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Title: Discovery, validation and diagnostic ability of multiple protein-based biomarkers in saliva and GCF to distinguish between health and periodontal diseases.

Running title: Protein biomarkers for periodontitis

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Conflict of Interests statement:

Kooijman, Rmaile and De Jager are employees of Philips Research who have contributed to the design and analysis of the study. Philips has been pursuing the Intellectual Property (patents) resulting from this research.

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Ethical approval:

The study protocol was approved by the South Birmingham NHS Research Ethics Committee, ref. 09/H1206/19 and by the County Durham & Tees Valley 1 NHS Research Ethics Committee, ref. 09/H0905/49.

Data availability statement:

The data that support the findings of this study are openly available in UBIRA eDATA repository at University of Birmingham at https://doi.org/10.25500/edata.bham.00000684., reference number 684.

Abstract

Aim: To discover and validate differential protein biomarker expression in saliva and gingival crevicular fluid (GCF) to discriminate objectively between periodontal health and plaque-induced periodontal disease states.

Materials and methods: 190 participants were recruited from two centres (Birmingham and Newcastle upon Tyne, UK) comprising healthy, gingivitis, periodontitis and edentulous donors. Samples from the Birmingham cohort were analysed by quantitative mass spectrometry proteomics for biomarker discovery. Shortlisted candidate proteins were then verified by enzyme-linked immunosorbent assay in both cohorts. Leave-one-out cross validation logistic regression analysis was used to identify the best performing biomarker panels.

Results: 95 proteins were identified in both GCF and saliva samples and 15 candidate proteins were selected based upon differences discovered between the donor groups. The best performing panels to distinguish between: health or gingivitis and periodontitis contained matrix metalloproteinase-9 (MMP9), S100A8, alpha-1-acid glycoprotein (A1AGP), pyruvate kinase and age (area under the curve (AUC) 0.970); health and gingivitis contained MMP9, S100A8, A1AGP, pyruvate kinase but not age (AUC 0.768); and mild-moderate and advanced periodontitis contained MMP9, S100A8, A1AGP, pyruvate kinase and age (AUC 0.789).

Conclusion(s): Biomarker panels containing four proteins with and without age as a further parameter can distinguish between periodontal health and disease states.

Key words: saliva, GCF, proteomics, biomarker

Clinical Relevance**:**

Scientific rationale for study: Objective point of care tests for distinguishing between periodontal health and disease would facilitate early case detection, prompt treatment and enhanced patient outcomes.

Principal findings: Protein biomarkers matrix metalloproteinase 9, S100A8, alpha 1 acid glycoprotein, pyruvate kinase in panels of 3-4 biomarkers can distinguish between health and gingivitis, health or gingivitis and periodontitis or between mild and advanced periodontitis.

Practical implications: This information could form the basis for a novel objective diagnostic approach for periodontal diseases.

1 Introduction

Periodontitis is a chronic infectious-inflammatory disease that causes the irreversible destruction of connective tissues which secure the teeth in the jaws. In its most severe or advanced form it affects 7-11% of the world's adult population (Kassebaum et al., 2014; Kassebaum et al., 2017), leading to tooth loss and negatively impacting speech, nutrition, self-confidence and overall quality of life (Chapple et al., 2017). The pathophysiology of periodontitis involves a dysfunctional immune-inflammatory response to a dysbiosis that starts within the dental plaque biofilm (Hajishengallis, 2015), and which then propagates an exaggerated and destructive inflammatory milieu, underpinned by certain genetic and lifestyle risk exposures such as smoking and hyperglycaemia (Meyle & Chapple, 2015).

Periodontitis presents initially with gingival bleeding and is not necessarily recognized by patients or the public as potentially significant to health. However, gingivitis is a necessary pre-requisite for periodontitis (Kinane, Attstrom, & European Workshop in Periodontology group, 2005) and the detection of transition from gingivitis to periodontitis requires a detailed periodontal examination by a dental healthcare professional. However, only 50% of the population routinely attend dental practice (Vernekar, Batchelor, & Heilmann, 2019) and the ability to identify the disease early in non-dental settings such as pharmacies and general medical practices offers a significant opportunity for early case detection and onward referral to dental professionals for definitive diagnosis and management.

Saliva is a recognized and validated diagnostic biological fluid, with sample collection being accessible to non-dental professionals; it therefore offers potential as a medium for the early detection of periodontitis. Importantly, periodontitis is a preventable disease and early detection improves patient outcomes (Sanz et al., 2020). Gingival crevicular fluid (GCF) is a serum transudate in health and tissue exudate in disease, enriched with biological signatures of the immune-inflammatory response to the plaque biofilm, as well as markers of the downstream effects on the connective tissue attachment

apparatus. Thus it can reflect in real time the state of the periodontal tissues and can be used to measure biomarkers of inflammation, tissue remodeling and bone metabolism.

A number of previous investigations have explored the potential for salivary and GCF biomarkers to detect periodontal disease (for reviews see Ghallab (Ghallab, 2018) or Taylor (Taylor, 2014)). In a systematic review, Kc et al. (Kc, Wang, & Gallagher, 2020) demonstrated that matrix metalloproteinase (MMP) 8 (all or active forms) and interleukin 6 (IL-6) exhibited the best diagnostic performance as individual biomarkers. However, biomarker combinations are reported to demonstrate improved sensitivity, specificity and diagnostic accuracy when compared to single biomarkers (Ebersole et al 2015). Quantitative proteomics offers a method to detect multiple protein biomarkers using a non-targeted approach. Biomarkers for periodontitis have been identified using this technique and a recent systematic review highlighted the need to follow up on these initial discoveries (Rizal et al., 2020).

The aim of the present study was therefore to explore the saliva and GCF proteomes using a non-presumptive discovery approach, to identify whether distinct biological signatures of health, gingivitis and periodontitis existed, and to determine whether these mapped to well-defined clinical phenotypes. The use of GCF and saliva was to determine site of inflammation specific biomarkers, i.e. those in GCF, that could be detected in saliva as saliva is ultimately an easier biofluid to sample. Our research question was whether distinct clusters of differentially expressed proteins could be employed for case identification using a 2-stage approach: initial discovery by mass spectrometry, followed by ELISA validation, sensitivity and specificity analysis of resulting clusters against clinical diagnostic criteria. Case identification focused on differentiating [1] health or gingivitis from periodontitis, [2] health from gingivitis and [3] mild periodontitis from advanced periodontitis. A pre-requisite to this study was a robust and unambiguous case definition for health and periodontal disease status, in order to avoid any significant clinical phenotypic overlap. This study was conducted prior to the 2017 world workshop classification (WWC) of periodontal and peri-implant diseases and

conditions, however thresholds used to define the different health/disease states in our study broadly

map to those of the 2017 WWC (Table 1).

2 Materials and Methods:

2.1 Study populations

For both the Birmingham and Newcastle cohorts, participants were recruited to one of 5 groups as defined in Table 1. All participants were recruited between 2009 and 2012. Population demographics can be found in Table 2.

Birmingham cohort: 50 medically healthy adult subjects (53% female) were recruited. The study was approved by South Birmingham NHS Research Ethics Committee, ref. 09/H1206/19 and all volunteers provided written informed consent.

Newcastle cohort: 140 medically healthy adult subjects (53% female) were recruited. The study was approved by County Durham & Tees Valley 1 NHS Research Ethics Committee, ref. 09/H0905/49 and all volunteers provided written informed consent.

Inclusion and exclusion criteria: Patients were excluded if they had less than 24 natural teeth (except edentulous patients), were current smokers, or had smoked up to within 5 years; wore removable dentures (partial or full) or bridges involving > 4 teeth (except for the edentulous group); wore orthodontic appliances; were on long term antibiotic/anti-inflammatory therapy or had taken antibiotic/anti-inflammatory medication during the month prior to baseline assessment; were pregnant, breast feeding or had medical/dental conditions incompatible with participation in study.

Patient characteristics can be found in Table 1.

2.2 Clinical Protocol

Volunteers in all groups (defined in table 1) were examined and had biological samples collected (GCF and saliva at Birmingham, saliva only at Newcastle) and clinical indices examined to confirm their periodontal status (Table 2). Clinical examination involved determination of clinical attachment level (CAL), probing pocket depths (PPD), bleeding on probing (BOP), gingival index (GI) (Loe, 1967) at

Birmingham or modified GI (Lobene, Weatherford, Ross, Lamm, & Menaker, 1986) at Newcastle and plaque scores (O'Leary, Drake, & Naylor, 1972), using a UNC-PCP15 periodontal probe. Participants with gingivitis received standard oral hygiene instruction and were provided with a professional prophylaxis. Participants with periodontitis received non-surgical periodontal therapy by a dental hygienist, until they achieved clinical outcomes that were consistent with successful periodontal therapy: endpoint of ≤4 sites with PD≥5mm and ≤10% of sites with BoP (Feres et al., 2020). Periodontitis patients returned for two further oral hygiene instruction reinforcement visits at approximately monthly intervals, as part of their routine clinical care. Post-operative review and post-therapy biological samples collection was performed 3-months following treatment completion.

Table 1. Definitions of participant groups.

Group	Definition Birmingham	Definition Newcastle
Health	• No sites with interproximal attachment loss • No sites with PPD >3mm • <10% sites with GI of 1 & no sites with G.I. of 2 or 3. • <10% sites with BOP.	• No sites with interproximal attachment loss • PD ≤ 3 mm in all sites (but would allow up to four 4 mm pockets at distal of last standing molars) • ≤ 10% sites with mGI of ≥ 2.0 • <10% sites with BOP.
Gingivitis	• No sites with interproximal attachment loss • >30% of sites with GI > 2 • BOP >30% • No sites with PPD > 4mm.	• No sites with interproximal attachment loss • >30% of sites with mGI ≥ 3.0 • BOP scores >10%. • No sites with PD > 4 mm.
Mild-moderate periodontitis (Stage I/II under 2017 WWC)	• interproximal CAL of 2-4mm at > 8 teeth with PPD of 5-7mm.	• interproximal PD of 5-7 mm (equating to approximately 2-4 mm CAL) at ≥ 8 teeth • % BOP scores >30%.
Advanced periodontitis (Stage III/IV under 2017 WWC)	• Interproximal CAL of >5mm at >12 teeth & PPD of >7mm.	• Interproximal PPD of >7mm (equating to approximately ≥ 5 mm CAL) at ≥ 12 teeth • % BOP scores >30%.
Edentulous	• Edentulous patients with no evidence of oral ulceration or erosive mucosal disease.	• Completely edentulous for >1 year with healthy oral tissues

Table 2 Clinical data for both cohorts. The Birmingham cohort was used for proteomics-based discovery and ELISA validation, the Newcastle cohort was used for ELISA validation. SD: standard deviation.

	Health	Gingivitis	Mild/Moderate Periodontitis (Stage I/II)	Advanced periodontitis (Stage III/IV)	Mild/Moderate Periodontitis after treatment (Stage I/II)	Advanced periodontitis after treatment (Stage III/IV)	Edentulous
Birmingham cohort							
Number in group	10	10	10	10	10	10	10
Age (years) mean (SD)	39 (9)	38 (11)	47 (6)	49 (7)	47 (6)	49 (7)	73 (7)
Gender (% female)	40%	50%	50%	59%	50%	59%	60%
Probing pocket depth (mm) mean (SD)	1.31 (0.25)	1.88 (0.21)	3.35 (0.61)	4.68 (0.75)	2.45 (0.36)	3.06 (0.45)	-
Probing pocket depth (mm) mean (SD) at sampled sites	1.80 (0.5)	2.12 (0.5)	3.50 (1.3)	4.63 (2.0)	2.42 (0.6)	2.82 (0.9)	-
Clinical attachment level (mm) mean (SD)	0	0	4.05 (0.50)	5.45 (0.82)	3.27 (0.44)	4.39 (0.69)	-
Clinical attachment level (mm) mean (SD) at sampled sites	0	0	3.72 (1.4)	5.15 (2.1)	2.88 (0.6)	4.15 (1.4)	-
Newcastle cohort							
Number in group	29	25	32	28	31	27	26
Age (years) mean (SD)	35 (11.9)	32.8 (9.7)	43.8 (7.2)	43.8 (7.2)	44.3 (6.5)	48.4 (9.2)	69.5 (8.7)

Gender (% female)	55%	48%	47%	57%	45%	59%	58%
Probing pocket depth (mm) mean (SD)	1.4 (0.2)	1.7 (0.2)	2.9 (0.5)	3.7 (0.8)	2.3 (0.4)	2.7 (0.7)	-
Clinical attachment level (mm) mean (SD)	0	0	3.5 (0.9)	4.9 (1.3)	3.3 (0.9)	4.1 (1.1)	-

2.3 Sample collection:

Participants were asked to refrain from brushing, eating or drinking (except water) for 2 hours prior to sample collection. Samples were collected prior to oral examination and probing. GCF samples were collected from mesiobucal aspect of upper right 6, 4, 3 & upper left 3, 4, 6 teeth on Periopaper™ strips and saliva production was stimulated using a sterilised marble. Further details on sample collection and processing are found in the supplemental section.

2.4 Sample preparation for proteomics and mass spectrometry analysis

Samples were pooled per group type prior to analysis. Pooling strategy is described in the supplemental methods section in detail. Samples were reduced, alkylated and digested using standard methods. They were then labelled with iTRAQ 8-plex labels and samples analysed using an Orbitrap Velos (Thermo Fisher Scientific, UK). Further details can be found in the supplementary methods. The RAW data files are https://doi.org/10.25500/edata.bham.00000684.

2.5 ELISAs

Selected analytes from the mass spectrometry discovery phase of the study were measured using commercially available ELISAs. The following ELISAs were from Cusabio: actin gamma 1; Rho GDP dissociation inhibitor beta; haemoglobin-beta; talin-1; plastin-2; carbonic anhydrase 1; profilin 1; and S100A12. The Keratin 4 ELISA was from Cloud clone. The following ELISAs were from Elab: myosin 9; and pyruvate kinase. Kits from Cusabio, Elab and Cloud Clone were supplied by 2B Scientific (Stonesfield, UK). R&D Systems (Biotechne, Abingdon, UK) ELISAs were used for: alpha-1-acid glycoprotein; S100A8; S100A9; and MMP9. ELISAs were performed following the manufacturers' instructions.

2.6 Data analysis

The quantitative profiles of the 95 proteins found in both GCF and saliva samples from the Birmingham cohort were analysed using PolySNAP3 software, (Barr, Dong, & Gilmore, 2009). Detailed information about the approach can be found in the supplemental material.

Quantified values of analytes measured in saliva obtained from ELISAs were used in logistic regression analysis programmed in R (Team., 2020). A leave-one-out cross-validation approach was used to determine the receiver operating characteristic and area under the curve for up to four biomarker combinations and four biomarker combinations plus age. Cut-offs were determined by selection of maximal accuracy and are shown as red dotted horizontal lines as indicated. Accuracy was defined as the sum of the true positives and true negatives divided by the sum of all positives and negatives (ie Accuracy = (true positives + true negatives)/(positives + negatives)) and was calculated for every cut off to find the maximum for each data set presented (see supplemental data for analysis). Graphs were prepared with GraphPad Prism (v 8.3, GraphPad Software, USA).

3 Results

3.1 Proteomic discovery of potential biomarkers

A mass-spectrometry based proteomics approach was used to reveal the protein profile of GCF and saliva from the different groups representing different periodontal conditions in the Birmingham cohort. In the analysis of the GCF samples, 270 proteins were identified with two or more peptides: this comprised 264 human and 6 bacterial proteins. From the analysis of the saliva samples 314 proteins were identified, including 307 human and 7 bacterial proteins. All data can be found in supplemental Tables 1 & 2.

The two data sets were compared to identify proteins observed in both saliva and GCF, yielding 95 proteins, and constituting approximately a third of the proteins identified in each individual dataset (35% GCF; 30% saliva). To analyse the pattern of protein abundance, the data from the two fluids were combined to provide one set of data per protein. This allowed for visualisation of combined protein patterns rather than patterns only in either saliva or GCF. Protein profiles were then clustered using weighted means of the Pearson parametric and Spearman non-parametric correlation coefficients using the Polysnap3 programme. Four rounds of clustering were performed, whereby after each round the group with the largest number of proteins present was taken to the next round of clustering as previously described (Grant et al., 2010). These are visualised in Figure 1 and listed in supplemental Table 3. Groups discriminating between periodontal health and disease were identified for validation via ELISA. Group C3a was of particular interest and then in addition the remaining proteins were identified by their pattern in other groups, particularly those that responded to treatment and were elevated in either gingivitis (e.g. haemoglobin), or in periodontitis (e.g. MMP9). It should be noted that there could potentially have been very many candidates and it was not possible to explore every protein and so the availability of commercially available ELISAs was also a factor in the selection of the potential biomarkers for further investigation. At the termination of clustering there were 15 candidates for which commercial ELISAs were available for further validation: actin gamma 1, alpha-

1-acid glycoprotein 1, Rho GDP dissociation inhibitor beta, carbonic anhydrase-1, haemoglobin subunit beta, keratin 4, MMP9, myosin-9, plastin-2, profilin-1, pyruvate kinase, S100A12, S100A8, S100A9 and talin1.
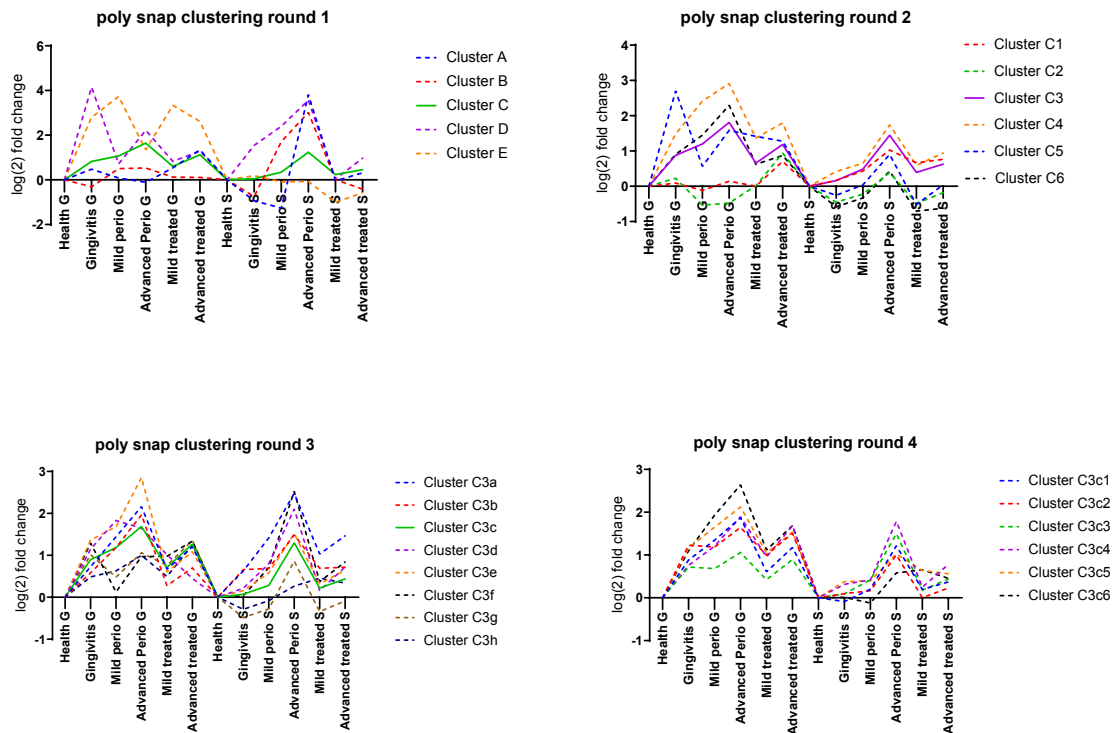

Figure 1. Clustering of the proteins discovered in both GCF and saliva to identify those with similar patterns in both oral fluids. Individual protein quantities spanning health to advanced periodontitis in GCF (G) and saliva (S) were clustered using PolySNAP3. Each line on the graphs represents the mean quantity for each cluster found and each graph represents a round of clustering. The solid lines on each graph represent the proteins taken forward to the next round: round 1 cluster C was taken forward; round 2 cluster C3 was taken forward; round 3 cluster C3c was taken forward.

3.2 ELISA validation and biomarker analysis

ELISAs for the shortlisted candidate biomarkers were evaluated for limits of detection, sensitivity and specificity with healthy and spiked healthy saliva samples. Validation of ELISAs was performed by spike recovery linearity analysis (Jaedicke, Taylor, & Preshaw, 2012); validation was performed before use of any clinical samples. Seven candidates had undetectable or low quantities of analyte in saliva using ELISA assays (actin gamma 1, carbonic anhydrase-1, Rho GDP dissociation inhibitor beta, myosin-9, plastin-2, S100A12 and talin-1) and a further one had levels of inter-assay variation >40% upon ELISA assay (haemoglobin subunit beta) and were not taken further. Seven were used to examine all saliva samples from both cohorts: alpha-1-acid glycoprotein, keratin 4, MMP-9, profilin-1, pyruvate kinase, S100A8, and S100A9. Validation data for these are shown in supplemental Table 4.

At this stage the remaining seven candidate biomarkers (alpha-1-acid glycoprotein, keratin 4, MMP-9, profilin-1, pyruvate kinase, S100A8, and S100A9) were measured in saliva in the original samples used for discovery (Birmingham cohort) and in addition they were measured in a second independent cohort (Newcastle cohort). Using a logistic regression approach, receiver-operating characteristics (ROC) and area under the curve (AUC) were calculated for each individual analyte and combinations of up to 4 different analytes. In addition, the effect of age was added to 4 analyte combinations. Age was added as an extra objective potential variable. A leave-one-out cross-validation approach was used to counteract overfitting of the data and to ensure the determined AUC values were robust estimates. Differentiation between health or gingivitis and periodontitis (HG v P) or health and gingivitis (H v G) or mild and advanced periodontitis (MP v AP) were explored in a subgroup analysis. Use of more than one analyte increased the AUC (Figure 2).
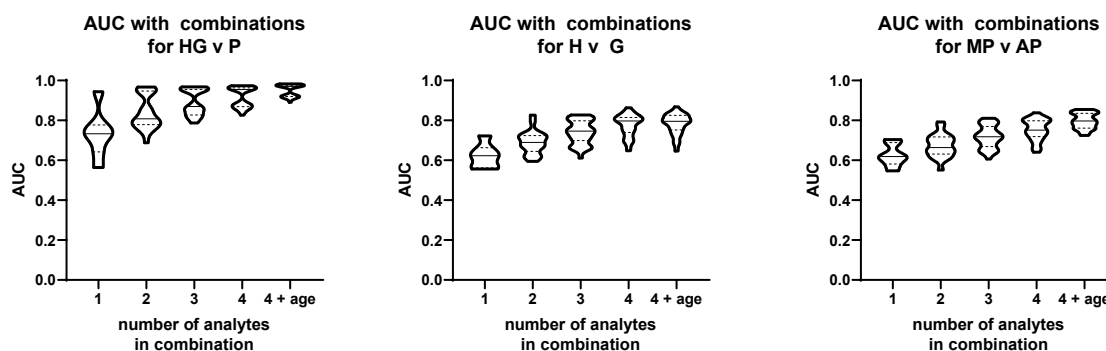
Figure 2. Change in AUC for all combinations of validated analytes for differentiating between health or gingivitis and periodontitis (HG v P), health and gingivitis (H v G) and mild and advanced periodontitis (MP v AP). Data represented as truncated violin plots, solid lines represent median and dashed lines represent quartiles. The width of each violin shape reflects the number of data points at each AUC.

The highest AUC combinations were selected and their performance investigated for diagnostic sensitivity and specificity. Cut-off values were selected for the best accuracy i.e. correctly assigned to the correct category (supplementary figures 1-9). These data are shown in table 3. Figure 3 shows the data calculated for each combination from all the different categories of donors along with the cut-offs selected from the two categories used in the logistic regression calculation, for example health or gingivitis v periodontitis. Each graph shows one panel, as described in table 3. The dotted line between the green and red shaded areas shows the cut-off between the two groups compared in the logistic regression in table 3, such that for example, in panel 1 for health v gingivitis (H v G) the majority of dots, representing individual saliva donors, in the health group sit within the green shaded area predicting that they are from healthy donors, whilst the majority of the gingivitis group sit within the red shaded area predicting that they are in the gingivitis group. Whilst the other groups were not used

in the logistic regression modelling the data are shown in the same graph using the resulting algorithm to predict in which category they would sit. In this example H v G panel 1, the periodontitis donors sit within the red shaded area flagging them as being more similar to gingivitis that health. Edentulous donors sit within the green shaded area predicting that they are more similar to healthy donors.

Whilst the primary aim of this study was the differentiation of health and disease states, and not prediction of treatment outcomes, periodontal treatment was undertaken and saliva samples were collected post-therapy for moderate periodontitis (MP + Tx) and advanced periodontitis (AP + Tx). Treatment resulted in changes (Figure 3) in the calculated biomarker value: for H v G the calculated value remained above the cut off suggesting the donors still retained a profile that was more similar to gingivitis than health; for HG v P the calculated value profile decreased, albeit variably across the individual donors and there may be potential for a bi-lobed distribution; for MP v AP values post-treatment decreased for a majority of AP individuals to levels below the cut off, creating a profile more closely mapped to a milder periodontitis phenotype. Supplemental data (Figures 1-9) show the individual ROC curves, probability plots and cut-off estimation plots for each combination reported.

Table 3. Area under the curve, confidence intervals and sensitivity and specificity for the highest combinations for differentiating between heath or gingivitis and periodontitis, between health and gingivitis or mild and advanced periodontitis. Health or gingivitis and periodontitis (HG v P), health and gingivitis (H v G) and mild and advanced periodontitis (MP v AP).

| Panel	Combination	AUC	95% confidence interval	Sensitivity (%)	Specificity (%)
HG v P					
1	MMP9 + A1AGP+PK	0.954	0.936-0.972	81	97
2	MMP9 + A1AGP+PK + S100A8	0.960	0.943-0.977	97	82
3	MMP9 + A1AGP+PK + S100A8 + Age	0.970	0.956-0.984	98	37
H v G					
1	MMP9 + A1AGP+PK	0.772	0.718-0.826	82	82
2	MMP9 + A1AGP+PK + S100A8	0.768	0.713-0.823	74	79
3	MMP9 + A1AGP+PK + S100A8 + Age	0.764	0.709-0.819	74	79
MP v AP					
1	MMP9 + A1AGP+PK	0.768	0.715-0.821	71	64

2	MMP9 + A1AGP+PK + S100A8	0.767	0.714-0.820	34	95
3	MMP9 + A1AGP+PK + S100A8 + Age	0.789	0.738-0.840	57	80

Figure 3 Visualisation of analytes in all saliva samples for the best performing combinations selected from leave-one-out cross-validation: top row H v G, middle row HG v P and bottom row MP v AP; first column panel 1 MMP9 + A1AGP+PK, second column panel 2 MMP9 + A1AGP+PK + S100A8 and third column panel 3 MMP9 + A1AGP+PK + S100A8 + Age. Each sample is represented as a mean value calculated from the algorithm determined for each comparison and shown as symbols, data have been cube root scaled to allow for all to be seen. H = health, G = gingivitis, MP = mild periodontitis, AP = advanced periodontitis, E = edentulous, Tx = post treatment. Cut-offs were determined by selection of maximal accuracy and are shown as red dotted horizontal lines as indicated. Green shading indicates the area below the cut-off (top row: health; middle row health or gingivitis: bottom row mild-moderate periodontitis) and red shading indicates the area above the cut-off (top row: gingivitis; middle row periodontitis: bottom row advanced periodontitis).
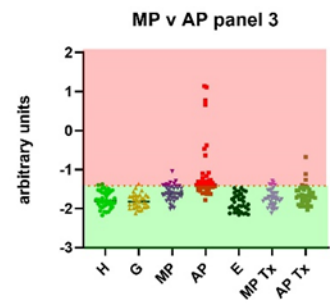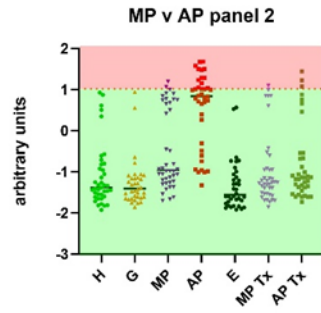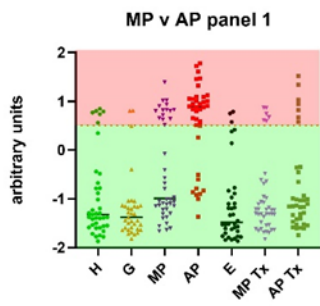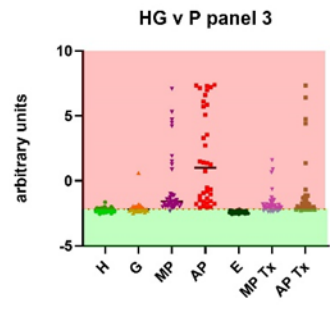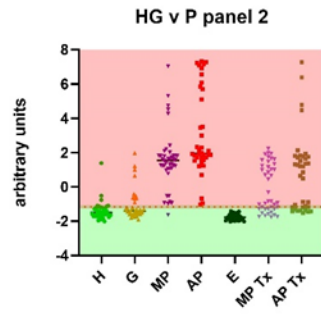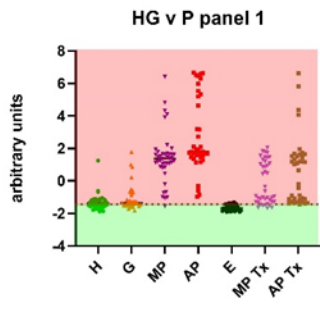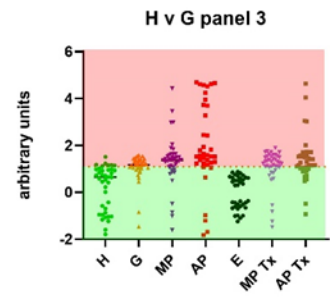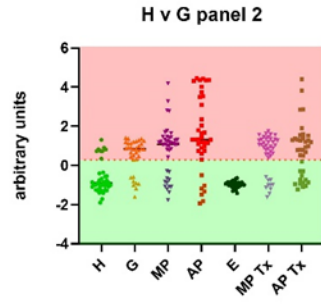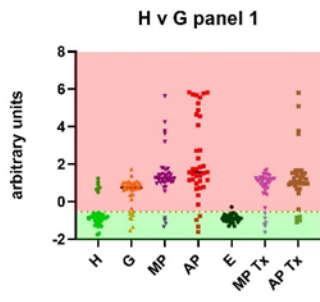
4 Discussion

The data presented demonstrate that saliva-based biomarkers can be used to differentiate between: health and gingivitis; health or gingivitis and periodontitis; or mild periodontitis and advanced periodontitis using the approach described. The addition of non-analyte data such as age can further improve the performance of these biomarkers in some cases. This was determined using logistic regression; overfitting of the data was minimised by use of leave-one-out cross-validation; AUCs ranged from 0.764 to 0.960 (table 3). Previously, it (Ebersole, Nagarajan, Akers, & Miller, 2015) has reported on the use of multiple biomarkers to distinguish between either health and periodontitis or gingivitis and periodontitis. These authors used a multiplex cytokine discovery approach. In the current study a non-presumptive proteomics-based approach was employed to discover potential biomarkers in GCF and saliva. The quantitative approach used an iTRAQ labelled method for pools of phenotyped samples: this has facilitated the examination of a large cohort of donors across a range of periodontal conditions. Similar approaches have been reported by Grant et al. (2010) to explore GCF from human donors experiencing experimental gingivitis and by Davies et al. (2016) to explore GCF from canine donors during the natural progression of gingivitis to periodontitis. Alternative proteomic approaches, such as label-free techniques have also been reported for other GCF and saliva protein profile discovery (reviewed by (Bostanci & Bao, 2017)). In our study we found 270 and 314 proteins in GCF and saliva respectively. This number is similar to that found previously (Davis et al., 2016; Grant et al., 2010), however Grassl et al. (Grassl et al., 2016) managed to discover 5500 proteins in a deep proteomic survey of saliva. This difference likely arises from the different instrumentation and methodologies used, however it implies that our data will have revealed more abundant proteins. It was also not possible to identify small signalling molecules, such as cytokines and chemokines. This was expected due to the dynamic range of proteins in saliva and GCF. Of the proteins discovered 95 were found in both GCF and saliva. As GCF flows into saliva this was anticipated, however the remaining proteins detected in GCF but not in saliva are likely to have been diluted sufficiently to prevent their detection in the methods employed. Additionally, while both human and bacterial

proteins were used in the search database there were very few bacterial proteins identified. This is likely due to the centrifugation of the samples prior to digestion: most bacteria will be associated with the desquamated cells or clumps of bacteria that will sediment and be removed during this step.

Following proteomic discovery, the GCF and saliva protein profiles were clustered and analysed to find profiles indicative of periodontitis. 15 proteins were identified that could also be validated by commercially available ELISAs. From the proteins that performed well in the ELISA it was possible to implement leave-one-out cross-validation logistic regression models to determine the best combinations for detection of health or gingivitis from periodontitis, health from gingivitis and mild periodontitis from advanced disease. Panels of 3-4 analytes or 4 analytes with the addition of age performed better than individual or pairs of analytes (Figure 2). The best performing panels (shown in Table 3) all included matrix metalloproteinase 9 (MMP9), alpha 1 acid glycoprotein (A1AGP) and pyruvate kinase (PK) with the possible addition of S100A8. The consistent return of these analytes highlights that these were the most reproducible individual analytes in the different groups of saliva donors. Higher content analysis, for instance using tissue biopsy and transcriptomic analysis might elucidate biomarker panels that include different biomarkers for each stage of the disease, as shown by (Kebschull et al., 2013) for aggressive periodontitis and chronic periodontitis. However, chronic inflammation is common to both gingivitis and periodontitis and therefore the emergence of similar biomarkers is to be expected as gingivitis and periodontitis are regarded as a continuum (Kinane et al., 2005). Key to the work presented here is the additional discovery of protein signatures that appear to differentiate periodontitis from the general inflammatory milieu of gingivitis. MMP9 is an enzyme produced by a wide range of cells including keratinocytes and neutrophils. Produced as an inactive pro-form, this enzyme is activated to degrade the extracellular matrix. MMP9 has previously been associated with periodontal destruction and has been detected in saliva for diagnosis of periodontitis (Kim et al., 2020; Kim, Shin, Kim, Kim, & Ahn, 2016). Another matrix metalloproteinase, MMP8, has been extensively researched for its use as a single biomarker for periodontitis (Sorsa, Gieselmann, Arweiler, & Hernandez, 2017). S100A8 is a small calcium or zinc binding protein, highly abundant in

neutrophils. It has also previously been found to be more abundant in saliva from patients with periodontitis (Karna, Shin, Kim, & Kim, 2019; Kim et al., 2016; Shin et al., 2019). Kim et al (Kim et al., 2020) have recently used MMP9 and S100A8 in combination to develop a test for diagnosis and prognosis of periodontitis. A1AGP is principally synthesised in the liver but can also be produced by neutrophils. It is anti-inflammatory and can bind small molecules including LPS and drugs (Huang & Ung, 2013). It has been shown to be increased in obese patients with severe periodontitis compared with obese patients with mild or moderate periodontitis (Shin et al., 2019). Pyruvate kinase is the enzyme responsible for the last step of glycolysis; however, it has also been shown to have other non-canonical nuclear and extracellular functions (Alves-Filho & Palsson-McDermott, 2016; Hsu & Hung, 2018). Pyruvate kinase has not been highlighted as a marker for periodontitis before. All of the highlighted proteins have the potential to be produced by the most abundant immune cell that travels through the periodontium in response to the dental biofilm: neutrophils. Neutrophil abundance in the periodontium has been explored using histochemistry of biopsies (Thorbert-Mros, Larsson, & Berglundh, 2015) and the implication of their presence is that there is more tissue destruction due to their activity within the tissues leading to bystander damage which further exacerbates the inflammatory response (Matthews, Wright, Roberts, Cooper, & Chapple, 2007; Roberts et al., 2015). Furthermore, this study additionally included saliva from edentulous patients. Previously elastase activity in edentulous patients has been found to be negligible (Uitto, Nieminen, Coil, Hurttia, & Larjava, 1996) further suggesting the role of neutrophils as a source of the biomarkers outlined in this study. This provides biologically plausible mechanisms for their presence in both GCF and saliva.

This was a two-centre study with 190 patients involving healthy donors and those with gingivitis and periodontitis. In addition, edentulous patients were included as non-GCF producing controls. It can be seen from Figure 3 that the edentulous patients consistently locate with healthy patients, which may be because they do not produce GCF and thus any inflammatory markers sourced from GCF could not contribute to saliva composition. Furthermore, periodontitis patients were treated per protocol and saliva biomarker levels measured post-therapy. However, biomarkers were selected based upon their

ability to discriminate between the defined health and diseases states and not as prediction tools for treatment efficacy, which warrants further study. Nevertheless, the analysis described here highlights that biomarker changes following periodontitis treatment created profiles better matched to milder pre-treatment disease states.

To generate sensitivity and specificity for the data presented, maximal accuracy was used to determine the cut off or threshold. This approach was chosen to minimise the misclassification of periodontitis samples. A different approach, which is very common, is to use Youden's Index. Youden's Index gives equal weighting to sensitivity and specificity which can also be expressed as equal weighting for false positives and false negatives (Shapiro 1999). This gave a greater false negative rate which would prevent some periodontitis patients from being classified correctly (supplemental figure 10). Further analysis of the utility of these cut offs will need to be tested in a further data set in the future.

There are some limitations associated with our study: proteomic and immuno-detection methods target total protein rather than enzyme activity, which may be more appropriate for MMP9 and PK. For MMP8 the detection of the active form has been used to detect periodontitis (Raisanen et al., 2018). There were minor differences between the threshold case definition criteria for the two cohorts, however, the four conditions (health, gingivitis, mild/moderate and advanced periodontitis) were tightly defined to ensure no risk of phenotype cross-over between each health/disease state. Although there were the slight differences in the recruitment to the healthy cohorts both were very healthy with less than 10% bleeding on probing making them as close to pristine health as was possible; and for the gingivitis group the Newcastle gingivitis donors had a mean percentage of bleeding on probing of 28.2% with 95% confidence intervals of 23.9-32.5% meaning that they were all clear gingivitis cases. This study was undertaken prior to the 2017 international classification system and whilst clinical phenotypes were robustly defined to avoid case cross-over, they do not match exactly to the stages defined in the 2017 system. It was necessary to emphasise current disease

activity, rather than historical attachment loss by placing a greater emphasis upon the presence of bleeding upon probing and probing pocket depth. However, our criteria do map to stage I/II for mild/moderate periodontitis and stages II/IV for severe/very severe periodontitis and outcomes therefore can be translated accordingly.

The concept of including both clinical and biological data into disease classification is embedded within the 2017 classification system, in order to future-proof for the emergence of predictive biomarkers. Further work will be needed to translate the findings from this study to pragmatic solutions that can be used in near-patient saliva tests to assist in the early detection of periodontal disease. In the future there is a need to evaluate such biomarker panels in studies with community dwelling non-stratified cohorts to further validate the panels described.

References

Alves-Filho, J. C., & Palsson-McDermott, E. M. (2016). Pyruvate Kinase M2: A Potential Target for Regulating Inflammation. *Front Immunol, 7*, 145. doi:10.3389/fimmu.2016.00145

Barr, G., Dong, W., & Gilmore, C. J. (2009). PolySNAP3: a computer program for analysing and visualizing high-throughput data from diffraction and spectroscopic sources. *Journal of Applied Crystallography, 42*, 965-974.

Bostanci, N., & Bao, K. (2017). Contribution of proteomics to our understanding of periodontal inflammation. *Proteomics, 17*(3-4). doi:10.1002/pmic.201500518

Chapple, I. L., Bouchard, P., Cagetti, M. G., Campus, G., Carra, M. C., Cocco, F., . . . Schulte, A. G. (2017). Interaction of lifestyle, behaviour or systemic diseases with dental caries and periodontal diseases: consensus report of group 2 of the joint EFP/ORCA workshop on the boundaries between caries and periodontal diseases. *J Clin Periodontol, 44 Suppl 18*, S39-S51. doi:10.1111/jcpe.12685

Davis, I. J., Jones, A. W., Creese, A. J., Staunton, R., Atwal, J., Chapple, I. L., . . . Grant, M. M. (2016). Longitudinal quantification of the gingival crevicular fluid proteome during progression from gingivitis to periodontitis in a canine model. *J Clin Periodontol, 43*(7), 584-594. doi:10.1111/jcpe.12548

Ebersole, J. L., Nagarajan, R., Akers, D., & Miller, C. S. (2015). Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Front Cell Infect Microbiol, 5*, 62. doi:10.3389/fcimb.2015.00062

Feres, M., Retamal-Valdes, B., Faveri, M., Duarte, P., Shibli, J., Soares, G. M. S., . . . Doyle, H. (2020). Proposal of a Clinical Endpoint for Periodontal Trials: The Treat-to-Target Approach. *J Int Acad Periodontol, 22*(2), 41-53.

Ghallab, N. A. (2018). Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva of periodontal diseases: Review of the current evidence. *Arch Oral Biol, 87*, 115-124. doi:10.1016/j.archoralbio.2017.12.022

Grant, M. M., Creese, A. J., Barr, G., Ling, M. R., Scott, A. E., Matthews, J. B., . . . Chapple, I. L. (2010). Proteomic analysis of a noninvasive human model of acute inflammation and its resolution: the twenty-one day gingivitis model. *J Proteome Res, 9*(9), 4732-4744. doi:10.1021/pr100446f

Grassl, N., Kulak, N. A., Pichler, G., Geyer, P. E., Jung, J., Schubert, S., . . . Mann, M. (2016). Ultra-deep and quantitative saliva proteome reveals dynamics of the oral microbiome. *Genome Med, 8*(1), 44. doi:10.1186/s13073-016-0293-0

Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol, 15*(1), 30-44. doi:10.1038/nri3785

Hsu, M. C., & Hung, W. C. (2018). Pyruvate kinase M2 fuels multiple aspects of cancer cells: from cellular metabolism, transcriptional regulation to extracellular signaling. *Mol Cancer, 17*(1), 35. doi:10.1186/s12943-018-0791-3

Huang, Z., & Ung, T. (2013). Effect of alpha-1-acid glycoprotein binding on pharmacokinetics and pharmacodynamics. *Curr Drug Metab, 14*(2), 226-238.

Jaedicke, K. M., Taylor, J. J., & Preshaw, P. M. (2012). Validation and quality control of ELISAs for the use with human saliva samples. *J Immunol Methods, 377*(1-2), 62-65. doi:10.1016/j.jim.2012.01.010

Karna, S., Shin, Y. J., Kim, S., & Kim, H. D. (2019). Salivary S100 proteins screen periodontitis among Korean adults. *J Clin Periodontol, 46*(2), 181-188. doi:10.1111/jcpe.13059

Kassebaum, N. J., Bernabe, E., Dahiya, M., Bhandari, B., Murray, C. J., & Marcenes, W. (2014). Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. *J Dent Res, 93*(11), 1045-1053. doi:10.1177/0022034514552491

Kassebaum, N. J., Smith, A. G. C., Bernabe, E., Fleming, T. D., Reynolds, A. E., Vos, T., . . . Collaborators, G. B. D. O. H. (2017). Global, Regional, and National Prevalence, Incidence, and Disability-Adjusted Life Years for Oral Conditions for 195 Countries, 1990-2015: A Systematic Analysis

for the Global Burden of Diseases, Injuries, and Risk Factors. *J Dent Res, 96*(4), 380-387. doi:10.1177/0022034517693566

Kc, S., Wang, X. Z., & Gallagher, J. E. (2020). Diagnostic sensitivity and specificity of host-derived salivary biomarkers in periodontal disease amongst adults: Systematic review. *J Clin Periodontol, 47*(3), 289-308. doi:10.1111/jcpe.13218

Kebschull, M., Guarnieri, P., Demmer, R. T., Boulesteix, A. L., Pavlidis, P., & Papapanou, P. N. (2013). Molecular differences between chronic and aggressive periodontitis. *J Dent Res, 92*(12), 1081-1088. doi:10.1177/0022034513506011

Kim, H. D., Kim, S., Jeon, S., Kim, S. J., Cho, H. J., & Choi, Y. N. (2020). Diagnostic and Prognostic ability of salivary MMP-9 and S100A8 for periodontitis. *J Clin Periodontol*. doi:10.1111/jcpe.13349

Kim, H. D., Shin, M. S., Kim, H. T., Kim, M. S., & Ahn, Y. B. (2016). Incipient periodontitis and salivary molecules among Korean adults: association and screening ability. *J Clin Periodontol, 43*(12), 1032-1040. doi:10.1111/jcpe.12607

Kinane, D. F., Attstrom, R., & European Workshop in Periodontology group, B. (2005). Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *J Clin Periodontol, 32 Suppl 6*, 130-131. doi:10.1111/j.1600-051X.2005.00823.x

Lobene, R. R., Weatherford, T., Ross, N. M., Lamm, R. A., & Menaker, L. (1986). A modified gingival index for use in clinical trials. *Clin Prev Dent, 8*(1), 3-6.

Loe, H. (1967). The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol, 38*(6), Suppl:610-616. doi:10.1902/jop.1967.38.6.610

Matthews, J. B., Wright, H. J., Roberts, A., Cooper, P. R., & Chapple, I. L. (2007). Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clin Exp Immunol, 147*(2), 255-264. doi:10.1111/j.1365-2249.2006.03276.x

Meyle, J., & Chapple, I. (2015). Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000, 69*(1), 7-17. doi:10.1111/prd.12104

O'Leary, T. J., Drake, R. B., & Naylor, J. E. (1972). The plaque control record. *J Periodontol, 43*(1), 38. doi:10.1902/jop.1972.43.1.38

Raisanen, I. T., Heikkinen, A. M., Siren, E., Tervahartiala, T., Gieselmann, D. R., van der Schoor, G. J., . . . Sorsa, T. (2018). Point-of-Care/Chairside aMMP-8 Analytics of Periodontal Diseases' Activity and Episodic Progression. *Diagnostics (Basel), 8*(4). doi:10.3390/diagnostics8040074

Rizal, M. I., Soeroso, Y., Sulijaya, B., Assiddiq, B. F., Bachtiar, E. W., & Bachtiar, B. M. (2020). Proteomics approach for biomarkers and diagnosis of periodontitis: systematic review. *Heliyon, 6*(6), e04022. doi:10.1016/j.heliyon.2020.e04022

Roberts, H. M., Ling, M. R., Insall, R., Kalna, G., Spengler, J., Grant, M. M., & Chapple, I. L. (2015). Impaired neutrophil directional chemotactic accuracy in chronic periodontitis patients. *J Clin Periodontol, 42*(1), 1-11. doi:10.1111/jcpe.12326

Sanz, M., Del Castillo, A. M., Jepsen, S., Gonzalez-Juanatey, J. R., D'Aiuto, F., Bouchard, P., . . . Wimmer, G. (2020). Periodontitis and Cardiovascular Diseases. Consensus Report. *Glob Heart, 15*(1), 1. doi:10.5334/gh.400

Shapiro, D.E. The interpretation of diagnostic tests. (1999) *Stat Methods Med Res*. 1999 8(2): 113-34. doi: 10.1177/096228029900800203

Shin, M. S., Kim, Y. G., Shin, Y. J., Ko, B. J., Kim, S., & Kim, H. D. (2019). Deep sequencing salivary proteins for periodontitis using proteomics. *Clin Oral Investig, 23*(9), 3571-3580. doi:10.1007/s00784-018-2779-1

Sorsa, T., Gieselmann, D., Arweiler, N. B., & Hernandez, M. (2017). A quantitative point-of-care test for periodontal and dental peri-implant diseases. *Nat Rev Dis Primers, 3*, 17069. doi:10.1038/nrdp.2017.69

Taylor, J. J. (2014). Protein biomarkers of periodontitis in saliva. *ISRN Inflamm, 2014*, 593151. doi:10.1155/2014/593151

Team., R. C. (2020). R: A language and environment for statistical computing. . *R Foundation for Statistical Computing, Vienna, Austria.*

Thorbert-Mros, S., Larsson, L., & Berglundh, T. (2015). Cellular composition of long-standing gingivitis and periodontitis lesions. *J Periodontal Res, 50*(4), 535-543. doi:10.1111/jre.12236

Uitto, V. J., Nieminen, A., Coil, J., Hurttia, H., & Larjava, H. (1996). Oral fluid elastase as an indicator of periodontal health. *J Clin Periodontol, 23*(1), 30-37. doi:10.1111/j.1600-051x.1996.tb00501.x

Vernekar, N., Batchelor, P., & Heilmann, A. (2019). Adult self-reported attendance for dental check-ups over a 16-year period in the UK. *Br Dent J, 226*(11), 883-888. doi:10.1038/s41415-019-0366-8