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A Pilot Study of Active Enzyme Levels in Gingival Crevicular Fluid of Patients with Chronic Periodontal Disease

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Running title: GCF Biomarkers of treatment outcome

Abstract

Aim: To determine whether combinations of enzymes in Gingival Crevicular Fluid (GCF) can act as improved biomarkers compared with single enzymes for predicting the outcome of treatment and also for diagnosing the clinical status of sites. .

Methods: 30 subjects with chronic periodontitis were recruited to a 12-month longitudinal pilot study. GCF samples from 3 representative sites: healthy (≤ 3mm), deep non-bleeding (NB) (≥6mm) and deep bleeding site (DB) (≥6mm) and clinical data were collected at baseline, 3-months, 6-months and 12 months following periodontal treatment. Active enzyme levels (MMP-8, cathepsin G, elastase, trypsin-like activity and sialidase) in GCF samples were assessed. The enzyme profiles and clinical data of each site were analysed for correlation and logistic regression was performed to find the predictive value of the active enzyme levels regarding the outcome of treatment.

Results: 22 individuals completed the study. All active enzyme levels were significantly higher in diseased sites than healthy sites. Logistic regression showed that the combination of MMP8, elastase and sialidase provided accurate predictions of treatment outcome (88% for NB, 86% for DB), which was significantly better than each enzyme alone (61%).

Conclusion: This pilot has suggested that combined active enzyme profiling could provide significant prediction of outcome of treatment.

Clinical Relevance

Scientific rationale for the study: Single clinical/laboratory measures are unable to determine the clinical status of periodontal disease and likely treatment outcome. The aims of this study were to assess the ability of a combined biomarker profile in GCF to predict the outcome of treatment compared with single biomarkers.

Principal findings: Combined profiles of GCF biomarkers provides a "fingerprint" that helps to predict the treatment outcome and better identifying diseased sites than can be achieved using single biomarkers alone.

Practical implications: Knowing the likely response to the treatment of diseased sites would provide information that helps direct therapeutic choices including minimizing unnecessary treatment.

Acknowledgement

Introduction

Oral bacteria are considered to be primary etiological factors in periodontal disease, although most of the tissue destruction is thought to be due to the host's response (Kinane 2001, Schenkein 2006). Furthermore, the disease does not necessarily progress linearly since affected sites may display periods of tissue destruction followed by episodes of quiescence (Socransky et al. 1984). Also, the severity of the disease varies, not only between teeth within the same person but also between sites around the same tooth (Baelum et al. 1996). These characteristics pose considerable diagnostic and prognostic difficulties for clinicians, which may result in inappropriate treatment

In contemporary clinical practice, diagnosis of disease is made on the assessment of clinical measurements and radiographic assessments. However, these largely provide information about past periodontal tissue destruction, rather than the current state of disease activity, the prediction of future disease progression or the likely outcome following treatment (Haffajee et al. 1983, Chapple 2009). Therefore, a major challenge in the field of periodontology is to discover methods that have improved diagnostic and prognostic capability.

There has been considerable progress over the years in the search for biomarkers of chronic periodontitis. Such biomarkers have been identified in saliva, plaque and GCF.

Amongst the most investigated biomarkers in GCF are levels of key enzymes, particularly those that are thought to lead to or be involved in tissue destruction (host or bacterial in origin). For example, elastase activity is considered to be a useful quantitative measure of gingival inflammation (Herrmann et al. 2001), cathepsin G and MMP-8 have been correlated with disease severity (Mailhot et al. 1998, Kinney et al. 2014), and trypsin-like activity (mainly of bacterial origin) has been significantly correlated with the Gingival Index, Plaque Index (PI) and Probing Pocket Depth (PPD) (Beighton and Life 1989). Certain other bacterial enzymes are just beginning to be studied in this context. For example, sialidases are produced by a number of periodontal pathogens and that of *Tannerella forsythia* has been found to promote biofilm formation (Roy et al. 2011). Also, sialidase activity has been found to be higher in people with periodontitis than gingivitis and this activity has been correlated with the clinical parameters (PI, BI, GI, PPD and CAL) (Beighton et al. 1992). However, there has been relatively little progress made in identifying markers that have value in predicting response to treatment and thereby assist in patient management. Owing to the complex nature of periodontitis, it is unlikely that a single clinical or laboratory test could address all issues related to the diagnosis and prognosis mentioned above (Ramseier et al. 2009, Kinney et al. 2014).

This longitudinal clinical study is directed at investigating whether an 'enzyme profile' has greater prognostic value than single enzymes alone in patients with chronic periodontitis. We report for the first time to our knowledge that the combined levels of elastase, MMP-8 and sialidase as a 'fingerprint' help to predict the outcome of conventional periodontal treatment of a site.

Materials and methods

Patient population

Subjects were recruited to a longitudinal study collecting whole mouth clinical periodontal data and GCF samples. The study protocol was approved by NRES Committee Yorkshire and Humberside, (study number: 13/YH/0114). Recruitment was performed among the patients attending the Periodontology Clinic at the Charles Clifford Dental Hospital, Sheffield between 2011 and 2012. Potential study participants were screened by the clinical investigator and then assessed for periodontal disease and general inclusion criteria before being invited to join the study. After obtaining written consent, subjects aged 18 and over participated in the study. They all had ≥ 20 teeth and were diagnosed with chronic periodontitis with at least one healthy site (≤ 3mm), one non-bleeding site (NB; ≥ 6mm) and one deep bleeding site (DB; ≥ 6mm).

Exclusion criteria were receipt of antibiotics or periodontal treatment 3 months prior to the study, pregnancy and lactation, a history of systemic disease or medication that may affect the periodontal condition and patients who did not have the capacity to consent for themselves.

Clinical measures and periodontal treatment

The measurements of probing pocket depth (PPD), plaque index (PI), bleeding on probing (BOP) and clinical attachment loss (CAL) were recorded as routine clinical practice for assessment of periodontal health by the clinical staff for all patients. The patient population acceded to the study was a cross-section of those referred to a secondary care centre for periodontal treatment and all had several diseased sites. A single consultant periodontist collected clinical data and samples, the enzyme parameters in the GCF samples were analysed by an independent single investigator. The presence of plaque was identified as present or absent using a Langer curette. The Williams probe was used to measure PPD, CAL and BOP within 30sec after probing. Three representative sites were randomly selected in each patient: one healthy site (≤ 3mm) and two deep diseased sites (≥ 6mm). The diseased sites selected for sampling were the deepest with or without bleeding that were accessible.

The clinical measurements were recorded at baseline, 3 months, 6 months and 12 months. At each interval, samples of GCF were also obtained from each representative site and volunteers received identical treatment for the management of their periodontal condition. This included

advice on plaque control, scaling and root surface debridement under local anaesthesia for sites ≥ 4mm in depth. All treatment was provided by a single staff hygienist, which ensured standardized care. The effects of this standard treatment were reviewed 3 months after completion and the need for further treatment assessed. Sites showing reduction in pocket depth of ≥ 2mm or more from the baseline measure were considered as having responded to the periodontal treatment. Sites not responding to initial treatment were re-treated. Intra-examiner reproducibility of PPD was assessed and compared for ±1mm agreement. The intra-examiner reliability was high (≥98%) and the literature indicates that 90% of PPD readings within ±1mm difference is considered to be good intra-examiner agreement (Badersten et al. 1984).

GCF collection and analysis

Prior to GCF collection, the area around the tooth was isolated with cotton rolls, dried with a short blast of air and supragingival plaque was removed with a sterile curette. GCF samples were collected from the three representative sites using a long micropipette tip. Resultant GCF samples were placed into small eppendorf tubes, centrifuged at 1000 x g for 5 minutes and stored at -80°C until analysed. On thawing, GCF samples were weighed to determine the volume of GCF collected, then diluted in Phosphate buffered saline (PBS) to a final volume of 105μl, and subjected to conventional colorimetric or fluorimetric assay using specific substrates for each of the 5 enzymes. The GCF samples were analysed on a site-specific basis.

Enzyme assays

The 5 enzyme levels were assayed using fluorescent and chromogenic substrates. Each reaction was measured in a microplate reader (FLUOstar Galaxy; BMG Labtech GmbH, Offenburg, Germany). All chemicals in this study were from Sigma-Aldrich Company Ltd, Dorset, UK unless otherwise stated.

Standard curves for each enzyme were produced using serial dilutions of the following enzyme solutions in PBS pH 7.3: 100ng/μl Pro-MMP8 (Enzo Life Sciences Inc. Lausen, Switzerland activated with 1μl of 20 mM 4-amino-phenyl-mercuricacetate for 5 minutes at room temperature), 20 ng/μl neutrophil elastase, 16.6 ng/μl leukocyte cathepsin G, 100ng/μl pancreatic trypsin (containing 5mM di-thiothreitol) and 51ng/μl Clostridial sialidase.

For the above standard enzymes and the diluted clinical samples (10μl GCF), substrate was added to each in duplicate as follows: 102 μM MMP8 substrate (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-

Ala-Arg-NH2; Enzo Life Sciences Inc. Lausen, Switzerland), 2mM elastase substrate (20mM) (N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide), 2mM cathepsin G substrate (N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide), 0.52mg/ml trypsin substrate (N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride) (BAPNA) in DMSO plus Tris saline (0.05M Tris + 0.2M NaCl pH7.5) and 0.2mM sialidase substrate (2mM 4-methylumbelliferyl-n-acetyl-α-d-neuraminic acid salt sodium in H_2O; (Neu5Ac; Carbosynth Ltd. Compton, Berkshire, UK).

The plates were incubated at 37°C for 18 hours (except MMP8, 4hrs) before being read at 405nm for elastase, cathepsin G and trypsin-like activity. The fluorescence generated from MMP8 and sialidase were monitored at excitation/emission wavelengths of $340_{ext}/380_{emm}$ nm and $355_{ext}/430_{emm}$ nm respectively. The levels of each enzyme in the clinical samples were determined by reference to the corresponding standard curve and adjusting for the sample dilution factor.

Statistical methods

Since this was a pilot study to inform a subsequent larger longitudinal study, the patient sample size was chosen as 30 to be in line with the number generally recruited to similar clinical studies. Only data applying to the subjects who completed the study were analysed. The "continuous" data were tested for normal distribution and thereafter subjected to appropriate statistical tests. Kruskal wallis test was used to explore statistically significant differences in enzyme values between the healthy and diseased sites. The correlations between clinical measures and enzyme values were determined with Pearson's correlation. 2mm improvement in probing pocket depth was used to dichotomise the outcome variable (pocket depth at the 12 month time point). The area under the curves (AUCs) of the receiver operating characteristic curve (ROC) were estimated non-parametrically. Threshold points for active enzyme levels were selected from the ROC curves with the highest sensitivity and specificity. Base line continuous values of enzymes (as predictors) were analysed by logistic regression against binary outcome measures to find their predictive values at 12 months after treatment. Regression analysis backward stepwise technique was used to exclude redundant enzyme biomarkers. Odds ratio estimates and their confidence intervals (CI) were calculated and statistical significance was defined as P≤0.05. All calculations were performed using the SPSS software package (version 20; SPSS Inc., Chicago IL, USA). The null hypothesis was that no combinations of enzyme biomarkers would predict the outcome of conventional periodontal treatment any better than each enzyme alone. Statistical advice for the data analysis was provided by the Statistical Services Unit, University of Sheffield.

Results

Forty seven subjects were screened between 2011 and 2012, 17 of which did not fulfil the inclusion criteria or declined to take part. 30 participants (14 male and 16 female) were recruited ranging in age between 40 to 70 years (40% 40-49 years, 30% 50-59 years, 30% >60 years), of whom 4 were smokers. Twenty-eight completed the 3-month appointment, 23 subjects completed the 6-month appointment and 22 completed the full study, only one of whom was a smoker. The other patients failed to attend further appointments with the exception of one participant who died during the period of the study. Most of the changes in PPD were detectable at the 6 month time point and did not differ from those found at the 12-month time point. No adverse events were reported as a consequence of the study.

The extent of disease ranged between moderate through to severe chronic periodontitis (1999 periodontal disease classification system). Full mouth clinical data (PPD and BOP) showed statistically significant improvement 3 months and 6 months after treatment. At the 3-month time point, 40% of sites with PPD of 4-5mm reduced to 3mm or below and showed further reduction at 6-months (55% sites). While 45% of sites with PPD ≥ 6mm, at the 3-month time point, reduced to 5mm or below and further reduced at 6-month (76% sites) (Table 1).

The sites selected for study were made randomly and their characteristics and response to treatment were similar to the patient based averages. At base line, the diseased sites chosen for the 22 subjects who completed the study showed a mean PPD of 7.11±1.12mm, which reduced 3 months after initial treatment to 5.72±1.56mm, and further decreased by the 6 month (4.5±1.84mm) and the 12 month (4.1±1.67mm), time points. However, on a site level, only 49% of sites selected for study had improved by 2mm reduction in PPD at the 3 month time point, whereas at 6 months and 12 months, 74.9% of the selected diseased sites showed 2mm reduction in PPD. Some sites (20.4%) reduced by less than 2mm, 1 site did not change at all and 1 site showed further pocket deepening (Figures 1 and 2). The patient based-percentage was 71.5% of sites with PPD ≥ 6mm showed 2mm reduction, 22.9% reduced by less than 2mm, 3.7% did not change and the remaining 1.9% showed further progression. Also, the mean percentage PI and BOP of DB sites showed significant reduction over the study period, however, the NB sites showed an increase in mean percentage of BOP over the study period (Table 1). The mean quantity of GCF collected were 1.82±0.52μl, 2.42±0.57μl, 2.99±0.66μl for healthy, NB and DB sites, respectively and the resultant dilution factor was used to adjust the final assessment of the active enzyme level.

Enzyme biomarkers

All enzyme biomarkers were detected at significantly higher levels in diseased sites than in healthy sites (Figure 3) (Kruskal wallis test). For the purposes of brevity, enzyme values for all diseased sites have been combined in figure 4, but these values are close to those found at each

individual site. Statistically significant correlations were found between all of the enzyme concentrations and probing pocket depth at baseline (Table 1 supplementary data). By the 12 month time points, all enzymes at diseased sites were correlated with the treatment outcome (2mm improvement) but only MMP8, elastase and sialidase were statistically significantly correlated (r= 0.68, 0.62 and 0.58 respectively) (Table 1 supplementary data). All biomarkers decreased through the course of the study but MMP8, elastase and sialidase showed the greatest reduction (Figure 4).

Threshold values

ROC curves, were used to evaluate whether active enzyme levels indicated that a site was diseased or healthy. Threshold points with the highest sensitivity and specificity were selected for each of the enzymes at baseline (Table 2 and Figure 1 supplementary data). MMP8, elastase and sialidase showed the greatest sensitivity and specificity and AUCs. In contrast, cathepsin G and trypsin-like activity showed the lowest sensitivity and specificity.

Predictive value

The odds ratio for MMP-8, elastase and sialidase as single enzymes in NB were 0.995, 0.995 and 0.995 and in DB sites were 0.995, 0.995 and 0.996 respectively (all 95% CI's: 0.99-1.1). Logistic regression analysis was conducted with continuous independent variables (baseline active enzyme levels) against the treatment outcome. At the 12 month time point, all the enzymes combined were able to predict the outcome of treatment (improved by ≥ 2mm PPD) with ≥85% certainty for all diseased sites. To identify redundant variables, each was excluded one by one from the model and analysed by stepwise logistic regression. It was found that the enzyme profile of MMP-8, elastase and sialidase combined had the best predictive value (Table 3).

Discussion

This study investigated the ability of combinations of enzymes in GCF to predict the outcome of non-surgical periodontal treatment in patients with chronic periodontitis. The patient population selected were a cross-section of those attending a secondary care centre for periodontal treatment and who had all been referred from primary care. The purpose of this pilot study was to determine whether certain enzymes might provide a 'finger print' to indicate the likely outcome of non-surgical periodontal treatment in a typical cohort of patients. The indications from this pilot study are that a combination of three GCF enzymes, MMP-8, elastase and sialidase, has a good predictive value (≥86%). To our knowledge this is the first time that this enzyme profile has been linked to the outcome of periodontal treatment.

The rationale for the investigation was based on the premise that single biomarkers are unlikely to reflect the complex nature of the disease sufficiently well and so would lack precision. Indeed no individual enzyme has as yet proved to be a suitably reliable biomarker for clinical use

(Buduneli and Kinane 2011, Kinney et al. 2014). Individual clinical parameters have failed to predict future disease progression (Haffajee et al. 1983), although Haffajee et al. (1991) found that by combining several clinical measures (11 variables as predictor) it is possible to correctly classify 80% of patients with a risk of future disease progression. Haffajee's study though was aimed at identifying patients who were at high risk for further disease rather than determining which sites within patients were at risk or were likely to fail to respond to treatment, as we have done here. This is important since periodontitis is largely a site-specific disease and for the clinician the major concern is how to differentiate between sites that will respond and sites that will not respond to treatment or which are at high risk for future deterioration. Indeed one of the commonest decisions that periodontists must make is whether to provide rigorous treatment, such as surgery plus systemic antibiotic therapy, or whether to limit treatment to more conservative measures (scaling and root surface debridement).

In this study, the GCF samples were analysed on a site specific basis and correlated with the clinical features of the same sites (pre and post treatment). The enzymes chosen for the study would have been very largely generated locally and based on the active enzyme levels found at the healthy sites, a systemic contribution was thought to be minimal. For the predictive value determinations, samples were not pooled together or correlated to the mean of the clinical measures as has been done in some previous studies (Kinney et al. 2014) and this was to ensure that any predictive value for outcome of treatment was not masked by averaging.

Deep sites that do not bleed have been considered to be more stable and more likely to respond to treatment and presence of bleeding has a 30% prediction value of future deterioration (Lang et al. 1990, Badersten et al. 1990). For this reason both bleeding and non-bleeding sites were included in this study. Here we found that bleeding on probing was not a good indicator of the subsequent response to treatment. Indeed we found a degree of variation in bleeding on probing even after treatment. This could be due to a residual effect of trauma from the first round of treatment and those sites had not healed sufficiently or that the sites had become more inflamed due to disease activity in the period since the previous treatment. In fact, the most accepted measure of response to treatment is change in PPD.

The clinical evaluation of sites showed that most of the changes in PPD had occurred by the 6 month time point and this is in agreement with several studies mentioned in a systematic review by Cobb (2002). The criterion we used as a measure of clinical improvement was a two millimeter reduction in PPD. This is beyond the clinician measurement error and so is considered to be acceptable as a real change (Cobb 1996). Consequently in this study we dichotomized the outcome data according to whether the PPD at 12 months had improved by 2mm or not.

Our finding that the active levels of these enzymes was higher in diseased sites than healthy sites is consistent with that reported in some previous studies (Beighton and Life 1989,

Beighton et al. 1992, Mailhot et al. 1998, Herrmann et al. 2001, Kinney et al. 2014). However, what has not been reported before is our finding that a combination of MMP8, elastase and sialidase had the greatest sensitivity and specificity to identify diseased and healthy sites (Table 2), and this combination had a higher predictive value than any single enzyme alone (Table 3). Other investigators have reported the diagnostic capability of MMP8 in saliva to identify different stages of disease (healthy, gingivitis and periodontitis patients) with 69% sensitivity and 70% specificity (Kinney et al. 2011). MMP8 in GCF has also been reported to differentiate healthy, gingivitis and periodontitis sites with 89% sensitivity and 87% specificity (Leppilahti et al. 2014) and this is in agreement with our results in which MMP8 showed greatest sensitivity/specificity (90%/90%) to differentiate healthy sites from diseased sites. However, in this study we examined the biomarkers to identify treatment outcome since distinguishing healthy from diseased subjects or sites can be achieved using clinical measures.

Elastase has been investigated for its ability to predict disease progression using bone loss as a indicator and showed sensitivity/specificity of 84%/66% in one study (Palcanis et al. 1992) and 77%/61% in another study (Armitage et al. 1994).

In contrast, cathepsin G and trypsin-like enzyme activity showed the lowest sensitivity and specificity amongst all the enzymes tested and they did not add any significant prognostic value. In previous studies cathepsin G and trypsin-like activity have only been evaluated in relation to disease status rather than as biomarkers for treatment outcome (Eley and Cox 1992, Kunimatsu et al. 1995). This could be due either to the fact that they do not have sufficient discriminatory ability individually or that they represent biological functions (e.g. neutrophil activity) that are better assessed by the other three enzymes. For example, cathepsin G was found to enhance tissue destruction by activating pro-MMPs (Kahari and Saarialho-Kere 1999) as well as working synergistically with elastase (Boudier et al. 1981).

There are few data on the presence and levels of sialidase activity in GCF in the literature. One report showed that it was higher in periodontitis subjects than gingivitis subjects and when it was coupled with other enzymes it was able to differentiate those two groups of patients with 61.3% sensitivity and 91.9%, specificity (Beighton et al. 1992). Our findings provide new evidence for the importance of this enzyme in predicting treatment outcomes.

MMP-8 and elastase are the only two enzymes that have each been studied in terms of their predictive value for periodontal disease progression and their ability to predict the outcome of treatment (Palcanis et al. 1992, Kinney et al. 2014). However, here we show that a combination of MMP8 and elastase has increased predictive value compared to each enzyme alone. Further enhancement of predictive value was achieved in this study by the inclusion of sialidase and the combination of these 3 enzymes, amongst all the combinations tried in this study, proved to be the best.

In conclusion this pilot study has shown that single enzymes alone were not able to predict the outcome of treatment more than the null hypothesis (61.8%), whereas our data suggest for the first time that combined enzyme profiling (MMP-8, elastase plus sialidase) in GCF could provide a significant predictive capability of a site's likely response to non-surgical periodontal treatment. These are preliminary conclusions because the study was insufficiently powered for statistical confidence, however, the data are sufficiently encouraging to support the undertaking of a larger prospective longitudinal study. Based on these pilot data showing that three enzymes are sufficient to provide a predictive profile and assuming 10 patients are required for each variable, such a prospective study would require participation of 70 subjects to be appropriately powered. We also found from this pilot study that a larger study would not need to continue beyond 6 months because the data for the 6-month and 12-month time point were almost identical.

It is anticipated that a larger prospective study should identify sites that require treatment other than conventional non-surgical treatment and if confirmed, the findings might lead to minimizing unnecessary treatment. It is anticipated that the proposed enzyme profiling would not be used for all sites but would be confined to those sites that had been identified as at risk using traditional clinical measures of periodontal disease.

References

Armitage, G. C., Jeffcoat, M. K., Chadwick, D. E., Taggart, E. J., Numabe, Y., Landis, J. R., Weaver, S. L. & Sharp, T. J. (1994) Longitudinal evaluation of elastase as a marker for the progression of periodontitis. *Journal of Periodontology* **65**, 120-128.

Badersten, A., Nilvéaus, R. & Egelberg, J. (1984) Reproducibility of probing attachment level measurements. *Journal of Clinical Periodontology* **11,** 475-485.

Badersten, A., Nilvéus, R. & Egelberg, J. (1990) Scores of plaque, bleeding, suppuration and probing depth to predict probing attachment loss 5 years of observation following nonsurgical periodontal therapy. *Journal of Clinical Periodontology* **17,** 102-107.

Baelum, V., Chen, X., Manji, F., Luan, W. M. & Fejerskov, O. (1996) Profiles of destructive periodontal disease in different populations. *Journal of Periodontal Research* **31,** 17-26.

Beighton, D. & Life, J. S. C. (1989) Trypsin-like, chymotrypsin-like and glycylprolyl dipeptidase activities in gingival crevicular fluid from human periodontal sites with gingivitis. *Archives of Oral Biology* **34,** 843-846.

Beighton, D., Radford, J. R. & Naylor, M. N. (1992) Glycosidase activities in gingival crevicular fluid in subjects with adult periodontitis or gingivitis. *Archives of Oral Biology* **37,** 343-348.

Boudier, C., Holle, C. & Bieth, J. G. (1981) Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin-g. *Journal of Biological Chemistry* **256,** 256-258.

Buduneli, N. & Kinane, D. F. (2011) Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *Journal of Clinical Periodontology* **38,** 85-105.

Chapple, I. L. (2009) Periodontal diagnosis and treatment–where does the future lie? *Periodontology 2000* **51,** 9-24.

Cobb, C. M. (1996) Non-surgical pocket therapy: mechanical. *Annals of periodontology*, 443-490.

Cobb, C. M. (2002) Clinical significance of non‐surgical periodontal therapy: an evidence‐based perspective of scaling and root planing. *Journal of Clinical Periodontology* **29,** 22-32.

Eley, B. M. & Cox, S. W. (1992) Cathepsin-b/l-like, elastase-like, tryptase-like, trypsin-like and dipeptidyl peptidase-iv-like activities in gingival crevicular fluid - a comparison of levels before and after periodontal surgery in chronic periodontitis patients. *Journal of Periodontology* **63,** 412-417.

Haffajee, A. D., Socransky, S. S. & Goodson, J. M. (1983) Clinical-parameters as predictors of destructive periodontal-disease activity. *Journal of Clinical Periodontology* **10,** 257-265.

Haffajee, A. D., Socransky, S. S., Lindhe, J., Kent, R. L., Okamoto, H. & Yoneyama, T. (1991) Clinical risk indicators for periodontal attachment loss. *Journal of Clinical Periodontology* **18,** 117-125.

Herrmann, J. M., Gonzáles, J. R., Boedeker, R. H., Vonholdt, J. & Meyle, J. (2001) Microassay for the detection of elastase activity in the gingival crevice. *Journal of Clinical Periodontology* **28,** 31-37.

Kahari, V. M. & Saarialho-Kere, U. (1999) Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Annals of Medicine* **31,** 34-45.

Kinane, D. F. (2001) Causation and pathogenesis of periodontal disease. *Periodontology 2000* **25,** 8-20.

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., Shelburne, C. E., Rayburn, L. A., Singh, A. K. & Giannobile, W. V. (2011) Saliva/pathogen biomarker signatures and periodontal disease progression. *Journal of Dental Research* **90,** 752-758.

Kinney, J. S., Morelli, T., Oh, M., Braun, T. M., Ramseier, C. A., Sugai, J. V. & Giannobile, W. V. (2014) Crevicular fluid biomarkers and periodontal disease progression. *Journal of Clinical Periodontology* **41,** 113-120.

Kunimatsu, K., Mine, N., Muraoka, Y., Kato, I., Hase, T., Aoki, Y. & Yamamoto, K. (1995) Identification and possible function of cathepsin-g in gingival crevicular fluid from chronic adult periodontitis patients and from experimental gingivitis subjects. *Journal of Periodontal Research* **30,** 51-57.

Lang, N. P., Adler, R., Joss, A. & Nyman, S. (1990) Absence of bleeding on probing an indicator of periodontal stability. *Journal of Clinical Periodontology* **17,** 714-721.

Leppilahti, J. M., Hernández‐Ríos, P. A., Gamonal, J. A., Tervahartiala, T., Brignardello‐Petersen, R., Mantyla, P., Sorsa, T. & Hernández, M. (2014) Matrix metalloproteinases and myeloperoxidase in gingival crevicular

fluid provide site‑specific diagnostic value for chronic periodontitis. *Journal of Clinical Periodontology* **41,** 348-356.

Mailhot, J., Potempa, J., Stein, S., Travis, J., Sterrett, J., Hanes, P. & Russell, C. (1998) A relationship between proteinase activity and clinical parameters in the treatment of periodontal disease. *Journal of Clinical Periodontology* **25,** 578-584.

Palcanis, K. G., Larjava, I. K., Wells, B. R., Suggs, K. A., Landis, J. R., Chadwick, D. E. & Jeffcoat, M. K. (1992) Elastase as an indicator of periodontal-disease progression. *Journal of Periodontology* **63,** 237-242.

Ramseier, C. A., Kinney, J. S., Herr, A. E., Braun, T., Sugai, J. V., Shelburne, C. A., Rayburn, L. A., Tran, H. M., Singh, A. K. & Giannobile, W. V. (2009) Identification of pathogen and host-response markers correlated with periodontal disease. *Journal of Periodontology* **80,** 436-446.

Roy, S., Honma, K., Douglas, C. W. I., Sharma, A. & Stafford, G. P. (2011) Role of sialidase in glycoprotein utilization by Tannerella forsythia. *Microbiology-Sgm* **157,** 3195-3202.

Schenkein, H. A. (2006) Host responses in maintaining periodontal health and determining periodontal disease. *Periodontology 2000* **40,** 77-93.

Socransky, S. S., Haffajee, A. D., Goodson, J. M. & Lindhe, J. (1984) New concepts of destructive periodontal disease. *Journal of Clinical Periodontology* **11,** 21-32.

Figure Legends

Figure 1. Changes in pocket depth of each site over the course of the study (NB = non-bleeding deep sites).

Figure 2. Changes in pocket depth of each site over the course of the study (DB = deep bleeding sites).

Figure 3. Median of the active levels of the five enzymes present in the healthy and diseased sites at base line (NB = non-bleeding deep sites; DB = deep bleeding sites, NS= not significant) (Kruskal wallis test).

Figure 4. Bar chart showing the changes in median active enzyme levels at diseased sites over the course of the study (data for deep non-bleeding and deep bleeding sites were combined at each of the time points to represent diseased sites).

Supplementary Figure. ROC curve for all tested biomarkers at baseline differentiating healthy from periodontitis sites.

Table 1. Full mouth and representative site-specific clinical data at study time points.

	Variable	Baseline (% sites ± SD)	3 months (% sites ± SD)	P value (baseline vs 3 month)	6 months (% sites ± SD)	P value (baseline vs 6 month)	12 months (% sites ± SD)	P value (baseline vs 12 month)
Full mouth clinical data	BOP	36.2 ±23.5	17.85 ± 10.4	0.0001	10.93 ± 7.78	0.0001	10.58 ±8.87	0.0001
	Mean PPD 4-5 mm	21 ±10.4	12.63± 5.82	0.0001	9.32 ± 3.6	0.0001	8.56 ± 5.41	0.0001
	Mean PPD ≥ 6 mm	12 ± 7.19	6.68 ± 6.14	0.0001	2.87±3.31	0.0001	2.4 ± 3.1	0.0001
Clinical data of sampled sites	PI (healthy site)	52	39	0.01	39	0.01	35	0.025
	PI (NB site)	91	47	0.001	47	0.001	42	0.001
	PI (DB site)	91	60	0.001	52	0.001	45	0.001
	BOP (healthy site)	0	4	0.7	0	1	0	1
	BOP (NB site)	0	47	0.001	30	0.01	33	0.01
	BOP (DB site)	100	52	0.001	21	0.001	23	0.001

Table 2. Diagnostic properties of specific thresholds of selected GCF Biomarkers.

Variable	Threshold	Sensitivity%/ Specificity%	Area under the curve	95% CI for OR		P value
				LCL	UCL	
MMP8	104	90/90	0.91	0.924	01	**0.0001**
Elastase	24	88/90	0.88	0.904	0.908	**0.0001**
Cathepsin G	0.8	55/50	0.63	0.509	0.75	**0.056**
Trypsin like	12	80/55	0.72	0.616	0.836	**0.001**
Sialidase	2.2	86/75	0.88	0.785	0.942	**0.0001**

Table 3. Logistic regression analysis with 2mm PPD improvement (at 12 months) as the dependent variable.

Method	NB sites	DB sites
	%Predictive	%Predictive
All variables	85%	85%
Stepwise	88%(MMP8, Elastase, Sialidase, Trypsin)	82%(MMP8, Elastase, Sialidase, Trypsin)
	88% (MMP8, Elastase, Sialidase)	86% (MMP8, Elastase, Sialidase)
	74% (MMP8, Sialidase)	70 %(MMP8, Elastase)
	61.8% (each single enzyme)	61.8% (each single enzyme)

Supplementary figure

ROC Curve

Diagonal segments are produced by ties.