque levels at test sites were assessed using the plaque index (PI) (16). Plaque was scored on a scale of 0–4, with 0 indicating an absence of plaque and 4 indicating abundant plaque deposits.
3 Probing depth. Periodontal pocket depths were measured at a constant force of 20 g, using an electronic Florida Probe® pocket probe (FP; Florida Probe® Company, Gainesville, FL, USA) with a tip diameter of 0.35 mm. Pocket-depth 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 pocket depth measurement. The RAL was measured at a constant force of 20 g using an electronic Florida Probe® disc probe (FP; Florida Probe® Company). 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 s 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 pocket depth and RAL measurements, a double-pass technique was used. Each site was probed twice and the average of the two values was taken. If the second measurement differed from the first by > 0.2 mm, a third measurement was taken and an average of the two closest values was taken. The five deepest sites in each patient were chosen based upon previous periodontal charts, and were used as test sites. Molar sites (except for the mesial surface) were excluded. Subjects received oral hygiene instruction and routine maintenance care for the duration of the study. At each visit, clinical indices were recorded for each test site, followed by gingival crevicular fluid collection and 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.

Gingival crevicular fluid collection

Gingival crevicular fluid was collected using sterile glass microcapillary tubes (Microcaps; Drummond Scientific, Brookmall, PA, USA) following the method previously described (17). The site where gingival crevicular fluid 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 using a sterile curette. A sterile glass capillary tube was then placed at the entrance to the periodontal pocket and left for 30 s. Crevicular fluid (0.2–1.5 μL) within the pocket was drawn into the glass tube through capillary action. Individual samples were transferred to microcentrifuge tubes and placed on ice until final storage at −70°C. Any samples visually contaminated with blood were discarded.

Gingival crevicular fluid 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 μL) 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 (Millipore, 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

Gingival crevicular fluid samples were analysed using an Ultraflex matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) instrument (Bruker Daltonics, Bremen, Germany). Preparation of gingival crevicular fluid for analysis involved a desalting step via the use of ZipTips (Millopore). TFA (0.1% aqueous) was used for washing, before direct elution with 50% acetonitrile and 0.1% aqueous TFA onto 600-μm anchors on an AnchorChip target plate (Bruker Daltonics). Matrix solution comprising 10 mg of α-cyano-4-hydroxycinnamic acid/1 mL of 50% acetonitrile in 0.1% aqueous TFA was prepared, 1.5 μL was added to the sample and the spot was left to dry before analysis. Gingival crevicular fluid samples from each site were analysed individually.

Samples were analysed with the mass spectrometer in linear mode. Each gingival crevicular fluid 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 (according to the Savitzky Golay algorithm) (18) with a mass-to-charge ratio (m/z) width of 0.2 for one cycle and baseline subtraction (median algorithm with a flatness of 0.8) (FlexAnalysis; Bruker Daltonics). All spectra were normalized to their total ion count using CLINPROTOOLS 2.1 software (Bruker Daltonics). For each spectrum the total ion count was 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 obtain 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 using a genetic algorithm (GA), as this allowed equal importance to be given to both small peaks and 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 the 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 (PI = 0–1; 112 samples) and high PI (PI = 2–4; 81 samples). For pocket-depth values, the shallowest and deepest sites in all subjects (the lowest and the highest 25th percentiles) 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 $\geq$ 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 before the increase in RAL was clinically detected. Control samples were matched paired samples (based on pocket depth, PI and GI) that 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 interpatient variation in gingival crevicular fluid profiles. Differences between patients may explain difficulties in the development of diagnostic tests, while similarities may highlight possible proteins that are similarly regulated in different patients.

Statistical analysis

Peak areas were the unit of analysis. Dependent variables included smoking status (nonsmokers vs. smokers), MGI, pocket depth, PI and gingival crevicular fluid. All variables were recorded before attachment loss, during attachment loss and after attachment loss and in controls. Mean values and standard deviations were calculated for individual peaks. p values of < 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 using a cross-validation (CV) procedure, which is a measure of the reliability of a calculated model (it determines the predicted number of false negatives). Recognition capability (RC) using the GA, and CV values were calculated using CLINPROTOOLS 2.2 software (Bruker Daltonics). This software was also used for the statistical analysis (t-test/analysis of variance) of peak areas.

Before statistical analysis and model generation, where possible gingival crevicular fluid 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 to provide sensitivity and specificity values.

Results

Forty-four subjects were initially enrolled in the study. Two subjects did not return after the initial screening examination, and one subject took antibiotics after the baseline examination; therefore, data for 41 subjects were included in the analysis. Twelve-month data were available for 31 patients, with 9-mo data for two patients, 6-mo data for six patients and 3-mo data for two patients. Of the subjects with partial data, three subjects took antibiotics during the course of the study, and so only data before this were included. Five subjects did not wish to continue with the study or failed to return during maintenance, one subject did not wish to continue with maintenance as a result of ill health and one patient moved interstate. Patient details are listed in Table 1.

Mass spectrometry

MALDI-MS analysis of gingival crevicular fluid 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 > 10) were seen in the mass spectra (Fig. 1). A total of 385 mass spectra were obtained from gingival crevicular fluid samples. Mass spectra were sorted into groups or classes based upon gingival inflammation (MGI), pocket-depth values (low pocket depth = 1.2–2.4 mm and high pocket depth = 4.2–10.8 mm) and plaque levels (PI = 0–1 and PI = 2–4).

Patient variation in gingival crevicular fluid profiles

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

Mass spectra and gingival inflammation

Samples were classified into two groups according to the MGI at the site at the time of collection: low (MGI = 0–1; 100 samples) and high (MGI = 2–4; 90 samples) (Tables 2 and 3). Using the MGI as the discriminating parameter, from the

Table 1. Patient demographics

Variable	Total population			Patients with attachment loss		
	Male	Female	All	Male	Female	All
Number of patients	12	29	41	2	12	14
Age (years)[a]	54.4 (9.6)	62.7 (11.0)	60.3 (11.2)	45.0 (4.2)	64.2 (9.3)	61.5 (11.1)
Number of smokers	2	3	5	1	2	3

[a]Values are given as *n* or as mean (standard deviation).
Basic demographic data for the total study population, as well as for patients who suffered attachment loss at one or more sites, are presented. 'Number of smokers' denotes current smokers.

training set data an overall RC of 86% was calculated (see Table 2). However, the large range in individual peak intensities from individual spectra meant that calculated CV values were low (Table 2), ranging from 54–57%. This low CV reflects the low sensitivity (63%) and specificity (50%) values when the model was tested with test data (169 samples, see Table 2). For sites with an MGI of 2–4, the majority of samples were incorrectly classified by the model as belonging to the low-MGI group.

Mass spectra and pocket depth

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

Mass spectra and plaque levels

Samples were classified into two groups based upon their PI: low PI (PI = 0–1; 112 samples) and high PI (PI = 2–4; 81 samples) (Tables 2 and 3). When these two groups were compared and analysed, overall RC and CV values were 88% and 61%, respectively. When the model was

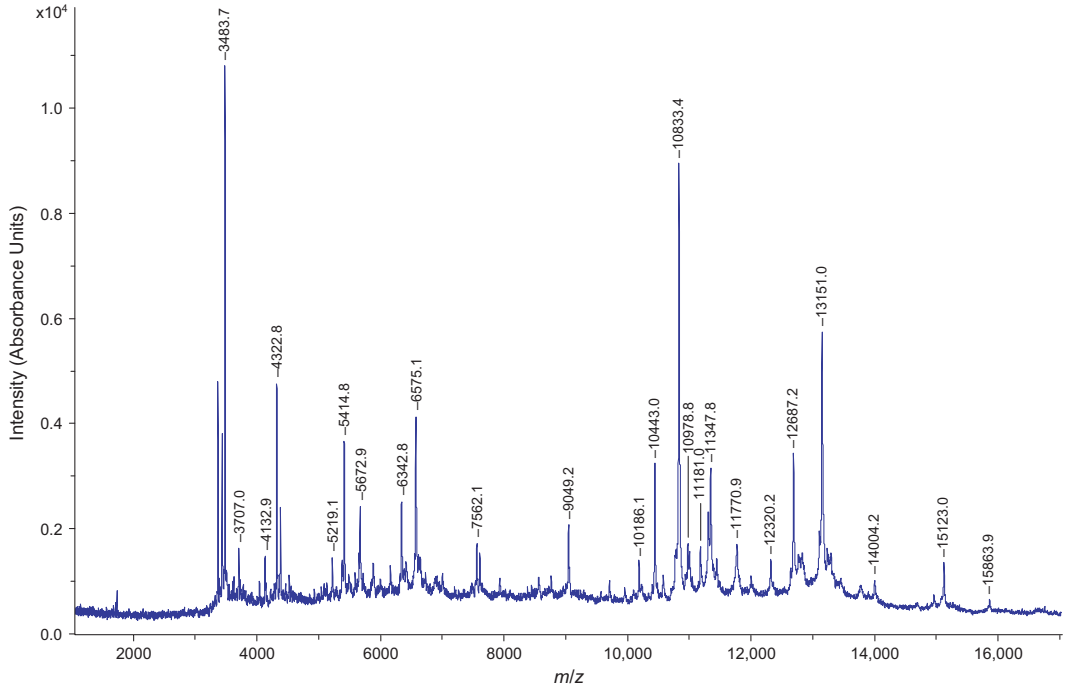

Fig. 1. Mass spectrum of a gingival crevicular fluid sample from a single site following desalting and analysis on a matrix-assisted laser desorption/ionization (MALDI) mass spectrometer with alpha-cyano-4-hydroxycinnamic acid as a matrix. The *x*-axis depicts the mass-to-charge ratio (*m*/*z*) value of the peptide/protein, while its relative intensity is shown on the *y*-axis. The major peaks have been labeled. The *m*/*z* range of 500–20,000 was used for MALDI analysis of gingival crevicular fluid samples.

Table 2. Model generation for gingival crevicular fluid samples based on clinical and subject criteria

	Training set			Test set		
Class	Samples	RC%	CV%	Samples	Correct	Incorrect
Modified gingival index						
0–1	100	86	54	169	59	38
2–4	90	86	57		34	38
Pocket depth						
Low	67	92	61	35	13	5
High	69	90	68		12	4
Plaque index						
0–1	112	88	76	169	71	27
2–4	81	87	47		31	40
Nonsmoker	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

CV, cross validation; RC, recognition capability.

Table 3. Modified gingival index and plaque index clinical data

Class	Mean pocket depth (mm)
Modified gingival index	
0–1	3.25
2–4	3.87
Plaque index	
0–1	3.46
2–4	3.62

validated with test set data, although a good sensitivity value of 72% was seen, the specificity value was 44%.

Mass spectra in nonsmokers vs. smokers

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

Attachment loss

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

A link was found between smoking and attachment loss, with three of five smokers in the study population losing attachment in at least one site over the 12-mo 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 (one to two cigarettes per day). Sixty per cent of smokers lost attachment compared with 34% of the total population.

Analysis of mass spectra from these sites allowed average spectra for test and control groups to be constructed (Fig. 3). One spectrum from each group could not be calibrated and these were excluded from the analysis. A total of 33 disease samples and 34 control samples were included in the averaged spectra and analysis. Owing to the small number of sites exhibiting attachment loss, data could not be divided into training and test sample sets. Hence, CV 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. RC values were calculated (the ability of the MS model to identify spectra that belongs to that group) for each group, as well as CV values (refer to Table 6). When comparing stable sites (control) and sites exhibiting attachment loss (test), a high overall RC of 87.9% was obtained, with an RC for test sites of 97% and for control sites of 78.8%. The higher RC obtained for test sites vs. control sites suggests that there is a high variability of crevicular fluid contents from a nonprogressing site. A total CV value of 64.2% was found.

As smoking was associated with attachment loss, an analysis was performed of gingival crevicular fluid obtained from smokers who had attachment loss vs. nonsmokers who had attachment loss (Table 7). A demonstrable difference was seen between gingival crevicular fluid obtained from smokers and nonsmokers, with an overall RC of 93.9% and a CV value of 77.6%. The higher CV value obtained for smokers suggests that smoking may play a role in the variability of gingival crevicular fluid constituents.

When gingival crevicular fluid from smokers (three patients) with attachment loss was examined, a recognition value of 100% for both attachment-loss sites and control sites was found. No CV values could be calculated because of 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. There was no similarity between any of the categories.

Discussion

Mass spectral analysis of gingival crevicular fluid samples in this study demonstrated that differences exist

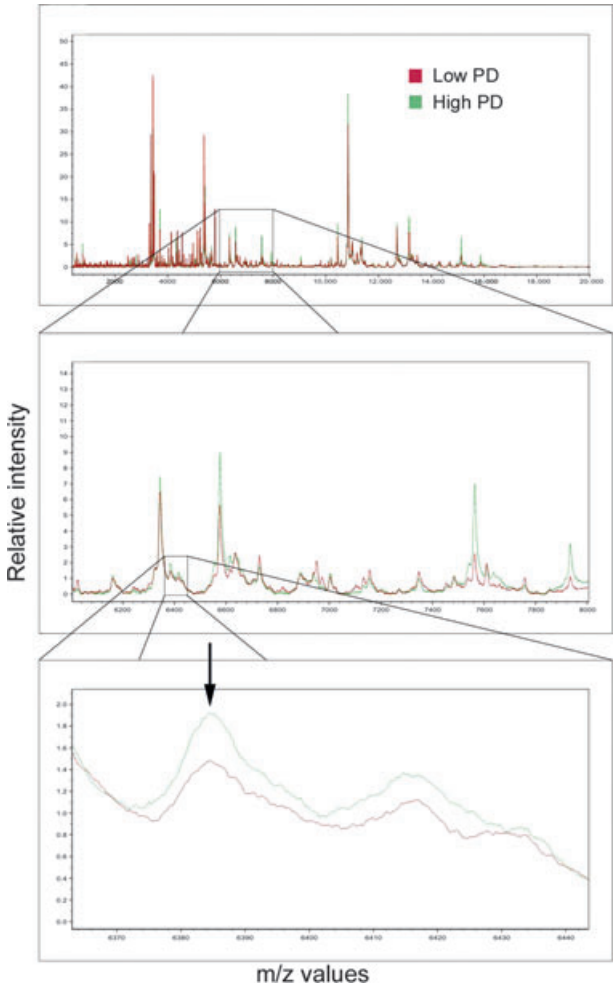

Fig. 2. Averaged mass spectra chromatogram for low pocket-depth and high pocket-depth classes. The averaged mass spectrum for low pocket depth (average pocket depth = 2.0 mm) and high pocket depth (average pocket depth = 5.7 mm) can be seen. The mass spectra have been magnified to identify the peak mass-to-charge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

Table 4. Clinical data for attachment-loss samples and control samples

			Modified gingival index				Plaque index			
	Number	Pocket depth mean (range)	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

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 pocket depth, modified gingival index and plaque index values is seen.

between gingival crevicular fluid obtained from stable and progressing sites. It was also clear from the MS analysis of gingival crevicular fluid 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 gingival crevicular fluid in this study represent a small fraction of the peptidome/proteome of gingival crevicular fluid.

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

Early studies of gingival crevicular fluid that used gel electrophoresis to visualize proteins (10–100 kDa mass range), demonstrated a difference in the quality of gingival crevicular fluid (28) from inflamed vs. healthy sites, as well as from sites with progressive destructive disease. This is supported by the results of the current study, which demonstrated that the mass spectra of gingival crevicular fluid samples (0.5–20 kDa) show some variation with gingival inflammation. In a cross-sectional study on untreated

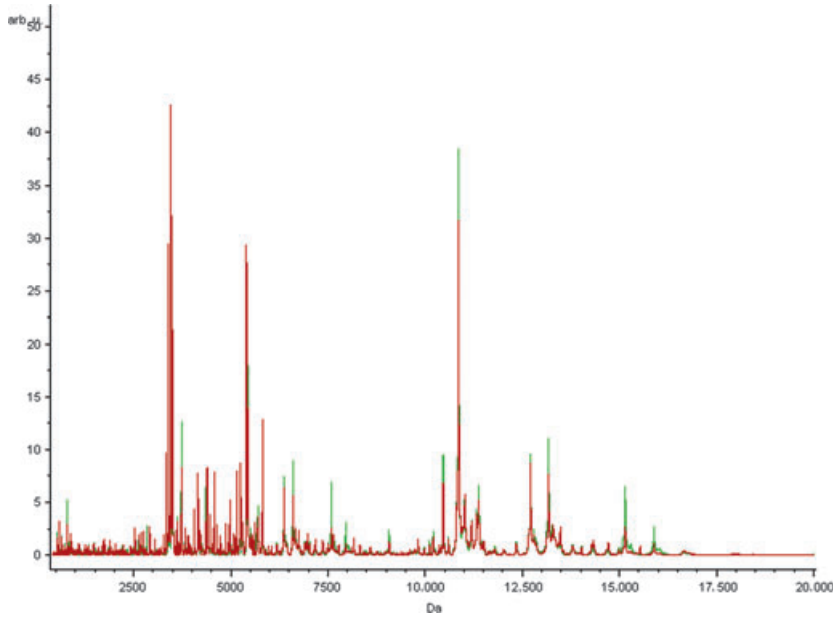

Fig. 3. Total averaged spectra for test and control groups. The averaged mass spectrum is shown for test samples (attachment loss; red line; 33 samples) and for control samples (green line; 34 samples). The *y*-axis depicts the intensity of the peak (absorbance units), while the *x*-axis depicts the mass value (Daltons). Peak areas from these averaged spectra were used by the genetic algorithm for model generation.

patients, a correlation was reported between cathepsin B/L-, elastase-, tryptase-, trypsin- and dipeptidyl peptidase IV-like activities in gingival crevicular fluid and clinical parameters (29). Studies have also demonstrated links between gingival crevicular fluid cytokine levels and pocket-depth values. Increased levels of transforming growth factor alpha, interleukin-1beta, 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 patients with untreated periodontitis, higher levels of interleukin-1beta were associated with deeper pocket depths, particularly in subjects with more severe periodontal disease (31). Increased levels of interleukin-1beta and C-reactive protein were also associated with clinical parameters (pocket depth, PI, GI and CAL) in an adult population (32).

Table 5. Mass spectra peaks used for model generation

		Average peak area		Standard deviation		
Mass	Weight	Test	Control	Test	Control	PTTA
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
10,939	0.125	3.17	3.4	1.34	1.3	0.907
12,833	0.251	1.53	1.82	0.81	0.81	0.907
14,008	0.502	0.96	1.3	0.4	0.55	0.449

The mass peaks used for the algorithm-generated disease "model" are listed. The difference in peak area between test and control samples, as well as the standard deviation, is shown. PTTA, *p*-value *t*-test/analysis of variance.

Table 6. Recognition capability values and cross-validation of model mass spectra obtained using the genetic algorithm

Sites	Cross-validation(%)	Recognition capability(%)
Test	68.3	97.0
Control	60.0	78.8
Total	64.2	87.9

Cross-validation utilizes an internal calibration system and reflects 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.

Similarly, a greater statistical significance was seen in this study when mass spectra from sites with low and high pocket depths were compared. The sensitivity and specificity values were greater for pocket-depth groups than for 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 the concentration of extracellular bacterial proteins in gingival crevicular fluid.

Samples from the same patient displayed the highest sensitivity and specificity. Gingival crevicular fluid mass spectra from the same patient showed the highest RC, CV and specificity values when compared with clinical indices (Table 2). 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). Distinct variations in gingival crevicular fluid profile between patients may be important in the interpretation of any gingival crevicular fluid-based test. These differences did not appear to be related to age, gender or degree of

Table 7. Recognition capability values and cross-validation of model mass spectra obtained, using the genetic algorithm, for all sites from smokers vs. nonsmokers who had attachment loss

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

Cross-validation utilizes an internal calibration system and reflects 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.

gingival inflammation, and may reflect individual variations in the host inflammatory response or subgingival bacterial profiles. In this study, the statistical algorithm demonstrated that there was more similarity in the gingival crevicular fluid profile between patients, than between sites with differing levels of inflammation. The differences in gingival crevicular fluid profiles between patients may help to explain the difficulty experienced so far in developing diagnostic tests for periodontal disease progression. The use of bioinformatics in this study demonstrates that even with these differences, gingival crevicular fluid mass-spectra profiles may be used to predict attachment loss.

The use of models generated from gingival crevicular fluid mass spectra in this study allowed for attachment-loss sites to be predicted (with 97% RC and 67% CV). Note that the prediction of attachment loss, as described in this study, differs significantly from cross-sectional studies that claim to identify potential biomarkers for periodontal disease but have not related the biomarkers to periods of clinical attachement loss. CV values would be expected to improve with increased sample size, or with the addition of more samples to the previously generated algorithmic model. The high RC reflects the ability of the model to correctly diagnose disease sites and compares favourably with previous studies that have used other methods to diagnose periodontal disease progression. In a 3-mo longitudinal study on 25 untreated periodontal patients (366 sites) it was reported that total crevicular fluid alkaline phosphatase (ALP) levels may serve as a predictor of future or current disease activity (34). The difference between ALP levels in active sites vs. control sites was significant ($p < 0.003$, paired *t*-test). Calculated sensitivity and specificity values for ALP levels were 68% and 64% for current or recent disease activity, respectively. Bacterial protease (gingipain and bacterial dipeptidyl peptidase) levels (35), and cathepsin B level (35) have been studied as possible markers of periodontal disease activity. The results from these studies showed a high correlation between the levels of gingipain and dipeptidyl peptidase ($p < 0.0001$) 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 dipeptidyl peptidase, gingipain and cathepsin B in gingival crevicular fluid 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 in 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 E_2, collagenase, ALP, alpha-2 macroglobulin, osteocalcin and antigenic elastase in gingival crevicular fluid have shown significant diagnostic values (80% sensitivity and 91% specificity) in identifying sites of future attachment loss (36). This previous study quantified six factors in gingival crevicular fluid using six individual assays, and while the results seem promising, the study involved only eight subjects and the results have not been validated. Also, the population studied by Nakashima *et al*. (36) comprised 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 owing to cost and time constraints. While the factors examined by Nakashima *et al*. (36) are known inflammatory mediators and tissue turnover/breakdown products, no attempt in this current study has been made to identify the proteins that are used in the algorithmic disease model.

In the present study, smoking was observed to have an influence on biomarkers in gingival crevicular fluid. Comparison of gingival crevicular fluid from smokers and nonsmokers who exhibited attachment loss demonstrated a notable difference, 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 were demonstrated in this current study, the standard deviations for all peaks were very large. The use

Table 8. Comparison of peaks used in model generation

Attachment loss sites vs. stable sites	Smokers vs. nonsmokers	Attachment loss in smokers	Low and high pocket depth	Gingival index
2023.50	448.88	540.88	1059.66	558.79
4042.80	505.45	705.44	3463.34	4525.11
4490.30	516.78	3797.44	5465.87	6888.79
4525.30	558.96	4133.51	10230.58	10981.80
5232.30	1436.92	4148.54	13537.89	12001.98
5502.20	3877.92	5695.27		
6890.10	4610.86	6952.80		
10939.00	5174.19	9050.99		
12833.00	6708.17	11004.63		
14008.00	11080.09	13537.12		

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.

of the genetic algorithm allows one to develop models that 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 MS, 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 that are known to be either involved in the inflammatory process or are breakdown products of connective tissue or bone destruction. MS, combined with the use of bioinformatics, negates the need for the identification of factors that 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 of 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-based differences in gingival crevicular fluid profile may enable the detection of individuals "at-risk" for periodontal disease. The analysis of gingival crevicular fluid samples, as undertaken in this study, has the potential for automation and high throughput, and may make large-scale screening for oral diseases economically feasible. At this stage, however, the high cost of sample analysis, as well as the difficulty in obtaining gingival crevicular fluid samples, will limit the clinical application. The collection of gingival crevicular fluid from individual sites for analysis on an individual basis is ideal for the detection of at-risk sites but may not be ideal for screening and detection of at-risk subjects, where salivary testing may be more convenient. The use of algorithmic models of disease means that the diagnosis of disease can be independent of the identification of individual biomarkers and their absolute amounts. In the future, the knowledge of disease biomarkers may facilitate the development of more rapid screening tests for periodontal disease progression based on a chairside multiplex assay.

Our results have demonstrated that with MS and bioinformatics, gingival crevicular fluid may be used to predict periodontal attachment loss at a site level with a high positive predictive value before it can be measured clinically. This can be achieved with a single test on individual gingival crevicular fluid samples.

Acknowledgements

The authors wish to thank Dental Health Services Victoria for providing a source of patients for gingival crevicular fluid sample collection. The authors would like to acknowledge the help of Dr S. Byrne for her clinical assistance. This work was funded by the National Health and Medical Research Council, the Australian Dental Research Foundation, and the Collaborative Research Centre for Oral Health Sciences, Melbourne Dental School, The University of Melbourne, Bio21 Molecular Science and Biotechnology Institute, Parkville, Australia. This work formed part of the PhD thesis of Luan Ngo.

References

1. Axelsson P, Nystrom B, Lindhe J. The long-term effect of a plaque control program on tooth mortality, caries and periodontal disease in adults. Results after 30 years of maintenance. *J Clin Periodontol* 2004;**31**:749–757.
2. Hirschfeld L, Wasserman B. A long-term survey of tooth loss in 600 treated periodontal patients. *J Periodontol* 1978;**49**: 225–237.
3. McFall WT Jr. Tooth loss in 100 treated patients with periodontal disease. A long-term study. *J Periodontol* 1982;**53**: 539–549.
4. Becker W, Berg L, Becker BE. The long term evaluation of periodontal treatment and maintenance in 95 patients. *Int J Periodontics Restorative Dent* 1984;**4**: 54–71.
5. Loos BG, Tjoa S. Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? *Periodontol 2000* 2005;**39**:53–72.
6. Badersten A, Nilveus R, Egelberg J. Scores of plaque, bleeding, suppuration and probing depth to predict probing attachment loss. 5 years of observation following nonsurgical periodontal therapy. *J Clin Periodontol* 1990;**17**:102–107.
7. Haffajee AD, Socransky SS, Goodson JM. Clinical parameters as predictors of destructive periodontal disease activity. *J Clin Periodontol* 1983;**10**:257–265.
8. Ngo LH, Veith PD, Chen YY, Chen D, Darby IB, Reynolds EC. Mass spectrometric analyses of peptides and proteins in human gingival crevicular fluid. *J Proteome Res* 2010;**9**:1683–1693.
9. Palys MD, Haffajee AD, Socransky SS, Giannobile WV. Relationship between C-telopeptide pyridinoline cross-links (ICTP) and putative periodontal pathogens in periodontitis. *J Clin Periodontol* 1998;**25**:865–871.
10. Kojima T, Andersen E, Sanchez JC *et al.* Human gingival crevicular fluid contains MRP8 (S100A8) and MRP14 (S100A9), two calcium-binding proteins of the S100 family. *J Dent Res* 2000;**79**:740–747.
11. Lundy FT, Orr DF, Shaw C, Lamey PJ, Linden GJ. 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* 2005;**42**:575–579.
12. Pisano E, Cabras T, Montaldo C *et al.* Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. *Eur J Oral Sci* 2005;**113**:462–468.
13. Baliban RC, Sakellari D, Li Z, DiMaggio PA, Garcia BA, Floudas CA. Novel protein identification methods for biomarker discovery via a proteomic

analysis of periodontally healthy and diseased gingival crevicular fluid samples. *J Clin Periodontol* 2012;**39**:203–212.
14. Tsuchida S, Satoh M, Umemura H *et al*. Proteomic analysis of gingival crevicular fluid for discovery of novel periodontal disease markers. *Proteomics* 2012;**12**:2190–2202.
15. Lobene RR, Weatherford T, Ross NM, Lamm RA, Menaker L. A modified gingival index for use in clinical trials. *Clin Prev Dent* 1986;**8**:3–6.
16. Silness J, Loee H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condtion. *Acta Odontol Scand* 1964;**22**:121–135.
17. Giannobile WV, Riviere GR, Gorski JP, Tira DE, Cobb CM. Glycosaminoglycans and periodontal disease: analysis of GCF by safranin O. *J Periodontol* 1993;**64**:186–190.
18. Savitzky A, Golay M. Smoothing and Differentiation of data by simplified least squares procedures. *Anal Chem (Wash)* 1964;**36**:1627–1639.
19. Adam BL, Qu Y, Davis JW *et al*. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res* 2002;**62**:3609–3614.
20. Petricoin EF, Ardekani AM, Hitt BA *et al*. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 2002;**359**:572–577.
21. Wong JW, Cagney G, Cartwright HM. SpecAlign–processing and alignment of mass spectra datasets. *Bioinformatics (Oxford, England)* 2005;**21**:2088–20900.
22. Grossi SG, Zambon JJ, Ho AW *et al*. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 1994;**65**:260–267.
23. Koch G, Lindhe J. The effect of supervised oral hygiene on the gingiva of children. The effect of toothbrushing. *Odontol Revy* 1965;**16**:327–335.
24. Lindhe J, Rylander H. Experimental gingivitis in young dogs. *Scand J Dent Res* 1975;**83**:314–326.
25. Alfano MC. The origin of gingival fluid. *J Theor Biol* 1974;**47**:127–136.
26. Brecx MC, Gautschi M, Gehr P, Lang NP. Variability of histologic criteria in clinically healthy human gingiva. *J Periodontal Res* 1987;**22**:468–472.
27. Egelberg J. The topography and permeability of vessels at the dento-gingival junction in dogs. *J Periodontal Res Suppl* 1967;**1**:1–39.
28. Curtis MA, Sterne JA, Price SJ *et al*. The protein composition of gingival crevicular fluid sampled from male adolescents with no destructive periodontitis: baseline data of a longitudinal study. *J Periodontal Res* 1990;**25**:6–16.
29. Eley BM, Cox SW. Cathepsin B/L-, elastase-, tryptase-, trypsin- and dipeptidyl peptidase IV-like activities in gingival crevicular fluid: correlation with clinical parameters in untreated chronic periodontitis patients. *J Periodontal Res* 1992;**27**:62–69.
30. Mogi M, Otogoto J, Ota N, Inagaki H, Minami M, Kojima K. Interleukin 1 beta, interleukin 6, beta 2-microglobulin, and transforming growth factor-alpha in gingival crevicular fluid from human periodontal disease. *Arch Oral Biol* 1999;**44**:535–539.
31. Engebretson SP, Grbic JT, Singer R, Lamster IB. GCF IL-1beta profiles in periodontal disease. *J Clin Periodontol* 2002;**29**:48–53.
32. Fitzsimmons TR, Sanders AE, Slade GD, Bartold PM. Biomarkers of periodontal inflammation in the Australian adult population. *Aust Dent J* 2009;**54**:115–122.
33. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25**:134–144.
34. Chapple IL, Garner I, Saxby MS, Moscrop H, Matthews JB. Prediction and diagnosis of attachment loss by enhanced chemiluminescent assay of crevicular fluid alkaline phosphatase levels. *J Clin Periodontol* 1999;**26**:190–198.
35. Eley BM, Cox SW. Correlation between gingivain/gingipain and bacterial dipeptidyl peptidase activity in gingival crevicular fluid and periodontal attachment loss in chronic periodontitis patients. A 2-year longitudinal study. *J Periodontol* 1996;**67**:703–716.
36. Nakashima K, Giannopoulou C, Andersen E *et al*. A longitudinal study of various crevicular fluid components as markers of periodontal disease activity. *J Clin Periodontol* 1996;**23**:832–838.
37. Cesar-Neto JB, Duarte PM, de Oliveira MC, Tambeli CH, Sallum EA, Nociti FH Jr. Smoking modulates interleukin-6: interleukin-10 and RANKL:osteoprotegerin ratios in the periodontal tissues. *J Periodontal Res* 2007;**42**:184–191.
38. Tiss A, Smith C, Camuzeaux S *et al*. Serum peptide profiling using MALDI mass spectrometry: avoiding the pitfalls of coated magnetic beads using well-established ZipTip technology. *Proteomics* 2007;**7**(suppl 1):77–89.