Quantitative proteomic mapping of gingival crevicular fluid from dogs progressing from mild gingivitis to periodontitis

Andrew W. Jones[1], Andrew J. Creese[1], Ian J. Davis[2], Iain L C. Chapple[1]

1. Periodontal Research Group, MRC Centre for Immune Regulation, College of Medical & Dental Sciences, University of Birmingham, Birmingham, B4 6NN, UK.
2. WALTHAM Centre for Pet Nutrition, Waltham-on-the-Wolds, Melton Mowbray, Leicestershire, LE14 4RS, UK

Overview

- Gingivial crevicular fluid (GCF) is a complex biological fluid exuded between the teeth and gums to deliver innate immune defences against colonising plaque bacteria in order to maintain gum health. It is a source of protein biomarkers for early diagnosis and classification of disease.
- Here we examine longitudinal variations in protein abundance longitudinally as mild gingivitis evolves into severe gingivitis, and ultimately periodontitis within the same individual.
- 1,071 proteins were identified with 2 or more peptides. Of these proteins 76 were shown to be ≥2-fold up-regulated in mild periodontitis in comparison to severe gingivitis.

Introduction

- GCF is a complex biological fluid with a large dynamic range. It is a rich source of proteins and its collection is non-invasive; however, changes in the relative abundance of these proteins across disease states is currently poorly defined, especially at the individual patient level.
- We used iTRAQ-based quantitative online data-dependent liquid chromatography (LC) MS/MS of GCF collected from dogs who exhibited natural periodontal disease progression from mild to severe gingivitis through to mild periodontitis.

Method

- GCF was collected on paper points from five teeth from five Miniature Schnauzers who exhibited natural periodontal disease progression.
- The samples were vortexed and centrifuged with the supernatant retained.
- The proteins were reduced and alkylated prior to overnight digestion with trypsin (Promega).
- Small equivalent fractions from all digests were pooled together to form a Mastermix (used as a comparable baseline).
- The digests, along with the Mastermix, were labelled with 4-plex iTRAQ (ABSciex) labels (114 to 117) and combined.
- The peptide mixtures were fractionated by strong cation exchange (SCX) chromatography. SCX was performed with a Polysulfoethyl A column. Peptides were eluted over a 60 minute salt gradient from 0-50 % 500 mM KCl (pH 3.0). 17 fractions were collected and pooled into four fractions according to peptide concentration.
- The fractions were loaded onto a 75 µm C_{18} reversed phase analytical column (LC Packings) and peptides were separated over a 60 minute gradient from 3.2 to 44 % acetonitrile (0.1 % formic acid).
- Samples were infused by use of an Advion Triversa Nanomate nanospray ionization source into a Thermo Fisher Scientific LTQ-Orbitrap Velos hybrid mass spectrometer.
- The mass spectrometer performed an initial high resolution survey scan (15,000 at *m/z* 400) in the Orbitrap and the three most abundant multiply charged ions were selected for collision induced dissociation (CID) MS/MS, detected in the ion trap. The same three ions were then fragmented by higher energy collisional dissociation (HCD), detected in the Orbitrap.
- Analysed ions were placed on an exclusion list for 60 seconds.
- Data were collected with Xcalibur 2.1 (Thermo Fisher Scientific) and analysed using the SEQUEST and Mascot search algorithms and the UniProt canine database.
- CID spectra were used to identify the peptides and HCD spectra were used for quantification.

Conclusions

- From GCF we have identified 1,071 canine proteins by two or more peptides at a 1 % false discovery rate; 327 (31 %) of these proteins were identified in all five teeth over time.
- In comparison to mild gingivitis 930 proteins were identified to be more abundant in severe gingivitis, and 460 were more abundant in mild periodontitis when compared to severe gingivitis.
- 76 of these proteins were observed to have a mean ≥2-fold increase in abundance in periodontitis in comparison to severe gingivitis.

Acknowledgements

This work was funded by WALTHAM Centre for Pet Nutrition, Mars Petcare UK. The Advion Triversa Nanomate, Dionex LC and Thermo Fisher Orbitrap mass spectrometer used in this research were funded through the Birmingham Science City Translational Medicine: Experimental Medicine Network of Excellence Project, with support for Advantage West Midlands (AWM). The authors thank Dr. Melissa Grant, Dr. Helen Cooper, Dr. Mark Marshall and Dr. Stephen Harris for their technical assistance and scientific input.

Results

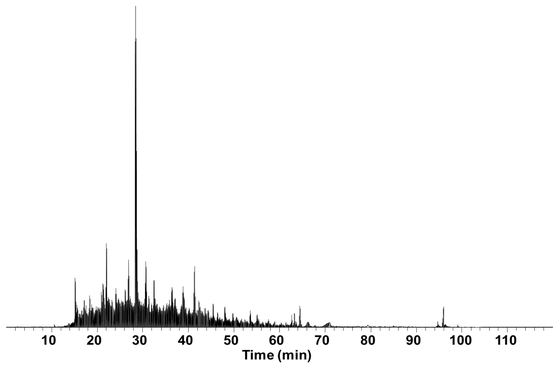

Figure 1: The total ion chromatogram of one of the pooled SCX fractions.

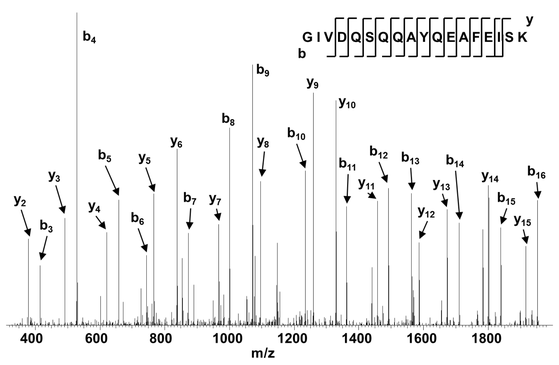

Figure 2: The CID mass spectrum of the doubly-charged peptide ion [iTRAQ-GIVDQSQQAYQEAFEISK-iTRAQ + 2H]$^{2+}$. Near complete peptide sequence coverage is observed.

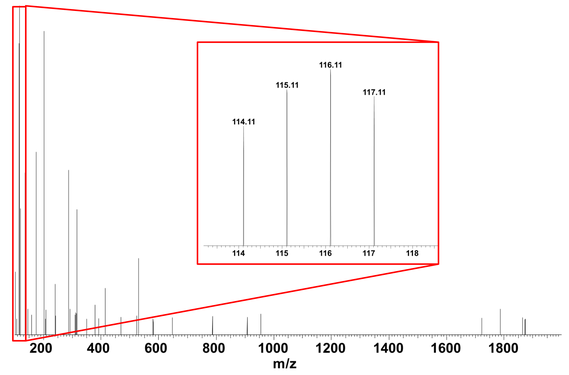

Figure 3: The HCD mass spectrum of the doubly-charged peptide ion [iTRAQ-GIVDQSQQAYQEAFEISK-iTRAQ + 2H]$^{2+}$. Inset: the *m/z* region containing the fragmented iTRAQ labels allowing quantification.

Total number of CID events	615,423
Total number of peptides identified	4,403
Total number of proteins identified	1,071
Proteins ≥2-fold upregulated (severe/mild gingivitis)	541
Proteins ≥2-fold upregulated (periodontitis/mild gingivitis)	474
Proteins ≥2-fold upregulated (periodontitis/severe gingivitis)	76

Table 1: The total number of CID mass spectra collected from all four pooled SCX fractions from each GCF sample, in triplicate (total of 72 LC-MS/MS runs), along with the number of peptides identified at a 1 % false discovery rate, the number of the proteins identified and the number of proteins which were quantified to be upregulated by ≥2-fold in different disease states.

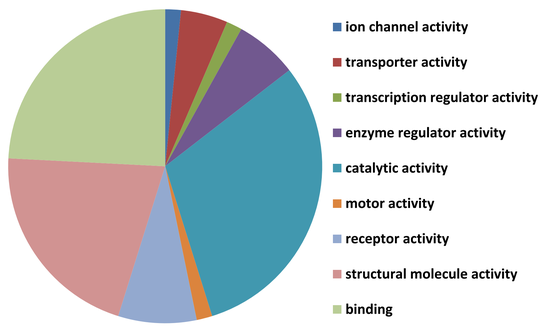

Figure 4: The gene ontology molecular function classification of 40 of the 76 proteins which were identified to be up-regulated by ≥2-fold in periodontitis in comparison to severe gingivitis.

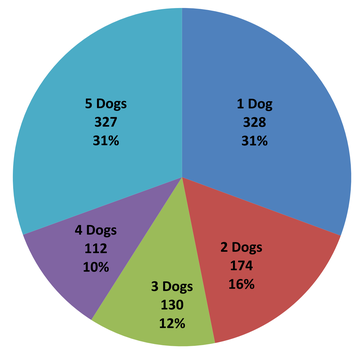

Figure 5: The proportion and number of proteins which were identified from each number of canine GCF samples.

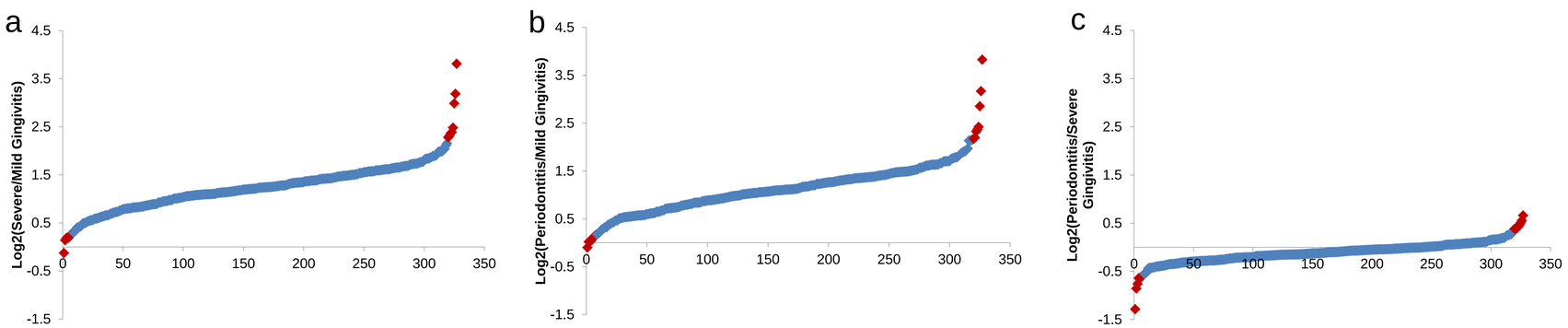

Figure 6: The observed log 2 fold change in concentration of the 327 proteins identified in all canine GCF samples from five teeth which exhibited disease progression from mild through to severe gingivitis and mild periodontitis. 4a: Log 2 fold change of proteins between severe gingivitis and mild gingivitis. 4b: Log 2 fold change of proteins between periodontitis and mild gingivitis. 4c: Log 2 fold change of proteins between periodontitis and severe gingivitis. In all cases, the upper and lower 2.5 % data points are shown in red.

Andrew Jones, Periodontal Research Group, College of Medical & Dental Sciences, University of Birmingham, Birmingham, B4 6NN, UK. E-mail: A.W.Jones@bham.ac.uk