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Predicted salivary human protease activity in experimental gingivitis revealed by endoProteo-FASP approach

Dimitri Mulkern 1,†, Amy Hewitt 1,†, Hadyn Parker 1,2, Joanna Batt 1, Zehra Yonel 1 and Melissa M. Grant 1

[1] Periodontal Research Group, School of Dentistry, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, B5 7EG

[2] School of Life Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom

[†] These authors contributed equally

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Corresponding author:

Melissa Grant

Periodontal Research Group, School of Dentistry, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, B5 7EG

Telephone: +44 121 446 5520

Email: m.m.grant@bham.ac.uk

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Abstract: Gingivitis is a highly prevalent oral condition that can be studied in humans via the 21-day experimental gingivitis model that allows for investigations into induction and resolution of gingivitis. In this study we used the autolysis of saliva as a source of peptides to predict the activity of human proteases in saliva during induction and resolution of inflammation. Healthy volunteers, with no remarkable oral or systemic conditions, were recruited into the study and stimulated saliva samples were collected at days 0, 21 and 35 of experimental gingivitis. Plaque and gingival indices were recorded to ensure clinical induction and resolution. Saliva was auto-digested at 37°C for 18 h before identification of peptides by mass spectrometry. Protease prediction was carried out using Proteasix *in silico* with the identified peptides. Comparison of day 0 to days 21 and 35 showed changes in predicted protease activity. Correlation network analysis revealed that at day 21 the proteases became less connected and showed a potential for a dysregulated system; by day 35 the connectivity was returning towards similar conditions at day 0. This study demonstrates that changes in predicted proteases are apparent even in saliva collected from donors experiencing inflammation at three teeth.

Key words: protease, saliva, gingivitis

Contact author:

Melissa Grant,

Periodontal Research Group, School of Dentistry, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, B5 7EG

Telephone: +44 121 446 5520

m.m.grant@bham.ac.uk

Periodontal disease is a very common inflammatory condition found worldwide (1). In its severest form, severe periodontitis, it affects over 10% of the population (1) and is a major cause of tooth loss. However the mild inflammation, gingivitis, is experienced by 90% of the population (2) and is easily reversible with proper tooth brushing and removal of dental plaque from the tooth surface. The mouth is bathed in saliva and this fluid is rich in antimicrobial defenses such as proteins and proteases. During inflammation the composition of saliva changes (3); this is due to changes originating from the salivary glands (4) and also from the gingival tissues (5). The periodontal tissues adjacent to the plaque covered tooth surface become inflamed and this will stimulate production of local mediators, such as cytokines and chemokines, which in turn drive recruitment of leukocytes, particularly neutrophils. Fluid from these tissues enters into the mouth and thereby saliva via the gingival crevice and gingival crevicular fluid (GCF).

Induction of gingivitis in the 21-day experimental gingivitis model allows for insights to be gained into human inflammation and resolution of inflammation in the oral cavity, a key mucosal surface. The first publication (6,7) of this model was in 1965 and since then it has been used to explore a wide variety of influences on inflammation, such as use of particular toothpastes (8-10). The composition of GCF has been extensively investigated for individual molecules (5,11-14) or for overarching changes using 'omic' technologies (3,15). Less information has been gathered about the changes in saliva (16) as often the model is used with only a few selected teeth being shielded from brushing, thereby allowing for contralateral teeth to be used in comparison.

There has been abundant interest in the role of proteases in periodontal disease. The induction of an inflammatory response requires proteases to facilitate access of leukocytes to the site of infection. The protease network is crucial for effective responses against infection, where swift deactivation of microbial proteins or activation of host proteins by proteases can be critical, but also has a key role in wound healing (17). They are essential instigators and executioners of responses and dysregulation may lead to excessive or non-resolving inflammation. With the experimental gingivitis model the change in abundance of cathepsins (6,18), elastase (5) and matrix metalloproteases (MMPs) (10,12) have been investigated.

There are more than 500 proteases (19) encoded in the human genome, making them the largest family of proteins (19), and study of their presence or activity requires a system-wide approach through degradomics. The prediction of protease sites requires knowledge of the substrates and products of protease action. There has been extensive research in this area and numerous databases exist that provide resources on proteolytic events (e.g., TopFIND (20), MEROPS (21), CutDB (22)). KLEIN *et al*. (23) developed the Proteasix tool to provide a method to predict protease activity from peptides derived from mass spectrometry data and TRINDADE *et al*. (24) developed the endoProteo-FASP technique to explore the protease activity in fluid samples,

such as saliva. Using the filter aided sample preparation (FASP) technique developed by WIŚNIEWSKI *et al*. (25), TRINDADE *et al.* (24) made use of the endogenous proteolytic activity of saliva samples to generate peptides that were then searched using a no enzyme search. This dramatically increases search time due to the increase in search complexity but can yield information about the non-traditional proteomics peptides in the sample. TRINDADE *et al.* explored saliva from chronic periodontitis patients vs controls donors (26) and also two healthy male donors (24). They demonstrated that degradomes were generated by autolysis of saliva over 18 or 115h at 37°C and that these peptides mapped across a wide range of proteins known to be present in saliva, including those not normally identified using trypsin mediated proteomics and that protease activity could be predicted using the beta version of Proteasix. In chronic periodontitis (26) patient samples they showed that there was more predicted activity of cathepsin L and Meprin 1a but lower activities predicted for MMP12, MMP7, MMP9 and Presequence protease (PREP or PITRM1) at 115h. TRINDADE *et al.* (24) also used zymography to explore the detectable protease activity. This complementary technique separates proteins in the sample by non-reducing SDS PAGE using gelatin (or other protein) containing acrylamide gels. After electrophoresis the separated proteins are allowed to renature and if protease activity is present that can degrade gelatin areas of gel with this activity will not be stained by conventional protein staining techniques, such as Coomassie blue staining. This is due to the degradation of the gelatin incorporated within the acrylamide gel: the rest of the gel stains due to the presence of the gelatin. TRINDADE *et al.* (24) demonstrated different levels of protease activity via gelatin zymography in chronic periodontitis patients.

In this study the aim was to explore, in a small number of saliva donors, the protease activity of saliva samples donated during the induction and resolution of inflammation in experimental gingivitis. The endoProteo-FASP technique was used to explore holistically the activity of proteases and shed light on the interconnected functions of proteases. This would not be possible with techniques that try to target individual proteases.

Material and Methods

Recruitment and protocol

The experimental gingivitis model previously described by CHAPPLE *et al.* (27) was used, and the study was approved by the South Birmingham Local Research Ethical Committee (LREC 2004/074). Nine non-smoking volunteers (mean age 20 yr; range 19–22, 3 males and 6 females) who had unremarkable medical histories, no known history of periodontal disease, were not undergoing orthodontic or prosthodontic appliance therapy or taking medication that may have affected results, were enrolled into the study, and informed consent was obtained. To induce gingivitis participants shielded three teeth on the opposite side from their dominant hand during brushing. These sites were the maxillary left 4, 5, and 6 (first and second premolars and first molar on the left-hand side of the upper jaw) in right-handed individuals (n = 8) and the equivalent teeth on the maxillary right side in left-handed individuals (n = 1). Contralateral sites were monitored at the corresponding teeth on the contralateral side to ensure induction and resolution of inflammation. Plaque accumulation was assessed using the Turesky modification of Quigley and Hein index (28) (PI; scores 0–5) and the gingival index (GI) of Löe (29) (scores 0–3) was used to measure gingival inflammation. A soft vinyl splint was constructed to cover five teeth, with a 5 mm clearance at each free surface from the marginal tissues. The splint was inserted gently over test teeth before brushing, to ensure against any mechanical or chemical cleaning. Participants were also asked to refrain from using mouth rinse, chewing gum, interdental cleaning aids (floss / brushes) for the duration of inflammation induction between day 0 and 21. The teeth were given a thorough prophylaxis and oral hygiene was closely monitored for 2 wk prior to the study commencing to ensure pristine gingival health at baseline. On day 21 volunteers were given a full mouth prophylaxis and recommenced brushing without using the splint, in order to resolve the experimentally induced inflammation at test sites. Fourteen days later (day 35), the final set of clinical indices was recorded.

Saliva collection

At days 0, 21 and 35 each participant donated a stimulated saliva sample. Samples were collected between 9-11am on the day of collection and participants were requested not to eat or drink 2 h prior to donation. Participants were asked to rinse with water 10 min prior to donation. This sample was stimulated by rolling a sterile marble around the mouth for 5 min; saliva was expectorated into a tube placed on ice throughout the collection time. The saliva was centrifuged at 1000 ***g*** for 10 min to remove any particulate material and then frozen at -80°C until use.

Saliva processing

On defrosting saliva was kept on ice. Protein quantity was measured using bicinchoninic acid assay (30). Saliva samples containing 150 µg protein were aliquoted into a 30 kDa molecular weight cutoff filter (Amicon; Merck-Millipore, Watford, UK) and the procedure for endoProteoFASP based on TRINDADE *et al.* (24) was followed. Briefly (Fig. S1), the saliva proteins in the Amicon filter were centrifuged at 14,000 ***g*** for 10 min and then ammonium bicarbonate (500µl, 50mM) was added to the filter. The filter was centrifuged again and the ammonium bicarbonate (500µl, 50mM) was replaced. This was repeated for a total of five times. After the fifth wash the Amicon filter was filled with ammonium bicarbonate (500µl, 50mM) and the filter was sealed with Parafilm and incubated at 37°C for 18 h. The resulting peptides were collected by centrifuging the Amicon filter at 14,000 ***g*** for 10min and then washing the filter, as before, four times with ammonium bicarbonate (500µl, 50mM). These five filtrates were combined, trifluoroacetic acid (400µl, 0.1%) was added and they were dried by vacuum centrifugation. Samples were stored at -80°C before analysis. Treatment of aliquots of saliva samples with dithiothreitol (20mM) at 100°C for 45 min completely inhibited proteolytic activity, as previously described (24).

Zymogram technique

Saliva (10 µg) was mixed with non-reducing SDS sample buffer (Thermo Fisher, UK) and loaded (without reduction or heating) onto Novex 10% Zymogram Plus (1 % Gelatin) Protein gel (Thermo Fisher, Gloucester, UK). Samples were electrophoresed for 90 min at 100V before incubation in Novex Renaturating buffer (30 min, Thermo Fisher). The gels were then incubated in Novex Zymogram Developing buffer (Thermo Fisher) for 30 min before Novex Zymogram Developing buffer was exchanged for fresh Novex Zymogram Developing buffer and the gels were incubated for a further 18 h at 24 °C. Gels were then rinsed (3x 5min) in distilled water before being immersed in to Imperial Blue protein stain (Expedeon, Harston, UK) for 1 h. Gels were destained for 30 min before imaging on a GS800 imaging system (BioRad, Watford, UK). Images were then processed with Image J (31).

Gel band protein processing

For identification of proteins in the bands from the zymogram gels bands not stained with Imperial Blue were excised and processed for mass spectrometry following in gel digestion as previously described (32).

Mass spectometry

UltiMate 3000 HPLC series (Dionex, Sunnyvale, CA, USA) was used for peptide concentration and separation. Samples were trapped on µPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 um, 100Å 300µm i.d. x 5mm (Dionex) and separated in Nano Series Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex). The gradient used was from 3.2% to 24% solvent B (0.1% formic acid in acetonitrile) for 30 min and then increased to 60% in 15 min. The column was washed with 80% solvent B for 15 min and equilibrated with 3.2% solvent B for another 15 min. The total run time was 75 min. Peptides were eluted directly (~ 350 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences, Ithaca, NY, USA) into a QExactive HF (QEHF) mass spectrometer (Thermo Fisher Scientific, Gloucester, UK). The data-dependent scanning acquisition was controlled by Xcalibur 4.0 software (Thermo Fisher Scientific, Gloucester, UK). The mass spectrometer alternated between a full FT-MS scan (m/z 375 – 1600) and subsequent high energy collision dissociation (HCD) MS/MS scans of the 20 most abundant ions. Survey scans were acquired in the QEHF with a resolution of 120 000 at m/z 200 and automatic gain control (AGC) $3x10^6$. Precursor ions were fragmented in HCD MS/MS with resolution set up at 60,000 and a normalized collision energy of 32. ACG target for HCD MS/MS was $1x10^5$. The width of the precursor isolation window was 1.2 m/z and only multiply-charged precursor ions were selected for MS/MS. Spectra were acquired for 60 min with dynamic exclusion time of 20s. The MS and MS/MS scans were searched against Uniprot database using Proteome Discoverer 2.2 (ThermoFisher Scientific) and the Sequest algorithm with a 1% false discovery rate (FDR) for both peptide and protein criteria and controlled by Percolator. The precursor mass tolerance was 10 ppm and the MS/MS mass tolerance was 0.02 Da. For saliva samples prepared by EndoProteoFASP no enzyme restriction was used during searching and only oxidation (M) and N terminal acetylation were set as variable modifications. For in gel digested samples trypsin enzyme was used and oxidation (M) and N terminal acetylation were set as variable modifications and cysteine carbamidomethylation on lysine and N-terminal as fixed modifications. Data have been deposited in PRIDE with accession PXD015121.

Data processing

Data files from Proteome Discoverer were used to search the Proteasix (23) database via the web tool (found at http://www.proteasix.org/). As Proteasix returns all possible proteases at a cleavage site the data were filtered for the most probable protease per peptide terminus. To generate networks graphs Pearson correlation coefficient matrices were determined by Prism (v 6.0) (Graphpad, San Diego, CA, USA) and networks were visualised by Fruchterman-Reingold algorithm

using Gephi (v 0.9.2) (33). Data for networks were selected as having p values of $p<0.001$. Statistical analysis was carried out using GraphPad Prism v 6.

Results

The nine volunteers enrolled in the 21-day experimental gingivitis study provided saliva at days 0, 21 and 35. At these time points the accumulation of plaque and induction of inflammation was recorded at the three shielded teeth where plaque was expected to accumulate and the three contralateral unshielded teeth. Fig. 1 shows that there was an accumulation of plaque at day 21 and that this was accompanied by an increase in inflammation as measured by the GI. Recommencing of oral hygiene measures at the shielded teeth decreased the amount of plaque and inflammation, indicating a resolution of inflammation. The saliva flow rate at each day was 0.98 (SD 0.2) ml/min on day 0; 0.87 (SD 0.3) ml/min on day 21; and 0.95 (SD 0.3) ml/min day 35. Additionally the protein content was measured at each day: 1.66 (SD 0.8) mg/ml on day 0, 1.73 (SD 0.8) mg/ml on day 21, and 1.66 (SD 0.5) mg/ml on day 35. No significant differences were found be one way ANOVA for either measurement.

Using the endoProteo-FASP technique saliva samples from the volunteers were auto-digested for 18 h at 37°C. The resulting peptides were analysed by mass spectrometry. 784 proteins were identified by a total of 73,334 peptides. The sequences were used to predict the proteases active in each of the samples using Proteasix. The profile of peptide signatures is shown in Fig. 2. Fifty-six proteases (Table 1 shows all identities, protease name abbreviations and accession numbers) were found in total. Many were at a very low level as seen in Fig. 2. Additionally, it can be seen that the detection of protease generated peptides changes from day 0; there is wide variation between the different proteases; however, statistical significance can be found between day 0 and day 35 for calpain subunit 2 (CAPN2), cathepsin S (CTSS), neutrophil elastase (ELANE) and MMP7; and between day 21 and 35 for MMP7. 36 proteases were found in 80% or more of the samples; 19 were found in all samples. Of these 36 predicted proteases 15 were identified in the LC-MS/MS data (Table 2).

Generation of correlation networks (Fig. 3) for each day showed that the network of proteases was highly connected at day 0 but that the network fractured into smaller modules (at p value threshold $p<0.001$) at day 21. By day 35 the network was more connected again but not to the same extent as at day 0.

To further investigate the activity of proteases in saliva from the volunteers undergoing 21-days of experimental gingivitis development and a further 14 days of resolution, the saliva samples were used with gelatin zymography. Bands of activity were visualized consistently at >175 kDa (band A), approximately 90 kDa (band B) and approximately 75 kDa (band C). There were also

occasional donor samples with activity at approximately 40 kDa (band D, n=3 donors only). An example gel is shown in Fig. 4. The band area was measured and the resulting data is shown in Fig. 4: bands A, B and C show some increases at day 21 and decreases at day 35, whereas band D shows greater variability. Overall, there was a definite variation associated with individual donors, when considering all the bands together. This appears to confirm the changes seen by the peptide analysis. The bands were additionally excised from the gels and in gel digested with trypsin to identify the proteases present. The results demonstrated the presence of MMP9 in bands A, B and C but not in D.

Discussion

In this study the activity of a network of proteases has been investigated using a proteomics (endoProteo-FASP) (24) and bioinformatics (Proteasix) (23) approach. In total 56 proteases showed some predicted activity. The proteases with the most activity (contributing to over 73% of the activity and at least 100 peptides each on average per sample) were calpain, cathespin L, S, K and G, pepsin A3, matrix metalloproteinase (MMP) 7, and elastase. Previously, TRINDADE *et al.* (24) and Sun *et al.* (34) have looked at the wide range of proteases activity in saliva samples. Sun *et al.* (34), using histatin containing zymograms detected 13 proteases, 8 of which were also detected in this study. When comparing data presented here and that from TRINDADE *et al*, TRINDADE *et al.* (26) predicted 66 proteases to be active in saliva using Proteasix. These matched to 45 of the proteases predicted in our dataset and there were a further 11 not in their data. This may be due to changes in the Proteasix algorithm since their publication. Indeed, the study presented here also found very similar patterns of zymogen activity on gelatin gels as compared to TRINDADE *et al.* (26) as well. The bands excised from the zymograms revealed MMP9 to be found in all but the lowest molecular weight band (approximately 45 kDa); as MMP9 is reported to be at least 78kDa this suggests that it is present either alone or in a complex, perhaps with an inhibitor or other proteins. The lower band (band D) could be associated with activity from one of the other smaller proteases predicted.

Thirty-six proteases were found in more than 80% of the samples. These proteases were examined further for their association with induction and resolution of inflammation in experimental gingivitis. No overt pattern could be seen with the data available here. This could be due to inter-individual variation in the responses to the accumulation of plaque and quantity of inflammation induced in the donors. TROMBELLI *et al.* (35) identified two groups responding to plaque induced inflammation whilst undergoing experimental gingivitis: low-responders and high-responders. The low responders displayed lower clinical measures of inflammation (gingival index and GCF volume) even though they accumulated similar levels of plaque. OFFENBACHER *et al.* (11), examining 21 day gingivitis GCF samples, adopted a hierarchical clustering technique based on changes in inflammatory mediator levels. Using this approach they saw associations between increased MMP-8 and MMP-9 levels (not activities) in group IC1, decreases in MMP-1 in group IC2 and decreases in MMP-1, MMP-3 and MMP-13 in group IC3. These were achieved by taking into account other mediators. Recently, NASCIMENTO *et al.* (36) identified slow and fast responders to plaque induced inflammation whilst undergoing experimental gingivitis. Further investigation by NASCIMENTO *et al.* (37) measured the salivary levels of MMP-8, MPO and TIMP-1 and suggested that the faster responders had greater neutrophil involvement. A larger number of participants in

future studies may help with identification of these kinds of groups using the methods presented in this study.

The 36 proteases predicted in more than 80% of the samples were used to create correlation networks and explore the interconnectivity of their activity. Each network, created for days 0, 21 and 35, demonstrated fairly sparse networks. At day 0 the network was highly connected; however, at day 21 there was a change, showing apparent removal of the network structure. At day 35 the network was once again more connected but not to the same extent as at day 0. Less connectivity in a network means that it is less flexible and there is less redundancy. In the case of these salivary protease networks this may indicate that the proteases are working more on their own and this may generate different peptides or effects. Further work on the peptides, for example predicting if they are antimicrobial (38, 39), could elucidate this further and could help drive development of therapeutics to target increases in antimicrobial peptides

The Proteasix tool was developed to allow for automatic retrieval of protease signatures from a variety of data sources including mass spectrometry peptide sequencing. This is the only tool of its type, though there are similarities with others that can predict substrate sequences or that store information about proteolytic events (20-22). The tool relies on an underlying cleavage site database that has been curated from information on proteases from the literature and other databases (23). These cleavage sites are represented by amino acid motifs of between 2-8 amino acids with a scissile bond between amino acids P1 and P1'. In particular the MEROPS (21) database has been used to generate probabilities, specificities and sensitivities associated between predicted protease and peptide. This is important as proteases are promiscuous and have pronounced overlaps in substrate recognition. FUCHS *et al.* (40) mapped the protease specificity of 61 proteases based on the similarity of their substrate sequences using sequence logos. They demonstrated a high number of less specific proteases and that these could be clustered together largely by catalytic type: for instance the MMPs clustered together and the caspases clustered together, however the cathepsins with their differing mechanisms were spread further apart. The Proteasix tool is set with high specificity (41), i.e., the percentage of sites correctly predicted not to be cleaved by a particular protease (see Table 2 for some examples). This goes a long way to ensure that the assignments are correct, though at the expense of sensitivity, the proportion of sites correctly predicted to be cleaved by a particular protease, in some cases. At present there is no automated tool for prediction of protease activity by bacterial proteases. Thus, it is difficult to take a wide approach to understanding subversion of the protease web by bacterial protease activity, however well-known proteases important in oral health such as gingipains from *Porphyromonas gingivalis*, karilysin from *Tannarella forsythia* and dentilisin from *Treponema*

denticola are listed in the MEROPS database and have sequence logos so it may be possible to examine data for specific bacterial proteases.

In previous research individual proteases or total activity have been investigated during 21 day experimental gingivitis models. Elastase presence, measured by ELISA, was found to increase from day 0 to 21 in GCF (5,14) and the activity of the enzyme was also shown to increase, by a factor of three even though the quantity of its inhibitors increased even further (5). SUN *et al.* (34) have also demonstrated the co-identification of proteases from human saliva with their protein inhibitors. Interestingly, they were using zymograms too and found proteases in multiple locations as we did for MMP9. Cathepsins B, L and G have also been detected by activity assays in GCF (13,18) with an increase across the 21-day experimental gingivitis course. MMPs have been extensively investigated in both GCF and saliva. In terms of experimental gingivitis MMP-1, -3, -7, -13, -8 and -9 were examined by multiplexed antibody based assay (Luminex) revealing little change across the 21 days of induction and 28 days of resolution of inflammation (11). The MMPs are particularly interesting as a lot of research evidence has been gathered around them and their use for the detection of periodontitis (see FRANCO *et al.* (42) and DE MORAIS *et al.* (43) for reviews). They work in a protease web to activate each other from their pro-forms and can work synergistically to breakdown the extracellular matrix. MMP-7 (44), MMP-8 (45) and MMP-9 (46) have been found in multiple forms: showing complexes, latent and active forms as well as the fragments released during activation. Here we have seen high predicted protease activity for MMP-7, a salivary MMP, whereas MMP-9 showed lower predicted activity and MMP-8 very low numbers of predicted generated peptides. These latter two are generated largely in the gingiva and may have much lower activities in saliva than for example GCF adjacent to these tissues. Furthermore, MMP-7 has a very different sequence logo compared to MMP-8 and MMP-9 (Table 3). Further research using dithiothreitol treated boiled saliva as a source of proteins and isolated MMPs may be able to give further evidence for the levels of protease activity and cross-over in identification. For total protease activity BIKKER *et al.* (47) reported the use of a fluorescence resonance energy transfer (FRET) assay. They could demonstrate higher total protease activity at day 21 and demonstrated that it remained elevated at day 35, 14 days after recommencement of oral hygiene.

The data presented here from protease prediction suggests little change in many proteases across the experimental gingivitis period, with some showing decreases on resolution of inflammation, whereas the zymogram data suggests an increase at day 21 compared to the other days. However, there is a wide variation in both. If it is possible to isolate individual activities of individual proteases from saliva in future experiments the similarities or disparities can be

explored in the future, bearing in mind the complex nature of the proteases present in terms of their specificities and that of the reporting assays.

One limitation of our study is that only three teeth underwent plaque accumulation, rather than the whole mouth as in the original experimental gingivitis set up. This will diminish the quantity of inflammation in the mouth as a whole; however, we have previously demonstrated that there are quantitative changes to the GCF protein composition under these conditions (3). This level of gingivitis may be similar to that seen in an individual consistently missing small areas within their mouth rather than not cleaning at all and could equate to similar levels of gingivitis in the wider population. That a small apparent change is seen, highlights that even mild changes in gingival inflammation could change the protease network and resilience.

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Conflicts of Interest statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of the article:

Fig. S1. Saliva processing for endoProteo-FASP protocol

Figure legends

Figure 1. Change in plaque index and gingival index over the course of the 21 day experimental gingivitis induction and resolution phases. Shielded side denotes the teeth covered with the removable splint to prevent brushing and contralateral side denotes teeth cleaned as normal and monitored for comparison.

Figure 2. Abundance of peptides detected with signatures for all proteases as predicted by Proteasix. Data represents all days and donors and is shown as mean+/- standard deviation. Data were analysed by oneway ANOVA. * $p<0.05$, ***$p<0.001$, ****$p<0.0001$

Figure 3. Correlation network graphs of each time point. A. day 0; B. day 21 and C. day 35. Size of node is proportional to the number of peptides predicted to be generated by that protease. Edges represent correlations with $p<0.001$.

Figure 4. Quantitation of bands found on zymograms. Representative example of zymogram, saliva samples (10ug) separated under non-reducing conditions on a 1% gelatin polyacrylamide gel. After renaturing the gel was developed for 18h and then stained with Coomassie Blue. m represents molecular weight markers, 7,8 and 9 represent the saliva donors and D0, D21 and D35 represent days 0, 21 and 35. A, B, C and D represent the bands of interest. Quantitation of all bands shown in labelled graphs. Box and whiskers plots: boxes represent 75th percentile with upper line, median with middle line and 25th percentile with lower line of box; whiskers represent upper and lower limits of all the data. Band D shown as individual data for the 3 individuals displaying this behavior. All bands shows summed data from each band for each individual.

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Table 1. Accession numbers and descriptions for all proteases predicted by Proteasix

Table 2. Proteases identified by peptides in mass spectrometry analysis

Table 1. Accession numbers and descriptions for all proteases predicted by Proteasix

Protein name	Accession number	Protein description
ADAMTS4	O75173	A disintegrin and metalloproteinase with thrombospondin motifs 4
BMP1	P13497	Bone morphogenetic protein 1
CAPN1	P07384	Calpain-1 catalytic subunit
CAPN2	P17655	Calpain-2 catalytic subunit
CASP1	P29466	Caspase-1
CASP 2	P42575	Caspase-2
CASP 6	P55212	Caspase-6
CASP7	P55210	Caspase-7
CASP 8	Q14790	Caspase-8
CMA1	P23946	Chymase
CTRC	Q99895	Chymotrypsin-C
CTSB	P07858	Cathepsin B
CTSD	P07339	Cathepsin D
CTSE	P14091	Cathepsin E
CTSG	P08311	Cathepsin G
CTSK	P43235	Cathepsin K
CTSL	P07711	Cathepsin L1
CTSS	P25774	Cathepsin S
ELANE	P08246	Neutrophil elastase
F2	P00734	Prothrombin
GZMA	P12544	Granzyme A
GZMB	P10144	Granzyme B
GZMK	P49863	Granzyme K
GZMM	P51124	Granzyme M
HPN	P05981	Serine protease hepsin
HTRA2	O43464	Serine protease HTRA2, mitochondrial
KLK3	P07288	Prostate-specific antigen
KLK4	Q9Y5K2	Kallikrein-4
KLK5	Q9Y337	Kallikrein-5
KLK6	Q92876	Kallikrein-6

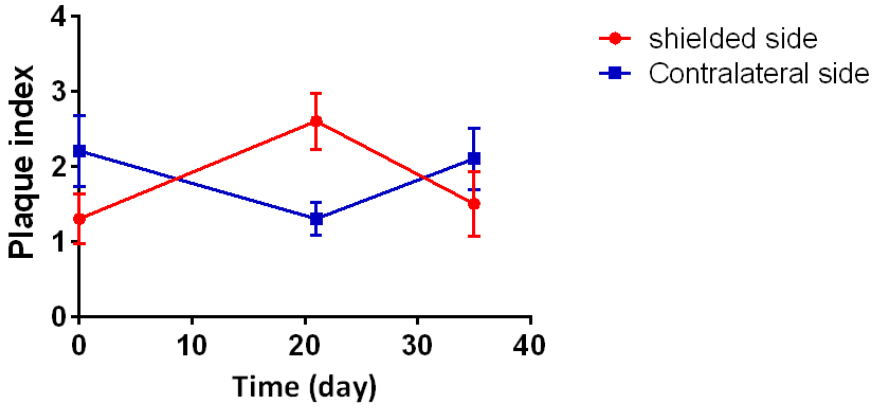
LGMN	Q99538	Legumain
MEP1A	Q16819	Meprin A subunit alpha
MME	P08473	Neprilysin
MMP1	P03956	Interstitial collagenase
MMP12	P39900	Macrophage metalloelastase
MMP13	P45452	Collagenase 3
MMP14	P50281	Matrix metalloproteinase-14
MMP2	P08253	72 kDa type IV collagenase
MMP25	Q9NPA2	Matrix metalloproteinase-25
MMP3	P08254	Stromelysin-1
MMP7	P09237	Matrilysin
MMP8	P22894	Neutrophil collagenase
MMP9	P14780	Matrix metalloproteinase-9
PCSK2	P16519	Neuroendocrine convertase 2
PCSK5	Q92824	Proprotein convertase subtilisin/kexin type 5
PGA3	P0DJD8	Pepsin A-3
PGC	P20142	Gastricsin
PITRM1	Q5JRX3	Presequence protease, mitochondrial
PLG	P00747	Plasminogen
PRTN3	P24158	Myeloblastin
ST14	Q9Y5Y6	Suppressor of tumorigenicity 14 protein
THOP1	P52888	Thimet oligopeptidase
TMPRSS11E	Q9UL52	Transmembrane protease serine 11E
TMPRSS6	Q8IU80	Transmembrane protease serine 6
TMPRSS7	Q7RTY8	Transmembrane protease serine 7
TRY3	P35030	Trypsin-3

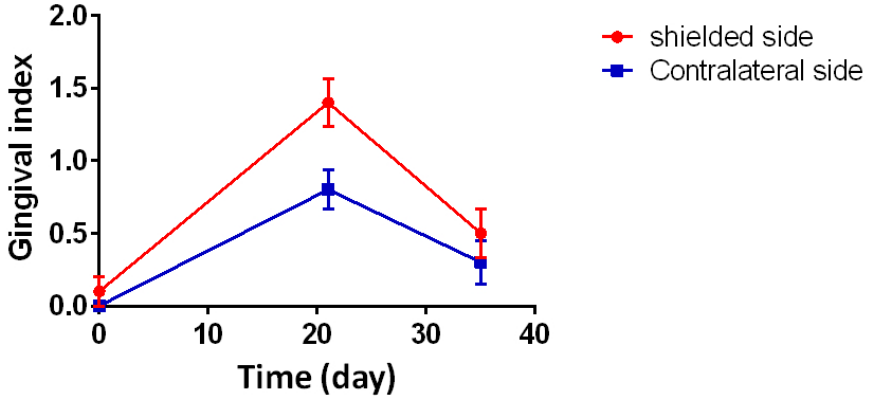
Table 2. Proteases identified by peptides in mass spectrometry analysis.

Protease symbol	Protease name	Protease UniProt Accession	Size (kDa)
ADAMTS4	A disintegrin and metalloproteinase with thrombospondin motifs 4	O75173	90
CTSD	Cathepsin D	P07339	45
CTSE	Cathepsin E	P14091	43
CTSG	Cathepsin G	P08311	29
ELANE	Neutrophil elastase	P08246	29
GZMM	Granzyme M	P51124	28
HTRA2	Serine protease HTRA2	O43464	49
KLK4	Kallikrein-4	Q9Y5K2	27
KLK6	Kallikrein-6	Q92876	27
MMP14	Matrix metalloproteinase-14	P50281	66
MMP7	Matrilysin	P09237	30
MMP9	Matrix metalloproteinase-9	P14780	78
PGA3	Pepsin A-3	P0DJD8	42
PLG	Plasminogen	P00747	91
TMPRSS7	Transmembrane protease serine 7	Q7RTY8	94

Table 3. Sequence logos for MMPs. Data extracted from MEROPS [21] (https://www.ebi.ac.uk/merops/). The letters represent the amino acid in that position, with lower case letters indicating lower specificity and capital letters demonstrating a higher specificity for that amino acid's presence. Hyphens represent any amino acid and the + represents the scissile bond (cleavage site). Amino acids P1 and P1' are to the left and right of the scissile bond respectively.

Matrix metalloproteinase	Sequence logo	Sensitivity of detection by Proteasix	Specificity of detection by Proteasix
MMP1	-/pa/-/-+li/-/-/-	31	98.45
MMP2	-/p/-/-+li/-/-/-	76	86.88
MMP3	-/p/ag/-+l/-/-/-	70	79.35
MMP7	-/pa/-/-+L/-/-/-	54	53.97
MMP8	g/pas/-/g+l/-/g/-	38	90.6
MMP9	g/pa/-/g+l/-/ga/-	63	63.68
MMP12	g/pag/ag/ga+l/-/ga/-	67	88.47
MMP13	g/P/-/g+l/-/ga/-	70	93.66
MMP14	-/p/-/-+l/-/-/-	75	80.66
MMP25	-/a/-/-+l/-/-/-	28	82.71





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80x91mm (300 x 300 DPI)

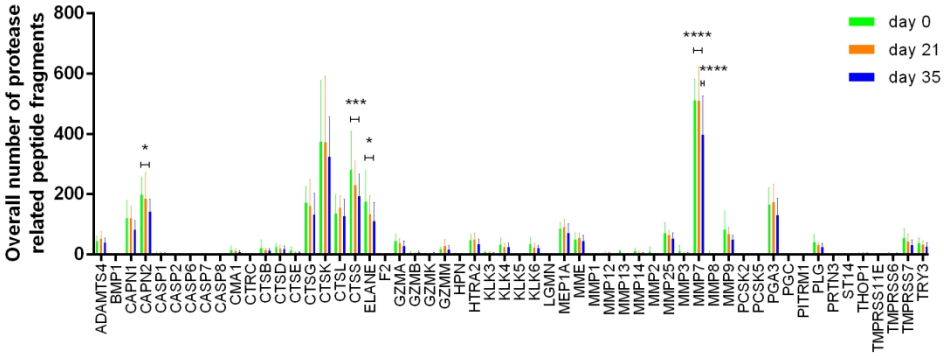

Figure 2. Abundance of peptides detected with signatures for all proteases as predicted by Proteasix. Data represents all days and donors and is shown as mean+/- standard deviation. Data were analysed by oneway ANOVA. * $p<0.05$, ***$p<0.001$, ****$p<0.0001$

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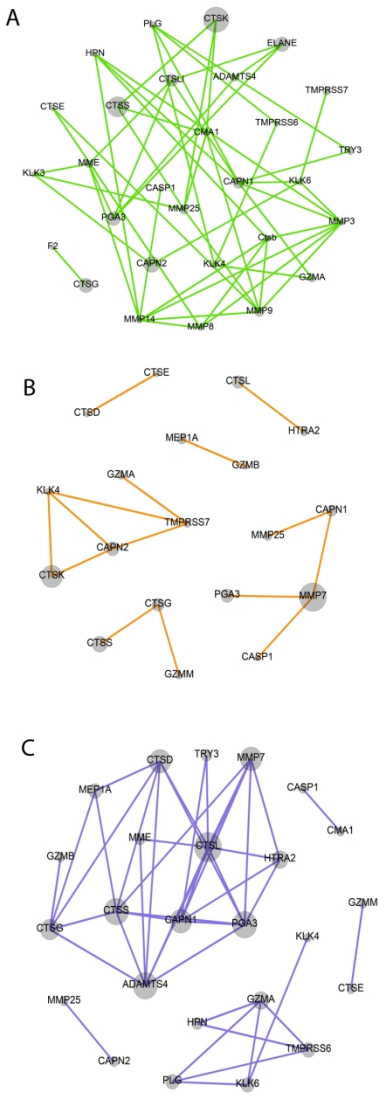

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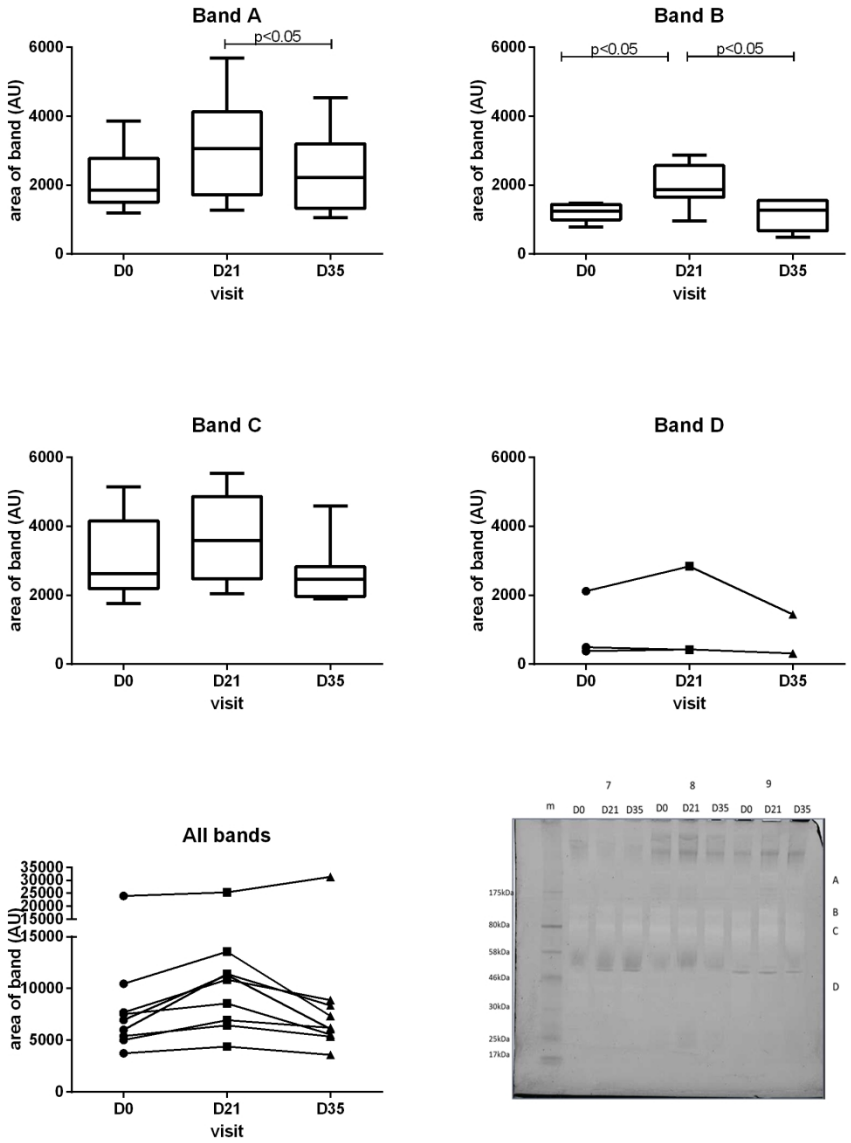

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170x217mm (300 x 300 DPI)

Supporting Information

Predicted salivary human protease activity in experimental gingivitis revealed by endoProteo-FASP approach

MULKERN D, HEWITT A, PARKER H, BATT J, YONEL Z, GRANT MM

School of Dentistry, University of Birmingham, Birmingham, United Kingdom

School of Life Sciences, University of Warwick, Coventry, United Kingdom

Fig. S1. Saliva processing for endoProteo-FASP protocol