
Article:
Gul, S.S., Griffiths, G.S., Stafford, G.P. et al. (3 more authors) (2017) Investigation of a novel predictive biomarker profile for the outcome of periodontal treatment. Journal of Periodontology, 88 (11). pp. 1135-1144. ISSN 0022-3492

https://doi.org/10.1902/jop.2017.170187

Investigation of a novel predictive biomarker profile for the outcome of periodontal treatment

Sarhang S. GUL, Gareth S. GRIFFITHS, Graham P. STAFFORD, Mohammed I. AL-ZUBIDI, Andrew RAWLINSON , Charles W.I. DOUGLAS.

School of Clinical Dentistry, University of Sheffield, Sheffield, UK.

Corresponding author:

CWI Douglas

Academic Unit of Oral and Maxillofacial Pathology,
School of Clinical Dentistry, University of Sheffield, Claremont Crescent,
Sheffield, S10 2TA, UK

Email: i.douglas@sheffield.ac.uk

Telephone: +44 (0) 114 236 1753

Fax: +44 (0) 114 271 7894

Counts: 3,607 words; 2 Figures and 4 tables; 48 References

Running title: Biomarkers for predicting treatment outcome

Key findings: A combination of the levels of five biomarkers gave a high level of prediction of the likely outcome of conventional, non-surgical treatment of periodontal sites.

Conflict of Interest and Sources of Funding Statement

This study was supported by a grant to SG by the Kurdistan Regional Government. Drs Gul, Stafford and Al-Zubidi and Professors Griffiths, Rawlinson and Douglas report no conflicts of interest related to this study.

Abstract (230 words)

Background: An ability to predict the response to conventional non-surgical treatment of a periodontal site would be advantageous. However, so far the biomarkers or tests devised to achieve this have lacked sensitivity. The aim of this study was to assess the ability of a novel combination of biomarkers to predict the outcome of treatment of patients with chronic periodontitis.

Methods: GCF and subgingival plaque were collected from 77 patients at 3 representative sites, 1 healthy (≤ 3mm) and 2 diseased (≥ 6mm) at baseline and at 3 and 6-months post treatment. Patients received standard nonsurgical periodontal treatment at each time point as appropriate. The outcome measure was improvement in pocket depth of ≥2mm. Concentrations of active enzymes (MMP 8, elastase and sialidase) in GCF and subgingival plaque levels of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Fusobacterium nucleatum* were analysed for prediction of the outcome measure.

Results: Using threshold values of MMP8 (94ng/µl), elastase (33ng/µl), sialidase (23ng/µl), *P. gingivalis* (0.23%) and *T. forsythia* (0.35%), Receiver Operating Characteristic curves analysis demonstrated that these biomarkers at baseline could differentiate healthy from diseased sites (sensitivity and specificity ≥77%). Furthermore, logistic regression showed that this combination of the above biomarkers at baseline provided accurate predictions of treatment outcome (≥ 92%).

Conclusion: The 'finger print' of GCF enzymes and bacteria described here offers a way to predict the outcome of non-surgical periodontal treatment on a site-specific basis.

Key words: Periodontitis; Gingival crevicular fluid; Prognosis;;; Microbiology

Introduction

While diagnosis of periodontal disease *per se* is well established and employs easy to use and relatively non-invasive procedures, there are a number of limitations. Specifically, an ability to predict the response to treatment or the likelihood of future tissue breakdown is absent[1,2,3]. Thus, there is a need for alternative diagnostic approaches that could be applied at initial triage and be used to direct treatment planning regimens. One approach is the use of biomarkers[3,4].

Various molecular biomarkers for periodontitis have been examined[5-7], particularly in saliva, plaque and gingival crevicular fluid (GCF). Although, a focus has been on GCF constituents[8,9], most of the tests devised so far have lacked sensitivity. This is likely to be due to the complex nature of periodontitis such that a single parameter is unlikely to be sufficiently discriminating[10,11]. Our team previously identified as part of a pilot study that a combination of high levels of three GCF enzymes, prior to treatment provided a predictive value of the outcome of conventional non-surgical treatment of 88%, compared to 61% for each enzyme alone[12]. The biomarkers were easily assayed and were representative of inflammation (MMP-8, elastase) and physiologically relevant bacterial community activity (sialidase).

As well as GCF biochemical biomarkers, presence and levels of key bacteria have been used as possible biomarkers of disease. Most attention has been directed at *P. gingivalis* and *T. forsythia*, members of the so-called 'red complex' of periodontopathogens[13,14], since these bacteria are generally found at higher levels in association with disease. In this longitudinal clinical study we have extended our earlier findings using MMP8, elastase and sialidase to a larger cohort of patients and determined whether addition of key bacteria to these GCF enzyme activities

could provide a profile with enhanced predictive value for the outcome of non-surgical periodontal treatment.

Materials and methods

Patient population

The prospective study was approved by the NRES Committee Yorkshire and Humberside, (study number: 13/YH/0114, 16th May 2013). Patients attended the Periodontology Clinic in the Charles Clifford Dental Hospital, Sheffield, UK between 2013 and 2015. Potential participants were screened by the consultant periodontist (AR) against the inclusion criteria of age ≥18 years, possession of ≥20 teeth, diagnosis of chronic periodontitis with several diseased sites. Three individual sites were chosen at random for study including one deep bleeding (DB; ≥6mm), one deep non-bleeding (NB; ≥6mm) and one healthy (≤3mm) site. For the diseased sites these were the deepest and most accessible sites available. Patient exclusion criteria were receipt of antibiotics or periodontal treatment in the 3 months preceding the study, pregnancy and lactation, a history of systemic disease or medication that may affect the periodontal condition. The subjects did not use chlorhexidine. Written consent was obtained from individuals entering the study.

Clinical measures and periodontal treatment

Full mouth clinical parameters of probing pocket depth (PPD), plaque index (PI)[15], bleeding on probing (BOP) and clinical attachment level (CAL) were recorded at six sites per tooth. The presence or absence of plaque was identified using a Langer curette and the parameters of PPD, CAL and BOP (within 30sec) were obtained using a UNC15 probe. Two dental therapists, who had been internally calibrated, provided standardised non-surgical treatment to manage their periodontal condition and also collected full mouth clinical data. These workers were blind to the clinical measurements recorded in the data capture forms of previous visits to avoid bias.

Clinical data and samples were collected at baseline, 3 and 6 months. Management included oral hygiene instruction, scaling and root surface debridement under local anaesthesia for sites ≥ 4mm in depth. A reduction of 2mm or more in PPD from the baseline was taken to indicate that the site had responded to treatment. Sites ≥ 4mm were reviewed and retreated 3 months and 6 months later as appropriate.

GCF and plaque sample collection and analysis

The sites selected for sampling were isolated and dried with cotton wool, and protected from salivary and blood contamination. Supragingival plaque was removed, the tooth air-dried and GCF collected using paper strips[*] placed in the entrance of the periodontal crevice or pockets for 30 sec[4,16].

The GCF volumes were immediately determined as described by Griffiths[17] or by weighing when their volume was outside the accurate range of the machine[†] (i.e. above 1.7 μl). To recover the enzymes from the samples, the paper strips were eluted for 1 hour in 105μl of sterile phosphate buffered saline (PBS, pH 7.3) containing 1% bovine serum albumin. The samples were then centrifuged at 10,000g for 15 minutes by centrifugal filtration[‡]. The GCF samples were analysed immediately for the concentration of active MMP8, elastase and sialidase as described by Gul[9]. Subgingival plaque samples were collected with a sterile curette from the same three representative sites at each time point, placed in 500 μl sterile PBS and stored at -80°C until DNA was extracted and analysed by 16srRNA qPCR for the levels of *P. gingivalis, T. forsythia* and *F. nucleatum*. (Supplementary methods). The enzyme and bacterial parameters in all samples were analysed by a single independent investigator. Similar methodology has been used by others[18].

[*] Periopaper® strips, Oraflow Inc., Plainview, NY, USA
[†] Periotron 8000, Oraflow Inc., Plainview, NY, USA
[‡] Millipore Ltd, UK

Statistical methods

All statistical methods employed were under the direction of a senior statistician of the Statistical Services Unit, The University of Sheffield. Analysis was undertaken to investigate the mean clinical mouth score and individual site scores at each time point to confirm that treatment was successful at most sites for the majority of patients. Subsequent analysis looked at the ability of the biomarker values to predict clinical outcome (PPD) at 6 months. Only data applying to subjects who completed the study were analysed.

The “continuous” data were tested for normal distribution and thereafter subjected to appropriate parametric/non-parametric testing (Shapiro-Wilk test). The Kruskal Wallis test was used to find the statistically significant differences in biomarker values in the three selected sites and at each time point. Correlations between clinical measures and biomarker values were evaluated using Spearman’s correlation. An improvement of ≥2mm in PPD was used to dichotomise the outcome variable at 6 months.

Receiver operating characteristic curves (ROC) were produced for each biomarker and the areas under the curves (AUCs) were used to determine threshold points that produced the highest diagnostic sensitivity and specificity.

To determine whether the ‘biomarker profile’ is a useful prognostic tool for treatment outcome, logistic regression analysis was performed with baseline continuous values of biomarker levels (as predictors) versus the binary outcome measure 6 months after treatment as dependent variable. Regression analysis with backward stepwise technique was used to exclude redundant biomarkers[12]. All variables included in the final multivariate model were determined to be independent through

the assessment of their co-linearity. Odds ratio (OR) estimates and their confidence intervals (CI) were calculated and statistical significance was defined as P≤0.05. Statistical power was calculated on the basis that 10 patients should be recruited for each of the 10 variables. These variables were the 2 types of sites investigated (DB and NB), 3 enzymes, 3 bacteria, 1 for the subject variable and 1 for the change from baseline to 6 months.

For validation against independent data, the baseline continuous values of biomarkers from the pilot study[12] were dichotomised using the threshold points from this study. Similarly, the current study was re-analysed using the threshold values derived from the pilot study[12]. Logistic regression was used with binary baseline enzyme values using these dichotomised prognostic cut-off points as predictors against binary treatment outcome data. All calculations were performed using a statistical software package [§].

Results

Patients

101 patients were invited to join the study, 2 patients were excluded according to the inclusion/exclusion criteria, 2 failed to attend further appointments and 8 declined to participate. The 89 remaining patients (44 males and 45 females) had a mean age of 49.7±8.9 years (range 30 - 70 years) (30% 30-39, 30% 40-49, 20% 50-59 and 20% 60 or above), 83 completed the 3-month review and 77 completed the full study, of which 8 were smokers. No adverse events were reported as a consequence of the study.

Clinical data

[§] SPSS version 20; SPSS Inc., Chicago IL, USA

The extent of disease ranged from moderate to severe chronic periodontitis as defined by the periodontal disease classification system of 1999[19].

Full mouth data

Following initial treatment, plaque (PI) and bleeding (BOP) reductions were statistically significant and retreatment at 3 months resulted in further reductions. At baseline, approximately two thirds of sites had PPD ≤3 mm while the remainder were almost equally distributed between PPD of 4-5mm and ≥6 mm. At 3 months, the mean percentage of sites with PPD ≤3 mm increased while the mean percentage of sites with PPD ≥6 mm had decreased ($p<0.001$). At 6 months, additional significant improvements were seen, with the mean percentage of healthy sites increasing to 82±11 % and the mean percentage of sites with PPD ≥6 mm decreasing to 3% ($P<0.0001$).

Diseased sites sampled

Of the sites that were sampled the mean PPDs at baseline for NB sites (6.7±1.1 mm) and DB sites (6.8±1.2 mm) were not significantly different from each other. At 3 months after initial treatment, the mean PPDs for both types of sites showed statistically significant decreases (ANOVA) to 5.3±1.6 mm ($p= 0.0001$) and 5.3±1.5 mm ($p= 0.0001$) respectively and further reductions to 4.4±1.6 mm ($p= 0.0001$) and 4.5±1.7 mm ($p= 0.0001$), respectively were seen following the second treatment phase.

The response to treatment of each of the individual DB and NB sites that were sampled are shown in Figures 1 and Supplementary Figure 1 respectively. At 6 months, 105 sites showed improvement (defined as ≥2mm improvement in PPD), however, 48 sites did not respond adequately. These included 6 NB sites (8%) and 13 DB sites (17%) that only partially improved (< 2mm), 12 NB sites (16%) and 8

DB sites (10%) that remained unchanged and 5 NB sites (7%) and 4 DB sites (5%) that deteriorated. Responses were similar amongst the subjects who smoked, with 63% of diseased sites (NB and DB) improving by ≥2mm in PPD over the period.

Biomarker levels

All of the biomarkers tested revealed significant differences in average levels between diseased and healthy sites, but bleeding and non-bleeding diseased sites at baseline did not differ (Supplementary Table 1). Sites that failed to respond to treatment adequately after 6 months (i.e. < 2mm reduction in PPD) generally had higher levels of all biomarkers at baseline (Table 1). Over the treatment period, comparing baseline measures with those at 6 months, all biomarkers showed a statistically significant decrease at diseased sites (Figure 2 and detailed in Supplementary Table 2) and the reductions in biomarkers correlated with reduction in PPD and outcome of treatment with the exception of levels of *F. nucleatum* (Supplementary Table 2).

Threshold values

ROC curves, were used to evaluate the ability of biomarkers in GCF and plaque to identify whether the site is diseased or healthy. Threshold points with the highest sensitivity and specificity were selected for each of the biomarkers at baseline (Table 2 and Supplementary Figure 2). Furthermore, the values of MMP8, elastase, sialidase *P. gingivalis* and *T. forsythia* showed high sensitivity (77 – 86%) and specificity (79 – 86%) and areas under the curves (0.79 – 0.92) for diagnosis of disease.

Predictive value

To determine whether the 'biomarker profile' is a useful prognostic tool for treatment

outcome, logistic regression analysis was performed with baseline biomarker levels as independent variables versus the outcome measure of ≥2mm improvement of PPD at the 6 month time point as dependent variable. The data are summarised in Tables 3 and 4. For all diseased sites, the three enzyme levels together (MMP8, elastase, sialidase) were able to predict treatment outcome with ≥80% certainty. Bacterial levels alone were able to predict treatment outcome with ≥74% certainty but, when combined with the enzyme biomarkers there was an increase in prediction value to ≥92% (Table 3). The odds ratio and confidence interval of these biomarkers are shown in Table 4. Backward stepwise logistic regression was used to exclude the variables that could not add any significant predictive value to the combination and it was found that *F. nucleatum* was a redundant variable ($p>0.05$) while the others were not. Furthermore, each individual biomarker alone was not able to predict treatment outcome at a level greater than the null hypothesis (61% in NB and 62.5% in DB sites; Table 3).

Validation of the predictive value of the 'biomarker profile'

The threshold levels of the GCF enzymes arrived at in this study were used to test their sensitivity and specificity for diagnosis and for prognostic value against an independent data set. The data used were those reported in the earlier pilot study[12] comprising 22 independent patients. Again ROC curves were used to identify specificity and sensitivity and logistic regression to determine predictive value but using only GCF enzymes. The diagnostic value of those thresholds was as high for the independent validating patient cohort as it was for the patients in this study (Supplementary Table 3). Also, the prognostic value of the enzyme threshold levels to predict the treatment outcome (≥2mm PPD improvement) was as high (84%) for the independent validation patient cohort as it was for the patients in this study (>80%) (Supplementary Table 4).

Discussion

The key finding of the study presented here is that combined high levels of three GCF enzymes and two bacteria provide a good prediction for the outcome of non-surgical treatment on a site specific basis. . While it is acknowledged that there are several limitations to the use of biomarkers for diagnosing/predicting disease outcome (e.g. appropriate marker selection, validation and robustness of analysis), and these can dramatically affect their predictive value, such limitations can be reduced by using combinations of different biomarkers, each of which alone may not be usefully predictive. The rationale for the study, therefore, was based on the premise that periodontal disease has a multifactorial aetiology and so combinations of several host and bacterial biomarkers are more likely to provide useful diagnostic and prognostic information than single biomarkers. Indeed no individual biomarker has yet been demonstrated to be sufficiently reliable for clinical use [5,6,7,11].

We did not seek to discover new, untried biomarkers but we utilised ones for which there has been clear evidence of association with disease. MMP-8 and elastase were selected because they are secreted by neutrophils[20]. Sialidase was selected as it is mainly of bacterial origin and produced by the red-complex pathogens *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* with evidence that it plays a role in pathogenesis[21,22]. Furthermore our pilot study identified raised sialidase in GCF of diseased sites[12], while others detected *Tannerella* sialidase gene (*nanH*) at high levels in periodontal plaque RNA[23]. These enzymes have been investigated individually by others in relation to periodontal disease[24-27] but not in combination and here we have also included assessment of the level of selected key bacterial species considered to be aetiologically important contributors to chronic periodontitis and its progression as potential biomarkers[28--30]. While ideally one would conduct a qPCR study of a larger number of bacterial *spp*., for practical reasons (particularly availability of control DNA material) and given their

prominence and close-association, we focussed on *P. gingivalis* and *T. forsythia* as representative 'red-complex' periodontal pathogens[14]. However, we acknowledge that ideally we would have included *T. denticola* or *Filifactor alocis*. We did, however, include *F. nucleatum* as a positive internal control since that would be expected to be present in all samples as it acts as a 'bridging' species in the oral biofilm[31,32]. Moreover, plaque samples were collected using curettes, which others have shown to yield higher levels of bacterial DNA than paper points[32].

Our findings strongly suggest that baseline concentrations of active MMP8, elastase and sialidase can not only be used to diagnose a diseased site but when combined also predict its likely response to treatment. The enzyme levels correlated with initial PPD, and with the exception of sialidase is in agreement with other information in the literature[34-38]. Our pilot study was the first to report the predictive value of sialidase in combination[12] and those data have been upheld in this larger cohort study. In both this and our pilot study we used the ROC curve to determine whether a given enzyme level could act as a threshold point to differentiate health from disease. The resultant diagnostic sensitivity and specificity for each enzyme was >78%, which for MMP-8 and elastase is in keeping with some earlier studies[6,7,39-41].

While these findings for diagnosis are useful, we feel that the primary value of a 'biomarker profile' would be if it is able to predict the outcome of non-surgical periodontal treatment or disease progression. Consequently in the current study we looked beyond the diagnostic value of the biomarkers and used backward stepwise logistic regression to evaluate each biomarker's contribution to the 'profile's' predictive value. The latter were judged against the primary treatment outcome measure (i.e. an improvement of 2mm in PPD[11,42])

These findings expand and improve upon recent reports that a raised MMP8 level is a good predictor of treatment outcome[7,11,41], and that elastase might be useful for predicting disease progression[43,44]. In addition, while Beighton[25] showed that sialidase could differentiate between gingivitis and periodontitis, to the best of our knowledge we are the first to report the correlation of initial GCF sialidase levels with treatment outcome.

The mere presence and absence of key bacterial species is not sufficient to distinguish healthy sites from diseased sites and it is generally accepted that an increase in the level of certain species is important[45-48]. Indeed we found that the proportions of *P. gingivalis* and *T. forsythia* above the critical threshold points of 0.23% and 0.35% respectively, were associated with disease (sensitivities and specificities > 77%). In terms of response to treatment, while there is much data indicating that the levels of *T. forsythia* and *P. gingivalis* at diseased sites reduce during treatment, there is a shortage of data on the usefulness of these bacteria as prognostic tools and the current study contributes to filling this research gap. Using logistic regression analysis we found that high levels of *P. gingivalis* and *T. forsythia* at were associated with sites that failed to respond to treatment compared with sites that did respond. This is in agreement with some findings in a study by Kinney[7]. Consequently by adding the levels of these species into the 'profile' of the three GCF enzymes, the predictive power for treatment outcome was raised from ~80% to >92%. It should be stressed, therefore, that our findings strongly indicate that high levels of the three enzymes, supplemented by high levels of *P. gingivalis T. forsythia,* at initial assessment predicts a poor outcome for those sites when conventional treatment is used. Thus, knowledge of the total combined profile of these biomarkers at patient assessment provides information that is useful in directing treatment.

To validate the predictive value of this 'biomarker profile, we tested the threshold values determined here against the clinical data of an independent cohort of 22 patients. These patients have been described previously by Gul[12] but only GCF enzyme data were available for these subjects so we could only validate against the three enzyme biomarkers. The enzyme threshold points determined here maintained a high level of sensitivity and specificity for predicting the outcome of treatment proving that they are reliable in different sample cohorts. Further testing is required, however, particularly in cohorts of challenging patients, for example those with diabetes mellitus.

We also acknowledge some limitations to the study, in addition to those mentioned earlier, but feel that despite these the work and the study not only stands alone but also presents novel aspects and avenues for study and diagnosis of periodontal disease. Firstly, our power calculation indicated an ideal study population of 100 would be required, but while we encountered some patient drop-out (12/101) we calculated early on in the work that both levels of *Fusobacterium* and a lack of differences in response between DB and NB sites essentially made them redundant variables and so our study was adequately powered. In addition we found during the study that bleeding on probing (BoP) was too variable a measure given its subjective nature and so employed a more rigorous primary outcome measure of $\geq$ 2mm improvement in PPD which is accepted as being outside inter-examiner variability[11]. .

Examining all sites in a patient would be the ultimate aim given the site-specific nature of chronic periodontitis, but this was not feasible in this study due to patient numbers and time required. However, finding that the data from this study patient cohort matches that from an independent study group provides strong validation and confidence in our findings. Finally, it would be of great interest to examine the relationship between the chosen biomarkers and disease onset, transition from

gingivitis to periodontitis, or whether a site is undergoing active destruction, all avenues that further work should explore to improve prognosis and guide treatment.

Conclusion

This study has shown that knowledge of the levels of three GCF enzymes plus two bacterial species at a site comprises a unique 'biomarker profile' or fingerprint that is useful for predicting the outcome of periodontal treatment. This is important since 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). While clearly taking this data forward to producing a chairside test is challenging, our data indicate that this is an avenue worth pursuing and that this 'biomarker profiling' might aid in treatment regimen decision making and thus improve patient outcomes for this chronic hard-to-treat but large global burden disease.

Acknowledgements

The authors would like to thank the Kurdistan Regional Government for funding S.S. Gul during this study. The help with collection of GCF and plaque samples by the Therapists, Nivan Al-Hammouri and Claire Vallance-Owen of the Charles Clifford Dental Hospital, Sheffield is gratefully acknowledged. Also, we gratefully acknowledge the assistance of Rosie Taylor, Senior Statistician, Statistical Services Unit, University of Sheffield

References

1. Goodson JM. Diagnosis of periodontitis by physical measurement- interpretation from episodic disease hypothesis. *J Periodontol* 1992;63:373-382.
2. Greenstein G. Contemporary interpretation of probing depth assessments: diagnostic and therapeutic implications. a literature review. *J Periodontol 1997;*68:1194-1205.

3. Korte DL, KinneyJ. Personalized medicine: an update of salivary biomarkers for periodontal diseases. *Periodontol 2000* 2016;70**:**26-37.
4. Griffiths GS. (2003). Formation, collection and significance of gingival crevice fluid. *Periodontol 2000* 2003;31:32-42.
5. Buduneli N, Kinane DF. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *J Clin Periodontol* 2011;38:85-105.
6. Kinney JS, Morelli T, Braun T et al. Saliva/pathogen biomarker signatures and periodontal disease progression. *J Dent Res* 2011;90:752-758.
7. Kinney JS, Morelli T, Oh M et al. Crevicular fluid biomarkers and periodontal disease progression. *J Clin Periodontol* 2014;41;113-120.
8. Embery G, Waddington RJ, Hall RC, Last KS. Connective tissue elements as diagnostic aids in periodontology. *Periodontol 2000* 2000;24;193-214.
9. Barros SP, Williams R, Offenbacher S, Morelli T. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontol 2000 2016;*70:53-64.
10. Xiang X, Sowa MG, Iacopino AM et al. An update on novel non-invasive approaches for periodontal diagnosis. *J Periodontol 2010;*81:186-198.
11. Sorsa T, Gursoy UK, Nwhator S et al. Analysis of matrix metalloproteinases, especially MMP8, in gingival creviclular fluid, mouthrinse and saliva for monitoring periodontal diseases. *Periodontol 2000* 2016;70:142-163.
12. Gul S S, Douglas CWI, Griffiths GS, Rawlinson A. A pilot study of active enzyme levels in gingival crevicular fluid of patients with chronic periodontal disease. *J Clin Periodontol* 2016;43:629-36.
13. Socransky S, Haffajee A, Smith C, Dibart S. Relation of counts of microbial species to clinical status at the sampled site. *J Clin Periodontol* 1991;18:766-775.
14. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992;63:322-331.
15. Galgut P. A comparison of different indices used in the clinical assessment of plaque and gingival bleeding. *Clin Oral Investig* 1999;3:96-99
16. Wassall RR, Preshaw PM. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontol 2000* 2016;70**:**65-79.
17. Griffiths GS, Wilton JMA, Curtis MA et al. Detection of high risk groups and individuals for periodontal diseases: Clinical assessment of the periodontium. *J Clin Periodontol* 1988;15:403-410.
18. Ata-Ali J, Flichy-Fernández AJ, Alegre-Domingo T, Ata-Ali F, Palacio J, Peñarrocha-Diago M. Clinical, microbiological, and immunological aspects of

healthy versus peri-implantitis tissue in full arch reconstruction patients: a prospective cross-sectional study. *BMC Oral Health*. 2015;15:43

19. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol 1999;*4:1-6.
20. Bender JS, Thang H, Glogauer M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J Periodontal Res 2006;*41:214-220.
21. Roy S, Honma K, Douglas CWI, Sharma A, Stafford GP. Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. *Microbiol-Sgm* 2011;157:3195–3202.
22. Li C, Kurniyati Hu B, Bian J et al. Abrogation of neuraminidase reduces biofilm formation, capsule biosynthesis, and virulence of *Porphyromonas gingivalis*. *Infect & Immun* 2012;80:3-13.
23. Duran-Pinedo AE, Chen T, Teles R et al. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *Inter Soc for Microl Eco J* 2014;8:1659-1672.
24. Beighton D, Life JSC. Trypsin-like, chymotrypsin-like and glycylprolyl dipeptidase activities in gingival crevicular fluid from human periodontal sites with gingivitis. *Arch Oral Biology* 1989;34;843-846.
25. Beighton D, Radford JR, Naylor MN. Glycosidase activities in gingival crevicular fluid in subjects with adult periodontitis or gingivitis. *Arch Oral Biology* 1992;37:343-348.
26. Mailhot J, Potempa J, Stein S et al. A relationship between proteinase activity and clinical parameters in the treatment of periodontal disease. *J Clin Periodontol* 1998;25:578-584.
27. Herrmann JM, Gonzáles JR, Boedeker RH, Vonholdt J, Meyle J. Microassay for the detection of elastase activity in the gingival crevice. *J Clin Periodontol* 2001;28:31-37.
28. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra-and subgingival plaque in health and periodontitis. *J Clin Periodontol* 2000;27**:**648-657
29. Tanner AC, Izard J. *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontol 2000* 2006;42**:**88-113.
30. Hajishengallis G, Lambris JD. Microbial manipulation of receptor crosstalk in innate immunity. *Nature Rev Immunol* 2011;11**:**187-200.
31. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 1993;175:3247-52.

32. Jakubovics NS, Kolenbrander PE. The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis* 2010;16**:**729-739.
33. Pérez-Chaparro PJ, Duarte PM, Pannuti CM, Figueiredo LC, Mestnik MJ, Gonçalves CP, Faveri M, Feres. M Evaluation of human and microbial DNA content in subgingival plaque samples collected by paper points or curette. *J Microbiol Methods*. 2015;111:19-20.
34. Kitawaki M, Iijima KI, Nakashizuka T, Hayakawa T. Neuraminidase activity in human crevicular fluid. *J Periodontal Res 1983;*18:318-320.
35. Nieminen A, Nordlund L, Uitto VJ. The effect of treatment of the activity of salivary proteases and glycosidases in adults with advanced periodontitis. *J Periodontol* 1993;64:297-301.
36. Pederson ED, Stanke SR, Whitener SJ, Sebastiani PT, Lamberts BL, Turner DW. Salivary levels of alpha(2)-macroglobulin, alpha(1)-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Arch Oral Biology* 1995*;*40:1151-1155.
37. Eley B, Cox S. 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.
38. Mantyla P, Stenman M, Kinane DF et al. Gingival crevicular fluid collagenase-2 (MMP8) test stick for chair-side monitoring of periodontitis. *J Periodontal Res* 2003;38:436-439.
39. Eley BM, Cox SW. A 2-year longitudinal study of elastase in human gingival crevicular fluid and periodontal attachment loss. *J Clin Periodontol* 1996;23:681-692.
40. Leppilahti JM, Kallio MA, Tervahartiala T, Sorsa T, Mäntylä P. Gingival crevicular fluid matrix metalloproteinase-8 levels predict treatment outcome among smokers with chronic periodontitis. *J Periodontol* 2014;85:250-260.
41. Leppilahti JM. Sorsa T, Kallio MA et al. The utility of gingival crevicular fluid matrix metalloproteinase-8 response patterns in prediction of site level clinical treatment outcome. *J Periodontol 2015;*86:777-787.
42. Cobb CM. Non-surgical pocket therapy: mechanical. *Ann Periodontol* 1996*;*1:443-90.
43. Palcanis KG, Larjava IK, Wells BR et al. Elastase as an indicator of periodontal disease progression. *J Periodontol* 1992*;*63:237-242.

44. Armitage GC, Jeffcoat MK, Chadwick DE et al. Longitudinal evaluation of elastase as a marker for the progression of periodontitis. *J Periodontol* 1994;65:120-128.
45. Byrne S, Dashper S, Darby I, Adams G, Hoffmann B, Reynolds E. Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol & Immunol* 2009;24:469-477.
46. Torrungruang K, Bandhaya P, Likittanasombat K, Grittayaphong C. Relationship between the presence of certain bacterial pathogens and periodontal status of urban Thai adults. *J Periodontol* 2009;80**:**122-129.
47. Ramseier CA, Kinney JS, Herr AE et al. V. Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol* 2009;80:436-446.
48. Lourenço TGB, Heller D, Silva-Boghossian CM, Cotton SL, Paster BJ, Colombo APV. Microbial signature profiles of periodontally healthy and diseased patients. *J Clin Periodontol* 2014;41:1027-1036.

Figure Legends

Figure 1

Chart showing changes in PPD of all deep bleeding (DB) sites from baseline to 6 months post treatment. ● Baseline PPD; ☐ Site did not change; ■ Site improved by ≥ 2mm; ■ Site improved by < 2mm; ■ Site deteriorated

Figure 2

Chart showing changes in the six biomarkers at all healthy, deep non-bleeding (NB) and deep bleeding (DB) sites from baseline to 6 months post treatment.

Tables

Table 1. Analysis of baseline median biomarker levels (ng/µl enzymes; % total bacteria) in respondent sites (n= 105) versus non-respondent sites (n= 48).

Disease sampled site	Biomarker (Baseline)	Respondent (median)	Non-Respondent (median)	p value*
NB sites	MMP8	122	231	0.003
	Elastase	68	307	0.001
	Sialidase	10	26	0.001
	Pg%	0.58	2.3	0.007
	Tf %	0.1	5.64	0.0001
	Fn%	4.03	4.19	0.6
DB sites	MMP8	138	352	0.001
	Elastase	53	447	0.001
	Sialidase	4.2	35	0.001
	Pg%	0.88	3.58	0.0001
	Tf %	0.3	7.13	0.0001
	Fn%	4.82	4.14	0.53

* Kruskal Wallis

Table 2. Diagnostic properties of specific thresholds of the three GCF enzymes and key bacteria in plaque

Variable	Threshold (ng/µl)	Sensitivity%/ Specificity%	Area under the curve	95% CI for OR LCL	UCL	p value
MMP8	94	86/83	0.92	0.89	0.95	0.0001
Elastase	33	78/80	0.87	0.83	0.91	0.0001
Sialidase	2.3	79/79	0.79	0.72	0.83	0.0001
Pg%	0.23	77/86	0.81	0.71	0.86	0.0001
Tf%	0.35	78/84	0.8	0.77	0.89	0.0001
Fn%	2.94	65/65	0.62	0.52	0.71	0.017

LCL lower confidence limit; UCL upper confidence limit

Table 3. Logistic regression analysis with 2mm PPD improvement (at 6 months) as the dependent variable.

	Method	NB sites Predictive	DB sites Predictive
GCF biomarkers	All variables	81.3%(MMP8, elastase, sialidase)	80.3% (MMP8, elastase, sialidase,)
Bacterial biomarkers	All	76% (*Pg%*, *Tf%, Fn%*)	76% (*Pg%*, *Tf%, Fn%*)
	Stepwise (backward conditional)	76% (*Pg%*, *Tf%*)	74% (*Pg%*, *Tf%*)
Combined GCF enzymes and bacterial biomarkers	(MMP8, elastase, sialidase, *Pg*%, *Tf*%)	92%	93.3%
Each single biomarker		61%	62.5%

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum

Table 4 Summary of logistic regression for each individual explanatory variable for response of diseased sites by 6 months post treatment.

Disease sampled site	Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR LCL	95% CI for OR UCL	p value *
NB	MMP8	-0.005	0.995	0.99	1.3	0.006
	Elastase	-0.006	0.994	0.99	1.2	0.002
	Sialidase	-0.002	0.998	0.99	1.2	0.03
	Pg%	-1.2	0.28	0.1	0.7	0.001
	Tf%	-0.6	0.53	0.3	0.7	0.001
	Fn%	-0.05	0.94	0.5	1.6	0.8
DB	MMP8	-0.005	0.995	0.99	1.3	0.007
	Elastase	-0.005	0.995	0.99	1.3	0.001
	Sialidase	-0.006	0.994	0.99	1.3	0.001
	Pg%	-0.32	0.68	0.4	1.1	0.01
	Tf%	-0.5	0.55	0.3	0.7	0.001
	Fn%	0.12	1.1	0.8	1.5	0.4

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum. * Mann Whitney test

Figure 1

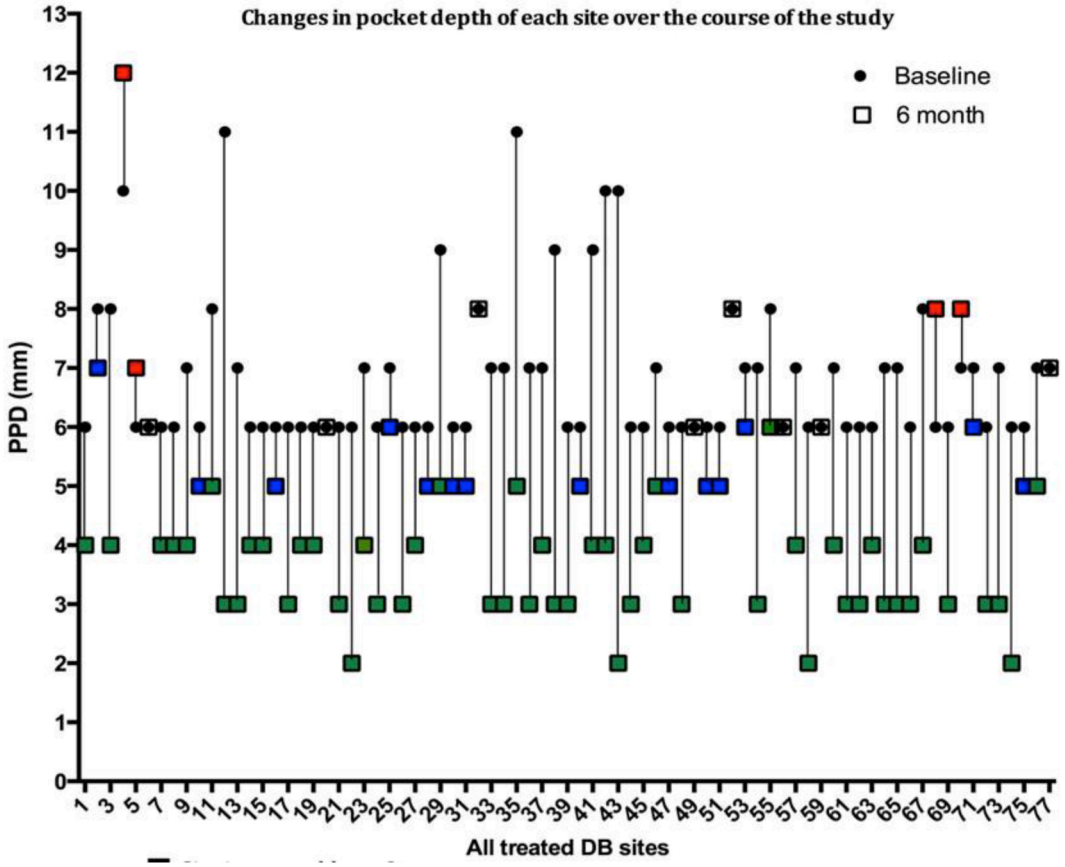
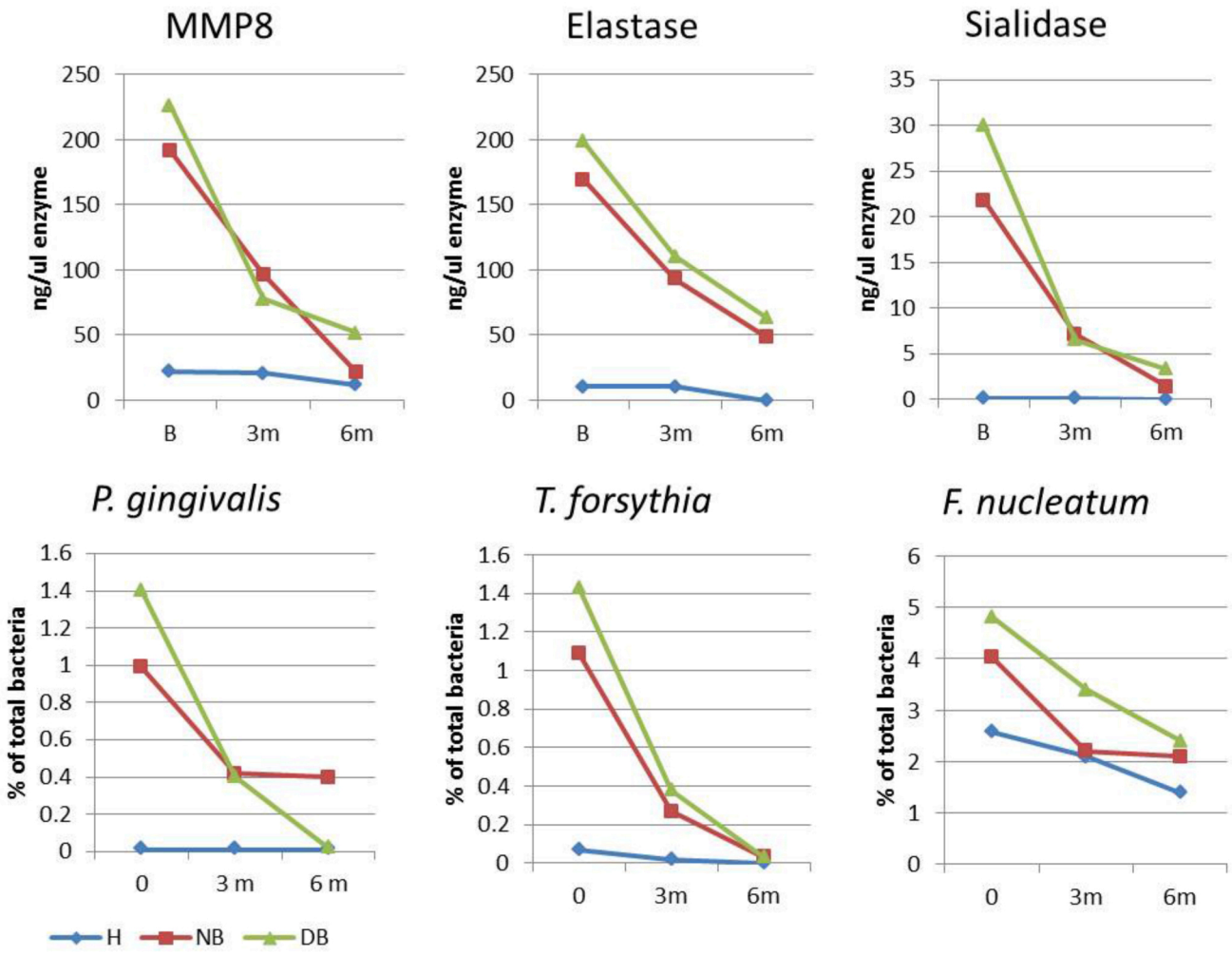

Changes in pocket depth of each site over the course of the study
Baseline
6 month
PPD (mm)
All treated DB sites

Figure 2


MMP8
Elastase
Sialidase
ng/ul enzyme
250
200
150
100
50
0
35
30
25
20
15
10
5
B
3m
6m
P. gingivalis
T. forsythia
F. nucleatum
% of total bacteria
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
6
5
4
3
2
1
0
3 m
6 m
H
NB
DB