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Comparative analysis of total salivary lipopolysaccharide chemical and biological properties with periodontal status

Running title: Salivary LPS profile in periodontal health and disease

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Highlights

- **Periodontal health and disease are characterised by distinct salivary LPS profiles**
- **Potent, highly inflammatory LPS isoforms are present in periodontitis patients**
- **Salivary LPS molecular signatures remain unchanged following periodontal therapy**

Abstract

Objective: Clinical manifestations of Gram-negative bacteria-mediated diseases can be influenced by how the host senses their major microbe-associated molecular pattern, the cell wall lipopolysaccharide (LPS). Keystone periodontal pathogens can produce a heterogeneous population of LPS molecules, with strikingly different host-microbiome interactions and immune outcomes.

Design: Structure-function correlations of salivary LPS extracts in patients with periodontitis before and after periodontal treatment and healthy volunteers were analysed by comparing its lipid A and carbohydrate chain chemical structure and evaluating its endotoxin activity and inflammatory potential.

Results: Salivary LPS extracts from periodontitis patients were characterised by high m/z lipid A mass-spectrometry peaks, corresponding to over-acylated and phosphorylated lipid A ions and by a combination of rough and smooth LPS carbohydrate moieties. In contrast, gingival health was defined by the predominance of low m/z lipid A peaks, consistent with under-acylated and hypo-phosphorylated lipid A molecular signatures, with long and intermediate carbohydrate chains as determined by silver staining. Total, diseased salivary LPS extracts were stronger inducers of the recombinant factor C assay and triggered significantly higher levels of TNF- α , IL-8 and IP-10 production in THP-1 cells, compared to almost immunosilent healthy samples. Interestingly, salivary LPS architecture, endotoxin activity, and inflammatory potential were well conserved after periodontal therapy and showed similarities to diseased samples.

Conclusions: This study sheds new light on molecular pathogenic mechanisms of oral dysbiotic communities and indicates that the regulation of LPS chemical structure is an

important mechanism that drives oral bacteria-host immune system interactions into either a symbiotic or pathogenic relationship.

Keywords: LPS, saliva, periodontitis, lipid A, host-microbiome interactions

Introduction

Salivary diagnostics in risk assessment and point-of-care patient management is an emerging field in periodontology. Gingivitis and periodontitis are preventable conditions and their timely treatment leads to reduced rates of tooth loss and improved quality of life (Chapple et al., 2015). To date, there is no single biomarker that is specific for periodontal disease and therefore there is a significant, unmet clinical need for novel indicators to be used in risk assessment, disease prevention and treatment planning.

The most important risk factor for periodontitis is the accumulation of a dysbiotic dental biofilm at and below the gingival margin, which is associated with an inappropriate and destructive host inflammatory immune response (Hajishengallis & Lamont, 2012). Periodontitis is initiated by a broadly-based, synergistic and dysbiotic microbial community in which keystone pathogens shape and stabilise a disease-provoking microbiota (Hajishengallis & Lamont, 2016). One of the core requirements for a potentially pathogenic community to arise involves the capacity of these keystone pathogens to elevate the virulence of the entire microbial community through interactive communication with accessory pathogens and expression of pro-inflammatory molecules that trigger and maintain a non-resolving and tissue-destructive host response (Hajishengallis, 2014). In periodontal disease, there are

substantial increases in the proportions of obligately anaerobic and proteolytic bacteria, many of which are Gram-negative (Naginyte, Do, Meade, Devine, & Marsh, 2019). Defining the precise composition of the oral microbiome in health and disease is difficult because the mouth is an open system and frequently exposed to exogenous bacteria and environmental factors. In functional terms, however, there is considerable redundancy among the oral microbiota and an emphasis on functional rather than phylogenetic diversity is required in order to fully understand host microbiome interactions (Wade, 2013). While bacteria are undoubtedly the principal cause of the initial inflammatory lesion leading to gingivitis, it is the host response, not the type of bacteria, which dictates whether disease progresses (Freire & Van Dyke, 2013).

Lipopolysaccharide (LPS) or endotoxin is a major component of the outer membrane of most Gram-negative bacteria and one of their most important virulence factors. It acts as an extremely strong stimulator of innate immunity in diverse eukaryotic species, ranging from insects to humans (Munford, 2008). The chemical architecture of the LPS molecule is strongly associated with its biologic activity. LPS consists of a poly- or oligosaccharide region (smooth (S) and rough (R) chemotypes respectively) that is anchored in the outer bacterial membrane by a specific carbohydrate-lipid moiety termed lipid A (Matsuura, 2013). Natural LPS from the bacterial cell wall is composed of a mixture of different molecules that can vary in the length and composition of their carbohydrate chain as well as in the chemical structure of their lipid A. In mammalian assay systems, maximal endotoxicity is induced by the hexa-acylated and bi-phosphorylated isoform lipid A structure (Pupo, Lindner, Brade, & Schromm, 2013).

Lipopolysaccharide provokes innate immune responses through Toll-like receptors (TLRs) and activates two main intracellular pathways: a MyD88-dependent pathway that acts via NF- κ B

to induce proinflammatory cytokines such as TNF- α and IL-8; and a MyD88-independent pathway that acts via type I interferons to increase the expression of interferon-inducible genes such as IP-10 (Broad, Kirby, Jones, Applied, & Transplantation Research, 2007). Many bacterial species carry enzymes that can modify their lipid A structure, either by changing the number of acyl chains or phosphate groups, resulting in altered activation of the TLR4/MD2 complex in innate immune cells. These modified lipid A structures subsequently induce differential intracellular signalling pathways and cytokine networks by preferential recruitment of the MyD88 or TRIF adaptor molecules (Zariri, Pupo, van Riet, van Putten, & van der Ley, 2016). Some members of the under-acylated and hypo-phosphorylated lipid A isoforms are even capable of antagonising the effects of strongly stimulatory LPS/lipid A forms (Darveau, Arbabi, Garcia, Bainbridge, & Maier, 2002). Differential recognition pathways leading to alterations in their inflammatory potential have also been suggested for smooth and rough chemotype LPS (Huber et al., 2006).

LPS has a potential to induce bone loss and excessive osteoclast formation by increasing expression of osteoclastogenic transcription factors and osteoclastic genes (Kassem et al., 2015). Periodontitis patients have increased salivary concentration of LPS, which has been implicated as a possible molecular mediator between periodontitis and coronary artery disease (Liljestrand et al., 2017). It has recently been shown that characteristic, high m/z ratios, lipid A isoforms are present in subgingival LPS extracts from patients with periodontitis, while these molecular signatures are absent from healthy and post-treatment samples (Strachan et al., 2018).

The aim of this study was to analyse the structure-function correlation of salivary LPS extracts in patients with periodontitis before and after periodontal treatment and healthy volunteers,

by comparing its lipid-A and carbohydrate chain chemical composition and evaluating its endotoxin activity and inflammatory potential.

Materials & Methods

Study population and saliva sample collection

Approval of the study protocol was obtained from the Health Research Authority, UK (NRES Committee South West - Cornwall and Plymouth 14/SW/0020). Thirty-two patients (11 female, 21 male, mean age 46) with moderate to severe periodontitis and 33 systemically and periodontally healthy persons (18 female, 15 male, mean age 31) were recruited from patients presenting to the Peninsula Dental School, University of Plymouth, UK.

Periodontitis patients were diagnosed in accordance to the following clinical criteria: probing pockets depth (PPD) of 4 mm or more, with bleeding on probing (BOP) and at least 50% alveolar bone loss in at least two quadrants (assessed radiographically). Periodontal health was defined as PPD \leq 3 mm and no more than 10% BOP. There were three smokers in the healthy group and fourteen in the periodontitis group. Patients with systemic diseases and conditions affecting the periodontal tissues as well as the patients with antibiotic or periodontal treatment in the previous 6 months were excluded from the study. Periodontitis patients underwent conventional, non-surgical periodontal therapy and clinical parameters were recorded three months after the completion of the therapy.

Unstimulated, whole saliva samples were collected into sterile universal tubes by expectoration for 5 minutes and not less than 30 minutes after eating, drinking or smoking. The samples were stored at -80°C until LPS extraction.

LPS extraction and lipid A isolation

LPS from salivary samples was extracted using the LPS extraction kit (iNtRON Biotechnology, S.Korea), following the manufacturer's instructions. Extracted LPS was re-suspended in 500 μ l of LPS-free water and stored at 4^oC. 50 μ l of LPS extracts from each patient were pooled together to form three groups of pooled samples: healthy, diseased and post-treatment. Lipid A moiety was isolated from LPS extracts by mild hydrolysis as described by Coats *et al.* (Coats *et al.*, 2009).

Lipid A Mass-spectrometry

Lipid A was desalted with 0.1M ammonium citrate and dissolved in methanol/dichloromethane (3:1, v/v). Mass spectrometric analysis of lipid A was performed on a 6530 Accurate Mass Quadrupole Time-Of-Flight (Q-TOF) MS system (Agilent Technologies, Singapore). Positive and negative ion mass spectra were recorded over the range of 1000–2100 m/z. The electrospray ion source (ESI) was operated using the following conditions: pressure of nebulizing gas (N₂) was 30 psi; temperature and flow rate of drying gas (N₂) were 300°C and 7 L/min, respectively; temperature and flow rate of sheath gas were 300°C and 11 L/min, respectively. The capillary voltage was set to 3.5 kV, the nozzle voltage to 2 kV, the fragmentor potential to 100 V and the skimmer potential to 65 V.

LPS Silver staining

Salivary LPS extracts and *P.gingivalis* LPS (InvivoGen, ATCC 33277) were treated for 5 min at 100°C in 0.05 M Tris hydrochloride buffer (pH 6.8), 2% SDS and 0.01% bromophenol blue, and fractionated on an SDS-polyacrylamide gel containing 4% and 10% acrylamide in the stacking and separating gels, respectively. SDS-PAGE-fractionated LPS preparations were stained by the conventional silver staining method (Merck).

Endotoxin activity

Salivary LPS extracts were diluted 1:100 in endotoxin free water and endotoxin activity was measured by an endpoint, fluorescent, recombinant Factor C assay according to manufacturer's instructions (EndoZyme, Hyglos, Germany).

Inflammatory potential of salivary LPS extracts

THP-1 cells (Human monocytic leukaemia cell line) were purchased from ECACC (European Collection of Cell Cultures) and maintained in RPMI 1640 medium (Invitrogen) supplemented by 2 mM Glutamine, 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen). The cells were cultured at 5% CO₂ atmosphere at 37° C.

THP-1 cells were stimulated with 25 µl of salivary LPS extracts per ml of cells (5×10^5) for 4 hours. Cells were centrifuged at 1000 rpm for 5 minutes and cell-free supernatants were collected. Concentrations of IL-8, TNF-α and IP-10 were measured by a multiplex assay (Merck Millipore).

Statistical Analyses

All samples, participants and clinical data were anonymised and locked before the codes were revealed. In order to analyse differences between examined groups of patients, one-way analysis of variance with Tukey post-hoc test was performed, using GraphPad Software, San Diego, CA. A p value below 0.05 was considered significant (*<0.05; **<0.01; ***<0.001). All experiments were performed at least three times in duplicates.

Results

Clinical parameters

The mean O'Leary plaque index in healthy, control participants was $14.68 \pm 5.02\%$, the mean probing depth was $1.45 \pm 0.45\text{mm}$ and the mean bleeding on probing $2.22 \pm 2.76\%$. Post-treatment plaque index, BOP and PPD in periodontitis patients were significantly lower compared to baseline measurements ($56.65\% \pm 21.52\%$ vs. $33.92\% \pm 14.20\%$, $41.47\% \pm 18.49\%$ vs. $23.06\% \pm 13.31\%$ and $5.78 \pm 0.61\text{mm}$ vs. $4.47 \pm 0.58\text{mm}$ respectively).

Mass spectrometric characterisation of salivary lipid A isolates

The mass spectra of salivary lipid As isolated from healthy individuals displayed the predominant ion peaks at low m/z ratios (below 1400), both at positive and negative mode analyses, that are consistent with less acylated and under-phosphorylated lipid A isoforms. In addition, some minor ion signals between 1400-1800 m/z were detected in the positive mode

(Figure 1a and 1b). In contrast to this, diseased lipid A isolates were characterised by exclusive, high m/z ion peaks (1600-2000), in the positive mode, likely to correspond to more acylated and phosphorylated lipid A isoforms and a range of ion peaks between 1000-2000 m/z in the negative mode (Figure 1c). Interestingly, the analysis of post-treatment lipid A isolates revealed the main, high m/z ion signals similar to diseased samples but which were enriched with lower m/z peaks found predominantly in the healthy lipid A isolates (Figure 1d).

LPS silver staining analysis

Analysis of the LPS carbohydrate profiles by silver staining revealed striking differences in the carbohydrate LPS moiety's architecture between healthy samples on one side and diseased and post-treatment samples on the other (Figure 2.). While LPS extracts from healthy individuals showed bands corresponding to long and intermediate O-polysaccharide chains, LPS extracts from diseased and post-treatment samples were characterised by a bimodal distribution, with intense bands corresponding to long polysaccharide O-chains and unique for these types of samples, low-molecular weight bands, representing rough-type LPS, composed of lipid A and core oligosaccharides only. Some indistinct bands at the levels of intermediate O-polysaccharide chains were also detected in post-treatment samples.

Endotoxin activity of salivary LPS extracts

Endotoxin activity of salivary LPS extracts was measured for each individual patient using the recombinant factor C assay. LPS extracts from periodontitis patients, both before and after treatment, showed significantly higher levels of endotoxin activity compared to healthy individuals (Figure 3.). Interestingly, endotoxin activities of salivary LPS extracts isolated from

periodontitis patients before treatment were segregated in two distinct groups: one in the lower range of EU and one within a higher range of endotoxin activity. Although the mean endotoxin activities of pre- and post-treatment salivary extracts were similar, majority of post-treatment salivary samples exhibited endotoxin activities in the range between these two sub-groups. Smoking status did not appear to significantly influence the level of salivary endotoxin activity in any of the three groups of patients (data not shown).

Inflammatory potential of salivary LPS extracts

Inflammatory potential of pooled salivary LPS extracts was assessed in the THP-1 cells model, by measuring the production of TNF- α , IL-8 and IP-10 after a 4-hour challenge (Figure 4.). TNF- α and IP-10 secretions were significantly higher by the THP-1 cells challenged with diseased and post-treatment extracts compared to LPS extracts from healthy individuals. IL-8 production was also higher by the cells treated with diseased and post-treatment LPS extracts but reached statistical significance only between healthy and post-treatment samples. In addition, post-treatment samples triggered higher production of all three cytokines compared to diseased LPS extracts.

Discussion

We show here for the first time that the lipid A chemical structure of LPS directly extracted from saliva is diametrically different between individuals with healthy periodontium and patients with periodontitis. Mass-spectrometric analyses of lipid As isolated from periodontitis patients revealed the presence of high m/z ratio peaks, likely to correspond to

highly immunogenic, hyper-acylated and phosphorylated lipid A isoforms. The healthy individuals were almost free from these isoforms and showed the prevalence of low m/z peaks, corresponding to under-acylated and hypo-phosphorylated lipid A isoforms, with immuno-modulatory properties, likely to contribute to immune homeostasis. Considering other human endotoxin- induced diseases, it has been shown that patients infected with *N.meningitidis* strains, producing under-acylated lipid As, presented significantly less frequently with rash and had higher thrombocyte counts, consistent with reduced cytokine induction and less activation of tissue-factor mediated coagulopathy (Fransen et al., 2009). Similarly, *P.aeruginosa* strains associated with severe cystic fibrosis lung disease lacked deacylated lipid A structures and the ratio of penta- and hexa-acylated lipid A had a profound impact on the disease progression (Cigana et al., 2009). Interestingly, our post-treatment saliva samples displayed a wide range of m/z peaks in both positive and negative ion mode, which appeared as a combination of peaks found in healthy and diseased samples. This could be a sign of a transition from diseased to healthy salivary lipid A profile or conversely from a healthy one, induced by the periodontal therapy, to the more pathogenic, leading to the remission of the disease. The observed changes in lipid A structure could also be the consequence of the increased microbial diversity in periodontitis patients and the higher prevalence of Gram-negative bacteria. We have already reported that the chemical composition of lipid As isolated from post-treatment subgingival plaque samples was similar to healthy samples (Strachan et al., 2018), indicating that the salivary lipid A profile is more resistant to changes induced by periodontal treatment compared to the subgingival niche. Temporal stability of salivary bacterial communities, especially in terms of its membership, has already been indicated both in studies with no treatment intervention and up to 2 years after periodontal therapeutic protocols (Yamanaka et al., 2012). We could speculate that

during the time course of pathogenic and adaptation mechanisms involved in the development of periodontitis, there might be a time point after which changes in salivary lipid A profile become well established and irreversible.

Microbiome-derived LPS has the ability to facilitate host tolerance, model host-microbiome interactions and influence the outcome of microbiome-linked diseases (d'Hennezel, Abubucker, Murphy, & Cullen, 2017). A holistic approach to periodontal care should account for our current understanding of the interplay between different microbial, environmental, behavioural and genetic risk factors in the aetiology and pathogenesis of periodontitis. As part of optimal patient management, precision dental medicine requires ongoing, individual risk assessment to evaluate the possibility of future disease development. Emerging evidence suggest that clusters of bacteria, rather than individual species, might be of use as diagnostic markers for periodontal disease; and that bacterial functions may be a more robust discriminant of disease than species (Trombelli, Farina, Silva, & Tatakis, 2018). It is increasingly acknowledged that periodontitis is caused by a synergistic and dysbiotic community of pathobionts rather than by “select periodontal pathogens” (Jiao, Hasegawa, & Inohara, 2014). Therefore, when studying associations between microbes and periodontitis, the focus should be on shared virulence factors and microbe-associated molecular patterns (MAMP) such as LPS.

LPS triggers numerous pathophysiological, immunostimulatory effects in mammalian organisms and in higher doses can also lead to life-threatening reactions such as the induction of septic shock. Cells of the myeloid lineage have been shown to be the principal cellular sensors for LPS (Munford, 2016). The lipid A component is the primary immunostimulatory centre of LPS. With respect to immunoactivation in mammalian systems, the classical group

of strongly agonistic (highly endotoxic) forms of LPS are characterised by a high degree of acylation and phosphorylation pattern. In addition, several natural or synthetic lipid A structures with lower levels of acylation and phosphorylation have been shown to display comparatively low or even no immunostimulatory effect. Some members of the latter are even capable of antagonizing the effects of strongly stimulatory lipid A isoforms (Steimle, Autenrieth, & Frick, 2016).

There is strong evidence that aggregates and not monomers of LPS play a decisive role at least in the initial stages of immune cell activation and that the length of the saccharide chain is an important determinant of the morphology, size distribution and aggregate structure of LPS (Richter et al., 2011). Using purified smooth (S) and rough (R) fractions of LPS, it has been shown that the R-LPS fraction induces a stronger immune response than the smooth LPS fraction (Vedrine et al., 2018) and that the highly glycosylated LPS fraction requires different receptors and molecular pathways to induce cell activation (Pupo et al., 2013). The R-LPS recruits a larger spectrum of cells in endotoxic reactions than S-LPS since it readily activates cells expressing the TLR4/MD-2 receptor complex, while the S-form requires further help of the LPS-binding proteins CD14 and LBP, which limits its activating capacity (Huber et al., 2006). Our results revealed the predominance of smooth LPS types with long and intermediate saccharide chains in saliva of healthy individuals and a mixture of smooth and rough LPS chemotypes in both diseased and post-treatment samples, indicating the presence of LPS isoforms with increased endotoxin potential in periodontitis patients which remained unchanged following periodontal treatment.

Total LPS produced by the healthy human gut microbiome, characterised by the prevalence of underacylated lipid A structural components is non-immunogenic and more importantly

inhibits TLR4- dependent cytokine production (d'Hennezel et al., 2017). There are only few studies considering salivary endotoxin activity and inflammatory potential with inconclusive results. Liukkonen *et al.* did not find differences in LPS activity in saliva samples from periodontitis patients and edentulous individuals and salivary LPS concentrations did not have significant associations with any of the tested cytokines (Liukkonen, Gursoy, Pussinen, Suominen, & Kononen, 2016). In contrast, Liljestrand *et al.* (Liljestrand et al., 2017) reported a direct association between alveolar bone loss and levels of salivary LPS and a moderate correlation with periodontal inflammatory burden. Decreased levels of pro-inflammatory cytokines were observed in saliva during the development of experimental gingivitis (Belstrom et al., 2017), while increased concentrations of IL-1 β , IL-4, IL-6 and IL-17 have been detected in saliva of periodontitis patients compared to healthy individuals (Marques et al., 2016). The main strength of our study is that we compared the chemical structure and biological activity of LPS directly extracted from saliva as opposed to extracting LPS from bacterial colonies isolated from the oral cavity, since LPS modification is a widespread occurrence and bacterial biofilm communities and environmental alterations are associated with profound physiological changes that lead to novel properties compared to the properties of planktonic bacteria (Chalabaev et al., 2014).

In our study, salivary endotoxin activity was significantly higher in diseased patients and remained high even after periodontal treatment compared to healthy individuals. The two sub-groups of diseased patients (one in the lower range of EU/ml and the other in the higher) may represent the episodic nature of periodontal disease progression, which is currently undetermined at point-of-care. The levels of TNF- α , IL-8 and IP-10, responsible for alveolar bone resorption and chemoattraction of inflammatory cells, secreted by THP-1 cells challenged with salivary LPS extracts, were significantly higher in diseased patients and

correlated well with their endotoxin activity measure by the recombinant factor C assay. Total salivary LPS from healthy patients was almost immune-silent and triggered measurable production of IP-10 only. These differences could be a combined result of LPS architectural modifications and alterations in total amounts of salivary LPS extracts but the influence of LPS contaminants, such as lipoproteins, cannot be ignored. Periodontal therapy and decreased post-therapy periodontal inflammatory burden had no influence on salivary LPS activity or inflammatory potential, the latter being even higher in post-treatment samples.

In conclusion, characteristic and divergent salivary LPS molecular signatures are present in patients with periodontitis compared to healthy individuals, resulting in increased salivary LPS activity and inflammatory potential in diseased patients. Moreover, salivary LPS architecture, endotoxin activity and inflammatory potential are well conserved after periodontal therapy, reflecting the influence of oral bacterial communities other than dental plaque on salivary LPS profile and emphasising the potential importance of effective oral mucosal microbiota management on long-term periodontal stability.

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Figures:

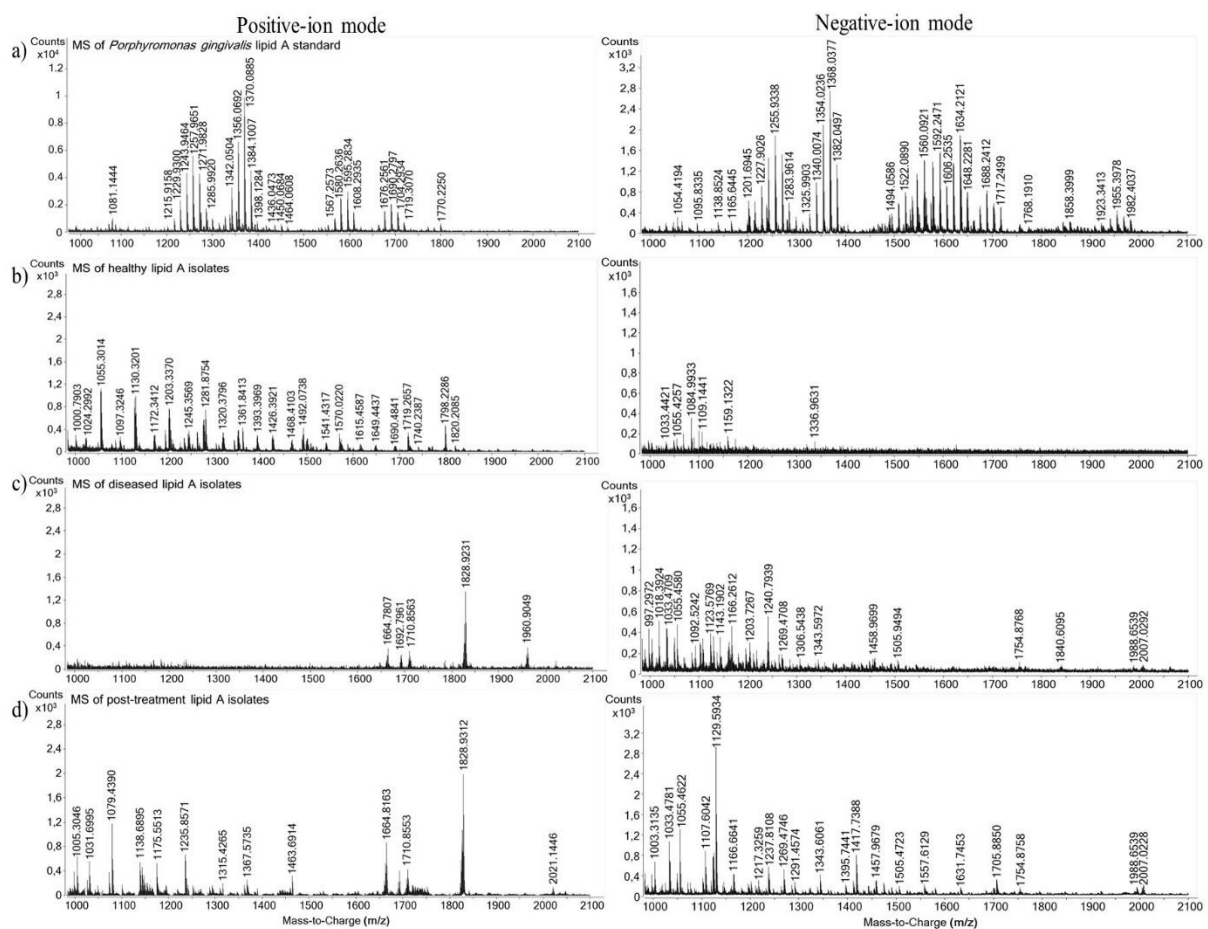


Figure 1. Illustrative electrospray ionisation mass spectra of lipid A isolated from a) *P. gingivalis* LPS (InvivoGen); and pooled salivary LPS extracts obtained from b) healthy

individuals; c) periodontitis patients; and d) periodontitis patients three months after periodontal therapy.

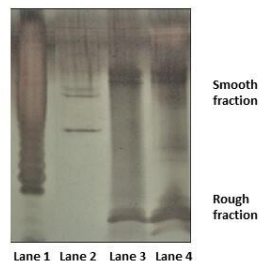


Figure 2. SDS-PAGE and silver staining analysis of *P. gingivalis* LPS (InvivoGen) (lane 1); and salivary LPS isolates from healthy individuals (lane 2); periodontitis patients (lane 3) and post-treatment samples (lane 4).

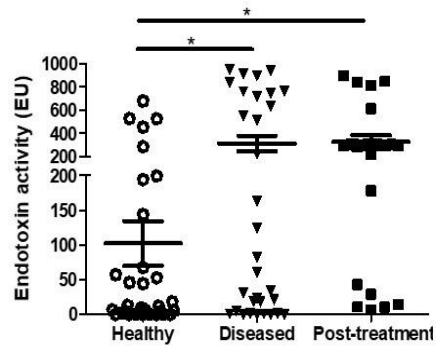


Figure 3. Endotoxin activity of salivary LPS extracts from individuals with healthy periodontium and periodontitis patients before and after periodontal treatment. Lines represent mean values with SEM.

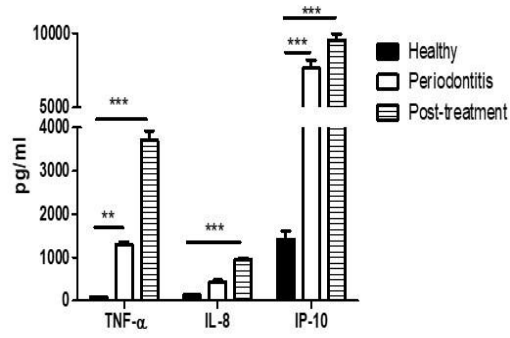


Figure 4. The production of TNF- α , IL-8 and IP-10 by monocytic cell line (THP-1) challenged with pooled LPS extracts from healthy, diseased and post-treatment saliva samples for 4 hours.