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**Title: The Effect of PMPR and Chlorhexidine Mouthwash on Salivary and Acquired Enamel Pellicle (AEP) Proteins and Vascular Function and Inflammation in People with Periodontal Disease.**

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## Introduction

Periodontal disease (PD) is a chronic inflammatory condition that affect the tissues supporting teeth, including the gingiva, bone, and ligaments (Dubey & Mittal, 2020). It begins with gingival inflammation (gingivitis), which if left untreated may progress into periodontitis with tissue destruction (Gasner et al., 2025) PD is not just limited to the mouth. It has been associated with 19% of an increased risk of cardiovascular diseases (CVD)(Beck et al., 1996b) , highlighting its broader systemic health implications (Simpson et al., 2024). The aetiology of PD is multifactorial, including-poor oral hygiene (Lertpimonchai et al., 2017) smoking (Borojevic, 2012), diabetes (Preshaw et al., 2012), genetic predisposition (Tettamanti et al., 2017), stress (Corridore et al., 2023) age (Clark et al., 2021) and certain medications (Nazir, 2017). PD has significant global economic impacts, costing \$54 billion in direct treatment and \$25 billion in indirect costs annually (Sanz et al., 2015; Simpson et al., 2024).

Professional mechanical plaque removal (PMPR) is a key non-surgical method that involves regularly removing supragingival and subgingival plaque to reduce tooth loss and stabilise attachment levels in PD patients. When non-surgical interventions like PMPR are insufficient or the disease progresses to an advanced stage, surgery is then reserved as the treatment option for more severe cases (Trombelli et al., 2015; Needleman et al., 2016; Boehm & Kim, 2024). Dental plaque is a biofilm of bacteria that forms on the teeth and gums, significantly contributing to PD. Chemical plaque control using antiseptic mouth rinses such as chlorhexidine mouthwash (CHX) can be used alongside PMPR to reduce bacterial load, but its long-term use is limited due to potential side effects, including staining and altered taste (Afennich et al., 2011; Brookes et al., 2020). The effectiveness of CHX in PD treatment is debated, and the S3 Level Clinical Practice Guidelines (West et al., 2021) do not completely endorse its role in active therapy due to lack of evidence. Despite being an effective antiplaque agent, CHX is primarily used for short-term acute periodontal disease management rather than for routine use (Bonito et al., 2005; *Good Practitioners Guide BSP*, 2016.). Nitric oxide (NO), a key signalling molecule, plays a vital role in immune response (Coleman, 2001), inflammation (Sharma et al., 2015; Sharma et al., 2016), PD (Simpson et al., 2024), and vascular function (Lima et al., 2024). Research indicates that CHX mouthwash not only disrupts the oral microbiome (Dejam et al., 2007; Kilian et al., 2016; Bescos et al., 2020b; Bartsch et al., 2024; Simpson et al., 2024) but also negatively disrupts the enterosalivary NO pathway, impacting vascular function- including raising the blood pressure (Lima et al., 2024), causing myocardial hypertrophy and affecting vascular stiffness (Mollace et al., 2023). Oral nitrate-reducing bacteria such as *Actinomyces*, *Neisseria*, *Rothia* promote ammonium production, reduce lactic acid accumulation, increasing denitrification (Hyde et al., 2014; Feng et al., 2023), *and regulate vascular function* (Fejes et al., 2024). Recent research indicates that nitrate reduction capacity (NRC) of oral microbiota is impaired in individuals with PD, which may affect overall health and contribute to the systemic consequences associated with the condition (Beck et al., 1996b; Bhuyan et al., 2022; Simpson et al., 2024).

Whole Mouth Saliva (WMS) contains organic components such as glucose, urea, cortisol, mucin, albumin, statherin, and amylase, contributing to enamel integrity, lubrication, acid neutralization, and antimicrobial action (Gurovich et al., 2009; Iorgulescu, 2009; Zimmerman, 2013; Mutahar et al., 2017; Pedersen et al., 2018). These salivary components contribute to the formation of the acquired enamel pellicle (AEP), a protective film that regulates calcium and phosphate levels, aiding enamel remineralization of enamel and shielding teeth from acid (W. L. Siqueira et al., 2012; Vukosavljevic et al., 2014). In addition to these protective functions, salivary proteins are important biomarkers for various oral disease. For instance, Matrix metalloproteinase-8 (MMP-8) is, a key biomarker for PD, is present in both saliva and Gingival Crevicular Fluid (GCF). MMP8 plays a significant role in tissue degradation, making it valuable for diagnosis and monitoring the progression of periodontal disease (Ghallab, 2018). During periodontitis, there is a notable shift in the oral microbiome, characterized by an increase in species associated with the disease at affected sites, along with a decrease in species, typically associated with oral health (T. Chen et al., 2022). For example, the red-complex bacteria, including *Porphyromonas gingivalis* (Simpson et al., 2024), *Tannerella forsythia* (Bao et al., 2022), and *Fusobacterium nucleatum* (Groeger et al., 2022), are key contributors to periodontitis (Kendlbacher et al., 2024). Salivary proteins have antimicrobial properties, helping to regulate the oral microbiome with most nutrient needs for oral bacteria, that can be supplied by saliva through predominantly proteolytic degradation (Pedersen et al., 2018). However, this process can be disrupted in periodontitis (Carpenter, 2020). Whilst, the salivary microbial proteins produced by these bacteria include- cysteine proteases (gingipains) and lipopolysaccharide (LPS) by *Porphyromonas gingivalis* (Hajishengallis, 2009); *Bacteroides* surface protein A (BsPA) by *Tannerella forsythia* (Bryzek et al., 2014) fascin-like adhesion protein (FadA) and major fimbrial adhesin (Mfa1) by *Fusobacterium nucleatum*, in people with PD (Fardini et al., 2011).

Saliva proteome is a complex mixture of proteins produced by the salivary glands (Lamy & Mau, 2012), with contributions from oral tissues and microorganisms (Scarano et al., 2010). This proteome focuses on protein structure (Castagnola et al., 2017), function (Scarano et al., 2010) and role in the biological systems (Lau et al., 2018). Saliva proteomics offers easier monitoring of an individual's physiology compared to blood tests and identifies prognostic and diagnostic biomarkers, which can improve patient quality of life and reduce healthcare costs for chronic diseases (Dongiovanni et al., 2023). Despite numerous studies investigating the unique properties salivary proteins and biomarkers (Messana et al., 2013; Castagnola et al., 2017; Wazwaz et al., 2023); the proteins in WMS and AEP in PD, with impact on vascular functions, have not been previously studied. Given their diagnostic potential in individuals with PD, this study will utilize a proteomic approach, supported by bioinformatics with other protein analysis techniques, as well as and vascular function tests, to identify and analyse the profiles of human and bacterial salivary proteins. This will help link PD activity and degradation patterns associated with cardiovascular changes.

### **Aim**

Investigate the effect of PMPR with and without CHX mouthwash on the salivary host and microbial proteins in Whole Mouth Saliva (WMS) and Acquired Enamel pellicle (AEP) in people with Periodontal Disease (PD) and assess its impact on vascular function.

### **Hypothesis**

This study proposes several hypotheses regarding the effects of PMPR and CHX mouthwash on individuals with PD. First, it is hypothesised that PMPR will modify salivary host and microbial proteins in both WMS and AEP in people with PD. Additionally, it is expected that the combination of PMPR and CHX mouthwash will further alter salivary and AEP protein profiles in this population. Beyond microbial and protein changes, it is also proposed that PMPR will have an impact on vascular function in individuals with PD. Finally, the combination of PMPR and CHX mouthwash is anticipated to influence vascular function as well. Together, these hypotheses are to investigate how PMPR and CHX mouthwash can affect both oral health and vascular functions in people with periodontal disease.

### **Methods**

### **Ethical Approval**

This document is intended to request approval from the NHS committee for the proposed study. The study will commence with an observational phase, followed by a double-blind randomized trial. The participants will receive a Participant Information Sheet (PIS) at least 24 hours before they are required to decide on participation. Written informed consent will be obtained from all participants before taking part in the study. A short screening will be conducted via phone call prior to participant recruitment to ensure eligibility. Participants will have the opportunity to ask questions before providing written informed consent, ensuring they fully understand what participation entails.

## **Study Design**

This study includes an observational assessment of PMPR treatment for PD conducted both before and after the intervention. It will be followed by a double-blind, randomised controlled trial visits (V1-V4) at the University of Portsmouth, using both placebo and CHX mouthwash, as shown in Figure 1.

The placebo mouthwash, designed to resemble commercially available products but without the active ingredient. The placebo mouthwash will be prepared in a clean kitchen area within the SDHCP building, using standardized containers and ensure it matches the appearance, colour, texture, and scent of the CHX mouthwash. Both mouthwash solutions will be stored in identical and plain bottles. The preparation of the placebo mouthwash will adhere to a detailed Standard Operating Procedure (SOP) under the supervision of Dr. Marta Roldo to ensure consistency with the existing mouthwashes, in accordance with our previous work (currently in preparation for publication). Additionally, pilot tests will be conducted with approximately 10 participants to confirm that the two mouthwashes are identical in all claimed aspects.

## **Recruitment of Participants**

### **Participants**

Thirty individuals with gingivitis and periodontal disease will need to be recruited for this study based on the power calculation (explained below). In accordance with the British Society of Periodontology (BSP) 2017 classification, including BPE codes 3 and 4 and periodontitis stages 1 and 2, individuals diagnosed with gingivitis and periodontal disease will be recruited from the University of Portsmouth (SDHCP) dental clinics. Under the supervision of the Chief Investigator (CI) and the Principal Investigator (PI), potential participants will be recruited through various channels. Internally, recruitment will target patients attending the University of Portsmouth Dental Academy, facilitated by the PhD researcher and clinicians (Dr. Anish Patel, Prof. Chris Louca, and Dr. Bhavin Dedha). Additionally, the PhD researcher will visit community centres and deliver talks aimed at engaging potential participants from the wider community. Participants will also be invited to volunteer for the study via word-of-mouth recommendations, emails distributed through departmental staff lists, announcements in the University Updates newsletter, and outreach during university departmental meetings. Presentations

about the study will also be conducted at the dental school's journal club events to raise awareness and encourage participation.

Individuals included in the study must be adults aged between 30 and 75 years who are recommended to undergo PMPR by their SDHCP clinician as part of their routine dental treatment. Participants should not have any other hard or soft tissue diseases of the oral cavity, and their participation is based on the following criteria (enlisted below).

The oral clinical examination will be conducted by trained clinical examiners. Participants will be screened for eligibility and will be required to complete medical, dental, and dietary questionnaires and forms. Calibration will involve training sessions and assessment through repeated pocket chart completion (by all three dental clinicians) to ensure consistent interpretation (Johnston et al., 2023). Reproducibility will be monitored by conducting repeated examinations on a randomly selected 5% of the volunteers (from the study). Both intra-examiner and inter-examiner agreement will be assessed using kappa statistics, with separate calculations for each examiner pairing (Kingman et al., 2008; Slate & Hill, 2012). Furthermore, for minimising inter-examiner reproducibility, each participant will be assigned a specific clinician for all 4 visits (as mentioned below, in the pre-experimental tests section).

### **Inclusion Criteria**

Participants must have a minimum of 2 natural teeth in each sextant (at least 12 teeth overall) (Costa et al., 2021) and should not have undergone any periodontal maintenance therapy (PMPR) treatment in the last 6 months, as most follow-up PD studies are between 1-5 months (Shi et al., 2015; C. Chen et al., 2018; Johnston et al., 2020, 2023). Should have gingivitis or early periodontal disease (as per BSP and BPE classification). They must not currently be wearing orthodontic appliances due to their significant impact on oral microbiota and altered plaque composition (Sukontapatipark et al., 2001; Naranjo et al., 2006; Freitas et al., 2014). Similarly, not using removable orthodontic appliances, due to increased plaque accumulation (Friedman et al., 1985) and should not have taken any antibiotic treatments for at least 3 months prior to visit to the study (L. Chen et al., 2012) Additionally, individuals must be able to provide written informed consent. Any individuals with dental erosion and caries will be matched during the baseline visit based on this and other criteria outlined below after being included in the study.

### **Exclusion Criteria**

Individuals will be excluded if they have any medical condition (Johnston et al., 2020) or regularly using medication within one month prior to the study's start, are pregnant or breastfeeding, require an interpreter or are non-English speakers, or are unable to provide written informed consent (D'Aiuto et al., 2018). Furthermore, participants who are part of another ongoing clinical study (other than an observational study) within 3 months preceding or during this study will be excluded. Additional exclusions apply to individuals who have used mouthwash or tongue scrapes in the last 6 weeks, individuals who consume more than >5 alcoholic drinks per day (Tezal et al., 2001) or smoke 20 or more cigarettes per day (Madi et al., 2023). Moreover, individuals with chlorhexidine allergy will also be excluded (Pemberton & Gibson, 2012; Chiewchalermsri et al., 2020).

### Participants Matching

Once screened for eligibility and after completions of the required questionnaires and forms, participants will be initially matched based on factors that affect salivary proteins at baseline, and will be assigned to different categories. These will include age (30 to 75 years)(Clark et al., 2021), smoking status (Beklen et al., 2022); PD classification (Johnston et al., 2020, 2023; Simpson et al., 2024), clinical severity of caries (Martignon et al., 2019) and dental erosion (Imfeld, 1996). Individuals with diabetes will also be taken into account (Caseiro et al., 2013; Fouani et al., 2021; Malathi et al., 2014). These variables will be represented as mean (SD) or percentage and the data will show the total amalgamated population recruited (Table 1). These individuals will then be randomised using concealed allocation via randomizer.org to receive either CHX mouthwash (Group 1) or placebo mouthwash (Group 2), following their PMPR treatment for PD. and forms. Participants will also be asked to continue their usual lifestyle, but for standardisation, they will be given the same toothpaste (which is neither marketed as a whitening toothpaste or to treat tooth sensitivity) throughout the study, as additional ingredients could affect the outcomes.

Criteria	Value		
<b>Age (years)</b>	Mean (SD)		
<b>Smoking status (%)</b>	Never	Former	Current
<b>Caries</b>	Initial	Moderate	Extensive
<b>Dental Erosion</b>	Class 1	Class 2	Class 3
<b>Periodontal Disease classification (%)</b>	Stage (1-2)		

Table 1: Participants matched in subgroups at baseline, visit 1 (N=30).

### Power Analysis

The primary outcome of this study is first to identify salivary proteins and measure fold changes before and after PMPR (observation). Using a t-test with an effect size of 0.87 (same as for ANOVA), 80% power, and a significance level set at  $\alpha < 0.05$ , approximately  $N = 6$  participants are needed for the observational component of the study. The 2<sup>nd</sup> part of the study (intervention), with and without mouthwash treatment, is based on previous studies examining the salivary biomarker MMP-8 (Wazwaz et al., 2023). An a priori power calculation (ANOVA: repeated measures, between factors) was performed to estimate the required sample size (n). For 90% power with an  $\alpha$ -level set at  $\alpha < 0.05$ , with an effect size  $f = 0.87$  with two groups, 12 people each (n) were required to detect a change in MMP-8 salivary biomarker, with a total sample size of  $N = 24$ . To account for a potential 14% dropout rate, including participants who may require a second PMPR treatment within the 3 months (commonly observed in clinical trials), a minimum of  $n = 15$  participants will be recruited in each group, resulting in a total sample size of  $N = 30$ .

### Pre-experimental Tests

Upon recruitment (V1), a Prof Chris Louca and Dr. Anish Patel and Mr. Bhavin Dedhia at William Beatty Building, SDHCP as part of their routine dental treatment. The oral clinical examination will include- clinical attachment loss (CAL), modified plaque index (PI), bleeding on probing (BOP), probing pocket depth (PPD) pocket depth ( $\geq 5\text{mm}$ ) in % (Simpson et al., 2024). A World Health Organisation (WHO) BPE probe 0.5mm will be used to assess participants' gingival and periodontal health. These values will be represented as median and inter-quartile ranges (Johnston et al., 2020, 2023; Simpson et al., 2024). The recruited participants will be assessed if they fit the inclusion and exclusion criteria and matched into groups at baseline (table 1). No X-rays will be taken specifically for the purpose of this research. However, only the results of clinical radiographs obtained as part of participants' standard dental care may be accessed as part of the study.

To ensure standardisation and consistency in periodontal treatment and evaluations, all participants will undergo supragingival PMPR, stage 1, using the same ultrasonic scaler tip design. The ultrasonic scaler tips will be maintained within 25% of their working efficiency and replaced as per manufacturer guidelines to ensure uniformity in mechanical action and minimise variability in treatment outcomes (Dentsply Sirona, 2017).

As periodontal measurements like probing depths and plaque scores are highly sensitive to examiner technique, maintaining consistency in probing force, angulation, and calibration is vital to minimise variability and enhance the reliability of the results (Van der Velden & De Vries, 1980; Walsh & Saxby, 1989; *Diagnosis and Examination - American Academy of Periodontology*, 2024). Therefore, to

avoid inter-examiner differences in recording periodontal indices and plaque scores and PMPR treatment, each participant will be assigned a dedicated clinician (amongst the three clinicians, as stated above) for the study duration.

The same clinician will conduct all evaluations and treatments for their assigned participant across all four study visits. This includes recording probing depths and clinical attachment levels during visits 1 and 4, and bleeding and plaque scores during all four visits. This standardised approach is essential for ensuring consistency in data collection, reducing examiner bias, and achieving reproducible and reliable results. Moreover, the instruments used for recording pocket depth, bleeding on probing clinical attachment loss and plaque scores will be discarded if they become damaged, excessively worn, or after prolonged use, in line with established standards for safe and effective dental practice (*Standards for Dental Team GDC, 2013; NHS England » National Infection Prevention and Control Manual (NIPCM) for England, 2021*).

Baseline samples will then be collected at the same dental clinic in UPDA (WMS and AEP). Further samples will be collected at the Spinnaker Building, School of Psychology, Sport and Health Sciences (SPSHS) on the same visit. These samples will include: Flow-mediated dilation (FMD), pulse wave velocity (PWV), pulse wave analysis (PWA), cardiac output (CO), Iontophoresis, blood sample collection and blood pressure (BP) measurements.

### **Observational Part**

Following the initial recruitment, consent, screening and baseline sample collection (V1) at the dental clinics at SDHP; the participants will undergo PMPR treatment during the same visit (as usual care). This part of the study will be purely observational, with no intervention based on their BPE score, PD indices and BSP 2017 classification.

The research team will:

- Not access, evaluate or use any existing radiographs
- Not take or request any radiographs
- Not alter any existing clinical diagnosis or make any new diagnoses
- Recruit participants solely based on their existing diagnosis of periodontal disease

### **Intervention**

After collection of the baseline samples and PMPR treatment in (V1), participants will be asked to come back the next day (V2) where they will be asked for the same samples as in (V1). Following this, they will be randomly divided (randomised) into two groups: [Group 1 will receive CHX mouthwash (n=15), while Group 2 will receive a placebo mouthwash (n=15)]. Each participant will be provided with 14 tubes containing 10 ml of their assigned mouthwash—either the antibacterial CHX mouthwash (Corsodyl Mint, GlaxoSmithKline, UK) containing 0.2% CHX or the placebo mouthwash. Both mouthwash types will be indistinguishable in taste, appearance, colour, texture, and scent, using identical, plain bottles. Participants will be instructed to rinse their mouth with 10 ml of mouthwash for 1 minute, twice daily, for 14 days.

## **Assessment Visits**

### **(V1) - Baseline**

V1 will involve the recruitment, screening, consent, and matching of participants (as outlined in the pre-experimental tests). Baseline sample collection will be conducted before and after PMPR. The sample will include The WMS and AEP samples will be collected at the SDHCP clinics, followed by vascular assessments, including- pulse wave velocity (PWV), pulse wave analysis (PWA), cardiac output (CO), endothelial function, flow-mediated dilation (FMD), blood pressure (BP), along with blood sample collection at SPSHS. Then, multiple scores will be collected, including plaque scores, pocket depths, clinical attachment level, and bleeding scores, along with oral hygiene instruction before the periodontal maintenance therapy (PMPR) treatment at the dental clinics (SDHCP); before PMPR (stage 1) treatment. Participants will be instructed to refrain from eating for at least 2 hours before sample collection and will be asked to shave their beard and chest to ensure clear vascular function data can be obtained. Throughout the experimental tests, participants will be guided through the procedures and will have the option to withdraw at any time.

### **(V2) - Day 1**

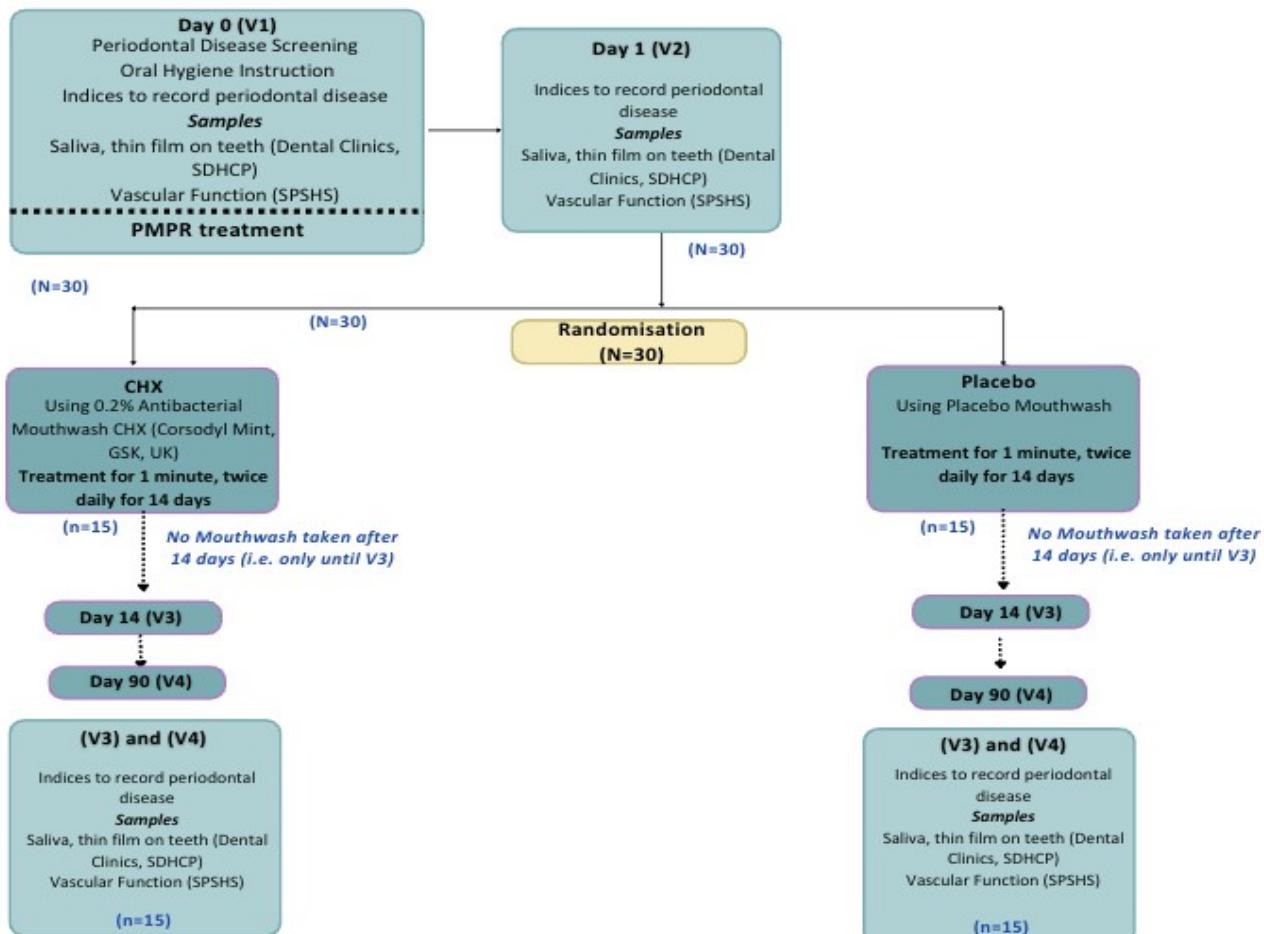
V2 will occur one day after the PMPR treatment. At this visit, the same samples collected during V1 will be recorded again, including the periodontal indices (except for probing depth and clinical attachment level). The samples collected in this visit will help analyse the changes acute changes (24 hours) in WMS, AEP and vascular function, before and after PMPR treatment.

### **(V3) - Day 14 and (V4) - Day 90**

V3 and V4 will be identical in nature and will take place after participants randomisation into 2 groups (into CHX mouthwash and placebo mouthwash groups) (Figure 1). V3 will take place 14 days (recommended mouthwash prescribed duration) and V4 will be 90 days after the PMPR treatment. The samples collected in these visits will mirror sample collection in V2. However, all periodontal indices including pocket depth and clinical attachment level will be recorded in V4. Moreover, the participants will not be using mouthwashes after 2 weeks following visit 3.

All visits will take approximately 1.5 hours (90 minutes), except PMPR in visit one (another 30 minutes), with appointments held in the morning between 9 am to 12 pm

*Recruitment, Screening, Consent and Matching*



**Figure 2: Study design of CHX Mouthwash and Placebo Mouthwash administration across 4 visits (V1-V4)**

**Outcome measures**

**1. Salivary Protein Analysis**

**PMPR**

UK adopted the BSP Implementation of Treatment of Stage (1-3)- (Sanz et al., 2015, 2020). This 2017 Classification scheme allowed an individualised treatment for each case- extent, severity, progression rates and local and systematic complicating factors (West et al., 2021). It usually involves 2 steps- 1 and 2 (Sanz et al., 2020; West et al., 2021). Step 1, PMPR involves removing supra- and subgingival plaque, focusing on visible or detectable subgingival deposits on the crown (*S3 Treatment Guidelines*

for Periodontitis / BSP, 2017.; West et al., 2021) Step 2 involves subgingival instrumentation (root surface debridement or PMPR) on the root surface, using either hand or powered instruments based on patient and site-specific needs (BSP 2017) and improves the oral microbiome (Di Stefano et al., 2022). All the clinical treatment will be carried out by experienced, calibrated dentists, Prof Chris Louca, Dr. Anish Patel and Mr. Bhavin Dedhia, respectively. The change in the salivary and AEP proteins (with impact on vascular function) will be assessed before and after PMPR treatment (24 hours, 14 days and 90 days). The 90 days period is suggested, as PMPR reduces inflammation of the periodontal tissues in 3 months and demonstrates decreased indices of periodontal disease severity with significantly improved endothelial function (Tonetti et al., 2007). At day 1, literature suggests, that there are significantly elevated levels of CRP, IL-6, alongside endothelial dysfunction after 24 hours (Mainas et al., 2022). And the 14-day period was selected because CHX is typically prescribed and recommended as an adjunct to PMPR for two weeks only, to mitigate the CHX side effects (Eley, 1999; Brookes et al., 2020).

## WMS

Upon arrival, (9 AM–12 PM), and after oral screening unstimulated WMS will be collected from all participants via passive drool into pre-weighed vials and sterilized Falcon tubes (Mutahar et al., 2020). The collection tubes will be pre- and post-weighed, and the time of collection recorded. Saliva pH will be measured immediately using a pH meter, and buffering capacity assessed with a saliva kit (from GC America Inc.). WMS will be collected over 5 minutes to calculate the salivary flow rate using the formula:

$$\text{Saliva Flow Rate (ml/min)} = \frac{\text{Weight of tube with saliva} - \text{Weight of empty tube}}{\text{Time of collection (min)}}$$

After use, WMS samples will be discarded according to the Human Tissue Act 2004.

## AEP

Based on previous studies, AEP will be collected from the teeth surface. (J. F. Siqueira et al., 2007; Zimmerman, 2013; Mutahar et al., 2017). After 2 hours of fasting, teeth will be isolated, washed, and

dried. AEP will be collected from buccal and occlusal surfaces of all quadrants using electrode filter papers soaked in 3  $\mu$ L of 0.5% SDS and rubbed for 15 seconds. Four strips (one per quadrant) will be stored at -20°C, then transferred to -80°C. For pellicle recovery, filter papers will be micro-centrifuged, and proteins eluted with 15  $\mu$ L of SDS and 5  $\mu$ L of LDS buffer, followed by centrifugation and the addition of DTT. Samples will be vortexed, heat-denatured, and stored at -20°C before transfer to -80°C for later analysis. The AEP samples collected, will be used to analyse AEP novel proteins and existing (albumin, cystatins, mucin and PRPs etc.) with focus on those proteins which overlap with findings from proteomic analysis of WMS; subsequent changes along the 4-end points will be compared.

## **Proteomics**

This proteomic analysis will be conducted according to a detailed protocol developed in collaboration with colleagues from Imperial College London (detailed protocol attached).

**Reduction & Alkylation:** A 10  $\mu$ L aliquot of saliva will be thawed and mixed with 7  $\mu$ L of ammonium bicarbonate buffer on ice. For reduction and alkylation, 5  $\mu$ L each of TCEP and CAA will be added, maintaining a pH of 7-8 to ensure proper modification of disulfide bonds and cysteine residues.

**SP4 Protocol:** Following reduction and alkylation, the SP4 protocol will be implemented by adding 80  $\mu$ L of LC/MS-grade acetonitrile to precipitate proteins; the mixture will be centrifuged to separate the supernatant, and the pellet will be washed three times with ethanol for thorough purification.

**Digestion:** The purified pellet will then be resuspended in stock trypsin by adding 20  $\mu$ L 25 mM ammonium bicarbonate to lyophilised powdered trypsin (powdered sequencing grade modified trypsin) and incubate overnight at 37°C, allowing trypsin to digest the proteins into peptides suitable for mass spectrometric analysis.

**Peptide Collection and Preparation for LC-MS/MS:** After digestion, formic acid will be added to halt enzymatic activity, and the peptides will be quantified to a concentration of 200 ng/20  $\mu$ L for LC-MS/MS analysis. This method will allow for the identification and quantification of salivary human proteins such as mucin, salivary amylase, statherin, and proline-rich proteins, as well as bacterial proteins produced by *P. gingivalis*, *T. forsythia*, and *F. nucleatum*, along with salivary biomarkers like MMP-8, MMP-9, MMP-2, IL-1 $\beta$ , and IL-6 from WMS samples. The fold changes of these proteins will also be measured (Taylor, 2014; Lundmark et al., 2017; Alftaikhah et al., 2023).

### **SDS-PAGE Electrophoresis and Western Blot**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), is a discontinuous electrophoresis method commonly used to separate proteins (in high resolution) with molecular weights between 5 and 250 kDa (LAEMMLI, 1970; Nowakowski et al., 2014). SDS functions as a surfactant, masking the proteins' natural charges and giving them nearly identical charge-to-mass ratios (Otter, 2003). When a constant electric field is applied, the proteins move toward the anode at speeds that vary according to their mass (Hansen et al., 2009). This straightforward process enables accurate separation of proteins based on mass (Zilberstein et al., 2007).

SDS-PAGE is also used alongside western blotting to detect the presence of a specific protein within a protein mixture (Mahmood & Yang, 2012; Mishra et al., 2017). Western blot is a key technique in molecular biology for detecting specific proteins from a complex mixture (Mahmood & Yang, 2012). Western Blot involves separating proteins by size, transferring them to a solid membrane, but by using specific antibodies to label and visualize the target protein (Khanna & Paoloni, 2007). This helps to identify and quantify the proteins of interest.

In this study, the AEP samples collected from all participants during the four visits will undergo SDS-PAGE for protein separation and identification, followed by western blotting to transfer proteins onto a nitrocellulose membrane (Mutahar et al., 2017). Each sample, with 7.5 µg of total protein, will be run in duplicate on a 14% polyacrylamide gel (Louro et al., 2021). The focus will be on quantifying proteins relevant to periodontal disease, including novel bacterial proteins (in AEP) and recently identified through proteomics (in WMS samples) (Cleaver et al., 2023) and standard proteins (as mentioned in the AEP section).

### **DNA Extraction and 16s Ribosomal RNA Analysis**

In this technique, bacterial genomic DNA is will be extracted from whole cells (from WMS and AEP samples in this study) using either a standard method or a commercial system, such as the PrepMan DNA extraction reagent (ABI) (Clarridge III, 2004). This extracted DNA serves as the template for PCR amplification of a 500 or 1,500 bp segment of the 16S rRNA gene sequence (Clarridge III, 2004).

The prepared saliva and pellicle samples will be sent to the Oral Microbiome Research Laboratory at Temple University (USA) for DNA extraction and 16S rRNA analysis. Material Transfer Agreement (MTA) will be obtained prior to send these samples.

Microbial DNA will be extracted from 1-3mL of samples using commercially available kits, such as Qiagen QIAamp or Vazyme VAMNE kits. DNA quantification will be performed using fluorometric methods (e.g., Qubit) and purity will be checked with an absorbance method. Additionally, 3 blank controls (sterile water) and 3 positive controls (*Escherichia coli*) will be used to monitor contamination and method effectiveness. The V4 hypervariable region of the 16S rRNA gene will be amplified using egSEQ Amplicon Library Kit with custom primers. The final library will be evaluated for fragment sizes and quantified prior to sequencing on an Illumina MiSeq (2x250bp configuration). Sequencing results will be compared between periodontitis patients and healthy participants. Bacterial DNA sequences will be analysed to profile diversity, and computational analyses will identify trends in periodontitis compared to controls.

## 2. Vascular Function

Vascular function refers to the health and performance of blood vessels in regulating blood flow and maintaining cardiovascular health, playing a key role in overall circulatory dynamics (Järvisalo & Raitakari, 2005). This involves assessing endothelial function, arterial stiffness, and blood flow dynamics, offering insights into vascular health, and allowing for the early detection of cardiovascular risks like hypertension and atherosclerosis (Huck et al., 2007.; Laurent et al., 2006). These tests help assess vascular adaptation and the role of the endothelium in maintaining vascular tone and elasticity (Green et al., 2011). In this study, vascular functions will be analysed as an impact of CHX mouthwash in people with PD (outlined below).

### Endothelial Function Test (Iontophoresis)

Iontophoresis is a non-invasive technique used to evaluate microvascular endothelial dysfunction through transdermal drug delivery using a low-intensity electric current to transport charged molecules (Roustit et al., 2014).

Participants will acclimatise for at least 30 minutes in a room at 23°C before receiving acetylcholine (ACh) and Sodium nitroprusside (SNP) via iontophoresis on the volar forearm, following established methods. The skin will be cleaned with water for injection, and two perspex rings will be placed on the skin—one as the anode and the other as the cathode—connected to the iontophoresis controller (MIC 2, Moor Instruments, UK). Each chamber will hold approximately 0.5 mL of ACh (1% concentration) or SNP (0.01% concentration). The electrical protocol consists of four pulses at 25 µA, followed by single pulses of 50 µA, 100 µA, 150 µA, and 200 µA, each lasting 20 seconds with 120-

second intervals without current. Skin blood flow will be measured using Laser Doppler probes (VP1T/7, Moor Instruments, UK) connected to a perfusion monitor (moor VMS-LDF, Moor Instruments, UK), with data recorded via Powerlab (AD Instruments, Australia) and LabChart 8 software. Cutaneous vascular conductance (CVC) will be calculated ( $CVC = \text{skin flux}/\text{MAP}$ ), and average skin blood flow will be determined over the final 20 seconds of each pulse interval. Maximal skin blood flow and area under the curve (AUC) will also be calculated for each participant. Blood pressure will be measured on the contralateral arm before and after the protocol to determine MAP.

### **Flow Mediated Dilation (FMD)**

Brachial artery FMD will be used to assess endothelial function through a 5-minute ischemic stimulus induced by forearm cuff inflation (Terason uSmart 3300, Terason, Burlington, MA, USA) (Thijssen et al., 2011). Measurements will be taken in the supine position on the right arm, with the cuff placed distal to the olecranon process. Using a 12-MHz multifrequency linear array probe, high-resolution duplex ultrasound will image the brachial artery in the distal upper arm while simultaneously recording B-mode images and Doppler blood velocity traces at a 60° angle. Settings for depth, focus, and gain will be consistent across assessments, and the transducer location will be documented with a tape measure and photograph or marked on the skin. After a 60-second baseline period, the cuff will be inflated to 220 mmHg for 5 minutes (moorVMS-PRES, Axminster, UK). Ultrasound recordings will continue during the inflation and for 3 minutes post-deflation. All scans will be conducted by the same researcher for each participant. Brachial artery diameter, blood flow, and shear rate will be analysed using custom edge-detection and wall-tracking software (Quipu Cardiovascular Suite, Pisa, Italy), minimizing investigator bias.

### **Cardiac Output (CO)**

The CO is a primary determinant of global oxygen transport from the heart to the body, to meet the metabolic demands of the tissues and is assessed to study cardiovascular insufficiency (central haemodynamics) (Pinsky, 2003). CO will be measured noninvasively using the Physio Flow PF-05 Lab1 device (Manatec Biomedical, Paris, France) with thoracic electrode placements. Two electrodes will monitor EKG (positions: sternal manubrium and left hip) for heart rate, while four additional electrodes (at the neck base and xiphoid process) will measure the impedance signal. Proper skin preparation, including shaving and cleaning, will ensure optimal signal quality. For patients with pacemakers, neck electrodes will be positioned opposite the device. Calibration involves acquiring stable signals over 30 heartbeats and simultaneous blood pressure measurement. The system will display heart rate, stroke volume, and other hemodynamic parameters, with data reviewed and printed as needed.

### **Pulse Wave Velocity (PWV) and Pulse Wave Analysis (PWA)**

The PWV is the gold standard for assessing arterial stiffness, a key indicator of hypertension and cardiovascular risk (Laurent et al., 2001; Nichols et al., 2022). This non-invasive measurement involves calculating the time delay between pressure waveforms from the carotid and femoral arteries. Participants will lie supine for 5 minutes before a femoral cuff is placed on the thigh, and carotid pressure is measured using a tonometer to determine PWV in m/s. A report will compare results with population data. While the PWA is a quicker and simpler method for evaluating arterial function, providing central aortic hemodynamic parameters such as pulse pressure (PP), augmentation pressure (AP), and augmentation index (AI75), which are important markers of arterial stiffness and predictors of atherosclerosis in type 2 diabetes (Laurent et al., 2006; Safar et al., 2008). After resting for 5 minutes, brachial pressure waveforms will be recorded and used to derive aortic waveforms via a transfer function, with results displayed as a graph comparing participant measurements to population norms using the SphygmoCor XCEL- ATCOR system.

### **Blood Pressure (BP)**

Blood pressure in the brachial artery will be measured after 30 minutes of seated rest in a quiet room, using an automated sphygmomanometer. Five consecutive readings will be taken, with a 1-minute rest between each measurement. The average of three readings will be recorded, including the mean values for SBP, DBP, and MAP.

## **3. Biochemical Analysis**

Saliva and blood samples, from the participants in the study, will be further undergo biochemical analysis, as given below. Moreover, the nitrate reducing activity (NRC) of the oral bacteria will be analysed from the WMS samples collected from the participants in all 4 visits.

### **Nitrate Reducing Activity of the Oral Bacteria**

To assess the nitrate-reducing activity of oral bacteria from the WMS samples, a water-nitrate solution will be prepared, following these instructions: A measured amount of 850 mg of sodium or 1011 mg of potassium nitrate will be weighed out. This nitrate will then be diluted into 1 litre of ultra-pure water. Subsequently, 10 ml of the resulting stock solution will be transferred into Falcon tubes (15 ml) and stored at a temperature of -20°C until the time of use. For the procedure, a Falcon tube containing 10 ml of the water-nitrate solution will need to be thawed. Participants will be instructed to rinse their

mouths with this solution for a precise duration of 5 minutes, with careful control over the timing. Once the rinsing is completed, the collected rinse will be gathered into a 50 ml Falcon tube. The rinse will then be transferred into one or two micro-centrifuge tubes as the procedure progresses. Following this, centrifugation will be carried out at 10,000 rpm for a period of 10 minutes. After centrifugation, the supernatant will be collected and transferred into a new microcentrifuge tube for subsequent freezing. This supernatant will be frozen for future analysis of nitrite concentration. This protocol will facilitate the evaluation of nitrate-reducing activity in oral bacteria through a meticulously outlined sequence of future steps

### **Biomarkers in Blood Sample**

Venous blood will be collected via venepuncture from the forearm in 4 mL aliquots in vacutainer tubes containing ethylene diamine tetra acetic acid. Samples of whole blood will be immediately centrifuged for 10 min at 4000 rpm at 4 °C following collection, to study biomarkers (using ELISA antibody kits, outlined below) that link PD to CVD, such as - IL-10, IL-6 and TNF $\alpha$  and CRP (Kampits et al., 2016; Caloian et al., 2024).

### **Enzyme-Linked Immuno Sorbent Assay (ELISA)**

In this study, DuoSet ELISA kits will be utilised to quantify blood biomarkers, including IL-6, IL-10, TNF $\alpha$ , and CRP, to assess systemic inflammation and its association with periodontal disease and vascular function (*Human IL-10 DuoSet ELISA DY217B-05: R&D Systems*). These kits will utilize a sandwich ELISA approach, where a capture antibody specific to the biomarker is pre-coated onto a microplate, followed by sample application (Kampits et al., 2016; Caloian et al., 2024).

This will allow for precise measurement of cytokines and acute-phase proteins from participants' blood samples. The kits ensure high specificity and sensitivity, allowing the detection of both pro-inflammatory (IL-6, TNF $\alpha$ , CRP) and anti-inflammatory (IL-10) markers (*Thermofisher ELISA*). The results will help elucidate the inflammatory response and its modulation post-PPMR, providing critical insights into the systemic effects of periodontal therapy.

### **Biomarkers in Saliva Sample**

The WMS will be placed in a chilled (4°C) centrifuge and spun at 4000 xg for 10 minutes immediately on collection. The samples will be frozen at a -80°C freezer until analysis. salivary cytokines (as mentioned in the WMS section) and novel biomarkers- such as mucin 4 and MMP-7, as observed in periodontitis patients and strongly associated with clinical parameters.

### **Ozone-based chemiluminescence (CLD)**

Ozone-based chemiluminescence detection is widely used in biomedical research to indirectly measure nitric oxide (NO), due to the short half-life of NO (Li et al., 2024) and to measure its metabolites (primarily nitrite ( $\text{NO}_2^-$ )). In this method, NO reacts with ozone to produce nitrogen dioxide ( $\text{NO}_2$ ) in an excited state, which can then be detected. Since NO is rapidly converted to nitrite and nitrate in biological systems, the measurement reflects primarily nitrite levels in fluids such as plasma, tissue homogenates, and cell cultures, as well as in gas mixtures (e.g., exhaled breath) (Kelm & Schrader, 1990; Di Fenza et al., 2022).

In this study, following centrifugation, the plasma samples will be immediately stored at  $-80^\circ\text{C}$  for later  $\text{NO}_3^-$  and  $\text{NO}_2$ -content analysis via ozone-based chemiluminescence. Deproteinised samples from saliva will also be analysed

for nitrite and nitrate concentrations using a Sievers nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham,

UK), a modification of the previous chemiluminescence technique.

### **Dissemination**

Findings will be shared through local groups, local and national media, and collaborative authorship of research papers. Our institutional partners have been instrumental in developing the protocol to this point and will continue to play a significant role in this project and the larger definitive trial that will follow. We plan to present our findings in the form of three papers as part of the PhD. Paper 1 will include a literature review linking oral health to cardiovascular health.

Paper 2 will present data from all participants (N=30) before and after the PMPR treatment.

Paper 3 will compare various parameters (WMS, AEP, and all vascular functions) between two groups: [PMPR + CHX (n=15) and PMPR + placebo (n=15)].

### **Study Team**

The School of Dental, Health and Care Professions (SDHCP) at the University of Portsmouth provides high-quality NHS dental care through undergraduate students under qualified supervision. This unique model facilitates collaborative learning and research across dental and community settings.

Dr. Mahdi Mutahar is a Senior Lecturer and Departmental Head of Postgraduate Research (DDPGR) holds a PhD in Biomaterials and Salivary Research from King's College London. His current research interests focus on erosive tooth wear and the role of salivary proteins, minerals, and pellicle in dental erosion and remineralization, as well as the function and modification of salivary proteins in oral health and disease.

The School of Psychology, Sport and Health Sciences (SPSHS) boasts advanced research facilities and a team of over 20 academics. Dr. Ant Shepherd, an Associate Professor, has published extensively on cardiovascular health. He aims to benefit patients and understand the pathophysiology of chronic diseases to identify therapeutic strategies.

With over 25 years of experience in dental education and research, Professor Louca supervises PhD students and has led numerous innovative dental courses. His research focuses on dental education and prosthodontics, particularly student assessment and novel teaching techniques. He will provide guidance on dental education in clinical settings according to UK standards for the research project.

Dr. Maria Perissiou is a Senior Lecturer in Clinical Exercise Physiology at the University of Portsmouth, where her research focuses on cardiovascular physiology and the impact of exercise on cardiovascular disease prevention and treatment. She will assist with training and guide in vascular function during the project.

Dr. Stephen Bailey is a Reader in Human and Exercise Physiology at the School of Sport, Exercise and Health Sciences, Loughborough University. He specializes in lifestyle interventions for health and well-being, as well as sport performance. With degrees from the University of Wales Institute Cardiff and a PhD from the University of Exeter, he is an active member of The Physiological Society and the European College of Sport Sciences.

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Dr. Marta Roldo, an Associate Professor in Biomaterials, has produced a placebo Chlorhexidine mouthwash and has significant funding and publication experience in oral biofilms and drug delivery.

Dr. Sassan Hafizi is an Associate Professor in Cell and Molecular Biology at the Faculty of Science & Health, School of Medicine, Pharmacy and Biomedical Sciences. He serves as Executive Editor for Biochemistry and Biophysics Reports, Reviews Editor for Frontiers in Drug Discovery, and Associate/Special Issues Editor for Cells (Basel). He will be involved in the SDS-PAGE and western blot technique for AEP samples.

Dr. Nezar Al-Hebshi, is an Associate Professor and Co-director of the Oral Microbiome Research Laboratory at Temple University Kornberg School of Dentistry, USA; with an adjunct role at the Lewis Katz School of Medicine. In this project, he will assist with oral microbiome analysis and studying host vs. oral microbial protein.

Prof Chris Louca, Dr. Anish Patel Dental, Mr Bhavin Dedhia are clinicians from SDHCP, will clinically screen and recruit participants with PD and collect AEP samples.

Saagarika Sharma PhD researcher at SDHCP, will assist in the participant recruitment and AEP sample collection, collect WMS samples from individuals, and conduct vascular assessments under Dr. Mutahar's and Dr. Shepherd's supervision.

Daniel Piccolo is a PhD student and part-time Teaching Fellow in SPSHS- focusing on ageing and clinical exercise physiology. His research examines extreme environments, like hot water, to improve health in older populations. He will assist with vascular function tests in the project.

Veronika Praskacova (UP2112670) is a placement year student at SPSHS, will be assisting with the measurement of various vascular functions and the collection of blood samples for this project.

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