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Pressure cycling technology-assisted mass spectrometric quantification of gingival tissue reveals proteome dynamics during the initiation and progression of inflammatory periodontal disease

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Abstract

Understanding the progression of periodontal tissue destruction is at the forefront of periodontal research. We aimed to capture the dynamics of gingival tissue proteome during the initiation and progression of experimental (ligature-induced) periodontitis in mice. Pressure cycling technology (PCT), a recently developed platform that uses ultra-high pressure to disrupt tissues, was utilized to achieve efficient and reproducible protein extraction from ultra-small amounts of gingival tissues in combination with liquid chromatography-tandem mass spectrometry (MS). The MS data were processed using Progenesis QI and the regulated proteins were subjected to METACORE, STRING, and WebGestalt for functional enrichment analysis. A total of 1614 proteins with 2 peptides were quantified with an estimated protein false discovery rate of 0.06%. Unsupervised clustering analysis showed that the gingival tissue protein abundance is mainly dependent on the periodontitis progression stage. Gene ontology enrichment analysis revealed an over-representation in innate immune regulation (e.g., neutrophil-mediated immunity and antimicrobial peptides), signal transduction (e.g., integrin signaling), and homeostasis processes (e.g., platelet activation and aggregation). In conclusion, we applied a PCT-assisted label-free quantitative proteomics workflow that allowed cataloging the deepest gingival tissue proteome on a rapid timescale and provided novel mechanistic insights into host perturbation during periodontitis progression.

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Associated Data

Data are available via ProteomeXchange with identifier PXD014608

Conflict of interest

The authors declare no conflict of interest

Keywords

Gingival tissue; experimental periodontitis; gingival inflammation; pressure cycling technology; proteome; label-free quantitation

Introduction

Periodontitis is an inflammatory disease of the gum and underlying tissues driven by a dysbiotic microbiota that colonizes subgingival tooth sites with a deregulated anti-inflammatory response of the human body [1–4]. Periodontitis represents the sixth-most prevalent chronic inflammatory disease affecting 10% to 15% of the world's population, and significant links have been elucidated between periodontal inflammation and systemic conditions, such as diabetes and cardiovascular disease [5].

Periodontitis is considered as a continuum of gingival inflammation to the deeper periodontal tissues. The human experimental gingivitis model enables the establishment of inflamed gingival lesions, but it does not permit their progression to periodontitis lesions [6, 7]. Therefore, the model confirms that tissue inflammation is initiated by oral biofilms, but cannot provide a deepened explanation on the conversion to a tissue-destructive lesion [8, 9]. Although a variety of animal models to study periodontal disease exist, none can reproduce its complexity, so that only specific aspects of the disease can be addressed by distinct models [10]. The most common *in vivo* periodontitis model involves procedures for oral gavage and placement of ligatures in rodents. In this regard, the placement of ligatures in molar teeth has been reported to simulate human periodontitis as it generates a subgingival biofilm-retentive milieu leading to dysbiotic inflammation and bone loss in mice [11–13]. An advantage of the ligature model is that the disease can be initiated at a precisely known time point, and progression can be monitored thereafter. Other periodontitis models, such as the lipopolysaccharides (LPS) injection model, is not appropriate for our purpose as it would only affect deeper tissues (as LPS is injected), whereas the ligature model also affects the epithelium due to the emergence of the dysbiotic microbiota. Earlier studies focused on histopathological alterations in gingival tissues, while more recent studies have utilized microarray approaches to decipher gene expression signatures in the affected gingival tissues [14, 15]. Although these studies have pointed to novel molecules potentially involved in the pathobiology of periodontitis, they cannot disclose unequivocally the actual effectors of gingival inflammation, as most biological functions are regulated on the protein level [16].

Several MS-based quantitative proteomic methods have intensively been reported as a comprehensive, high-throughput, and robust approach, with the potential of identifying and quantifying essentially all expressed proteins in cells, organisms, and tissues [17–19]. Such proteomic approaches have been successfully applied for uncovering proteomes of oral biological fluids obtained from patients with periodontal health or disease but not gingival tissues [16, 20–23]. To understand the mechanisms of the progression of the disease, a global view of the “measurable biological drivers” of periodontitis is needed. This can undoubtedly be delivered by the use of contemporary proteomic technologies [24, 25].

However, the output of these technologies can be hindered by inefficient ways to process the experimental tissue, concealing the breadth and depth of the proteins present in the tissue.

Usual protein isolations from gingival tissues have been performed using mechanical and biochemical disruption strategies, including freeze-thawing, homogenization, sonication, or bead beating [26–29]. In the field of tissue proteomics, the pressure cycling technology (PCT), using a barocycler instrument, is one of the emerging sample preparation methods. This method uses alternating cycles between atmospheric and ultra-high pressure to achieve effective physical tissue disruption [30], tissue solubilization, and fast proteolytic digestion of proteins into peptides [30–32]. By the use of PCT, ultra-small tissue samples can be processed, and the digested peptides can be ready for the MS in 3–6 h [33, 34]. In fact, tissue samples, prepared under the PCT procedure, have been shown to yield a more complete and reproducible set of proteins as compared with the other extraction methods [30, 35].

There is yet no broad-scale quantitative and integrative analysis of gingival tissue proteomes in experimental periodontitis models. As the periodontitis model allows the dissection of the cause-and-effect relationship between bacterial accumulation and tissue destruction in a longitudinal manner [36–42], the main goal of the present study was to examine protein expression and the respective signaling pathways during the establishment of periodontitis in mice. Collectively, our data indicated that PCT-assisted label-free quantitative proteomics workflow is an efficient pipeline to reproducibly dissect the gingival tissue proteome and leads to a reliable quantification and catalog of more than 1600 proteins in a 0.5 mg tissue. The present findings may shed light on the understanding of periodontitis and, potentially, other inflammatory diseases of polymicrobial etiology.

Materials and Methods

Ligature-induced periodontitis model in mice

The study aims to develop a PCT-assisted LFQ workflow for detecting and quantifying gingival tissue proteome changes during the initiation and progression of inflammatory periodontal disease (Figures 1A & 1B). The ligature-induced periodontitis model in specific-pathogen-free C57BL/6 mice (n=9) was used as described previously [43]. All experiments were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and performed in compliance with established federal and state policies for animal protection. Briefly, a 5–0 silk ligature was tied around the maxillary left second molar to induce periodontitis. Gingival tissue from palatal sites surrounding the ligated second molar was collected for analysis. Gingival tissue was also collected from unligated contralateral molar sites from the same mice to serve as the baseline control. Tissues were immersed in the RNAlater stabilization solution (Invitrogen) according to the manufacturer's instructions and stored at –80 °C before further usage. Skulls of the mouse were collected for bone loss determination. Periodontal bone height was determined as described previously [36]. Briefly, freshly harvested skulls were defleshed by boiling, and then, subjected to bleaching and staining with 0.5% eosin (Ricca Chemical Company, TX, USA) and 1% methylene blue (Ricca Chemical Company, TX, USA). The periodontal bone was assessed under a Nikon SMZ800 microscope (Nikon Instruments Inc., NY, USA) using a 40 ×

objective. The images were captured using a Nikon Digital Sight DS-U3 camera controller (Nikon Instruments Inc.) and then subjected to NIS-Elements software (Nikon Instruments Inc.) to measure bone heights (the distances from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC)). CEJ-ABC distances were measured at six predetermined points [36]. To calculate bone loss, the six-site total CEJ-ABC distance for the ligated site of each mouse was subtracted from the six-site total CEJ-ABC distance of the contralateral unligated site. The results were presented in millimetres, and negative values indicate bone loss relative to the baseline (unligated control). Normality of the data was assessed by Shapiro-Wilk normality test. Two-tail student *t*-tests were performed using the Prism 7 software (GraphPad Software, La Jolla, USA). P values ≤ 0.05 were considered statistically significant.

Gingival tissue homogenization and protein extraction using PCT

A total of fourteen samples (Experiment 1 (Day 0 to Day 8) (n=4), Experiment 2: Day 5 (n=10)) were lysed and digested as described by Guo et al [31] using a Barocycler NEP2320 (Pressure BioSciences) at 33 °C. In brief, mouse gingival tissues (typically 0.5 (± 0.1) mg from each mouse) were collected. Each sample was transferred into an individual PCT Micro Cape (Pressure BioSciences) loaded with 30 μ l of lysis buffer (8M urea (Sigma), 0.1M ammonium bicarbonate (Sigma) and complete protease inhibitor (Roche)). These samples were lysed with a 60-cycle barocycling process, each cycle consisting of 50 seconds at 45000 pounds per square inch (PSI) following with 10 seconds at ambient pressure. Tris (2-carboxyethyl) phosphine (Sigma) and iodoacetamide (Sigma) were added to the lysate solution at final concentrations of 10 mM and 40 mM, respectively. These solutions were incubated in the dark at thermomixer at 33 °C 1000rpm for 30 minutes, followed by diluting urea concentration in samples from 8 M to 6 M with 0.1 M ammonium bicarbonate. The diluted samples were first digested using Lys-C (Wako) at an enzyme-to-protein ratio (protein estimated to be 10% of wet sample weight) of 1:45 in the barocycler with a 45-cycle barocycling process, each cycle consisting of 50 seconds at 20000 PSI following with 10 seconds at ambient pressure. Subsequently, the urea concentration of the Lys-C samples was further diluted from 6M to 1.6M using 0.1 M ammonium bicarbonate. Then, trypsin (Promega) was used with an enzyme to protein ratio at 1:45. Tryptic digestion was performed with a 90-cycle barocycling process, each cycle consisting of 50 seconds at 20000 PSI following with 10 seconds at ambient pressure. After the digestion, the solution was acidified by trifluoroacetic acid (TFA) to a final concentration of 0.8 % w/v to stop the reaction. The peptide mixtures were desalted using reverse-phase cartridges Finissterre SPE C18 (Wicom International AG) and dried with vacuum centrifuge. Samples were kept in -20 °C freezers and reconstituted right before loaded on MS.

NanoLC-MS/MS analysis

Peptides were suspended in 3% ACN in 0.1% formic acid. Their concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher), and adjusted to 0.3 μ g/ μ l. 0.6 μ g (2 μ l) of peptides were injected into an Orbitrap Fusion MS (Thermo Fisher Scientific) for proteomic analysis as described previously [44] (Figure 1B). Briefly, peptides were separated using a Thermo Scientific™ EASY-nLC™ 1200 system (Thermo Fisher Scientific) coupled to a 15 cm-long and 75 μ m-diameter silica emitter, packed with a

ReproSil-Pur C18-AQ 120 A and 1.9 μm resin (Dr. Maisch HPLC GmbH). Peptide mixes were separated with two linear gradients of acetonitrile/water (first gradient from 2 to 30% acetonitrile in 60 minutes, second gradient from 30% to 97% in 10 minutes, and then 97% for 10 minutes), containing 0.1% formic acid, at a flow rate of 300 nL/min. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS using a top 12 method. The MS spectra were acquired in the Orbitrap analyzer with a mass range of 300–1500 m/z. The peptide fragmentation was performed using higher-energy collisional dissociation at 28 normalized collision energy and analyzed at high resolution in the Orbitrap.

Label-free proteome quantification, data search and protein identification

The protein label-free quantification was performed by Progenesis QI for proteomics software (Nonlinear Dynamics) as described previously [44]. In brief, the raw files of each sample were aligned with a pooled sample for peak picking and creating Mascot files (.mgf). Best five tandem mass spectra with 200 fragment ion count of each feature were chosen as well as deisotoping and charge deconvolution optioned were applied for spectrum export. The .mgf file was searched with Mascot (version 4.0, Matrix Science) against a mouse database (contains 119,566 sequences and 54,692,468 residues). The database, included isoforms and reversed sequence as a decoy, was downloaded from Uniprot on Sep 2016. Following search parameters were set for the search: precursor tolerance: ± 10 ppm; fragment ion tolerance: ± 0.6 Da; enzyme: trypsin; maximum missed cleavages: 2; fixed modification: carbamidomethylation of cysteine; variable modification: deamidated (NQ), Gln $\rightarrow$ pyro-Glu (N-term Q), oxidation (M) and acetylation on protein N-termini. The resulting .dat file was imported into Scaffold (version Scaffold_4.2.1, Proteome software) to solve protein conflicts, with protein false discovery rate (proFDR) of 10%, minimal one peptide and peptide false discovery rate (pepFDR) of 5%. FDR was calculated using the decoy sequences. Then, the Scaffold spectrum report was imported back into Progenesis for quantifying proteins. For quantitative protein expression analysis, significant differences between the pair-wise group comparisons were done in the Progenesis QI for proteomics using normalized protein abundances in arcsinh transformation. Differences were considered as statistically significant at p -value ≤ 0.05 or fold change (FC) ≥ 2 , p -value ≤ 0.05 as a more stringent cut-off for proteins with at least two unique quantified peptides. Progenesis reported the q -value as an adjusted p -value using optimized FDR approach [45]. Additionally, Benjamini-Hochberg (BH) adjusted p -values were also calculated by R using the number of the identified proteins with at least two unique quantified peptides as the number of comparisons. The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium via the PRIDE [46] partner repository with the dataset identifier PXD014608.

Data clustering and heat maps

To obtain global visualization trends of protein profiles, clustering analysis and heat maps were generated using the R software (R: A Language and Environment for Statistical Computing, R Development Core Team) in particular the Quantable package (<https://cran.r-project.org/web/packages/quantable/index.html>). Apparent outliers were excluded from the quantification. Modified normalized abundances (one was added to all the normalized

abundance values to eliminate the value of zero, which is indication proteins undetectable in this condition) of all quantified proteins were used. Distance matrices were generated based on normalized abundance using the R software. Abundance intensity of quantified protein was log 2 transformed and presented. In addition, log 2 transformed fold changes of the normalized abundance of comparison with baseline (Day 0) or consecutive time points (i. e., Day 8/Day 5, Day 5/ Day 1, Day 1/ Day 0) were also calculated and generated in R.

Bioinformatics analysis for the regulated proteins

The regulated proteins were subject to METACORE online database (updated on 28th of November 2017) for ontology enrichment of molecular functions and localizations. These proteins applied with FC ≥ 2 , p -value ≤ 0.05 were further subjected to STRING version 10.5 (<http://string-db.org/>, updated on 6th of July 2018) for protein-protein interaction network analysis with the highest confidence (0.9). Only interacted proteins were showed in the analysis. Functional analysis was performed using the online tool WebGestalt (for overrepresentation enrichment analysis method, updated on 25th of March 2019) based on the Reactome platform.

Results:

Dynamics of gingival tissue proteome over consecutive time points during the initiation and progression of periodontitis

As the alveolar bone loss is a hallmark of periodontitis, we first assessed alveolar bone levels in response to dental biofilm accumulation at four different time points in the ligature-induced murine periodontitis model. Reduction in bone levels was observed at Day 5 and Day 8, which was accelerated from Day 1 to Day 5. Representative images of the defleshed jaws were provided in Figure 2A.

To establish a quantitative map of the mouse gingival tissue proteome during the initiation and progression of the periodontitis, we prepared gingival tissue protein extracts using the PCT platform, as outlined in Figure 1A. Briefly, a piece of gingival tissue (typically around 0.5 mg) was placed into a MicroTube and subjected to three identical barocycler cycles for tissue lysis and protein digestion. The resulting peptide was purified and later quantified by Progenesis QI (Figure 1B). To reduce the impact of possible false-positive identifications, the proteins with less than two peptides were excluded. This led to the quantification of a total of 1614 unique proteins (including 1596 mouse proteins, 17 contamination, and 1 decoy) with an estimated protFDR of 0.06% (Figure 1C, Table S1, Table S4).

To explore the pattern of protein changes over time ($n=1$ for day 0, 1, 5, and 8), we first performed an unsupervised hierarchical clustering analysis based on the modified normalized abundance intensities (Figure 2A). The cluster analysis indicated considerable variations among these proteins at different time points. The unsupervised hierarchical clustering of gingival tissue samples showed that sample Day 0 did not cluster well with the other days, while protein expressions on Day 1 and Day 5 showed considerable overlaps. The distance matrix showed Day 0 was distant to the other samples chronologically (Table S2).

Fold changes of these proteins were subsequently calculated based on these normalized abundance intensities ((Figures 2c & 2.D, n=1 for day 0, 1, 5, and 8). These changes were presented in log 2 ratio and compared either with the baseline (i.e., Day 1/Day 0, Day 5/Day 0, and Day 8/Day 0) (Figure 2C) or between pairs of consecutive time points (i.e., Day 1/Day 0, Day 5/Day 1, and Day 8/Day 5) (Figure 2D). Of 1614 quantified proteins, 254 (15.1% of all quantified proteins, Day 1/Day 0), 296 (17.6%, Day 5/Day 0), and 500 (29.8%, Day 8/Day 0) proteins were considered as upregulated ($\log_2(\text{FC}) \geq 1$) when compared with Day 0 (Table S3). On the contrary, 113 (6.7%, Day 1/Day 0), 155 (9.2%, Day 5/Day 0), and 90 (5.4%, Day 8/Day 0) proteins were downregulated ($\log_2(\text{FC}) \leq -1$). Myocilin lysozyme g-like protein 1 was downregulated over time, while lactotransferrin, receptor-type tyrosine-protein phosphatase C, and ribonuclease 2B were among the top upregulated proteins (Table 1A). The comparison between the consecutive time-points showed that 254 (15.1% of all quantified proteins, Day 1/Day 0), 104 (6.2%, Day 5/Day 1), and 320 (19.1%, Day 8/Day 5) proteins were upregulated (Table S3). In contrast, 113 (6.7%, Day 1/Day 0), 123 (7.3%, Day 5/Day 1), and 67 (4.0%, Day 8/Day 5) proteins were downregulated. Among those three consecutive time-points, the most strongly upregulated proteins were lactotransferrin, protein BC100530, and hematopoietic lineage cell-specific protein, while myocilin, aldehyde dehydrogenase 4 and EGF-containing fibulin-like extracellular matrix protein 1 were the most strongly downregulated proteins (Table 1B). The overlap between the regulated proteins in each comparison was shown with Venn diagrams (Figure 2E, F).

Dynamics of gingival tissue proteome after five days of ligation

Our initial data indicated that a PCT-assisted sample preparation protocol is efficient for the dissection of gingival tissue proteome in mice. To examine the variability among mice and validate the reproducibility of PCT-assisted peptide extraction, we performed similar analysis in a new set of healthy and diseased gingival tissue samples (n = 5 for healthy and n=5 for disease), which were obtained at Day 5 in which bacterial accumulation peaks and significant inflammatory bone loss occurs [36]. Alveolar bone level measurements indicated that there were significant changes ($p = 0.0284$) in bone levels (mean [standard deviation] mm) at the diseased sites (-1×10^{-5} [0.236]) compared with the healthy sites (-0.035 [0.106]) (Figure 3A).

We first used unsupervised hierarchical clustering analysis to separate samples and elucidate groups based on their protein abundance, which recapitulated the similarities between the same types of gingival tissues (Figure 3B) (Table S4 & S5). To identify differentially expressed proteins, we used two approaches. With an ANOVA test to assess differential expression at the protein level (was used as a cut-off for the p -value at $p \leq 0.05$), 520 proteins were considered differentially expressed between two groups (Figure 3C). Among these, 338 proteins were upregulated ($\log_2(\text{FC}) \geq 0$, $p \leq 0.05$) in the periodontitis sites compared with the healthy sites, while 182 proteins were downregulated ($\log_2(\text{FC}) \leq 0$, $p \leq 0.05$) (Table S6). When applying a more stringent cut-off (the $p \leq 0.05$, abs ($\log_2(\text{FC}) \geq 1$)), the analysis of the protein expression profiles showed that a total of 210 proteins were differentially expressed between healthy and diseased tissues (Table S7). Among these, 122 (54 of which with BH p -value < 0.05) proteins were upregulated ($\log_2(\text{FC}) \geq 1$, $p \leq 0.05$) in the periodontitis sites compared with the healthy sites, while 88 (41 of which with BH p -

value <0.05) proteins were downregulated ($\log_2(\text{FC}) \leq -1$, $p \leq 0.05$). 20 top regulated proteins were listed in Table 1B.

Enrichment analysis for the regulated proteins

In the subsequent bioinformatics analyses, we focused on the differentially expressed proteins to provide an overview of their role during the periodontitis pathogenesis. The regulated proteins were annotated using METACORE online software (<https://portal.genego.com>, Thomson Reuters). Although the majority of the differentially expressed proteins were recognized by the METACORE software, approximately 10% were excluded from the analysis (58/520 and 27/210 for ANOVA and stringent cut-off, respectively) (Table S8). All identified proteins (including those with only one peptide, 2142 proteins were recognized by METACORE) (Table S9) were used as a background to calculate the statistical significance based on the hypergeometric distribution algorithm.

The public ontologies function in METACORE enriched the Gene Ontology (GO) cellular processes, GO molecular functions, and GO localization of the corresponding regulated proteins and listed according to their $\log_2$ transformed statistical significance ($-\log_{10} p$ value) (Table S10). Interestingly, eight or nine of the top 10 GO terms were assigned for secretory or extracellular functions (Table S10). This tendency also reflected on their GO term of process category; the top three enriched terms were “exocytosis” and “secretion” related. Besides, many of the regulated proteins showed a close correlation with the innate immune responses such as “antimicrobial humoral response” (the 4th enriched GO term for stringent cut-off) or “neutrophil mediated immunity” (the 5th and 9th enriched GO term for ANOVA and stringent cut-off, respectively). In addition, many top regulated proteins involved molecular functions such as “actin binding,” “RNA binding,” “C5L2 anaphylatoxin chemotactic receptor binding,” and “endopeptidase regulator activity.”

Functional protein association networks

To obtain a further understanding of the relationships between the regulated proteins, protein-protein interaction analysis was conducted using the STRING search tool. With the highest confidence cut-off (0.9), a network was retrieved for 63 functional associations between 54 regulated proteins (Table S11). Although most of these associations only involved two or three proteins, some of them involved multiple proteins and formed three protein-network clusters (Figure 3E). The largest cluster included 18 different members. The second-largest cluster of these three, with 11 different proteins, involved “oxidation-reduction,” “small molecule metabolic,” and other metabolic processes. The smallest cluster of these three was a group of four ribosomal proteins.

Pathway analysis

The WebGestalt online tool was used for overrepresentation enrichment analysis based on the computational pathways from the Reactome database (Table 2). Within this database, the regulated proteins (210) assigned to 178 (Table S12) unique Entrez Gene IDs against a reference list of 2046 IDs (Table S9). The top pathway was “regulation of toll-like receptors (TLRs) by an endogenous ligand” that included five regulated proteins (Table S13). The other enriched functions involved “antimicrobial” and integrin-related pathways such as

“antimicrobial peptides pathway,” “GRB2:SOS provides linkage to MAPK signaling for Integrins,” “Platelet Aggregation (Plug Formation),” and “Integrin alphaIIb beta3 signaling.”

Discussion

In the present study, we successfully applied the PCT technology in combination with liquid chromatography-tandem MS for the first time to dissect gingival tissue proteomes in ultra-small tissue samples (typically 0.5 mg) from the experimental periodontitis mouse model. Our findings shed new highlights on the inflammatory periodontal disease progression by quantifying and cataloging more than 1600 proteins. In addition to higher coverage in protein identification and quantification, the unsupervised clustering analysis for the protein abundance showed that the healthy gingival tissue proteome was distinct from the diseased one. The unsupervised hierarchical clustering of gingival tissue samples across the experimental time course did not co-segregate chronologically. This indicated significant alterations in the gingival tissue proteome during different stages of the progression of periodontitis in response to microbial accumulation.

The entirety of proteomics findings has suggested that affected gingival tissue during progression contained a defense response that was regulated by many proteins and through various pathways and networks. Although we could not find any earlier data regarding the global quantitative proteome of mouse gingiva, the number of identified or quantified proteins in this study was considerably higher than that reported earlier in the human gingival tissue [26–28] or the gingiva of mini pigs [47]. The mouse genome reportedly encodes 48,709 genes, of which about one-half are protein-coding (22,018 genes) [48]. As about 80% of all protein-coding genes are shared between human and mouse genomes, the present findings may complement available data and be valuable to shed insights in periodontitis progression in humans.

The next analyses were conducted to survey the differentially expressed proteins. Comparisons made between consecutive time points showed that the number of upregulated proteins was increased during the progression of the disease, an observation that is in agreement with the current literature on transcriptomics changes in the gingival tissues during the course of experimental periodontitis [15]. Another commonly used approach for investigating differences between the studied groups is to place the differentially regulated proteins in the context of pathways or processes and place an emphasis on the proteins in the top enriched categories [49, 50]. Many of the top enriched GO terms in the protein localization category were “extracellular” or “secretory related.” This may not be surprising since oral biofilms are known to stimulate the secretion of several inflammatory molecules [17]. The gingival proteins were known to be released into a gingival crevice, which could be collected as gingival crevicular fluid in different experimental disease models [20, 23, 50].

When considering the GO classifications for biological process networks affected in the gingival tissue over the progression of the disease, these involved the innate immune responses, primarily the ones associated with antimicrobial humoral response and neutrophil-mediated immunity. These observations on the chemotactic migration of

neutrophils were in agreement with the well-established early innate immune responses of the gingiva in response to subgingival biofilms [51, 52]. In mice, alterations in neutrophil recruitment disrupted the oral microbial-host tissue homeostasis leading to inflammatory bone loss [38, 53, 54].

GO cellular process analysis of these proteins also suggested that periodontitis progression was associated with exocytosis. The exocytosis of granules (or degranulation) containing pre-formed mediators, such as proteases, lipases, and inflammatory mediators by neutrophils, is an essential element of host defense against microbial infection, but can also instigate tissue damage in inflammatory disease [12, 23, 50].

Over five days, “RNA binding,” “actin binding,” “endopeptidase regulator activities,” and “C5L2 anaphylatoxin chemotactic receptor binding” were among the top enriched functions for regulated proteins. RNA binding proteins (RBPs) regulate target cytokine transcription and expression [55], therefore modulate inflammatory pathways [56]. Actin binding proteins (ABPs) contribute to apoptosis [57, 58]. However, the importance of the RBPs and ABPs in periodontitis is poorly understood. The complement anaphylatoxin C5a is a proinflammatory component of host defense that functions through two identified receptors, C5a receptor 1 (C5aR1) and C5aR2, also known as C5L2 [59]. C5L2 is a regulator of C5aR1-mediated signal transduction but also has immunomodulatory action on TLRs and the NLRP3 inflammasome [60].

Since more than 1600 proteins were simultaneously quantified in the present experimental setting, the application of an arbitrary fold change threshold (i.e., 2-fold), along with *p*-value cut-offs, may result in loss of information [61]. Among the missed information were complement pathway-related proteins. Four complement proteins, complement C3 (CO3_MOUSE), complement C4-B (CO4B_MOUSE), complement factor H (CFAH_MOUSE), and complement 1 Q subcomponent-binding protein (Q8R5L1_MOUSE) were identified and quantified. Among them, CO3_MOUSE and CO4B_MOUSE were significantly upregulated, with fold changes of 1.74 and 1.91 times, respectively. Complement is activated in naturally occurring periodontitis in humans and non-human primates, as well as in experimental mouse periodontitis (such as the ligature-induced and the oral gavage models) and is a major driver of inflammatory bone loss [50, 62, 63]. Pharmacological targeting of the central complement component C3 (where all initiation pathways of complement converge) inhibits periodontitis in non-human primates and is currently considered as an approach to treat human periodontitis [62]. Although complement activity is predominantly regulated post-translationally at the level of proteolytic activation [64], the current finding that important complement components, such as C3, increased in abundance during experimental periodontitis suggests that complement activation can persist in the chronic setting of periodontitis.

Interestingly, two other proteins involved in lipid metabolism, prostaglandin E synthase 3 (TEBP) and leukotriene A-4 hydrolase (LKHA4), were significantly upregulated in ligated tissues compared to their unligated counterpart, although they did not reach the threshold of two-fold regulation. TEBP and LKHA4 contribute to the production of prostaglandin E2 [65] and leukotriene B4 [66] that triggers a number of pro-inflammatory pathways. Both

compounds are well described to be elevated in gingival tissue with inflammation [67, 68]. Therefore, the present findings provided additional experimental evidence that the PCT-assisted protein extraction method provided consistent biological data to study periodontal disease progression.

Further pathway analysis based on the Reactome database identified “TLR by endogenous ligand” as the top enriched pathway. These findings are in line with earlier literature that potential oral pathogens, including *Porphyromonas gingivalis* [69] and *Aggregatibacter actinomycetemcomitans* [70], are known to elevate the expression of TLRs [71]. Hence, the present data are consistent with the reported crosstalk between complement and TLRs in periodontal disease pathogenesis [72].

In conclusion, we developed a PCT-assisted LFQ workflow that allowed cataloging of the deepest gingival tissue proteome on a rapid timescale. These findings suggested mechanistic insights into host perturbation during the progression of periodontitis that can be specifically addressed in future studies targeting molecules of interest. In a further perspective, this workflow may prove to be useful in the unraveling of the gingival proteome map of human or other animals. Potential pathogens were known to be inactivated under these high-pressure cycles in PCT [30], which could be an additional advance to minimize the potential risk of contamination from gingival tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ABC	Alveolar bone crest
ABP	Acting binding protein
BH	Benjamini-Hochberg
CEJ	Cemento-enamel junction
ECM	Extracellular matrix
FC	Fold change
LFQ	Label-free quantification
MMP	Matrix metalloproteinase
PCT	Pressure cycling technology

PRR	Pattern recognition receptor
PSI	Pounds per square inch
RBP	RNA binding protein
TLR	Toll-like receptors

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Significance statement

The present study deals with periodontitis, the most prevalent chronic inflammatory disease of the gum and underlying tissues driven by a dysbiotic microbiota that colonizes tooth surfaces together with a deregulated anti-inflammatory response of the human body. To understand the mechanisms of the disease progression, a global view of the “measurable biological drivers” of periodontitis is needed, which can undoubtedly be provided by the use of contemporary proteomic technologies. However, current methodologies do not process the experimental tissue efficiently and can hinder the output of these technologies, therefore underestimating the complexity of the proteins present in the tissue. In the present study, we applied the combination of pressure cycling technology, a disruption technique for ultra-small amounts of specimen, in combination with liquid chromatography-Orbitrap-mass-spectrometry to capture the dynamics of gingival tissue proteome during initiation and progression of experimental periodontitis in mice. This workflow allowed us to catalog the deepest gingival tissue proteome on a rapid timescale. The present findings may shed light on the understanding of periodontitis and, potentially, other inflammatory diseases.

Figure 1: Experimental flow of the PCT label-free quantification of gingival tissue.

(A) Tissue lysis and protein digestion. The gingival tissues from mouse (typically around 0.5 mg) were lysed in barocycles for 60 min. The extracted proteins were reduced, alkylated and diluted for Lys-C digestion in the barocycler for another 45 min. The protein solutions were further diluted prior to trypsin digestion for an additional process of 90 min in the barocycler. The resultant peptide solutions were desalted and dried for LC-MS/MS analysis.

(B) Mass spectrometry and data analysis. The peptides were separated by liquid chromatography in an 80-min gradient and then analyzed by Orbitrap fusion mass spectrometer. The raw data were quantified by Progenesis Q1 and processed with various functional analyses. (C) Overview of the experiment

Figure 2: Dynamic of gingival tissue proteome in ligature-induced periodontitis model over consecutive time points.

(A) Representative images of time-dependent bone loss in unligated and ligated sites in ligature-induced periodontitis model over consecutive time points. (B) The heatmap of normalized abundance for all quantified proteins among four different time points (n=1 for each day, Table S1). The distance matrix of the heatmap was shown in Table S2. Dot plots of log₂ (FC) for all identified proteins in baseline (C) or consecutive time points (D).

Upregulated, downregulated, and unregulated proteins were plotted in red, blue, and grey, respectively. Venn diagrams showed the overlap of quantified proteins in baseline (E) or consecutive time points (F) (Table S3). The numbers of upregulated protein and downregulated protein were labelled in red and blue color, respectively. The numbers of

protein that did not regulate in any of condition were list on the right side of each Venn diagram with black color.

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Figure 3: Alveolar bone loss of ligature-induced periodontitis model after five days of ligation. (A) Kinetics of total bone loss “and representative images of time-dependent bone loss in unligated and ligated sites. * $p < 0.05$ (B) The normalized abundance of all these quantified proteins among 10 samples (Table S4). Samples from the unligated side and the ligated side were highlighted in green and red, respectively. The distance matrix of the heatmap was shown in Table S5. (C) Volcano plots of log 2(FC) and log 10 p -value of ANOVA test for all identified proteins. Upregulated ($p < 0.05$, Log2(FC) > 1), downregulated ($p < 0.05$, Log2(FC) < -1), and unregulated proteins ($p < 0.05$) were plotted in orange, green, and grey in periodontitis compared to healthy gingival tissues, respectively; proteins that significant

regulated ($p < 0.05$) but not reaches stringent cut off ($FC \geq 1$) were labelled in red or blue. Regulated proteins that also had Benjamini-Hochberg p -value less than 0.05 were plotted in crosses. The labels of regulated proteins were shown in Figure S1. Proteins involved in top 10 pathways based on Reactome database were labelled with black plots and their Swissprot IDs (Table S13). (D) Fold change distribution of regulated proteins in mouse gingival tissues. (E) Network established using STRING 10.5 (Table S11) based on the highest confident score (0.9) of regulated tissue proteins with stringent cut-off. Lines indicated different types of protein-protein interactions. Blue and purple lines indicated known interaction determined from the curated database and experimental results, respectively. Green, red, and dark blue lines indicated predicated interaction determined from gene neighbourhood, gene fusions, and gene co-occurrence, respectively. Yellow, black and light blue lines indicated interactions were from textmining, co-expression and protein homology, respectively. An enlarged version of this protein-protein interaction network was updated as Figure S2.

Table 1:

List of representative proteins in (A) ligature-induced periodontitis model overtimes (n=1 per timepoint, top 5 proteins) or (B) ligature-induced periodontitis model (n=5 for periodontitis model, n= 5 for healthy control, top 20 proteins) after five days of ligation.

A	Protein name				
	Upregulated proteins	Downregulated proteins			
Day 1 Vs Day 0	Lactotransferrin	Myocilin			
	Isoform 2 of Receptor-type tyrosine-protein phosphatase C	Lysozyme g-like protein 1			
	Isoform 2 of Fibrinogen alpha chain	Beta-catenin-interacting protein 1			
	Isoform 2 of Membrane primary amine oxidase	Protein Ankrd35			
	Cathelicidin antimicrobial peptide	Filaggrin (Fragment)			
Day 5 Vs Day 0	Protein BC100530	Myocilin			
	Ribonuclease 2B	Lysozyme g-like protein 1			
	Isoform 2 of Receptor-type tyrosine-protein phosphatase C	Aldehyde dehydrogenase 4			
	Isoform 2 of Ig mu chain C region	Protein-arginine deiminase type-1			
	Fatty acid-binding protein, adipocyte	Keratin, type I cytoskeletal 9			
Day 8 Vs Day 0	Lactotransferrin	Myocilin			
	Neutrophil elastase	Lysozyme g-like protein 1			
	Prothrombin	Carboxylesterase 1D			
	Cathelicidin antimicrobial peptide	Keratin, type I cytoskeletal 9			
	Ribonuclease 2B	Keratin, type II cytoskeletal 2 oral			
Day 5 Vs Day 1	Protein BC100530	Aldehyde dehydrogenase 4			
	Lysosomal alpha-mannosidase	Alpha-2-antiplasmin			
	Fatty acid-binding protein, adipocyte	Golgi phosphoprotein 3			
	Ribonuclease 2B	Keratin, type I cytoskeletal 9			
	Isoform E2 of Drebrin	Selenium-binding protein 1			
Day 8 Vs Day 5	ATP-dependent RNA helicase DDX19A	EGF-containing fibulin-like extracellular matrix protein 1			
	Hematopoietic lineage cell-specific protein	Isoform 2 of Membrane primary amine oxidase			
	Prothrombin	T-complex protein 1 subunit delta			
	Isoform 2 of Heterogeneous nuclear ribonucleoprotein U-like protein 1	Collagen alpha-2(I) chain			
	Prostaglandin E synthase 3	Collagen alpha-1(I) chain			
* Proteins that undetectable in one of the conditions (normalized abundance value is 0) were excluded.					
B					
Swissprot accession	Description	Unique peptides	Log ₂ (fold change) *	Anova (p)	Benjamini-Hochberg p-value
D6REU3	Protein BC100530	2	6.9	5.78E-06	6.44E-03
Q3UP87	Neutrophil elastase	3	6.12	2.09E-05	6.76E-03
P31725	Protein S100-A9	18	5.18	1.05E-04	9.45E-03
P08071	Lactotransferrin	17	4.86	9.88E-04	3.01E-02

B

Swissprot accession	Description	Unique peptides	Log ₂ (fold change) *	Anova (p)	Benjamini-Hochberg p-value
Q9JHW9	Aldehyde dehydrogenase family 1 member A3	7	4.5	8.42E-04	2.77E-02
P27005	Protein S100-A8	5	4.47	2.96E-04	1.65E-02
P51437	Cathelicidin antimicrobial peptide	2	4.33	1.11E-04	9.45E-03
O08692	Neutrophilic granule protein	9	3.81	1.78E-04	1.30E-02
A0A077S2U6	Lysozyme	3	3.79	1.41E-05	6.44E-03
P41245	Matrix metalloproteinase-9	9	3.55	1.07E-03	3.01E-02
Q05117	Tartrate-resistant acid phosphatase type 5	3	3.15	9.01E-03	6.33E-02
D3YUU6	Protein Crnn	23	-3.26	5.79E-05	9.45E-03
P50114	Protein S100-B	2	-3.26	6.12E-03	5.47E-02
Q9JJV1	Beta-crystallin A2	5	-3.31	1.13E-02	7.02E-02
P15089	Mast cell carboxypeptidase A	2	-3.53	2.28E-03	3.96E-02
Q8CEZ4	Uncharacterized protein C2orf54 homolog	2	-3.61	6.49E-03	5.59E-02
Q3UV17	Keratin, type II cytoskeletal 2 oral	89	-3.72	2.82E-04	1.65E-02
O70624	Myocilin	6	-3.83	3.49E-03	4.61E-02
P00329	Alcohol dehydrogenase 1	2	-4.62	4.78E-02	1.51E-01
P16015	Carbonic anhydrase 3	2	-6.11	5.13E-03	5.24E-02

* Log₂ (FC) was calculated based on the normalized abundance in periodontitis compared to healthy gingival tissues.

Table 2:

Pathway analysis of all regulated proteins with the WebGestalt analysis tool based on Reactome database

Gene set	Description	Nr. of Genes	FDR
R-MMU-5686938	Regulation of TLR by endogenous ligand	5	0.002592
R-MMU-6803157	Antimicrobial peptides	6	0.015752
R-MMU-354194	GRB2:SOS provides linkage to MAPK signalling for Integrins	5	0.015752
R-MMU-76009	Platelet Aggregation (Plug Formation)	6	0.017111
R-MMU-354192	Integrin alphaIIb beta3 signalling	5	0.019341
R-MMU-9006921	Integrin signalling	5	0.019341
R-MMU-1474244	Extracellular matrix organization	17	0.019341
R-MMU-211945	Phase I - Functionalization of compounds	7	0.041501
R-MMU-372708	p130Cas linkage to MAPK signalling for integrins	4	0.041501
R-MMU-76002	Platelet activation, signalling and aggregation	15	0.041501