Coronary Artery Disease Risk is Increased by Periodontal Pathobionts

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Short title: Periodontal pathobionts as a novel risk factor for myocardial infarction

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Abstract

Objective:

To investigate the independent predictive risk of periodontal indicators in coronary artery disease (CAD).

Methods/results:

Acute myocardial infarction (MI) patients (n=160) and 50 controls of same age/gender distribution were assessed for periodontal inflamed surface area (PISA), probing pocket depths (PPD), clinical attachment loss (CAL), periodontal pathobionts' loads and virulence genes expression. The proportion of variation (ΔR^2) in two MI outcome variables- myocardial injury [troponinI (TnI)] and CAD burden (SYNTAX-I scores) caused by each periodontal indicator was assessed using hierarchical linear regression. Results showed MI patients had worse periodontitis and higher pathobionts' loads (all $p<0.05$ *vs* controls). In MI, after CAD risk factors adjustment, increased PISA (ΔR^2=5%), mean-PPD (ΔR^2=3%), mean-CAL (ΔR^2=2%), *P. gingivalis* load (ΔR^2=4%) and expression of virulence genes *bioF-3*, *fimA*, *prtH*, *prtP*, *ltxA*, *cdtB* (ΔR^2=4-9%) were associated with increased TnI, all $p<0.05$. Increased mean-PPD (ΔR^2=5%), mean-CAL (ΔR^2=4%), *P. gingivalis* load (ΔR^2=5%) and expression of *bioF-3*, *fimA*, *prtP* and *ltxA* (ΔR^2=4-9%) were associated with higher SYNTAX-I score, all $p<0.05$. In MI patients, intra-subject *P. gingivalis* and *T. forsythia* loads from aspirated intra-coronary thrombi and subgingival plaque showed a strong correlation (r=0.6, $p<0.05$).

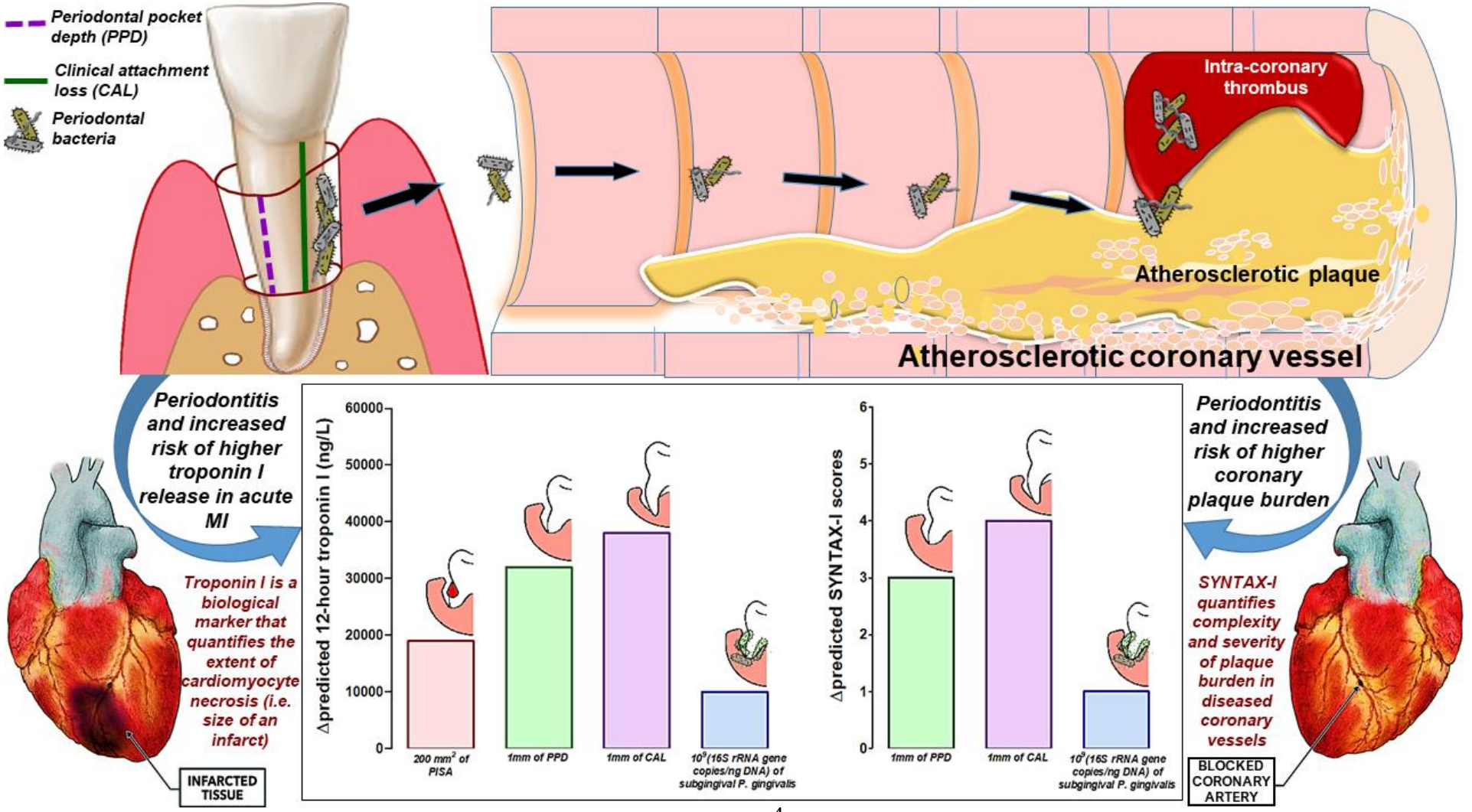
Conclusion:

Periodontitis should be considered in CAD secondary prevention strategies and periodontal pathobionts may offer novel targets for CAD risk reduction.

Trial registration: ClinicalTrials.gov Identifier: NCT04719026

Keywords: acute myocardial infarction, coronary artery disease, periodontitis, periodontal bacteria, periodontal virulence factors, pathobiont

Central illustration/graphical abstract: The current study demonstrated that the extent of acute myocardial injury and the severity of coronary artery plaque burden increased with higher subgingival pathobiont load and with upregulated virulence gene expression of periodontal pathobiont species, along with the higher quantitative clinical indicators of periodontitis (PISA, CAL and PPD). Several periodontal pathobionts' DNA was detected in coronary thrombi



Introduction

Periodontitis has an increasingly recognised association with atherosclerotic cardiovascular disease, particularly with coronary artery disease, stroke and essential hypertension (1). Case-control epidemiological studies have shown a relationship between suboptimal periodontal health and an increased risk of developing an acute myocardial infarction (2-6). However, all previous reports have relied on surrogate markers of periodontitis exposure, either as clinical assessments (inflamed area, attachment loss, pocket depths), radiographic assessment of bone loss or self-reported questionnaires (3, 4, 6, 7). No studies examined directly the relationship between coronary artery disease outcomes and bacterial species which colonise the periodontal environment and can become pathogenic under altered conditions, also known as "pathobionts". Five bacterial species are lead candidates for transition to pathobiont status in the periodontal environment, promoting oral dysbiosis and the chronic inflammatory responses of periodontitis: *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* (8, 9). Transcriptomic studies demonstrated they express virulence factors that contribute to periodontitis progression and possibly to systemic bacterial dissemination (10, 11). Indeed, recent data identified DNA of bacteria involved in chronic infections, inclusive of some periodontal species, in the occlusive intra-coronary thrombi from patients with acute myocardial infarction (MI), supporting the possibility that such bacteria could translocate from other locations to atherosclerotic plaques (12, 13).

In the current study, we first established if increased periodontitis severity as assessed by clinical indicators of periodontal disease progression and inflamed periodontal surface area relates to worse cardiovascular status, such as infarct size and/or increased coronary artery plaque burden in patients presenting with an acute MI. Second, we investigated if amongst the pathobionts assayed, bacterial load and expression of bacterial virulence genes are predictors of the extent of acute myocardial injury or the underlying coronary artery plaque burden. Finally, in a subset of acute ST-elevation MI patients, we explored whether pathobiont species can be detected *at the intra-patient level* from both their subgingival plaque and the coronary thrombus extracted during the primary percutaneous intervention.

Methods

Study populations

Consecutively consenting patients hospitalised with type-1 MI (14) at Aberdeen Royal Infirmary between March 2018 and September 2020 were enrolled in this single centre, prospectively recruited cross-sectional investigation. Additionally, subjects habitually attending Dental School at the University of Aberdeen and free of any history or symptoms of coronary artery disease were invited

as controls. All those with a history of recent infection, inflammatory or allergic conditions, renal or hepatic impairment, malignancy, pregnant women, or anyone with prior use of antibiotics, anti-inflammatory medication, calcium-channel blockers, phenytoin or cyclosporin were excluded. This study complies with the Declaration of Helsinki and was approved by the Cambridge east research ethics committee (16/EE/0283). The written informed consent was obtained from all participants according to the World Health Organisation guidelines for good research practice.

Study protocol

MI patients (n=160) underwent assessment within 3 days of acute MI presentation. Study assessments included a comprehensive periodontal examination with the collection of subgingival dental plaque, saliva, blood samples. Routine 12-hour Troponin I (TnI) and coronary angiography were included in the consented assessments for the study. In a sub-group of patients presenting with ST-elevation (n=10), aspirated coronary thrombi and the balloons used for coronary stent deployment were collected. Fifty controls of similar age and gender distribution underwent periodontal examination and blood sampling but not coronary angiography. The control group received an ECG and echocardiogram to rule out any significant subclinical findings of coronary disease.

Periodontal examination

A full-mouth assessment following the British Society of Periodontology guidelines (15) measured gingival recession as probing pocket depth (PPD) (16) and clinical attachment loss (CAL) (17) in millimetres, presence of bleeding on probing (BoP) and dental plaque around six sites (disto-buccal, mid-buccal, mesio-buccal, disto-lingual, mid-lingual, mesio-lingual) per tooth, excluding 3rd molars, dental implants and retained roots. The site-specific measurements were added to calculate mean PPD, mean CAL, percentage bleeding (18) and plaque indices (PI) (19) for each patient. Periodontitis was diagnosed according to "the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions" case definition (20). The number of missing teeth was recorded. Subsequently, Periodontal Inflamed Surface Area (PISA) (21) score, which quantifies the BoP-positive periodontal pocket epithelium surface area in mm^2, was calculated for each patient. A graphic representation of periodontal measurements is described in the **Supplementary file, Figure 1**. A single examiner (CJ) screened eligible participants and carried out the clinical periodontal examination of all study participants using a computerised periodontal Florida Probe (Florida Probe Corporation, Gainesville, Florida, USA). The probe imparts a constant force reducing intra-examiner variability and thereby, provides standardisation as per manufacturer guarantee. Inter-study variability was performed in 10 controls in consecutive days and was 1±1% for both PPD and CAL.

Subgingival plaque collection

In each oral quadrant, one deepest periodontal pocket was selected, dried and isolated using sterile cotton rolls before sampling. A sterile ProTaper Next® absorbent paper point X3 (Dentsply Sirona, Surrey, UK) was inserted passively to the bottom of each periodontal pocket for 30s and discarded if any bleeding was encountered. Subsequently, all four paper points were pooled in a sterile Greiner Bio-One™ microtube (Thermo Fisher Scientific Inc., UK) containing 200µL of phosphate-buffered saline (PBS) (Sigma-Aldrich Company Ltd., Dorset, UK) and stored at -80˚C. In every 4th recruited MI patient (n=40), an additional sample was collected from the second deepest periodontal pocket per oral quadrant following the same procedure.

Saliva sample collection

A 2mL unstimulated saliva sample was collected in the morning after a 1 hour fast (before the periodontal examination) using a passive drool method (22) and stored at -80˚C in Nalgene™ cryogenic tubes (Thermo Fisher Scientific Inc).

Aspirated coronary artery thrombi and primary percutaneous intervention balloons

When clinically judged appropriate by the interventional cardiology operator, a high thrombus load was aspirated prior to primary percutaneous stenting using a Pronto V3 or Export extraction catheter. The aspirated thrombi from the culprit coronary artery and the primary percutaneous balloons (n=10 each) were retrieved in an aseptic environment, transferred immediately into sterile Greiner Bio-One™ microtubes containing PBS buffer and stored at -80˚C. A venous blood sample from the same patient was used as a comparator.

Blood sampling

10mL venous blood was collected in serum separator tube (BD, Oxford, UK), centrifuged (3000 rpm for 10 minutes, 4˚C) and serum aliquoted in Greiner Bio-One™ microtubes for storage at -80˚C.

Quantification of primary periodontal bacterial loads

Genomic bacterial DNA from subgingival plaque samples (n=210), aspirated intra-coronary thrombi, the respective deployment balloons and venous blood (n=10 each) was extracted using the GenElute™ Kit (Sigma-Aldrich Company Ltd). Primers and probes were designed to target hypervariable regions (V) of the 16S ribosomal RNA gene of the selected periodontal bacterial species: *P. gingivalis* (V3), *T. forsythia* (V4), *A. actinomycetemcomitans* (V2) *and P. intermedia* (V7) - **Supplementary Table 1.** The full quantitative polymerase chain reaction (qPCR) protocol is reported in **Supplementary file, section 1**. In each clinical sample, bacterial loads of the aforementioned periodontal bacterial species were quantified using absolute quantification and reported as 16S rRNA gene copies/ng DNA (23) (**Supplementary file, section 1).** The positivity

threshold for bacterial detection was set at the 35th quantification cycle (Cq), above which a sample was considered negative for the tested bacterial species, **Supplementary file, Figure 2**.

Virulence genes expression assays

Total bacterial RNA was extracted from the additional subgingival plaque collected from a subset of 40 MI patients (n=40) using the GenElute™ Total RNA Purification Kit (Sigma-Aldrich Company Ltd) and subsequent DNase treatment (on-Column DNase-I Digestion Set, Sigma-Aldrich Company Ltd). Purified RNA was used as template in one-step quantitative reverse transcription PCR (RT-qPCR) assays to target thirteen recognised genes encoding major virulence factors of *P. gingivalis (kgp, rgpA, fimA, bioF-3)*(24-26)*; T. forsythia (prtH, bspA, siaHI)*(27, 28)*; T. denticola (prtP, msp)*(29)*; A. actinomycetemcomitans (ltxA, cdtB)*(30, 31) *and P. intermedia (clpB, dnaK)*(32), (**Supplementary file, section 2**). The biological functions of the selected virulence factor genes are described in the respective papers previously (24-31). The primer-probe sequences used to target these virulence genes are described in **Supplementary Table 2**, respectively. Virulence gene expression was quantified using the ΔΔCT method (33) against 3 reference genes (*recA, glyA* and *groL)* and expressed as fold change in compliance with 'the minimum information for publication of quantitative real-time PCR experiments (MIQE)' guidelines (34).

Local and systemic host immune profile against Porphyromonas gingivalis

Serum IgG, IgM, IgA and salivary secretory IgA2 isotype antibody responses against *P. gingivalis* lipopolysaccharide (LPS) were quantified using enzyme-linked immunosorbent assay (ELISA) (**Supplementary file, section 3**).

Cardiac biomarkers and Coronary angiography

For each recruited patient, troponin I (TnI) value with the lower cut-off/reporting limit (99th centile) of 40ng/L (Siemens ADVIA Centaur® TnI Ultra® assay, Surrey, United Kingdom) was collected 12 hours from the onset of chest pain and used as a surrogate marker of acute myocardial injury (35). The invasive coronary angiography study performed during the index admission was analysed using quantitative coronary arteriography analysis (QCA) by an investigator (MG), blinded to the periodontal status. The Synergy between PCI with TAXUS drug-eluting stent and Cardiac Surgery score (SYNTAX-I, http://www.syntaxscore.com/) (36) was calculated to quantify the complexity and severity of coronary artery disease burden in the native coronary vessels.

Statistical analyses

The normality of continuous variables was assessed using the Kolmogorov-Smirnov test supplemented with histograms. Normally distributed variables were summarised using means and standard deviations (SD) and skewed variables with medians and interquartile ranges (IQR).

Descriptive categorical measures were expressed as frequencies (n) and percentages (%). For case-control comparison, the differences between categorical variables were examined using the Chi-squared test. Normally distributed continuous variables were compared using Student's t-test and skewed continuous variables with Mann-Whitney U test. In the MI population, the predictive relationship of clinical, microbial and immunological periodontal indicators on the outcome measures (12-hour TnI levels and SYNTAX-I scores) was analysed using hierarchical linear regression. Firstly, baseline risk factors *common to periodontitis and MI*, such as age, gender, Scottish Index of Multiple Deprivation (SIMD), body mass index (BMI), diabetes, smoking were included in the basic regression model-I. Additionally, to account for MI presentation type (ST-elevation vs non-ST-elevation MI), the basic model-I was further adjusted for ST-elevation status. Subsequent models were constructed separately by adding one predictor of interest in each model (PISA, mean PPD, mean CAL, subgingival bacterial loads and antibody levels, each added to the basic model-I separately). Similarly, the effect of virulence gene expression levels on the outcome variables was tested in hierarchical linear regression. Since virulence gene expression levels were analysed in a patient subgroup (n=40), to reduce the risk of overfitting, a parsimonious basic model-I was constructed with the most important MI confounders based on the previous analysis findings *i.e.* age, BMI, diabetes, smoking and ST-elevation. The change in R^2 indicated the improvement in the proportion of variation in the outcome variable explained by adding one predictor of interest. Based on the linear regression models, we plotted the incremental change in acute myocardial injury (12-hour TnI) and coronary artery disease burden (SYNTAX-I score) corresponding to a per-unit change in PISA, mean PPD, mean CAL, and bacterial loads, respectively. The periodontal bacterial loads from aspirated intra-coronary thrombi and subgingival plaque samples collected from the same individuals were compared by Spearman's correlation. Variabilities were calculated as mean±SD of the percentage ratios between differences and means of the 2 independently measured variables. Data were analysed using IBM SPSS Statistics v.25 (IBM Corp., Armonk, NY, USA) software and graphs were prepared using GraphPad Prism v 5.04 (GraphPad Software, San Diego, CA, USA). The level of statistical significance was set at 5%. The corresponding author has full access to all the datasets used for analysis and it will be shared at a reasonable request.

Results

Baseline characteristics of the 160 type-1 MI patients and 50 controls are described in **Table 1**. The majority of MI patients were middle-aged men from a mix of socio-economic backgrounds, current or former smokers, with high body mass index (BMI) and typical comorbidities for such a cohort. Most MI patients had mild leucocytosis with neutrophilia and mildly elevated C-reactive protein levels, typical of systemic pro-inflammatory status associated with acute MI. MI patients were more

likely to have a history of hypertension, diabetes or hypercholesterolaemia, as such, a higher proportion received statins pre-admission and thus had lower levels of total and LDL-cholesterol compared to controls. Two-thirds presented with ST-elevation MI, reflected by the significant degree of acute myocardial injury measured as 12-hour TnI. Two-thirds of the MI patients and one-third of controls demonstrated the presence of clinical periodontitis. There were no differences between the ST-elevation and non-ST-elevation patients regarding the clinical periodontal measurements, bacterial loads or antibody levels. When compared to controls, MI patients had significantly higher values of periodontitis-related clinical indicators suggesting the presence of a more severe form of periodontitis, accompanied by significantly higher bacterial loads and anti-*P.gingivalis* serum and salivary antibody levels.

Additive prognostic value of quantitative indicators of clinical periodontitis and periodontal bacterial load in subgingival plaque in the acute MI-patient population

The basic model-I showed that age, BMI, diabetes, smoking and ST-elevation were significant predictors of the extent of myocardial injury (all $p<0.05$), whereas age, smoking and ST-elevation were predictors of the coronary disease burden (all $p<0.05$) (**Table 2, upper panel**). Subsequently (**Table 2, lower panel**), the hierarchical linear regression analysis revealed an increase in the percentage of variation explained (ΔR^2) for the extent of myocardial injury (TnI release) of 5% when PISA was added to the basic model (model II, $p=0.02$), 3% for mean PPD (model III, $p=0.03$), 2% for mean CAL (model IV, $p= 0.01$) and 4% for subgingival *P. gingivalis* bacterial load (model V, $p= 0.01$). Similarly, the increase in R^2 for the model predicting coronary artery plaque burden (SYNTAX-I score) was 5% when mean PPD was added to the basic model (model III, $p=0.02$), 4% for mean CAL (model IV, $p= 0.03$) and 5% for subgingival *P. gingivalis* bacterial load (model V, $p= 0.003$). The independent addition of PISA had no significant effect on the SYNTAX-I score. The addition of subgingival *T. forsythia, A. actinomycetemcomitans* and *P. intermedia* bacterial loads had no significant effect on 12-hour TnI levels or SYNTAX-I scores.

The linear equations of models II-V (adjusted for the established risk factors) showed that with every 200 mm^2 incremental change in PISA, there was a 19,000 ng/L increase in the 12-hour TnI level. Similarly, every 1 mm increase in either mean PPD or mean CAL corresponded to 32,000 and 38,000 ng/L increase in 12-hour TnI levels, respectively. An increase of 10^9 (16S rRNA gene copies/ng DNA) of *P. gingivalis* load in subgingival plaque equated to a 10,000 ng/L increase in 12-hour TnI level. In terms of total coronary plaque burden, every 1mm increase in either mean PPD or mean CAL equated to a 3 and 4 unit increase in the SYNTAX-I scores, respectively. An increment

of 10^9 (16S rRNA gene copies/ng DNA) of *P. gingivalis* load in subgingival plaque was responsible for a 1 unit increase in SYNTAX-1 score (**Fig. 1**).

Additive prognostic value of increased expression of periodontal bacterial virulence genes in subgingival plaque in the acute MI-patient population

In a subgroup of 40 MI patients, the hierarchical linear regression models revealed that age, BMI, diabetes, smoking and ST-elevation were significant predictors of the extent of myocardial injury (all $p<0.05$), whereas age, smoking and ST-elevation were predictors of the coronary disease burden ($p<0.05$ for both) (**Table 3, upper panel**). Subsequent models (**Table 3, lower left panel**) showed an increase in the percentage of variation explained (ΔR^2) for the extent of myocardial injury (TnI release) of 4% and 9% for *P. gingivalis*-related *fimA* (model IV) and *bioF-3* (model V) gene expression ($p=0.002$ for both). *T. forsythia prtH* expression (model VI) increased it by 7% ($p=0.006$) and *T. denticola prtP* (model IX) by 9% ($p=0.002$). Lastly, expression of *A. actinomycetemcomitans ltxA* (model XI) and *cdtB* (model XII) increased the percentage of variation in TnI release by 8% and by 7% ($p=0.007$ and $p=0.009$, respectively). Similarly, the increase in R^2 for the model predicting coronary artery plaque burden (SYNTAX-I score) after independent addition of *P. gingivalis fimA* (model IV) and *bioF-3* (model V) expression was 4% ($p=0.002$) and 9% ($p=0.005$), respectively. *T. denticola prtP* expression (model IX) increased the percentage of variation in SYNTAX-I score by 9% ($p=0.001$) and *A. actinomycetemcomitans ltxA* (model XI) gene expression by 6% ($p=0.01$) (**Table 3, lower right panel**).

Simultaneous detection of periodontal pathobiont bacteria in subgingival plaque and intra-coronary thrombi in acute MI patients with clinical periodontitis

In the subgroup of 10 acute ST-elevation MI patients, whose coronary thrombi were aspirated, 3 patients were periodontally healthy and their coronary thrombi samples were negative for DNA of the four tested bacterial species (>35 Cq value). The remaining 7 patients had clinical periodontitis with varying severity and the frequency of periodontal bacteria detection in their coronary thrombus was highest for *P. gingivalis* (7/10), followed by *T. forsythia* (6/10) and *P. intermedia* (5/10) and lowest for *A. actinomycetemcomitans* (2/10). Bacterial loads of the four assayed pathobiont species in aspirated coronary thrombi and subgingival plaque collected from the 10 patients are shown in **Figure 2**. A strong correlation was observed between bacterial loads detected in coronary thrombi and the corresponding subgingival plaque from the same patient for *P. gingivalis* and *T. forsythia* (Spearman's rho=0.6 and $p<0.05$, for both) but not for *P. intermedia or A. actinomycetemcomitans* (**Fig. 2**). No bacterial DNA of the tested four species was detected in percutaneous intervention balloons or venous blood samples.

Local and systemic immune host profile against *Porphyromonas gingivalis*

No significant relationship between any of the anti-*P. gingivalis* LPS antibody isotypes (serum/salivary) (models IX-XII) (**Table 2, lower panel**) and cardiac outcome measures (12-hour TnI levels and SYNTAX-I scores) was observed.

Discussion

In a prospectively recruited cross-sectional study of acute MI patients, who had significantly worse clinical periodontal disease compared to a control group, we showed that: 1) quantitative clinical indicators of active periodontitis (PISA, CAL and PPD), increased subgingival bacterial load of *P. gingivalis*, and increased expression of periodontal pathobionts' virulence genes are significant predictors of acute myocardial injury extent and underlying coronary artery plaque burden; 2) periodontal bacterial DNA was detected in intra-coronary thrombi; there was a strong within-subject correlation for the bacterial loads of *P. gingivalis* and *T. forsythia* isolated from subgingival plaque and coronary thrombus, respectively. Taken together, these findings provide new supporting evidence that periodontal pathobionts which underlie the process of periodontitis are novel targets as risk reduction strategies in coronary artery disease, which need to be addressed in clinical cardiology and dental practice.

Study Rationale

Despite major advances in primary and secondary prevention of coronary artery disease through risk factors modification, coronary artery disease remains responsible for most deaths worldwide. Identification of additional contributory risk factors may provide further risk reduction. Periodontitis is one such suggested risk factor but it remains unknown if periodontal pathobionts contribute to risk increase.

Periodontitis: prevalence, case definitions and methodological variation

The percentage of individuals affected by periodontitis in our MI cohort (66%) was higher than controls (30%) and the overall prevalence of periodontitis in our MI patient group was much higher than previously reported studies (3, 6). Whilst dental care differs between countries, there are also differences with the use of periodontitis case definitions and periodontal examination methods. For example, Ryden *et a*l.(6) based their diagnosis on the percentage of radiographic alveolar bone loss, while Gomes-Filho et al., used a case definition developed by their own group previously (3). Whilst X-ray examinations are ideally suited for large throughput studies, we opted for the latest case definition of periodontitis (20) as we were able to apply detailed examination in our chosen sample size. To mitigate variability, we used a computerised Florida periodontal probe addressing the methodological issues associated with manual periodontal probes, which either over or under-estimate disease severity (37). The Florida probe imparts a constant probing pressure reducing the

intra and inter-examiner variability while increasing reproducibility (38), as demonstrated by our low intra-study variabilities. Therefore, our findings are likely to be generalisable.

Contribution of clinical indicators of periodontitis, subgingival periodontal pathobiont load and expression of their virulence factors

The collective contribution of inflammatory processes occurring in the periodontal milieu was assessed by 3 quantitative clinical indicators of periodontitis (PISA, PPD and CAL) as done by previous investigators (3). All 3 significantly predicted the extent of acute myocardial injury, while PPD and CAL were significant predictors for the underlying chronic coronary artery plaque burden. PISA delineates BoP-positive periodontal epithelial surface area and was only associated with acute cardiac outcomes (Tn I). This measure of increased periodontal bleeding could be a reflection of a generalised low-grade inflammatory state, known to be present during an acute coronary event (39). Although a confounder role of dual antiplatelet therapy cannot be ruled out, previous reports suggest that patients on dual antiplatelet therapy generally have no increased periodontal bleeding compared to controls (40).

Among the primary periodontal pathobiont bacterial species, our study showed that the bacterial load of *P. gingivalis* independently predicted both a worse myocardial injury after acute MI as well as a higher/more severe chronic plaque burden accumulation. However, the presence of high bacterial loads of *P. gingivalis* or other pathobionts (41) is not solely causative of inflammatory disease *per se* (locally and systemically) unless accompanied by gene expression shifts in favour of well-characterised virulence factors(10, 11). The overexpression of virulence factors is the basis of the transition of periodontal bacteria from a commensal to pathogenic status driving tissue destruction through proteolytic activity, inflammatory responses, immune evasion. Animal studies based on ApoE knock-out mice have also suggested a role for these genes in pro-inflammatory responses leading to atherosclerosis (42). In this study, for the first time, we demonstrate that the upregulation of genes encoding pathogenic phenotypes in four periodontal bacteria is an independent predictor of the extent of myocardial injury and severity of underlying coronary atherosclerotic plaque burden. More specifically, the genes implicated are responsible for a range of phenotypes relevant to atherosclerosis, namely: i) mounting pro-inflammatory responses *via* TLR-2 pathway (*bioF-3* gene; *P. gingivalis*) (26, 43, 44), *ii)*) bacterial adhesion leading to intracellular and transepithelial invasion (*fimA* gene; *P. gingivalis*) (45, 46), iii) degradation of the intercellular adhesion proteins facilitating bacterial invasion beyond epi/endothelium (*prtH* and *prtP* genes; *T. forsythia, T. denticola*) (28, 47, 48), and iv) evading host-defences (*ltxA* and *cdtB* genes; *A. actinomycetemcomitans*) (30, 31). Future studies investigating mechanistic links between these factors and coronary artery disease are warranted.

Simultaneous detection of periodontal pathobionts in subgingival plaque and intra-coronary thrombi of MI patients with clinical periodontitis

As discussed above, using a repertoire of virulence factors, periodontal bacteria breach the confines of periodontal pockets. Here we recovered their DNA in aspirated intra-coronary thrombi responsible for acute MI. This is in keeping with previous reports detecting *P. gingivalis*, *P. intermedia* (13) and *A actinomycetemcomitans* (12) in aspirated intra-coronary thrombi. However, we showed for the first-time simultaneous recovery of these bacteria in both subgingival plaque and coronary thrombi, during acute MI presentation in patients with objective evidence of clinical periodontitis. The coronary thrombus bacterial load was generally lower than that in the subgingival plaque, but in two patients the loads were comparable.

None of the venous blood or coronary stent deployment balloons were positive for periodontal bacterial DNA, in keeping with previous findings (12, 13, 49). This is expected as the low-grade systemic bacteraemia typically associated with daily oral activities, such as tooth-brushing, mastication or professional periodontal therapies is short-lived and clears rapidly (50).

Study limitations

A larger size control group would have allowed a case-control evaluation of pathobionts bacterial loads in addition to the prospective cross-sectional evaluation presented here, however, the recruitment of healthy volunteers was severely impacted by covid pandemic conditions.

Conclusion

We demonstrate that quantitative clinical indicators of periodontitis (PISA, mean PPD, mean CAL), increased subgingival bacterial load of the periodontal pathobiont *P. gingivalis* and increased expression of genes encoding virulence factors of four keystone periodontal pathobiont species are predictors of the extent of acute myocardial injury as well as of the severity of underlying coronary artery plaque burden. Bacterial DNA of *P. gingivalis* and *T. forsythia* was simultaneously recovered from both intra-coronary thrombi and subgingival plaque in patients with ST-elevation MI. These data further support the role of periodontitis, and its causative pathobiont species, as an independent risk factor for myocardial infarction. Periodontitis treatment should be considered for coronary artery disease risk prevention strategies.

Clinical perspective

What Is New?

- Quantitative clinical indicators of periodontitis, the subgingival pathobiont load of *P. gingivalis* and upregulated expression of virulence genes of several periodontal pathobiont species independently predict the extent of acute myocardial injury and the severity of coronary artery plaque burden, after adjusting for well-established CAD risk factors.
- Periodontal pathobiont species are detected in coronary thrombi, in lesser or equal number to bacterial loads detected in subgingival plaque samples from the same acute MI patients, strengthening the associative relationship between periodontitis, its causative pathobionts and myocardial infarction.

What Are the Clinical Implications?

- The concept of periodontitis as an additional, treatable risk factor for myocardial infarction should be further emphasised. Periodontal pathobionts which underlie the process of periodontitis are novel targets as risk reduction strategies in coronary artery disease which need to be addressed in clinical cardiology and dental practices.
- Increased patient awareness regarding oral health and its potential contribution to coronary artery disease progression should be widely promoted both in the at-risk and the general population. Regular periodontal care to reduce periodontitis severity and associated microbial burden may offer novel approaches for coronary artery disease risk reduction.

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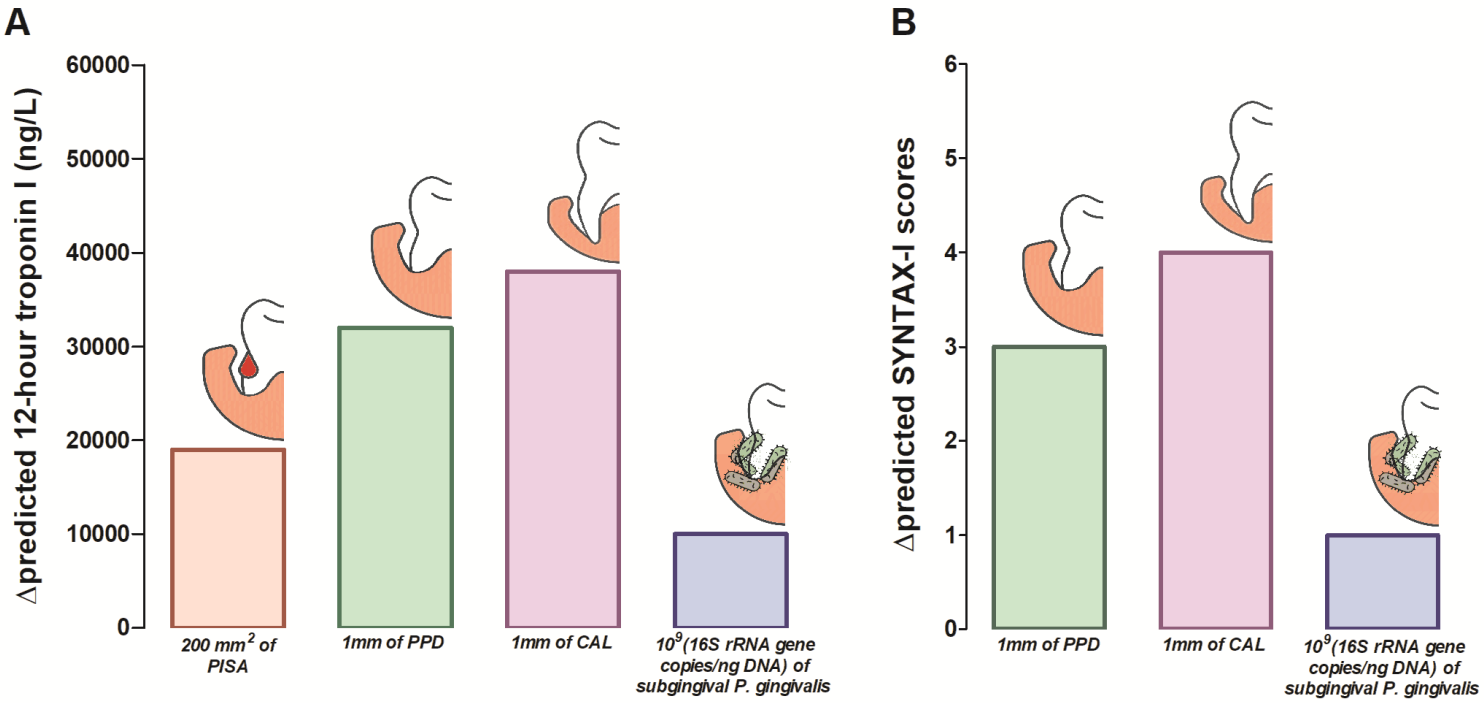
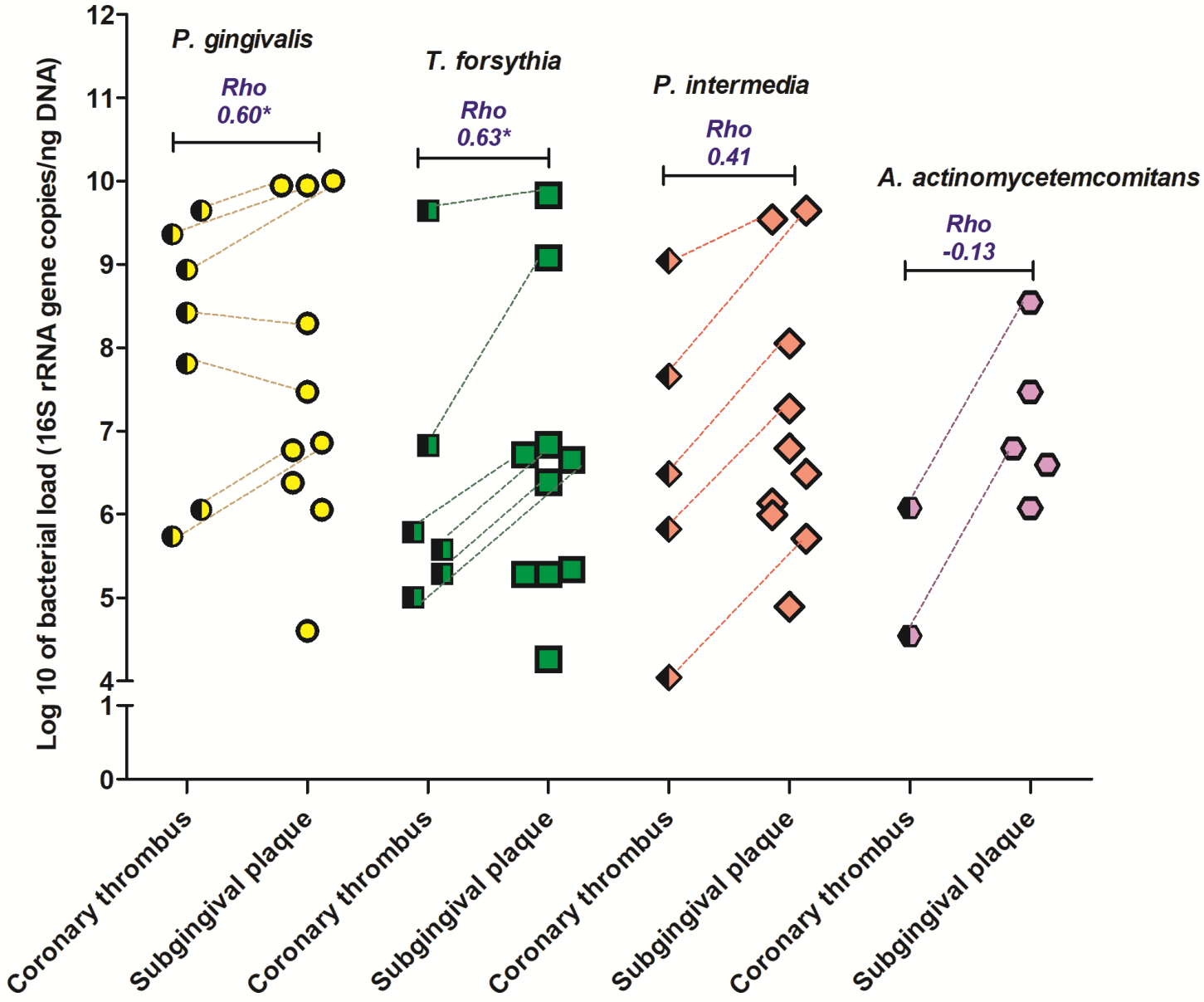
Figures

Figure 1: Schematic representation of incremental change in **A**) predicted 12-hour TnI levels and **B**) predicted SYNTAX-I scores, with worsening of periodontitis



The linear regression analysis revealed that **A**) with every 200mm^2 change in periodontal inflamed surface area (PISA) and 1 mm increase for in either mean probing pocket depths (PPD) or mean clinical attachment loss (CAL), there was approximately 19,000 ng/L, 32,000 and 38,000 ng/L increase in 12-hour TnI levels, respectively. Similarly, every 10^9 (16S rRNA gene copies/ng DNA) increase in subgingival *P. gingivalis* load leads to an increase of 10,000 ng/L in 12-hour TnI levels. **B**) Every 1mm increase in mean probing pocket depths (PPD) or clinical attachment loss (CAL) corresponded to 3 and 4 units increase in SYNTAX-I scores, respectively. Every 10^9 (16S rRNA gene copies/ng DNA) increase in subgingival *P. gingivalis* load corresponds to 1 unit increase in SYNTAX-I score.

Figure 2: Within-subject detection, quantification and correlation of periodontal bacterial pathobiont loads in intra-coronary thrombi and subgingival plaque sampled from ST-elevation MI patients



Corresponding bacterial loads (16S rRNA gene copies/ng DNA) of four periodontal pathobiont bacteria, namely *P. gingivalis* (yellow), *T. forsythia* (green), *P. intermedia* (red) and *A. actinomycetemcomitans* (purple) in aspirated intra-coronary thrombi retrieved from 10 ST-elevation MI patients and their subgingival plaque samples (dashed lines show paired samples from each patient). Rho- spearman's correlation coefficient, * = statistically significant (p<0.05)

Tables

Table 1: General characteristics of the study population

	Myocardial infarction population (n=160)	**CAD-free control population (n=50)**	***p* value**
Males, n (%)	137 (86)	45 (90)	0.1
Age in years, (median, IQRs)	60 (54-67)	60 (49-68)	0.2
BMI in kg/m^2, n (%)			
Obese (≥30 kg/m^2) and overweight (25-29.9 kg/m^2)	131 (82)	36 (72)	0.09
Normal (≤24.9 kg/m^2)	29 (18)	14 (28)	
Scottish Index of Multiple Deprivation (SIMD), n (%)			
Deprived (quintiles 1 to 3)	62 (39)	20 (40)	0.2
Less deprived (quintiles 4 and 5)	98 (61)	30 (60)	
Smoking status, n (%)			
Current and former smokers	94 (59)	25 (50)	0.2
Non-smokers	66 (41)	25 (50)	
Increased alcohol consumption, (%)	127 (79)	40 (80)	0.7
Hypertension, n (%)	68 (42)	11 (22)	0.03[a]
Diabetes (type-1 and 2), n (%)	34 (21)	2 (4)	0.01[a]
Hypercholesterolemia, n (%)	41 (26)	5 (10)	0.02[a]
Self-reported physical activity, n (%)			
Sedentary activity	19 (12)	4 (8)	0.6
Moderate activity	139 (87)	46 (92)	
High activity	2 (1)	0 (0)	
Family history of cardiovascular disease, n (%)	102 (64)	24 (48)	0.06
Family history of periodontitis, n (%)	21 (13)	3 (6)	0.06
Prior statin use, n (%)	41 (26)	5 (10)	0.02[a]
Presenting electrocardiogram (ECG), n (%)			
ST-elevation MI	94 (59)	N/A	

Non-ST-elevation MI	66 (41)	N/A	
Blood biochemistry, (median, IQRs)			
White cell count (normal range: 4-10x10^9/L)	10.4 (8.5-12.2)	6.6 (5.8-8)	0.02[a]
Neutrophils (normal range: 1.5-7x10^9/L)	7.7 (5.6- 9.6)	4 (3-4.6)	0.01[a]
Lymphocytes (normal range: 1.5-4x10^9/L)	1.6 (1.2-2.1)	1.5 (1.2-2)	0.03[a]
Platelets (normal range:140-400x10^9/L)	258 (218-303)	259 (223-279)	0.4
C-Reactive protein (normal range: 0-4 mg/L)	17 (3-43)	N/A	
Total cholesterol (normal range: 3.4-5.2 mmol/L)	4.8 (3.7-5.7)	5.4 (4.7-6)	0.03[a]
Low-density lipoprotein (normal range: 1.4-5 mmol/L)	2.9 (1.9-3.7)	3.2 (2.7-3.9)	0.02[a]
12-hour TnI in ng/L, median (IQRs)	24482 (2869-91983)	N/A	
SYNTAX-I score, median (IQRs)	17 (10-26)	N/A	
Echocardiography-derived left ventricular Ejection Fraction (EF), n (%)			
Severely impaired (EF<30%)	6 (4)	0 (0)	
Moderately impaired (EF 31-45%)	32 (20)	0 (0)	
Mildly impaired (EF 46-54%)	68 (42)	0 (0)	
Normal (EF>55%)	54 (34)	50 (100)	
Pro-bleeding post-MI medications, n (%)			
Dual anti-platelet therapy	132 (82)	N/A	
Dual anti-platelet therapy+anticoagulant	28 (18)	N/A	
Number of diseased coronary vessels, n (%)			
Single vessel disease	66 (41)	N/A	
Two vessel disease	64 (40)	N/A	
Three vessel disease	30 (19)	N/A	
Periodontitis status, n (%)			
Periodontally-healthy	54 (34)	35 (70)	0.01[a]
Periodontitis	106 (66)	15 (30)	
Mild periodontitis (Stage I)	4 (2)	0 (0)	0.01[a]
Moderate periodontitis (Stage II)	53 (33)	6 (12)	

Severe periodontitis (Stage III and IV)	49 (31)	9 (18)	
Periodontal clinical indicators, median (IQRs)			
PISA[b] (mm^2)	330 (190-428)	125.5 (67.6-165.6)	0.02[a]
mean Probing Pocket Depth (mm)	1.92 (1.59-2.31)	1.48 (1.34-1.71)	0.01[a]
mean Clinical Attachment Loss (mm)	1.99 (1.66-2.43)	1.58 (1.43-1.81)	0.01[a]
Number of missing teeth	6 (3-11)	7 (3-11)	0.6
Percentage plaque index (%)	44 (31-56)	20 (12-30)	0.02[a]
Percentage bleeding index[b] (%)	45 (37-56)	19 (12-29)	0.02[a]
Subgingival periodontal bacterial loads in 16S rRNA gene copies/ng DNA, median (IQRs)			
Porphyromonas gingivalis	$2.4x10^6$ ($4x10^4$-$9.8x10^9$)	$1.3x10^5$ ($1.9x10^4$-$1.1x10^6$)	0.01[a]
Tannerella forsythia	$1.5x10^6$ ($2.4x10^4$-$6.6x10^9$)	$3.3x10^5$ ($2.6x10^4$-$3.1x10^7$)	0.02[a]
Aggregatibacter actinomycetemcomitans	$4.5x10^5$ ($1.2x10^5$-$5.1x10^6$)	$4.8x10^4$ ($3.7x10^4$-$7.1x10^5$)	0.02[a]
Prevotella intermedia	$2.8x10^5$ ($1.9x10^4$-$5.8x10^9$)	$1.1x10^6$ ($7.4x10^4$-$2.2x10^7$)	0.01[a]
Anti-*P. gingivalis* LPS antibodies in ELISA units (EU), median (IQRs)			
Serum IgG	16.6 (12.7-19.6)	12.1 (10.8-13.3)	0.01[a]
Serum IgM	9.9 (7.6- 12.2)	5.9 (4.6-7.3)	0.02[a]
Serum IgA	9.5 (7.5-12.1)	8.2 (7-9.6)	0.02[a]
Salivary IgA2	8.2 (5.6-15.1)	7.5 (4.9-10.9)	0.03[a]

Descriptive categorical measures are expressed as number (n) and corresponding percentage (%). Quantitative measures are expressed as medians and interquartile ranges (IQRs). Periodontitis indicators measured as: site-specific (6 measurements per tooth) - PPD, CAL, BoP; subject-specific- PISA, mean PPD, mean CAL, percentage plaque and bleeding indices. N/A- Not applicable, a= statistically significant ($p<0.05$), b= values of these variables may be influenced by the antiplatelet therapy given in acute MI patients.

Table 2: Hierarchical multiple linear regression analysis of periodontal disease clinical variables, bacterial loads and antibody responses for 12-hour TnI and SYNTAX-I scores as outcome variables

	Entire acute MI patient cohort (n=160)					
Established risk factors (basic model I)	**12-hour Troponin I**			**SYNTAX-I score**		
	β	**(95 % CI)**	***p***	**β**	**(95 % CI)**	***p***
Age	-2356.2	(-4114.1 to -598.3)	0.001[a]	0.2	(0.1 to 0.4)	0.007[a]
Gender (male vs female)	10927.4	(-37401.6 to 59257.1)	0.42	-1	(-5.5 to 3.5)	0.66
SIMD quintiles (≤3 vs >3)	-8496.2	(-23279.6 to 6287.2)	0.55	-0.3	(-1.6 to 1.1)	0.68
BMI (obese and overweight vs normal)	23328.9	(5631.3 to 47289.2)	0.04[a]	-0.4	(-2.6 to 1.7)	0.69
Diabetes (presence vs absence)	-9001.3	(-17148.1 to -3715.1)	0.001[a]	0.6	(-1.2 to 2.4)	0.53
Smoking (current and former smokers vs non-smokers)	12655.6	(9350.1 to 34661.4)	0.02[a]	1.9	(0.2 to 3.9)	0.03[a]
ST elevation (STEMI vs NSTEMI)	-108398.9	(-142139.9 to -74657.9)	<0.001[a]	1.2	(0.3 to 2.8)	0.02[a]
Adjusted R^2 for model-I (established risk factors plus ST-elevation)	0.54			0.52		
Subsequent models (II to XII)						
	12-hour Troponin I			**SYNTAX-I scores**		
Clinical indicators of periodontitis	**β**	**(95% CI)**	***p***	**β**	**(95% CI)**	***p***
Model II (model I+PISA)	94.8	(33.2 to 134.7)	0.02[a]	0.003	(-0.001 to 0.007)	0.13
Model III (model I+mean PPD)	32000.3	(6681.3 to 51581.1)	0.03[a]	3.1	(2.5 to 4.8)	0.02[a]
Model IV (model I+mean CAL)	38110.4	(9154.5 to 55662.1)	0.01[a]	3.5	(1.1 to 5.1)	0.03[a]

Subgingival periodontal microbial load						
Model V (model I+*Porphyromonas gingivalis* load)	$6.8x10^{-6}$	($1.1x10^{-7}$ to $2.1x10^{-5}$)	0.01[a]	$3.8x10^{-9}$	($3.6x10^{-10}$ to $1.8x10^{-8}$)	0.003[a]
Model VI (model I+*Tannerella forsythia* load)	$1.2x10^{-8}$	($-6.2x10^{-2}$ to $3x10^{-6}$)	0.7	$8.4x10^{-9}$	($-1.2x10^{-3}$ to $2.9x10^{-7}$)	0.4
Model VII (model I+*Aggregatibacter actinomycetemcomitans* load)	$1.5x10^{-6}$	($-1.5x10^{-2}$ to $3.4x10^{-4}$)	0.1	$2.1x10^{-7}$	($-1.1x10^{-2}$ to $4.3x10^{-5}$)	0.1
Model VIII (model I+*Prevotella intermedia* load)	$1.8x10^{-6}$	($-1.1x10^{-2}$ to $3.2x10^{-4}$)	0.3	$1.8x10^{-7}$	($-1.3x10^{-2}$ to $3.1x10^{-5}$)	0.4
Anti-*P. gingivalis* LPS antibody isotypes						
Model IX (model I+serum IgG antibody levels)	-1158.6	(-4448.5 to 2131.3)	0.5	0.1	(-0.1 to 0.4)	0.2
Model X (model I+serum IgM antibody levels)	1432.6	(-2635.3 to 5500.6)	0.4	0.3	(-0.1 to 0.6)	0.2
Model XI (model I+serum IgA antibody levels)	6274.3	(-1843.7 to 10704.8)	0.3	0.3	(-0.08 to 0.7)	0.1
Model XII (model I+salivary IgA2 antibody levels)	751.4	(-1666.1 to 3169)	0.5	0.2	(0.03 to 0.4)	0.1

The established risk factors were introduced in model I followed by individual additions of clinical periodontal disease indicators: periodontal inflamed surface area (PISA) (model II), mean probing pocket depths (PPD) (model III), and mean clinical attachment loss (CAL) (Model IV); followed by subgingival microbial loads of: *P. gingivalis* (Model V), *T. forsythia* (Model VI), *A. actinomycetemcomitans* (Model VII), *P. intermedia* (Model VIII) and then levels of *anti-P. gingivalis LPS* antibody isotypes: serum IgG (Model IX), serum IgM (Model X), serum IgA (Model XI), salivary IgA2 (Model XII). β=unstandardised regression coefficient, CI=confidence interval. a= statistically significant ($p<0.05$).

Table 3: Hierarchical multiple linear regression analysis between expression levels of primary periodontal bacterial virulence genes as exposure and 12-hour TnI as well as SYNTAX-I scores as outcome variables

	Subgroup MI patient cohort (n=40)					
Established risk factors (model I)	**12-hour Troponin I**			**SYNTAX-I score**		
	β	**(95 % CI)**	***p***	**β**	**(95 % CI)**	***p***
Age	-3814.4	(-7401.2 o -227.6)	0.02[a]	0.1	0.08 to 0.2	0.03[a]
BMI (obese and overweight vs normal)	-5239	(-13132.1 to -2781.8)	0.02[a]	0.2	-4 to 4.3	0.94
Diabetes (presence vs absence)	1254.1	(829.3 to 16591.4)	0.01[a]	4.2	-1.7 to 10.1	0.51
Smoking (current and former smokers vs non-smokers)	19820.6	(1941.8 to 25231.9)	0.04[a]	-4.4	-7.9 to -1.6	0.02[a]
ST elevation (STEMI vs NSTEMI)	116030.1	(42959.6 to 189100.7)	0.003[a]	6.3	1 to 11.8	0.02[a]
Adjusted R^2 for model-I (established risk factors plus ST-elevation)	0.54			0.49		
Subsequent models (II to XIV)						
	12-hour Troponin I			**SYNTAX-I scores**		
Expression of virulence genes						
Porphyromonas gingivalis	**β**	**(95% CI)**	***P***	**β**	**(95% CI)**	***p***
Model II (model I+*kgp*) (upregulated in 16 patients out of 40)	1.7	(-0.7 to 4.1)	0.2	0.02	(-0.001 to 0.1)	0.2
Model III (model I+*rgpA*) (upregulated in 16 patients out of 40)	2.2	(-0.2 to 4.5)	0.7	0.01	(-0.001 to 0.1)	0.1
Model IV (model I+*fimA*) (upregulated in 10 patients out of 40)	14.3	(1.5 to 27.1)	0.002[a]	1.2	(1.1 to 2.1)	0.002[a]
Model V (model I+*bioF-3*) (upregulated in 12 patients out of 40)	7.8	(1.1 to 12.3)	0.002[a]	1.1	(1 to 5.2)	0.005[a]
Tannerella forsythia						

Model VI (model I+*prtH*) (upregulated in 15 patients out of 40)	1107.8	(235.6 to 2451.3)	0.006[a]	0.003	(-0.01 to 0.01)	0.7
Model VII (model I+*bspA*) (upregulated in 11 patients out of 40)	22.1	(-58.2 to 102.2)	0.6	0.04	(-0.005 to 0.09)	0.4
Model VIII (model I+*siaHI*) (upregulated in 17 patients out of 40)	39.9	(-36.5 to 116.4)	0.3	0.09	(-0.04 to 0.22)	0.3
Treponema denticola						
Model IX (model I+*prtP*) (upregulated in 14 patients out of 40)	6772.8	(2418.7 to 11126.9)	0.002[a]	3	(1.3 to 4.6)	0.001[a]
Model X (model I+*msp*) (upregulated in 8 patients out of 40)	322.5	(-310.5 to 955.6)	0.3	0.3	(-0.1 to 0.7)	0.9
Aggregatibacter actinomycetemcomitans						
Model XI (model I+*ltxA*) (upregulated in 4 patients out of 40)	1811.8	(217.1 to 3840.8)	0.007[a]	1.5	(1.2 to 2.5)	0.01[a]
Model XII (model I+*cdtB*) (upregulated in 3 patients out of 40)	568.3	(113.4 to 1250.1)	0.009[a]	0.002	(-0.005 to 0.01)	0.5
Prevotella intermedia						
Model XIII (model I+*clpB*) (upregulated in 10 patients out of 40)	-54.1	(-331.5 to 223.2)	0.8	0.04	(-0.11 to 0.09)	0.9
Model XIV (model I+*dnaK*) (upregulated in 13 patients out of 40)	-7.2	(-1730.3 to 1715.8)	0.9	0.004	(-0.01 to 0.02)	0.64

The established common risk factors were introduced in model I followed by individual additions expression levels of *P. gingivalis*-related virulence genes: *kgp* (model II), *rgpA* (model III), *fimA* (model IV), *bioF-3* (model V); followed by *T. forsythia*-related virulence genes*: prtH* (model VI), *bspA* (model VII), *siaHI* (model VIII); followed by *T. denticola*-related virulence genes: *prtP* (model IX), *msp* (model X); followed by *A. actinomycetemcomitans*-related virulence genes: *ltxA* (model XI), *cdtB* (Model XII); followed by *P. intermedia*-clpB (model XIII), dnaK (model XIV). β=unstandardised regression coefficient, CI=confidence interval. a= statistically significant ($p<0.05$).

Supplementary file

Section 1: Quantitative polymerase chain reaction (qPCR) for detection and quantification of periodontal bacterial load

Quantitative polymerase chain reaction assays were conducted on genomic bacterial DNA purified from all clinical samples. All samples were analysed in triplicate using 384-well plates (Sigma-Aldrich Company Ltd., Dorset, UK). Each well contained a 10µL reaction mixture that included: 5µL of 2X Takyon™ qPCR dTTP MasterMix (Eurogentec Ltd., Hampshire, UK), 1µL of template genomic DNA, 0.5µL of forward and reverse primers each, 0.2µL of the probe (Eurogentec Ltd., Hampshire, UK) and 2.8 µL nuclease-free water. The primer and probe sequences are listed in **Supplementary Table 1**. The primer (500nM) and probe (100nM) concentrations were optimised in a pilot assay (data not shown). In each plate, genomic DNA of *P. gingivalis* 33277 was used as a positive control and nuclease-free water (Sigma-Aldrich Company Ltd) as a negative control. All the qPCR assays were performed on a LightCycler® 480 Real-Time PCR (Roche Molecular Systems, Inc, UK) device. The thermocycling program was set for: 10min at 95°C (initial denaturation), 40 cycles of 30s at 95°C, 1 min at 60°C. The increase in fluorescence was monitored during PCR amplification and all data was analysed using LightCycler® 480 Software (Roche Molecular Systems, Inc, UK). The absolute quantification method (23) was utilised to quantify the bacterial load of all selected periodontal bacterial species. *P. gingivalis* (ATCC 33277), a previously characterised *T. forsythia* isolate (51), *A. actinomycetemcomitans* isolated from a periodontitis patient at Aberdeen Dental Hospital and *P. intermedia* (ATCC 25611) were used as reference strains to construct the respective standard curves (**Supplementary Figure 2**).

Section 2: Reverse transcription PCR (RT-qPCR) for quantification of the virulence genes expression

RT-PCR assays were conducted on purified RNA extracted from subgingival plaque and tested in triplicate. The expression levels of three genes per PCR-well were multiplexed using a combination of three dual labelled probes as follows: i) channel 1: 6FAM and Eclipse® Dark Quencher, ii) channel-2: HEX and Eclipse® Dark Quencher, iii) channel-3: Cy®5 and DDQII. Each PCR-well contained: 5µL of 2X Takyon™ qPCR dTTP MasterMix (Eurogentec Ltd.), 0.25 µL of forward and reverse primers of 3 distinct target genes, respectively; 0.1µL of probes of 3 distinct target genes, respectively; 1µL of the template (RNA), 0.1µL of Takyon™ One-Step Kit Converter (Eurogentec Ltd.) and 2.1µL of nuclease-free water. The primer and probe sequences targeting virulence genes are listed in **Supplementary Table 2.** The concentrations of primers (500nM) and probes (100nM) were optimised in a pilot assay (data not shown). Each plate contained purified *P. gingivalis* 33277 RNA as a positive control and nuclease-free water (Sigma-Aldrich Company Ltd.) as a negative control. All RT-qPCR assays were performed in a LightCycler® 480 Real-Time PCR (Roche Molecular Systems, Inc, UK).

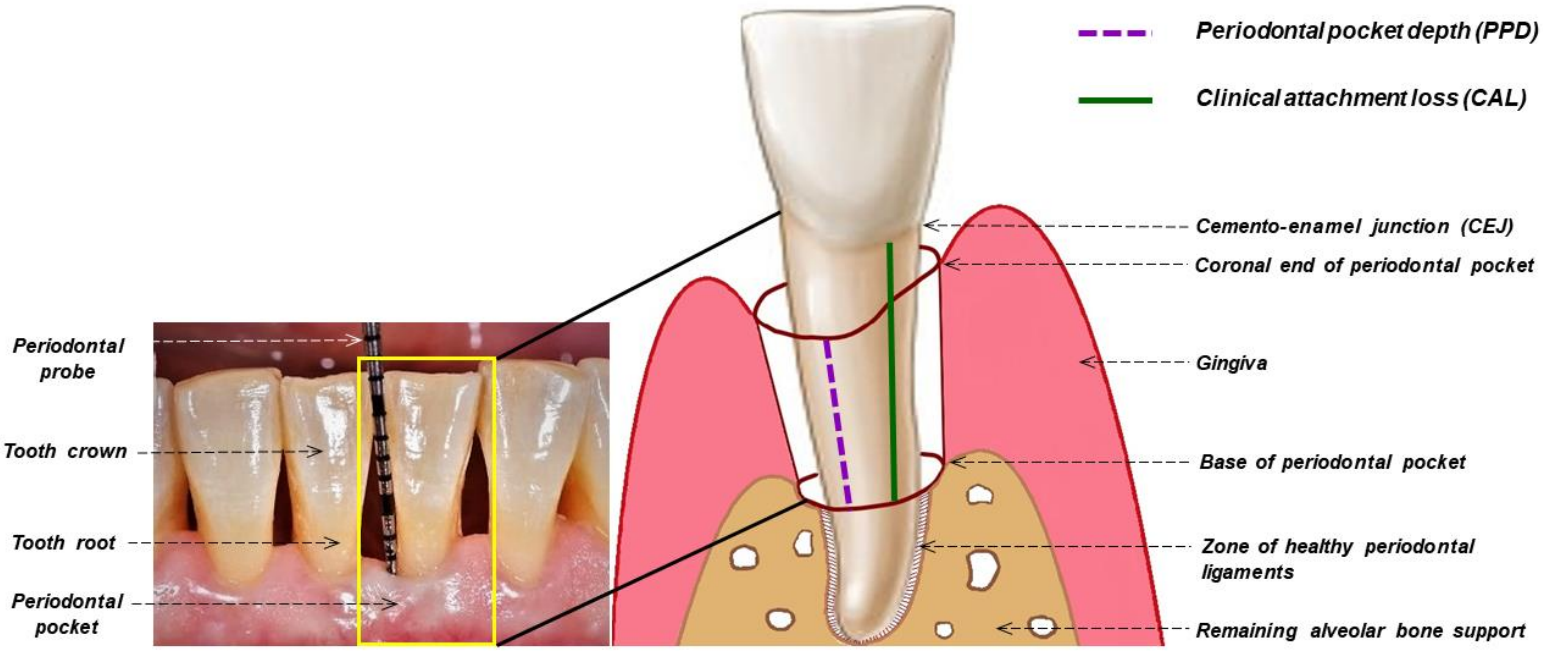
The thermocycling program was: i) step-1 (Reverse transcription):10 min at 48 °C, ii) step-2 (cDNA amplification): 10 min at 95°C (initial denaturation), 40 cycles of 30 s at 95°C, 1 min at 60°C. The increase in fluorescence was monitored during PCR amplification and all data was analysed using LightCycler® 480 Software (Roche Molecular Systems, Inc, UK). The expression levels were normalised against 3 reference genes (*recA*, *glyA* and *groL*) for relative quantification using the ΔΔCT method (33).

Section 3: Enzyme-linked immunosorbent assay (ELISA) to quantify different anti-*P. gingivalis* antibody isotypes

High binding polystyrene 96-well plates were coated with *P. gingivalis*-purified lipopolysaccharide (InvivoGen, Toulouse, France) at 1µg/mL concentration and stored overnight at 4˚C. Thereafter, plates were blocked with 5% bovine serum albumin in PBS at room temperature for 30 minutes. Each serum and saliva sample at 1:10 dilution was incubated in duplicate for 2 hours at room temperature. The bound serum IgG, IgM and IgA antibodies were detected by horseradish peroxidase-conjugated, anti-human IgG (A0170, Sigma-Aldrich Company Ltd) diluted at 1:30000, anti-human IgM (A6907, Sigma-Aldrich Company Ltd) diluted at 1:10000, and anti-human IgA (A18781, Thermo Fisher Scientific Inc.) at 1:2500 concentrations, respectively. Similarly, for the plate containing saliva samples, an anti-human IgA2 antibody (BT91-4005, Generon Ltd, Dublin, Ireland) was added at 1:1000 concentration. Samples were visualised spectrophotometrically at 492 nm using o-Phenylenediamine dihydrochloride (SIGMAFAST™ OPD, P9187 Sigma-Aldrich Company Ltd). The dilution factors for test sera, saliva samples and HRP-conjugated antibodies were optimised before the main experiment (data not shown). Spectrophotometry values were corrected for non-specific binding of the HRP-conjugated antibody to LPS, measured in wells that contained no serum/saliva.

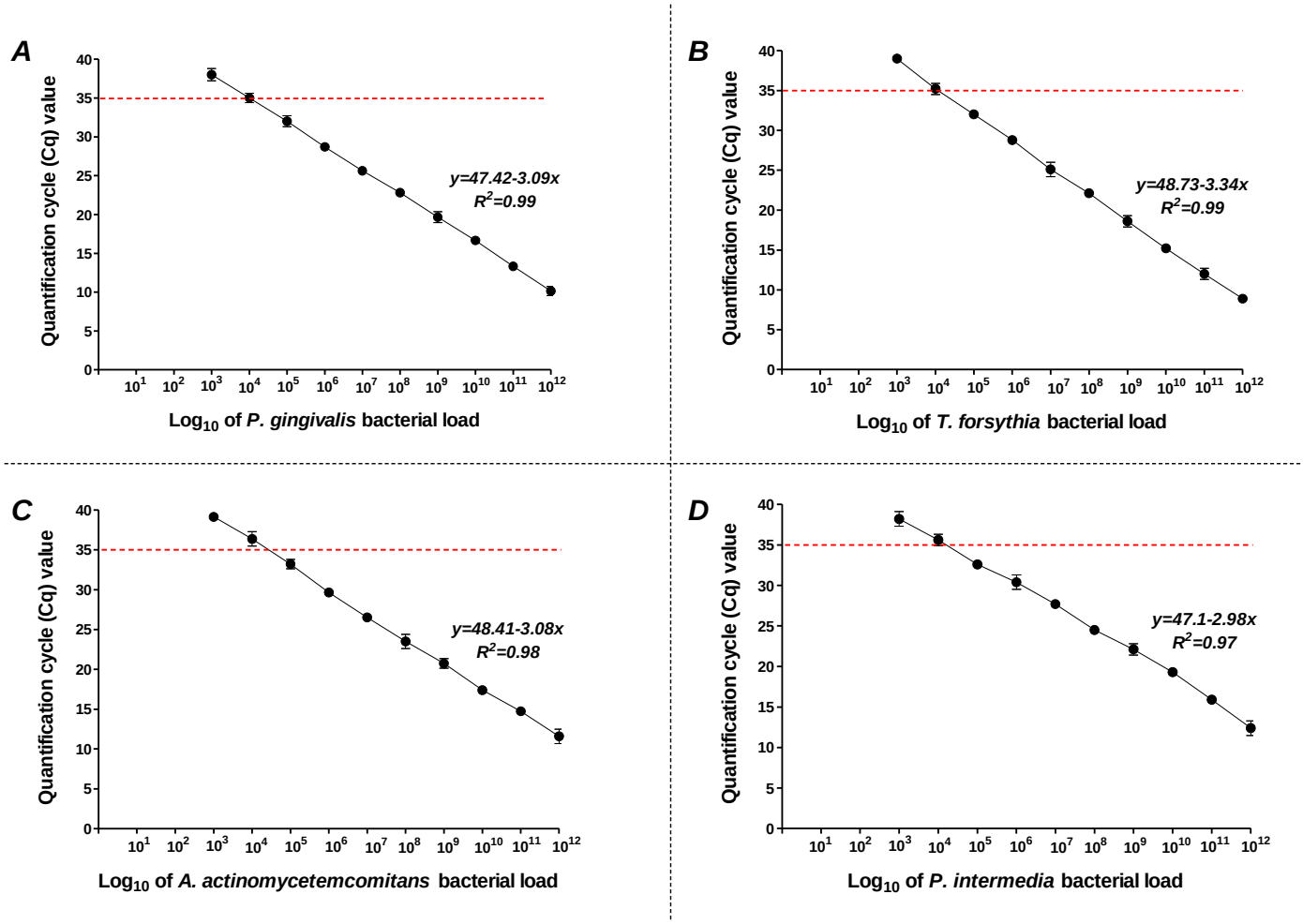
Supplementary Figures

Supplementary figure 1: Illustration detailing periodontal pocket anatomy and periodontal measurements recorded for calculation of periodontal inflamed surface area (PISA).



Periodontal pocket depth (PPD), clinical attachment loss (CAL) and a number of bleeding on probing (BoP)-positive sites were entered into the excel spreadsheet containing inbuilt formulas to calculate periodontal inflamed surface area (PISA). http://www.parsprototo.info (21)

Supplementary figure 2: Standard curves generated for the reference strains of primary periodontal bacterial species: *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia,* using qPCR assays



The log_{10} transformed serially diluted bacterial loads and the corresponding quantification cycle (Cq) values generated in qPCR assay plotted for: A) *P. gingivalis*, B) *T. forsythia*, C) *A. actinomycetemcomitans* and D) *P. intermedia.* The linear regression equations generated by each standard curve were used to calculate the bacterial load of the respective species in the tested samples. R^2=squared correlation coefficient. The red dotted red lines in each plot represent the upper cut-off limit of 35 Cq, set for bacterial detection.

Tables

Supplementary Table 1: The primer-probe sequences used to target and amplify 16S rRNA gene hypervariable regions of four periodontal bacteria: *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* in qPCR assays.

16S ribosomal RNA gene	Primers 5'→ 3'	Probe 5'→ 3'	Amplicon length (bp)
1. *Porphyromonas gingivalis* ATCC 33277, GenBank accession: L16492.1, Region: 1-1474	Forward: CGTGAAGGAAGACAGTCCTAAG Reverse: CGGAGTTAGCCGATGCTTATT	[6FAM]TACGGGAATAACGGGCGATACGAGTA[TAM]	128
2. *Tannerella forsythia* ATCC 43037, GenBank accession: NR_040839.1, Region: 1-1466	Forward: CGGTGAAAGTTTGTCGCTTAAC Reverse: GGAGTTCTGCGTGATCTCTATG	[6FAM]AAGTAGGCGGAATGCGTGGTGTAG[TAM]	130
3. *Aggregatibacter actinomycetemcomitans* ATCC *29523*, GenBank accession: M75038.1 Region: 1-1480	Forward: ACGGGAAACTGTCGCTAATAC Reverse: CCCACCAACTACCTAATCACAC	[6FAM]GTAGAGTCGGGAGACGAAAGTGCG[TAM]	105
4. *Prevotella intermedia* ATCC 25611, GenBank accession: NR_113107.1, Region: 1-1380	Forward: CAGAGGGACGGTGTAATGTAAA Reverse: CGCGATTACTAGCGAATCCA	[6FAM]ATCCAATCTTGAAAGCCGGTCCCA[TAM]	106

ATCC-American Type Culture Collection, bp-base pairs, 6FAM- reporter dye and TAM-quencher

Supplementary Table 2: The primer and probe sequences used to target and amplify thirteen genes (1-13) that encode the major virulence factors of 5 primary periodontal bacteria. Primer and probe sequences of three (14-16) reference genes used for normalisation in ΔΔCt method (34) are listed.

Targeted genes	Primers 5'→ 3'	Probe 5'→ 3'	Amplicon length (bp)
Virulence genes of five periodontal bacteria			
1. *kgp*, *Porphyromonas gingivalis* strain W83, GenBank accession:CP025932, Region:1939820-1940915	Forward: GGAGACCAAAGGTGGTACTTTC Reverse: GCACAGCAATCAACTTCCTAAC	[6FAM]GGTGAGGTTGGTTCTCCCGAAGTG[Eclipse® Dark Quencher]	139
2. rgpA *Porphyromonas gingivalis* strain W83, GeneBank accession:CP025932, Region: 2119432-2125762	Forward: CTTCCACCACCTTCGCTTATAG Reverse: GGACCGACGAAAGAAGATGATTA	[6FAM]TTCAGTTCCATCACCGCTACCCAT[Eclipse® Dark Quencher]	143
3. fimA *Porphyromonas gingivalis*, GeneBank accession: AB195793, Region:103-1257	Forward: ATGTTGACTGGTTGGGAAGAG Reverse: CCTGCATTCTGAGCGTATGT	[6FAM]AGCCTTCCAATAATGCTCCACAAGGT[Eclipse® Dark Quencher]	141
4. bioF-3 *Porphyromonas gingivalis* strain W83, GeneBank accession:AE015924, Region:1868926-1870113	Forward: CGACACCTATTATCCCGCTTT Reverse: GCACAGCAATCAACTTCCTAAC	[HEX]TCCGCAACAACGAGAAGACATTCCA[Eclipse® Dark Quencher]	97

5. *prtH* *Tannerella forsythia* strain ATCC 43037,GeneBank accession: AB001892, Region:193-1464	Forward: GGCTATCCCACTTCTTTCACTC Reverse: CACCGCATCCATTCCGTATAA	[6FAM]TGGTGTAAGTGTTTGGATGTATGAAGAGGC[Eclipse® Dark Quencher]	117
6. *bspA* *Tannerella forsythia* strain ATCC 43037,GeneBank accession: AF054892, Region: 112-3357	Forward: TCCCAAAGACGCGGATATCA Reverse: ACGGTCGCGATGTCATTGT	[HEX]CCGCGACGTGAAATGGTATTCCTC[Eclipse® Dark Quencher]	66
7. *siaHI* *Tannerella forsythia* strain ATCC 43037,GeneBank accession: AY069941, Region: 193-1590	Forward: GGGCGACCAGTATGATAACTTC Reverse: TTGGCGCAACCGTCTATC	[Cy®5]TGGAGAACGGGATTCATCTGCACA[DDQII]	93
8. *prtP* *Treponema denticola* strain ATCC 35405, GeneBank accession:D83264, Region:1515-3683	Forward: AAGCAAGGCTCCGAATCAA Reverse: GCCTCAGGGTCATCCAAATAA	[6FAM]ACGATAGCCCTGTAGACCCTTCCA[Eclipse® Dark Quencher]	96
9. *msp* *Treponema denticola* strain ATCC 35405, GeneBank accession:KJ671481, Region:455610- 457241	Forward: CTGTTGACGGTCTTGCTCTAA Reverse: ACCGAAATAGGCACCAAGAG	[HEX]TAGGCACGGATTCAAAGGTCGCTT[Eclipse® Dark Quencher]	128
10. *ltxA* *Aggregatibacter actinomycetemcomitans* strain ATCC 29524, GeneBank accession: KY965314, Region: 636- 3802	Forward: CAGCACAGAAGTTAGGGATTGA Reverse: CTCGTTCGGTAAGACCAAGTAAT	[Cy®5]AAGGGAAAGACGGCCCAGCATTAA[DDQII]	108
11. *cdtB* *Aggregatibacter actinomycetemcomitans* strain serotype a, GeneBank accession: AY366473, Region:11-862	Forward: GTAGGTATCCGCATTGGTACTG Reverse: GGTGATGATGGTGATGAGGTAAA	[6FAM]ACAGGTGGTTCTGATGCGGTAAGT[Eclipse® Dark Quencher]	122

12. clpB *Prevotella intermedia* strain 17, GeneBank accession: CP019302, Region:1688488-1691076	Forward: GAGAGCAGCCATAACAGAAC Reverse: CCAAGTTGCGTGCGTATTTC	[HEX]ACACAAAGCGGCGACG AGAACTAT[Eclipse® Dark Quencher]	99
13. dnaK *Prevotella intermedia* strain strain 17, GeneBank accession: CP019302, Region: 779640-781541	Forward: GATGAAGAAGACCGCTGAAGA Reverse: CGCTGTGAGTCAGAGAAGTAAG	[Cy®5]TGGACAAGAGGTAACA GACGCAGT[DDQII]	87
Reference genes used for normalisation in ΔΔCt method			
14.RecA *Porphyromonas gingivalis* ATCC 33277, GenBank accession: CP025930, Region: 1178008-1179030	Forward: TTGGGCGTCAATGTGGATAA Reverse: GACAGCAGAAGAGCGAATCA	[6FAM]CGGCGATTTCCAAAGC CTGTTCAC[TAM]	137
15.glyA *Porphyromonas gingivalis* ATCC 33277, GeneBank accession: CP025930, Region:46617- 47897	Forward: AGGCTATGGGTAGCTGTATGA Reverse: GATACGGTCGATGGCGATTT	[HEX]TATGCCGAAGGTTATCC CGGCAAA[Eclipse® Dark Quencher]	113
16.groL *Porphyromonas gingivalis* ATCC 33277, GeneBank accession: CP025930, Region:1649350- 1650987	Forward: GTGAGCGTAGCGAAAGAGATAG Reverse: CATTGGTCTTGGAGGCTACTT	[Cy®5]ATTGGAGTGCCCGTTC GAGAACAT[DDQII]	88

ATCC-American Type Culture Collection, bp-base pairs, 6FAM, HEX, Cy®5- reporter dyes and TAM, Eclipse® Dark Quencher, DDQII-quenchers

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