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University of Glasgow

The host and microbial response to non-surgical periodontal therapy

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Submitted in fulfilment of the requirements of the Degree of Doctor of Philosophy

School of Medicine, Dentistry and Nursing

College of Medical, Veterinary and Life Sciences

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October 2021

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

William Johnston

Abstract

Background: Periodontitis is one of the most prevalent diseases of mankind, characterised by dysbiotic subgingival plaque biofilms and chronic gingival inflammation. If left untreated, inflammation results in irreversible bone resorption and tooth loss, with both nutritional and psychosocial consequences for patients. Periodontitis has become increasingly associated with systemic co-morbidities including rheumatoid arthritis, suggesting it's impact may extend beyond the oral cavity. Periodontal treatment involves mechanical biofilm disruption, which is often only partially successful. Moreover, potentially detrimental systemic effects have been reported following intensive full-mouth periodontal debridement. This thesis sought to evaluate the host and microbial response to non-surgical periodontal therapy.

Methods: Clinical parameters and samples were obtained from two independent clinical studies. The first was a longitudinal cohort study (n=42), the second was a randomised controlled trial with patients receiving full-mouth debridement performed exclusively with hand (n=19) or ultrasonic instruments (n=18).

Results: The cohort study demonstrated widespread improvement in periodontal clinical parameters. There were no changes in surrogate markers of systemic inflammation or autoimmunity at 90 day follow-up (**chapter 2**). There were notable alterations to local cytokine profiles (saliva/GCF), which were accompanied by shifts in the subgingival plaque microbiota (**chapter 3**). The second study evaluated the systemic inflammatory response following full-mouth debridement. Although treatment induced an increase in serum CRP at day 1, there were no differences between groups and the magnitude of response was markedly lower than has been previously reported (**chapter 4**). Hand and ultrasonic instruments also induced comparable clinical and microbial outcomes (**chapter 5**). Notably, in both studies, differences in the post-treatment subgingival plaque composition appeared independent of residual disease.

Conclusion: There were no differences in the systemic inflammatory response following hand or ultrasonic instrumentation, and the clinical significance of this systemic response must be further substantiated. There was disparity in microbial outcomes following NSPT, and future studies should seek to establish whether compositional differences dictate long-term disease recurrence.

Acknowledgements

I would firstly like to thank my primary supervisor Professor Shauna Culshaw who has continually guided, supported and believed in me throughout my PhD. Shauna, you have taught me so much and I will be forever grateful for your knowledge and wisdom, I appreciate everything you have done for me. I would also like to thank my second supervisor Dr. Andrea Sherriff who had the difficult task of guiding me through the world of statistics and has been instrumental in the completion of my PhD. I am immensely grateful for both of your support these past few years and it was an honour to work under your supervision. The support and knowledge from Professor Gordon Ramage is also greatly appreciated and helped shape the wonderful experience I had during my PhD.

I would also like to express a huge thank you to my industry sponsors Dentsply Sirona and particularly Marilyn Goulding who has been a massive help both within and outside of the laboratory. You offered positivity and encouragement throughout my studies, and it was an honour to work and collaborate alongside yourself, Ken Guaragno and Gail Malone. You were always willing to hear my ideas and helped turn them into reality! I also extend a massive appreciation to our collaborators in the oral microbiome lab in Valencia, particularly Alex Mira, Bob Rosier, Miguel Carda-Diéguez and Alejandro Artacho. Thank you for teaching me about the microbiome and letting me visit your wonderful lab and city, it was a fantastic experience that I will never forget. You have went above and beyond to support me and I am very grateful for your encouragement, support and friendship!

None of this work would have been possible without the support of the immensely talented clinical staff - Michael Paterson, Rob Reilly, Clare Brown and Debbie McKenzie. It was a pleasure to work alongside an exceptionally talented team of clinicians, you have kept me going throughout my PhD and were always there to explain what a pocket chart is over and over! I wish to also thank all members of the Oral Sciences group past and present who have made GDH a fantastic place to work and study over these last few years. A special thanks to Dr. Jason Brown who was tasked with teaching me techniques in the laboratory and has been a brilliant footballing companion and friend throughout my studies. Dr. ‘Del’ Delaney, Mark Butcher, Emily Mckloud and Bryn Short have kept me

laughing throughout my PhD and always made sure we had ample coffee supplies! Khawlah Albashaireh, Om Alkhir Alshanta, Summaya Abusrewil, Abeer Alghamdi and Saeed Alqahtani have continually improved my Arabic reading and writing, and provided friendship, laughter and support during many late nights in the lab! Thank you all for a wonderful experience at GDH.

To my Mum, Dad, Nat and Julie – thank you for all your support. You have been fantastic and I would not have finished this thesis without you. You have put up with my constant stress and listened to my presentations 100 times in our living room! Last but certainly not least I would like to thank Isla, the only person more relieved than me that this thesis is being submitted. You have always supported me, kept me going when I needed it the most and were always willing to hear me rant! You have provided unwavering love and encouragement for years and I am eternally grateful.

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Abbreviations

AAP	American Academy of Periodontitis (AAP)
ACPA	Anti-citrullinated protein antibody
ANOSIM	Analysis of similarities
ASV	Amplicon sequencing variant
AU	Arbitrary units
AUC	Area under the curve
BoP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment level
CBA	Columbia blood agar
CDC	Centre for Disease Control and Prevention
CEJ	Cementoenamel junction
CFU	Colony forming unit
CRP	C-Reactive protein
CVD	Cardiovascular disease
DGES	Dentogingival epithelial surface area
EFP	European Federation of Periodontology
ELISA	Enzyme linked immunosorbent assays
EPH	Ecological plaque hypothesis
EUs	ELISA units
FAA	Fastidious anaerobic agar
FBS	Foetal bovine serum
FDR	False discovery rate
FISABIO	Foundation for the Promotion of Health and Biomedical Research
FISH	Fluorescent in-situ hybridisation
FMBS	Full-mouth bleeding index
FMD	Full-mouth debridement
FMPS	Full-mouth plaque index
GCF	Gingival crevicular fluid
GDP	General dental practitioner
GLM	General linear model
GWAS	Genome wide association study
HI	Hand instrumentation
HRP	Horse radish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KPH	Keystone pathogen hypothesis
LGM	Location of gingival margin

LLOQ	Lower limit of quantification
LOD	Limit of detection
LtxA	Leukotoxin A
mm	Millimetres
MMP	Matrix metalloproteinase
NGS	Next generation sequencing
NHANES III	National Health and Nutrition Examination Survey
NSPH	Non-specific plaque hypothesis
NSPT	Non-surgical periodontal therapy
OPG	Osteoprotegerin
OR	Odds ratio
OTU	Operational taxonomic unit
PAD	Peptidylarginine deiminase
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PCoA	Principal coordinate analysis
PD	Periodontitis
PEA	Proximity extension assay
PESA	Periodontal epithelial surface area
PIL	Patient information leaflet
PISA	Periodontal inflamed surface area
PPAD	*P. gingivalis* peptidylarginine deiminase
PPD	Periodontal probing pocket depth
PRRs	Pattern recognition receptors
PSD	Polymicrobial synergy and dysbiosis
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RCT	Randomised controlled trial
ROC	Receiver operating characteristic curve
rRNA	Ribosomal RNA
SCH	Schaedler anaerobic broth
SPH	Specific plaque hypothesis
SRA	Sequencing read archive
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TSB	Tryptic soy broth
U-NSPH	Updated non-specific plaque hypothesis
UI	Ultrasonic instrumentation
ULOQ	Upper limit of quantification

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Preface

Chapter 1 provides a general introduction to the thesis, outlining the current understanding of the clinical, microbial and immunological aspects of periodontitis. The methods by which periodontal treatment is performed are also discussed, alongside the techniques used to study clinical samples.

Chapter 2 investigates whether periodontal treatment can reduce serum markers of inflammation, anti-CCP2 IgG titres, and IgG antibodies reactive against periodontal bacteria. Patients suffering from periodontitis received treatment over various visits (n=42), with serum collected at baseline and 90 days after the last treatment visit.

Chapter 3 evaluates local cytokine profiles (GCF, saliva) and the subgingival plaque microbiota within the same cohort as chapter 2. The relationship between these variables and clinical disease severity at baseline, longitudinal alterations following treatment and associations with treatment response were evaluated.

Chapter 4 presents the primary outcomes from a randomised controlled trial, designed to establish whether the systemic inflammatory response to full-mouth debridement differs following instrumentation with hand (n=19) or ultrasonic instruments (n=18). Clinical parameters and local inflammatory cytokines were also compared between treatment groups.

Chapter 5 investigates the impact of different instruments on periodontal biofilms both *in vitro* and *in vivo*. The *in vivo* section compares the subgingival plaque microbiota from patients within the randomised controlled trial of chapter 4. Additionally, the reformation of this polymicrobial biofilm community was assessed with longitudinal profiling at baseline, days 1, 7 and 90 post-treatment.

Chapter 6 provides context to the major findings from this thesis, highlights general limitations of the work presented and outlines possible avenues of future research.

Chapter 1

General introduction

1.1. Periodontal disease

1.1.1. Overview

Periodontal disease refers to a range of conditions impacting the support apparatus of teeth, such as the gingival tissue, alveolar bone and periodontal ligament. The most common periodontal diseases are gingivitis and periodontitis (PD), often collectively termed 'gum-disease'. Gingivitis is primarily caused by the accumulation of dental plaque biofilms, although may also be modified by other local or systemic stimuli (Trombelli et al., 2018). Common symptoms of gingivitis including swelling, tenderness and enlargement of the gingival tissue (Tonetti et al., 2015). Most cases of gingivitis are mild and painless, with many patients being unaware of having the disease (Blicher et al., 2005). Unlike PD, gingivitis is reversible upon effective removal of the aetiologic agent (usually the dental plaque biofilm), and there is no permanent loss of tooth supporting structures (Murakami et al., 2018).

If left untreated, gingivitis may progress to PD in susceptible patients (Ammons et al., 1972, Page and Schroeder, 1976). In PD, inflammation results in irreversible destruction of periodontal ligament and alveolar bone, with corresponding loss of attachment and formation of deep periodontal pockets (**figure 1.1**). Deep pockets allow biofilms to form beneath the gumline (subgingivally), where they can no longer be effectively removed via routine oral hygiene procedures such as toothbrushing and flossing. As such, periodontal pockets create an anaerobic and protected environment for biofilms to grow and mature causing further inflammation in a continuous cycle (Van Dyke et al., 2020). Ultimately, destruction of the tooth supporting apparatus results in tooth loss which negatively impacts patients quality of life with both nutritional and psychosocial consequences including physical appearance, mastication and speech (Chapple, 2014).

The following chapter outlines the epidemiology, clinical manifestations, host and microbial mechanisms and treatment of PD, with a particular emphasis on studies involving human subjects.

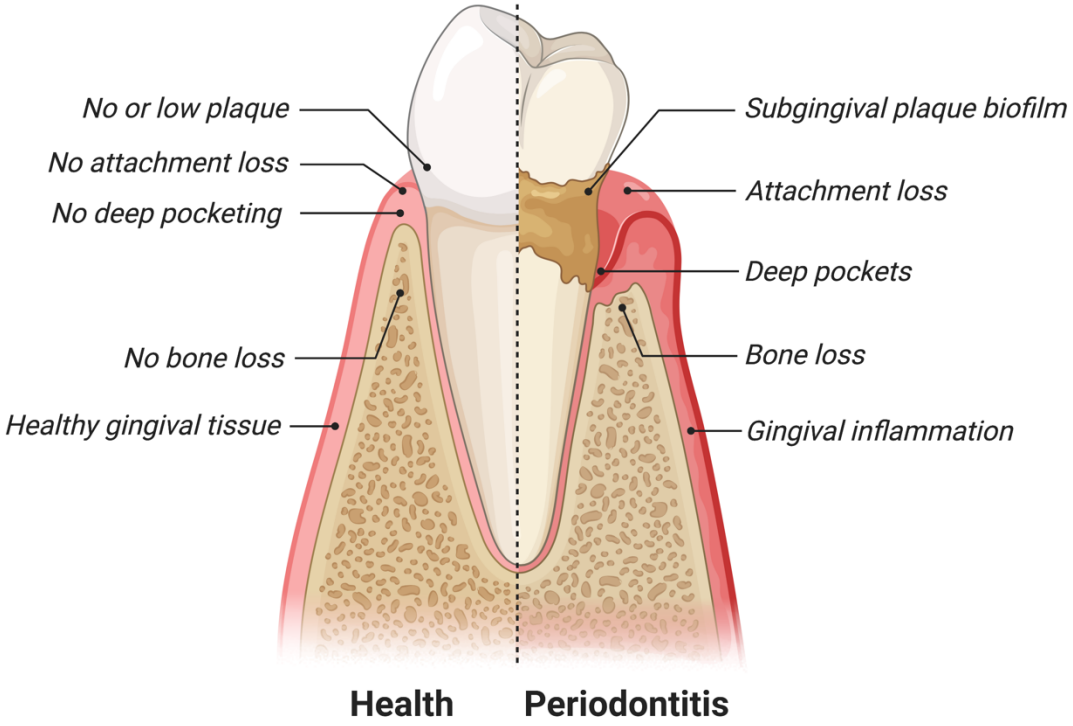

Figure 1.1. The clinical differences between health and periodontitis. Periodontitis is associated with the development of subgingival plaque biofilms, periodontal pocketing, alveolar bone loss and gingival inflammation. Image created using BioRender.

1.1.2. Epidemiology and economic burden

Periodontal disease is the most ubiquitous bone-destructive, inflammatory disease of mankind, affecting 20-50% of the total global adult population (Sanz et al., 2010). As of 2016, severe PD is ranked eleventh among all diseases for global prevalence (Global Burden of Disease., 2017, Nazir et al., 2020). It is estimated that roughly 796 million individuals suffer from severe PD worldwide with an age-standardised prevalence of 9.8% (Bernabe et al., 2020). The prevalence of severe PD peaks at 60-64 years of age, after which it plateaus (Bernabe et al., 2020). In the United Kingdom roughly 6.4% of adults suffer from severe PD (Kassebaum et al., 2014), whilst only a minority (17%) have 'very healthy' periodontal tissue according to the Adult Dental Healthy Survey 2009 (White et al., 2012).

The high prevalence of PD, particularly in severe forms, contributes to a substantial global economic burden. The indirect costs associated with all dental diseases are estimated to be $144.25 billion per year, with severe PD contributing $53.99 billion (37%) of this cost (Listl et al., 2015). Indirect costs largely reflect lost productivity or missing work through this condition, with a

combined global rate of 3.5 million years lived with disease throughout 2016 (Bernabe et al., 2020, Nazir et al., 2020). Severe PD also accounts for a large portion of the $295.67 billion per year that is estimated to be spent on direct treatment costs of dental diseases, of which Western Europe accounts for $91.05 billion (Listl et al., 2015, Tonetti et al., 2017). Recent modelling from the European Federation of Periodontology (EFP) suggest that the cost of diagnosing and treating 90% of PD cases in the United Kingdom may exceed €175 billion, however, does provide a positive return on investment over a 10 year period (European Federation of Periodontology, 2021). With an aging population and increased tooth retention these costs continue to rise, with a 57.3% increase in the burden of periodontal disease between 1990 and 2010 (Jin et al., 2016).

1.1.3. Risk factors

Whilst dental plaque biofilms are required for development of PD, they are not solely sufficient for its progression. A seminal study by Löe *et al* documented that the association between PD and plaque was relatively weak among 480 participants across 15 years (Löe et al., 1986). In this study, workers at tea plantations in Sri Lanka were classified into those with rapidly progressing PD, those with moderately progressing PD and those with no progression of PD beyond gingivitis, despite no participants performing conventional oral hygiene procedures. It was found that groups did not differ with respect to plaque indices, indicating that PD is not simply a result of dental plaque. Indeed, additional factors have been shown to increase the risk of PD development including age, smoking, systemic conditions and genetics.

Ageing is associated with an increased incidence of PD (Grossi et al., 1994, Grossi et al., 1995, Bernabe et al., 2020), although this is believed to be a reflection of the cumulative damage induced over time rather than increased rates of destruction (Van Dyke and Sheilesh, 2005). In contrast, smoking is a major risk factor for PD and can influence the extent and severity of disease. Evidence from over 12,000 participants in the third National Health and Nutrition Examination Survey (NHANES III) in the United States found that this response is dose dependent, with heaviest smokers being at the highest risk (odds ratio [OR]: 5.88), followed by milder smokers (OR: 2.79) and former smokers (OR: 1.68) compared with non-smokers (Tomar and Asma, 2000).

Furthermore, this response is not limited to cigarettes with similar findings observed with cigar and pipe smoking (Albandar et al., 2000). The mechanism by which smoking elevates risk of PD is thought to be multifactorial, related to both impairment of the host immune system (Ryder, 2007, Lee et al., 2012) and modulation of the subgingival plaque composition (Haffajee and Socransky, 2001, Jiang et al., 2020). Whilst epidemiological evidence pertaining to electronic cigarettes is still in its infancy, there is evidence to suggest that they may also modify the host immune response and the subgingival plaque microbiome towards that comparable with disease (Ganesan et al., 2020).

The most well established systemic condition associated with PD development is diabetes mellitus caused by a lack of insulin production or response, resulting in elevated blood glucose levels. Diabetic patients are roughly three times more likely to suffer PD than non-diabetic controls, with suggestion that a bi-directional relationship may exist (Preshaw et al., 2011). Evidence from NHANES III found poorly controlled diabetic patients have a greater prevalence of PD (OR: 2.90) compared with well controlled patients (OR: 1.56) (Tsai et al., 2002). As such, poorly controlled diabetes has been included as a modifiable risk factor in the most recent PD classification guidelines (Caton et al., 2018, Dietrich et al., 2019). It is believed that diabetes may increase periodontal inflammation and thereby predispose patients to PD, with additional roles in immune functioning and neutrophil activity (Taylor et al., 2013).

A genetic predisposition to PD development has also been reported. An early study profiling clinical parameters in monozygotic and dizygotic twins indicted that roughly 50% of PD susceptibility is inherited, which was not the case for gingivitis (Michalowicz et al., 2000). Later work employing a similar study design confirmed a role for genetics in PD development, although suggest that the magnitude of genetic influence has been previously overestimated (Torres De Heens et al., 2010). Evidence for the genetic basis of PD has been strengthened in recent years by large-scale analysis of single nucleotide polymorphisms. For example, a metanalysis of over 71,000 participants documented that polymorphisms in genes encoding proinflammatory cytokines (interleukin family) and matrix metalloproteinases (MMPs) are associated with PD development (Da Silva et al., 2017), whilst genome wide association studies (GWAS) have identified several additional risk loci (Munz et al., 2017, Munz et al., 2019).

1.2. Clinical classification of periodontitis

1.2.1. Periodontal examination

1.2.1.1. Clinical assessment

Several indices are employed to measure the severity of PD, assessed using a six point pocket chart in which clinicians measure different variables around teeth (**figure 1.2**). The most common index is periodontal probing pocket depths (PPD) - representing the depth from the top to the base of the pocket measured using a periodontal probe. The top of the pocket is also termed the location of gingival margin (LGM) which can be measured relative to the cementoenamel junction (CEJ). From here PPD and LGM can be combined to measure clinical attachment level (CAL). These parameters are measured in millimetres (mm) with a generally accepted error of 1 mm in either direction. Clinicians also measure bleeding on probing (BoP) scores, usually by dichotomously measuring the presence or absence of bleeding, which is presented as a percentage of total sites (Ainamo and Bay, 1975). Similarly, several plaque indexes are employed in clinical use. In the studies within this thesis, the plaque index is measured by assigning a binary score (0, 1) at each site (O'leary et al., 1972). More recent incorporation of several parameters has also led to the development of a novel parameter – the periodontal inflamed surface area (PISA) - which is discussed extensively within **chapter 2**.

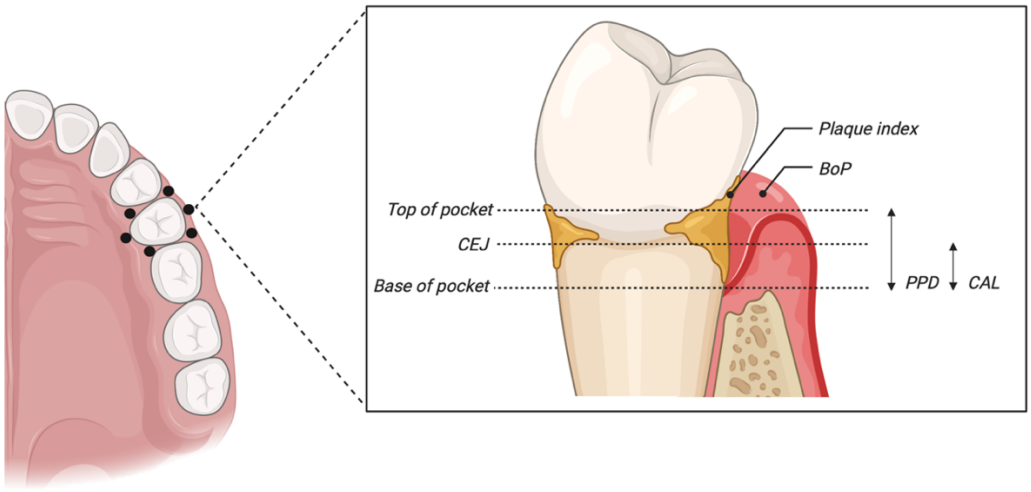

Figure 1.2. Parameters assessed during a periodontal examination. CEJ; cementoenamel junction, BoP; bleeding on probing, PPD; periodontal probing pocket depth, CAL; clinical attachment level. Black dots around the tooth represent sites at which clinical parameters are recorded. Image created using BioRender.

1.2.1.2. Radiographic assessment

Radiographs may be used to aid in the diagnosis and monitoring of PD. This technique is used to image the underlying bone structure, offering information such as root length and residual bone support which cannot be gathered by periodontal probes (Corbet et al., 2009). For many patients, radiographs may not be required and clinicians must therefore justify its use against the risks posed by ionising radiation (Tugnait et al., 2000).

1.2.2. Clinical classification

The clinical classification of PD underwent two major changes in the past 22 years, being updated in 1999 (Armitage, 1999, Lang et al., 1999, Lindhe et al., 1999) and 2017 (Caton et al., 2018, Papapanou et al., 2018, Dietrich et al., 2019) to align with new clinical and biological knowledge in the field. The 1999 classification of periodontal diseases classified PD as chronic or aggressive depending on the rate of disease progression, and either localised or generalised depending on the extent of disease. In the new 2017 classification the groupings of chronic and aggressive were removed on the basis that each form represents differing stages of the same disease (Dietrich et al., 2019). Instead, the new system features a staging (I-IV) and grading (A-C) criteria for disease severity and rate of progression respectively. The staging criteria is based on the extent of bone loss, which may be measured either via radiographic assessment or by CAL. The grading criteria factors in patients bone loss relative to their age in order to determine the rate of disease progression. The new classification also assesses the current periodontal status and risk factors for future progression. The current periodontal status is classified as stable, in remission or unstable based on BoP and PPD (Chapple et al., 2018, Papapanou et al., 2018, Caton et al., 2018, Dietrich et al., 2019). Under this new classification, the mildest form of PD is Stage I Grade A representing a mild degree of bone loss with a slow rate of progression, whilst the most severe form is Stage IV Grade C representing a very severe degree of bone loss (apical third of root) with a rapid rate of progression.

1.2.3. Case definition

In addition to clinical classification, there is a requirement to define the case definition of PD for use in epidemiological and biological research studies. Previous studies have employed differing minimum disease criteria, which may have a downstream impact on cross-study comparisons, systematic reviews and meta-analyses. The universal adoption of a standardised case definition is essential to standardise inclusion criteria on an independent study and population scale. Early case definitions, such as the Russell's periodontal index (Russell, 1956) and periodontal disease index (Ramfjord, 1959), focussed largely on the presence of inflammation under the assumption that untreated gingivitis would eventually progress to PD in all cases. Since then, several case definitions have been employed using CAL and PPD in isolation or in combination, although there is a distinct lack of uniformity between different criteria (Beck et al., 1990, Machtei et al., 1992, Tomar and Asma, 2000). To address this issue, the Centre for Disease Control and Prevention (CDC) and American Academy of Periodontitis (AAP) outlined threshold values for PPD and CAL which constitute PD (Page and Eke, 2007). These values have been widely adopted for use in periodontal research, and allow differentiation between no/mild, moderate and severe disease (**table 1.1**). Additional criteria including cumulative PPD, representing the cumulative sum of elevated probing depths either >3, >4 or >5 mm, are also becoming more frequently used in combination to this case definition to ensure a minimum level of disease in clinical studies (Dietrich et al., 2008).

Table 1.1. Case definition of PD proposed by CDC and AAP working group. This table is modified from Page and Eke (2007) in the Journal of Periodontology.

Case	Criteria		
	Clinical attachment level (CAL)		Periodontal probing pocket depth (PPD)
Severe periodontitis	≥2 interproximal sites ≥6 mm at non-adjacent sites	and	≥1 interproximal site with ≥5 mm at non-adjacent sites
Moderate periodontitis	≥2 interproximal sites ≥4 mm at non-adjacent sites	or	≥2 interproximal sites ≥5 mm at non-adjacent sites
No or mild periodontitis	Does not meet criteria for moderate or severe periodontitis		

1.3. Microbial aspects of periodontitis

1.3.1. Dental plaque biofilms

Bacteria rarely exist as isolated free-floating planktonic organisms, despite this being the most well-studied form in conventional microbiology research. Instead, they primarily exist within complex polymicrobial communities termed 'biofilms'. Biofilms are communities of microorganisms which attach to surfaces and become embedded within a self-produced extracellular matrix (Costerton et al., 1981a, Costerton et al., 1981b). These communities are ubiquitous in nature and can form within human hosts at a range of sites including the mouth, gut, skin and implants. Embedded organisms benefit from extensive protection against antimicrobials and the host immune system, causing a substantial burden in healthcare settings (Patel, 2005, Lindsay and Von Holy, 2006, Ramage et al., 2010). For example, it has been estimated that biofilms contribute to over 65% of all microbial diseases and up to 80% of chronic infections (Jamal et al., 2018).

The role of biofilms has become increasingly recognised and appreciated within several disciplines, and none more so than periodontology. Bacteria were first observed in dental plaque over three centuries ago by Antoni van Leeuwenhoek, who depicted various morphologies originating from the 'white matter between teeth'. Since then, it has been recognised that dental plaque is a microbial biofilm which adheres to the tooth surface playing an important role in health and disease (Socransky and Haffajee, 2002, Marsh, 2004). These biofilms are rich in both the quantity and diversity of bacteria, with over 700 species estimated to inhabit the oral cavity (Aas et al., 2005, Human Oral Microbiome Database, 2016). Of these, individuals are thought to harbour roughly 100-200 species, primarily belonging to Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes and Fusobacteria phyla (Dewhirst et al., 2010, Griffen et al., 2012).

In healthy sites, dental plaque biofilms form a symbiotic relationship with the host, providing a colonisation barrier against more pathogenic species and regulating local host defences (Marsh, 2000, Devine et al., 2015). Commensal biofilms aid in the development of the immune system via constant exposure to a diverse array of microbial antigens and are capable of metabolising nutrients - including nitrate - for use by the host (Seneviratne et al., 2011, Hyde et al., 2014, Rosier et al., 2018). However, perturbations in the composition of dental

plaque are associated with PD and several 'periodontal pathogens' have been implicated in disease development. The following sections outline the maturation and composition of dental plaque biofilms, and hypotheses describing a microbial origin of PD.

1.3.2. Adhesion and maturation of dental plaque biofilms

Dental plaque biofilms form rapidly on the tooth surface, to the extent that it is recommended individuals brush their teeth at least twice per day to prevent biofilm accumulation and maintain oral health. It has been demonstrated that biofilms quickly reform following professional cleaning, with some species recolonising the tooth surface within a 2 hour period (Li et al., 2004, Hartenbach et al., 2018). Additional studies have demonstrated that individual species recolonise at different timepoints ranging from days (Teles et al., 2012) to months (Haffajee et al., 2008). These studies confirmed the concept of 'early' and 'late' colonisers which was initially documented in the 1960s (Ritz, 1967). From these studies, it is known that dental plaque biofilms form rapidly and undergo dynamic alterations at different timepoints during development.

Indeed, dental plaque formation is a sequential process involving multiple stages (**figure 1.3**). The first stage involves the formation of a protein rich pellicle, termed the salivary or acquired pellicle, which begins to coat the tooth surface within minutes of cleaning (Al-Hashimi and Levine, 1989, Kolenbrander et al., 2002). The pellicle arises from deposition of glycoproteins and mucins contained within saliva onto the tooth surface (Lindh et al., 2014). This functions to protect, lubricate and hydrate the enamel, although also provides a substrate for the attachment of early colonising bacteria (Marsh and Bradshaw, 1995). Early colonisers are primarily Streptococci species such as *S. sanguinis, S. mitis* and *S. oralis*, which equate to roughly 80% of the initial biofilm composition (Nyvad and Kilian, 1987, Aruni et al., 2015a). The remaining biofilm is mainly composed of *Actinomyces*, *Haemophilus*, and *Neisseria*, capable of binding to glycoprotein receptors on the exposed salivary pellicle via outer membrane adhesins. This is driven by electrostatic attractions, van der Waals forces, hydrogen bonds and calcium bridges (Hannig and Hannig, 2009). At this stage, attachment between bacteria and pellicle is weak and reversible. Soon after binding, bacteria excrete extracellular polymeric substances which helps

maintain firm adherence both to the salivary pellicle and each other (Huang et al., 2011).

Once adhered to the salivary pellicle, early colonisers provide a base from which the mature biofilm can form. Later colonising species are able to recognise receptors present on the surface of early colonisers and begin to attach on to the biofilm in a sequential manner (Huang et al., 2011). Under the spatiotemporal model it was thought that *Fusobacterium* species, characterised by long filamentous rods, were key bridging organisms that linked early and late colonisers (Kolenbrander et al., 2002). This analysis was largely based on co-aggregation studies between *Fusobacterium* other late colonisers. However, more recent analysis using fluorescent in-situ hybridisation (FISH) probes to outline the biogeography of human dental plaque have suggested that this role may also be performed by *Corynebacterium* species (Welch et al., 2016). The same study suggested that *Corynebacterium* species anchor themselves within the biofilm and grow outwards from the tooth, thereby providing a crucial support structure in the biofilm architecture. Co-aggregation among bacteria is also central to the development of dental plaque biofilms. Binding of early colonisers to each other, or to bridging organisms, provides a substrate for additional organisms to attach to the biofilm. Obligate anaerobes including *Prevotella*, *Porphyromonas*, *Treponema* and *Tannerella* species recognise receptors in the other biofilm layers and begin to colonise.

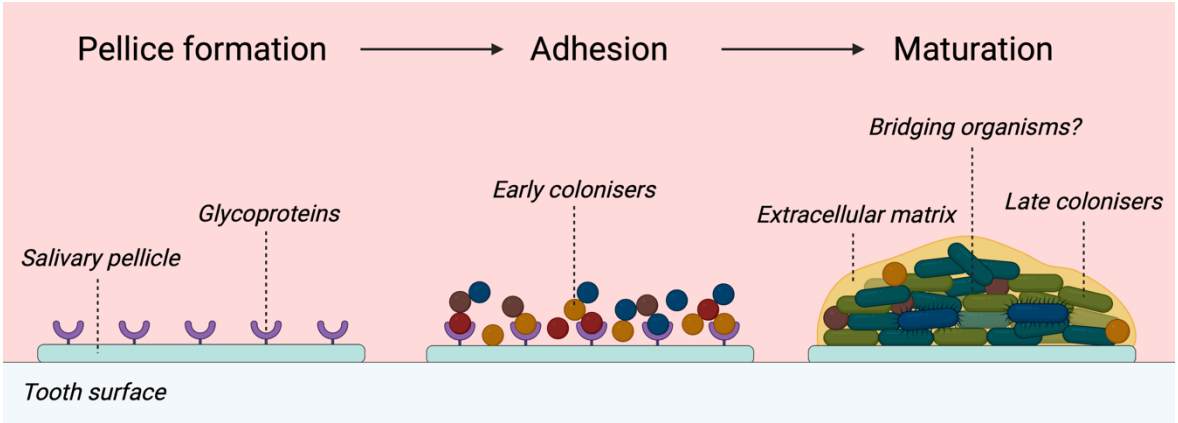

Figure 1.3. Simplified diagram detailing the stages involved in the formation and development of dental plaque biofilms. The salivary pellicle rapidly coats the tooth surface and provides a substrate for attachment of early colonisers. From here, co-aggregation and bridging species allow attachment of late colonisers and formation of mature dental plaque biofilms. Image created using BioRender.

1.3.3. Supra and subgingival biofilms

Dental plaque biofilms fall into one of two categories; supragingival which forms above the gingival margin, and subgingival which forms below it (within the periodontal pocket). Current understanding is that subgingival biofilms are a continuation of supragingival biofilms, although they are not entirely interdependent despite being physically connected (Aruni et al., 2015a). In previously healthy sites, subgingival biofilms likely arise from supragingival plaque as it extends into the periodontal pocket. However, a drastic shift in the environment from an openly exposed oxygen rich atmosphere to an anaerobic microenvironment flushed with inflammatory infiltrate dictates alterations in biofilm composition (Marsh, 1994). The lack of interdependence between supra and subgingival biofilms is most readily evidenced by the finding that supragingival biofilm removal is not sufficient to halt disease progression in deep periodontal pockets, and induces little alteration in the subgingival biofilm composition (Teles et al., 2006, Aruni et al., 2015a). As such, the majority of periodontal microbiology research has focussed on subgingival plaque biofilms.

1.3.4. Hypotheses on microbial origin of disease

The role of dental plaque in the development of PD has been extensively studied for over 100 years. During this timeframe, several hypotheses have been proposed which link features of the subgingival plaque biofilm with disease initiation and progression. As knowledge of the field has advanced and technology has improved these theories have been gradually updated, forming a cumulative understanding of PD aetiopathogenesis.

1.3.4.1. Non-specific and specific plaque hypotheses

The earliest reported hypothesis relating to a microbial origin of PD is the original non-specific plaque hypothesis (NSPH), dating back to the 19th century (Black, 1884, Miller, 1890). At this time, identification of specific bacteria were limited and researchers were unable to identify a consistent pathogen responsible for disease. As such, it was assumed that the quantity rather than composition of plaque was responsible for disease. In this model it was thought that the host was capable of tolerating and detoxifying bacterial products via salivary neutralising acid. However, once plaque accumulated above a certain

threshold, this tolerance would become overwhelmed resulting in the development of PD (Black, 1884, Miller, 1890, Rosier et al., 2014). Therefore, treatment was aimed at removing the entire plaque biomass irrespective of composition, which is still routinely used as a first-line therapy for PD.

In the 1970s, advancement in microbial techniques such as culture and microscopy led to better and more accurate species identification. The ability to identify species associated with disease led to the specific plaque hypothesis (SPH), which was originally associated with dental caries (Loesche, 1976). In this model, certain pathogenic organisms were solely responsible for the onset and progression of disease such as *Lactobacillus* species and mutans Streptococci in dental caries (Loesche and Nafe, 1973, Loesche, 1976). The SPH coincided with development of the anaerobic cabinet, which allowed for culture and identification of anaerobes associated with PD. Thus, the SPH was further extended to incorporate PD, although identification of several 'causative' pathogens in the proceeding decade questioned it's applicability (Rosier et al., 2018, Fragkioudakis et al., 2021). Nonetheless the SPH was the first theory to appreciate the variance in species virulence, which still applies to the current understanding of PD.

In recognition of differences in species virulence, the NSPH was revisited in the 1980s with the development of the updated NSPH (Theilade, 1986). This theory gained insight from the finding that many pathogens in the SPH were common members of dental plaque in both health and disease. Therefore, it was suggested that all bacteria functioned in the transition from health to disease and displayed varying degrees of virulence in doing so. Evidence for this hypothesis stemmed from the finding that plaque accumulation would at least induce gingivitis, and the progression to PD was dependent upon the virulence of specific communities (Rosier et al., 2014). The U-NSPH framed future research by revisiting the role of the entire community in the development and onset of PD. Additional support for this theory arises from the finding that non-specific mechanical removal and disruption of the entire biofilm community remains the most effective way to treat and manage PD.

1.3.4.2. Ecological plaque hypothesis

Nearly a decade after the U-NSPH, the ecological plaque hypothesis (EPH) was developed by Philip D Marsh which combined aspects of preceding theories (Marsh, 1994). To explain the shift towards oral disease (caries and PD), it was proposed that environmental pressures may selectively enrich pathogenic species. In the case of PD, it was proposed that plaque accumulation at the gingival margin triggers inflammation, which results in an elevated flow of gingival crevicular fluid (GCF) and gingival bleeding. GCF is a serum transudate released from the gingival tissue and it has been suggested that proteins present in GCF and blood may be utilised by bacteria as sources of nutrition, thereby shifting the plaque biofilm composition (Marsh, 1994). Furthermore, GCF has a neutral or slightly alkaline pH, which has been shown to enhance the growth of organisms associated with PD including *Prevotella intermedia* and *Porphyromonas gingivalis* (Bickel and Cimasoni, 1985). Marsh also suggested that bacteria can influence the environment to create a more suitable environment for growth. In this case, aerobic early colonisers consume oxygen to produce carbon dioxide and hydrogen (Marsh, 2003), which lowers the redox potential and allows obligate anaerobes to grow and survive. Environmental influence has since been widely demonstrated in the case of smoking, diet and inflammation. However, the pitfall of the EPH is that it fails to explain inter-individual differences in PD susceptibility (Löe et al., 1986, Rosier et al., 2014).

1.3.4.3. Keystone pathogen hypothesis

A more recent hypothesis for the microbial origin of PD is the keystone pathogen hypothesis (KPH) which focuses on *P. gingivalis* as a driver of biofilm modulation and disease initiation (Hajishengallis et al., 2012). Using mouse models, Hajishengallis et al. demonstrated that this low-abundance bacterium is able to markedly shift the composition of the oral microbiome and drive alveolar bone loss upon colonisation (Hajishengallis et al., 2011). Notably, bone loss was not observed within germ-free mice colonised with *P. gingivalis*, indicating that the commensal microbiota is required to initiate disease. Furthermore, C3a and C5a receptor deficient mice did not develop bone loss, suggesting that the host complement pathway is also essential to this process. To date, the KPH has been convincingly demonstrated in mice but human evidence is still lacking. For

example, whilst *P. gingivalis* is frequently isolated from diseased sites, it is also found in 20-30% of healthy individuals (Griffen et al., 1998, Yang et al., 2004). Nonetheless, the essential role of the host immune response incorporated within this hypothesis is an important feature which had been previously overlooked.

1.3.4.4. Community dysbiosis

The current paradigm is that PD arises as a result of biofilm 'dysbiosis'. Dysbiosis refers to an imbalanced interaction between bacteria or bacterial community and the host, which is detrimental to the host (Lamont et al., 2018). Evidence for dysbiosis comes largely from 16S ribosomal RNA (rRNA) sequencing studies where the diversity, richness and relative abundances of species is altered in PD compared with health (Griffen et al., 2012, Abusleme et al., 2013, Hong et al., 2015). Therefore, the question remains as to how dysbiosis arises within the subgingival plaque community. One hypothesis which has been developed to outline the shift from 'eubiosis' to dysbiosis is the polymicrobial synergy interaction and dysbiosis (PSD) model (Hajishengallis and Lamont, 2012, Lamont and Hajishengallis, 2015).

In the PSD model, synergy within biofilm communities is important for the shift to dysbiosis and disease development. Given the heterogeneity of the oral microbiota it is proposed that different species may be able to fill distinct roles which converge to stabilise the microbiota in a synergistic state through co-adhesion, interspecies signalling, physiological compatibility and community adaption. From here, colonisation of keystone pathogens (e.g. *P. gingivalis*) is required to induce the shift to dysbiosis through immune modulation, cross-talk with accessory pathogens and increased expression of virulence factors (Lamont and Hajishengallis, 2015). This model links to microbiome studies and explains the heterogeneity in the biofilm composition from subgingival plaque samples. Additionally, whilst *P. gingivalis* was given as an example, it was noted that other organisms may similarly fill this role.

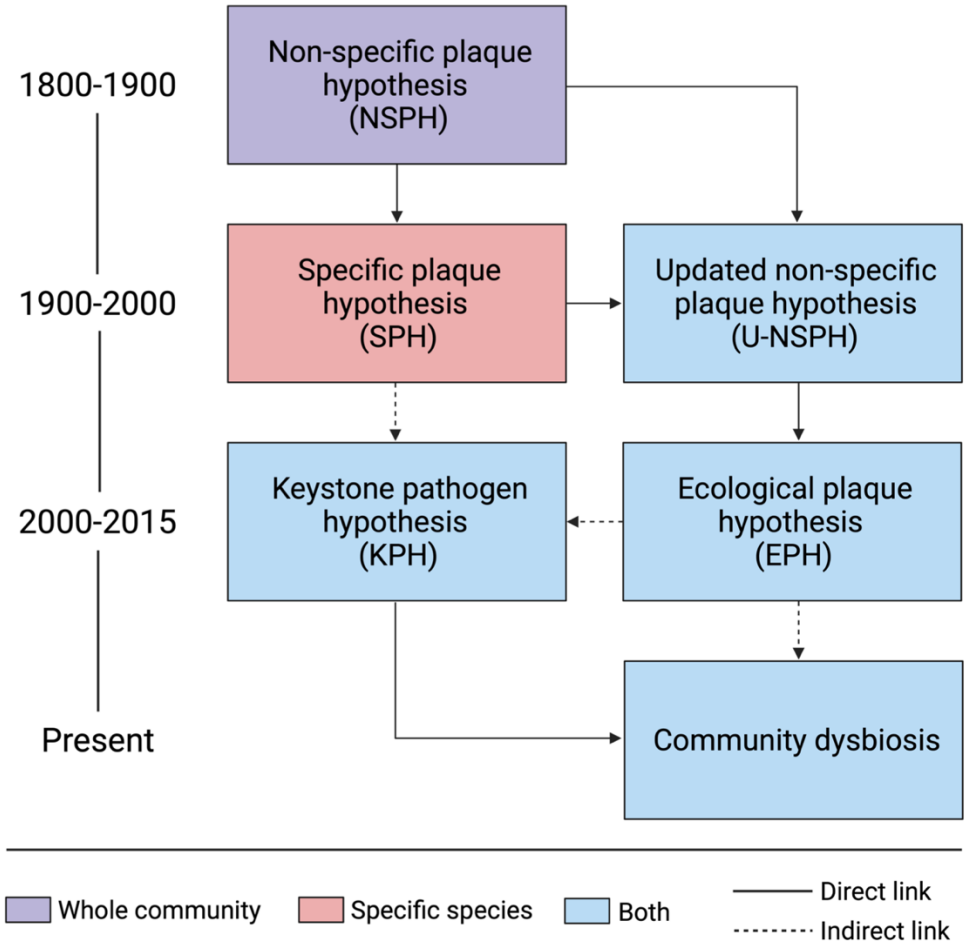

Figure 1.4. Timeline of changing dental plaque hypotheses in periodontal research. As early as the 19th century, these hypotheses began to shape periodontal microbiology research. With advancements in research techniques, new models have been developed to reflect current knowledge and build upon preceding paradigms. Purple boxes indicate whole community involvement, pink boxes indicate specific species involvement and blue boxes indicate both. Image created using BioRender.

1.3.5. Studying subgingival plaque composition

The hypotheses outlined above are a reflection of techniques and knowledge available to researchers at set points in time. The data gathered from microbial analysis of subgingival plaque samples has been continually used to form and advance the understanding of dental plaque biofilms in health and disease. Several techniques have been used in the analysis of subgingival plaque, including culture, microscopy, DNA-DNA hybridisation and most recently, 16S rRNA sequencing. Each technique has its own advantages and disadvantages but have undoubtably contributed to the modern day understanding of PD.

1.3.5.1. Culture and microscopy

Distinct compositional changes in the subgingival plaque of PD patients were initially discovered using culture and microscopy techniques. Early microscopy studies highlighted that the composition of healthy plaque was predominantly composed of Gram-positive cocci, whilst PD plaque was characterised by an increase in Gram-negative flagellated cells and spirochetes (Listgarten, 1976). In line with these findings, incorporation of culture techniques identified a predominance of streptococci and *Actinomyces* species in health (Slots, 1977, Syed and Loesche, 1978). Development of the anaerobic cabinet allowed identification and culture of Gram-negative bacilli predominant in PD such as *Bacteroides, Campylobacter* and *Capnocytophaga* species (Newman et al., 1976, Van Palenstein Helderman, 1981). These pioneering studies formed a foundation for periodontal microbiology research, although they were inherently limited by bias towards easily cultivatable species (Rosier et al., 2014).

1.3.5.2. DNA-DNA hybridisation

Advancement in molecular identification in the 1990s led to improvements in species identification, allowing simultaneous quantification of up to forty bacteria in subgingival plaque samples through 'checkerboard' DNA-DNA hybridisation. This technique works by adding microbial DNA to a nylon membrane, which is then hybridised with whole-genomic digoxigenin labelled DNA probes against target species (Socransky et al., 1994). Antibodies directed against digoxigenin can next be applied and quantified to estimate bacterial quantities.

In 1998, Socransky et al. quantified 40 bacterial species in over 13,000 subgingival plaque samples from 185 patients with (n=160) and without (n=25) PD (Socransky et al., 1998). Among the 40 species, the authors evaluated associations between each species and between species and periodontal disease severity. From this study, bacterial complexes were identified and each was designated a colour (**figure 1.5**). The yellow complex consisting of streptococci species were strongly associated with one another and formed part of the core composition, present in comparable levels in both healthy and diseased samples. This was additionally true for the purple complex consisting of *Actinomyces odontolyticus* and *Veillonella parvula*, and green complex consisting of

Eikenella, Capnocytophaga and *Aggregatibacter* (serotype a) species. Originally ungrouped, the '*Actinomyces*' complex (colloquially termed 'blue' complex) also appears part of this core composition. In contrast, the orange complex primarily consisting of *Fusobacterium, Prevotella* and *Campylobacter* species was found to mildly associate with pocket depth, whilst the most pronounced association with disease severity was observed for the red complex comprising *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*.

After this discovery, colour complexes became the cornerstone of periodontal microbiology and checkerboard DNA-DNA hybridisation is still routinely used in clinical trials (Duarte et al., 2018, Feres et al., 2018, Teles et al., 2021). The included species in these assays have been carefully selected over several years to quantify 40 major constituents of the ecosystem (Gellen et al., 2007). This technique offers a massive improvement on conventional culture and microscopic identification, although it remains limited by dependency on *in vitro* growth. For organisms to be included in checkerboard arrays, they must be isolated, grown and standardised so that standard curves can be generated. It has been estimated that 40-60% of oral bacteria have yet to be cultivated, and as such cannot be incorporated into these systems (Siqueira and Rocas, 2013). Recent advances in culture techniques have started to allow growth of previously uncultivatable organisms (Vartoukian et al., 2016), although the field has gradually transitioned towards high-throughput culture independent technologies for assessment of subgingival plaque.

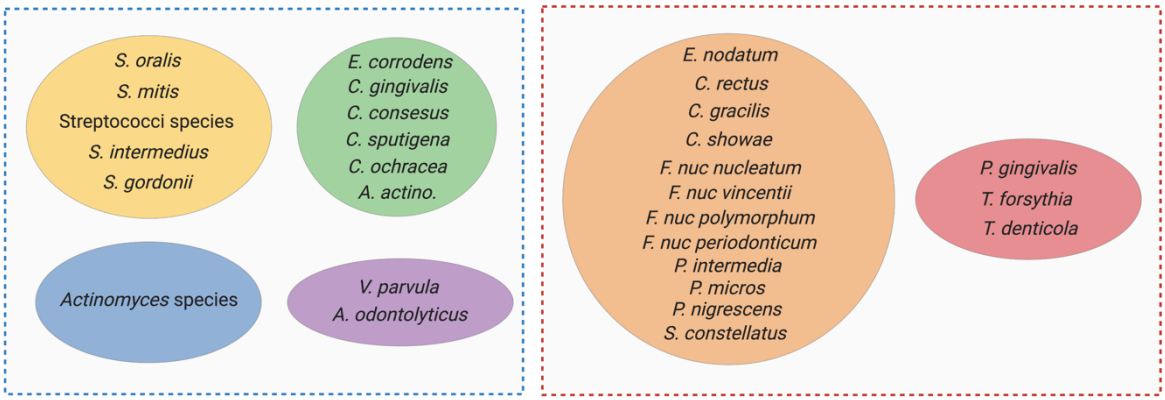

Figure 1.4. Subgingival bacterial complexes outlined by Sigmund Socransky. Outer boxes indicate complexes in the core composition (blue) and those associated with disease (red). This figure is modified from Socransky et al., (1998) in the Journal of Clinical Periodontology. 'A. actino' in the green complex represents *Aggregatibacter actinomycetemcomitans* serotype a. Image created using BioRender.

1.3.5.3. The microbiome

In the last 20 years, considerable strides in the accessibility, affordability and throughput ability of sequencing has facilitated a new area of extensive research – the microbiome. The microbiome relates to all genetic material within a microbial community, although this thesis focussed solely on the bacterial component. This has been of particular relevance in the oral cavity given large diversity and low proportion of cultivatable species. For example, of the 700 species predicted to inhabit the oral cavity, only 280 are estimated to have been cultured (Dewhirst et al., 2010). Therefore, culture independent assessment of subgingival plaque samples has allowed for a deeper understanding of PD than was previously possible. Species identified only via culture-independent sequencing technologies are often termed 'phyla' or 'taxa', although in the interest of simplicity this thesis has termed any anything classified at species-level as a 'species'. To facilitate microbiome research, several next generation sequencing (NGS) platforms have emerged (Roche 454, Ion torrent, Nanopore, Illumina). This section will primarily discuss Illumina systems, which have recently become the most frequently used in microbiome research due to high quality reads and affordable cost (Hodzic et al., 2017).

Microbiome research has largely focussed on short-read sequencing using fragments of the 16S rRNA gene, which encodes the RNA component of the 30S subunit of the bacterial ribosome. This 1,500 basepair gene is conserved within all bacteria and contains nine hypervariable regions (V1-V9), which are interspaced among various conserved regions (Chakravorty et al., 2007). Current practices involve sequencing only a fragment of this gene containing one or several of these hypervariable regions to allow for discrimination of bacterial genera or species. The most widely used Illumina protocols target the V3-V4 region (Wade and Prosdocimi, 2020), which was employed throughout this thesis. These regions have been extensively applied to study the oral microbiome (Dzidic et al., 2018, Carda-Diéguez et al., 2019, Ferrer et al., 2020). Recently, the V1-V3 region has also gained popularity, which was previously limited to the Roche 454 platform that provided longer reads (Zheng et al., 2015). The trade-off of hypervariable region selection appears to be that V3-V4 is more reproducible (Teng et al., 2018), although V1-V3 may better discriminate certain genera (streptococci) at the species-level (Wade and Prosdocimi, 2020).

After DNA extraction, the selected hypervariable regions (V3-V4) are amplified by PCR which attaches overhang adapters containing sample barcodes and regions complimentary to the Illumina flow-cell. These DNA fragments are then hybridised to oligos present in the flow-cell and undergo bridge amplification to generate fragment clusters (**figure 1.5**). Fluorescently labelled nucleotides are next introduced and bind complimentary to the fragment. Through excitation by a light source, the base can be determined and used to generate a forward and reverse sequence. After sequencing, forward and reverse reads are aligned and sorted into samples using unique barcodes. The sequences produced from 16S rRNA sequencing can either be grouped into operational taxonomic units (OTUs) or used as amplicon sequencing variants (ASVs). The OTU approach groups sequences typically based on a 97% similarity threshold, whilst the ASV approach denoises the data to determine the true biological sequences from PCR and sequencing errors, and uses this final sequence as an ASV. Thus, the ASV approach generates exact sequences with high confidence that these sequences are not due to error. In recent years ASV methods such as DADA2 have superseded the use of OTUs as more reliable and translatable protocols for analysis of marker gene sequencing (Callahan et al., 2017).

Applying these technologies in PD has revealed that the diversity of subgingival plaque is far higher than previously assumed (Bik et al., 2010). Studying subgingival plaque from 29 healthy controls and 29 PD patients, it was found that the abundance of 72 genera and 176 species significantly differed between groups (Griffen et al., 2012). Although this analysis confirmed the association of the red-complex with PD, roughly half of the species elevated in disease were uncultured. Likewise, one of the largest elevations in disease was observed for *Filifactor alocis,* a Gram-positive anaerobe, suggesting that previous segregation of Gram-positives as 'health-associated' was too simplistic in nature. Ensuing analysis demonstrated an increase in taxonomic diversity in subgingival plaque from PD patients compared with health (Abusleme et al., 2013). This analysis involving 10 healthy controls and 22 PD patients characterised the core subgingival microbiota in each group. It was found that *Streptococcus, Actinomyces* and *Rothia* species predominated in health, whilst anaerobes including *Porphyromonas, Tannerella, Treponema, Parvimonas, Mogibacterium, Filifactor* and *Eubacterium* were highly abundant in disease. The same analysis

suggested that a core microbiota was found in all samples, consisting of *Lautropia, Corynebacterium, Eikenella, Veillonella* and *Fusobacterium* species.

Collectively, these findings highlight that the microbiota shifts in PD extend well beyond the conventional red-complex and involve several unculturable organisms. In line with these findings, a systematic review to identify novel 'periodontal pathogens' found moderate evidence for the association of 17 additional species including *Fretibacterium fastidiosum, Mogibacterium timidum* and *Filifactor alocis* (Pérez-Chaparro et al., 2014). Many of these species have been independently confirmed in a recent meta-analysis of 10 microbiome studies outlining distinctive changes in subgingival plaque composition between health, gingivitis and PD (Abusleme et al., 2021).

Short-read 16S rRNA sequencing has allowed for a wider analysis of subgingival plaque communities than was previously possible. However it is limited by issues such as reduced classification accuracy at species level and lack of absolute quantification. The former is particularly true for *Streptococcu*s, which is the most abundant genera in the oral cavity and contains several species with nearly identical 16S rRNA sequences (Wade and Prosdocimi, 2020). Moving forward, such issues may be overcome as shotgun metagenomic sequencing of the entire 16S rRNA region become more accessible. In this regard, newly developed technology such as the Nanopore MinION offer promising protocols for long-read benchtop sequencing, although are currently hampered by higher error rates (Rang et al., 2018).

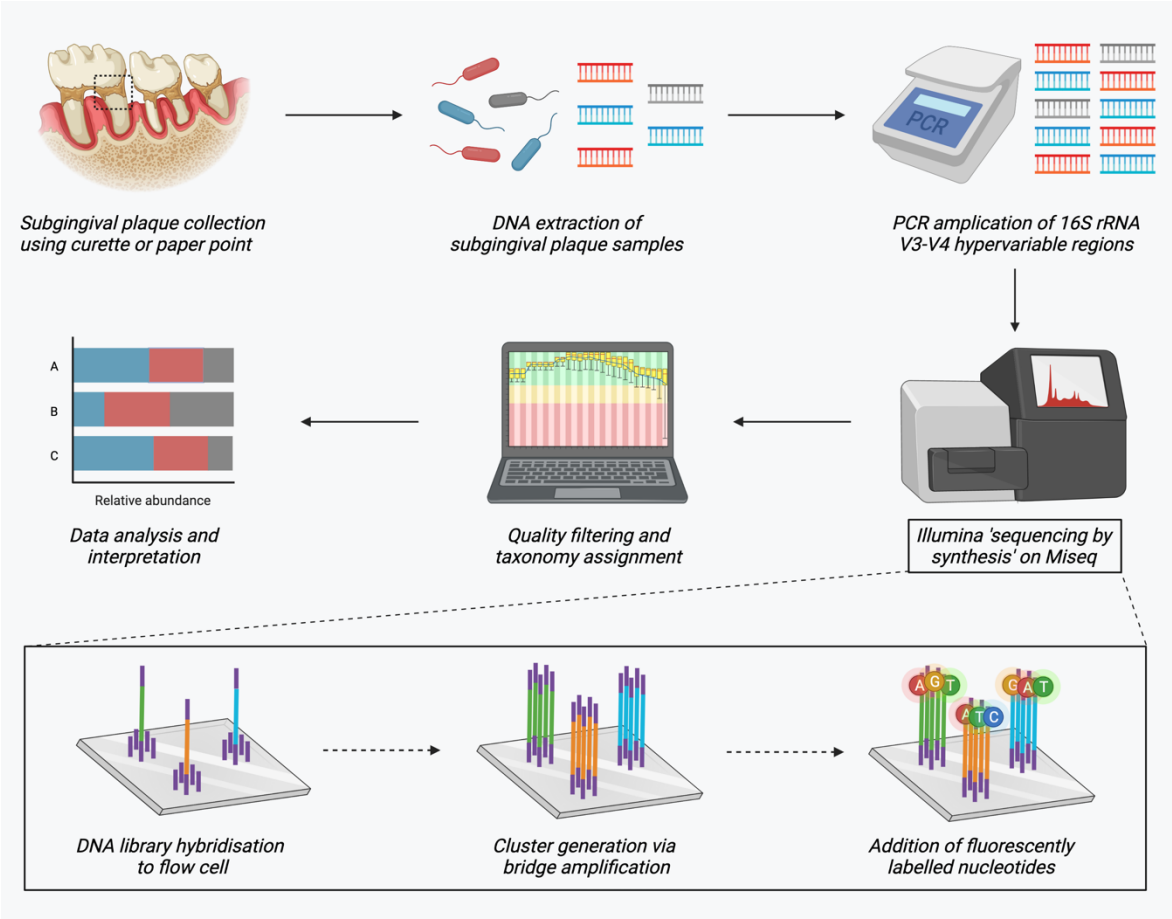

Figure 1.5. Basic summary of the steps involved in analysing the subgingival plaque microbiome. Plaque samples are harvested via curettes or paper points. DNA is extracted and selected hypervariable regions (V3-V4) amplified via PCR. DNA is normalised and pooled prior to Illumina sequencing which involves library hybridisation, cluster generation and sequence determination via fluorescent nucleotides. Resulting sequences are assigned against taxonomy databases and relative abundances can be analysed. Image created using BioRender.

1.4. Host response in periodontitis

1.4.1. Importance of the immune response

As evidenced by the Löe et al. seminal study, dental plaque biofilms are necessary but not sufficient for PD development, and such biofilms do not directly induce alveolar bone loss (Löe et al., 1986). Instead, the immune response plays a pivotal role in disease progression, driving host-mediated tissue destruction and bone resorption. Therefore, the host response is instrumental in the progression of disease. The following section will discuss the immunological features of PD primarily focussing on cytokines and chemokines which modulate this response, the interplay between inflammation and biofilm dysbiosis, and how the host response is assessed within clinical studies.

1.4.2. Immune cell networks in health and disease

The initiation and progression of PD from an immunological perspective has been conventionally broken down into four distinct stages based on histological analysis; the initial lesion, early lesion, established lesion and advanced lesion (Page and Schroeder, 1976). More recently, it has been suggested that this model is too arbitrary and simplistic, with a large degree of overlap between each stage (Cekici et al., 2014). This early immunological model does however provide a useful way to simplify and compartmentalise the various features of PD progression and many aspects remain fundamentally valid to this day (Hajishengallis and Korostoff, 2017).

Within the oral cavity, the immune response is constantly active and functions to maintain tissue homeostasis. In pristine periodontal health, whilst there is an absence of clinical inflammation (e.g. BoP) there is a constant level of immunological surveillance, evidenced by immune cell infiltration and production of low levels of inflammatory mediators (Lang and Bartold, 2018, Chapple et al., 2018). Neutrophils are one of the key cell types which function in maintaining gingival tissue homeostasis. This cell type appears crucial in this regard, with neutropenic patients (via neutrophil deficiency and/or trafficking deficiency) being highly susceptible to PD progression and elevated disease severity (Deas et al., 2003, Darveau, 2010).

Neutrophils are also one of the characteristic cell types of the initial lesion identified by Page and Schroeder (1976). Within 2-4 days of plaque accumulation there are minimal signs of clinical inflammation although early features of an immune response are visible histologically. Bacterial antigens and metabolic products trigger adjacent epithelial cells to produce cytokines and chemokines via recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), resulting in increased neutrophil and monocyte migration into the gingival tissue (Cekici et al., 2014). Importantly, this initial response is unable to remove or neutralise dental plaque biofilms inducing further inflammation. As a result, the initial lesion progresses into the early lesion, where macrophages, lymphocytes and mast cells begin to accumulate. During formation of the early lesion, GCF flow is increased and clinical signs of inflammation are observed commensurate with gingivitis (Page and Schroeder, 1976).

Despite comparable biofilm exposure, some patients will never progress beyond this early lesion. In cases where this response does progress into the established lesion, then plasma cells, T-cells, B-cells and macrophages begin to predominate in the inflammatory infiltrate. This was originally thought to represent a distinct switch from the innate to adaptive immune response, although current understanding is that pathways of preceding lesions still function during progression (Cekici et al., 2014). For example, neutrophils are now believed to mediate a large portion of periodontal tissue destruction, and neutrophil loads positively correlate with PD disease severity (Hajishengallis and Korostoff, 2017). Thus, the pathways involved in the initial lesion are not simply replaced by ensuing cell networks.

Similar to the early lesion, the established lesion may remain stable or progress to the advanced lesion, representing clinical PD. In this stage, the inflammatory lesion extends into the periodontal ligament and alveolar bone, resulting in irreversible attachment loss and an influx of plasma cells. MMPs function in combination with osteoclasts to destroy the periodontal ligament and resorb alveolar bone. The result is a transition in the immune response from that of protective surveillance in periodontal health, to destructive chronic inflammation in PD (**figure 1.6**).

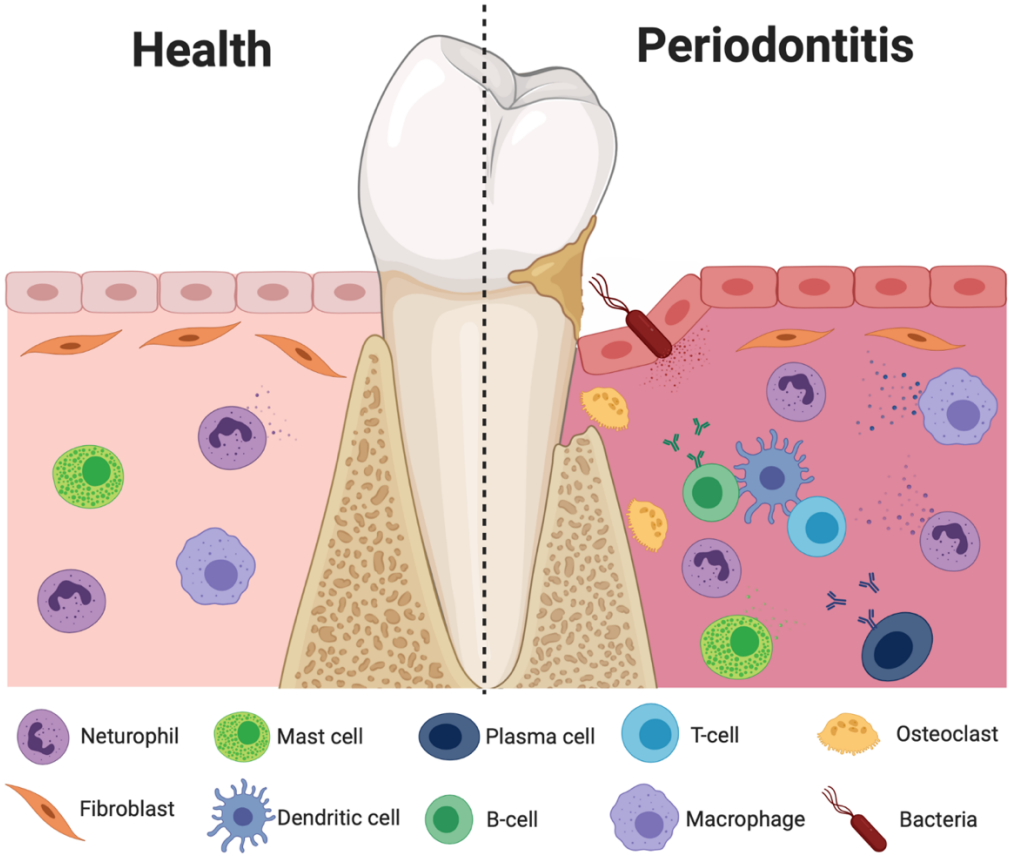

Figure 1.6. General overview of the cell types involved in periodontal health and disease. In health, the immune system is constantly exposed to external antigens and functions to maintain tissue homeostasis by immune surveillance. In PD, a dysregulated immune response occurs via failure to resolve dental plaque biofilms. There is an influx of various cell types and production of inflammatory mediators, with osteoclast activation resulting in alveolar bone resorption. Image created using BioRender.

1.4.3. Proinflammatory cytokines and chemokines

Throughout the response to subgingival plaque biofilms, various cell types function in concert to mount a collective response against targets. These cells communicate via small soluble mediators such as cytokines and chemokines. Cytokines are peptides that are produced by specific cells and function in a paracrine (nearby cells) or autocrine (same cell) manner. Several cytokine families exist including interleukins (ILs), interferons (IFNs) and tumour necrosis factors (TNFs). Chemokines are a family of cytokines which specifically function as chemoattractant molecules to regulate trafficking of immune cells and are distinguished based the location of conserved cysteine residues (CC, CXC, XC and CX3C). Nearly all of these cytokine families are involved at different stages in the host response against subgingival plaque biofilms (**figure 1.7**), forming

complex interlinking networks spanning the innate and adaptive responses (Kinane et al., 2011, Pan et al., 2019).

Cytokines may function to promote inflammation, regulate inflammation or have a dual role supporting both. In line with the key role of inflammation in PD pathogenesis, several proinflammatory cytokines such as IL-1β, TNFα, IL-6 and IL-17A have been implicated in periodontal inflammation, tissue injury and bone resorption (Cheng et al., 2020). IL-1β belongs to the IL-1 superfamily and is primarily expressed by activated macrophages, although gingival epithelial cells and fibroblasts have also been shown to produce this cytokine (Liu et al., 2010). In GCF samples, several studies demonstrate elevated IL-1β expression in PD compared with healthy subjects (Buduneli and Kinane, 2011). Following production, IL-1β increases the expression of MMPs which function in breakdown of the periodontal ligament (Cheng et al., 2020). Additionally, IL-1β has been shown to upregulate receptor activator of nuclear factor kappa-B ligand (RANKL) and thus plays a key role in osteoclast recruitment and alveolar bone resorption (Huynh et al., 2017).

It has been suggested that IL-1β is synergistic with TNFα in driving bone resorption (Stashenko et al., 1987, Graves and Cochran, 2003), with IL-1β increasing *TNFα* gene expression (**figure 1.7**). TNFα is a member of the TNF superfamily and is predominantly expressed by activated monocytes and macrophages. This cytokine stimulates elevated expression of adhesion molecules (ICAM-1 and VCAM-1) which are involved in immune cell recruitment (Hosokawa et al., 2006). In a similar manner to IL-1β, TNFα appears to be directly involved in bone resorption in PD by promoting osteoclast activity and inducing MMP secretion by gingival fibroblasts (Azuma et al., 2000, Graves and Cochran, 2003). Additionally, both IL-1β and TNFα stimulate the production of IL-6 (**figure 1.7**), which is expressed by a range of immune cells including macrophages, T-cells, B-cells, endothelial cells, gingival fibroblasts, osteoblasts and periodontal ligament cells (Sell et al., 2017). IL-6 functions to regulate the activity of key immune cells (T-cells, B-cells, **figure 1.7**), although also appears to be involved in stimulating MMP secretion and RANKL upregulation (Irwin and Myrillas, 1998, Taylor, 2014).

IL-1β, TNFα and IL-6 are well-characterised and central in the control of osteoclast activation, and the development of bone loss and tissue destruction

(**figure 1.7**). In contrast, IL-17A (often termed 'IL-17') has also been suggested to play a role in this process. Originally deemed a hallmark cytokine of Th17 cells, more recent immunofluorescence evidence indicates that mast cells may in fact be the primary source of IL-17A in periodontal tissues (Parachuru et al., 2018). Interestingly, both protective and destructive effects of IL-17A have been observed in relation to PD (Zenobia and Hajishengallis, 2015). On one hand, IL-17A is an important regulator of neutrophil recruitment and induces the production of antimicrobial peptides, which are protective against PD (Liang et al., 2006, Gorr, 2009). Alternatively, this cytokine also appears to be potent stimulator of osteoclastogenesis, and has been deemed a 'double edged sword' for this reason (Zenobia and Hajishengallis, 2015).

Along with the cytokines described above, the most well characterised chemokine in PD is IL-8 (CXCL8) - which is produced by a range of cell types including gingival epithelial cells, fibroblasts and macrophages. The primary function of IL-8 is neutrophil recruitment and activation and thus it plays a prominent role in maintaining tissue homeostasis and responding to the initial challenge from subgingival plaque biofilms (Dommisch et al., 2015, Hajishengallis and Korostoff, 2017). In turn, neutrophils recruit Th17 cells involved in disease progression (Pelletier et al., 2010) and a role in osteoclast activation has been reported *in vitro* (Chakravarti et al., 2009).

As mentioned above, it is believed that the activities of these cytokines and chemokines may directly or indirectly contribute to a state of dysregulated osteoclastogenesis and ultimately alveolar bone loss. Several of these cytokines (IL-1ß, TNFα, IL-6, IL-17A) have been shown to upregulate RANKL production from T-cells, B-cells, fibroblasts and osteoblasts. As a result, RANKL triggers osteoclast differentiation from macrophages and subsequent activation resulting in elevated levels of bone resorption (Bartold et al., 2010). Osteoprotegerin (OPG) is a decoy receptor of RANKL and can block it's effects. In line with dysregulated osteoclastogenesis in PD, it has been observed that GCF levels of RANKL are higher, and OPG lower, in PD patients compared with healthy controls (Belibasakis and Bostanci, 2012, Baltacioglu et al., 2014).

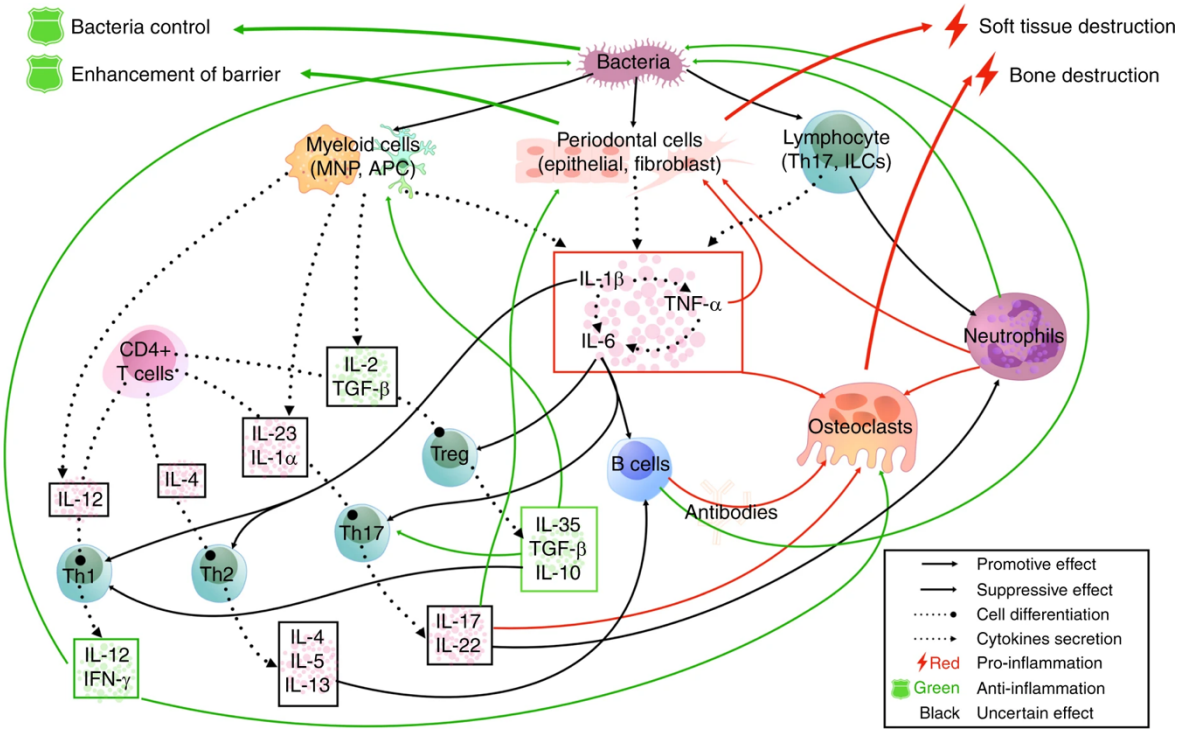

Figure 1.7. The cytokine network in periodontitis. Spanning the innate and adaptive responses, cytokines are essential soluble molecules which function to regulate the immune response. Cytokines are important for maintaining tissue homeostasis (green) and mounting a defence against dysbiotic biofilms (red). In the onset of disease, IL-1ß, TNFα and IL-6 are produced by cells within the gingival epithelium and function as key drivers of osteoclast activation, immune cell recruitment and bone loss. More recent evidence also demonstrates that IL-17A and neutrophils are involved in stimulating osteoclastogenesis. The image is reused from Pan et al., (2019) in the International Journal of Oral Science under Creative Commons Attribution 4.0 International (CC BY 4.0) license (https://creativecommons.org/licenses/by/4.0/).

1.4.4. Interplay between inflammation and dysbiosis

It has long been assumed that biofilm dysbiosis is the starting point of PD, followed by inflammation and eventually bone loss. However, recent ideology contests this sequence of events, suggesting that inflammation actually precedes biofilm dysbiosis. In the recent 'inflammation-mediated polymicrobial-emergence and dysbiotic-exacerbation (IMPEDE)' model by Thomas E Van Dyke and colleagues, it was suggested that gingival inflammation both precedes and subsequently induces biofilm dysbiosis (Van Dyke et al., 2020). In support of this theory, 'periodontal pathogens' implicated in biofilm dysbiosis are primarily observed in established disease, likely through factors explained in the EPH

(Marsh, 1994, Kirst et al., 2015). It is well accepted that an overgrowth of the predominantly Gram-positive healthy microflora triggers gingivitis (Page and Kornman, 1997). In the IMPEDE model it is proposed that the inflammatory state of gingivitis then triggers elevated diversity and dysbiosis through enhanced nutrient acquisition and a change in the periodontal pocket microenvironment. As a result, biofilm dysbiosis triggers further inflammation and vice-versa in a continuous cycle throughout subsequent disease progression. Therefore, PD cannot be viewed as a disease solely of microbiological or immunological origin, but rather as a continuous interplay between each of these factors.

1.4.5. Studying the immune response

As discussed in the preceding sections, the immune response in PD involves several cell types and soluble mediators. As a result, investigating the immune response can be an extremely complex procedure. One option is to directly assess cells, gene and protein expression in the periodontal tissue using flow-cytometry, immunohistochemistry, immunofluorescence and quantitative polymerase chain reaction (qPCR). However, this is a difficult and complicated procedure which requires invasive tissue biopsies from patients and healthy controls, or repeated biopsies for longitudinal assessment. Therefore, an often favoured alternative is to assess soluble mediators (cytokines, chemokines, antibodies) in non or less-invasive sample types (GCF, saliva, serum). These mediators give an indirect indication of the immune response both locally and systemically.

1.4.5.1. Gingival crevicular fluid

Several sample types may be used to investigate these soluble mediators in PD. The most localised of which is GCF, often defined as an inflammatory serum exudate derived from periodontal tissues. This fluid is initially derived from blood vessels, although as it travels to the junctional epithelium it acquires local immune cells, by-products of tissue breakdown and inflammatory mediators (Kinney et al., 2014). Periodontal pockets are constantly flushed with GCF which both aids in defence against subgingival plaque biofilms by carriage of these components into the subgingival microenvironment, and expels bacteria outwards from the gingival sulcus (Bostanci and Belibasakis, 2018). Accordingly, it has been demonstrated that the level of GCF in health is extremely low, and

the flow-rate increases up to 5.5-fold with the development of experimental gingivitis (Kowashi et al., 1980). Increased flow occurs due to elevated permeability of local blood vessels, thus resulting in elevated fluid leakage and entrance into the pocket (Page and Schroeder, 1976, Barros et al., 2016).

Several methods of GCF collection have been reported, including washing pockets with an isotonic solution or timed absorption onto paper strips (Subbarao et al., 2019). The latter is the most common of these methods, and a direct comparison revealed higher cytokine recovery (IL-6, IL-8) using paper strips compared with sodium chloride wash (Guentsch et al., 2011). However, it is currently debated whether data from paper strips should be reported as a total quantity over time, or standardised to the volume of GCF collected (Wassall and Preshaw, 2016). Nonetheless, several putative biomarkers have been recorded in the GCF using these methods (Buduneli and Kinane, 2011). In-line with the immunological understanding of PD, GCF levels of IL-1ß, MMPs and RANKL appear to be the most robust and are consistently elevated in disease compared with health (Beklen et al., 2006, Silva et al., 2008, Teles et al., 2010).

1.4.5.2. Saliva

Whilst GCF provides a localised sample to the periodontal tissue, the measured volume and corresponding protein composition is specific for the sampled site. In contrast, saliva has been utilised as a more general sample type to study local immunological markers and compare against full-mouth clinical variables, which has been suggested to be more clinically useful (Taylor, 2014). Saliva comprises secretions from the minor and major salivary glands, GCF, aspects of diet and bacteria (Baum et al., 2011). A myriad cytokines and chemokines have been detected and studied in saliva samples of PD patients (Jaedicke et al., 2016), and it has been demonstrated that the primary origin of these proteins is GCF. For example, one study measured IL-1α, IL-6, IL-8 from whole-saliva, parotid gland secretions and submandibular-sublingual secretions, finding significantly higher levels of all markers in whole-saliva across 31 periodontally healthy controls and patients with gingival over-growth (Ruhl et al., 2004). In fact, these proteins were virtually undetectable in salivary gland secretions, leading authors to conclude that their most likely source is GCF.

Along with providing a full-mouth reflection, saliva has several benefits over GCF including its abundancy and ease of collection, processing and analysis. Similar to GCF however is that several methods may be used to collect saliva samples and there is considerable heterogeneity in the published literature. Saliva samples may be collected after stimulation by paraffin wax or an alternative, or unstimulated samples may be collected using the passive-drool method. The latter sampling technique is generally preferred in order to limit the impact of increased flow rate and pH changes associated with stimulation (Lee and Wong, 2009, Granger et al., 2007). Further support for salivary cytokines arising in the GCF comes from the finding that certain analytes are consistently elevated in PD regardless of sample type. For example, there is evidence that salivary IL-1ß and MMP-8 are elevated in PD compared with health, although these results do appear less consistent than in GCF (Taylor, 2014, Jaedicke et al., 2016). Likewise, it should be noted that whole saliva does display an inhibitory effect against analytes *in vitro* (Wozniak et al., 2002), which may explain why some cytokines (TNFα, IL-6) are only observed in very low levels (Taylor, 2014).

1.4.5.3. Serum

Although more distant to the site of disease, serum samples are regularly assessed in PD clinical studies. Certain serum markers have the benefit of defined reference ranges, which are seldom available for GCF and salivary analytes. One of the most extensively studied serum markers is C-Reactive protein (CRP), an acute-phase pentamer produced by liver hepatocytes primarily in response to IL-6 (Pepys and Hirschfield, 2003). CRP is a non-specific marker of systemic inflammation which binds to damaged cells and bacteria to act as an opsonin and activate the classical complement cascade via C1q (Du Clos, 2000). Serum CRP levels of <1 mg/L are observed in the majority of healthy Caucasian adults but may rise to several hundred mg/L during an infection (Sproston and Ashworth, 2018). The level of circulating CRP has also been promoted as a marker of cardiovascular disease (CVD) risk by the American Heart Association and CDC, with levels <1 mg/L constituting a low risk, 1-3 mg/L equating to a moderate risk and >3 mg/L indicating a high risk (Pearson et al., 2003).

In relation to PD, it has been suggested that locally derived inflammatory cytokines (IL-6) or bacteria may transition from the periodontal tissues into

circulation and stimulate the release of CRP. Independent cross-sectional studies have documented an increase in serum CRP and IL-6 in systemically healthy PD patients in comparison with systemically and periodontally healthy controls (Ebersole et al., 1997, Loos et al., 2000). Furthermore, evidence from meta-analyses demonstrate that periodontal treatment effectively lowers circulating CRP and IL-6 levels in-line with a reduction in gingival inflammation (Teeuw et al., 2014, Machado et al., 2021).

Another common output from serum samples are antibacterial antibody titres. For this analysis, immunoglobulin G (IgG) is employed as it is the most abundant antibody in serum and boasts a long half-life (2-3 weeks) to evaluate chronic exposure to certain bacteria (Curtis and Bourne, 1973). Most studies have investigated antibodies directed against PD associated organisms such as *P. gingivalis* and *A. actinomycetemcomitans* (associated with aggressive PD), and provide a useful indication that organism has colonised and stimulated an immune response (Haffajee and Socransky, 1994). These titres readily reflect carriage within subgingival plaque samples (Pussinen et al., 2011), and have been shown to distinguish between healthy and PD subjects with good accuracy (Kudo et al., 2012, Damgaard et al., 2021). Additionally, these antibodies have been used to evaluate associations between specific bacteria and systemic disease (Pussinen et al., 2004, Lappin et al., 2013), along with future disease progression (Sakai et al., 2001). Other antibacterial antibody isotypes (IgA, IgM) have also been assessed, although their short half-life of 2-3 days means they more readily reflect recent rather than chronic colonisation (Sweier et al., 2009, Gadekar et al., 2018, Isola et al., 2020). For this reason, serum IgG titres are the most accurate and preferred method for studying past exposure.

As evidence for GCF, saliva and serum markers continues to grow, there is hope that they may aid the diagnosis of PD under the 2017 classification criteria. Serum CRP has been added as a potential marker of the systemic impact of PD (Tonetti et al., 2018). Similarly, a space has been left for GCF, saliva and serum biomarkers to reflect the different rates of disease progression (**table 1.2**). This reflects a general movement in the field to supplement clinical diagnoses with accurate and specific biomarkers, although it was noted that there is a need to further substantiate this evidence before firm inclusion in clinical guidelines.

Table 1.2. The potential integration of immunological markers into the periodontal grading system. Excerpt taken from Tonetti et al., (2018).

Periodontitis grade	Grade A: Slow progression	Grade B: Moderate progression	Grade C: Rapid progression
Clinical evidence of progression	No evidence of bone loss over 5 years	<2 mm bone loss over 5 years	≥2 mm bone loss over 5 years
Systemic impact of periodontitis	Serum CRP <1 mg/L	Serum CRP 1 to 3 mg/L	Serum CRP >3 mg/L
Biomarkers (GCF, saliva, serum)	?	?	?

Question marks are used as placeholders by Tonetti et al. until substantiated evidence becomes available on markers. Taken from the Journal of Clinical Periodontology.

1.5. Periodontitis and systemic disease

1.5.1. Epidemiology of associated diseases

PD is viewed as a chronic inflammatory disorder of the mouth, although collective evidence has emerged over the past 20-30 years indicating that the impact of disease may extend well beyond the oral cavity. It has been suggested that PD is associated with a multitude of inflammatory comorbidities including type 2 diabetes mellitus, rheumatoid arthritis (RA), Alzheimer's disease, hypertension, CVD, colorectal cancer and adverse pregnancy outcomes (Preshaw et al., 2011, Dominy et al., 2019, Czesnikiewicz-Guzik et al., 2019, Bartold and Lopez-Oliva, 2020, Hajishengallis and Chavakis, 2021). A summary of the association between PD, CVD and RA is provided below and further explored in the subsequent chapters.

There is a recognised association between PD and CVD, an umbrella term for a range of conditions impacting the heart and blood vessels (coronary heart disease, peripheral arterial disease, aortic atherosclerosis). One of the largest studies supporting this association is from the Academic Centre for Dentistry Amsterdam, which evaluated over 60,000 adult patient records (>35 years old) between 1998 and 2013 (Beukers et al., 2017). From this study, it was observed that the incidence of atherosclerotic CVD was 4.7% in patients with PD (n=9730) compared with 1.9% in non-PD patients (n=50,544). Notably, this association remained even after adjustment for potential confounders such as economic

status, sex, age, smoking, diabetes and hypertension (OR: 1.59). These findings are supported by an earlier systematic review of 12 cohort and case-control studies, which documented an increased incidence of atherosclerotic CVD in PD patients across all but one study (Dietrich et al., 2013). Data from the Scottish Health Survey found that the rate of CVD events (primarily coronary heart disease) were associated with poor oral hygiene (n=11,869, hazard ratio: 1.7) and serum CRP in a subset of patients (n=4830) (De Oliveira et al., 2010).

Epidemiological studies also support a link between PD and RA, a debilitating autoimmune disease characterised by destruction of synovial joints. Independent studies have observed a positive and potentially bidirectional relationship between these conditions. Studying 4461 patients in NHANES III demonstrated a higher prevalence of PD among RA patients (n=103) compared with non-RA subjects (n=4368) when adjusting for age, sex, ethnicity and smoking status (OR: 1.82) (De Pablo et al., 2008). Conversely, a nationwide case-control study in Taiwan observed that newly diagnosed RA (n=13,779) was positively associated with a history of PD after adjustment for confounders such as diabetes, geographical region and Sjögren's syndrome (OR: 1.16) (Chen et al., 2013). Notably, the latter study did not account for smoking which is a major shared risk factor of PD and RA, although similar findings have been observed in smaller single-centre studies (n=1412) (Mercado et al., 2000).

1.5.2. Correlation or causation?

It is clear from the above epidemiological studies that a positive association exists between PD and extra-oral inflammatory diseases. However, whether this is a correlative or indeed causative relationship remains unclear, particularly in consideration of the high prevalence and shared risk factors between diseases.

It is widely documented that inflammation is key in the pathogenesis of several diseases that have been associated with PD. Therefore, it is possible that an elevated level of systemic inflammation and immune cell recruitment at distal sites may predispose susceptible patients to comorbidities (Genco and Van Dyke, 2010, Paul et al., 2020). Accordingly, it has been found that periodontal treatment reduces systemic markers of inflammation and improves features of some associated systemic diseases. Periodontal treatment has been shown to improve long-term endothelial function (Tonetti et al., 2007), lower blood

pressure in hypertensive patients (Czesnikiewicz-Guzik et al., 2019), reduce disease activity scores in RA patients (Okada et al., 2013, Erciyas et al., 2013) and improve glycaemic control in type 2 diabetics (D'aiuto et al., 2018). It is worth noting that these studies have specifically assessed surrogate markers of disease, given the inherent ethical implications in hard endpoints such as cardiovascular events.

A more recent theory has been proposed suggesting that release of systemic cytokines (IL-6) may prime bone marrow precursor cells to favour osteoclast differentiation, which links PD with other bone destructive disorders such as RA (Hajishengallis and Chavakis, 2021). This has largely been demonstrated in rodent models (Zhao et al., 2020), although some human evidence has been reported. Peripheral blood mononuclear cells isolated from PD patients more readily differentiate into osteoclasts when compared with the same cells from healthy individuals (Herrera et al., 2014). As such, these cells may traffic to alternative sites of bone resorption and increase osteoclastogeneis (Hajishengallis and Chavakis, 2021). Although feasible, direct human evidence for this mechanism remains limited and thus future research is paramount to establish the strength of such a mechanism in the causality of systemic disease.

Another theory explaining the link between PD and associated comorbidities is through the direct effects of periodontal bacteria. This concept is not necessarily new, as the ‘focal infection theory’ and ‘oral sepsis hypothesis’ gained mass popularity in the early 20th century (Hunter, 1900, Kumar, 2017). These concepts proposed that multiple chronic diseases (RA, CVD, cancer, mental illness) were caused by the systemic translocation of bacteria and their by-products from oral infections to distal sites. As such, total tooth extractions were advocated as a treatment for systemic diseases including RA (Billings, 1930). Subsequently, it was noted that tooth extraction did not cure, and in many cases worsened RA symptoms (Vaizey and Clark-Kennedy, 1939, Kumar, 2017). As a result of these findings, the theory was largely disregarded by medical and scientific communities alike.

Despite this historic controversy, the causative role of oral bacteria in RA has been revisited in the past 20 years. Emerging studies consistently associate members of the subgingival plaque microbiome with RA pathophysiology (Scher et al., 2014). This concept is not related to bacterial translocation, but instead

postulates that the oral cavity may be the initial site of autoimmunity through the actions of specific periodontal bacteria. Of these, several studies demonstrate a possible role for *P. gingivalis* and *A. actinomycetemcomitans* in this process, which is further discussed within **chapter 2**.

1.6. Periodontal treatment

1.6.1. Non-surgical periodontal therapy

Periodontal treatment encompasses several processes including oral hygiene guidance and patient-performed oral hygiene control, non-surgical and surgical therapy. Of those performed clinically, non-surgical periodontal therapy (NSPT) has been the first-line treatment for over a century (Ower, 2013). In some cases NSPT may be supplemented with local and systemic antimicrobial adjuncts, however, current guidelines state there is insufficient evidence to warrant routine use of antibiotics compared with NSPT alone (Scottish Dental Clinical Effectiveness Programme, 2014). Historically, NSPT involved scaling and root planning whereby supra and subgingival biofilms, calculus and 'contaminated root cementum' were removed through the use of extensive hand instrumentation – leaving a smooth root surface free of bacterial agents. Root planning is an invasive procedure featuring removal of tooth structure, and was implemented to detach bacterial endotoxins believed to have penetrated the cementum (Fine et al., 1980). Under this protocol, treatment was performed separately in each quadrant of the mouth over several visits, and required substantial time and technical expertise (Ower, 2013).

Subsequent studies demonstrated that root planing is not justified in NSPT as bacterial endotoxins were found to minimally penetrate exposed roots and only loosely adhered to extracted teeth (Nakib et al., 1982). Additionally, it was found that complete removal of calculus from deep pockets ≥5 mm was rare in any circumstance and was not required for successful treatment (Brayer et al., 1989, Robertson, 1990). Protocols for root surface debridement were thus implemented as an alternative to root planing, involving lighter instrumentation to obtain a clean surface without removal of the tooth structure (Ower, 2013). If NSPT is unsuccessful then surgical therapy may be employed, and this can be beneficial in pockets exceeding 6 mm (Heitz-Mayfield et al., 2003). Within this thesis, NSPT is used as a general term to describe non-surgical instrumentation

phase of treatment, while full mouth debridement (FMD) is used when relating to the completion of all subgingival instrumentation within a 24 hour period.

1.6.2. Types of instrumentation

Root surface debridement may be performed using hand and ultrasonic instruments in combination or in isolation (**figure 1.8**). Hand instruments comprise various specially designed curettes with a sharpened blades to mechanically remove plaque and calculus. These instruments offer greater tactile awareness and benefit from a lack of aerosol production, although require greater treatment time and expertise, repeated sharpening and induce greater levels of operator fatigue (Krishna and De Stefano, 2016).

Ultrasonic instruments are a subset of 'machine driven instruments' used in NSPT. Various ultrasonic tip inserts are available depending on requirements, which feature vibrating metallic tips that are cooled via irrigation (commonly water). Ultrasonic instruments benefit from faster treatment times, reduced root surface damage, flushing effects of coolant irrigation and greater operator comfort, although generate extensive aerosol and leave a rougher surface compared with hand instruments (Kocher et al., 2001). For this reason, hand instruments may be used to smooth root surfaces following ultrasonic treatment (Ruppert et al., 2002). Although different in their mechanism of action, extensive analysis has revealed no significant differences in the clinical efficacy when using hand or ultrasonic instruments as monotherapies (Ioannou et al., 2009, Suvan et al., 2019).

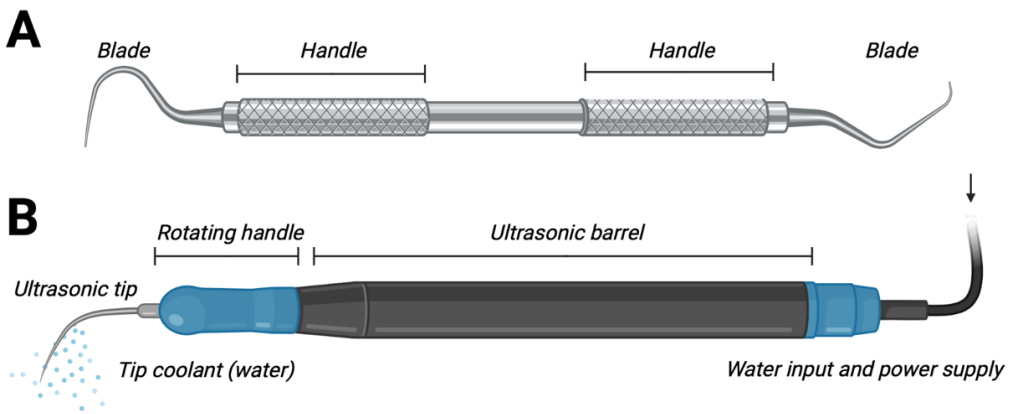

Figure 1.8. Different instruments used in non-surgical periodontal therapy. Hand (A) and ultrasonic (B) instruments with labelled sections. Image created using BioRender.

1.7. Summary and thesis overview

The literature reviewed throughout this chapter has outlined the multifaceted nature of PD. Previously considered a disease dictated solely by subgingival plaque biofilms, it has become increasingly clear that the host immune response plays a pivotal role in PD onset, progression and potential susceptibility. Despite increased understanding of PD pathophysiology, it remains one of the most prevalent and costly diseases of mankind. Current treatment protocols, particularly NSPT, are beneficial but not ubiquitously successful with several patients requiring further treatment.

There is therefore an unmet need to better understand the response to periodontal treatment - both in terms of host and microbial changes. This can potentially help identify novel and improved treatment modalities, identify patients who require specific targeted treatment adjuncts, and offer insight into the systemic implications of treating PD. As such, this thesis aimed to further explore the host and microbial response to NSPT using two independent clinical studies. The first is a longitudinal cohort study presented in **chapters 2 and 3**, the second is a randomised controlled trial (RCT) presented in **chapters 4 and 5.** Detailed aims are provided within each chapter, and an overview schematic of each study is provided below (**figure 1.9**).

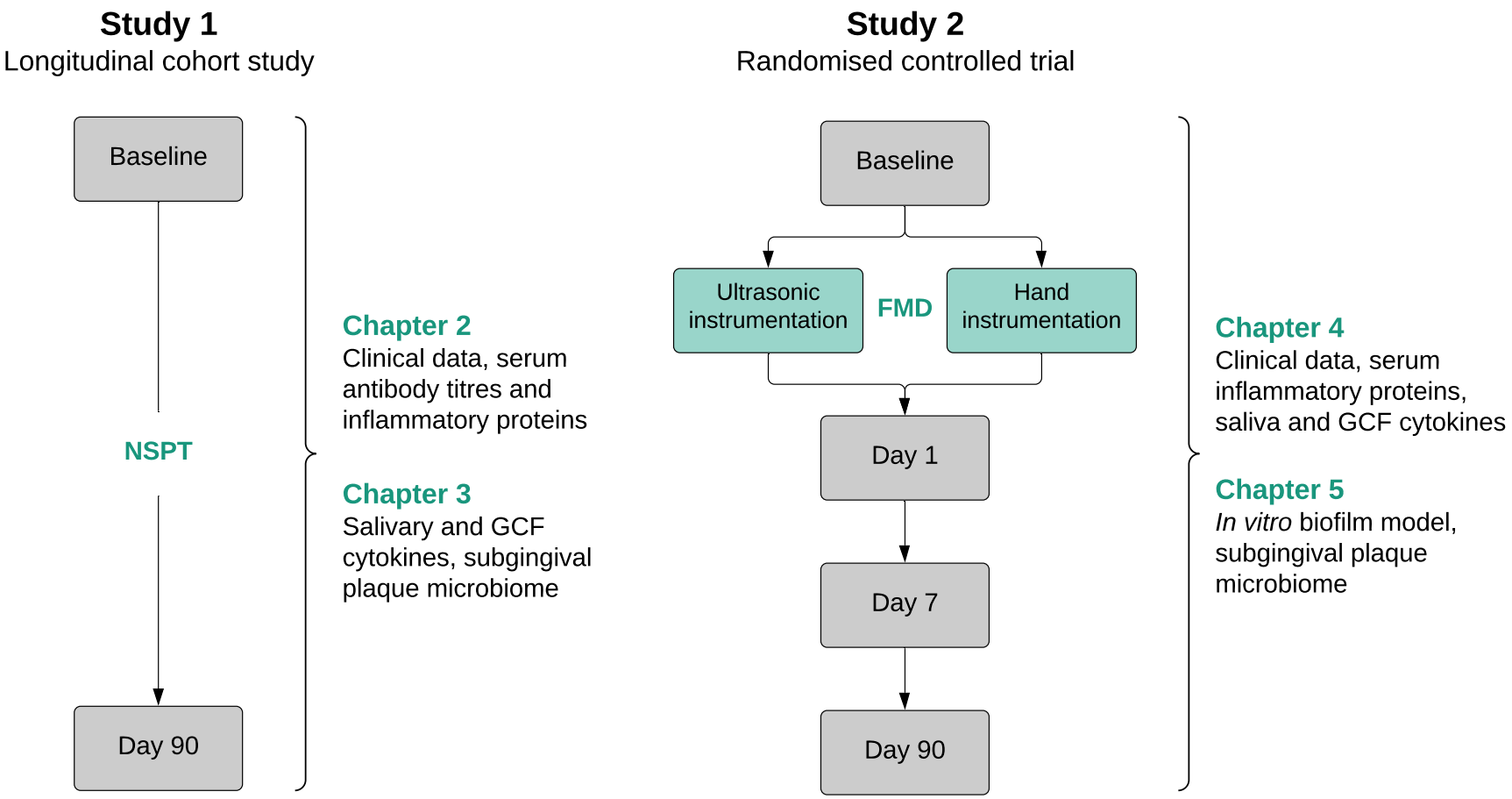

Figure 1.9. Overview of the two studies used throughout this PhD thesis. Study 1 is a longitudinal cohort study presented within chapter 2 and 3. Study 2 is a randomised controlled trial presented within chapter 4 and 5. NSPT; Non-surgical periodontal therapy, FMD; Full-mouth debridement.

Chapter 2

Evaluating the impact of periodontal inflammation, smoking status and non-surgical treatment on anti-citrullinated protein antibody titres

Work from this chapter has been presented at the following meetings;

'Biological impacts of NSPT' by W. Johnston presented at the University of Glasgow Dental School PGR day, May 2018, Glasgow, Scotland, UK. Awarded runner-up prize for best PGR presentation.

'Systemic antibodies following NSPT' by W. Johnston presented at the Sao Paulo School of Advanced Science on the Molecular Basis of Inflammatory Disease, November 2019, Sao Paulo, Brazil.

Work from this chapter has been published in the following journals;

Review;

Reilly, R.; **Johnston, W.;** Culshaw, S. Autoimmunity and the Oral Cavity. *Current Oral Health Reports* (2019), 6, 1-8. https://doi.org/10.1007/s40496-019-0203-9

Research;

*Davison, E.; ***Johnston, W.;** Piela, K.; Rosier, B.T.; Paterson, M.; Mira, A.; Culshaw, S. The Subgingival Plaque Microbiome, Systemic Antibodies Against Bacteria and Citrullinated Proteins Following Periodontal Therapy. *Pathogens* (2021), 10, 193. https://doi.org/10.3390/pathogens10020193

*Equal contributions.

Manuscript included as **appendix i**

2.1. Introduction

Despite being historically viewed as a disease limited to the oral cavity, PD has become increasingly associated with systemic diseases involving chronic inflammation and immune dysregulation. Notably, as mentioned throughout **chapter 1**, numerous studies highlight a positive and potentially bidirectional relationship between PD and RA. Both diseases share genetic and environmental risk factors, along with similar pathophysiological processes. For example, both PD and RA involve cellular infiltration at an inflammatory focus, express similar cytokine profiles and result in irreversible immune-mediated destruction of bone and connective tissue (Culshaw et al., 2011).

RA is an autoimmune disease of unknown aetiology, with systemic effects that result in a 54% higher mortality rate compared with that of the general population (Van Den Hoek et al., 2017). Several systemic inflammatory markers have been well characterised in RA patients, including pro-inflammatory cytokines (TNFα, IL-6) that drive the production of CRP, which can be employed diagnostically (Anderson et al., 2012). The same systemic cytokines and acute phase proteins also appear to be elevated in PD patients compared with periodontally healthy controls (Slade et al., 2000, Noack et al., 2001, Hajishengallis and Chavakis, 2021), although the strength of relationship is far less robust than in RA.

Alongside common risk factors and pathologic processes, there is evidence to suggest a causal mechanism between PD and RA. A theory which has gained considerable traction in recent years is that certain members of the oral microbiota may trigger the generation of anti-citrullinated protein antibodies (ACPAs). ACPAs are autoantibodies that target citrulline containing peptides. Citrulline, the deiminated form of arginine, arises only via post-translational modification. Importantly, arginine is a positively charged amino acid whilst citrulline is neutral (Yamada et al., 2005). This means that citrullination induces an overall net change in protein charge, conformation and potentially function, thereby increasing the antigenicity of citrullinated proteins (Raptopoulou et al., 2007).

Serum IgG ACPAs are observed in 70% of RA patients and have been used as sensitive and specific diagnostic markers (Rantapää-Dahlqvist et al., 2003, Farquharson et al., 2012). Under normal conditions, citrullination is catalysed by five host peptidylarginine deiminase (PAD) isoenzymes (PAD1-4, 6) in the presence of calcium. This process has been found to occur during apoptosis where membrane damage causes an influx of extracellular calcium, activating PAD enzymes which likely lead to a loss of protein function. It is believed that dysregulated, or indeed off-target, citrullination may drive the formation of ACPAs and development of RA (Darrah and Andrade, 2018). Accordingly, positive serum ACPA titres may be found up to 10 years prior to the clinical onset of RA, suggesting that autoantibody generation may occur out with the joints (Bizzaro et al., 2013, Guo et al., 2018).

As described in **chapter 1**, *P. gingivalis* is a disease-associated anaerobic bacterium frequently isolated from subgingival plaque samples. Remarkably, this bacteria is the only known prokaryote to express a PAD enzyme, termed *P. gingivalis* peptidylarginine deiminase (PPAD) (Mcgraw et al., 1999). This enzyme is capable of citrullinating both *P. gingivalis* and host proteins (Wegner et al., 2010), and PPAD activity has been suggested as one possible link between PD and the development of RA (Cheng et al., 2017). Gingipains are another potent virulence factor produced by *P. gingivalis*. Two of these gingipains (RgpA and RgpB) are known to cleave host proteins at arginine residues, thus creating an appropriate substrate for PPAD induced citrullination (Farquharson et al., 2012) which may be followed by ACPA generation in the periodontium. In line with this theory, citrullinated proteins have been found in periodontium of PD patients (Nesse et al., 2012), and ACPA positive individuals display an elevated abundance of *P. gingivalis* compared with healthy controls (Mankia et al., 2019, Cheng et al., 2021). Some studies have further demonstrated that RA patients harbour a more 'dysbiotic' subgingival plaque microbiota (Lopez-Oliva Santa Cruz et al., 2018), with others showing elevated abundance of specific genera (*Prevotella, Leptotrichia*) compared with health (Scher et al., 2012).

Although *P. gingivalis* has received much attention and attribution for the link between PD and RA, it is not the only periodontal bacterium to do so. Another PD-associated bacteria, *A. actinomycetemcomitans,* has also been recently shown to trigger ACPA generation. This bacterium has received substantial

attention in periodontal research due to its association with aggressive forms of PD (Aberg et al., 2015). In contrast to *P. gingivalis*, there is no PAD expression by *A. actinomycetemcomitans*. Instead, another virulence factor known as leukotoxin A (LtxA) is thought to be responsible for the generation of ACPAs. It has been demonstrated that this pore forming toxin is capable of inducing neutrophil lysis, causing hyperactivation (via calcium influx) of host PADs which facilitate citrullination (Konig et al., 2016). Clinical evidence for this mechanism has also been reported in a case study involving an ACPA-positive RA patient with endocarditis induced by a leukotoxic *A. actinomycetemcomitans* strain (Mukherjee et al., 2018). It was found that joint symptoms resolved and ACPA titres normalised following antibiotic therapy, albeit this is limited to a single patient.

Following generation, ACPAs are believed to play a key role in RA pathogenesis by binding naturally citrullinated human proteins (fibrinogen, enolase, vimentin) in synovial joints. In a 'two-hit' model, it has been suggested that the initial trigger for ACPA generation may be through bacterial induced citrullination in the oral cavity (first hit), which is followed by epitope spreading to synovial joints where antibodies cross-react and bind naturally citrullinated proteins (second hit) (Wegner et al., 2010, Lundberg et al., 2010). Given these previous epidemiological and mechanistic associations between PD and RA, this chapter sought to evaluate the association between systemic ACPAs, antibacterial antibodies directed against periodontal bacteria and inflammatory proteins (CRP, IL-6, TNFα) in a cohort of PD patients. Additionally, longitudinal alterations in these variables following NSPT were assessed. Specifically, this chapter sought to answer the following research questions;

- In a cohort of PD patients, are serum ACPAs associated with elevated antibacterial antibody titres and inflammatory proteins?
- Does NSPT reduce serum ACPAs, antibacterial antibody titres and inflammatory proteins?
- Are serum ACPAs, antibacterial antibody titres and/or inflammatory proteins influenced by periodontal inflammation or smoking status?

2.2. Materials and methods

2.2.1. Study outline

This longitudinal cohort study was carried out within Glasgow Dental Hospital from 2017 until September 2018. The study was designed as an exploratory study to evaluate changes in serum antibody titres and inflammatory proteins following periodontal treatment.

2.2.1.1. Recruitment and ethical considerations

The study received ethical approval from London – Stanmore Research Ethics Committee (REC reference: 14/LO/2064) and was conducted in accordance with the Declaration of Helsinki 1975 (revised in 2013). The study was sponsored by NHS Greater Glasgow and Clyde Research & Development (GN16DN404). For recruitment, patients were referred to the Unit of Periodontics at Glasgow Dental Hospital. At the initial appointment at Glasgow Dental Hospital, patients were approached regarding participation in this study and supplied with a patient information leaflet (PIL). Patients could withdraw at any time without compromising their periodontal treatment.

2.2.1.2. Inclusion and exclusion criteria

Inclusion criteria were PPD of ≥ 5 mm on 2 or more teeth at non-adjacent sites excluding third molars, as has been described previously (Page and Eke, 2007); requiring periodontal treatment at Glasgow Dental Hospital; written informed consent; male or female ≥ 18 years of age. Exclusion criteria were patients with a known diagnosis of RA or other condition likely to notably impact the systemic immune response; known or suspected risk of tuberculosis; hepatitis B or HIV infections; requiring interpreter or non-English language written material to provide written, informed consent; history of bleeding diathesis.

2.2.1.3. Sample size

This exploratory study sought to further investigate the effect of periodontal treatment on serum ACPAs. A previous study comparing serum ACPAs in 39 PD patients pre and post NSPT observed a statistically significant reduction in non-smokers (n=23) only, but no difference in current smokers. The current study

sought to confirm these findings using a study with similar design. With the exception of our own previous study, there was limited published appropriate data to allow a formal calculation of sample size, therefore, the analysis conducted must still be viewed as pilot and exploratory in nature. A pragmatic group size of n=45 allowed for potential drop out and this, along with our previous data, could be used to power future trials.

2.2.2. Treatment and visit scheduling

A flow chart outlining the study is provided in **figure 2.1**. All patients attended an initial treatment visit where baseline clinical data and samples (serum, saliva, GCF and subgingival plaque) were collected. During this visit, patients were provided with detailed oral hygiene advice and supragingival scaling. Following this visit, NSPT was performed over multiple visits. Patients were generally treated in quadrants (4 visits), or according to patient and clinician preference for treatment scheduling. All patients returned 90 days (±14 days) after the last treatment visit, where samples were re-taken for analysis.

Non-surgical treatment was administered by a single experienced dental hygienist in Glasgow Dental Hospital Clinical Research Facility. Treatment consisted of detailed oral hygiene instruction, and supra and subgingival scaling using a combination of hand and ultrasonic instruments, with local anaesthetic if required. At each treatment visit, oral hygiene instructions and dental health education were reinforced by the clinician. Three patients were excluded due to a new diagnosis of systemic disease throughout the study period.

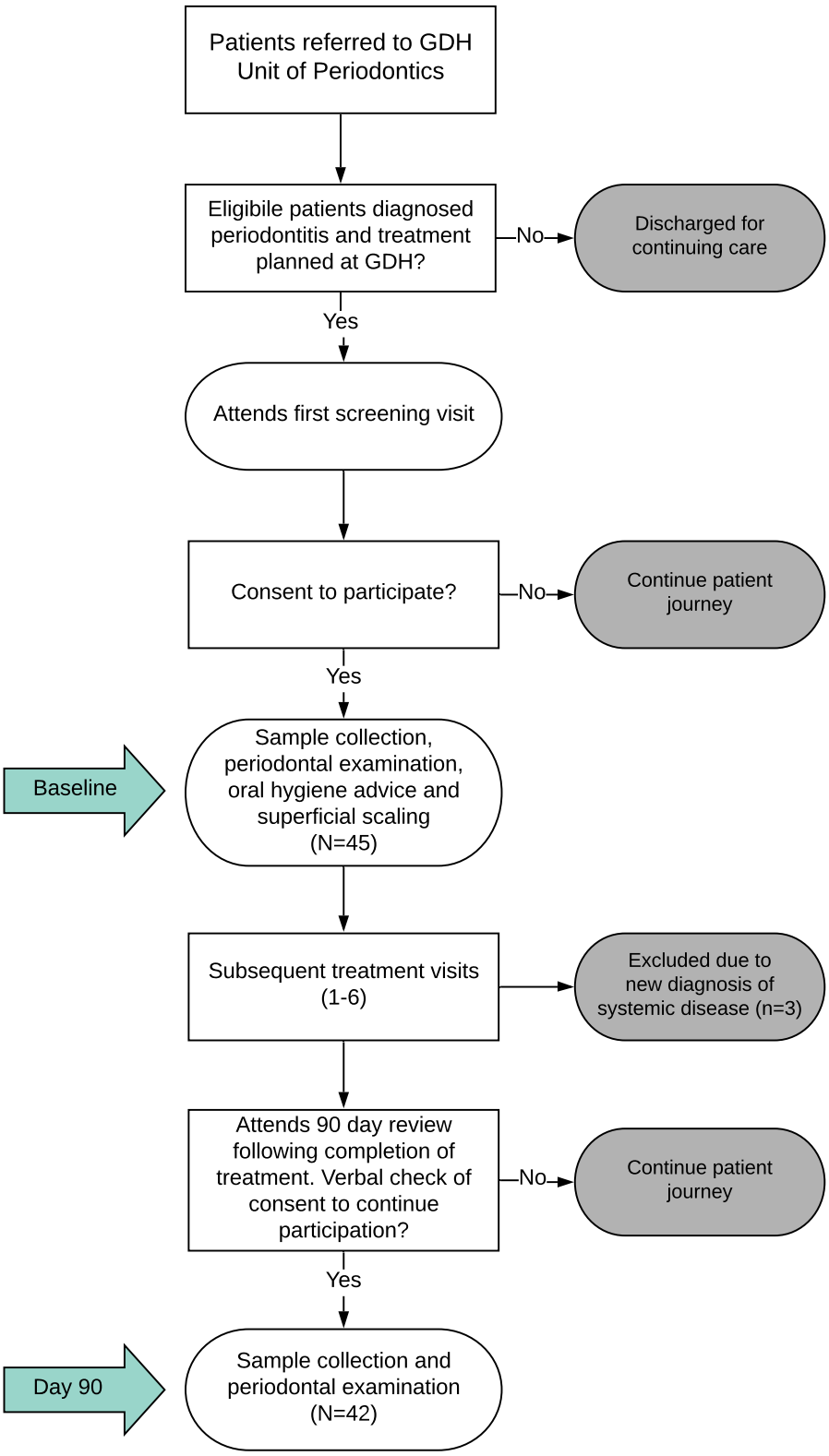

Figure 2.1. Study flow chart. Eligible patients were recruited among referrals to the Unit of Periodontics at Glasgow Dental Hospital (N=45). Blue arrows indicate sample timepoints during the study. During the treatment period, three patients were excluded due to new diagnosis of systemic disease (N=42). GDH; Glasgow Dental Hospital.

2.2.3. Clinical examination

The LGM, PPD, BoP, and presence of dental plaque ('periodontal parameters') were assessed by a single experienced dental hygienist at six sites per tooth at the initial screening visit prior to any treatment (baseline) and 90 days following the last treatment visit (day 90). Full-mouth plaque scores (FMPS) were assessed by assigning a binary score to each site and are presented as the percentage of total sites in the mouth with the presence of plaque (O'leary et al., 1972). Similarly, BoP was assessed by dichotomously measuring the presence of bleeding and are presented as a percentage of total sites (Full-mouth bleeding index; FMBS) (Ainamo and Bay, 1975). Both PPD and LGM were recorded to the nearest millimetre per sites and divided by the total number of sites to give full-mouth scores. The CAL was then calculated by addition of PPD and LGM per site and divided by the total number of sites to give full-mouth scores. The PISA was calculated as described previously (Nesse et al., 2008) using BoP and PPD.

2.2.4. Sample collection and processing

2.2.4.1. Serum

Samples were collected at baseline and day 90 prior to periodontal measurements. Blood was collected via venepuncture into 'serum-collection tubes' containing silica particles which activate coagulation (Vacuette, Grenier bio-one, Gloucestershire, UK). Samples were incubated at room temperature for 30 minutes to facilitate the coagulation process. After this period, tubes were centrifuged at 2500 RPM for 10 minutes to separate serum (MegaStar 1.6R, VWR, Lutterworth, UK). Serum samples were then aliquoted into sterile Eppendorf tubes (500 µL per tube) and stored at -80°C until analysis.

2.2.5. Analysis of serum

2.2.5.1. Antibacterial IgG antibody titres

Bacterial strains: *P. gingivalis* ATCC BAA-308 (W83), *P. intermedia* ATCC 25611, *F. nulceatum* subsp. *polymorphum* ATCC 10953 and *A. actinomycetemcomitans* ATCC 43718 (Y4, serotype b) were used throughout these experiments. All selected strains have been used previously to assess serum antibacterial IgG in PD patients within various clinical studies (Noble et al., 2014, Nishi et al., 2020,

Shelburne et al., 2008, Aoki et al., 2020, Konig et al., 2016). Frozen bacterial stocks (stored on 10% glycerol beads at -80°C) were cultured onto suitable agar media. For *P. gingivalis, P. intermedia* and *F. nulceatum,* Fastidious Anaerobic Agar (FAA) was used (Oxoid, Hampshire, UK). Beads were streaked onto agar plates and incubated in a 37°C anaerobic cabinet (Don Whitley Scientific, Shipley, UK) with anaerobic gas influx (5% CO_2, 10% H_2 and 85% N_2) for 48 hours.

Following growth, 2-3 isolated colonies from each strain were inoculated into 25 mL of de-oxygenated Schaedler anaerobic broth (SCH) (Oxoid, Hampshire, UK) and incubated for a further 48 hours. For *A. actinomycetemcomitans,* Columbia Blood Agar (CBA) was used (Oxoid, Hampshire, UK), and plates were incubated at 37°C in a 5% CO_2 incubator. Following growth, individual colonies were inoculated into 25 mL Tryptic Soy Broth (TSB) (Oxoid, Hampshire, UK) for a further 48 hours. Following growth of all broths, organisms were pelleted by centrifugation at 4000 RPM for 10 minutes (AWEL C20 model, Blain, France) and washed with PBS. This washing process was repeated 3 times, and pellets were stored at -80°C until use.

Frozen stocks of bacteria were heat-killed by incubation at 70°C for 2 minutes and then transferred immediately to an icebox. Serum IgG antibody titres against periodontal bacteria were determined using indirect enzyme linked immunosorbent assays (ELISAs) as outlined previously (Mooney et al., 1993). Briefly, Imunolon 1B low-binding microtiter plates (Thermofisher, Loughborough, UK) were coated overnight at 4°C with 100 µL heat-killed whole-cells in carbonate bicarbonate buffer at pH 9.6 (Sigma Aldrich, Gillingham, UK). Optimal coating concentrations of each organism were previously determined using serum from 10 healthy controls and 12 PD patients. Serum from healthy patients was taken from a historic study (University of Glasgow MVLS ethics committee number 2011002), with the 12 patients in the current study with the highest PISA used for PD controls. Additional optimisation steps included comparing the use of whole cells in EDTA versus non-EDTA, and foetal bovine serum (FBS) versus bovine serum albumin (BSA) as blocking buffers. The final standard curves for optimised assays displaying healthy and PD patient control serum are displayed in **figure 2.2**.

The selected concentrations were OD_{550nm} 0.06 for *P. gingivalis* and *Prevotella intermedia,* 0.08 for *Fusobacterium nulceatum* and 0.04 for *A.*

actinomycetemcomitans. After coating, wells were washed using 0.05% tween20 in PBS and blocked using 200 µL 10% FBS in PBS (Gibco, Thermofisher, Loughborough, UK) for one hour at 37°C. After blocking, 100 µL serum dilutions were added to wells ranging from 1:50 to 1:400 for patient samples in duplicate. The same PD and healthy control serum, alongside blank samples (dilution buffer only) were assayed on every plate. Following sample addition, plates were incubated for 2 hours at 37°C. Plates were then washed and bound antibody was detected using 100 µL biotin conjugated anti-human IgG antibodies (γ-chain specific) for 1 hour, 100 µL extravidin peroxidase for 1 hour and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma Aldrich, Gillingham, UK). The TMB reaction was stopped after approximately 15 minutes using 2N hydrochloric acid, and absorbance read at 450_{nm} with correction at 570_{nm} (FLUOstar Omega Microplate Reader, BMG Labtech, Ortenberg, Germany). All data were blank corrected prior to interpolation.

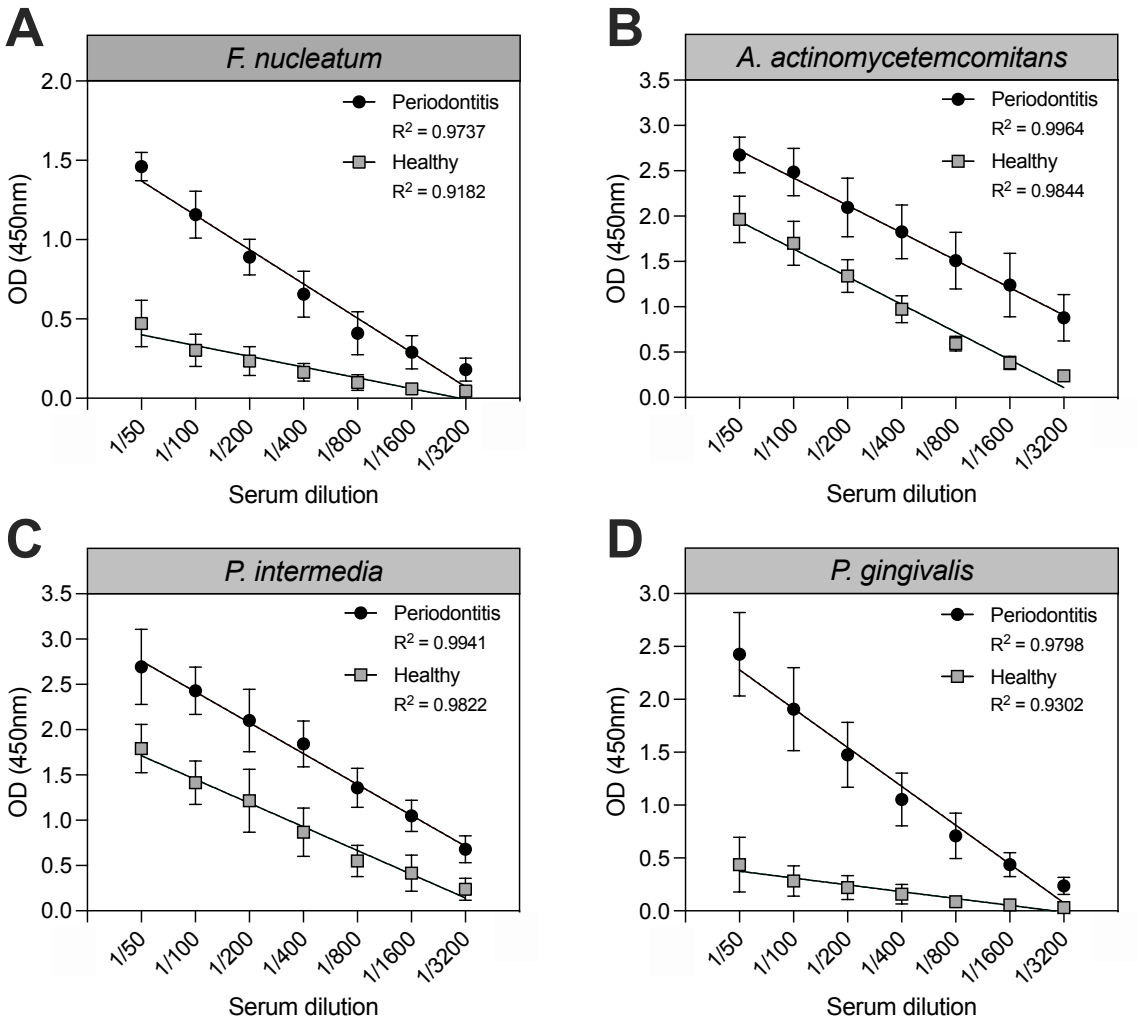

Figure 2.2. Optimisation curves for serum antibacterial IgG antibody titres. To optimise the antibacterial ELISA assays, IgG antibodies directed against *F. nulceatum* (A), A. *actinomycetemcomitans* (B), *P. intermedia* (C) and *P. gingivalis* (D) were assessed in the pooled serum of 12 PD and 10 healthy patients. Results are representative of three independent experiments (each run in duplicate). Linear regression was performed to establish reproducibility of optimised coating concentrations for each organism. Values plotted are means ± standard deviation.

2.2.5.2. Calculating ELISA units

Using the optimisation curves outlined in **figure 2.2** as guidance, patient and control serum were added to plates in 1:50, 1:100, 1:200 and 1:400 dilutions. To account for plate-to-plate variation, and standardise results to allow for comparison, antibody titres were calculated as ELISA units (EUs, **figure 2.3**). These were determined by assessing the intercept produced from patient samples against the intercept of control serum, as described previously (Mooney et al., 1993, Lappin et al., 2013).

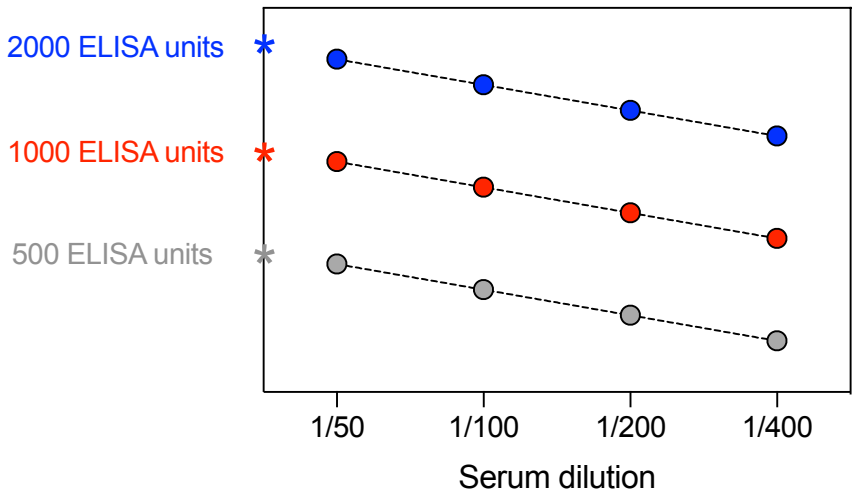

Figure 2.3. Theoretical calculation of ELISA units for antibacterial antibody titres. EUs were determined for patient samples by comparison to a PD control serum. The intercept produced from diluting PD control serum (red) is arbitrarily defined as 1000 EUs. The intercept from diluting patient samples is then compared to this value. For example, a patient sample with exactly double the IgG titre of the control (blue) would be 2000 EUs. Similarly, a sample with exactly half the IgG titre of the control (grey) would be 500 EUs.

2.2.5.3. Serum ACPAs

Serum ACPAs were determined using a semi-quantitative, commercially available anti-CCP2 ELISA assay 'Immunoassay CCPlus®' (EuroDiagnostica AB, Malmö, Sweden). Assays were performed according to the manufacturer's instructions. In brief, patient samples, standards and controls were diluted 1in5 (20 µL serum, 80 µL dilution buffer) and added to wells precoated with citrullinated synthetic peptides, alongside concentration standards (3200-12.5 AU/mL). Plates were incubated at room temperature for 2 hours, before being washed with 0.05% tween20 in PBS (PBST). After washing, 100 µL horse radish peroxidase (HRP) labelled anti-human IgG was added to wells. Wells were washed with PBST and 100 µL TMB substrate was added for 15 minutes. The reaction was stopped using 100 µL sulphuric acid (0.5M). Absorbance was measured at 450_{nm} with correction at 570_{nm} (FLUOstar Omega, BMG Labtech). All samples were assayed in duplicate, and a standard curve was constructed by plotting the mean absorbance for each standard against anti-CCP2 concentration. The level of anti-CCP2 antibodies in patient samples were interpolated using a 4-parameter curve fit (GraphPad PRISM, V8). All data were blank corrected prior to standard curve interpolation. As per assay guidelines, the diagnostic cut-off for positivity was 25 AU/mL. The limit of detection (LOD) for this assay was 2.29 AU/mL, determined by addition of the mean blank value plus the standard deviation of blanks multiplied by two. Seven samples (8.3%) were <LOD (4 at baseline, 3 at day 90), these were assigned a value of LOD/2 for statistical analysis. Herein, 'ACPAs' are referred to as anti-CCP2 IgG. These assays were conducted alongside Emily Davison.

2.2.5.4. Total serum IgG

Total serum IgG was quantified using a commercially available sandwich ELISA assay (Invitrogen, Paisley, UK). In brief, microtiter plates (Corning™ Costar™) were coated with 100 µL purified anti-human IgG monoclonal antibody (capture antibody) overnight at 4°C. Following incubation, wells were blocked for 2 hours using 1% BSA. Patient samples were then diluted between 1:250,000 and 1:500,000 and 100 µL was added to wells for 1 hour. Recombinant human IgG was used for generating a standard curve (ranging 1.6-100 ng/mL). After sample incubation, 100 µL HRP-conjugated anti-human IgG monoclonal antibody

(detection antibody) was added for 1 hour, followed by 100 µL TMB substrate solution for 15 minutes to detect signal. The reaction was stopped using 100 µL 1M phosphoric acid (H_3PO_4) and absorbance was measured at 450_{nm} with correction at 570_{nm}. The concentration of serum IgG in patient samples were interpolated from the standard curve using a 4-parameter curve fit (GraphPad PRISM, V8). All data were blank corrected prior to standard curve interpolation.

2.2.5.5. C-Reactive Protein (CRP)

Serum hsCRP (herein termed CRP) was quantified at the British Heart Foundation Glasgow Cardiovascular Research Centre. Levels of serum CRP were measured via immunoturbidometry using the Cobas C311 analyser (Cobas, Roche Diagnostic, Mannheim, Germany). This method incorporates anti-CRP antibodies which bind the analyte and cause turbidity. Sample preparation and loading was performed as per the manufacturer's instructions using the high-sensitivity CRP kit. All samples fell within the recommended measuring range (0.15 – 20.0 mg/L) and had been freeze-thawed on one occasion prior to analysis. A random subset of samples were assayed on multiple occasions to assess variability, alongside positive control samples with known CRP concentration. Repeated assaying generated results within 0.01 mg/L of the original concentration, with control samples falling within 0.03 mg/L of expected concentrations.

2.2.5.6. Proximity extension assays (IL-6 and TNFα)

Serum IL-6 and TNFα were determined using high-sensitivity ProQuantum qPCR immunoassays (Thermofisher, Loughborough, UK) measured on a StepOnePlus real-time PCR system (Applied Biosystems). These kits are proximity extension assays (PEAs), which amplify oligonucleotide-labelled antibodies that bind to the target protein. Assays were performed according to manufacturer's guidance. In brief, patient samples were initially diluted 1:5 with assay dilution buffer (2 µL patient sample, 8 µL of assay dilution buffer). Antibody conjugates directed against each analyte of interest were diluted 1:60 with antibody conjugate dilution buffer (5 µL antibody conjugate A, 5 µL antibody conjugate B, 290 µL antibody conjugate dilution buffer). Once mixed, diluted samples, standards, controls and antibodies were added to 96-well qPCR plates in a 1:1 ratio (2 µL diluted sample, 2 µL antibody conjugates). The plate was mixed thoroughly and incubated at room temperature for 1 hour. Following incubation, a qPCR

mastermix was prepared by adding 15 µL ligase to 2.5 mL pre-prepared mastermix supplied with the kit. Once mixed, 16 µL of mastermix was applied to each well (20 µL in total) and plates were centrifuged at 1000 RPM for 1 minute. The qPCR was then performed using the following reaction settings: 25°C for 20 minutes to facilitate ligation, 95°C for 2 minutes to inactivate ligase followed by 40 amplification cycles of 95°C for 3 seconds and 60°C for 30 seconds. Once complete, the Ct values from samples was compared to the Ct values of standard curves (1000-0.0128 pg/mL for both assays) using a 4-parameter curve fit. The LODs for IL-6 and TNFα were 0.12 pg/mL and 0.01 pg/mL respectively. Seventeen samples (20%) were <LOD for TNFα (10 at baseline, 7 at day 90) and none were <LOD for IL-6 assays. Samples <LOD were given as LOD/2 for statistical analysis.

2.2.6. Statistical analysis

2.2.6.1. Clinical data

Prior to statistical analysis, data distribution was visually assessed using histograms. Due to their skewed distribution, clinical data are presented as median (Q1 [25th percentile] – Q3 [75th percentile]) for continuous data or n (%) for categorical data. Differences in clinical data between baseline and day 90 was assessed using paired samples t-tests (parametric distribution) or Wilcoxon signed-rank tests (non-parametric distribution). Likewise, correlations between PISA and conventional periodontal parameters were assessed using Pearson (parametric distribution) or Spearman-Rho (non-parametric distribution) correlation co-efficient. Data analysis was conducted using GraphPad PRISM version 8 (GraphPad Software, California, USA). For post-hoc analysis, patients were split into mild-moderate or severe disease groups according to baseline PISA values, using reference ranges outlined previously (Leira et al., 2018).

For the purposes of this thesis a general rule was used to describe the strength of correlation coefficients in comparison to previous thresholds (Mukaka, 2012). Correlation coefficients between 0 and ±0.3 are considered negligible, ±0.3 to ±0.5 are considered weak, ±0.5 to ±0.7 are considered moderate and anything greater than or equal to ±0.7 considered strong. Given the arbitrary nature of this threshold and exploratory nature of the analyses, this general rule was considered in the context of the specific comparison when describing results.

2.2.6.2. Serum analytes

All serum analytes followed a non-parametric distribution. Therefore, longitudinal differences in these variables were assessed using Wilcoxon signed-rank tests. Correlations between serum analytes and periodontal clinical parameters were assessed using Spearman-Rho correlation co-efficient. Comparisons between groups was performed using Kruskall-Wallis tests with Dunn's post-hoc (smoking categories) or Mann-Whitney U-tests (PISA groups). Data analysis was conducted using GraphPad PRISM version 8 (GraphPad Software, California, USA).

2.3. Results

2.3.1. Study population

2.3.1.1. Baseline demographics and clinical parameters

In total, 45 patients with PD who required specialist periodontal treatment at Glasgow Dental Hospital Unit of Periodontics were recruited to this study. During the course of the study, 3 patients were excluded due to a new diagnosis of systemic disease, meaning 42 patients were included for analysis. The baseline demographics and periodontal parameters for included patients are outlined in **table 2.1**.

The median age of patients was 50 years old, with 12 males and 30 females. The median number of teeth was 27, which remained unchanged throughout the course of the trial. Of the 42 patients, 15 had never smoked (36%), 17 were former smokers (40%) and 10 were current smokers (24%). All patients received NSPT with varying numbers of treatment visits, dependent on baseline disease severity and patient preference for treatment scheduling (median; 4 visits, ranging from 1-6). One patient attended 1 treatment visit, 10 patients attended 2 treatment visits, 9 patients attended 3 treatment visits, 17 attended 4 treatment visits, 2 patients attended 5 treatment visits attended 3 patients had 6 treatment visits.

Table 2.1. Baseline demographics and clinical variables of patients completing the study. Data are presented as median (Q1, Q3) or n (%). n=42.

Variable	Median (Q1, Q3)	Min, Max
Total patients included	42	N/A
Age in years	50.00 (41.25, 58.50)	27.00, 75.00
Sex: Male, Female (n, %)	12 (29%), 30 (71%)	N/A
Smoking never, former, current (n, %)	15 (36%), 17 (40%), 10 (24%)	N/A
Treatment visits	4.00 (2.00, 4.00)	1.00, 6.00
Number of teeth	27.00 (24.75, 28.00)	17.00, 32.00
Full-mouth plaque score (%)	71.00 (46.75, 76.50)	12.00, 92.00
Full-mouth bleeding score (%)	61.00 (32.75, 76.25)	2.00, 100.00
Clinical attachment loss (mm)	4.43 (3.73, 5.33)	3.17, 7.12
Periodontal probing pocket depth (mm)	3.59 (3.08, 4.16)	2.53, 5.54
Pockets ≥5 mm (%)	27.50 (18.75, 41.75)	7.00, 65.00
Periodontal inflamed surface area (mm^2)	1224.60 (754.80, 1687.08)	45.60, 3655.90

2.3.1.2. Periodontal diagnosis

There was heterogeneity in the extent of disease at baseline, evident from the spread of values for periodontal parameters (**table 2.1**). For example, the median percentage of pockets ≥5 mm was 27.50% but ranged from 7.00% to 65.00%. Post hoc, patients were re-diagnosed according to the 2017 classification of periodontal disease (Papapanou et al., 2018). Almost all patients (n=40) were diagnosed with generalised PD, with the remaining patients suffering from Localised Stage IV Grade C PD (n=2).

Assessing the entire cohort, the majority of patients were diagnosed with Stage III or Stage IV PD (n=38, 90.50%), indicative of severe disease. A single patient was diagnosed with Stage I, with 3 patients diagnosed with Stage II. Likewise, over half of patients (n=26, 61.90%) were diagnosed with Grade C PD, representing a rapid rate of disease progression. Whilst 13 (31.00%) and 3 (7.10%) were diagnosed with Grade B and Grade A respectively (**Table 2.2**).

Table 2.2. Periodontal diagnosis of patients included in this study according to the 2017 classification of periodontal disease (Papapanou et al., 2018). Data is presented as a matrix where each value indicates the number of patients with each diagnosis.

		Stage			
		I	II	III	IV
Grade	A	1	2	-	-
	B	-	1	9	3
	C	-	-	6	20

2.3.2. Clinical improvement following NSPT

2.3.2.1. Longitudinal analysis of clinical periodontal parameters

Periodontal parameters (FMPS, FMBS, CAL, PPD and PPD ≥5 mm) were measured at baseline and 90 days following NSPT (**figure 2.4**). Based on data distribution at both timepoints, paired samples t-tests were used to assess longitudinal alterations in PPD and CAL, with Wilcoxon signed-rank tests used for FMPS, FMBS and PPD ≥5 mm. At day 90, a significant improvement was noted in all clinical parameters when compared with baseline. Median plaque scores decreased from 71.00% to 12.00% following treatment ($p<0.001$, **figure 2.4A**), with bleeding scores reducing from 61.00% to 8.00% ($p<0.001$, **figure 2.4B**). Patients recorded a median gain in attachment levels of 0.81 mm across all recorded sites ($p<0.001$, **figure 2.4C**). The mean PPD of all sites improved from 3.67 mm (±0.74) at baseline to 2.78 mm (±0.45) at day 90 ($p<0.001$, **figure 2.4D**), with median deep sites (≥5 mm) reducing from 27.50% to 9.00% following treatment ($p<0.001$, **figure 2.4E**).

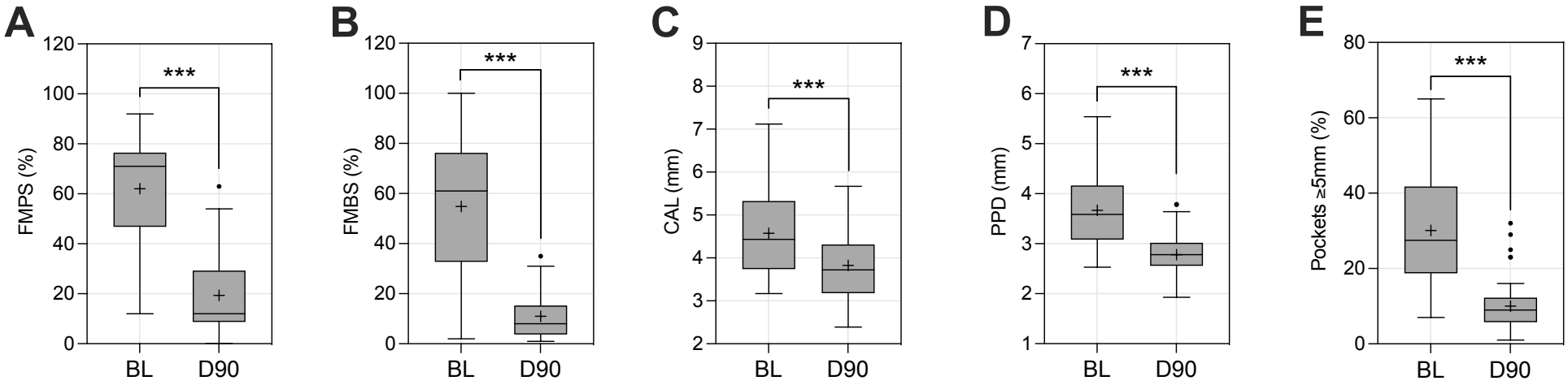

Figure 2.4. Comparison of periodontal parameters at baseline and day 90. Data represent full-mouth plaque scores (A), full-mouth bleeding scores (B), clinical attachment loss (C), periodontal probing pocket depths (D) and pockets ≥5 mm (E). Tukey boxplots at each timepoint are shown, with individual points highlighting potential outliers, horizontal line representing median and '+' representing mean. Statistical analyses refer to either paired samples t-test (CAL, PPD) or Wilcoxon signed-rank test (FMPS, FMBS, Pockets ≥5 mm), depending on data distribution (***$p<0.001$). BL; baseline, D90; day 90 (n=42).

2.3.2.2. Clinical success criteria

To evaluate the degree of treatment success in this study in the wider context, clinical improvement was compared to published success criteria. Previous studies have used various criteria to identify NSPT treatment success or determine high and low response groups. Nine different success criteria were identified that had been previously published in peer-reviewed journals or routinely clinically used throughout Glasgow Dental Hospital (**table 2.3**).

Included criteria fell into one of two categories. The first category was criteria dependent on set values relating to different periodontal indices (Scottish Dental Clinical Effectiveness Programme, 2014, Lang and Tonetti, 2003, Hughes et al., 2006, Eick et al., 2017, Suvan et al., 2019, Greenwood et al., 2020, Feres et al., 2020). The second category was cohort dependent, meaning response criteria depended on the mean or median of the specific cohort under study (Bizzarro et al., 2016, Grande et al., 2020).

The SDCEP guidelines which are used in clinical practice across Scotland focus on three parameters: FMPS, FMBS and site-specific PPD. Similar parameters are utilised by Lang and Tonetti (2003), with the exception of FMPS. The remaining 'set-value' criteria all focussed on the proportion of responding or remaining deep sites (≥5 mm) following treatment. In addition to studies explicitly outlining criteria for treatment success, the average pocket closure from a recent systematic review, defined as ≥57.00%, was included to provide a general reference to several previous studies involving NSPT.

In contrast to studies outlining set criteria, which mainly focussed on deep sites, the cohort dependent criteria included alternative periodontal parameters. From the two studies included, one was based on above the cohort median CAL improvement (Bizzarro et al., 2016), whilst the other termed 'good responders' those with above mean FMBS improvement (Grande et al., 2020).

2.3.2.3. Treatment outcomes against clinical success criteria

For evaluating treatment success, patients were assigned a number 1 through 45, with patients 3, 5 and 35 excluded as mentioned previously (**figure 2.5A**). If patients met the threshold of clinical success they were deemed 'high responders' (red – set criteria, blue – cohort dependent), with those not meeting the criteria termed 'low responders' (grey). Despite the highly significant reductions in clinical parameters observed previously (**figure 2.4**), no patient fulfilled all of the SDCEP criteria (Scottish Dental Clinical Effectiveness Programme, 2014). The FMPS criteria was met by 25 patients (59.50%), 26 patients met the FMBS criteria (61.90%) and no patients met the PPD criteria (all sites <4 mm). Although at least one pocket ≥4 mm remained in every patient, there was an overwhelming trend for the number of these deep sites to reduce following treatment (**figure 2.5B**). Furthermore, the number of sites ≥4 mm at day 90 strongly correlated with the number of sites ≥4 mm at baseline (Pearson r = 0.705, p<0.001, **figure 2.5C**).

Assessing the clinical outcomes of this trial with other set criteria yielded variable results. For example, every patient met the criteria outlined by Greenwood et al. (2020) and all but one patient met criteria outlined by Hughes et al. (2006). Similarly, 26 patients met the criteria outlined by Eick et al. (2017). In contrast, very few patients met the criteria outlined by Lang and Tonetti (2003) and Feres et al. (2020), with 10 and 5 patients deemed 'high-responders' respectively. As was the case with sites ≥4 mm, there was a positive correlation between sites ≥5 mm at baseline and day 90 (Spearman r = 0.567, p<0.001, **figure 2.5D**). This makes it difficult to delineate true 'high-responders' using this criteria, as patient's pre-treatment disease severity carries substantial weight on these metrics.

Other studies have used cohort dependent variables (Bizzarro et al., 2016, Grande et al., 2020). When comparing these categories in our current cohort, an agreement was met in only half of the patients (13 termed 'high responders' by both criteria, 8 termed 'low responders' by both criteria, n=21). A more accurate representation of treatment outcome may be comparison to a systematic review which outlined typical clinical responses among a number of previous NSPT studies (Suvan et al., 2019). Using the average pocket closure rate

identified by this study (57.00%), it was found that 24 out of 42 patients met this criteria in the current cohort (study average: 65.23% pocket closure). Therefore, despite varying agreement with other set criteria, the data from the current trial does appear commensurate with previous trials conducted with a similar treatment regime and follow-up period (3 months).

Table 2.3. Overview of treatment success criteria used in previous studies ranging from 2003 to 2020.

Reference	Success criteria
Set criteria	
Scottish Dental Clinical Effectiveness Programme (2014)	FMPS <15% FMBS <10% All sites <4 mm PPD
Lang and Tonetti (2003)	<8 sites with PPD ≥5 mm <25% FMBS
Hughes *et al.*, (2006)	A reduction of ≥2 mm in ≥30% of 'deep sites' (≥5mm at baseline)
Eick *et al.*, (2017)	A reduction of ≥1 mm in ≥60% of 'deep sites' (>4mm at baseline)
†Suvan *et al.*, (2019)	Pocket closure ≥57%
Greenwood *et al.*, (2020)	≥25% 'responding sites' (defined at sites ≥ 5mm at baseline that achieved at least 2 mm reduction)
Feres *et al.*, (2020)	≤4 sites with PPD ≥5 mm
Cohort dependent	
Bizarro *et al.*, (2016)	Above median CAL improvement
Grande *et al.*, (2020)	Above mean FMBS improvement

†Suvan et al., (2019) refers to a systematic review outlining the average pocket closure 3 months after periodontal treatment and was not used as a treatment success criteria in the original study

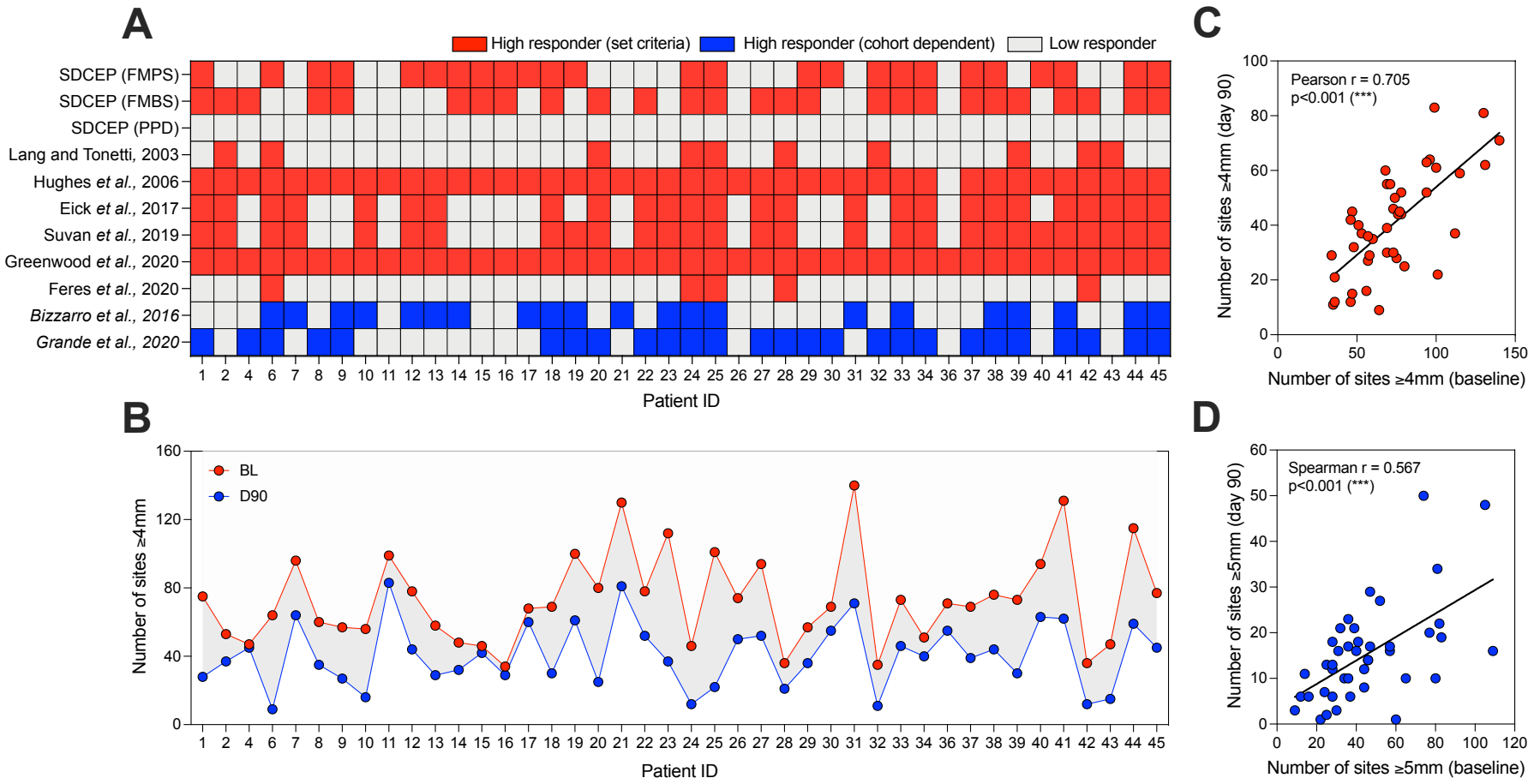

Figure 2.5. Schematic representation of patients fulfilling published criteria of treatment success. (A) Patients are classified into those meeting different success criteria. (B) The number of sites ≥4 mm at each timepoint per patient (n=42). (C) Pearson's correlation between the number of sites ≥4 mm at baseline and day 90. (D) Spearman correlation between the number of sites ≥5 mm at baseline and day 90. Raw p-values are shown.

2.3.2.4. Calculation of the periodontal inflamed surface area

Whilst the periodontal parameters outlined in **table 2.1** and **figure 2.3** provide useful metrics to assess disease severity of patients, none accurately quantify the degree of inflamed periodontal tissue. Given links between PD and other inflammatory disorders (RA, CVD), quantifying inflamed periodontium has become increasingly important. Furthermore, defining a sole metric would allow for more accurate evaluation of the relationship between periodontal disease and indicators of local and systemic inflammation.

To address this issue, the PISA was developed (Nesse et al., 2008), which reflects the surface area of bleeding pocket epithelium in square millimetres and thus provides an estimate of the local inflammatory burden posed by PD. The calculation of PISA builds upon an earlier metric (Hujoel et al., 2001) termed the 'dentogingival epithelial surface area' (DGES, **figure 2.6A**) which incorporates the CAL and data from a meta-analysis of root surface areas to quantify the root surface area that has become exposed due to disease (Hujoel, 1994). The periodontal epithelial surface area (PESA) expands on DGES to encompass PPD rather than CAL. Multiplication of PESA by the proportion of bleeding sites around a particular tooth generates PISA (Nesse et al., 2008).

Using the formula developed previously (Nesse et al., 2008), PISA was calculated at baseline and day 90 (**figure 2.6B**). As expected, there was a large spread in baseline PISA values (median: 1224.60mm^2, range: 45.60mm^2 to 3655.90mm^2). Matching with reductions found in other periodontal parameters, PISA significantly reduced 90 days following treatment highlighting lower levels of periodontal inflammation ($p<0.001$, Wilcoxon signed-rank test).

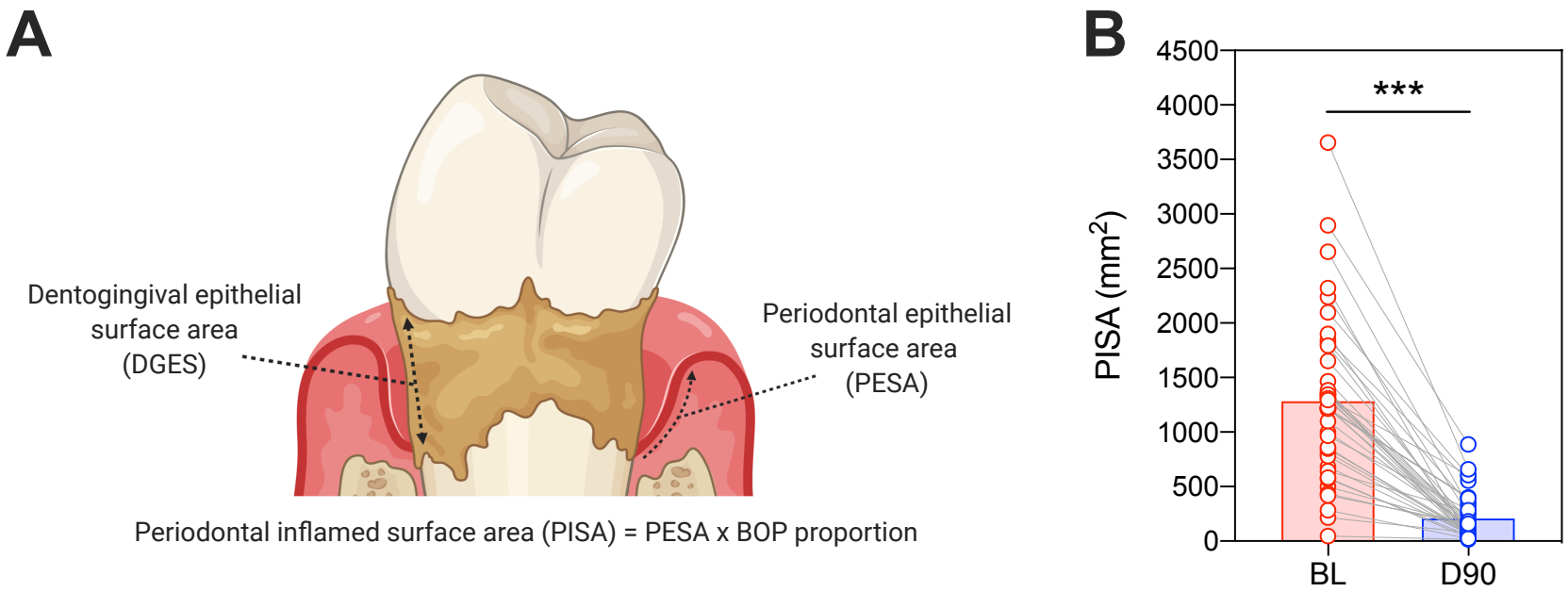

Figure 2.6. The periodontal inflamed surface area (PISA). Schematic outlining the calculation of PISA with reference to the dentogingival epithelial surface area (A), as outlined previously (Nesse et al., 2008). Schematic prepared using the Biorender software. Comparison of PISA at baseline (BL) and day 90 (D90) in the current patient cohort (B). Bars represent medians with lines connecting individual patients. Statistics refer to Wilcoxon signed-rank test where ***p<0.001.

2.3.2.5. Validating PISA as a metric to indicate disease severity

PISA has been adopted for use in periodontal research (Leira et al., 2018, Sakanaka et al., 2017, Park et al., 2017); however it's applicability as a sole metric to indicate disease severity remains elusive. To understand whether PISA would provide a useful indication of disease severity, we investigated the relationship between PISA at each timepoint, and the change in PISA following treatment (Δ) against standard periodontal parameters (**figure 2.7**). Correlation analysis was performed using Pearson or Spearman-Rho correlation coefficient's depending on data distribution.

At baseline, there were positive associations between PISA and all conventional periodontal parameters ranging from weak to strong (**figure 2.7A**). Whilst this may be expected for some parameters directly involved in the calculation of PISA (FMBS and PPD), positive associations were also observed with parameters not associated with its calculation. For example, weak positive associations were observed between PISA and FMPS (Spearman R = 0.483, p=0.0012, **figure 2.7Ai**) along with PISA and CAL (Spearman R = 0.475, p=0.0015, **figure 2.7iii**). Similar results were observed at day 90 (**figure 2.7B**) with the exception of CAL (Spearman R = 0.124, p=0.43, **figure 2.7Biii**).

There was a positive association between ΔPISA and the changes in all standard periodontal parameters to varying degrees (**figure 2.7C**). As expected, the weakest correlations were found for FMPS (Pearson R = 0.369, p=0.016, **figure 2.7Ci**) and CAL (Pearson R = 0.425, p = 0.005, **figure 2.7Ciii**). Considering these results and the strong association with PPD, FMBS and pockets ≥5 mm, PISA was subsequently employed throughout this study as an estimate of local inflammatory status and disease severity.

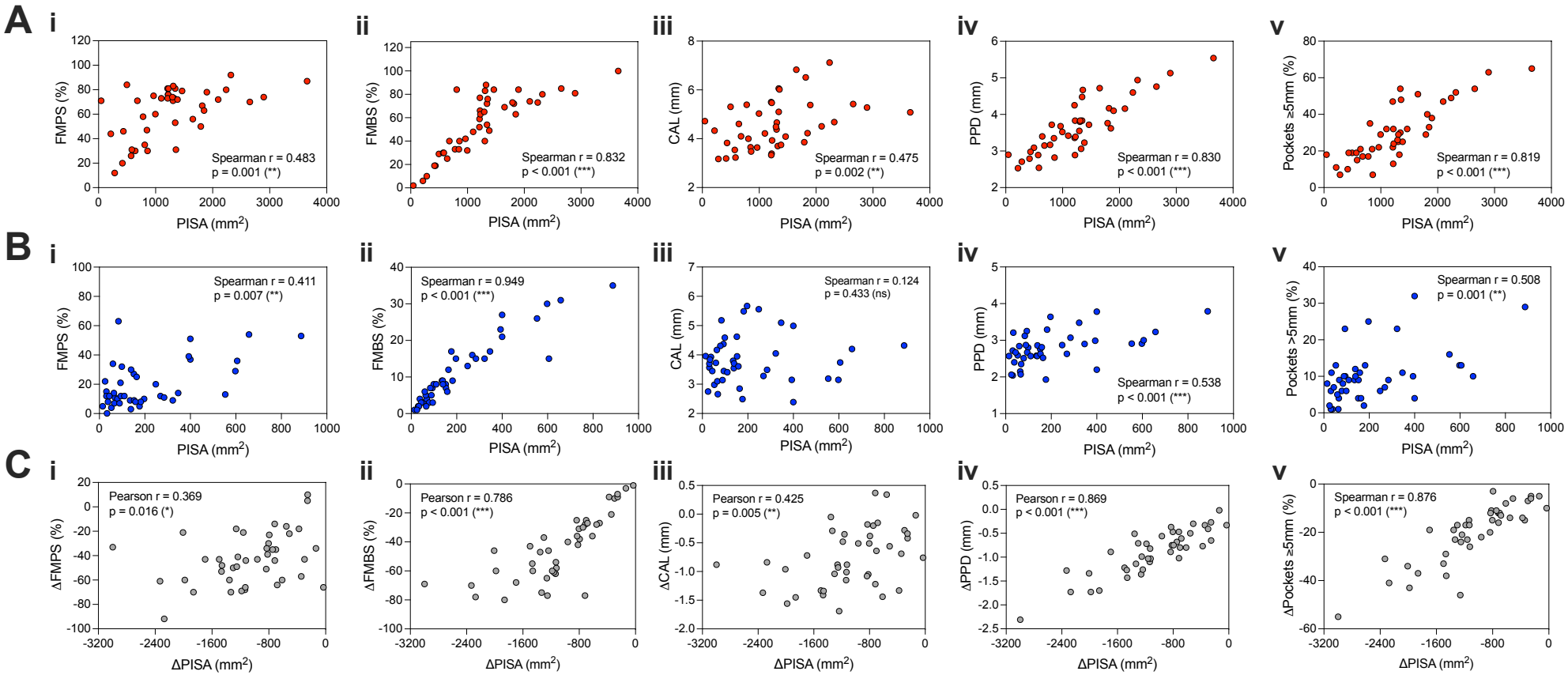

Figure 2.7. Association between PISA and conventional periodontal parameters. Correlations were performed at baseline (A), day 90 (B) or using the delta (day 90 minus baseline) values (C). Depending on data distribution, r and p-values refer to either Pearson (parametric distribution) or Spearman rank correlation coefficient (non-parametric distribution), where *p<0.05, **p<0.01 and ***p<0.001. Correlations were performed across all patients (n=42).

2.3.3. The systemic response to NSPT

2.3.3.1. Association between systemic analytes at baseline

Throughout this study six serum antibody titres (anti-*P. gingivalis* IgG, anti-*P. intermedia* IgG, anti-*A. actinomycetemcomitans* IgG, anti-*F. nucleatum* IgG, anti-CCP2 IgG, total IgG) and three inflammatory mediators (CRP, IL-6, TNFα) were evaluated. To establish whether any of these were associated with periodontal disease severity, a correlation analysis was performed between serum antibody titres, inflammatory proteins and periodontal clinical parameters at baseline. All serum analytes followed a non-parametric distribution; therefore this analysis was performed using Spearman-Rho correlation coefficients (**figure 2.8**).

From this analysis, there were no strong associations between any systemic antibody titre or inflammatory marker with any periodontal parameter. All positive associations were < 0.7; all negative associations were < -0.7 (**figure 2.8**). Several weak associations were observed which may become relevant in larger powered studies. For antibody titres, weak associations were observed between anti-*A. actinomycetemcomitans* IgG and PISA (R=-0.315, p=0.045), anti-CCP2 IgG and FMPS (R=0.328, p=0.034) and total serum IgG and PPD (R=0.359, p=0.019).

For systemic inflammatory mediators, CRP showed weak associations with several periodontal parameters, including FMPS (R = 0.368, p=0.016), PPD (R = 0.315, p=0.042), PPD≥5mm (R=0.357, p=0.02) and PISA (R=0.346, p=0.025). Similarly, IL-6 showed weak associations with FMBS (R=0.319, p=0.04), PPD (R=0.328, p=0.034) and PISA (R=0.344, p=0.025). Within the current study, there was insufficient evidence for an association between serum TNFα and any periodontal parameter.

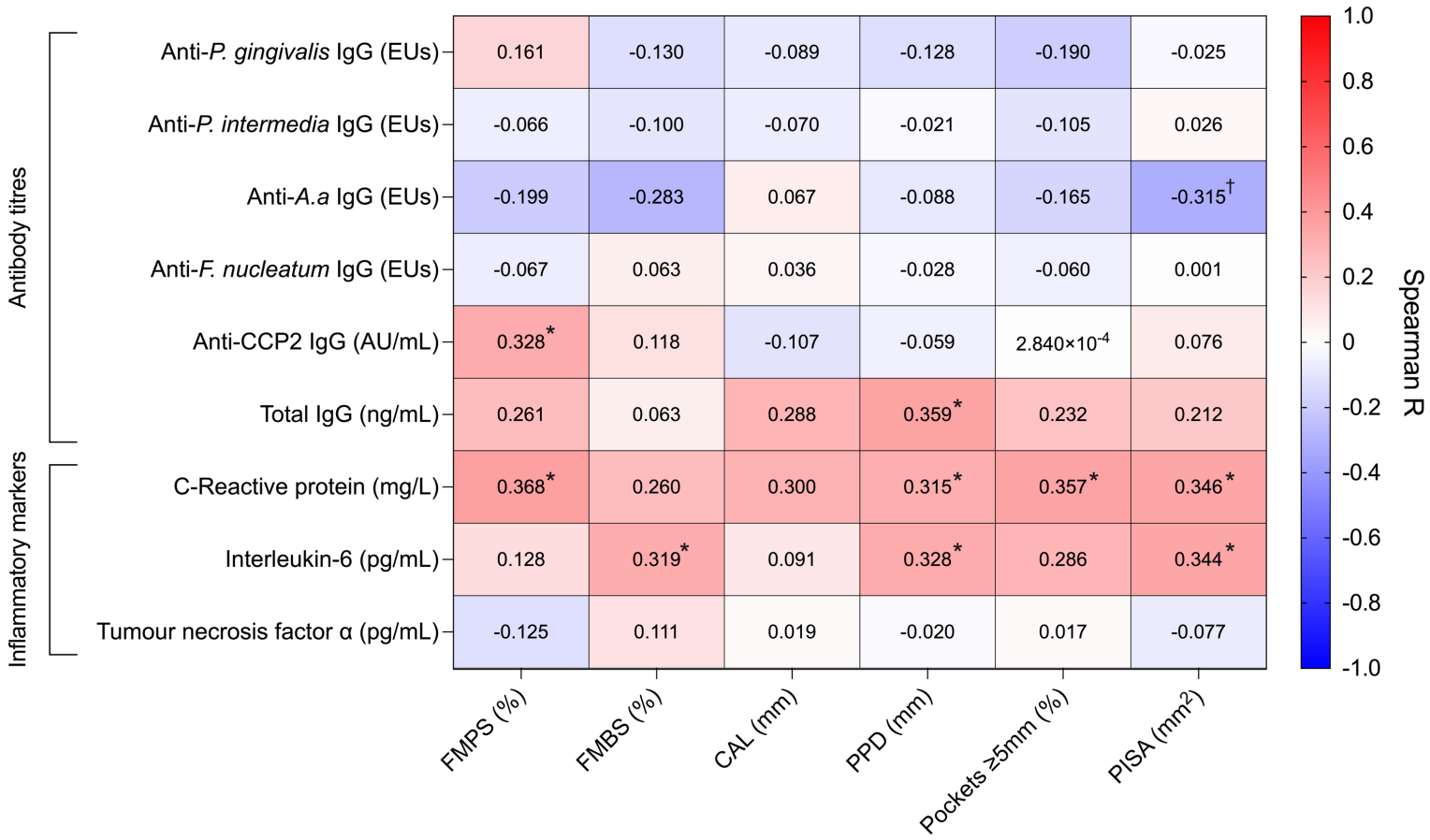

Figure 2.8. Correlation between systemic antibody titres, inflammatory mediators and periodontal clinical parameters at baseline. Correlations were performed using Spearman-Rho method, with individual R values displayed in a heatmap. *associated p-value <0.05. N=42 excluding anti-*P. intermedia*, *A.a* and *F. nucleatum* (n=41). A.a = *Aggregatibacter actinomycetemcomitans*.

2.3.3.2. Total serum IgG antibodies

Prior to analysing antibacterial IgG and ACPAs, total serum IgG was quantified at baseline and day 90 to establish whether a longitudinal alteration in specific IgG's may be driven by a decrease in the total level (**figure 2.9**). However, no significant differences were observed following treatment across all patients (p=0.42, Wilcoxon signed-rank test). Recorded median values of total serum IgG were $2.73x10^6$ ng/mL at baseline and $2.52x10^6$ ng/mL at day 90.

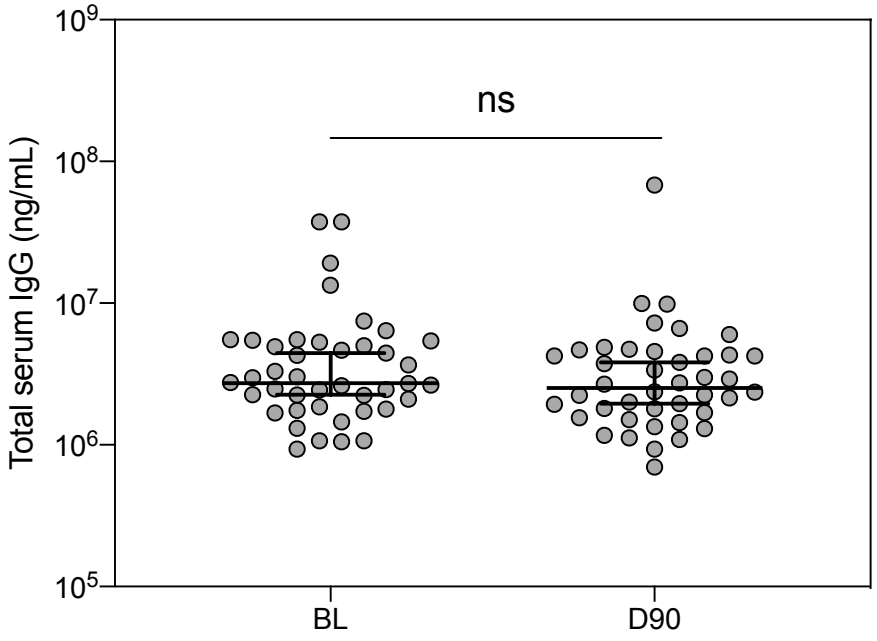

Figure 2.9. Total serum IgG at baseline (BL) and day-90 (D90). Individual values are shown, error bars representing median ±95% confidence interval. Statistics are Wilcoxon signed-rank test where ns means no statistically significant difference (p>0.05). Y-axis is log-transformed.

2.3.3.3. Shifts in ACPAs and association with antibacterial antibodies

Having observed no association between ACPA titres and periodontal disease severity at baseline, longitudinal alterations in ACPAs and other antibody titres were evaluated. Despite no patients in this study being diagnosed with RA, three patients at baseline, and six patients at day 90, met the assay positivity threshold of 25 AU/mL (red circles, **figure 2.10A**). When comparing ACPAs across all patients, there was no significant difference observed following NSPT ($p=0.12$, Wilcoxon signed-rank test, **figure 2.10A**). Median ACPA levels were 6.84 AU/mL at baseline and 7.86 AU/mL at day 90.

Correlation analysis was performed between antibacterial IgG antibodies, total serum IgG and ACPAs at baseline (**figure 2.10B-F**). One patient (#2) had limited serum volume and was only included in analysis of ACPAs, anti-*P. gingivalis* IgG and total serum IgG (n=42 for **figure 2.10A,B,F**, n=41 for **figure 2.11C,D,E**). Negligible associations were observed between ACPAs and other IgG titres within the current study (all $p>0.05$, $R>-0.3<0.3$). Thus, elevated antibody titres reactive against periodontal bacteria, in particular *P. gingivalis* and *A. actinomycetemcomitans*, did not appear strongly related to elevated serum ACPA titres in this current study.

2.3.3.4. Longitudinal alterations in antibacterial antibody titres

Following treatment, there were no significant alterations in antibodies directed against *F. nulceatum*, *A. actinomycetemcomitans* or *P. intermedia* (all $p>0.05$, **figure 2.11A-C**). In contrast, there was a significant reduction in anti-*P. gingivalis* IgG ($p<0.001$, **figure 2.11D**). Median EUs decreased from 381.5 (25^{th} to 75^{th} percentile: 181.2 to 793.3) at baseline to 307.3 (156.1 to 690.8) at day 90, representing a change of -11.54% across all patients (**figure 2.11E**).

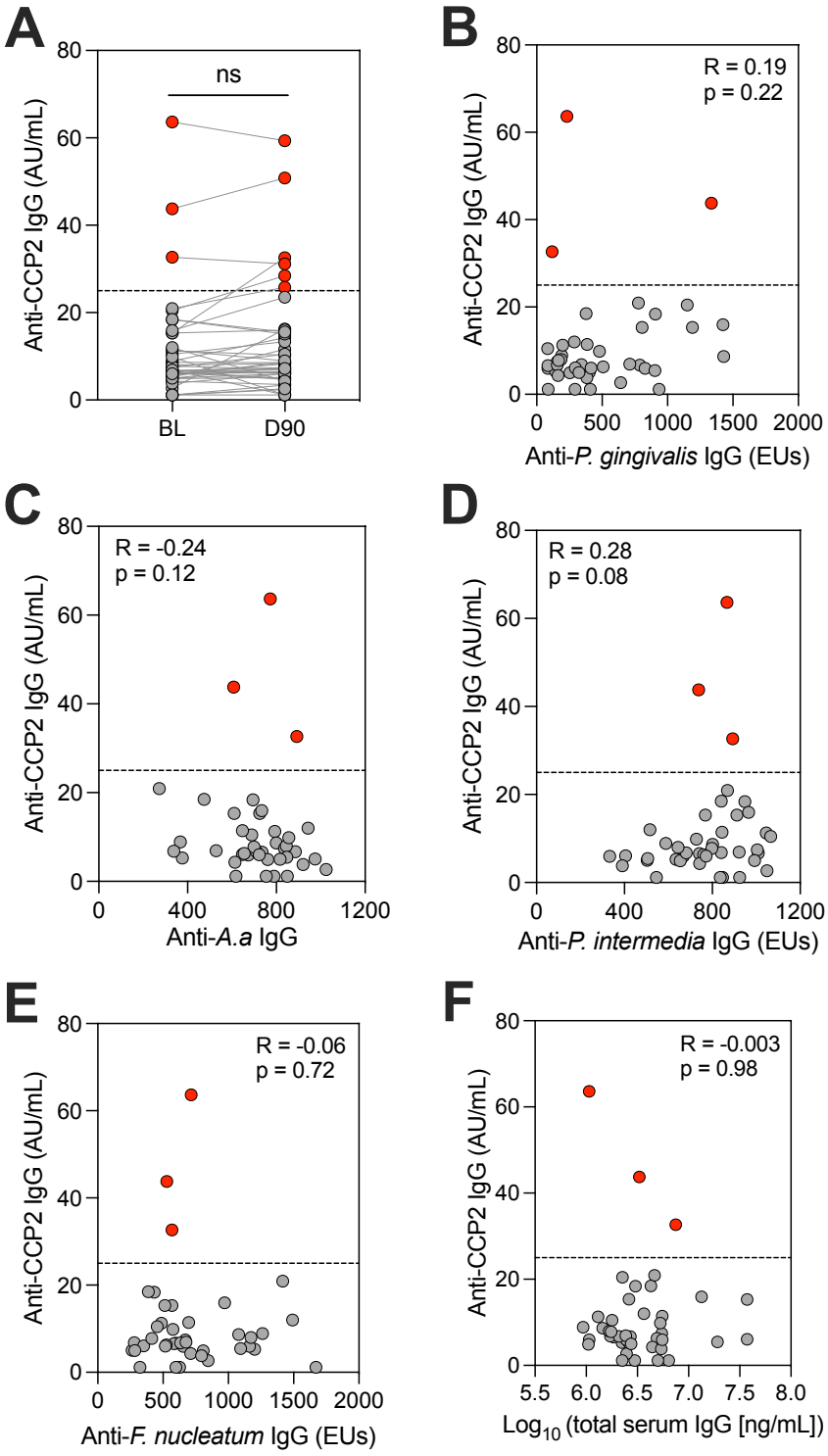

Figure 2.10. Serum ACPAs in PD. Comparing ACPAs longitudinally following NSPT where lines connect individual patients (A). Dashed black line indicates the 'ACPA-positivity' threshold (>25AU/mL), positive samples shown in red. Statistics are Wilcoxon signed-rank test, ns means no statistically significant difference. Serum ACPAs were correlated against other IgG using Spearman-Rho correlation coefficients, anti-*P. gingivalis* IgG (B), anti-*A.a* IgG (C), anti-*P. intermedia* IgG (D), anti-*F. nucleatum* IgG (E) and total serum IgG (F). A,B,F: n=42, C,D,E: n=41. A.a: *Aggregatibacter actinomycetemcomitans*. ACPA experiments conducted with Emily Davison.

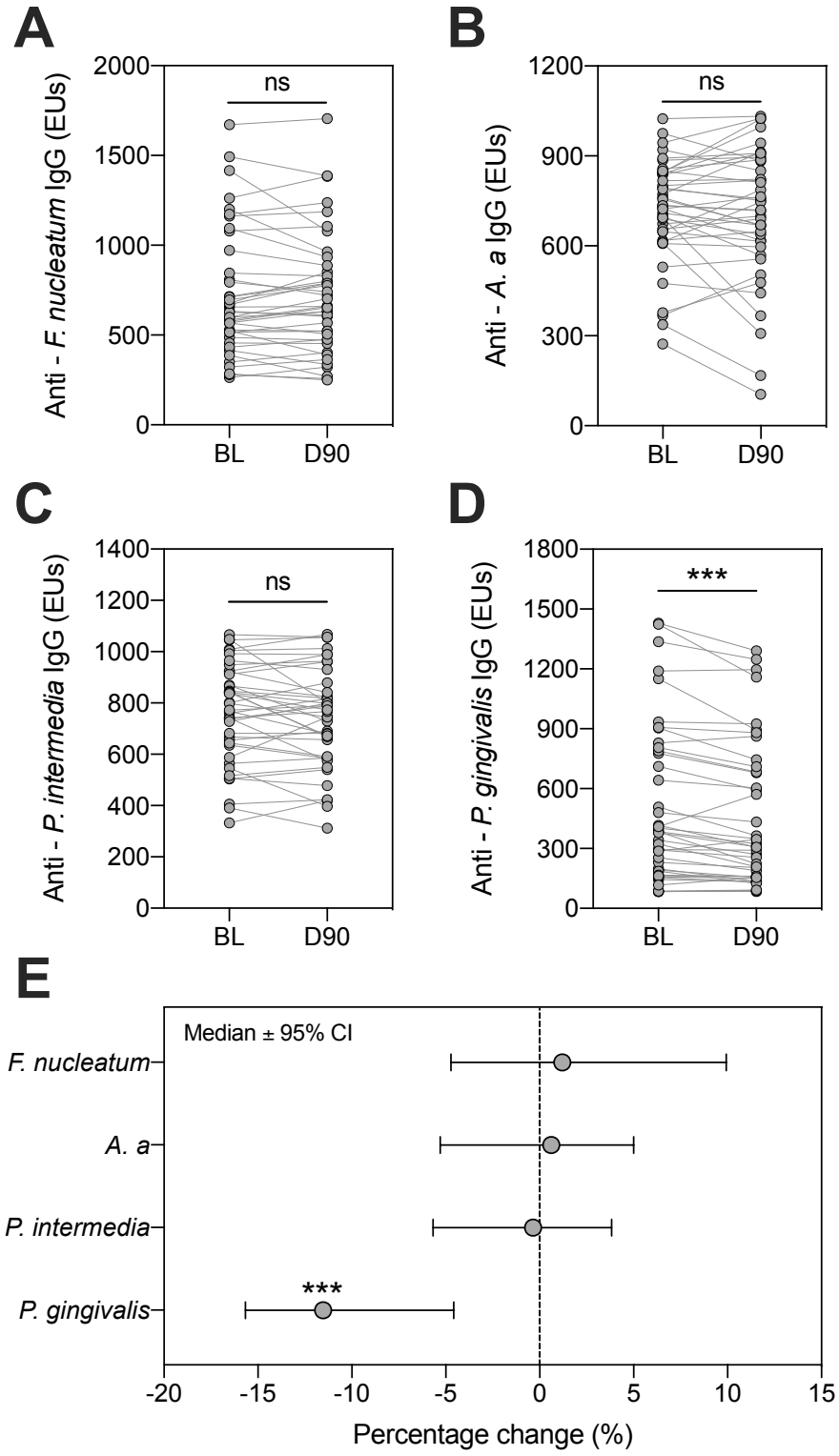

Figure 2.11. Serum IgG against periodontal bacteria at baseline (BL) and day-90 (D90). Anti-*F. nucleatum* (A), *A.a* (B), *P. intermedia* (C) and *P. gingivalis* (D). Individual values are shown, with lines connecting each patient. Statistics are Wilcoxon signed-rank test where ***p<0.001, and ns means no statistically significant difference (p>0.05). The percentage change across all patients is highlighted (E), where dots indicate medians and error bars are 95% confidence intervals. A, B, C: n=41, D: n =42. A.a = *Aggregatibacter actinomycetemcomitans*.

2.3.3.5. Subgingival plaque colonisation and systemic antibody titres

There were no strong positive or negative association between antibacterial antibody titres and periodontal clinical parameters at baseline (**figure 2.8**). Yet, we observed a large spread in baseline antibody titres (**figure 2.10**). Serum levels of anti-*F. nulceatum* IgG ranged from 263.6 EUs to 1671.0 EUs, anti-*A. actinomycetemcomitans* IgG ranged from 272.6 EUs to 1024.0 EUs, anti-*P. intermedia* IgG ranged 333.0 EUs to 1067.0 EUs and anti-*P. gingivalis* IgG ranged from 84.12 EUs to 1429.0 EUs.

To investigate whether species colonisation in the subgingival plaque impacted these antibody titres, IgG titres were compared between patients with detectable and undetectable levels of each species using 16S rRNA sequencing (**section 3.2.7**). All patients in the current cohort had detectable levels of *F. nulceatum*. Only 3 (7%) had detectable levels of *A. actinomycetemcomitans* at baseline, although an additional patient was positive at day 90. Twenty-three patients (55%) had detectable levels of *P. intermedia*, and 13 patients (31%) had detectable levels of *P. gingivalis* in sampled sites.

Patients with detectable *P. gingivalis* displayed significantly higher levels of anti-*P. gingivalis* IgG in their serum when compared to those with undetectable levels of *P. gingivalis* in subgingival plaque ($p<0.0001$, Mann-Whitney U test, **figure 2.12C**). This trend was not maintained for other bacteria. ACPAs were also compared according to subgingival plaque colonisation status of each organism (**figure 2.12E-H**). For *A. actinomycetemcomitans* and *P. intermedia*, no significant differences in ACPA titres were observed between positive and negative patients. For *P. gingivalis*, positive patients appeared to display a slight elevation in ACPA titres at baseline ($p=0.03$, **figure 2.12H**), however, it is worth noting that 2 out of 3 ACPA-positive patients were in-fact *P. gingivalis* negative. The implications of elevated ACPA levels below the positivity threshold are not clear, and the small magnitude of median increase (+8.8 AU/mL) limits the extent to which this increase may be further interpreted.

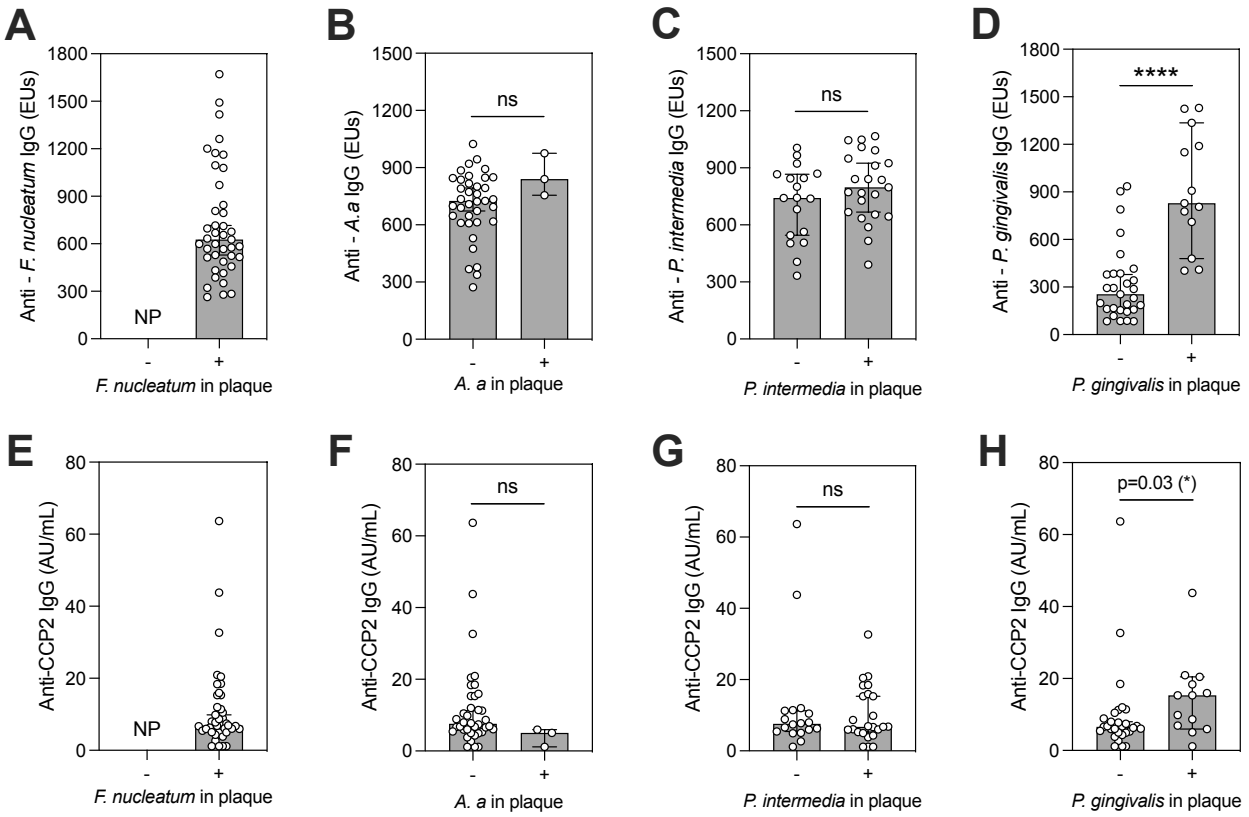

Figure 2.12. Antibacterial antibody titres and ACPAs according to subgingival plaque colonisation status. Patients were split according to whether *F. nulceatum*, *A. a*, *P. intermedia* and *P. gingivalis* were detectable (+) or undetectable (-) using 16S rRNA sequencing. (A-D) Antibody titres and (E-H) ACPAs were compared according to colonisation status. Statistics represent Mann-Whitney U tests, where ns means no significant difference ($p>0.05$) and ****$p<0.0001$. NP means no patients fulfilled this criteria. A.a = *Aggregatibacter actinomycetemcomitans*. Individual datapoints are displayed, bars represent medians. ACPA experiments conducted with Emily Davison.

2.3.3.6. Systemic antibodies according to *P. gingivalis* colonisation

To further explore the relationship between *P. gingivalis* and systemic antibody titres, a subgroup analysis was performed to assess longitudinal alterations in ACPAs and anti-*P. gingivalis* IgG in patients positive (n=13) and negative (n=29) for *P. gingivalis* in subgingival plaque samples (**figure 2.13**). Despite observing a slight elevation in ACPAs for patients positive for *P. gingivalis*, there was no significant difference in ACPA titre in either group following treatment (**figure 2.13A,B**). Surprisingly, significant reductions in anti-*P. gingivalis* IgG were observed regardless of plaque colonisation (**figure 2.13C, D**). Together these results demonstrate that *P. gingivalis* detection in subgingival plaque samples did not impact longitudinal alterations in ACPAs and was not a prerequisite for a reduction in anti-*P. gingivalis* IgG titre following NSPT.

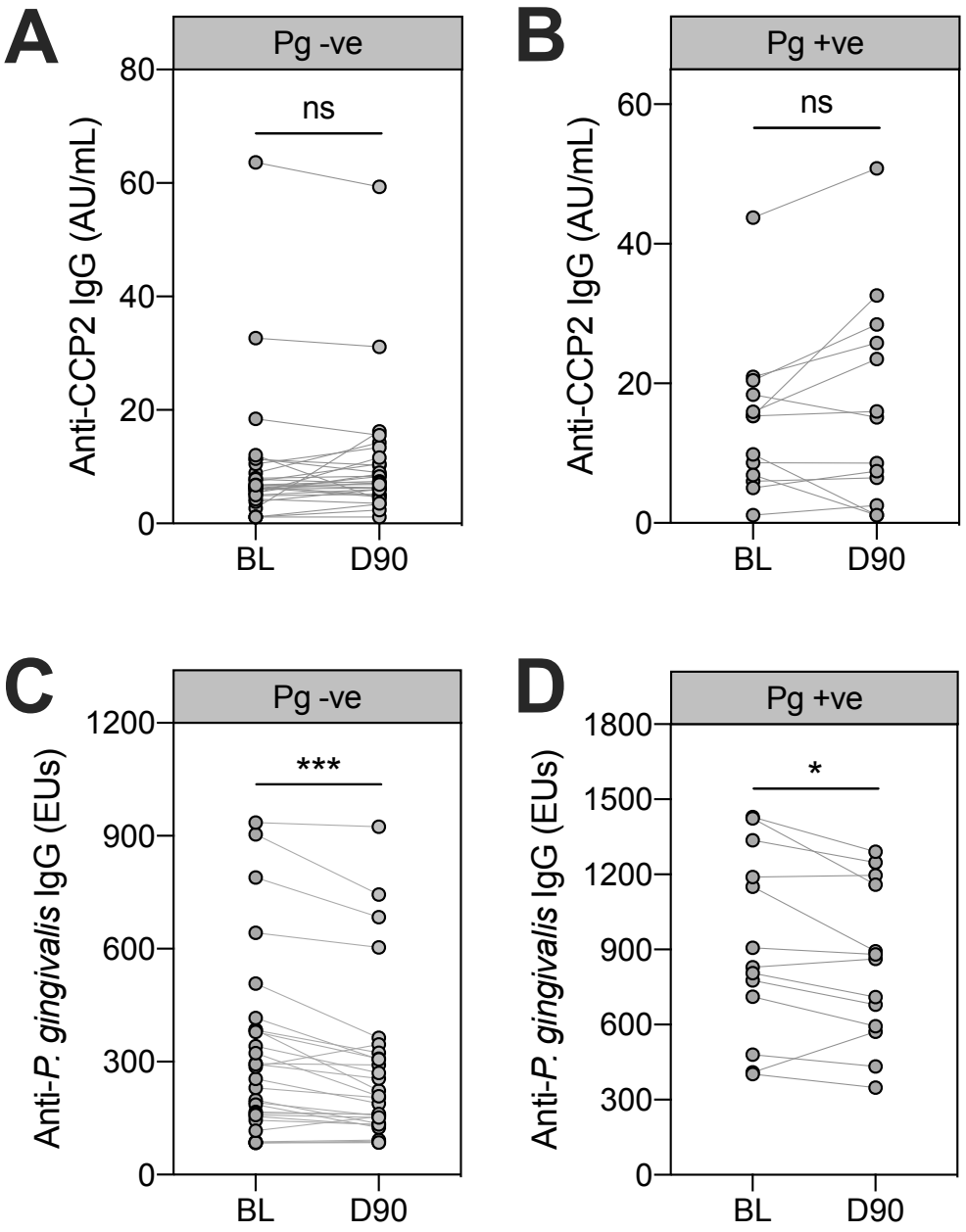

Figure 2.13. *P. gingivalis* plaque status at baseline with ACPAs and anti-*P. gingivalis* IgG antibodies. ACPAs (A, B) and anti-*P. gingivalis* IgG (C,D) at baseline (BL) and day 90 (D90) in patients negative (A, C; n=29) and positive (B, D; n=13) for *P. gingivalis* in subgingival plaque. Statistics refer to Wilcoxon signed-rank tests, were ns means no significant difference, *p<0.05 and ***p<0.001. Individual datapoints displayed with lines connecting each patient at BL and D90. ACPA experiments conducted with Emily Davison.

2.3.3.7. Longitudinal alterations in systemic inflammatory proteins

Having evaluated systemic antibody titres, inflammatory proteins were quantified in serum to establish their association with serum ACPAs and changes following treatment. Three systemic inflammatory proteins (CRP, IL-6 and TNFα) were selected based on previous studies finding reductions following NSPT and associations with RA (D'aiuto et al., 2004b, Dag et al., 2009, Anderson et al., 2012). Longitudinal analysis was performed using Wilcoxon signed-rank tests, with the hypothesis that reductions in periodontal inflammation may reduce levels of systemic inflammation.

At baseline, median levels were CRP: 1.33 mg/L; IL-6: 2.64 pg/mL; and TNFα 0.03 pg/mL. Negligible associations were observed between ACPAs and systemic inflammatory markers using Spearman-Rho correlation coefficients (R = 0.23 [CRP], R=0.24 [IL-6], R=0.27 [TNFα], all p>0.05, **figure 2.14D-F**). Additionally, despite weak associations between CRP and IL-6 with PISA at baseline, there were no significant alterations for any marker following treatment; CRP (p=0.49, **figure 2.14A**), IL-6 (p=0.98, **figure 2.14B**) or TNFα (p=0.054, **figure 2.14C**).

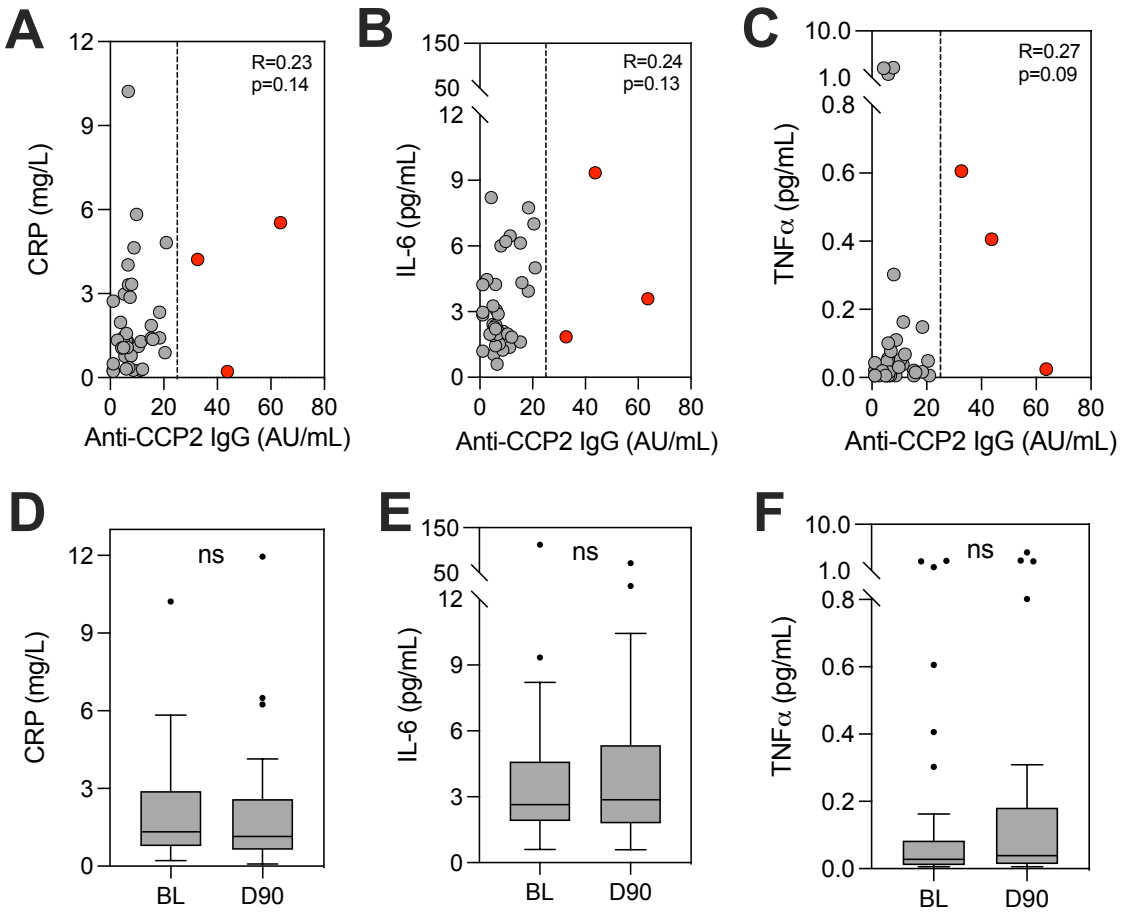

Figure 2.14. Associations between serum ACPAs and systemic inflammatory proteins. Graphs display CRP (A), IL-6 (B) and TNFα (C). Statistics refer to Wilcoxon signed-rank test; ns means no statistically significant difference. (D-F) Tukey boxplots with potential outliers highlighted by individual datapoints. Correlation between serum ACPAs and CRP (D), IL-6 (E) and TNFα (F), statistics refer to Spearman-Rho correlation coefficients, raw p-values are displayed. Dashed black line indicates ACPA-positively threshold (>25 AU/mL). ACPA experiments conducted with Emily Davison.

2.3.4. Impact of local inflammation on the systemic response

2.3.4.1. Grouping patients according to baseline PISA

Given the large spread in baseline PISA (45.60 mm^2 to 3655.90 mm^2), it was hypothesised that the lack of alterations in systemic analytes may be partially skewed by patients with low baseline values. Previous work by Leira et al., (2018) compared PISA values across healthy patients and patients suffering from mild, moderate and severe PD. From this study, ranges of PISA values were identified that were representative of previously established disease severity categories. A range of 934.71 to 3274.96 mm^2 was identified for severe PD; 521.58 to 790.30 mm^2 for moderate PD; 110.16 to 447.01 mm^2 for mild PD and 10.22 to 62.78 mm^2 for healthy participants.

Overlaying these values on top of baseline PISA values in the current cohort, it was found that one patient exceeded the range of severe PD, with a PISA value of 3655.90 mm^2 (**figure 2.15A**). Twenty-seven patients fell within the threshold of severe PD, whilst 3 fell between moderate and severe disease. Five patients fell within the criteria of moderate PD, and one fell between moderate and mild disease. Four patients fell within the criteria of mild disease and surprisingly, one patient recorded a baseline level commensurate with health (45.6 mm^2).

Using these PISA categories, 28 patients were classified as having severe disease at baseline, whilst 14 were classified as mild-moderate. As expected, patients presenting with severe disease at baseline demonstrated a significantly greater reduction in periodontal inflammation following treatment when compared with patients suffering from mild-moderate forms of disease (median reduction: 1255.50 mm^2 vs. 445.35 mm^2, $p<0.001$, Mann-Whitney U test, **figure 2.15B**).

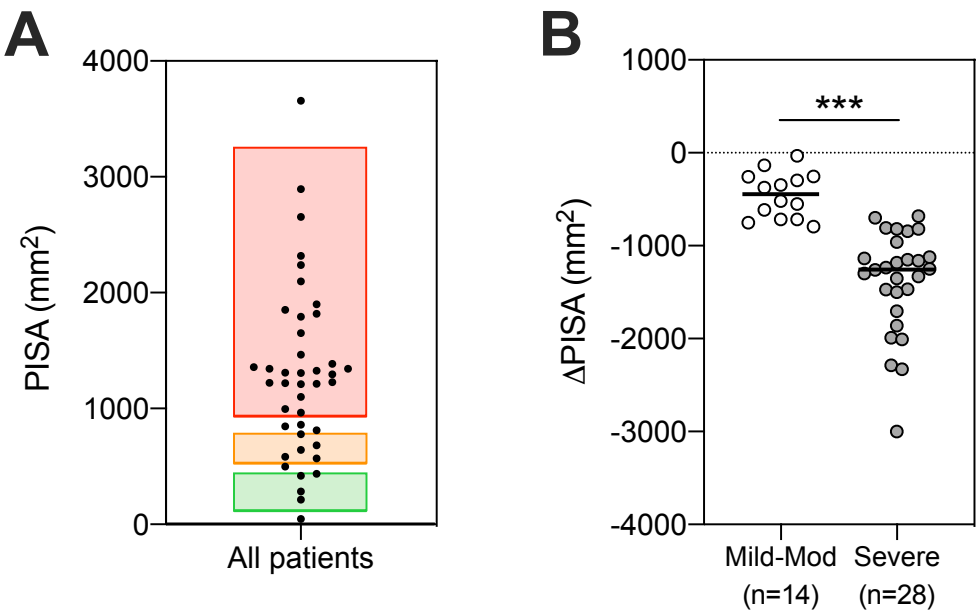

Figure 2.15. Grouping patients according to baseline PISA values. (A) PISA values at baseline for the cohort with overlaid reference values from (Leira et al., 2018). In relation to periodontal disease severity, the red box indicates 'severe', yellow box indicates 'moderate' and green box indicates 'mild' disease. (B) Change in PISA values following treatment depending on whether patients had mild-moderate or severe disease at baseline. Statistics refer to Mann-Whitney U test, where ***p<0.001. Individual datapoints are displayed.

2.3.4.2. Systemic antibodies according to baseline disease severity

Systemic antibody titres were compared between 'mild-moderate' and 'severe' groups at baseline and day 90, and longitudinally within each group following treatment (**figure 2.16**). Reductions in anti-*P. gingivalis* IgG were observed following treatment in both the mild-moderate (p=0.006) and severe groups (p<0.001, **figure 2.16F**). In contrast, no significant difference in anti-*P. gingivalis* IgG was observed between groups at baseline or day 90. No longitudinal alterations were observed within groups for ACPAs, total serum IgG, anti-*P. intermedia* IgG, anti-*A. actinomycetemcomitans* IgG, anti-*F. nucleatum* IgG, suggesting that a larger reduction in periodontal inflammation did not impact changes in these antibody titres. A trend was observed whereby the severe PISA group showed lower anti-*A. actinomycetemcomitans* IgG, albeit this did not reach statistical significance at either timepoint.

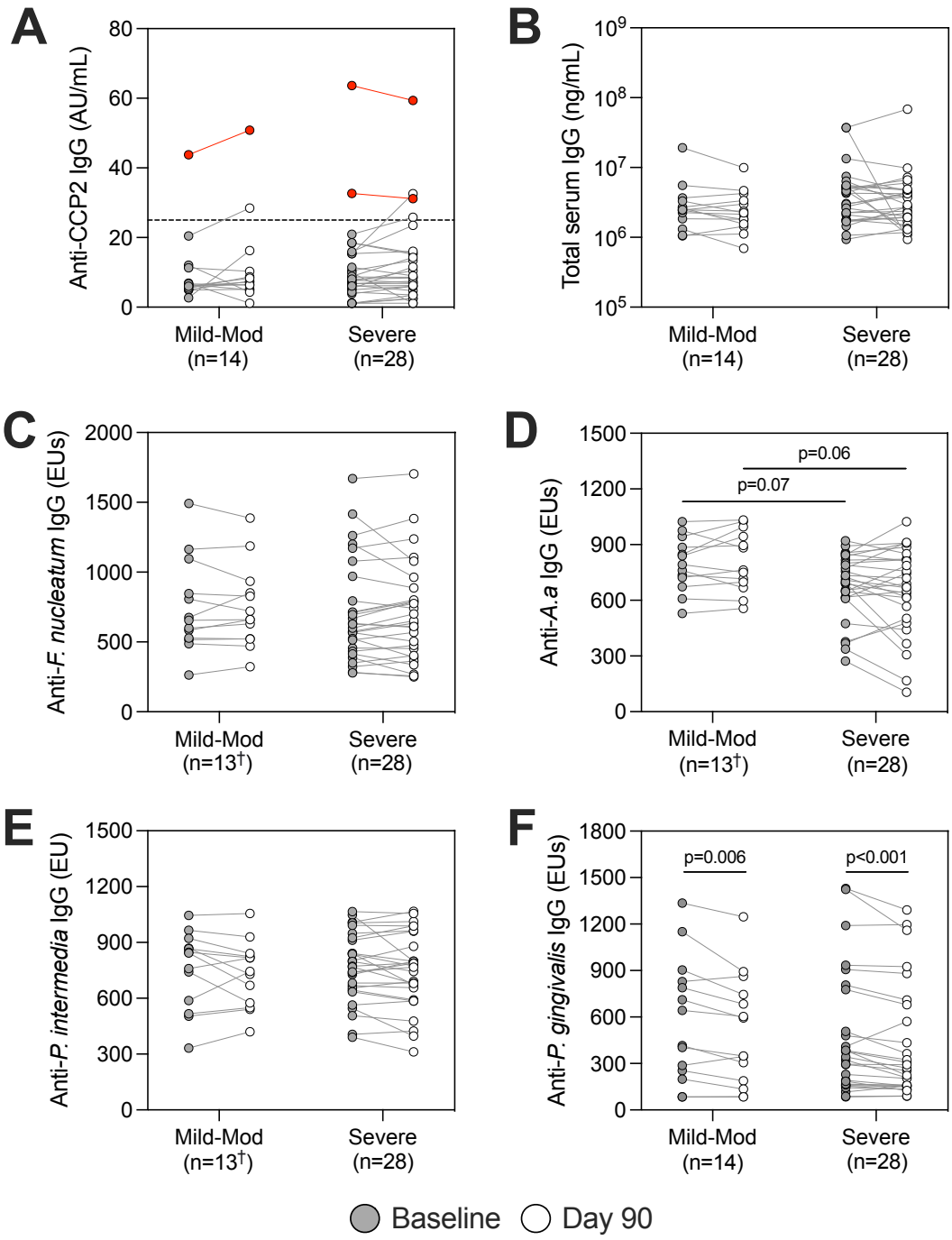

Figure 2.16. Systemic antibodies at baseline (BL, grey circles) and day 90 (D90, white circles) according to PISA groups. ACPAs (A), total serum IgG (B) and antibodies directed against *F. nucleatum* (C), *A. a* (D), *P. intermedia* (E), *P. gingivalis* (F) were compared between disease severity groups at each timepoint (Mann-Whitney test), and longitudinally within groups following NSPT (Wilcoxon signed-rank test). ACPA positivity threshold (25 AU/mL) is indicated by dotted line (A). Raw p-values are displayed. *A.a*: *Aggregatibacter actinomycetemcomitans*. †One patient had limited serum volume and was not included in analysis of anti-*F. nucleatum*, anti-*P. intermedia* or anti-*A.a* IgG, n=13. Individual datapoints displayed with lines connecting each patient at BL and D90.

2.3.4.3. Systemic inflammation according to baseline disease severity

Similar to antibacterial antibody titres, systemic inflammatory proteins were compared between mild-moderate and severe PISA groups. Having confirmed that patients who started with severe disease showed significantly larger reductions in periodontal inflammation following treatment, it was hypothesised that this may equate to a consistent reduction in systemic inflammation. Patients with severe disease had significantly higher levels of serum CRP at baseline (medians: 1.52mg/L vs. 2.22 mg/L, p=0.008, Mann-Whitney U test, **figure 2.17A**). Surprisingly, despite a greater reduction in PISA, no significant changes were observed following treatment in the mild-moderate or severe disease groups for CRP, IL-6 or TNFα (**figure 2.17A-C**). Thus, in the current cohort, a larger reduction in periodontal inflammation did not equate to a consistent reduction in systemic inflammatory markers.

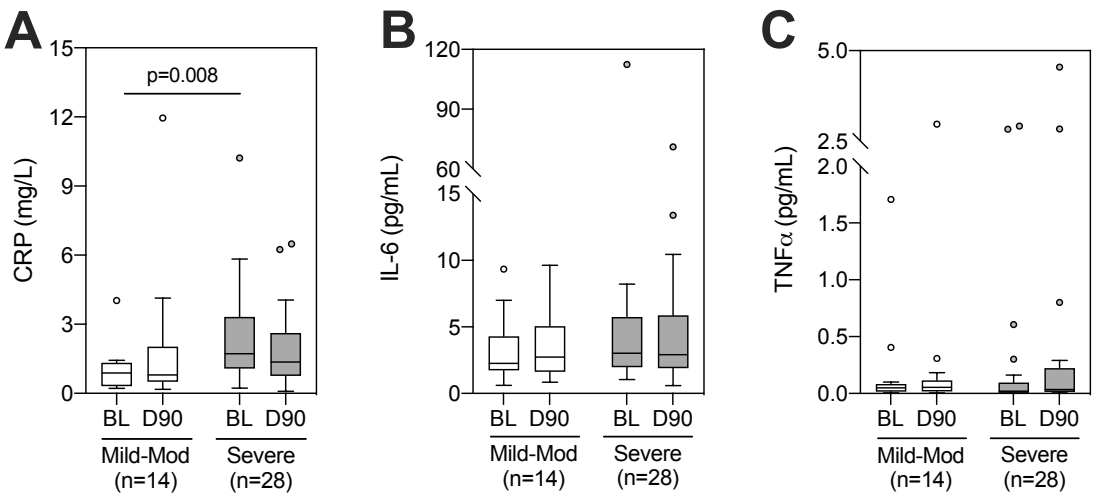

Figure 2.17. Systemic inflammatory markers at baseline (BL) and day 90 (D90) according to PISA groups. (A) CRP, (B) IL-6 and (C) TNFα were investigated. Statistical analysis was performed longitudinally within groups (Wilcoxon signed-rank test) or between groups at each timepoint (Mann-Whitney test). Raw p-values are shown when p<0.05. Graphs display Tukey boxplots with potential outliers highlighted by individual datapoints.

2.3.5. Impact of smoking on the clinical and systemic responses

2.3.5.1. PISA according to smoking status

Lastly, it was hypothesised that smoking status may influence the systemic alterations in antibodies and inflammatory proteins, as has been observed previously (Lappin et al., 2013). Within the current study, patients were grouped according to smoking status, including never (n=15), former (n=17) and current smokers (n=10). To establish whether smoking impacted the clinical response to NSPT, PISA values were compared cross-sectionally between smoking categories at each timepoint, and longitudinally within each group (**figure 2.18**). No differences in PISA were observed at baseline (Kruskall-Wallis p=0.48) or day 90 (Kruskall-Wallis p=0.78). Significant reductions were observed within all groups following treatment suggesting that, within the limitations of small sample sizes, smoking did not appear to influence the clinical response to NSPT in the current cohort.

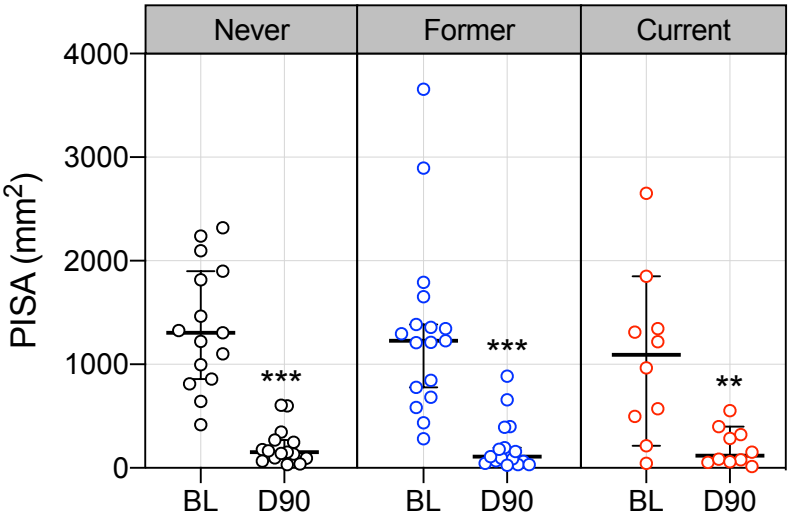

Figure 2.18. PISA values at baseline (BL) and day 90 (D90) according to smoking status. Patients were split into never (n=15), former (n=17) and current smokers (n=10). Cross-sectional analysis was performed using Kruskall-Wallis test at each timepoint with Dunn's post-hoc, longitudinal analysis was performed using Wilcoxon signed-rank tests (**p<0.01, ***p<0.001). Individual data points are shown with bars representing median ±95% CI.

2.3.5.2. Systemic antibodies according to smoking status

Systemic antibody titres were evaluated according to smoking status. In a previous study, smoking was shown to influence both anti-*P. gingivalis* IgG and ACPAs, with the latter showing reductions after treatment only in patients who had never smoked (Lappin et al., 2013). In this cohort, no significant differences were observed in ACPAs with respect to smoking status at baseline or day 90. However, in contrast to previous work showing a reduction in ACPAs in never smokers following treatment, this current study actually found an increase at day 90 ($p=0.003$, Wilcoxon singed rank test, $n=15$, **figure 2.19A**). Total serum IgG was significantly higher in former smokers compared with current smokers at baseline ($p=0.012$, Kruskall-Wallis test), and significantly higher in former smokers compared with never smokers at day 90 ($p=0.017$, Kruskall-Wallis test). However, no longitudinal alterations were observed for any group (**figure 2.19B**).

Current smokers also appeared to display lower anti-*P. intermedia* IgG when compared to former smokers at day 90 (Kruskall-Wallis $p=0.012$, **figure 2.19E**). Despite previous studies observing significantly lower anti-*P. gingivalis* IgG in current smokers compared with never smokers (Lappin et al., 2013), this finding was not replicated in the current cohort (**figure 2.19F**). Anti-*P. gingivalis* IgG significantly reduced following treatment in both never ($p=0.0006$) and current smokers ($p=0.003$), but not former smokers ($p=0.12$, Wilcoxon signed-rank test, **figure 2.19F**).

It should be reiterated that these data are based on an exploratory subgroup analysis, and the current study was not powered nor designed to detect a significant finding in these comparisons. Nonetheless, these findings do suggest that further work is required to identify any causal impact between smoking, ACPAs and antibacterial antibodies in PD patients. Furthermore, this study found no evidence that NSPT can reduce serum ACPA titres irrespective of periodontal disease severity or smoking status, although the majority of this cohort were diagnostically ‘ACPA-negative’ at baseline.

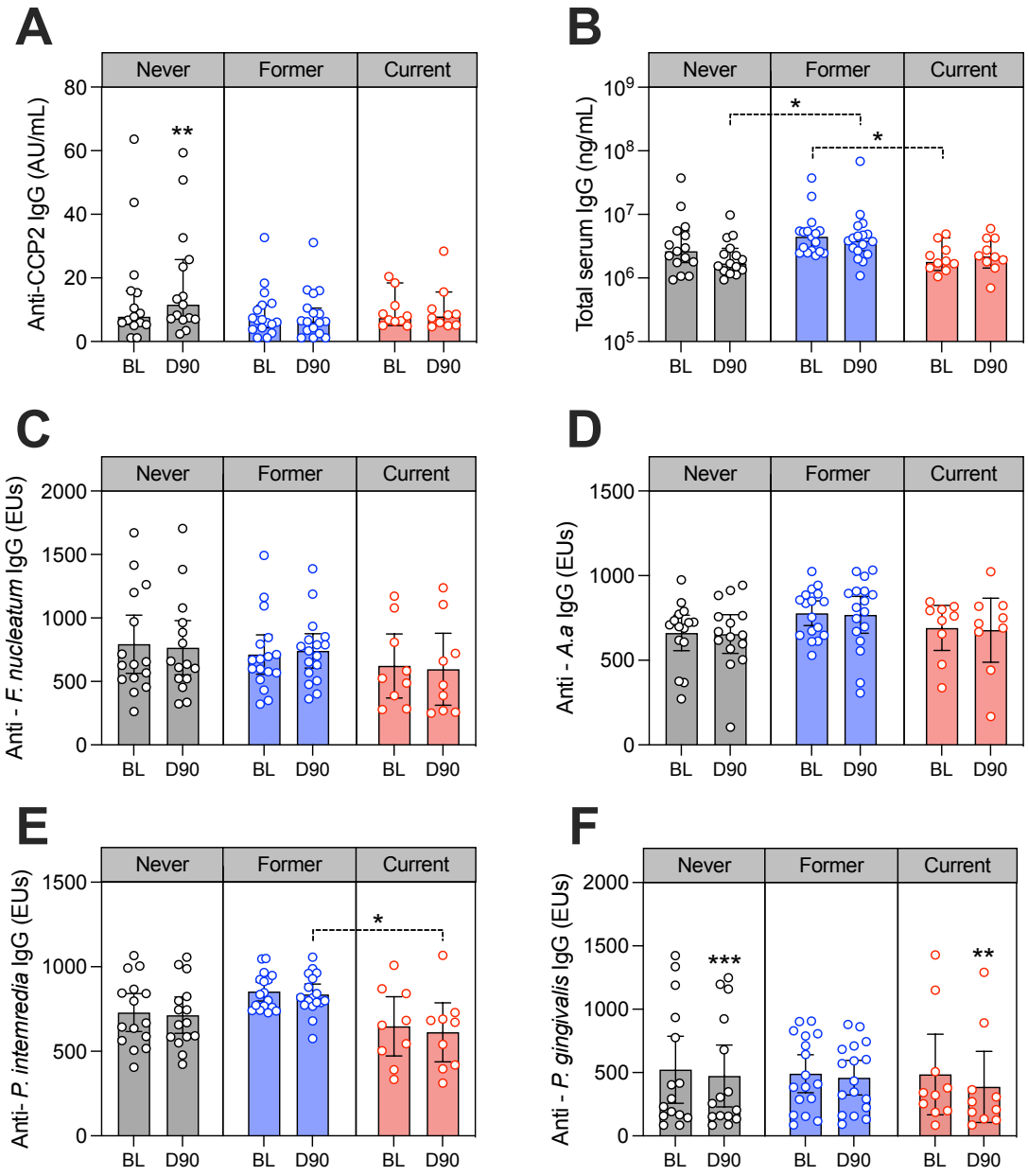

Figure 2.19. Serum IgG antibodies according to patients smoking status. Never smokers (n=15), former smokers (n=17) and current smokers (n=10†). Graphs represent ACPAs (A), total serum IgG (B), anti-*F. nulceatum* (C), *A. a* (D), *P. intermedia* (E) and *P. gingivalis* (F). Bars display medians (± 95% CI) with individual value shown. Statistics are Wilcoxon signed-rank test (within group comparison) or Kruskall-Wallis test with Dunn's post-hoc (between group comparison). *p<0.05, **p<0.01, ***p<0.001. † One patient (current smoker) had limited sample volume and was not included in analysis of anti-*F. nulceatum*, *P. intermedia* or *A. a* (n=9). A. a: *Aggregatibacter actinomycetemcomitans*.

2.3.5.3. Systemic inflammation according to smoking status

Systemic inflammatory proteins were compared among smoking categories. No significant differences were observed between groups at baseline or day 90 for CRP, IL-6 or TNFα (**table 2.4**). Assessing each marker longitudinally within groups, the only difference was found for TNFα in former smokers, in which a quantitively small (0.02 pg/mL) increase was observed at day 90. Serum CRP showed a reducing trend following treatment in never-smokers albeit this did not reach statistical significance (p=0.06).

Table 2.4. Systemic inflammatory proteins according to smoking status. Data displayed as median (Q1, Q3).

Analyte	Timepoint	Never (n=15)	Former (n=17)	Current (n=10)	Between group p-value†
CRP (mg/L)	Baseline	1.44 (0.78, 4.64)	1.34 (0.63, 2.60)	1.18 (0.73, 1.89)	0.59
	Day 90	1.13 (0.32, 2.47)	1.57 (0.59, 2.72)	1.00 (0.72, 3.14)	0.63
	Within-group p-value‡	0.06	0.23	0.99	-
IL-6 (pg/mL)	Baseline	2.42 (1.96, 4.33)	2.89 (1.91, 5.30)	2.61 (1.34, 6.26)	0.87
	Day 90	2.86 (1.71, 7.98)	2.66 (1.78, 5.22)	3.61 (2.06, 5.50)	0.87
	Within-group p-value‡	0.99	0.75	0.63	-
TNFα (pg/mL)	Baseline	0.02 (0.01, 0.11)	0.03 (0.01, 0.07)	0.04 (0.01, 0.07)	0.99
	Day 90	0.03 (0.01, 0.27)	0.05 (0.02, 0.18)	0.04 (0.01, 0.06)	0.46
	Within-group p-value‡	0.30	**0.009**	0.65	-

†Kruskall-Wallis test, ‡Wilcoxon signed-rank test. Exact p-values displayed.

2.4. Discussion

This chapter explored the association between systemic ACPAs, antibacterial antibodies directed against periodontal bacteria and inflammatory proteins in a cohort of PD patients. Patients with PD only (rather than PD and RA) were evaluated to avoid the confounding factors associated with RA and RA-related treatments such as biologic disease-modifying anti-rheumatic drugs (bDMARDs). Across the entire patient cohort, no strong association was observed between serum ACPA titres and other serum antibodies or inflammatory mediators, or periodontal clinical parameters. Notably, this included antibodies directed against *P. gingivalis* and *A. actinomycetemcomitans* despite mechanisms linking both of these oral bacteria to ACPA generation. Additionally, whilst NSPT induced widespread reductions in overall disease severity and anti-*P. gingivalis* IgG, there was no significant alteration in serum ACPAs or systemic inflammatory proteins across all patients.

A link between serum ACPAs and PD severity has been shown in studies involving RA patients (Gonzalez-Febles et al., 2020) or first-degree relatives of RA patients (Loutan et al., 2019). Both previous studies included a far higher proportion of ACPA-positive individuals equating to 66% (Gonzalez-Febles et al., 2020) and 34% (Loutan et al., 2019) of the respective cohorts. Within the current study, three (7%) ACPA-positive individuals were found and only a weak association between ACPAs and FMPS was recorded. Notably, no association was observed with the PISA, which was independently validated as an indicator of disease severity. The lack of a monotonic relationship between ACPAs and PISA may be partially explained by the majority of ACPA titres being constrained within a relatively small and diagnostically negative range (5-25 AU/mL). Importantly, the meaning of ACPA titres which fall below the positivity threshold (<25 AU/mL), relating to the majority of the samples in the current study, is currently unclear. The data from this chapter are, however, supported by a recent study involving a clinically diverse cohort of patients suffering from RA and PD, RA only, PD only and healthy controls (Lew et al., 2021). Despite a far larger spread in serum ACPA titres, no correlation was found with periodontal disease severity (PISA) in any of the clinical groups within this study.

In line with these findings, there was also no significant alterations in serum ACPA titres following NSPT when assessing the entire cohort. Despite widespread clinical improvement, ACPA titres remained relatively consistent with three ACPA-negative samples at baseline increasing above the assay positivity threshold throughout the study. Two previous studies have evaluated longitudinal shifts in serum ACPAs following NSPT, finding significant reductions only when stratifying cohorts into non-smokers at 6 month follow-up (Lappin et al., 2013) or patients suffering from generalised PD at 8 weeks follow-up (Yang et al., 2018). Neither of these findings were maintained within the current study at 3 months (90 days), with ACPA titres actually increasing in non-smokers upon stratification. Two major limitations of the current study and those preceding are the small sample sizes and low baseline ACPA levels. Within the current study 42 patients were included, which is slightly higher than Lappin et al. (n=39) and Yang et al. (n=31). Small sample sizes from each study, combined with the exploratory nature of these analyses undoubtably limit the extent to which these results can be extrapolated to a population scale. However, data presented in this chapter suggest that whilst a minority of PD patients appear to be ACPA positive, these trends are not fully consistent across different studies and independent cohorts. To this extent, future work in an ACPA-positive PD cohort would be useful to delineate whether periodontal therapy in isolation is sufficient to reduce serum ACPA titres.

Antibacterial antibodies were evaluated in this study to test whether an elevated systemic immune response against certain periodontal bacteria, such as *P. gingivalis* or *A. actinomycetemcomitans,* may be associated with elevated serum ACPA titres. This hypothesis was not confirmed as no antibacterial antibody titre was positively or negatively associated with ACPAs. There are several possible explanations for these findings. The first is the lack ACPA specificities (CEP, citFib, non-cirtullinated native protein) in this study. Inclusion of these titres in future work would help establish whether ACPA titres are specific for bacterial citrullinated epitopes or cross react with human citrullinated epitopes. The next limitation is the employment of whole-cell antibacterial ELISAs. Reactive antibodies in this assay do provide a useful indication that these bacteria were once present and an initiated an immune response (Haffajee and Socransky, 1994), and anti-*P. gingivalis* antibodies have been shown to increase prior to the onset of RA symptoms (Johansson et al.,

2016). However, for *P. gingivalis* and *A. actinomycetemcomitans*, simply identifying exposure to these bacteria may be insufficient given strain variation in virulence factor expression. Indeed, this is known for *A. actinomycetemcomitans* where LtxA expression has been shown to massively vary among different isolates (Claesson et al., 2020). Although the strain used in this study is an LtxA producing isolate (Y4, serotype b), assessing antibodies directed specifically against this toxin, or against PPAD in the case of *P. gingivalis*, may be of greater relevance in ensuing studies.

Following treatment, anti-*P. gingivalis* IgG titres were reduced, and the slight but consistent reduction is in line with several previous studies (Wang et al., 2006, Lappin et al., 2013, Morozumi et al., 2018). For example, Morozumi et al. reported a statistically significant 14.2% reduction in anti-*P. gingivalis* titres which is of comparable magnitude to the present study (11.5%), and no alterations in anti-*P. intermedia* IgG at 6 weeks follow up. This result was maintained within the current study, as there were no alterations in antibodies directed against other periodontal bacteria (*F. nucleatum, A. actinomycetemcomitans, P. intermedia*). Whilst the majority of studies agree with a reduction in anti-*P. gingivalis* IgG following treatment, the literature on other bacteria is less consistent. For example, some studies show a reduction in anti-*A. actinomycetemcomitans* IgG following NSPT (Rebeis et al., 2019) whilst others find no change (Horibe et al., 1995). One reason for these findings may be that antibodies are cross-reactive against other bacterial epitopes, possibly within the same genera or family. Only four patients within the current study had detectable *A. actinomycetemcomitans* in sampled periodontal pockets, yet all patients had detectable levels of anti-*A. actinomycetemcomitans* IgG. Moving forward, the incorporation and correlation between antibodies directed against different species, and even specific bacterial proteins, within the same genera would be useful to establish the accuracy and specificity of these titres.

With respect to systemic inflammatory proteins, some studies have recorded significant reductions in serum CRP, IL-6 and TNFα following NSPT (Dag et al., 2009, D'aiuto et al., 2004b). At baseline, a weak association was observed between serum CRP and PISA; however it should be noted that potential confounders such as body mass index were not available from the current study and may influence these results. Nonetheless, this relationship was not

confirmed longitudinally regardless of the reduction in periodontal inflammation. The inconsistency in results may relate to the baseline levels of systemic inflammatory proteins, which were higher in previous studies. In general, the baseline level was low within the current cohort (79% of CRP values <3 mg/L, 76% of IL-6 values <5 pg/mL, all TNFα values <5 pg/mL), and thus it could be considered unlikely that a consistent reduction would be observed. This is supported by a rigorous systematic review which concluded that reductions in systemic inflammation following periodontal treatment are most readily observed in patients with systemic co-morbidities (Teeuw et al., 2014).

Shifting the focus to subgingival plaque, this study did suggest that patients harbouring *P. gingivalis* display highly elevated anti-*P. gingivalis* and slightly elevated (below the positively threshold) ACPA titres. The former has been previously observed in several studies evaluating *P. gingivalis* carriage and antibacterial antibodies (Seror et al., 2015, Fisher et al., 2015), with one study showing that current carriage of this bacteria is a stronger predictor of antibody titres than PD diagnosis (Pussinen et al., 2011). The slight elevation in ACPA titres is supported by previous studies reporting similar comparisons (Lappin et al., 2013), whilst others demonstrate a higher abundance of *P. gingivalis* in ACPA positive individuals (Cheng et al., 2021, Mankia et al., 2019). Furthermore, a large study of 600 participants found that the level of salivary *P. gingivalis* DNA positively associates with serum ACPAs in patients with baseline CRP levels >3 mg/L (Oluwagbemigun et al., 2019). Nonetheless, it is important to critically evaluate the magnitude of difference in the current study (+8.8 AU/mL). This small increase resulted in ACPA titres which remained below the positivity threshold in all but one patient. Likewise, two out of three ACPA-positive patients were *P. gingivalis* negative, and these observations must be carefully considered when interpreting this result.

Within this study, colonisation was based on one single periodontal pocket, and it is possible that negative patients may be colonised at alternative sites. In support of this theory, antibodies directed against *P. gingivalis* reduced following treatment regardless of colonisation status, suggesting likely alternative site colonisation or cross-reaction with other bacterial epitopes. To rule this out, future studies would benefit from screening several sites such as several periodontal pockets, tongue scrapings and saliva for *P. gingivalis*

carriage. Additionally, it would be useful to evaluate whether these results are maintained in periodontally healthy subjects to establish whether *P. gingivalis* carriage in isolation can elevate serum ACPAs irrespective of periodontal status.

In summary, this study cohesively analysed associations between serum ACPAs, antibacterial antibodies, inflammatory proteins and periodontal clinical parameters – longitudinally following treatment - in a PD cohort. This study did not find any association between ACPAs and antibacterial antibody titres or systemic inflammatory proteins. Similarly, despite a 12-fold reduction in PISA, only 47% of patients achieved a level of periodontal inflammation commensurate with periodontal health (Leira et al., 2018). Low levels of residual periodontal inflammation may contribute to the lack of longitudinal alterations in serum ACPAs or systemic inflammatory proteins following treatment. There was evidence that *P. gingivalis* carriage may be associated with a slight elevation in serum ACPAs, but these titres generally remained low and *P. gingivalis* colonisation was not a prerequisite for ACPA positivity. Although a reduction in anti-*P. gingivalis* IgG was recorded following treatment, similar findings were not observed for other antibacterial antibody titres. It would be of benefit to repeat this study in a cohort of non-RA ACPA positive PD patients to further explore ACPA positivity in PD.

2.5. Chapter summary

This chapter provides a cohesive analysis of serum ACPAs, antibacterial antibodies and systemic inflammatory proteins in a clinically diverse PD cohort, before and after periodontal treatment. The main findings from this chapter are highlighted below;

- The PISA is a useful indicator of periodontal disease severity.
- There was insufficient evidence from the current study to suggest an association between serum ACPA titres and antibodies directed against periodontal bacteria (*P. gingivalis* and *A. actinomycetemcomitans*), or RA related inflammatory proteins in a predominantly ACPA-negative cohort.
- No longitudinal shifts in ACPA titres or systemic inflammatory proteins were observed following NSPT across the current cohort.
- ACPA titres increased following NSPT only in never-smokers, which contrasts with previous literature.
- Anti-*P. gingivalis* IgG reduced following treatment commensurate with previous studies, however, the current exploratory study did not observe an association with periodontal disease severity.
- A weak association between PISA and serum CRP was observed, which may warrant further investigation in a larger cohort.

Chapter 3

Longitudinal changes in local host and microbial parameters following non-surgical periodontal therapy

Work from this chapter has been presented at the following meetings;

'Microbial impacts of periodontal treatment' poster presented at the Eurobiofilms conference (European Society of Clinical Microbiology and Infectious Diseases), September 2019, Glasgow, Scotland, UK.

'Longitudinal host and microbial alterations following non-surgical periodontal therapy' presented at the British Society for Oral and Dental Research (BSODR) annual meeting, September 2020, Virtual presentation. Awarded first place for the Septodont poster prize.

Work from this chapter has been published in the following journal;

Johnston, W.; Rosier, B.T.; Artacho, A.; Paterson, M.; Piela, K.; Delaney, C.; Brown, J.L.; Ramage, G.; Mira, A.; Culshaw, S. Mechanical biofilm disruption causes microbial and immunological shifts in periodontitis patients. *Scientific Reports* 2021. 11, 9796. https://doi.org/10.1038/s41598-021-89002-z.

Manuscript included as **appendix ii**

3.1. Introduction

In PD there is a continuous cycle of host-bacterial interplay whereby inflammation facilitates greater dysbiosis of the microbiome, and vice-versa (Rosier et al., 2018, Van Dyke et al., 2020). As such, local host and microbial factors have been extensively studied in periodontology to uncover their relationship with disease, particularly within cross-sectional study designs. To date, far less attention has been paid to analysing whether these variables are altered in response to improvements in clinical status.

As mentioned throughout **chapter 1**, the term 'dysbiotic' is frequently used to describe subgingival plaque biofilms in PD. These communities have been found to be more diverse and display an increased abundance of facultative anaerobic species compared with subgingival plaque from periodontally healthy subjects (Griffen et al., 2012, Abusleme et al., 2013). Chronic inflammation in response to these biofilms is a consistent feature of PD, and several microbial and inflammatory mediators have been termed 'biomarkers' of disease. The evidence for these markers is based largely on cross-sectional studies comparing PD with periodontal health. However, whether these markers reflect disease severity and respond to improvements in clinical status is largely unknown, both of which represent key features of a robust disease biomarker.

Saliva and GCF are frequently used to evaluate inflammation in the oral cavity and results generally support an increase of pro-inflammatory cytokines in PD (Ebersole et al., 2015, Miller et al., 2006, Stadler et al., 2016). However, when evaluating trends within individual analytes the results become less consistent. For example, Ebersole et al. found that salivary interleukin-1β and interleukin-6 were significantly elevated in 101 PD patients compared with 65 healthy controls (Ebersole et al., 2015). In contrast, Teles et al. found no significant differences when the same cytokines in the saliva of 74 PD patients and 44 healthy controls (Teles et al., 2009). Therefore, characterising longitudinal alterations in these cytokines following improvement in clinical status may provide an alternative insight into their association with PD.

Recently there has also been interest in whether baseline host or microbial variables may be used to identify patients at risk of a poorer treatment

response. Although NSPT induces a reduction in overall disease severity (Suvan et al., 2019), it is often only partially successful when assessed against firm clinical success endpoints. A recent study reported a success rate of roughly one third when using pocket closure as an endpoint in a retrospective analysis of 1182 patients (Van Der Weijden et al., 2019). Identifying poorer responding patients or sites at an early stage in the treatment plan could permit more efficient use of additional treatment and/or the addition of adjuncts (Shaddox and Walker, 2010). Both host and microbial variables offer promising avenues in this regard, representing aspects of the microbiota challenge and corresponding host inflammatory response. Indeed, there is evidence that each may have *bona fide* prognostic utility (Lee et al., 2018, Bizzarro et al., 2016); however, there is a need to delineate the site-specific versus whole mouth response and evaluate these variables against clinically translatable endpoint criteria.

This study sought to evaluate how the subgingival plaque microbiota and local inflammatory cytokines associate with periodontal disease severity, and whether these variables are altered following NSPT. Additionally, the association of these variables with a poor treatment response was assessed at baseline. Specifically, this chapter sought to answer the following research questions;

- Are concentrations of inflammatory cytokines in saliva and GCF related to periodontal disease severity?
- Do the concentrations of inflammatory cytokines in saliva and GCF change following NSPT?
- Is the composition of the subgingival plaque microbiota related to periodontal disease severity?
- Is there a compositional shift in the subgingival plaque microbiota following NSPT?
- Is there an association between baseline host and microbial variables and treatment response of patients or sites following NSPT?

3.2. Materials and methods

3.2.1. Patient selection

This chapter presents local inflammatory (saliva, GCF) and microbial (subgingival plaque) alterations in the same cohort described in **chapter 2**. Baseline demographics, whole mouth clinical parameters and study design are described within **chapter 2**.

3.2.2. Sample collection and processing

3.2.2.1. Clarified saliva

Whole-saliva was collected using the passive drool method into 50 mL Falcon tubes (Grenier Bio-One, Gloucestershire, UK). Using a sterile Pasteur pipette, 1 mL aliquots were made into Eppendorf tubes. Samples were then clarified via centrifugation at 13,000 RPM for 5 minutes (Eppendorf model 5415C, Hamburg, Germany). The supernatant was aliquoted into sterile Eppendorf tubes and immediately stored at -80°C until analysis.

3.2.2.2. Gingival crevicular fluid

GCF was collected from a single site (preferentially ≥5 mm) in each quadrant at baseline and day 90. Sites were dried and isolated using cotton rolls to avoid saliva contamination. PerioPaper® strips were placed into each pocket for 30 seconds (timed). Following collection, strips were placed into a sterile Eppendorf and stored immediately at -80°C until analysis.

3.2.2.3. Subgingival plaque

Subgingival plaque samples were collected from each quadrant at baseline and day 90. For sampling, supragingival plaque was removed and subgingival plaque was carefully harvested using a Gracey curette. Samples were placed into four Eppendorfs containing 250 µL of sterile pre-filtered (0.1 µM) phosphate buffered saline (PBS; pH 7.4, Gibco, Thermofisher, Loughborough, UK). Tubes were centrifuged at 13,500 RPM for 10 minutes (Eppendorf model 5415C, Hamburg, Germany) to isolate the bacterial pellet and the supernatant was discarded. Samples were stored immediately at -80°C until analysis.

3.2.3. Analysis of saliva

One patient had limited saliva volume and was not included in any salivary analysis (n=41 for all cytokines). Levels of salivary TNFα, IL-6, and IL-1ß were determined using commercially available sandwich ELISA assays (Thermofisher, Loughborough, UK). For IL-6, samples were initially evaluated using a normal sensitivity assay; however, roughly 50% were undetectable. Therefore, these samples were repeated on high-sensitivity kits. Salivary IL-8 was quantified using commercially available DuoSet sandwich ELISAs (Biotechne – R&D systems, Abingdon, UK). Salivary IL-17A was quantified using high-sensitivity ProQuantum immunoassays, as described previously in **section 2.2.5.5**. A summary of the kits used to quantify salivary cytokines is shown in **table 1.1**.

3.2.3.1. ELISA buffers and reagents

For all ELISA assays, a stock 10X PBS solution was prepared each week by dissolving 80 g sodium chloride (NaCl), 14.4 g sodium phosphate dibasic dihydrate (Na_2HPO_4 $_2H_2O$), 2.4 g potassium dihydrogen phosphate (KH_2PO_4) and 2 g potassium chloride (KCl) into 1 litre of sterile distilled water. The solution was mixed thoroughly until all chemicals had dissolved and stored at room temperature. Wash buffer was prepared by adding 200 mL 10xPBS to 1800 mL sterile distilled water to create a 1xPBS solution. Once mixed, 1 mL of Tween20 (Sigma Aldrich, Gillingham, UK) was added to create 0.05% PBST.

For all sandwich ELISA assays, 1xPBS solution was used as a coating buffer, and capture antibodies supplied with the kit were diluted 1:250. For kits supplied by Thermofisher (TNFα, IL-6, IL-1ß) 1xELISPOT diluent in distilled water was used as a blocking buffer. This solution was supplied by the manufacturer and contains FBS for blocking wells. For kits supplied by Bioteche (IL-8), 1% BSA in PBS was used as a blocking buffer.

Reagent diluents differed according to manufacturer. This was either 1xELISPOT diluent in distilled water (Thermofisher) or 0.1% BSA in PBS (Biotechne). Biotin-conjugated detection antibodies supplied with each kit were diluted 1:250 using the appropriate reagent dilution. To bind biotin, avidin-HRP (Thermofisher) or strepavidin-HRP (Biotechne) were used. For all assays, TMB was used as a substrate solution provided with the kit. For IL-8, TMB was mixed 1:1 with

'colour reagent A' (containing H_2O_2) immediately prior to use. Stop solutions consisted of 1M phosphoric acid (H_3PO_4, Thermofisher) and 2N sulphuric acid (H_2SO_4, Biotechne). ELISA plates were washed by filling each well entirely with wash buffer, which was allowed to soak for 1 minute before being decanted. This process was repeated 3-5 times at every wash step. After the final wash, plates were gently blotted onto paper towels to remove excess liquid.

3.2.3.2. Sandwich ELISA methods

Patient samples, standards and negative controls were assayed in duplicate according to the manufacturer's instructions. In brief, 100 µL of capture antibody was added to 96-well high-binding flat-bottom microtiter plates and incubated overnight at 4°C (Corning™ Costar, Thermofisher, Loughborough, UK). The following day, wells were washed and 200 µL of blocking buffer was added. Plates were incubated for 1 hour at room temperature before further washing. Samples were added at appropriate dilutions alongside standard curves and negative controls, and plates were incubated for 2 hours at room temperature. Following incubation, plates were washed and 100 µL of biotin-conjugated detection antibodies were added for 1 hour. Plates were washed and 100 µL avidin-HRP (TNFα, IL-6, IL-1ß) or streptavidin-HRP (IL-8) was added to each well for 30 minutes. Plates were washed and 100 µL TMB substrate was added to each well. Following colour development, the reaction was stopped using 100 µL of phosphoric acid (TNFα, IL-6, IL-1ß) or 50 µL of sulphuric acid (IL-8). Plates were immediately read at 450_{nm} with correction at 620_{nm}. Signal from negative controls was subtracted from sample and standard wells, and the concentration of samples was determined by interpolation from a standard 5-parameter fit curve of standards using PRISM (version 8.0).

3.2.3.3. Limit of detection

The LOD was assigned as the concentration given by the mean absorbance of negative controls plus the standard deviation multiplied by 2. The in-house calculated LOD for cytokines was IL-6: 0.04 pg/mL, IL-1ß: 1.35 pg/mL, IL-8: 6.78 pg/mL, TNFα: 0.14 pg/mL and IL17-A: 2.75 pg/mL. Detectable levels of salivary IL-6, IL-1ß and IL-8 were observed in all samples. TNFα was <LOD in 3 samples (1x baseline, 2x day 90) and IL-17A was <LOD in 12 samples (10x baseline, 2x day 90). For statistical analysis, samples <LOD were assigned as LOD/2.

3.2.4. Gingival crevicular fluid (GCF)

3.2.4.1. Elution of GCF strips

Collected GCF strips were pooled together in a single sterile Eppendorf and eluted using a method adapted from previously published work (Wassall and Preshaw, 2016, Fitzsimmons et al., 2010). This consisted of eluting strips in 150 µL 0.1% BSA in PBS. Tubes were placed on a rocking shaker (100 RPM) at 4˚C for 20 minutes (3D rocking platform STR9, Stuart Scientific, Staffordshire, UK). After rocking, tubes were centrifuged at 10,000 RPM for 2 minutes (Eppendorf model 5415C, Hamburg, Germany). The resulting liquid was aliquoted into new sterile Eppendorf's and stored at -80˚C until use.

3.2.4.2. GCF cytokine analysis

GCF IL-17A was measured using high-sensitivity ProQuantum immunoassays described previously in **section 2.2.5.5.** These assays were performed on a new StepOnePlus real-time PCR system (Applied Biosystems). The assay LOD was redetermined and found to be 0.48 pg/mL. Standard curve values were similar regardless of analyser, but blank values were notably lower (Ct mean of blanks; 29.52 vs. 27.10). GCF IL-6, IL-1ß and IL-8 were determined using the same commercially available ELISA assays described in **section 3.2.2**. TNFα was undetectable in all samples (n=84) and was thus excluded from analysis. IL-6 was <LOD in 2 samples (1x baseline, 1x day 90), IL-8 was <LOD in 1 sample (day 90), IL-17A was <LOD in 14 samples (13x baseline, 1x day 90) whilst IL-1ß was >LOD in all samples. Samples <LOD were assigned LOD/2 for statistical analysis.

According to the clinical protocol, blood contaminated GCF strips were discarded during sampling. During sample processing GCF strips were visually inspected for blood contamination. Contaminated strips were discarded prior to pooling to avoid interference with cytokine measurements. Therefore, a small subset of patients had three strips available for analysis at a specific timepoint. The elution volume (150 µL) was kept consistent regardless of the number of eluted strips. To account for this difference and potential dilution of these samples, GCF analysis was performed two-fold and both are semi-quantitative measurements. Firstly, analysis was performed only across patients with four eluted strips at each timepoint (n=33). This analysis is termed 'total' and given

as the total measured cytokine concentration per 30 seconds (pg/30s). Secondly, analysis was performed across the entire cohort using values standardised to the number of eluted strips (n=42). This analysis is termed 'standardised' and values are given as arbitrary units (AU/30s), determined using the equation below;

$$Standardised\ cytokine\ concentration\ (AU/30s) = \frac{Total\ measured\ cytokine}{Number\ of\ eluted\ strips}$$

A summary of all assays used to measure salivary and GCF cytokines is provided below (**table 3.1.**), alongside the corresponding dilution factor and in-house calculated LOD.

Table 3.1. Summary of assays used for saliva and GCF analysis.

Analyte	Supplier (catalogue #)	Assay type	Standard curve (pg/mL)	Dilution	LOD (pg/mL)
TNFα	Thermofisher (BMS223HS)	Sandwich ELISA	0.31 - 20	1in2	0.14
IL-6	Thermofisher (88-7066-22)	Sandwich ELISA	3.125 - 200	Neat	5.93
IL-6†	Thermofisher (BMS213HS)	Sandwich ELISA	0.078 - 5	1in2	0.04
IL-1β	Thermofisher (88-7261-88)	Sandwich ELISA	1.17 - 150	1in2	1.35
IL-17A	Thermofisher (A35611)	ProQuantum immunoassay	0.32 - 25,000	1in5	2.75
IL-8	R&D systems (DY208-05)	Sandwich ELISA	15.625 - 2000	1in3	6.78

†Samples that were undetectable on normal sensitivity IL-6 assay were repeated using a high-sensitivity kit.

3.2.5. Analysis of subgingival plaque

3.2.5.1. Sample selection

Subgingival plaque from a single quadrant was selected from each patient for sequencing. Samples were preferentially selected from the upper right quadrant (n=35), unless unable to sample the same site at day 90. In which case a sample from the remaining three quadrants was selected; upper left (n=1), lower left (n=3), lower right (n=3).

3.2.5.2. DNA extraction

DNA extraction and sequencing were performed at the Foundation for the Promotion of Health and Biomedical Research (FISABIO) in Valencia, Spain. The full methodology for these processes has been described previously (Dzidic et al., 2018, Carda-Diéguez et al., 2019, Rosier et al., 2020). In brief, subgingival plaque pellets were initially resuspended in 100 µL PBS and sonicated for 30 seconds in a sonicator bath at low ultrasound intensity (model VCI-50, Raypa, Barcelona, Spain). Following sonication, DNA extraction was performed automatically by the MagNA Pure LC 2.0 instrument using the MagNA Pure LC DNA isolation kit III for bacteria and fungi (Roche Diagnostics, Mannheim, Germany) with an additional in-house lysis step; subgingival plaque samples were resuspended in 100 µL PBS, 130 µL lysis buffer and 2.5 µL enzyme mix. The enzyme mix consisted of 20 mg/mL lysozyme (Thermomixer comfort, Eppendorf, Hamburg, Germany), 5 mg/L lysostaphin (Sigma-Aldrich, Madrid, Spain) and 0.625 mg/mL mutanolysin (Sigma-Aldrich, Madrid, Spain). Following extraction, DNA was resuspended in 100 µL of elution buffer and frozen at -20°C until further analysis.

3.2.5.3. DNA sequencing

An Illumina amplicon library was prepared following the 16S rRNA gene Metagenomic Sequencing library preparation protocol (Part #15044223 Rev. A). Gene-specific primers used in this protocol target the V3-V4 regions of the 16S rRNA gene (**table 3.2**), resulting in a single amplicon of 460 basepairs. Dual indices and sequencing adapters were added using the Nextera XT kit (Illumina, San Diego, California, USA). Amplicons were confirmed using 1.4% agarose gel alongside no template controls, purified using AMPure XP beads and quantified using the Quant-iT PicoGreen dsDNA assay kit with a Qubit™ 3 Fluorometer (Thermofisher, Waltham, Massachusetts, USA). Amplicons were pooled in equimolar ratios and sequenced on an Illumina MiSeq using the 2x300 base paired-ends protocol according to manufacturer's instructions. Reads from this study are deposited in the publicly available NCBI Sequencing Read Archive (SRA) under BioProject PRJNA725103.

Table 3.2. Primer sequences used for subgingival plaque sequencing.

Name	Primer sequence	Reference
Illumina_16S_341F	TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG	(Illumina, 2013)
Illumina_16S_805R	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC	

3.2.5.4. Taxonomic classification

Following sequencing, the DADA2 pipeline (v1.8) was used to filter, end-trim, denoise and merge paired reads before generating an ASV table (Callahan et al., 2016). The was applied using R scripts developed at the FISABIO institute and was performed by experienced bioinformaticians with processes described previously (Carda-Diéguez et al., 2019, Ferrer et al., 2020, Rosier et al., 2021). After sequencing, forward and reverse reads were trimmed and truncated to 251 and 206 bases respectively, to remove primers from the beginning of each read and low-quality bases from the end. Reads with any ambiguous 'N' bases or >5 expected errors were discarded, and error rates for each base transition were estimated. Dereplication was then performed to combine all identical reads into unique sequences. Using dereplicated reads and error estimations, sequence variants were inferred. Next, forward and reverse pairs were merged together with a minimum overlap of 12 bases and a maximum mismatch of 1 base in the overlapping region to obtain single denoised variants. From here, chimeras and singletons were detected and eliminated. ASVs were then mapped onto the Homo sapien genome (GRCh38.p13) using Bowtie2 (v2.3.5.1) to remove host reads (Langmead and Salzberg, 2012). The SILVA database (v138) was then used to assign taxonomy to remaining ASVs (Quast et al., 2013). Classification up to genus-level was achieved using the DADA2 naïve Bayesian classifier (Wang et al., 2007) and species initially assigned by exact matching (100%). ASVs with genus-level classification but without exact species matching were aligned using the BLASTn tool (v2.10.0) against the SILVA database with a minimum of 97% matching (Altschul et al., 1990). This assignment was only used if the difference between the first and the second-best matches was ≥2%.

3.2.6. Data analysis

3.2.6.1. Analysis of salivary and GCF cytokines

Analysis of salivary and GCF cytokines was performed using GraphPad PRISM (v8). All salivary and GCF cytokines displayed a non-parametric data distribution upon visual inspection of histograms. Therefore, correlations with periodontal clinical parameters were performed using Spearman-Rho correlation coefficients. Delta values in salivary cytokines and PISA were normally distributed, and thus correlation between these variables were conducted using Pearson's correlation coefficients. Longitudinal alterations were assessed using Wilcoxon signed-rank tests, and cross-sectional differences assessed using either Mann-Whitney U tests (PISA groups) or Kruskall-Wallis test with Dunn's post-hoc for multiple comparisons (smoking categories). The total level of GCF cytokines were compared for patients with 4 eluted strips, or standardised GCF cytokine levels were compared across the entire cohort, both of which are semi-quantitative.

3.2.6.2. Analysis of the subgingival plaque microbiome

For analysis of the subgingival plaque microbiome, R programming language (v3.4+) was used for statistical computing (R Core Team, 2014) using scripts developed at the FISABIO institute. The lowest number of classified reads at species-level was 9284. Therefore, α-diversity indices (observed species, shannon, ACE) were calculated rarefying to 9000 reads per sample using the Vegan library in R (Oksanen et al., 2019). This process was repeated 5 times and the average values were analysed. All indices were normally distributed and longitudinal differences were assessed using paired t-tests in GraphPad PRISM (v.8). Differences between demographic and behavioral subgroups were assessed using Mann-Whitney or Kruskall-Wallis tests where appropriate.The ß-diversity was analysed using Bray-Curtis based principal co-ordinate analysis (PCoA) generated within the MicrobiomeAnalyst software using the phyloseq R package (Mcmurdie and Holmes, 2013, Dhariwal et al., 2017, Chong et al., 2020). The Analysis of Similarities (ANOSIM) tool in MicrobiomeAnalyst was used to evaluate differences in ß-diversity, which tests whether differences within-groups are greater than between-groups (Chong et al., 2020).

Although the DADA2 pipeline can produce species-level taxonomic assignment, the classification accuracy is reduced given the short length Illumina reads (Claesson et al., 2010). Therefore, microbiota analysis was performed at both genus (more accurate) and species (more informative) level. For longitudinal analysis, only genera and species with a minimal signal of detection were included. Specifically, a genus or species was included if it was present in 50% of the samples at either timepoint with an abundance superior to five times the smallest percentage above zero. For univariate longitudinal analysis, paired tests (i.e., wilcox.test function of stats library of R) were performed to test differences in the relative abundance of genera and species between baseline and day 90, corrected for multiple comparisons using a false discovery rate (FDR) of 5% (Benjamini and Hochberg, 1995). To compare the abundance of known health and disease-associated organisms, species were grouped according to previously defined complexes (Socransky et al., 1998, Pérez-Chaparro et al., 2014), and analysed using Wilcoxon signed-rank tests in GraphPad PRISM (v.8).

For association networks, pairwise associations were computed between genera based on a multivariant approach described previously (González et al., 2012), which was implemented in the 'mixOmics' R package (Rohart et al., 2017). Using this method, associations between genera and species are obtained based on their projection onto a correlation circle plot derived from a principal components analysis. In the association networks, only negative associations below -0.4 and positive associations above 0.4 were considered. The network graphs were obtained with the R ggraph package (Pedersen, 2020).

3.2.6.3. Analysis of treatment response

Treatment response analysis was conducted two-fold to account for the site-specific nature of subgingival plaque samples. For microbiota analysis, treatment response was defined as sites which achieved ‘pocket closure’ following treatment (conversion of a periodontal site measuring ≥5 mm at baseline to a site measuring ≤4 mm at day 90). This analysis was conducted only within sites ≥5 mm at baseline, and therefore two samples were excluded from the analysis.

For salivary analysis, full-mouth clinical parameters were employed to classify treatment response. Only patients with PISA values commensurate with at least

moderate PD based on previous thresholds were included (Leira et al., 2018), meaning six patients were excluded from this analysis. Response to treatment was then defined as >median percentage PISA improvement following treatment, leaving equal sized groups of high (n=18) and low responders (n=18). Median improvement has been previously used to determine clinical success groups following NSPT (Bizzarro et al., 2016, Grande et al., 2020).

For both analyses, differences in clinical parameters were assessed using general linear models (GLMs). This was performed in the SPSS software (v26), and baseline levels of disease severity were included as covariates. Microbiome analysis (α and ß-diversity, differential abundance) was conducted as described above (Chong et al., 2020). Bray-Curtis dissimilarity for each sample pair were calculated in the Paleontological statistics (PAST) software (v4.01) (Hammer et al., 2001) and compared using a Mann-Whitney U test between groups. For salivary analysis, receiver operating characteristic (ROC) curves and classifier evaluation metrics were generated using SPSS software (v26).

3.3. Results

3.3.1. Salivary cytokines

Throughout this section, five salivary cytokines were investigated to establish their relationship with disease severity and alterations following NSPT (TNFα, IL-6, IL-8, IL-17A and IL-1ß). This non-exhaustive panel of analytes was selected based on the cytokines already assessed in serum from this cohort (TNFα, IL-6). In addition, IL-1, IL-8 and IL-17A were included as these have been commonly assessed within previous cross-sectional studies (Jaedicke et al., 2016). This panel allowed comparison with previous work and systemic findings in the current study. The Luminex™ panel in **chapter 4** was also considered in selection.

3.3.1.1. Association between salivary cytokines and disease severity

To evaluate whether cytokines were associated with disease severity at baseline, a correlation analysis was performed with clinical parameters using Spearman-Rho correlation coefficient's (**figure 3.1A**). Salivary IL-1ß showed moderate positive associations with clinical parameters, including PISA ($r=0.534$, $p<0.001$). A weak positive association was observed between salivary IL-8 and full-mouth pocket depth, proportion of deep periodontal pockets and PISA ($r=0.423$, 0.424, 0.372 respectfully), whilst a weak negative association was observed between IL-17A and plaque index ($r=-0.375$, $p=0.049$). In contrast, there was insufficient evidence for an association between salivary TNFα or IL-6 with any clinical parameter within the current study.

3.3.1.2. Alterations in salivary cytokines following treatment

Assessing longitudinal alterations following treatment, there were no shifts in the level of salivary TNFα, IL-6 or IL-8 within the current study (all $p>0.05$, Wilcoxon signed-rank test, **figure 3.1**). Interestingly, salivary IL-17A appeared to increase following treatment, albeit a quantitatively small change was recorded (median: 7.02 pg/mL at baseline to 12.25 pg/mL at day 90, $p<0.001$, **figure 3.1E**). In contrast, salivary IL-1ß showed highly consistent and significant reductions following treatment ($p<0.001$, **figure 3.1F**).

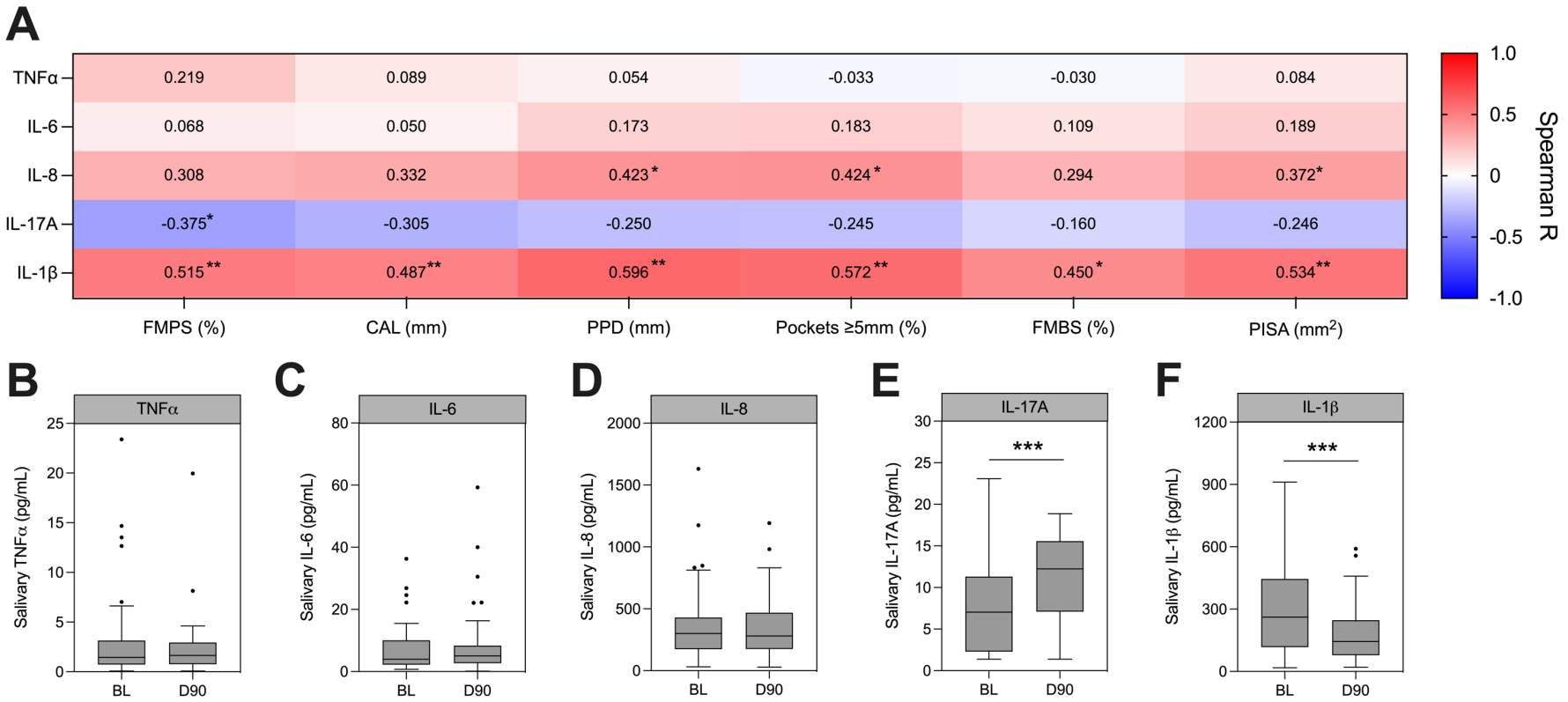

Figure 3.1. Investigating salivary cytokines. (A) Correlating the levels of salivary cytokines at baseline with clinical periodontal parameters, values represent Spearman-Rho correlation. P-values were corrected using FDR (5%) where *p(adjusted)<0.05 and **p(adjusted)<0.01. (B) Comparing changes in the levels of TNFα, IL-6, IL-8, IL-17A and IL-1ß at baseline (BL) and day-90 (D90). Graphs are Tukey boxplots where horizontal line indicates the median and individual points indicate potential outliers. Statistics are Wilcoxon signed-rank test, where ***p<0.001. n=41.

3.3.1.3. The impact of local inflammation on salivary cytokines

To further explore the relationship between periodontal disease severity and salivary cytokines, a subgroup analysis was performed. Patients were divided into mild-moderate and severe disease groups based on their baseline PISA values using the thresholds outlined previously in **chapter 2** (Leira et al., 2018). This analysis suggested no differences in salivary TNFα, IL-6 or IL-8 between mild-moderate and severe groups at either timepoint, or within groups following treatment (**figure 3.2A-C**). Patients with severe disease displayed a trend towards lower salivary IL-17A at baseline ($p=0.06$), and levels significantly increased following treatment only within this group ($p<0.001$, **figure 3.2D**). Patients with severe disease also displayed higher salivary IL-1ß at baseline ($p<0.01$), which significantly reduced following treatment ($p<0.001$, **figure 3.2E**). For both IL-17A and IL-1ß, no longitudinal alterations were observed in the mild-moderate group, suggesting that alterations in these cytokines were heavily influenced by the degree of baseline periodontal inflammation and subsequent reductions following treatment.

The change (Δ) in each salivary cytokine was also correlated against the change in PISA (**figure 3.2F-J**). The change in salivary IL-1ß showed a weak positive association with the change in the PISA ($r=0.42$, $p=0.006$, **figure 3J**). Despite the lack of change at day 90, salivary IL-8 showed a similar relationship ($r=0.45$, $p=0.003$, **figure 3H**), whereby those with the greatest reduction in PISA tended to show a slight reduction in salivary IL-8 following treatment, whilst patients with less pronounced changes showed similar levels compared with baseline.

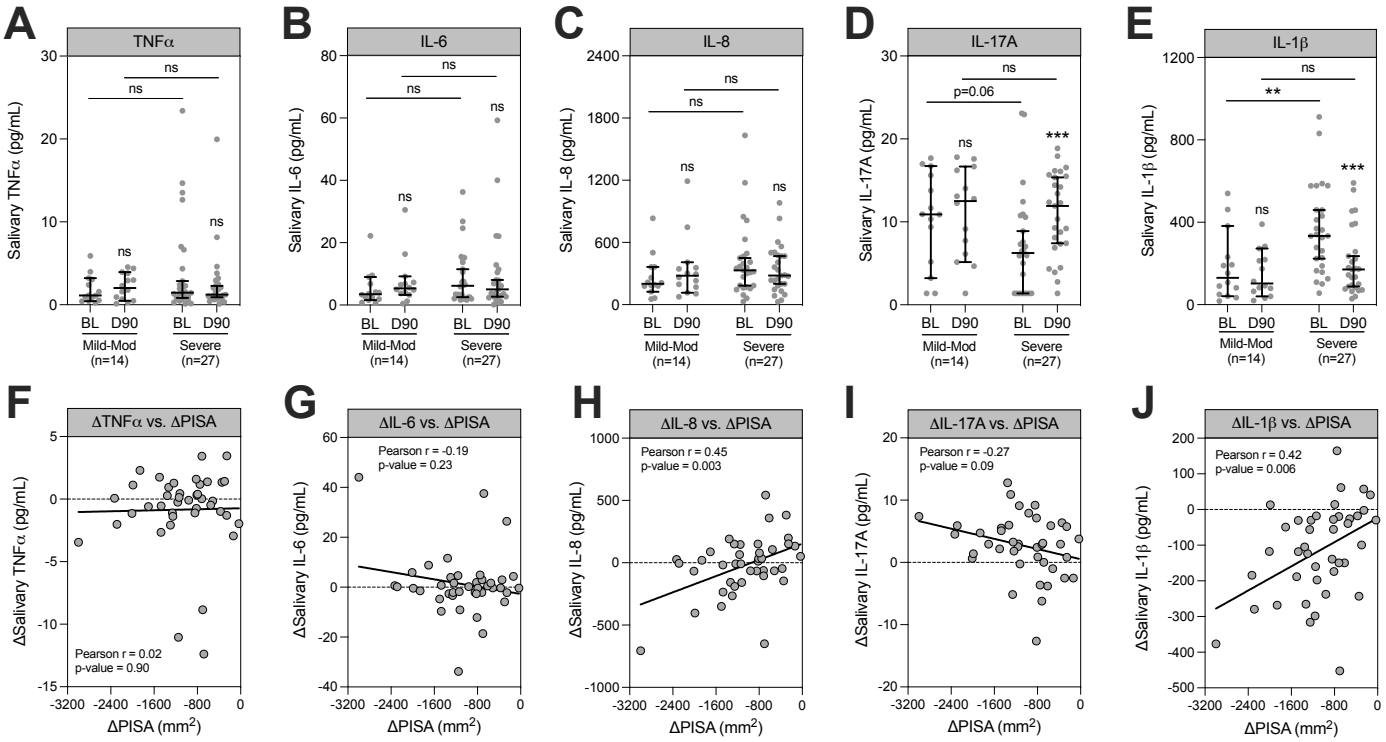

Figure 3.2. The relationship between salivary cytokines and disease severity. Comparing TNFα (A), IL-6 (B), IL-8 (C), IL-17A (D) and IL-1ß (E) between disease severity groups at baseline (BL) and day 90 (D90), and longitudinally within each group following treatment. Statistics refer to Mann-Whitney U test for between group comparisons and Wilcoxon signed-rank test for within-group comparisons, where ns means no significant difference, *p<0.05, **p<0.01 and ***p<0.001. The change (Δ) in PISA was correlated against ΔTNFα (F), ΔIL-6 (G), ΔIL-8 (H), ΔIL-17A (I) and ΔIL-1ß (J) using Pearson's correlation co-efficient. Raw p-values are shown, n=41.

3.3.1.4. The impact of smoking on salivary cytokines

Smoking has previously been shown to influence cytokine concentrations within saliva and GCF of PD patients (Rodríguez-Rabassa et al., 2018, Buduneli et al., 2008, Boström et al., 1999, Tymkiw et al., 2011), therefore an additional subgroup analysis was performed where patients were divided according to smoking status; never smokers (n=15), former smokers (n=16) and current smokers (n=10).

Assessing cross-sectional differences between groups and longitudinal alterations within groups, there were no consistent alterations with respect to salivary cytokines. Within group analysis suggested a reduction in salivary TNFα within current smokers (median: 2.26 pg/mL [baseline] to 0.92 pg/mL [day 90], p=0.027, **figure 3.3A**) and this trend was not maintained in former- or never-smokers. Salivary IL-6 appeared greater in never-smokers in comparison with current smokers at baseline (p=0.01, **figure 3.3B**), and appeared to reduce within this group following treatment (6.52 pg/mL [baseline] to 5.02 pg/mL [day 90], p=0.002). The current analysis did not demonstrate any cross-sectional or longitudinal differences in salivary IL-8 (**figure 3.3C**).

When patients were divided according to smoking status, the increase in salivary IL-17A following treatment was consistent only within never-smokers (5.95 pg/mL [baseline] to 9.20 pg/mL [day 90], p=0.007, **figure 3.3D**), however a similar trend was maintained across all groups. Salivary IL-1ß was reduced following treatment in both never- and former smokers following treatment and there was a weak trend within current smokers at day 90, particularly for patients who displayed higher levels at baseline (p=0.08, **figure 3.3E**). The small sample size of smoking subgroups must be considered in this analysis and it is noteworthy to mention that trends in IL-17A and IL-1ß appeared to be maintained regardless of smoking status. For the remaining cytokines, it could be suggested that smoking status influenced salivary IL-6 levels at baseline, although the magnitude of difference was generally low (6.52 pg/mL [never-smokers] vs. 2.27 pg/mL [current smokers]).

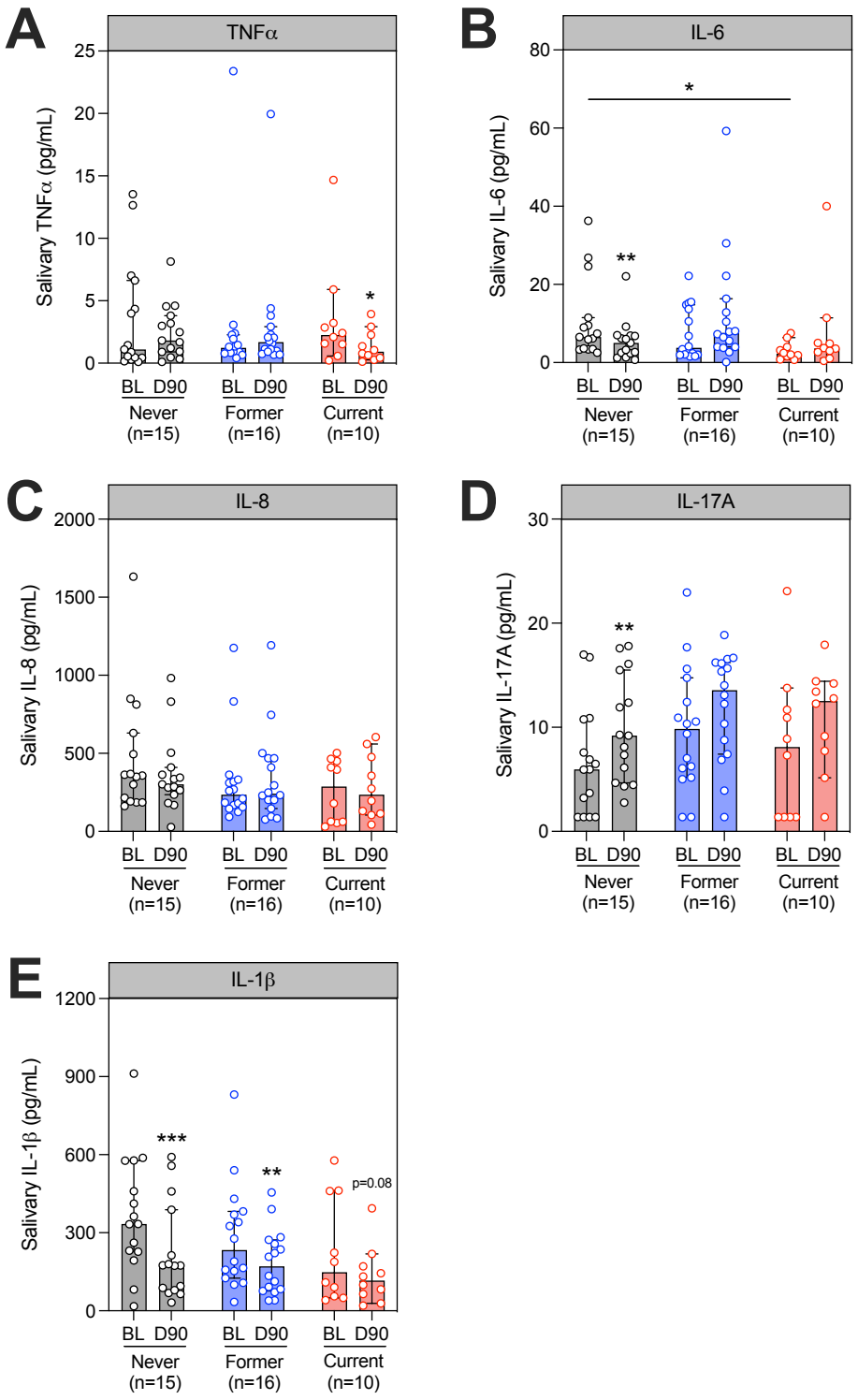

Figure 3.3. The influence of smoking on salivary cytokines. Never smokers (n=15), former smokers (n=16†) and current smokers (n=10). Comparing TNFα (A), IL-6 (B), IL-8 (C), IL-17A (D) and IL-1ß (E) between smoking categories severity groups at baseline (BL) and day 90 (D90), and longitudinally within each group following treatment. Statistics refer to Kruskall-Wallis test with Dunn's post-hoc (between group) or Wilcoxon signed-rank test (within-group), where ns means no significant difference. †One patient had limited saliva volume and was not included in any salivary analysis.

3.3.2. Cytokines in gingival crevicular fluid (GCF)

To establish whether alterations in salivary cytokines were consistent in a localised site, GCF was evaluated at baseline and day 90. According to the clinic protocol, blood contaminated strips were discarded at the time of collection. However, during sample processing GCF strips were visually inspected for blood contamination and an additional 10 strips were found to be contaminated (**figure 3.4A**). These strips were subsequently discarded prior to sample-pooling. At baseline, 37 patients had 4 strips pooled together for final analysis, whilst 5 patients had 3 strips pooled together. At day 90, 38 patients had all 4 strips pooled together, 3 patients had 3 strips pooled together and one patient had 2 strips pooled together (**figure 3.4B**).

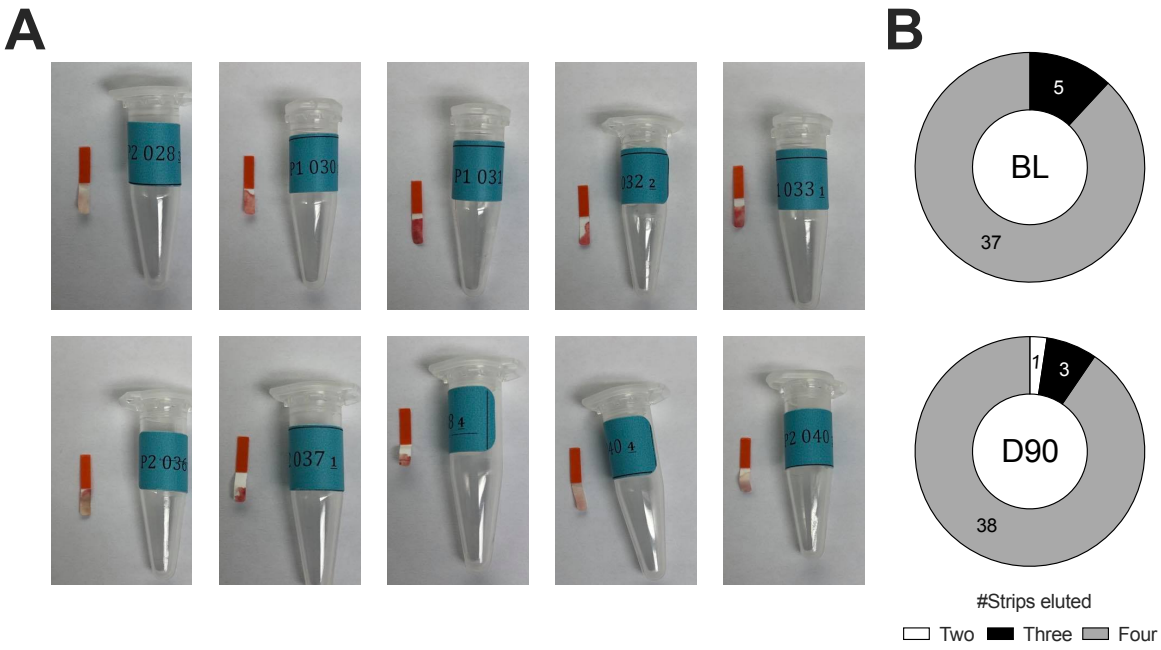

Figure 3.4. Pooling of GCF strips. Images of GCF strips which were discarded from analysis due to blood contamination (A). Pie-charts showing the numbers of GCF strips eluted per patient (n=42) at baseline (BL) and day 90 (D90). Grey indicates 4 strips eluted, black indicates 3 strips eluted and white indicates two strips eluted.

3.3.2.1. Clinical characteristics of sampled sites

In contrast to saliva, GCF samples are site-specific. Therefore, clinical changes at sites sampled for GCF were initially evaluated to ensure clinical improvement. Cumulative values of PPD and CAL were compared only across patients with 4 strips eluted at both timepoints (n=33, **table 3.3**). Within these sites, significant reductions were observed in both PPD and CAL following treatment ($p<0.001$ for both). The changes in clinical sites were then evaluated for the whole cohort (n=42). In this 'whole cohort' analysis, clinical parameters were averaged within each patient, allowing a control for patients from whom 2 or 3 strips were eluted. For example, if two strips were eluted then clinical characteristics from these two sites were averaged. This analysis also showed significant reductions in PPD and CAL across all patients ($p<0.001$) and reflected clinical improvement across every patient.

Table 3.3. Periodontal clinical parameters for GCF sampled sites. Table represents the cumulative values of PPD and CAL of the 4 sites from which samples were collected (n=33), or the average values from all patients (n=42).

Variable	Baseline (n=42)	Day 90 (n=42)	p-value
Cumulative values (4 sites, n=33)			
PPD (mm)	21.46 (±4.21)	14.76 (±3.48)	<0.001
CAL (mm)	24.32 (±5.60)	18.97 (±4.73)	<0.001
Averaged values (2-4 sites, n=42)			
PPD (mm)	5.51 (±1.15)	3.75 (±0.89)	<0.001
CAL (mm)	6.28 (±1.51)	4.88 (±1.29)	<0.001

Data are displayed as mean (±SD); statistics refer to paired t-test.

3.3.2.2. Longitudinal alterations in GCF cytokines

The concentration of GCF cytokines were correlated against site specific clinical data at baseline, which yielded negligible associations for all cytokines (all R >-0.3 <0.3 and p>0.05). These cytokines were next assessed longitudinally, initially across patients with 4 eluted strips at each timepoint, and subsequently within every patient standardised to the number of strips eluted. When assessing the total measured cytokine concentrations within patients with 4 eluted strips, the current study did not observe changes in IL-6 or IL-8 following treatment (**figure 3.5A,B**). However, there was an increase in GCF IL-17A (median: 3.72 [baseline] to 5.17 total pg/30s [day 90], p<0.001, **figure 3.5C**), and a reduction in GCF IL-1ß (1145.00 [baseline] to 428.70 total pg/30s [day 90], p<0.001, **figure 3.5D**), matching with alterations in saliva.

Similar results were observed when standardised GCF cytokine concentrations were assessed across the entire cohort, with an increase in IL-17A (1.01 [baseline] to 2.21 AU/30s [day 90], p<0.001, **figure 3.5G**) and reduction in IL-1ß (264.1 [baseline] to 99.96 AU/30s [day 90], p<0.001, **figure 3.5H**) following treatment. There were no longitudinal alterations in GCF IL-6 or IL-8 when standardised for the number of eluted strips (**figure 3.5E,F**). Thus, trends remained consistent regardless if total or standardised cytokine levels were employed.

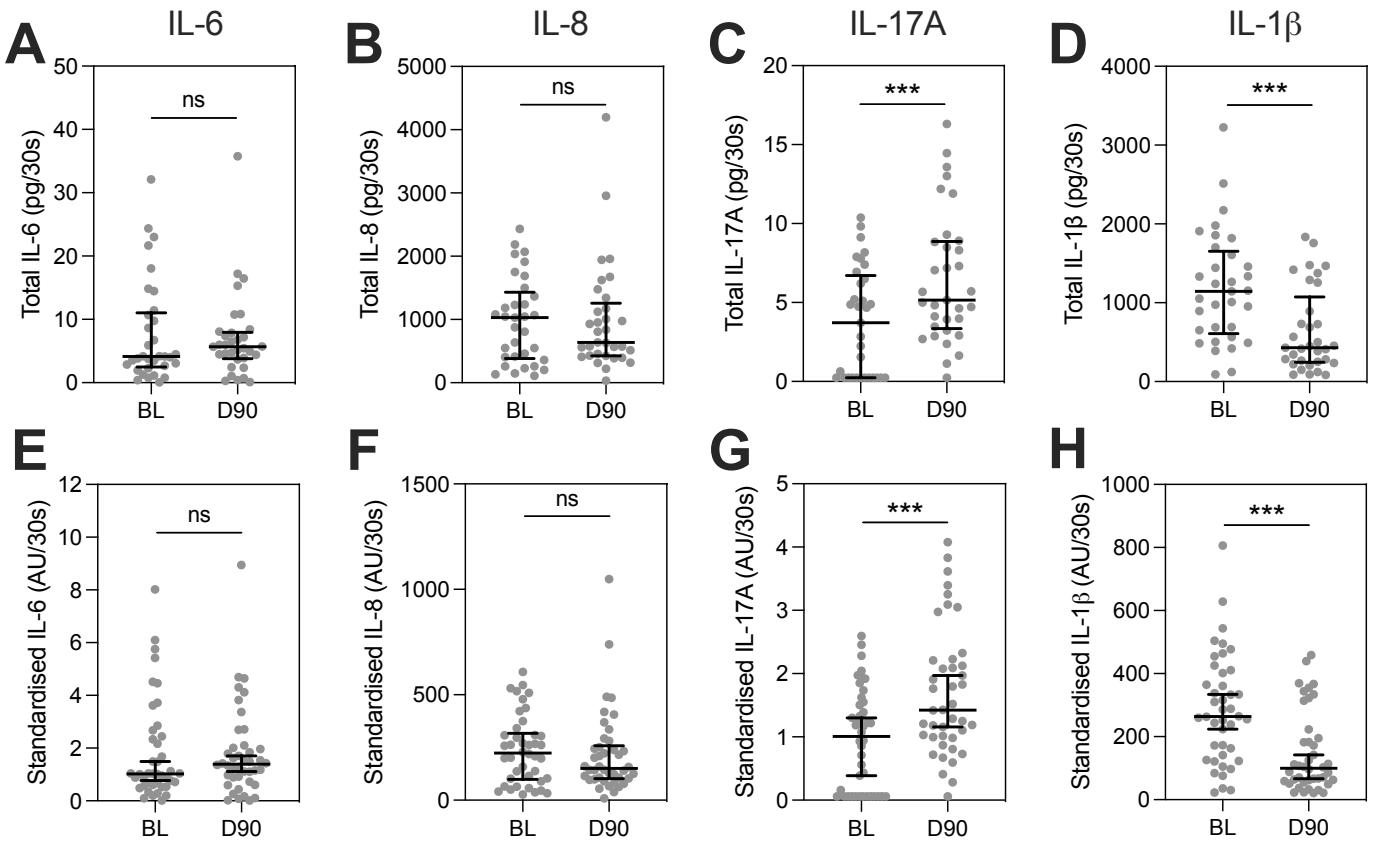

Figure 3.5. Longitudinal alterations in GCF IL-6, IL-8, IL-17A, IL-1ß. Total cytokine levels were compared across patients with 4 eluted strips at each timepoint and reported as total cytokine level per 30 seconds (A-D, n=33). Graphs represent Tukey boxplots with outliers highlighted as individual datapoints. Standardised cytokine levels were compared across the entire cohort (E-H, n=42). Individual values are plotted with error bars representing median ± 95% confidence interval. Statistics are Wilcoxon signed-rank tests were ns means no significant difference and ***p<0.001. Values are reported as picograms (pg) per 30 seconds (30s) sampling time.

3.3.2.3. The impact of smoking on GCF cytokines

GCF cytokines were next assessed across different smoking categories. Previous studies have suggested that cytokines within GCF, such as IL-6 and IL-1β (Tymkiw et al., 2011, Binshabaib et al., 2019) may be heavily influenced by cigarette smoking. Within the current study, cross-sectional analysis suggested no differences in GCF cytokine levels among smoking groups at either baseline or day 90 (**figure 3.6**). This was consistent irrespective of whether total (**figure 3.6A-D**) or standardised (**figure 3.6E-H**) cytokine values were compared.

Exploratory longitudinal analysis showed no apparent impact of smoking status on GCF IL-6 (**figure 3.6A, E**). In contrast, GCF IL-8 appeared to decrease following treatment within former-smokers (**figure 3.6B, F**). This finding only reached statistical significance when standardised cytokine levels were compared across the entire cohort (median: 258.0 [baseline] to 143.3 AU/30s [day 90], $p<0.001$, **figure 3.6F**), although a comparable trend was observed using total levels across a smaller number of patients (**figure 3.6B**).

Elevated IL-17A and reduced IL-1β were observed following treatment across the entire cohort, and this was largely consistent when stratifying according to smoking status. When assessing total cytokine levels, GCF IL-17A increased in both never- and former-smokers following treatment, but not current-smokers (**figure 3.6C**). GCF IL-1β reduced in both former- and current-smokers, but not never-smokers (**figure 3.6D**). When standardised cytokine levels were compared, GCF IL-17A increased significantly following treatment in all three smoking categories, whilst GCF IL-1β reduced significantly in all three smoking categories (all $p<0.05$, **figure 3.6G-H**). Therefore, smoking did not appear to markedly influence the trends in GCF cytokines within this small exploratory subgroup analysis.

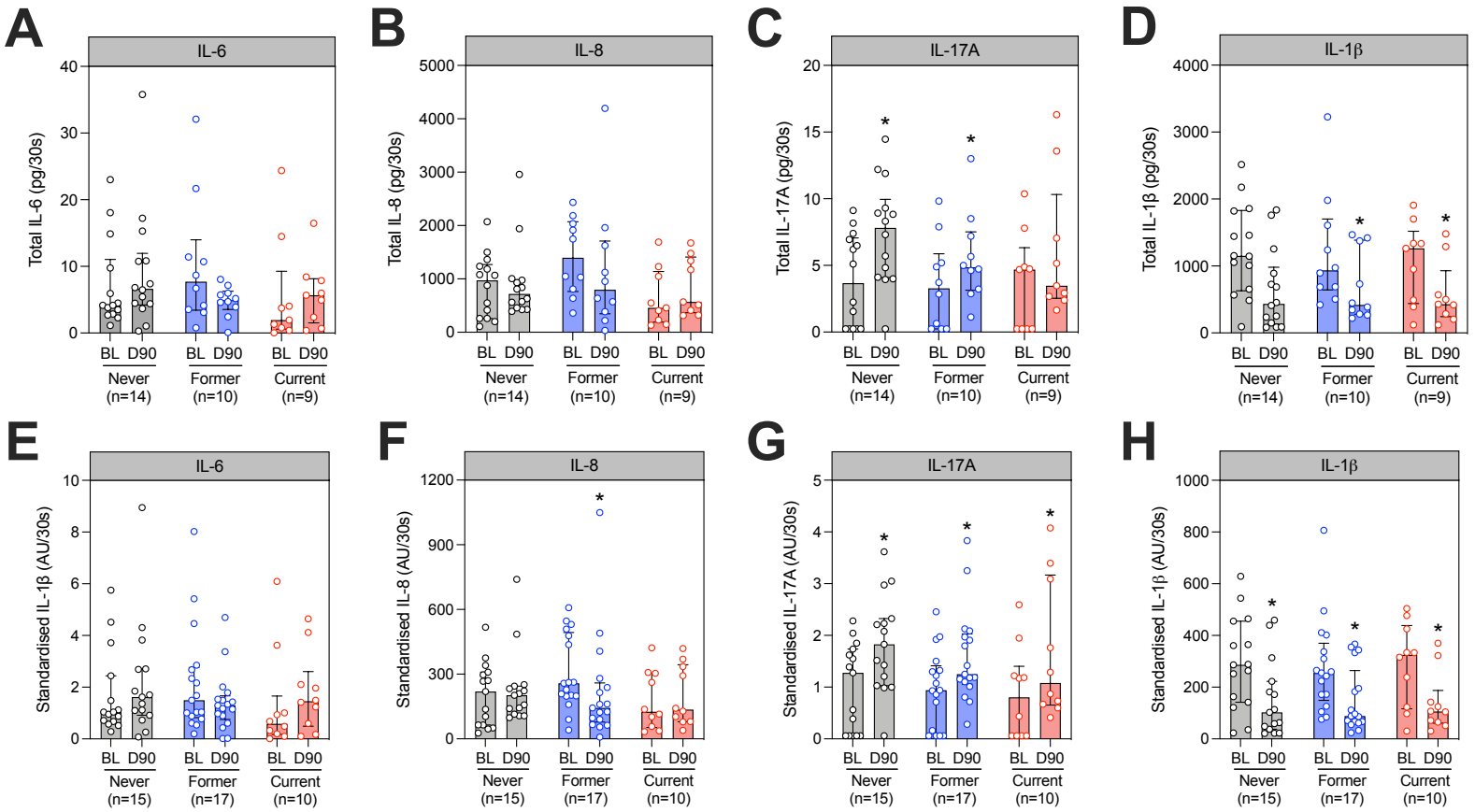

Figure 3.6. The influence of smoking on GCF cytokines. Total IL-6, IL-8, IL-17A and IL-1β were compared across smoking categories among patients with 4 eluted GCF sites (A-D, n=33), and standardised levels among all patients (E-H, n=42). Statistics refer to Kruskall-Wallis test with Dunn's post-hoc (between group) or Wilcoxon signed-rank test (within-group), where ns means no significant difference. Raw values are displayed, bars represent median with 95% confidence intervals.

3.3.2.4. Association between salivary and GCF cytokines

As longitudinal shifts remained consistent within GCF and saliva with regard to IL-17A (increase) and IL-1ß (decrease), it was hypothesised that the levels of cytokines may correlate within each sample type. Although extensive analysis has characterised cytokines in GCF and saliva of PD patients, this analysis has largely been conducted independently (Teles et al., 2009, Ebersole et al., 2015, Stadler et al., 2016, Zein Elabdeen et al., 2017). Establishing whether associations exist between GCF and salivary cytokines is currently lacking, particularly within a cohort of PD patients. Such a finding, although limited to four cytokines, could be important to support the use of saliva as an ideal sample type for screening periodontal disease, and offer a preliminary insight into the relationship between saliva composition and more local cytokine production at specific sites at the gingivae.

Analysis between salivary and GCF cytokines was performed using Spearman-Rho correlation coefficients at baseline. Assessing correlations between total GCF cytokine level (4 strips, n=33) with salivary cytokines, a moderate positive correlation was observed for IL-6 levels at baseline (Spearman r=0.498, p=0.002, **figure 3.7A**). Similar results were observed across the entire cohort using standardised GCF levels (Spearman r=0.514, p<0.001, **figure 3.7E**); however, this did appear skewed by a cluster of samples with very low quantities of IL-6.

No other associations were observed between GCF and salivary cytokines at baseline, using both total and standardised GCF quantities, and many factors may contribute to these results. For example, GCF samples are collected over a 30 second period, whereas saliva collection is standardised for volume rather than time. In addition, there are several difficulties in collecting and analysing cytokines within GCF samples, as has been observed throughout this study. Therefore, the results from this study for GCF cytokines are semi-quantitative estimates given that results were standardised to the number of eluted strips, and no measurement of collected GCF volume were available.

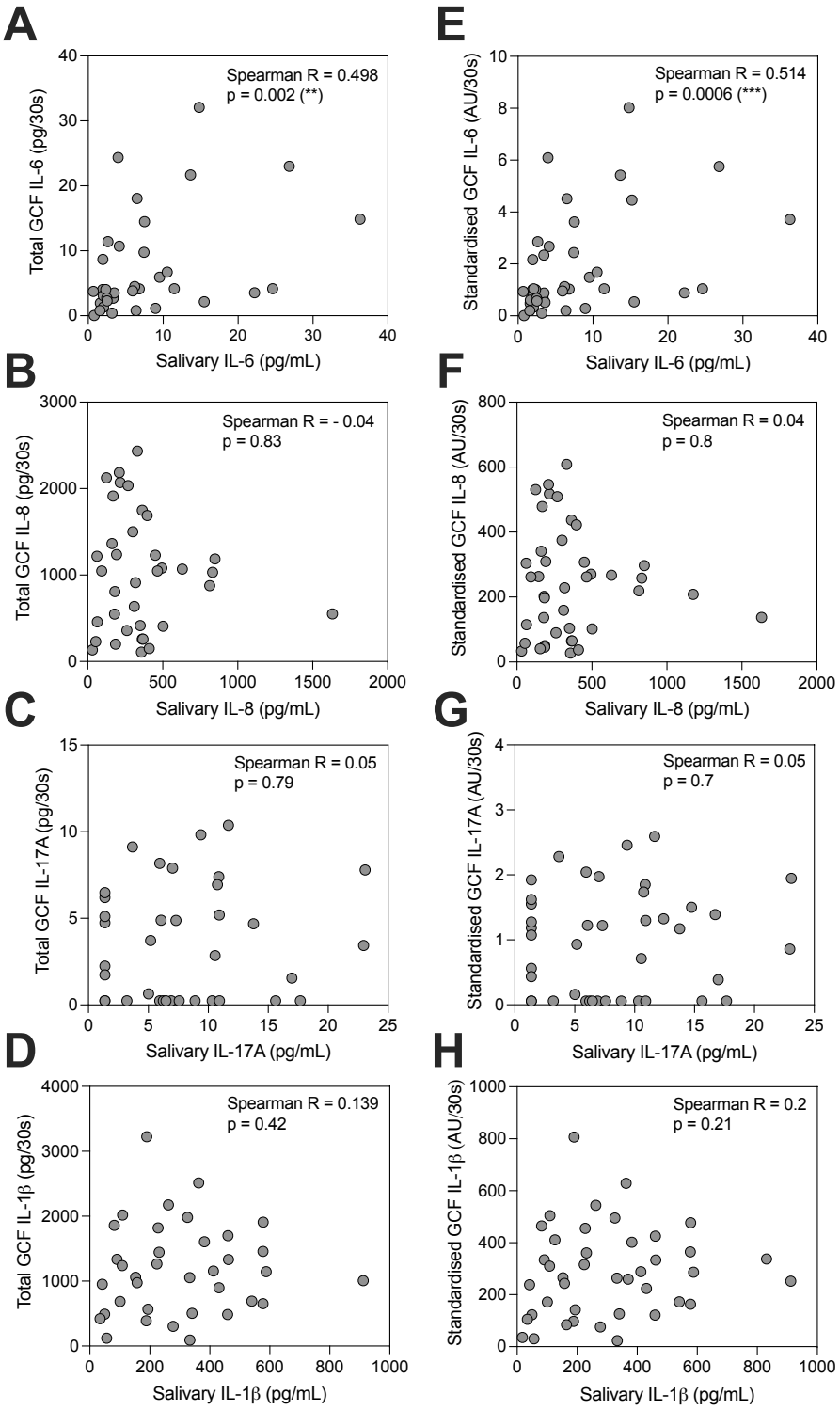

Figure 3.7. Association between cytokine levels in the saliva and GCF at baseline. Correlation analysis was performed for IL-6, IL-8, IL-17A and IL-1ß across patients with 4 eluted strips at baseline (A-D, n=33) and across all patients with GCF levels standardised per strip eluted (E-H, n=42). Statistics refer to Spearman-Rho correlation coefficients with raw p-values displayed.

3.3.3. Subgingival plaque microbiome

Given the widespread clinical improvements and alterations in local inflammatory cytokines observed throughout this study, the subgingival plaque microbiome was compared at baseline and day 90 to investigate the microbial response to NSPT.

3.3.3.1. Clinical characteristics from sampled sites

One site from each patient was selected for 16S rRNA sequencing. Clinical parameters (PPD and CAL) are shown in **table 3.4** for sampled sites. The median PPD and CAL of sampled sites was 7 mm and 8 mm respectively. Following treatment, PPD reduced to 4 mm, and CAL reduced to 6 mm, which was statistically significant for each parameter ($p<0.001$ for both). The deepest site in each quadrant was sampled, and therefore sampled sites included molar and non-molar teeth.

Table 3.4. Periodontal clinical parameters for subgingival plaque sampled sites. Data represent sites from which subgingival plaque samples were harvested. Data are displayed as medians (Q1, Q3), statistics are Wilcoxon signed-rank tests. n=42.

Variable	Baseline (n=42)	Day 90 (n=42)	p-value
Site PPD (mm)	7.00 (6.00, 8.00)	4.00 (4.00, 5.00)	<0.001
Site CAL (mm)	8.00 (7.00, 9.00)	6.00 (5.00, 7.00)	<0.001

3.3.3.2. Sequencing depth and rarefaction analysis

The 16S rRNA sequencing analysis of subgingival plaque yielded 4745 ASVs across 84 samples. The average number of reads obtained was 90,830 per sample, which ranged from 35,372 to 343,259 reads. After removing singletons, ASVs were classified into 206 genera and 379 species. Prior to calculating α-diversity indexes, rarefaction analysis was performed which plots the observed number of species against different rarefied read depths. This analysis rarefies samples to a pre-defined number of reads, to demonstrate that differences in diversity are not due to sequencing depth. At low reads, it is expected that the number of observed species will increase rapidly as more common species are identified. However, once only rare species remain the curve will gradually plateau indicating that sample diversity has been adequately captured.

Across the samples in the current study, the lowest number of reads classified to species-level was 9,103. As such, 9,000 was used as an upper boundary for rarefaction analysis to be used in calculation of α-diversity indices (**figure 3.8**). The results of this analysis showed that the observed species from all samples gradually plateaued upon rarefaction, suggesting that a reliable estimate of taxonomic diversity was feasible when rarefying to 9,000 reads per sample.

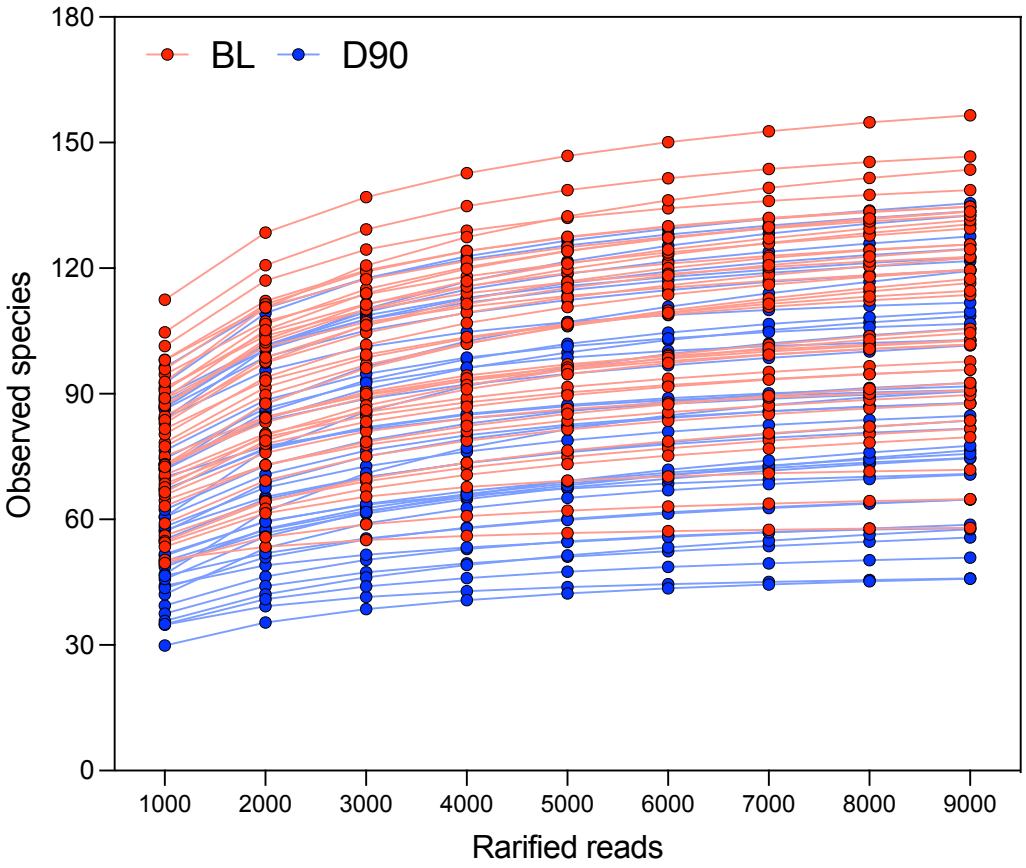

Figure 3.8. Rarefaction curves for α-diversity indices. Graph displays the observed species at baseline (red) and day-90 (blue) (A) for all samples when rarefying from 1000-9000 reads. N=84.

3.3.3.3. Baseline associations with α-diversity

After rarefying to 9,000 reads, the Shannon diversity index was calculated at species-level. This index is a measure of both richness and diversity, which factors in how many species are present and their corresponding abundance. Low values indicate dominance from a few number of species, whilst higher values indicate many species with similar abundances. These results were supplemented with an additional α-diversity index known as abundance-based coverage estimators (ACE index), which uses the number of rare and common species to estimate how many more undiscovered species may exist in samples. Thus, it is possible to estimate how many species are present in samples with a higher degree of accuracy in comparison to using observed species alone.

It was initially investigated whether the diversity of subgingival plaque samples was associated with the pocket depth or attachment level of sampled sites at baseline. Previous studies have shown that the diversity of the subgingival

plaque microbiome is elevated in PD compared with periodontal health (Abusleme et al., 2013, Griffen et al., 2012), indicating that increased pocket depths, inflammation and/or modulation of the host immune response allow for growth of a more diverse plaque community. Given these previous findings, it was hypothesised that the pocket depth of sampled sites may correlate with α-diversity indices.

No strong association was observed between either of these clinical variables (PPD and CAL) and the Shannon index (**figure 3.9A, C**). However, the data generally supported a weak positive association for all parameters with comparable trends. This was only considered statistically significant between the ACE index and PPD of sampled sites (Pearson R = 0.352, p=0.02, **figure 3.9B**) and appeared driven by extreme values.

Along with site-specific clinical variables, α-diversity indices were compared across different demographic and behavioural variables to establish the impact on subgingival plaque diversity (**figure 3.10**). This analysis suggested no alterations in the Shannon and ACE indices at baseline when the sites were sampled from molars or non-molars (**figure 3.10A, E**), different age groups (**figure 3.10B,F**) or smoking categories (**figure 3.10C, E**). A slight elevation in ACE index was found when comparing mild-moderate and severe PISA groups (mean[SD]: 107.5 [28.51] vs. 124.7 [20.90], p=0.03, **figure 3.10H**). The Shannon index also appeared higher in the severe PISA group; however this did not maintain statistical significance and variance appeared larger within each group (**figure 3.10D**).

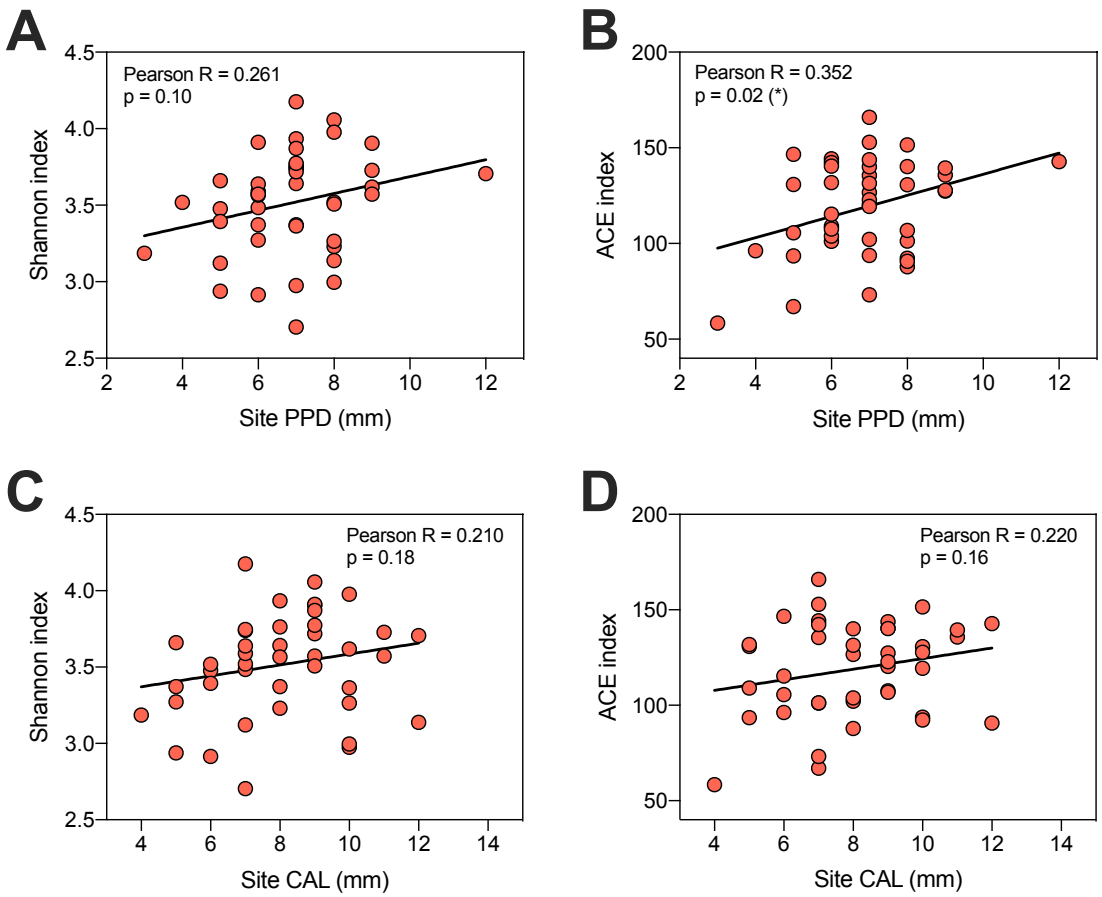

Figure 3.9. Association between α-diversity indexes at site-specific clinical variables at baseline. Graphs display Shannon (A,C) and ACE (B, D) indices with PPD (A,B) and CAL (C,D). Associations were assessed using Pearson correlation coefficients. Raw R and p-values are displayed, *p<0.05. N=42.

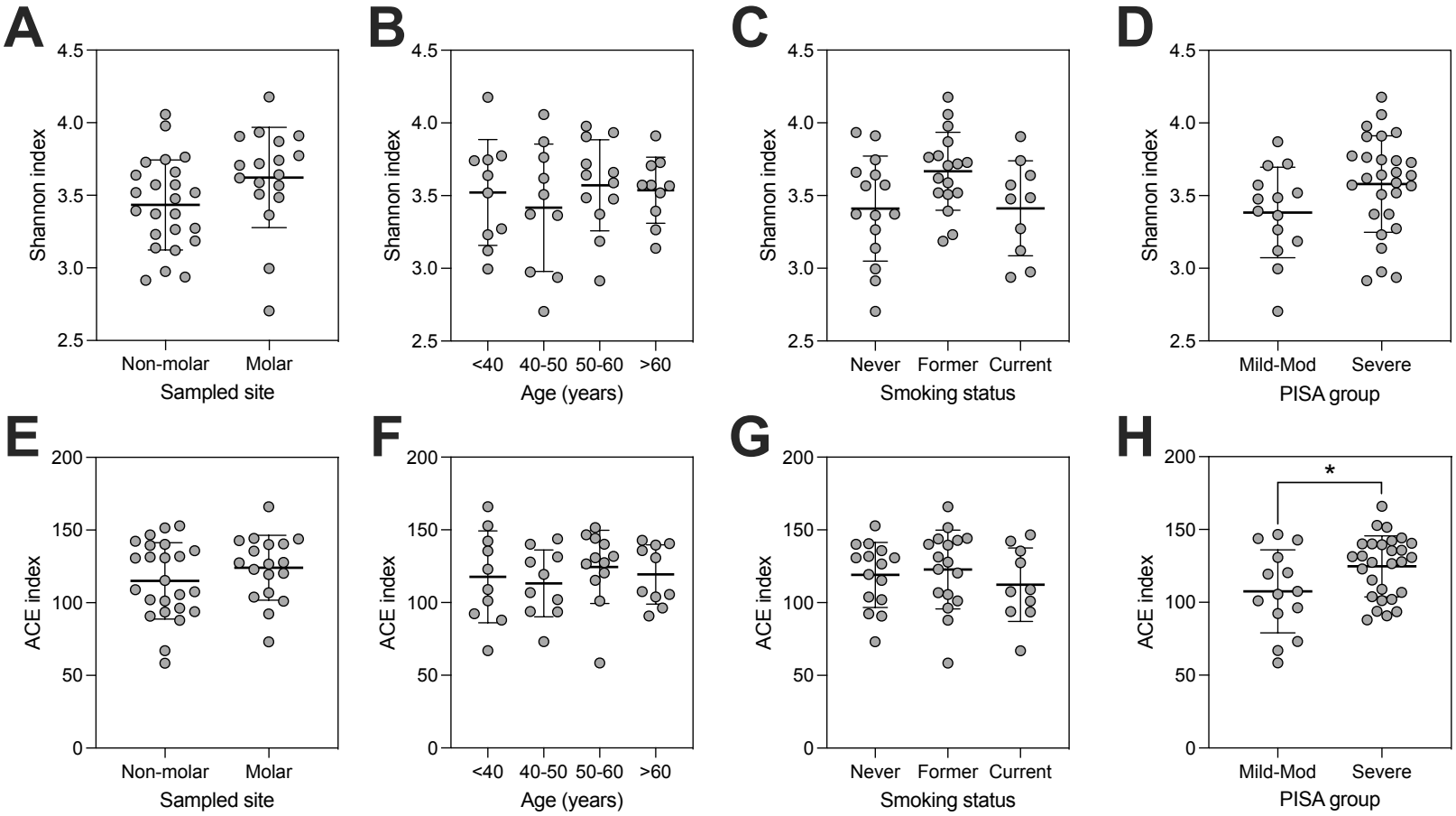

Figure 3.10. α-diversity indexes in samples stratified for demographic, behavioural and clinical groupings at baseline. Shannon and ACE indices were compared across molar vs non-molar sites (A, E), age (B,F), smoking (C,G) and PISA groups (D,H). Statistics represent one-way ANOVA with Tukey post-hoc (A-D) or unpaired t-test (E,F), *p<0.05. Horizontal bars represent mean, error bars show standard deviation, each circle represents one site.

3.3.3.4. Baseline associations with ß-diversity

Whilst α-diversity measures the diversity of a single sample, ß-diversity assesses how different samples vary against one another. One method to measure ß-diversity is using Bray-Curtis dissimilarity which uses abundance data to discern differences in microbial community composition. To reduce the dimensionality of the data, results can be plotted using a Principal Coordinate Analysis (PCoA), capable of handling dissimilarity rather than Euclidean distances (used by principal components analysis). Hence, ß-diversity is able to identify factors contributing to variation in the microbiome community. This analysis was conducted at genus- and species-level to ensure reliability of results.

It was hypothesised that the microbiota composition may differ according to sampled site disease severity. This was visualised between sites with different PPD and CAL values at baseline using a Bray-Curtis dissimilarity based PCoA. Interestingly, there appeared to be no distinct clustering of different PPD and CAL values at both genus- (**figure 3.11A, C**) or species-level (**figure 3.11B, D**). This visual assessment was confirmed statistically using the ANOSIM function (all $p>0.05$).

One factor which may explain this lack of clustering is that all samples were collected from patients with PD, and predominantly from sites with PPD values between 6 and 8 mm (>75% of samples). Although extreme values were recorded (up to 12 mm), these equated to only a very low proportion of samples. Evaluating these data, it is perhaps unsurprising that no widespread, consistent alterations were observed when clinical variables were largely confined within a relatively low range.

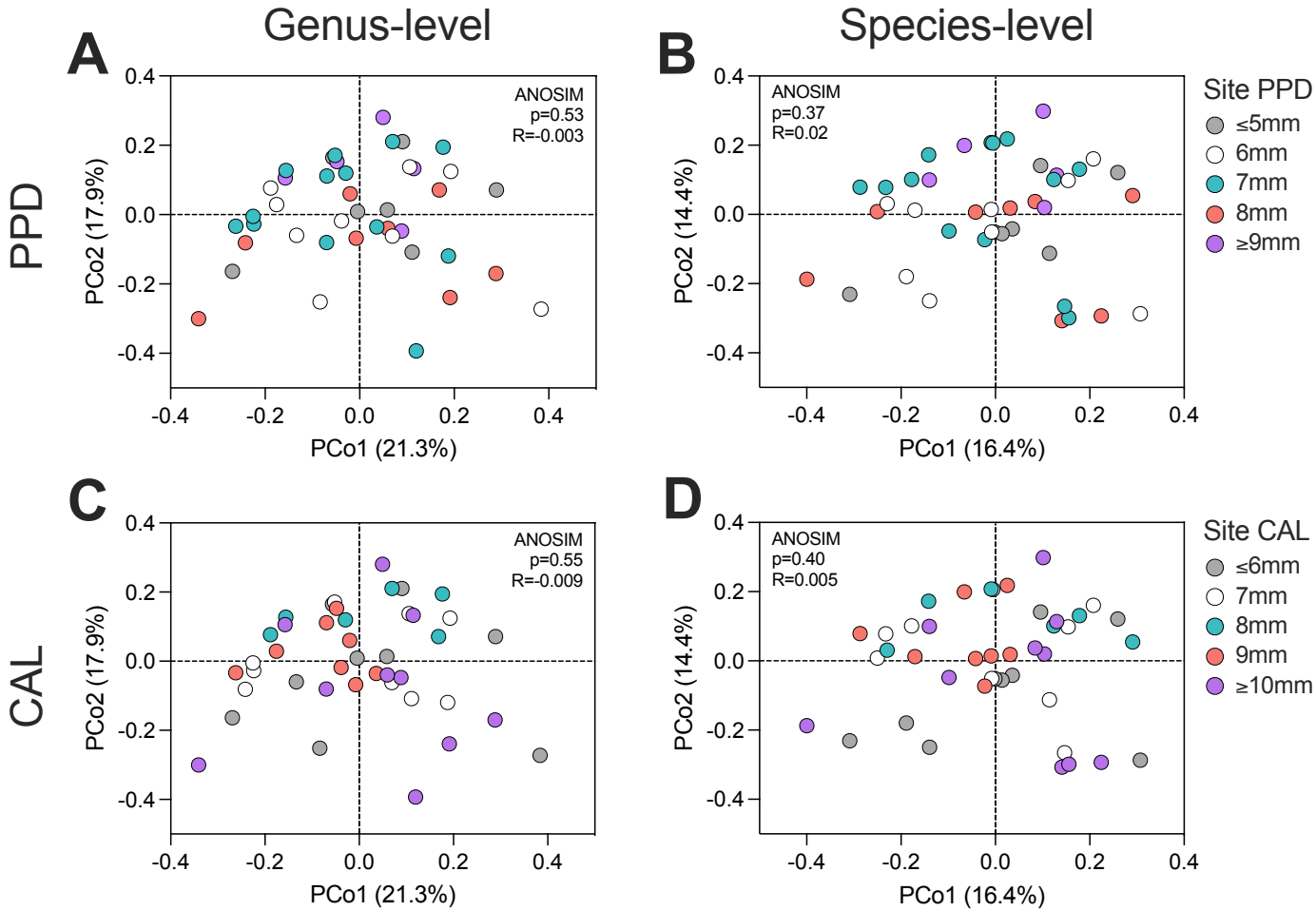

Figure 3.11. ß-diversity when stratified for site-specific disease severity at baseline. PCoA plots based on Bray-Curtis dissimilarity at genus- (A,C) and species-level (B,D). ß-diversity across sampled sites with different PPD (A, B) and CAL (C,D) at baseline. Statistics are ANOSIM with raw p- and R-values displayed.

Differences in baseline ß-diversity were also assessed in relation to sampled site anatomy (molar/non-molar), age, smoking status and PISA groups. When assessed using ANOSIM, no significant differences were observed when comparing molar and non-molars (**figure 3.12A,B**), different age (**figure 3.12C,D**) or smoking (**figure 3.12E,F**) categories ($p>0.05$ for all). Similar to the ACE index, this analysis did suggest subtle differences in PISA groups ($p=0.06$ [genus-level], $p=0.03$ [species-level], **figure 3.12G,H**). However, differential abundance analysis did not identify any differences in genera or species between each category.

Thus, whilst variation in the baseline subgingival plaque microbiome of PD patients did exist, this did not appear to be solely driven by demographic or behavioural variables within the current cohort. Similar to the clinical analysis described previously, it is important to note that all patients in the current study suffered from PD and sampled predominantly from deep sites between 6 and 8 mm in depth, which may mask any influence of these factors on the subgingival plaque microbiota.

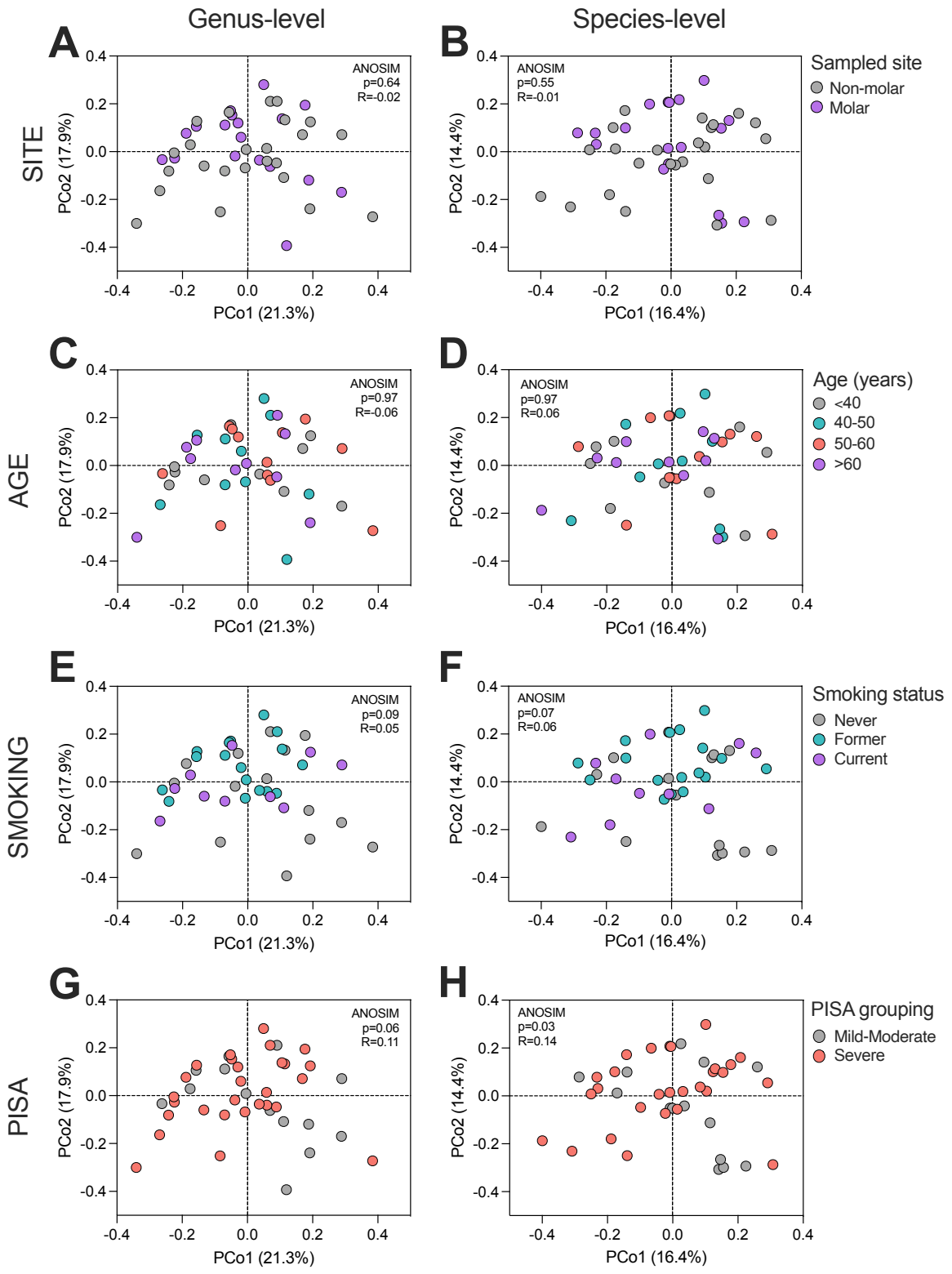

Figure 3.12. ß-diversity when stratified for demographic, behavioural and clinical variables at baseline. PCoA plots based on Bray-Curtis dissimilarity at genus- (A,C,E) and species-level (B,D,F). Comparing ß-diversity across age, smoking and PISA groups at baseline. Statistics are ANOSIM with raw p- and R-values displayed.

3.3.3.5. Longitudinal alterations in α-diversity

Longitudinal analysis was collectively performed across all 42 patients. The α-diversity indices were compared at baseline and day 90 using a paired analysis (paired t-test), which showed reductions in both the Shannon (mean [SD]: 3.52 [0.33] baseline to 3.05 [0.56] day 90, p<0.001, **figure 3.13A**) and ACE indexes (119 [24.77] baseline to 96.94 [26.83] day 90, p<0.001, **figure 3.13B**) following treatment. Visual inspection suggested that these reductions were highly consistent across the majority of patients. Indeed, of the 42 patients analysed; 35 (83%) showed a reduction in the Shannon index, and 32 (76%) showed a reduction in the ACE index at day 90.

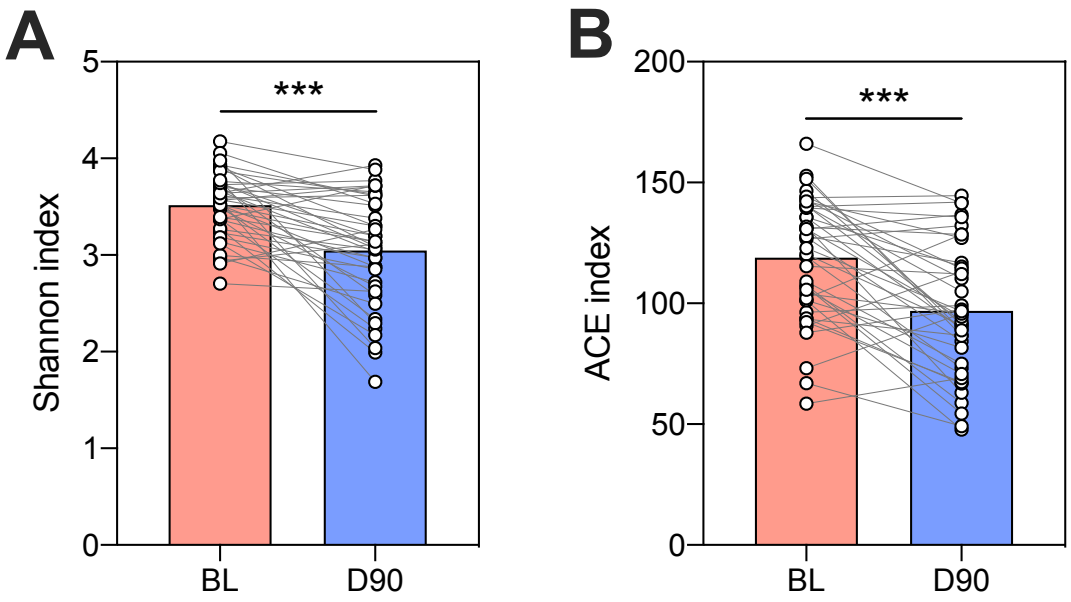

Figure 3.13. Shifts in α-diversity following treatment. Shannon (A) and ACE (B) indexes longitudinally following NST. Bars represent means with lines connecting individual patients. Statistics are paired t-test where ***p<0.001. N=42.

3.3.3.6. Longitudinal alterations in ß-diversity

Shifts in the total microbiome community following treatment were next investigated using a Bray-Curtis based PCoA. This analysis was performed across all 42 patients, comparing baseline and day 90 samples at genus- and species-level. At genus-level, PCo1 and PCo2 equated to 47.4% of the variance in the dataset. There appeared to be distinct clustering of samples from each timepoint which existed largely across PCo1, although some degree of overlap remained (**figure 3.14A**). This was confirmed statistically using the ANOSIM function, outlining larger differences between groups than within groups ($p<0.001$). At species-level, PCo1 and PCo2 equated to less variance than at genus-level (36.1%), although distinct clustering between baseline and day 90 samples was visually more apparent (**figure 3.14B**). Similar to genus-level, variation between timepoints was largely across PCo1, confirmed statistically using the ANOSIM function ($p<0.001$, $R=0.20$).

3.3.3.7. Microbiome composition at baseline and day 90

To supplement the ß-diversity analysis, the most abundant genera and species were plotted to visualise the microbiota composition (**figure 3.15A-B**). The most abundant genera were *Fusobacterium* and *Prevotella* representing 10.8% and 9.6% of the baseline composition respectively (**figure 3.15A**). The abundance of both genera reduced to 6.3% (*Fusobacterium*) and 6.0% (*Prevotella*) at day 90. *Streptococcus*, which represented 9.2% of the composition at baseline increased to 16.4% at day 90. Large increases were also observed for *Actinomyces* (3.2% at baseline, 8.8% at day 90), *Corynebacterium* (3.0% at baseline, 7.7% at day 90) and *Rothia* (2.5% at baseline, 7.7% at day 90). Similar data was observed at species-level (**figure 3.15B**) where *Fusobacterium nucleatum* was the most abundant species at baseline (9.2%), which reduced to 4.5% at day 90. Unclassified *Streptococcus* increased from 8.0% at baseline to 13.9% at day 90.

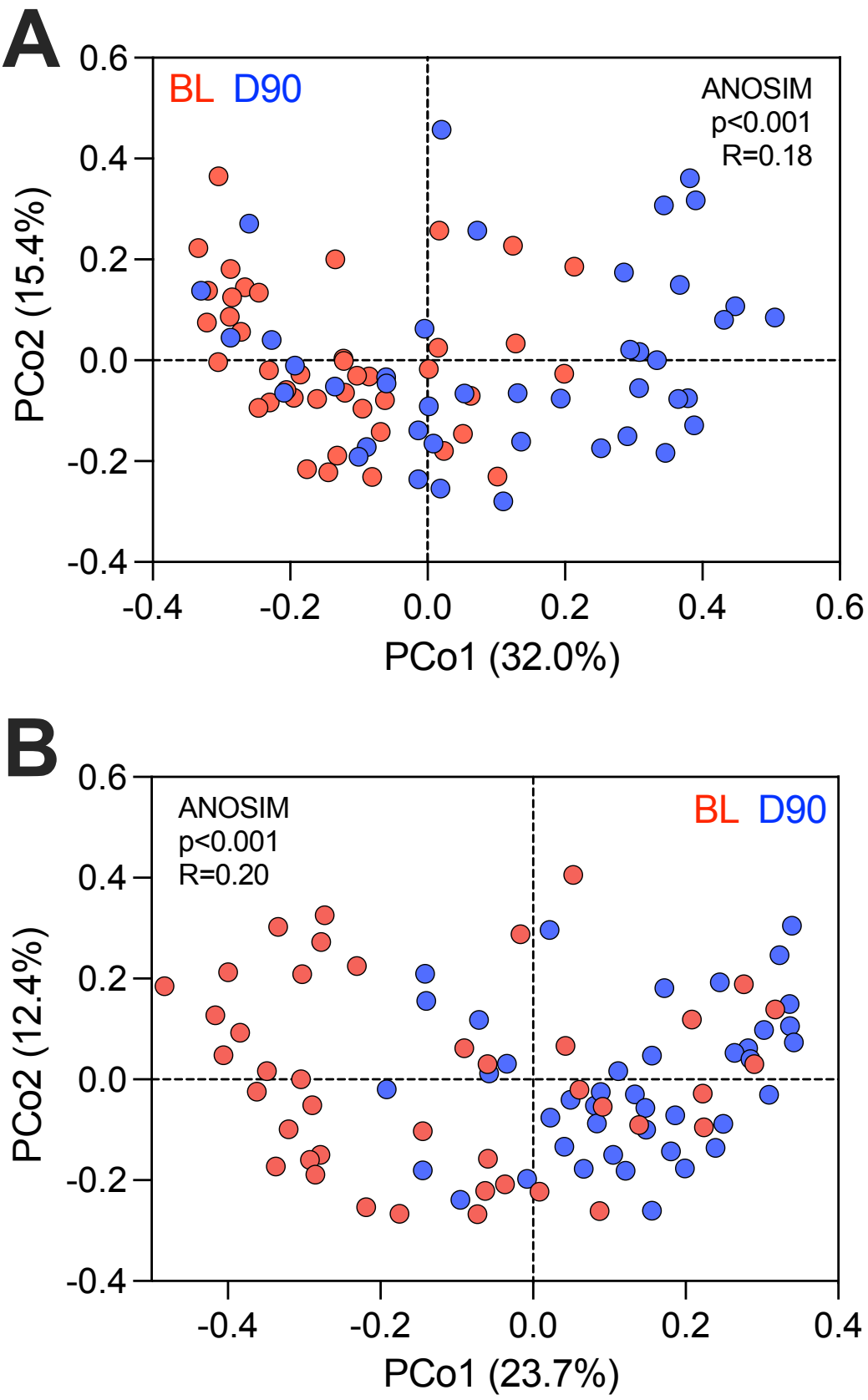

Figure 3.14. Shifts in ß-diversity following treatment. PCoA plots based on Bray-Curtis dissimilarity at genus- (A) and species-level (B). Comparing baseline (BL) and day 90 (D90) samples. Statistics are ANOSIM with raw p- and R-values displayed.

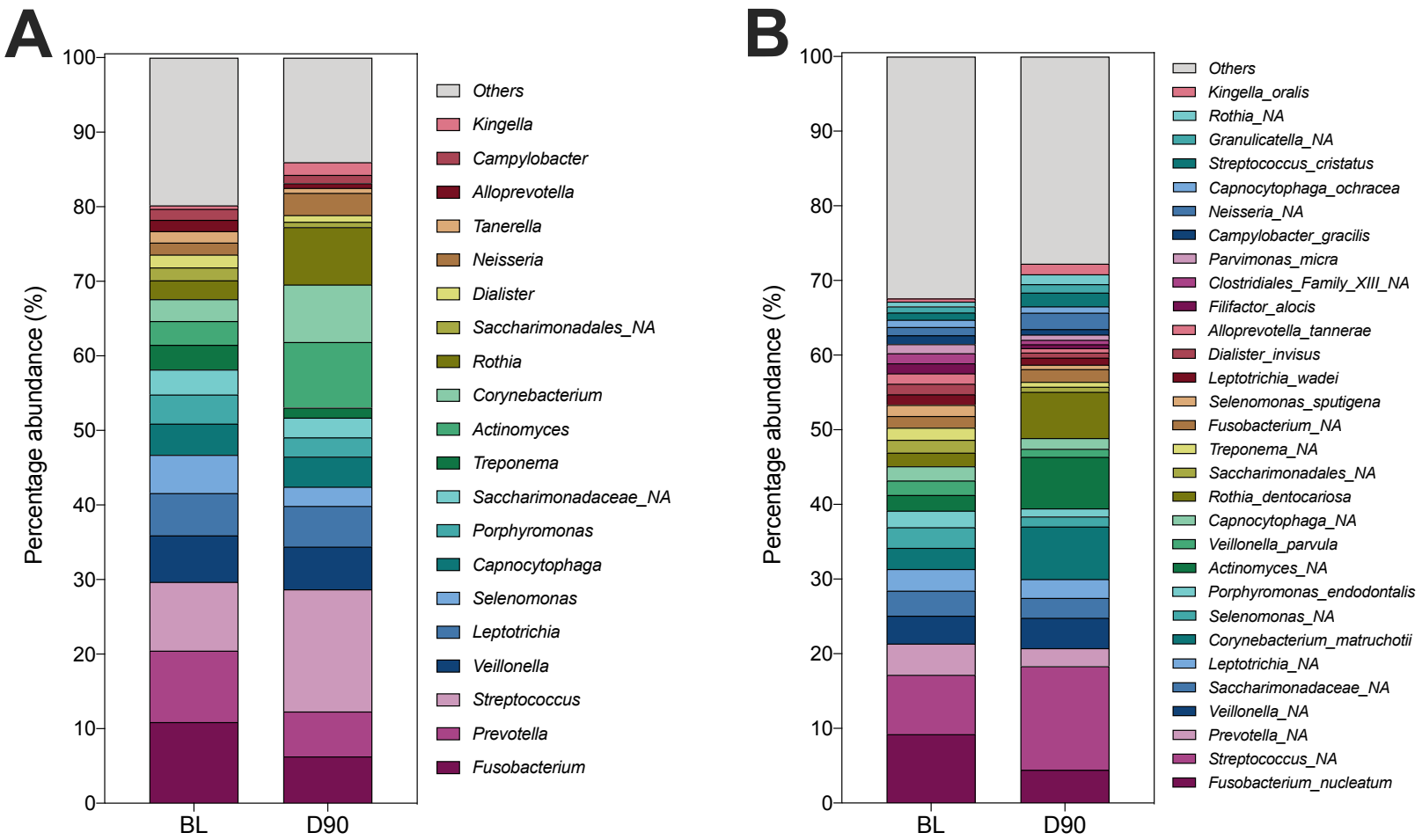

Figure 3.15. Microbiome composition at each timepoint. Displaying the top 20 most abundant genera (A) and top 30 most abundant species (B) at baseline (BL) and day-90 (D90) across all patients. All other genera and species are grouped together as 'Others' shown in grey. N=42 both timepoints.

3.3.3.8. Longitudinal alterations in genera and species abundance

To establish which genera and species significantly differed following NSPT, ranked abundances were compared using paired Wilcoxon tests adjusted for multiple comparisons using the FDR (5%) approach (Benjamini and Hochberg, 1995). Only genera and species with a minimum signal of detection were included in this analysis, as outlined in the methodology section (**3.2.7**). The results of this analysis at genus- and species-level are plotted as volcano plots. In total, 68 genera and 128 species met the pre-determined abundance threshold and were assessed longitudinally. This analysis demonstrated significant changes in the abundance of 42 genera (**figure 3.16A**) and 61 species (**figure 3.16B**) comparing baseline and day 90 samples. Of the 42 genera, 34 decreased whilst 8 increased in abundance; at species level 46 species decreased and 15 species increased in abundance following treatment. One genera; *Centipeda*, significantly reduced following treatment but displayed a positive fold-change due to several extreme outliers (not plotted).

To identify genera and species contributing most to the compositional alterations, a further filter of an absolute median difference of >0.25% (genus-level) or >0.1% (species-level) between baseline and day 90 samples was applied. Once this filter was applied, 18 of the 42 significantly-different genera and 29 of the 61 significantly different species remained, with abundances plotted in **figure 3.17A-B**. This additional filter allowed exclusion of genera and species where only slight compositional alterations were observed.

After this additional filtering step, five genera displayed a significant increase following treatment. The most abundant of which were *Streptococcus* and *Actinomyces*, both grouped in colour complexes and associated with a commensal microbiota (Socransky et al., 1998). Additionally, significant increases were observed in other ungrouped genera such as *Corynebacterium, Rothia* and *Kingella*. These results were largely consistent at species-level, despite a large proportion of organisms being unclassified. For example, the most abundant species-level alterations were observed for unclassified *Streptococcus* and *Actinomyces* species. Of those classified to species-level, increases were observed for *Corynebacterium matruchotii* (2.00 vs. 4.19%),

Rothia dentocariosa (0.56 vs. 1.64%), *Streptococcus cristatus* (0.24 vs. 1.41%), and *Kingella oralis* (0.18 vs. 0.30%).

In contrast, 13 genera significantly decreased following treatment and met the additional difference filter, suggesting that a large proportion of the reductions initially observed represented only a minor compositional shift. Nearly all reductions were observed for strict-anaerobic genera. Notable reductions were observed for genera previously found to be strongly associated with PD, including; *Treponema* (2.54 vs. 0.28%), *Porphyromonas* (2.50 vs. 0.72%), *Tannerella* (1.28 vs. 0.14%) and *Filifactor* (0.98 vs. 0.03%). Likewise, anaerobic genera which are known to be abundant in health but increase in PD such as *Fusobacterium* and *Prevotella* (Chen et al., 2018) displayed significant reductions following treatment. At species-level, 27 species showed significant reductions and met the additional filtering criteria. Whilst many of these species are known to be associated with PD (*Tannerella forsythia, Treponema denticola, Filifactor alocis*), less abundant species such as *Fretibacterium feline* (0.15 vs. 0.001%), *Dialister invisus* (0.75 vs. 0.03%) and *Selenomonas sputigena* (0.69 vs. 0.12%) also showed comparable trends.

Collectively, these data highlight longitudinal alterations in the abundance of several genera and species following non-surgical treatment. The majority of reductions were observed for strict anaerobes, whilst increases were mainly found in facultative anaerobes or aerobes. Importantly, these alterations were present 90 days following treatment, suggesting that NSPT induces a lasting impact on the subgingival plaque microbiota composition across a relatively large number of patients (n=42). It is also noteworthy to mention that although several significant alterations were observed, large-scale compositional changes were driven by relatively few genera; reductions in *Fusobacterium, Prevotella, Selenomonas, Porphyromonas, Treponema, Tannerella* and increases in *Rothia, Corynebacterium, Actinomyces* and *Streptococcus*.

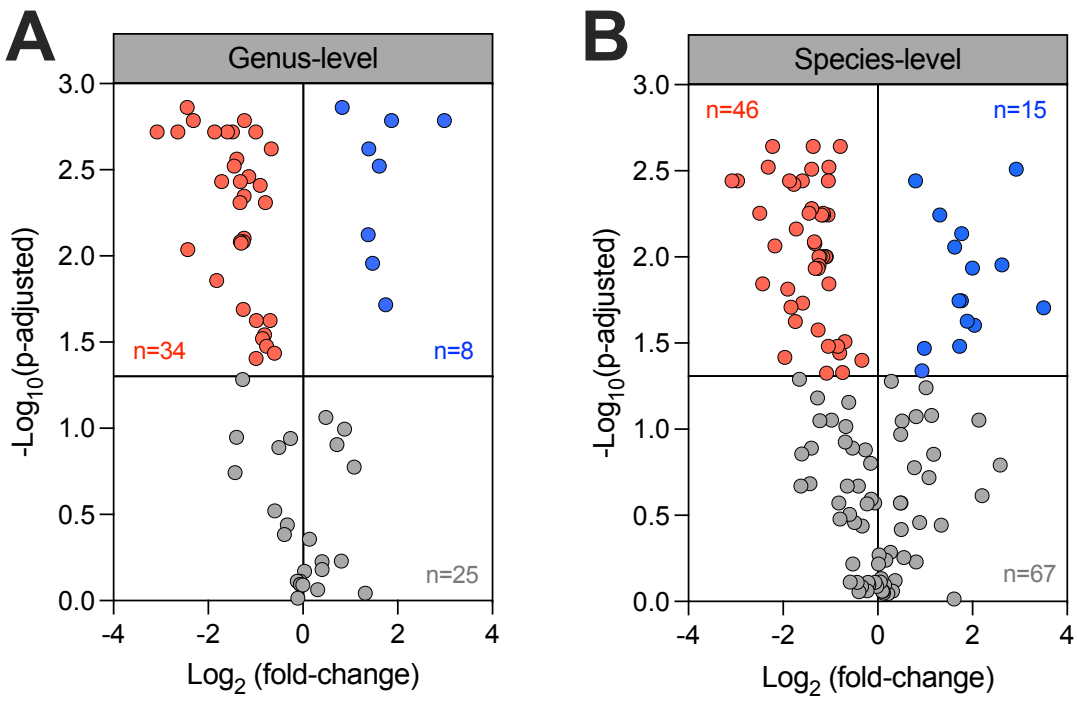

Figure 3.16. Longitudinal shifts in abundance following NSPT. Volcano plots of the longitudinal analysis of 67 genera (A) and 128 species (B) comparing baseline and day 90 samples. Only genera and species which met the pre-determined prevalence threshold were included in this analysis. Data are displayed as the -$\log_{10}$ transformed adjusted p-value (y-axis) generated through Wilcoxon-signed rank tests with FDR (5%) adjustment, and $\log_2$ fold-change in the average abundance of each genera and species (x-axis). Red dots indicate a significant decrease and blue dots indicate a significant increase in abundance following treatment, grey dots displayed no significant alterations. N=42.

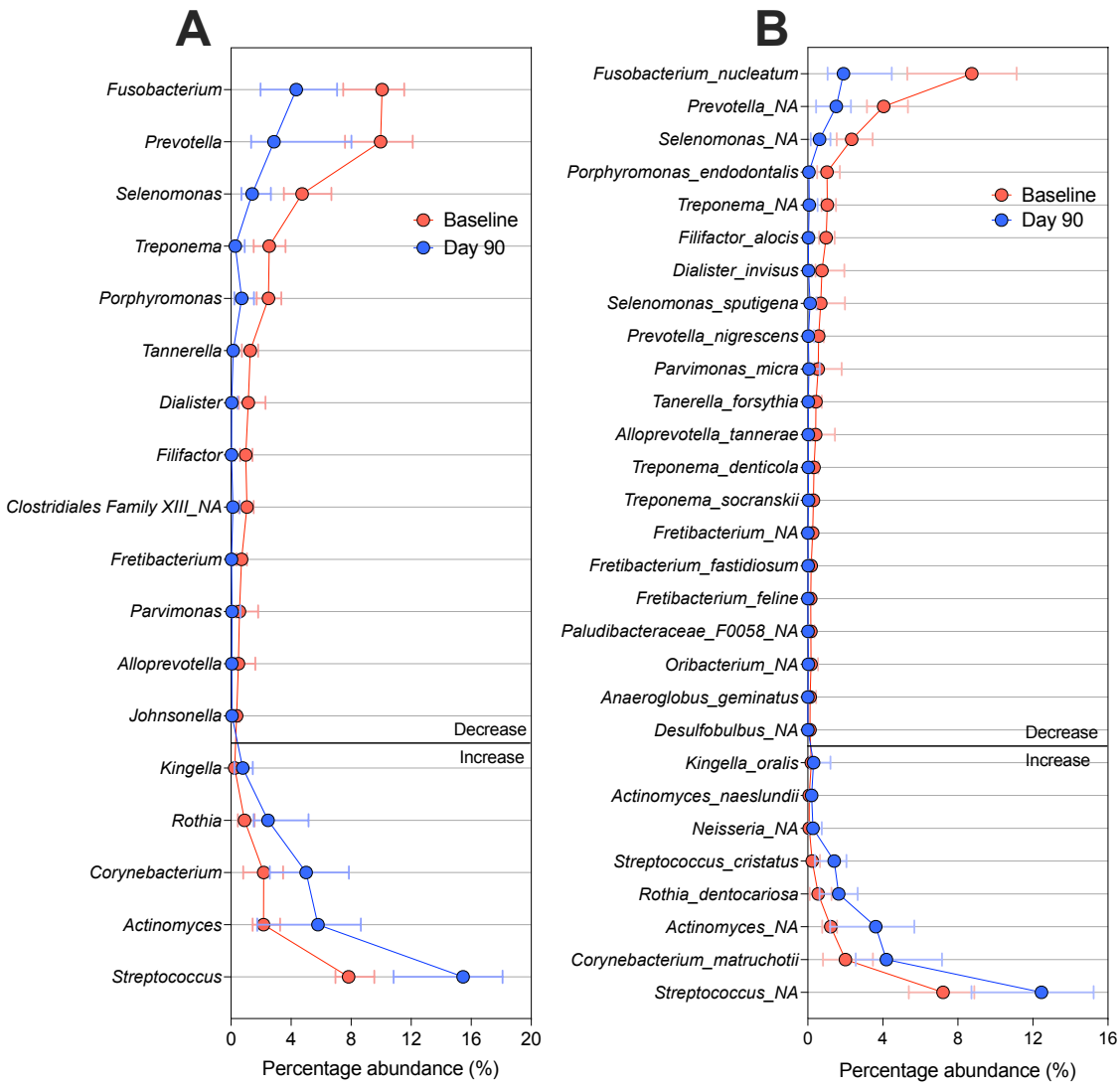

Figure 3.17. Percentage abundance of genera (A) and species (B) with the largest alterations following treatment. This threshold was set at significantly different and median >0.25% difference for genera and >0.1% difference for species between timepoints. Statistics are Wilcoxon-signed rank test, all displayed genera and species are p(adjusted)<0.05. Data are shown as median ± 95% confidence interval. N=42.

3.3.3.9. Longitudinal alterations in grouped complexes

PD is associated with dysbiosis of the subgingival plaque microbiome, with groups of bacteria believed to contribute to disease development and shift the subgingival plaque from a 'eubiotic' to a dysbiotic state. As such, groups of bacteria, rather than individual species, have been suggested as microbial biomarkers. To confirm that the reductions observed throughout the current study following treatment were indicative of a health-associated, rather than random microbial shift, various predefined bacterial groups were compared at baseline and day 90 (**table 3.5**).

Historically, Socransky *et al.*, identified distinct complexes and assigned each a colour (red, orange, green, purple, blue and yellow) depending on their strength of association with periodontal disease (Socransky et al., 1998). Red and orange complexes contain groups of disease-associated organisms, whilst the green, purple, blue and yellow complexes contain species associated with a commensal microbiome. Following treatment there were significant reductions in the abundance of the red and orange complexes ($p<0.001$ for both). No differences were observed in green and purple complexes, whilst significant increases were found in the blue ($p<0.01$) and yellow ($p<0.001$) complexes at day 90.

A more recent review by Perez-Chaparro *et al.*, identified a more complex community of disease associated bacteria (Pérez-Chaparro et al., 2014). When grouped together, there was a similar reduction in the abundance of these species following treatment ($p<0.001$). Thus, whilst comparison with subgingival plaque from periodontally healthy subjects was not available during this study, a reduction in disease-associated complexes and an increase in complexes associated with health was observed. Together, these data represent a clear health associated shift in the subgingival plaque microbiome composition following NSPT.

Table 3.5. The abundance of health and disease associated species (n=42). Abundances are median (Q1, Q3).

Bacterial species	Baseline (%)	Day 90 (%)	Adjusted p-value
Red complex[1]			
Porphyromonas_gingivalis	1.396 (0.491, 4.965)	0.059 (0.000, 0.626)	0.0005
Treponema_denticola			
Tannerella_forsythia			
Orange complex[1]			
Prevotella_intermedia	12.440 (8.151, 18.52)	3.287 (1.366, 12.970)	0.0009
Prevotella_nigrescens			
Fusobacterium_nucleatum			
Fusobacterium_periodonticum			
Parvimonas_micra			
Campylobacter_gracilis			
Streptococcus_constellatus			
Campylobacter_showae			
Green complex[1]			
Aa[#]	0.999 (0.456, 3.534)	1.079 (0.360, 3.049)	0.8430
Eikenella_corrodens			
Capnocytophaga_ochracea			
Capnacytophaga_gingivalis			
Capnocytophaga_sputigena			
Purple complex[1]			
Actinomyces_odontolyticus	1.160 (0.503, 2.761)	0.790 (0.329, 1.716)	0.1458
Veillonella_parvula			
Blue (*Actinomyces*) complex[1]			
Actinomyces_cardiffensis	2.162 (1.023, 4.040)	5.768 (1.417, 10.560)	0.0053
Actinomyces_israelii			
Actinomyces_graevenitzii			
Actinomyces_meyeri			
Actinomyces_hongkongensis			
Actinomyces_oris			
Actinomyces_gerencseriae			
Actinomyces_NA			
Actinomyces_naeslundii			
Actinomyces_massiliensis			
Actinomyces_georgiae			
Yellow complex[1]			
Streptococcus_NA	7.832 (5.702, 12.700)	15.460 (10.280, 19.910)	0.0002
Streptococcus_cristatus			
Streptococcus_anginosus			
Streptococcus_gordonii			
Streptococcus_mutans			
Streptococcus_oralis			
Streptococcus_parasanguinis			
Streptococcus_sobrinus			
Streptococcus_salivarius			
Streptococcus_sinensis			
Streptococcus_massiliensis			

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Table 3.4. continued…

Bacterial species	Baseline (%)	Day 90 (%)	Adjusted p-value
Novel disease-associated species[2]			
Treponema_medium	8.472 (3.296, 13.610)	1.542 (0.055, 5.057)	0.0002
Peptostreptococcus_stomatis			
Prevotella_denticola			
Mogibacterium_timidum			
Filifactor_alocis			
Selenomonas_sputigena			
Alloprevotella_tannerae			
Anaeroglobus_geminatus			
Fretibacterium_fastidiosum			
Porphyromonas_endodontalis			
Treponema_vincentii			
Treponema_lecithinolyticum			
Dialister_pneumosintes			

[1]As outlined in Socransky et al., (1998)
[2]As outlined in Perez-Chaparro et al., (2014), excluding those previously classified[1]
FDR corrected Wilcoxon signed-rank test; adjusted p-values are displayed.
[#]Aa = *Aggregatibacter actinomycetemcomitans*.

3.3.3.10. Association network construction: Correlation circle plots

Having evaluated compositional shifts, bacterial association networks were constructed to evaluate change in the overall community structure, and gain insight into the subgingival plaque ecology in PD. Networks were constructed using a method outlined previously (González et al., 2012) utilising correlation circle plots derived from PCAs. Both genera (**figure 3.18**) and species (**figure 3.19**) were analysed at baseline and day 90.

This method plots variables (genera/species) onto correlation circle plots with two radii of 0.5 (inner circle) and 1.0 (outer circle) around a standardised centre point. The association between two genera or species can then be determined by calculating the cosine angle between each variable and the centre point. If the angle is sharp (45°) then a positive association exists, if the angle is obtuse (180°) then a negative correlation exists and if a right angle is present then no association exists (cos[90]=0).

Given that variables are standardised around the centre point, the greater the distance to the origin, the stronger the relationship between variables. This method allows computation of positive and negative associations between different genera and species, without reliance on parametric (Pearson's) or non-parametric (Spearman's) correlation coefficients, which may be skewed by matching '0' data that is common in microbiome datasets (Faust and Raes, 2016). Throughout the current analysis, only negative associations <-0.4 and positive associations >0.4 were considered in network analysis. As such, the majority of genera and species fell between the two radii when plotted on correlation circle plots.

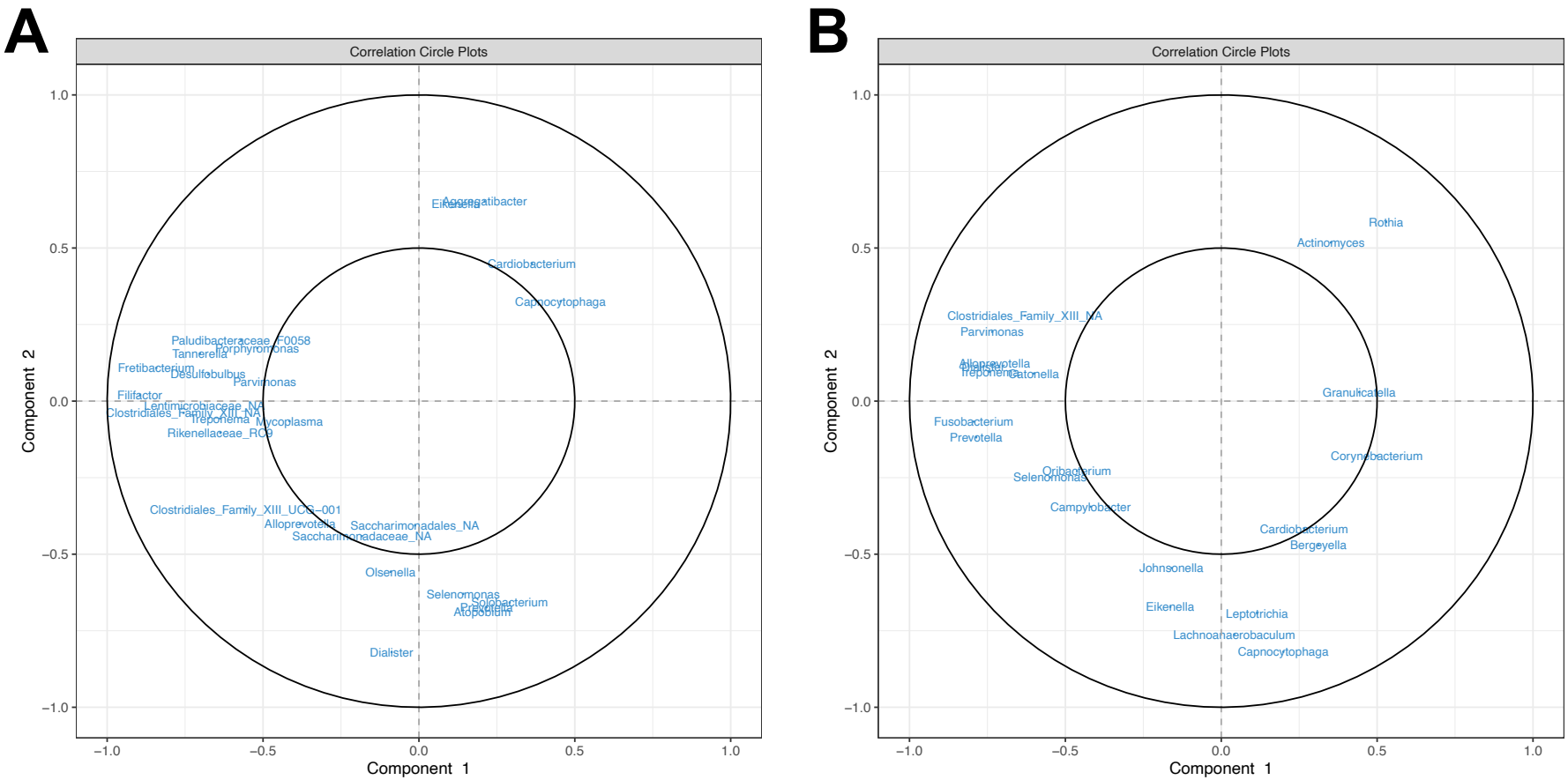

Figure 3.18. Correlation circle plots for genus-level association networks. Association between genera were obtained based on their projection onto a correlation circle plot derived from a principal component analysis, as described by González et al (2012). This was performed at baseline (A) and day 90 (B).

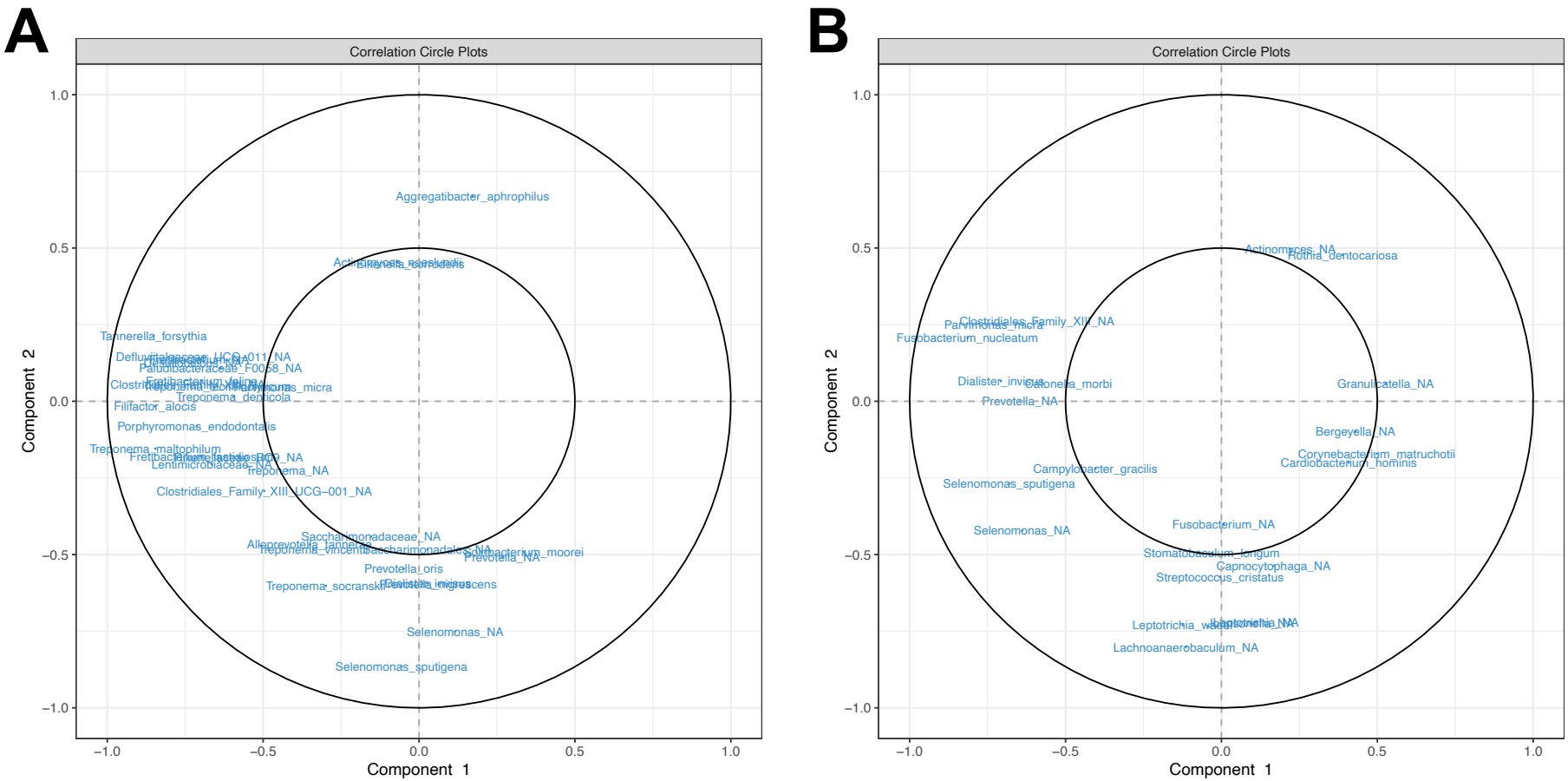

Figure 3.19. Correlation circle plots for species-level association networks. Association between species were obtained based on their projection onto a correlation circle plot derived from a principal component analysis, as described by González et al (2012). This was performed at baseline (A) and day 90 (B).

Networks were next constructed to visualise associations between different genera and species at each timepoint. At baseline there were two clear disease-associated clusters consisting of anaerobic genera, highlighting the polymicrobial aetiology of PD (**figure 3.20A**). The first pre-treatment network consisted of *Prevotella, Selenomonas, Dialister, Solobacterium, Olsenella* and *Atopobium,* whilst *Eikenella* and *Aggregatibacter* showed negative associations with some of these genera (**figure 3.20A**, red lines). Within the second cluster, *Filifactor* and *Fretibacterium* were central players, both correlating positively with all three red-complex genera (i.e., *Porphyromonas*, *Treponema, Tannerella*). After treatment (**figure 3.20B**), a new disease-associated network was observed consisting of *Treponema, Prevotella, Parvimonas, Fusobacterium, Alloprevotella, Selenomonas, Dialister* and *Catonella*. Importantly, *Rothia* showed negative associations with *Selenomonas, Fusobacterium* and *Prevotella* of this cluster, whilst a positive correlation was found between *Rothia* and *Actinomyces*.

Similar results were observed at species-level, where *Filifactor alocis*, *Fretibacterium fastidiusum* and *Fretibacterium feline* were central in a closely associated network of anaerobic bacteria, forming strong connections to various PD-associated organisms at baseline (**figure 3.21A**). Following treatment (**figure 3.21B**), *Rothia dentocariosa* negatively associated with unclassified *Selenomonas* and *Lachnoanaerobaculum*, whilst *Corynebacterium matruchottii* showed negative associations with *Fusobacterium nulceatum* and *Parvimonas micra,* two orange-complex species.

Clear alterations were observed in the abundance of specific genera/species and the overall community structure following treatment. However, there appeared to be an anaerobic core present in day 90 samples which was particularly evident at genus-level. *Fusobacterium, Prevotella, Treponema, Parvimonas* and *Catonella* have been shown to increase in PD compared with periodontally healthy patients (Griffen et al., 2012, Abusleme et al., 2013), and our data suggest that whilst reduced, these genera do persist and highly associate with one another following NSPT.

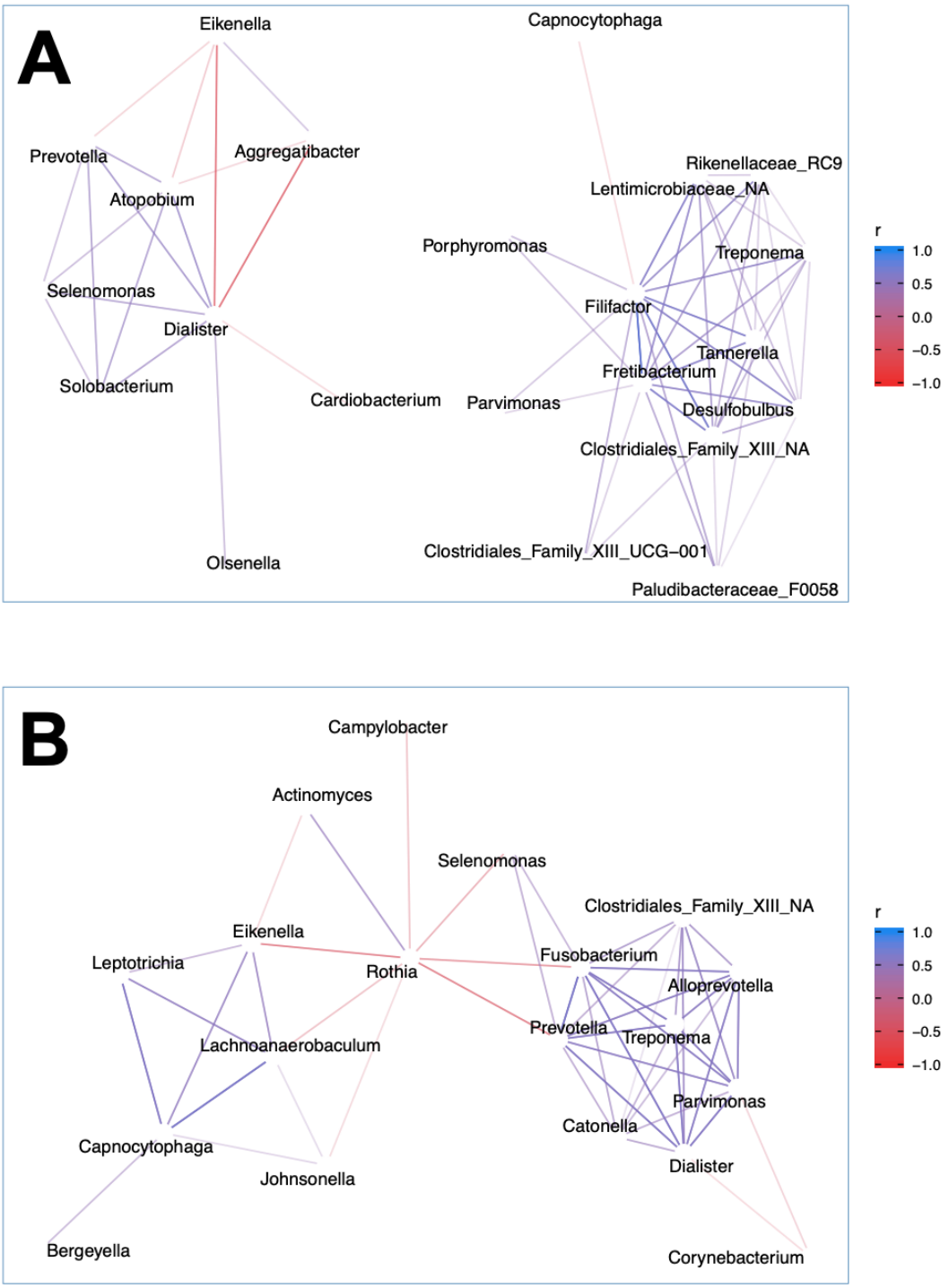

Figure 3.20. Genus-level association networks. Associations between abundant genera at baseline (A) and day-90 (B) based on correlation circle plots derived from principal component analysis. Only associations <-0.4 (negative associations) and >0.4 (positive associations) are shown.

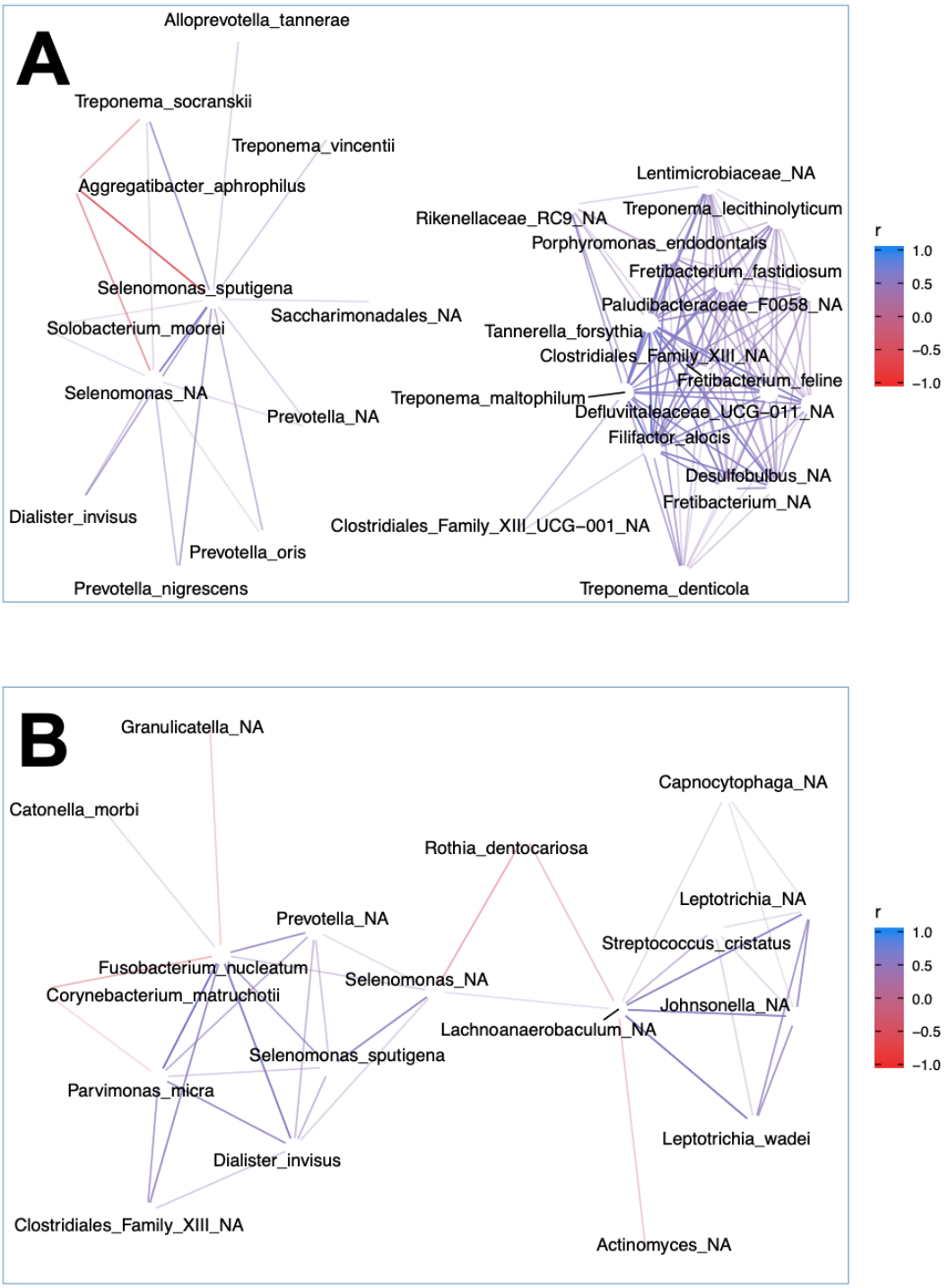

Figure 3.21. Species-level association networks. Association networks between abundant genera at baseline (A) and day-90 (B) based on correlation circle plots derived from principal component analysis. Only associations <-0.4 (negative associations) and >0.4 (positive associations) are shown.

3.3.4. Is the microbiome associated with treatment response?

Throughout this chapter thus far, the local microbial and inflammatory alterations following NSPT have been evaluated in 42 patients. Although widespread clinical improvement was recorded, there remained some degree of heterogeneity in the extent of the clinical response. The ability to identify patients with a poor response to NSPT at an early stage and modify their treatment plan accordingly could be hugely beneficial for patients, as this may allow incorporation of more regular maintenance visits and/or additional treatment adjuncts. Such targeted, precision medicine-type additions to periodontology carries potential to improve the overall standard of care.

3.3.4.1. Classifying patients site-specific treatment response

As previously described (**section 2.3.2.3**), several criteria have been employed by previous studies to evaluate treatment success in PD patients. Most of these have focussed on full-mouth parameters normally involving varying cut-offs for residual 'deep' periodontal pockets (≥4 mm or ≥5 mm) to investigate the relationship between different inflammatory or microbial biomarkers with treatment response. From a microbial perspective, subgingival plaque is often collected from a single periodontal pocket, as was the case in the current study. Therefore, the microbiota under study is that of a specific subgingival microenvironment, and whilst there may be some degree of overlap, classifying patients based on full-mouth parameters may not truly capture this site-level clinical change.

As an exploratory investigation into whether the baseline subgingival plaque microbiota may associate with treatment response, patients were split depending on whether the outcome of 'pocket closure' was achieved in sampled sites. Pocket closure is defined as the conversion of a periodontal site measuring ≥5 mm to a site measuring ≤4 mm and is a clinically meaningful threshold which determines the need for further active treatment (Badersten et al., 1984, Suvan et al., 2019). Sites <5 mm were unavoidably sampled in two patients at baseline, and these patients were excluded from this current exploratory analysis. Of the sites evaluated (n=40), 21 sites achieved pocket closure at day 90 and were termed 'responding', whilst 19 sites did not and were termed 'non-responding'. Comparing patients from which responding and non-responding sites were

sampled, there were no differences in baseline demographic (age, sex), clinical (site PPD, CAL and number of teeth) or behavioural (smoking) variables (**table 3.6**). Trends were observed in which the number of treatment visits appeared slightly higher for those with responding sites (p=0.08), and molar distribution appeared higher in the non-responding group (p=0.06) – both of which must be considered when interpreting the data.

Table 3.6. Demographic, behavioural and clinical variables according to whether pocket closure was achieved in sites selected for subgingival plaque sampling. Data are presented as median (25^{th}, 75^{th} percentile) or n (%) at baseline.

Variable	Site-specific pocket closure		p-value (NR vs. R)
	Non-responding (n=19)	Responding (n=21)	
Age in years	47.00 (39.00, 57.00)	52.00 (40.50, 62.50)	0.19†
Sex: Male, female (n, %)	4 (21.1%), 15 (78.9%)	7 (33.3%), 14 (66.7%)	0.49‡
Smoking: Never, former, current (n, %)	7 (36.8%), 6 (31.6%), 6 (31.6%)	8 (38.1%), 9 (42.9%), 4 (19.0%)	0.62#
Treatment visits (median, range)	3.00 (1.00 – 6.00)	4.00 (2.00 – 6.00)	0.08†
Number of teeth	27.00 (26.00 – 29.00)	27.00 (24.00 – 28.00)	0.54†
Molar, non-molar (sampled sites)	12 (66.7%), 7 (33.3%)	6 (33.3%), 15 (66.7%)	0.06‡

Statistics refer to unpaired t-test (†), Fisher's exact test (‡) or Chi-squared analysis (#). NR: Non-responding, R: Responding.

3.3.4.2. Clinical differences according to treatment response

When evaluating treatment response, it is important to establish whether 'responding' and 'non-responding' sites displayed similar levels of disease severity at baseline. To rule out the possibility that non-responding sites simply started with higher disease severity, site-specific PPD and CAL were assessed. Both variables followed a normal distribution at each timepoint, and were compared between groups at day 90, adjusting for baseline levels using a GLM (**figure 3.22A,B**). This analysis demonstrated significantly greater clinical improvement in responding compared with non-responding sites for both PPD and CAL ($p<0.001$ for both), irrespective of baseline disease. Importantly, longitudinal analysis showed significant reductions in both PPD and CAL for both groups ($p<0.001$ for both). Thus, even non-responding sites did display significant, albeit less pronounced, clinical improvement following treatment.

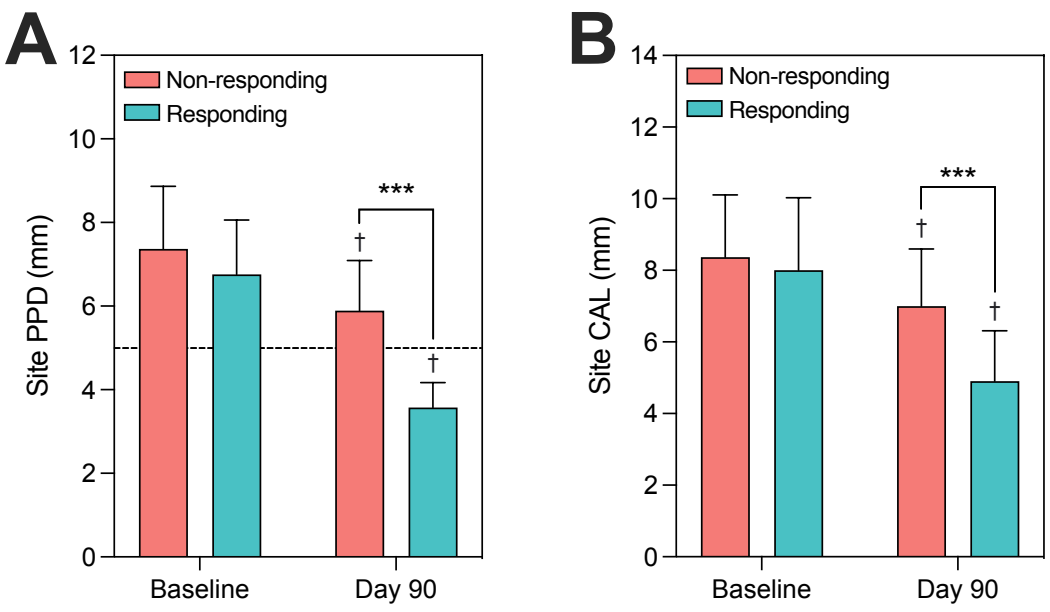

Figure 3.22. Clinical response of sampled sites. PPD (A) and CAL (B) of sampled sites at baseline and day 90 in responding (n=21) and non-responding (n=19) sites. Dotted black line indicates 5 mm. Between group comparisons refer to a GLM comparing day 90 variables adjusted for baseline PPD and CAL (***$p<0.001$), within-group comparisons refer to paired t-tests (†$p<0.001$), graphs display mean ± standard deviation.

3.3.4.3. Evaluating the microbiota between groups

To evaluate whether the baseline subgingival plaque microbiota was associated with treatment response, α-diversity indices (Shannon, ACE) were compared between groups at each timepoint. It has been suggested that the diversity of the subgingival plaque may positively correlate with treatment response when utilising full-mouth CAL as an indicator of clinical improvement (Bizzarro et al., 2016). In the current study, no differences in α-diversity were observed between responding and non-responding sites at baseline or day 90 (**figure 3.23A,B**). Each group showed a reduction in both indices following treatment, matching with reductions observed across the entire cohort.

Analysis of ß-diversity yielded similar results. Genus- and species-level Bray-Curtis PCoAs were constructed at baseline and day 90 to compare the overall microbial composition between each group (**figure 3.24A-D**). The corresponding ANOSIM suggested no differences between groups at baseline (p=0.77 genus-level and p=0.70 species-level) or day 90 (p=0.26 genus-level, p=0.13 species-level). This data suggests that α- and ß-diversity were comparable between treatment response groups at baseline. Surprisingly, these results were consistent at day 90, despite differing clinical profiles.

Although ß-diversity is a useful technique for visualising factors which contribute to variation in the microbiome, it is heavily influenced by large-scale variations. Therefore, this analysis was supplemented with differential abundance testing using univariate Mann-Whitney U tests comparing groups at each timepoint. No alterations in the abundance of any genus or species were recorded when comparing groups at baseline or day 90 (**figure 3.25**). This result remained unchanged if the linear discriminant analysis effect size (LEfSe) method – a commonly used approach for microbiota biomarker analysis - was employed between groups at either timepoint (Segata et al., 2011).

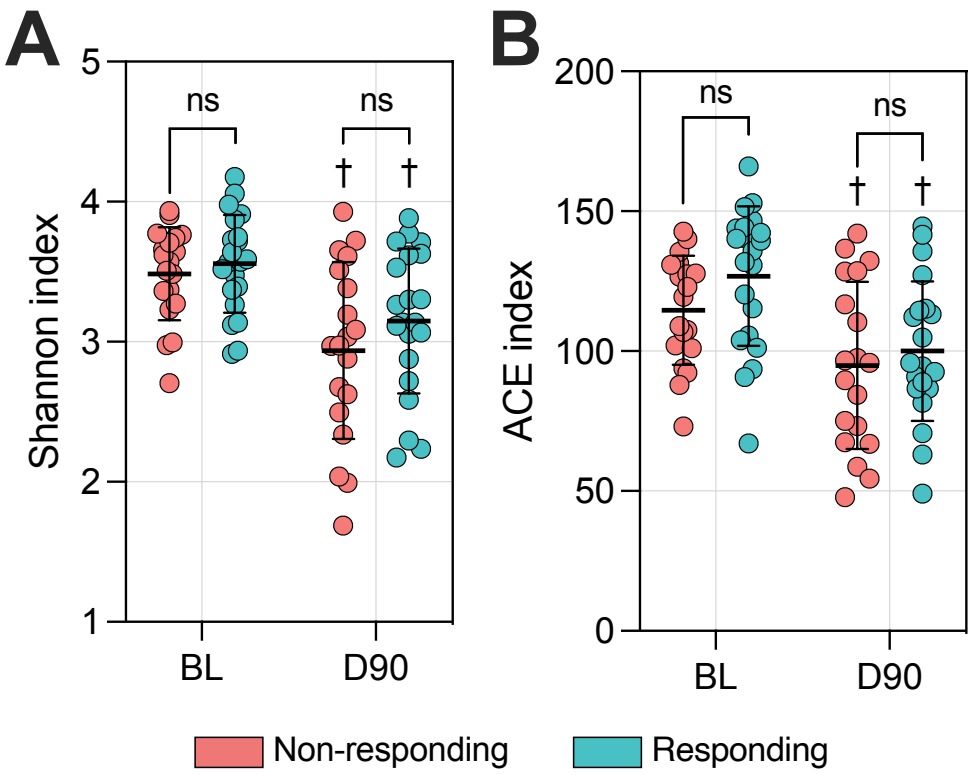

Figure 3.23. α-diversity by treatment response. Shannon (A) and ACE (B) indexes in responding (n=21) and non-responding (n=19) sites. Analysis was performed at baseline (BL) and day 90 (D90). Individual values shown with lines displaying mean ± standard deviation. Between group comparisons refer to unpaired t-tests (ns: non-significant), within-group comparisons refer to paired t-tests (†p<0.001).

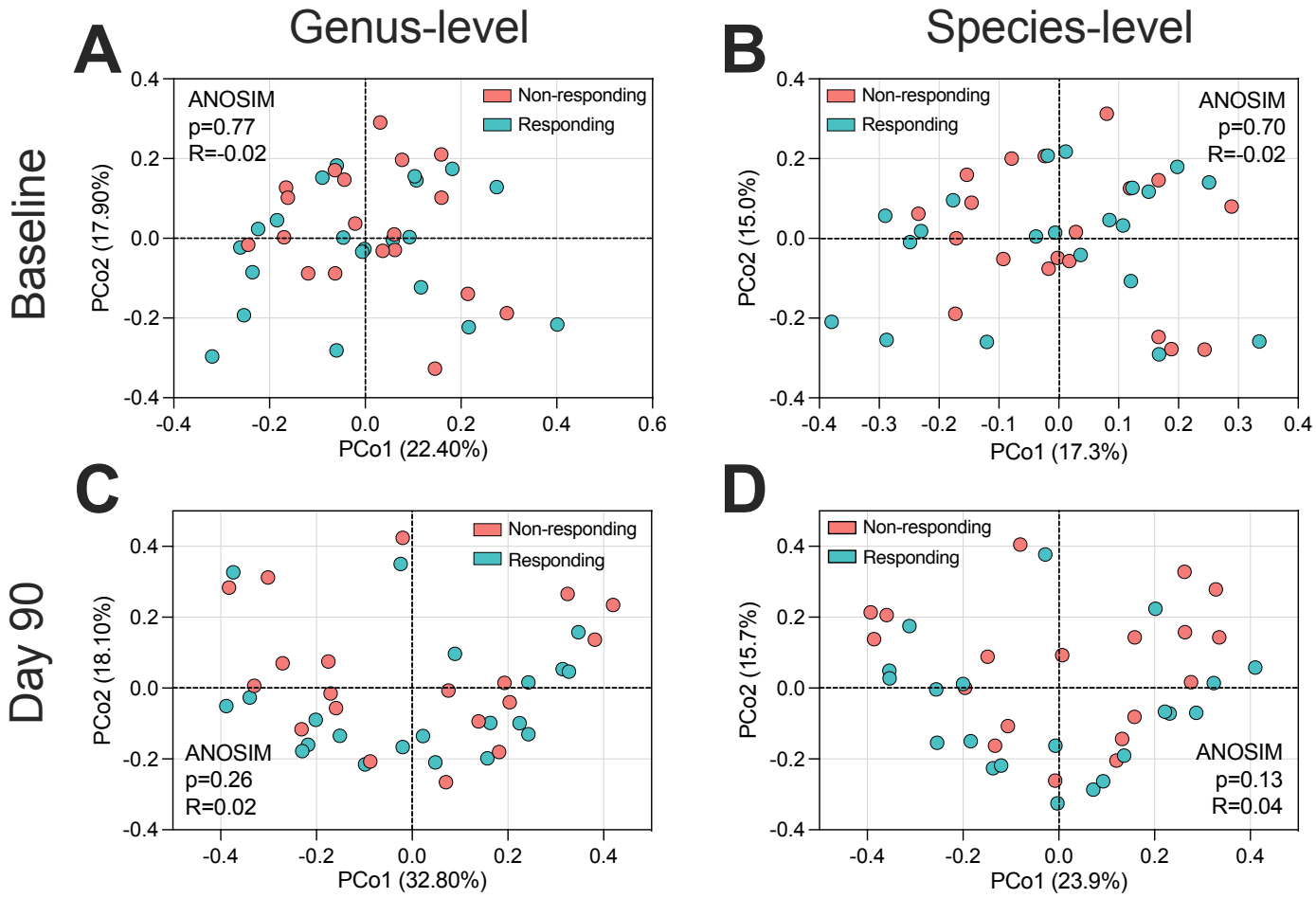

Figure 3.24. ß-diversity by treatment response. Bray-Curtis based PCoAs comparing responding (n=21) and non-responding (n=19) sites. Genus- (A,C) and species-level (B, D) Bray-Curtis based PCoA of each group at baseline (A,B) and day 90 (C,D). Statistics are ANOSIM with raw p- and R-values displayed.

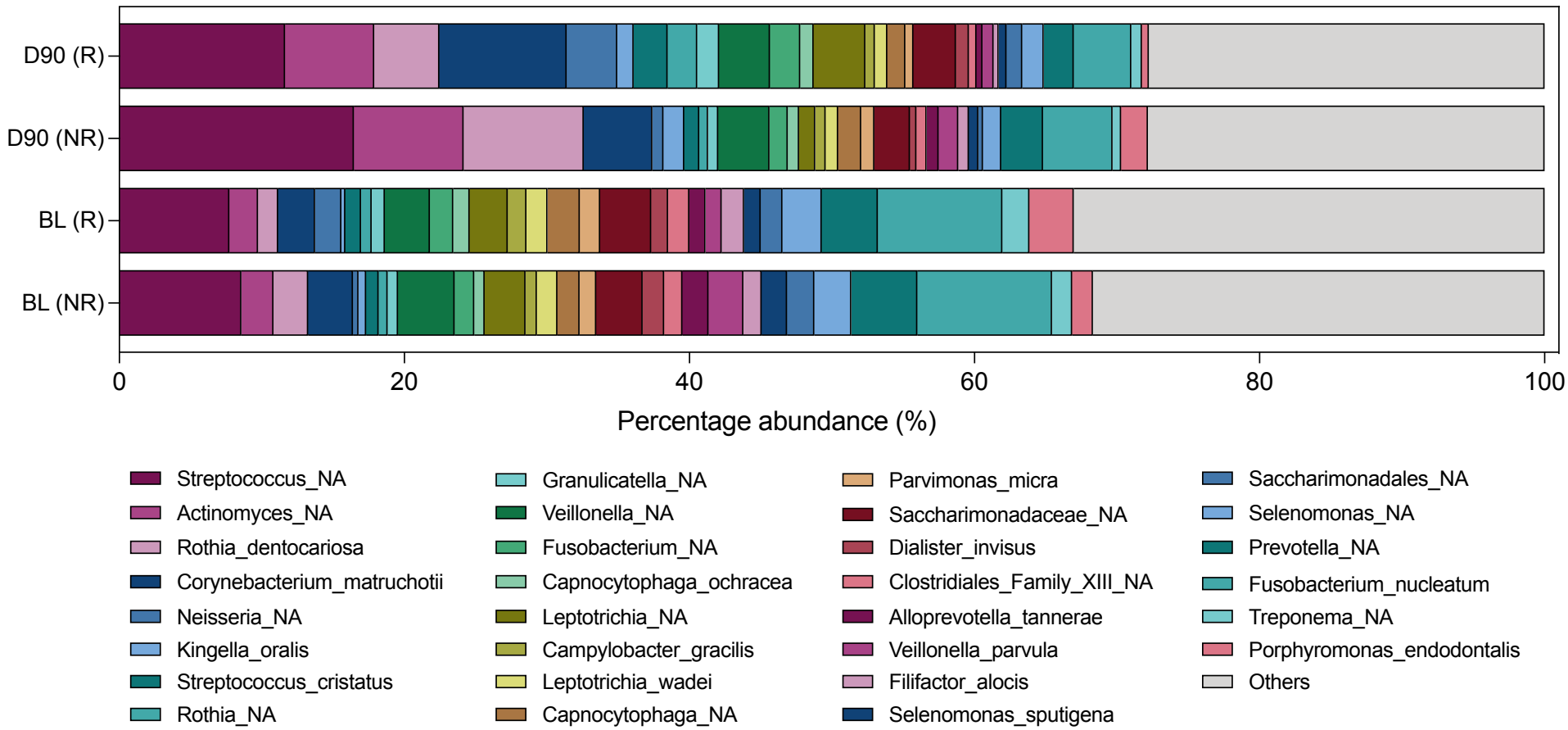

Figure 3.25. Plotting the species composition at baseline (BL) and day (D90) across treatment outcome groups. Patients were split according to whether pocket closure was achieved (n=21) or not achieved (n=19) in sites sampled for subgingival plaque. The top 30 most abundant species are displayed, with all others grouped together in grey as 'Others'.

3.3.4.4. Colour complexes as markers of treatment response

While a large advantage of the current study was the incorporation of an open-ended technique (16S rRNA sequencing), it has been suggested that bacteria within the subgingival plaque act as a consortium to drive disease progression. As such, numerous studies focus solely on species within known health and disease-associated complexes. Previous work has suggested that levels of *P. gingivalis* and *T. denticola*, both red-complex members (Eick et al., 2017), or *P. intermedia* and *P. micra*, two orange-complex members (Mombelli et al., 2017), negatively predict treatment response in PD patients. Therefore, the conventional Socransky colour complexes and the more recent disease-associated group outlined by Perez-Chaparro were compared at baseline and day 90 (Socransky et al., 1998, Pérez-Chaparro et al., 2014).

From this analysis, it was hypothesised that an elevated abundance of disease associated species at baseline may be capable of differentiating clinical response groups. However, no differences were observed for any microbial complex at baseline (all $p>0.05$, Mann-Whitney U test, **figure 3.26A,B**), which remained consistent following treatment (**figure 3.26C,D**). As expected, disease-associated species only equated to a minimal proportion of the day 90 microbiota within both groups and no differences were observed between groups. Other complexes (blue, yellow) were present in far higher abundances after treatment, although no significant alterations were observed in the blue (R[3.14%] vs. NR[8.07%], $p=0.59$) or yellow-complex (R[14.00%] vs. NR[16.67%], $p=0.59$). Collectively, this pilot exploratory analysis suggests that the baseline subgingival plaque microbiota was unable to differentiate responding and non-responding sites either prior to, or following, NSPT.

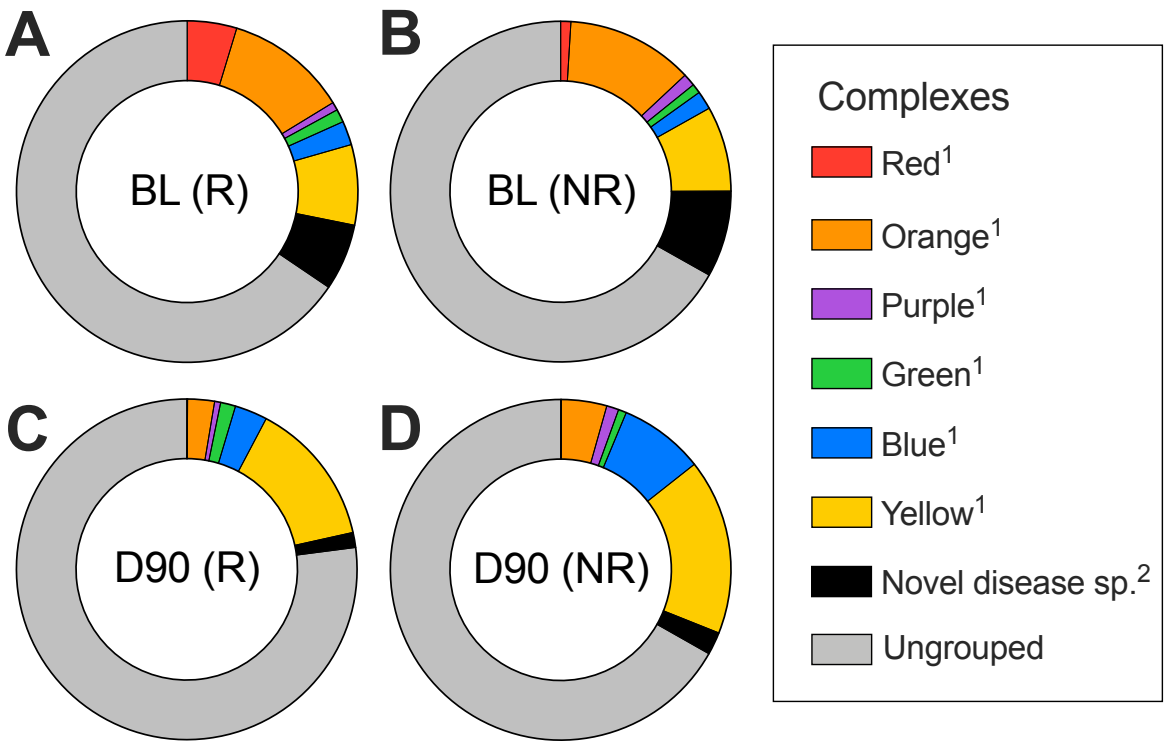

Figure 3.26. Grouped complexes in treatment response groups. Graphs display the median abundance of different bacterial complexes associated with health and disease between responding (n=21) and non-responding (n=19) sites. Displaying the median abundance of complexes identified by [1]Socransky *et al.* and [2]Perez-Chaparro *et al.* at baseline (BL; A, B) and day 90 (D90; C, D). No significant differences were observed between groups at either timepoint using Mann-Whitney U test. R; Responding, NR; Non-responding.

3.3.4.5. Microbiota similarity between treatment success groups

No individual feature of the subgingival plaque microbiome was associated with the clinical response of sampled sites. Previous work has suggested that inter-patient variation in the subgingival plaque microbiota may mask consistent alterations following treatment, or compositional differences between groups of patients (Schwarzberg et al., 2014). Although consistent alterations following treatment were observed in the current study, this variation may potentially have a larger impact on this subgroup analysis with smaller sample sizes.

To overcome this issue, Bray-Curtis similarity was computed for every patient. This metric compares the similarity of each day 90 sample with the corresponding baseline sample and is the basis of PCoA analysis described previously (comparing between sites). Similar analysis has been applied to the salivary microbiome to delineate microbial markers of treatment response (Greenwood et al., 2020). Based on this analysis, it was hypothesised that samples collected from non-responding sites would be more resilient to largescale alterations and thus may display a post-treatment microbiome with a higher similarity to baseline, irrespective of inter-patient variation in exact compositions.

Both responding and non-responding sites displayed comparable genus- and species-level similarity with baseline (**figure 3.27A-B**, p=0.79 and p=0.77, Mann-Whitney U test), with large variations in each group. For example, species-level Bray-Curtis similarity values ranged from 0.20 to 0.61 in non-responding sites, and from 0.09 to 0.68 in responding sites. Therefore, some sites with minimal clinical improvement underwent large microbial alterations following treatment, whereas other sites with large clinical improvement displayed a microbiome more similar with baseline.

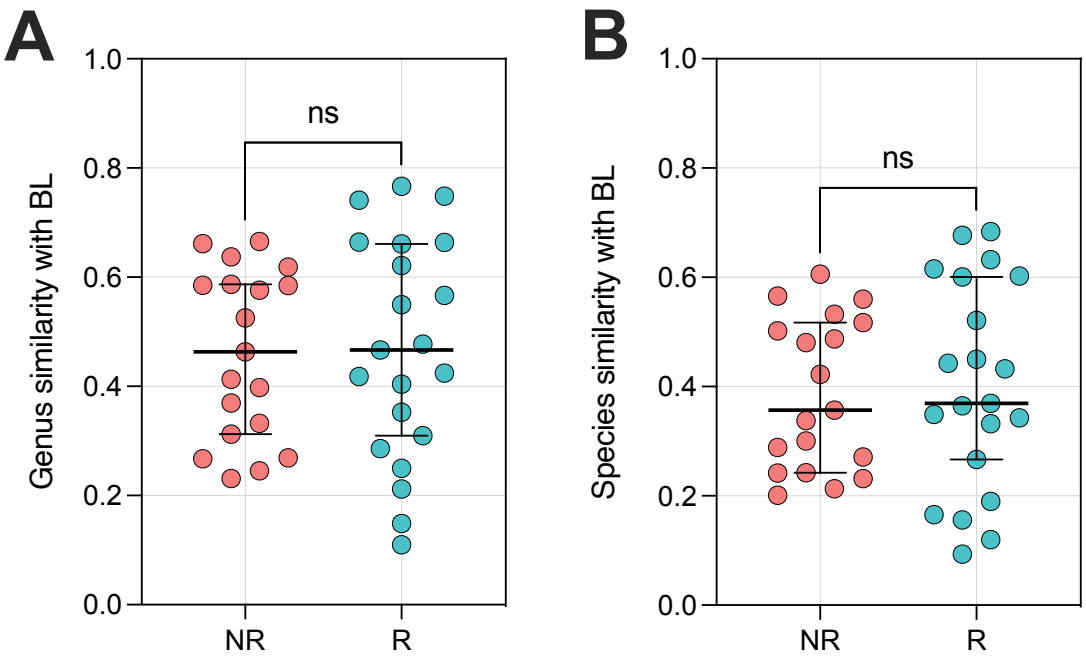

Figure 3.27. Bray-Curtis similarity of day 90 samples with paired baseline samples in responding and non-responding sites. Similarity was calculated at genus- (A) and species-level using the inverted Bray-Curtis index, where a value of 1 is the highest similarity and 0 is the lowest similarity with baseline. Statistics refer to Mann-Whitney U test, ns: non-significant. NR: Non-responding, R: Responding.

3.3.5. Are salivary cytokines associated with treatment response?

Much attention has been paid to the utilisation of subgingival plaque bacteria as prognostic markers of treatment response, however the extrapolation of any subgingival microbial marker to full-mouth level on a large-scale represents a difficult technical and analytical challenge. Additionally, our data suggest that the baseline subgingival plaque microbiome is not associated with the clinical response to NSPT within the limitations of the current analysis. In contrast to subgingival plaque, saliva can be easily collected and analysed using high-throughput assays, making it a much more suitable sample type for clinical diagnostic and/or risk assessment purposes. Throughout this chapter, salivary IL-1ß showed positive associations with periodontal clinical parameters and a link between salivary IL-1ß and treatment response has also been previously reported (Lee et al., 2018). Therefore, this exploratory analysis aimed to expand upon previous work and explore the associations between salivary IL-1ß and treatment outcome. Additionally, other commonly assessed cytokines (TNFα, IL-6, IL-8, IL-17A) were investigated. It was hypothesised that patients who respond poorly to treatment may exhibit a differing salivary cytokine profile from those with a high treatment response at baseline, and thus salivary cytokines may be employed prognostically.

3.3.5.1. Classifying full-mouth treatment response

Salivary cytokines are thought to reflect the full-mouth inflammatory status (Jaedicke et al., 2016, Taylor, 2014), which is supported by earlier results in this chapter. Therefore, it is possible to assess treatment response using full-mouth clinical variables and form a more accurate clinical profile in comparison with site-specific values used for subgingival plaque analysis. To assess full-mouth treatment response, PISA was selected as an appropriate clinical indicator given the association with other periodontal parameters and local inflammatory markers observed within **chapter 2**, and the nature of cytokines in saliva samples. In order to accurately classify patients and ensure a similar level of baseline disease severity, only patients with PISA scores commensurate with at least moderate PD (baseline PISA >521.58 mm^2, n=36) using previously published thresholds were included (Leira et al., 2018). Inclusion of patients with moderate to severe disease has been previously utilised in studies assessing

periodontal treatment success (Eick et al., 2017), and excludes bias introduced by patients with lower starting levels of disease severity. In the current study, 6 patients did not meet this PISA threshold and were excluded from this analysis.

The remaining 36 patients were classified into high and low treatment response using median percentage PISA improvement (88.67%). Patients with a percentage improvement <median were termed low responders (n=18), and >median termed high responders (n=18). Low responders had PISA improvement scores of 75.90%, compared with 93.10% in the high responders. No differences in the age, sex, smoking status or number of teeth were observed between each group at baseline (**table 3.7**).

Table 3.7. Demographic, behavioural and clinical variables at baseline according to treatment response (determined by PISA improvement). Data are presented as median (25^{th}, 75^{th} percentile) or n (%). N=36.

Variable	PISA improvement		p-value (Low vs. High)
	Low (n=18)	High (n=18)	
Age in years	47.00 (39.00, 57.00)	51.00 (37.75, 63.25)	0.38†
Sex: Male, female (n, %)	7 (38.9%), 11 (61.1%)	5 (27.8%), 13 (72.2%)	0.72‡
Smoking: Never, former, current (n, %)	6 (33.3%), 9 (50%), 3 (16.7%)	8 (44.4%), 6 (33.3%), 4 (22.2%)	0.59#
Treatment visits (median, range)	4.00 (2.00 – 6.00)	3.00 (1.00 – 6.00)	0.13†
Number of teeth	27.00 (26.00, 28.25)	26.50 (23.00, 28.00)	0.34†

Statistics refer to unpaired t-test (†), Fisher's exact test (‡) or Chi-squared analysis (#).

3.3.5.2. Full-mouth clinical parameters between groups

Full-mouth clinical parameters were compared between groups to confirm the classification of 'high' and 'low' response to treatment. This analysis was performed at day 90 using GLMs to adjust for the baseline level of each variable. Both CAL and PPD displayed normal distribution upon visual inspection of histograms and were directly compared, whereas FMPS, FMBS, pockets ≥5mm and PISA were initially ln-transformed and shown to follow a ln-normal distribution.

Nearly all full-mouth parameters were significantly lower at day 90 within the 'high-response' group when adjusting for baseline disease severity. As expected, PISA displayed highly significant differences between groups ($p<0.001$ for both, GLM), indicating higher levels of residual inflammation in the 'low response' group following treatment (**table 3.8**). An important finding was that these clinical differences were not limited to PISA, with significant differences observed between groups for FMBS, PPD and pockets ≥5 mm (all $p<0.05$, GLM).

Whilst no significant finding was observed between groups for FMPS, a trend was observed with higher median FMPS observed for 'low-responders' at day 90 ($p=0.085$, GLM). In contrast, CAL was remarkably similar between groups at day 90 and showed no significant differences when adjusting for baseline ($p=0.94$, GLM). These data are unsurprising given that both FMPS and CAL showed the weakest associations with PISA within **chapter 2**. Despite this, significant differences between groups for PISA, FMBS, PPD and pockets ≥5 mm support the use of percentage PISA improvement as an appropriate indictor of full-mouth treatment response.

Table 3.8. Comparing full-mouth clinical parameters at baseline and day 90 according to treatment response (determined by PISA improvement). Data are presented as median (25th, 75th percentile). N=36.

Variable	Timepoint	PISA improvement		p-value† (Low vs High)
		Low (n=18)	High (n=18)	
FMPS (%)	Baseline	72.50 (62.25, 74.25)	71.50 (49.25, 80.25)	0.085
	Day 90	17.50 (9.75, 37.50)	12.00 (7.75, 16.25)	
FMBS (%)	Baseline	68.50 (46.50, 81.75)	64.50 (38.25, 74.75)	**<0.001**
	Day 90	16.50 (13.25, 26.25)	4.50 (2.75, 8.00)	
CAL (mm)	Baseline	4.50 (3.93, 5.32)	4.38 (3.73, 5.41)	0.94
	Day 90	3.73 (3.18, 4.33)	3.72 (3.34, 4.28)	
PPD (mm)	Baseline	3.70 (3.42, 4.36)	3.74 (3.17, 4.20)	**0.019**
	Day 90	2.95 (2.77, 3.31)	2.67 (2.39, 2.87)	
Pockets ≥5 mm (%)	Baseline	32.50 (22.00, 47.25)	29.00 (18.50, 40.75)	**0.017**
	Day 90	11.00 (9.00, 23.00)	7.50 (4.75, 10.00)	
PISA (mm^2)	Baseline	1301.00 (1074.00, 1912.00)	1327.00 (830.20, 1687.00)	**<0.001**
	Day 90	334.50 (171.90, 564.70)	67.05 (42.30, 101.40)	

† General linear model adjusting for baseline levels of each variable. FMPS, FMBS, Pockets ≥5 mm and PISA were ln transformed. FMPS; full-mouth plaque score, FMBS; full-mouth bleeding score, CAL; clinical attachment level, PPD; periodontal probing pocket depth, Pockets ≥5mm; percentage of pockets ≥5mm, PISA; periodontal inflamed surface area.

3.3.5.3. Receiver operating characteristic curve for salivary cytokines

To establish whether any of the five salivary cytokines evaluated in this study were associated with treatment response, ROC curves were generated using baseline concentrations. This method has been previously used to study the ability of different salivary markers to discriminate between high and low responders to NSPT (Sexton et al., 2011, Grande et al., 2020). ROC curves plot the sensitivity (true positive rate) and 1-specificity (false positive rate) at various cut-off values. From here, an area under the curve (AUC) is generated ranging from 0-1, which can be used to evaluate classification accuracy (Fawcett, 2006).

Within the current study, ROC curves were generated for all five salivary cytokines. This was initially performed using a positive test direction, under the hypothesis that poor responders would display higher levels of salivary cytokines at baseline (**figure 3.28A**). Using this positive test direction, no salivary cytokine was able to discriminate high from low responders at baseline, with AUC values of 0.637 (IL-1β), 0.601 (IL-6), 0.556 (IL-8) and 0.654 (TNFα) (**figure 3.28B**, all $p>0.05$). Unlike other cytokines, the AUC for IL-17A was surprisingly low (0.194), suggesting that the opposite may be true, i.e. that low responders may display lower levels of this marker at baseline.

To test this, the same ROC curves were generated with a negative test direction (inverse analysis, **figure 3.28C**). Indeed, the results demonstrated that salivary IL-17A levels were able to discriminate between the two groups at baseline. The AUC for IL-17A under a negative test direction was the reciprocal of the positive test direction (AUC: 0.806, 95% CI: 0.656 to 0.956, $p<0.001$), suggesting moderate classification accuracy (Akobeng, 2007). Classifier evaluation metrics indicated that a salivary IL-17A concentration of 7.44 pg/mL at baseline was the optimum cut-off to discriminate between high and low responders, with 83.3% sensitivity and 70.6% specificity within the current subgroup.

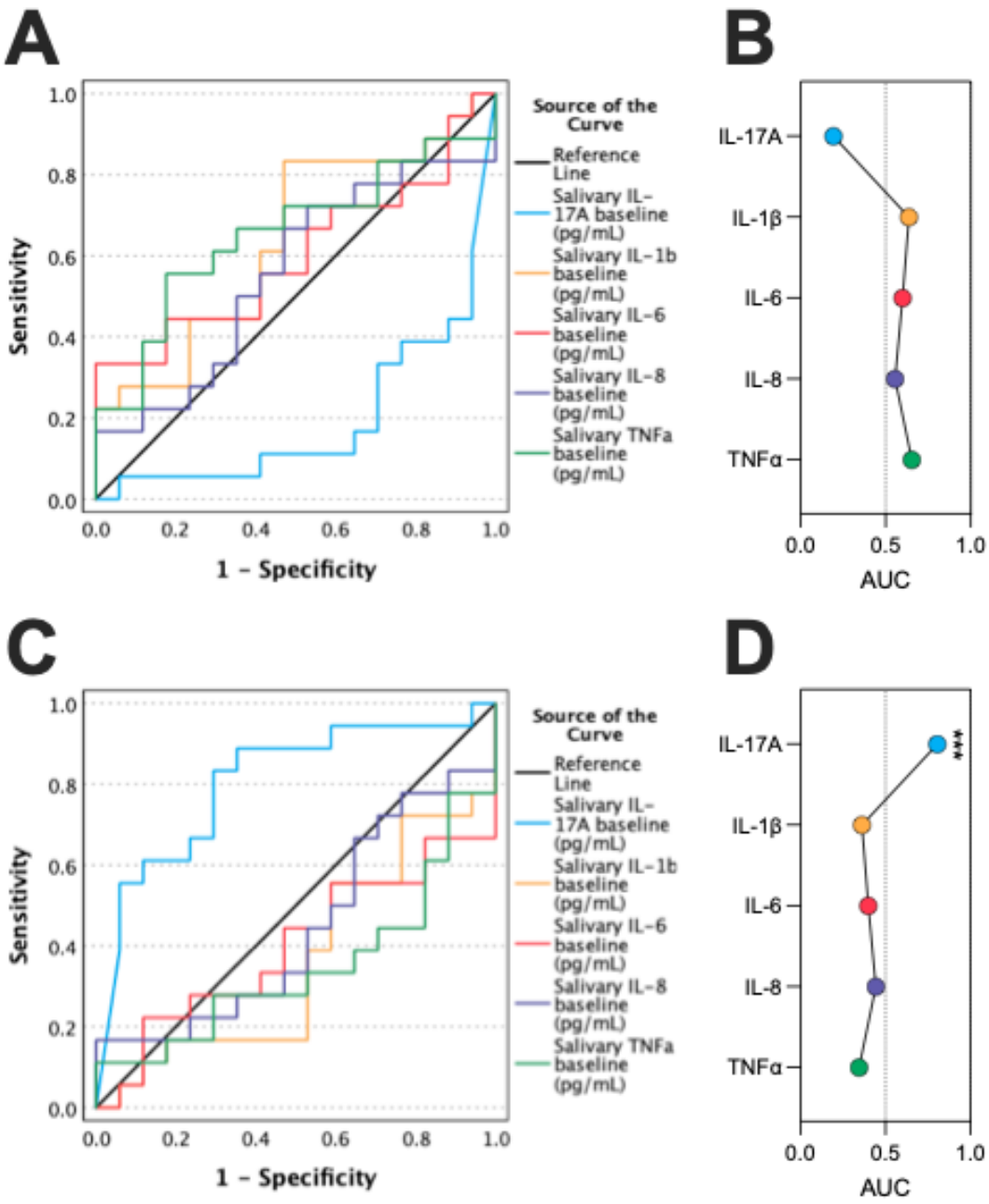

Figure 3.28. Salivary cytokines as indicators of treatment response. Receiver operating curves (ROC) for identifying poor responders to treatment using salivary cytokines with positive (A) and negative (C) test direction. ROC performed using baseline salivary IL-6, TNFα, IL-8, IL-17A and IL-1ß as test variables. Area under the curve (AUC) values for each variable using positive (B) and negative (D) test directions. Low responders (n=18) and high responders (n=17).

3.3.5.4. Comparison of IL-17A between high and low responders

To supplement ROC curves, levels of salivary IL-17A in both saliva were directly compared between groups (**figure 3.29**). As expected, high-responders displayed significantly greater salivary IL-17A at baseline when compared with low-responders (median; 4.35 vs. 10.56 pg/mL, p=0.002, Mann-Whitney U test, **figure 3.29**). Similar results were observed at day 90 with high responders displaying significantly greater levels of salivary IL-17A (8.45 vs. 14.20 pg/mL, p=0.009), although this cytokine showed a tendency to increase following treatment within both groups (p=0.01 low responders, p=0.06 high responders, Wilcoxon singed-rank test). There was no difference in the magnitude of this increase between groups (p=0.26 comparing BL vs. D90 delta values, Mann-Whitney U-test). Taken together, this analysis indicates that within the limitations of this subgroup analysis, elevated salivary IL-17A at baseline was associated with a larger improvement in local inflammation. Separate ROC curves were not performed on GCF cytokines as pooled samples mean they are not reflective of site nor whole-mouth disease. Additionally, using estimate values it is not possible to translate a clinically meaningful result from this data.

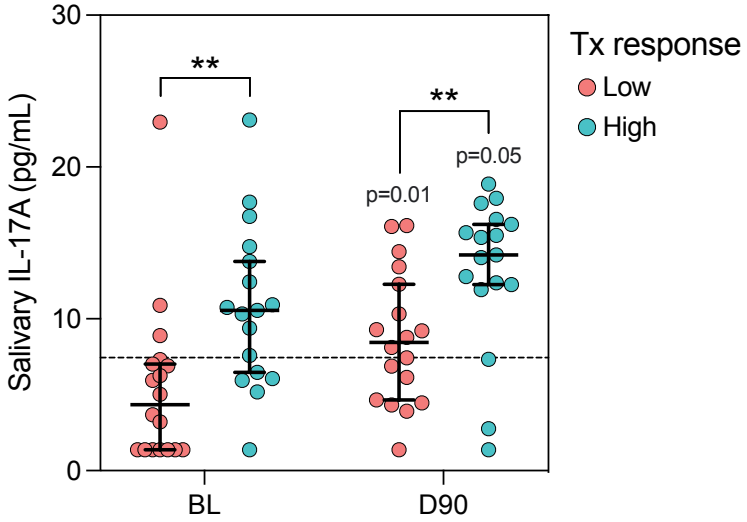

Figure 3.29. Salivary IL-17A and the response to NSPT. Salivary IL-17A between low (n=18) and high (n=18) responders at baseline and day 90. Dotted line indicates optimum cut-off concentration. Between group comparisons are Mann-Whitney U tests (**p<0.001), within-group comparisons are Wilcoxon signed-rank tests (raw p-values displayed above day 90 values). n=37 as one patient (high responder) had limited saliva volume.

3.4. Discussion

This chapter evaluated local host and microbial variables in PD patients in relation to baseline disease severity and longitudinally following NSPT. Salivary IL-1β was positively associated with periodontal inflammation, and proportional reductions were observed following treatment. Surprisingly, salivary and GCF IL-17A increased following NSPT and higher salivary IL-17A at baseline was associated with a better treatment response within the current cohort. In subgingival plaque samples, NSPT induced clear and consistent reductions in the abundance of disease associated organisms in the microbiota, which was accompanied by reductions in overall richness and diversity. Despite this, the current study was unable to demonstrate any association between the microbiota and site specific treatment response.

The association between salivary IL-1β and PD is in line with previous cross-sectional studies, showing higher levels of this cytokine in PD saliva compared with periodontal health (Miller et al., 2006, Tobon-Arroyave et al., 2008, Gursoy et al., 2009). IL-1β is one of the few salivary cytokines considered to show robust discrimination between PD and healthy subjects (Taylor, 2014). In the current study, it was demonstrated that the level of salivary IL-1β is associated with the degree of periodontal disease severity at baseline. The level of IL-1β was also shown to be reactive to clinical improvement following NSPT, which is supported by previous studies demonstrating comparable longitudinal shifts (Kaushik et al., 2011, Sanchez et al., 2013). Uniquely, the current study also demonstrates that the reduction in salivary IL-1β is proportional to the reduction in periodontal inflammation, adding further strength to the utilisation of this cytokine as a biomarker of PD.

Commensurate with previous studies, there were no consistent trends for salivary TNFα, IL-6 or IL-8. Previous studies have failed to establish a relationship between salivary IL-8 and PD (Rathnayake et al., 2013) and its association with disease is generally considered uncertain for this reason (Taylor, 2014). In contrast, the concentrations of salivary TNFα and IL-6 were low which is supported by vast majority of previous studies (Jaedicke et al., 2016, Taylor, 2014). Saliva is a harsh environment for proteins, containing bacteria and their by-products, mucins and various proteolytic enzymes. As

such, it is possible that the low level of these mediators (TNFα and IL-6) merely represents their inability to survive in these extreme conditions, rather than lack of production in surrounding tissues. This is supported by *in vitro* work showing that human gingival epithelial cells challenged with *P. gingivalis* produce high-levels of IL-1ß, but not IL-6 despite increased gene-expression (Stathopoulou et al., 2009). Within this previous study, the authors speculate that IL-1ß survival may arise as it can be secreted within vesicles (Mackenzie et al., 2001) or it's tertiary structure may block cleavage sites of *P. gingivalis* lysine gingipains. Although *P. gingivalis* is unlikely to solely contribute to the values found in this study, heightened susceptibility to degradation may explain the low levels of salivary IL-6 and TNFα consistently observed within saliva samples.

A surprising result from this chapter was that IL-17A increased following NSPT in both saliva and GCF samples. Primarily produced by Th17 and mast cells, IL-17A functions in the host defence against external pathogens at mucosal surfaces (Jin and Dong, 2013). Although this function appears co-aligned with the pathophysiology and development of PD, the relationship between IL-17A and PD remains elusive. Both protective and destructive effects have been reported (Zenobia and Hajishengallis, 2015) and conflicting results found within independent cross-sectional studies showing elevated levels in health (Ozçaka et al., 2011, Prakasam and Srinivasan, 2014) and disease (Awang et al., 2014, Techatanawat et al., 2020). However, all of these studies have compared established PD with pristine periodontal health. Some studies have expanded this analysis and stratified patients according to disease severity, which has demonstrated that salivary IL-17A actually peaks in the early stages of disease development and decreases in established PD (Liukkonen et al., 2016). This is supported by earlier work assessing gingival biopsies, where IL-17A expression was significantly higher in gingiva adjacent to 4-5 mm pockets compared with pockets ≥6 mm (Johnson et al., 2004). In relation to the current study, day 90 samples represent a post-treatment state of stability rather than complete periodontal health. As such, it is possible that NSPT induces a shift back to an early inflammatory state whereby IL-17A is a driving force. This is indeed a speculative theory, although IL-17A plays a key role in neutrophil recruitment and survival, which is the predominant cell type in 'initial lesions' (Page and Schroeder, 1976, Hajishengallis and Korostoff, 2017).

In agreement with the shifts in salivary cytokines; GCF IL-1β reduced and IL-17A increased following NSPT, with no alterations in the level of IL-6 or IL-8. The link between GCF IL-1β and PD has been considered strong from a rigorous systematic review (Buduneli and Kinane, 2011), and longitudinal increases in GCF IL-17A have been recorded following treatment (Buduneli et al., 2009). A limitation of the GCF analysis in this chapter is the difficulties in collecting and processing GCF samples, meaning cytokines were standardised to the number of eluted strips. Although it was validated that the trends remained consistent with patients where 4 strips were eluted at each timepoint, these data remain as estimate values for both outputs. A Periotron® unit was not available and therefore GCF volumes could not be measured on strips. There is no gold standard for performing analysis of GCF cytokines, and it is debated whether these analytes should be reported as a total measured value across time, or standardised to eluted GCF volumes (Wassall and Preshaw, 2016). Nonetheless, measurement of GCF volume is valuable in and among itself and would be a useful addition to future studies. The trends in GCF cytokines in the current study match those reported previously and shifts in the same analytes were found in saliva. However, until the collection, processing and reporting of GCF analytes is standardised it remains difficult to establish the strength of putative biomarkers using this sample type.

In relation to the subgingival plaque, this chapter demonstrates consistent reductions in the richness and diversity in the microbiota following NSPT. In contrast to typical bacterial 'infections' – PD differs in that development of disease has been associated with an increase in microbial diversity (Abusleme et al., 2013, Griffen et al., 2012, Shi et al., 2018). This elevation has been conventionally related to an anaerobic environment induced by deep periodontal pockets and increased nutrient availability through inflammatory infiltrate (Marsh and Bradshaw, 1995, Tanaka et al., 1998, Loesche, 1996), supporting growth of more fastidious microorganisms. In agreement with these findings, there was differential abundance of 42 genera and 61 species following NSPT. The most abundant organisms that decreased following treatment were strict anaerobes (e.g. *Fusobacterium, Prevotella, Porphyromonas, Filifactor*), with increases found typically in facultative anaerobes or aerobes (e.g. *Streptococcus, Kingella, Rothia, Actinomyces*).

In line with the polymicrobial aetiology of PD, large clusters of positively associated anaerobes were observed at baseline. Notably, *Fretibacterium* and *Filifactor* formed strong positive associations with one another at genus-level, and with all three red-complex associated genera (*Porphyromonas, Treponema, Tannerella*). Despite challenges in species-level classification using 16S rRNA sequencing, our data suggest that these associations were most likely driven by *Fretibacterium feline, Fretibacterium fastidiosum* and *Filifactor alocis*. All three of these species have been suggested as emerging 'periodontal pathogens' (Pérez-Chaparro et al., 2014, Aruni et al., 2015b). Similarly, these associations support an earlier study demonstrating that a *F. alocis* centred co-occurrence group (including *P. gingivalis, T. forsythia* and *Fretibacterium* species) can be used to accurately diagnose PD (Chen et al., 2015). Following treatment, these associations were lost and a newly formed network was formed, including genera that are abundant in health but increase in PD (e.g., *Fusobacterium* and *Prevotella*) (Chen et al., 2018), along with *Treponema*, *Alloprevotella*, *Selenomonas*, *Parvimonas*, *Dialister* and *Catonella*. Whether this group of organisms may persist to drive future disease recurrence longer term (>3 months) remains unclear and requires a longer study period with routine microbiological monitoring.

Longitudinal shifts in the subgingival plaque microbiome have been previously reported following NSPT, confirming the reductions in the abundance of previously ungrouped and uncategorised genera such as *Dialister* and *Olsenella*, and elevations in *Rothia* and *Corynebacterium* (Shi et al., 2015, Chen et al., 2018). However, far more alterations were observed within the current study compared with those conducted previously. Within the study by Shi et al., an increase in the abundance of 4 genera and a reduction in the abundance of 8 genera were observed across 12 patients. The study by Chen et al. included 19 patients and observed a large patient-to-patient variation in microbial outcomes. Differences in sample size and follow-up periods may explain why this study observed far larger microbial alterations. In previous studies, subgingival plaque was resampled at 4 to 19 weeks (Shi et al., 2015), or at least 4 weeks after NSPT (Chen et al., 2018). The lack of a standardised follow-up period may influence the stage of biofilm redevelopment and thus composition. In the current study subgingival plaque was resampled at 90 days (±14 days) across every patient, which is a recommended clinical follow-up period (Darcey and

Ashley, 2011). Collectively, a larger cohort and a more standardised follow-up period may explain why a greater number of microbial alterations were observed. Additionally it is worth reiterating that whilst several longitudinal alterations were observed, large compositional differences between timepoints were driven by relatively few (*Prevotella, Fusobacterium, Selenomonas, Porphyromonas, Treponema, Tannerella, Rothia, Corynebacterium, Actinomyces* and *Streptococcus*).

Interestingly, in spite of the marked changes in the microbiome following treatment, this study did not find an association between the microbiota and treatment response. Two previous studies have evaluated the subgingival plaque microbiome with respect to treatment response following NSPT (Bizzarro et al., 2016, Chen et al., 2018). Each study has reported differing results, with Bizzarro et al., suggesting that lower taxonomic diversity and increased abundance of non-oral taxa (*Pseudomonas*) at baseline is associated with poor treatment response, whilst Chen et al., found that the microbial response was not associated with clinical improvement. The findings from the current study agree with the latter, although inherent differences in sample collection, study design and clinical success criteria were present. In both previous studies, subgingival plaque was collected using paper points and pooled from various sites, compared with the single-site analysis using curettes in the current study. Differing sample collection methods may impact on biofilm composition and it has been suggested that curettes sample contents of the entire pocket, whereas paper points reflect the outer layers of the biofilm (Loomer, 2004, Perez-Chaparro et al., 2015). Differences in response classification were also present with Bizzarro et al., employing median full-mouth CAL improvement as an indicator of clinical success at 12 month follow-up, compared with site-specific PPD, CAL and BoP by Chen et al., at >4 week follow-up, and site-specific pocket closure at 3 month follow-up in the current study. A collection of these factors may therefore explain differences in observed results, and there is a desperate need to standardise treatment success criteria in periodontology.

One restriction within the current study is that sites were selected based on disease severity rather than location. This meant that responding and non-responding groups contained samples originating from both molars and non-molars. Although it was confirmed that sampled site morphology did not alter

the baseline microbial composition, it has been suggested that molar sites may respond worse to NSPT in general and this should be considered (Citterio et al., 2021). Nonetheless, sites ≥5 mm are considered 'diseased' and are associated with future disease progression regardless of tooth morphology. In addition, standardising this site selection can be challenging, particularly when a predefined site will not be the most severe in the mouth or may not be suitable for repeated sampling. It should be reiterated that the non-responding group also displayed significant, albeit less pronounced, clinical improvement following treatment. To this extent, the incorporation of sites which do not improve at all, or even worsen, following NSPT would be useful to establish the relationship between the baseline subgingival plaque microbiota and treatment response.

Given the small sample size of the current exploratory analysis, it was investigated whether aspects of the microbiome merely associate with a poor treatment response. In reality, far more complex prediction methods may be applied which could be better suited for this analysis. One example are machine learning approaches such as the random forest technique, which have been used to predict clinical phenotypes based on the microbiome (Zhou and Gallins, 2019). These methods could be applied at baseline with a known day 90 clinical outcome, to determine whether certain features may truly 'predict' (rather than associate with) response to treatment. However, such techniques require extremely large sample sizes to prevent data overfitting which are seldom available from single studies. There has recently been a call for the development of an international repository for oral microbiome data, and such an effort would greatly improve the applicability of prediction modelling of treatment response (Seneviratne et al., 2020).

From an inflammatory perspective, baseline levels of salivary IL-17A were able to differentiate between high and low responders with moderate sensitivity and specificity, using median PISA improvement as a threshold for treatment success. This threshold was not limited to improvement in PISA and reflected reduced levels of several clinical parameters (PPD, FMBS, pockets ≥5 mm), forming a better picture of total clinical response. The results for salivary IL-17A imply that higher baseline levels are associated with a higher response to NSPT and provides pilot data for a more rigorous investigation which is wholly required. This finding is intriguing because whilst IL-17A drives bone destruction

in RA via stimulating osteoclastogenesis (Kotake et al., 1999, Sato et al., 2006), mouse models indicate that IL-17A actually plays a protective role in PD via neutrophil recruitment and regulation (Yu et al., 2007).

The current study merely highlights a potential association between elevated salivary IL-17A at baseline and enhanced response to NSPT. The reason for this association is unclear – although a very speculative theory may be that elevated IL-17A reflects an immunological state closer to healthy subjects, and thus the inflammatory response may be more readily resolved in this cohort. As such, future work could seek to characterise salivary IL-17A in patients with long-term disease recurrence. The lack of association between salivary IL-1β and clinical outcome is contrary to previous work (Lee et al., 2018), but is perhaps unsurprising given that this marker was associated with baseline inflammation which was standardised in this subgroup analysis.

Including those mentioned thus far, this chapter has several limitations which must be stated. One of which is the lack of a periodontally healthy control population for comparison with post-treatment samples. Although reductions in disease-associated microbial and immunological variables were assessed, it should be further evaluated whether these resort back to a state comparable with periodontally healthy individuals. Additionally, whilst an open-ended technique was used for microbial analysis, only a small number of saliva/GCF cytokines were assessed. Considering these data, it would be useful to assess additional cytokines such as IL-23 considering its relationship with IL-17A production, along with other non-cytokines such as MMP-8 and -9 which have been established as robust salivary biomarkers in PD (Taylor, 2014). In a similar sense, GCF was not sampled from the same sites as subgingival plaque samples, and this should be considered moving forward to delineate accurate site-specific host-microbial relationships following NSPT.

Additionally, whilst 16S rRNA sequencing is the most widely used technique for profiling microbiome communities, it is not devoid of disadvantages. A major pitfall of fragment 16S rRNA sequencing is the reduced accuracy in classifying and discriminating at species-level (Do et al., 2013, Wade and Prosdocimi, 2020), and inability to accurately assess important low-abundant organisms (De Cena et al., 2021). Moreover, this technique only outlines the relative abundance genera/species, rather than their quantity or function. Moving

forward, metagenomic studies will help to overcome these issues particularly as shotgun sequencing becomes more accessible and affordable. Although at present, the cost and complexity of this sequencing is simply out with the scope of many research projects. Lastly, the oral cavity harbours a diverse range of microorganisms including fungi and archaea which were not assessed during this study. Integrating these communities into future studies would be beneficial to understand whether interkingdom interactions (such as generation of anoxic conditions by fungal species) may contribute to the pathogenesis of PD.

In conclusion, this chapter outlines alterations in local inflammatory cytokines and the subgingival plaque microbiota. These findings lend further credence to the use of salivary IL-1ß as a sensitive biomarker of PD, positively associating with periodontal inflammation and reducing following treatment. Whilst the abundance of disease-associated organisms was reduced within the subgingival plaque, a tightly clustered network of anaerobic genera persisted. Entirely removing such organisms from the microbiome is unlikely with any treatment and the importance of residual disease-associated organisms must be critically evaluated. The baseline microbiota was unable to discriminate sites with residual disease at day 90, and widespread microbial alterations were observed in some patients regardless of clinical improvement. Baseline salivary IL-17A was able to discriminate high and low responders within the current cohort, and future work should be directed towards understanding the role of IL-17A in health and disease.

3.5. Chapter summary

This chapter demonstrates lasting immunological and microbiological shifts in PD patients following NSPT. The association of these variables with clinical disease severity and response to treatment was also investigated, although the exploratory nature of this analysis must be reiterated. Moving forward, the data presented may be used to power future studies and delineate true effects.

The main findings from this chapter are highlighted below.

Salivary and GCF cytokines;

- Salivary IL-1β positively associates with PISA at baseline, and proportional reductions were observed following treatment.
- Salivary and GCF IL-17A increased following treatment, potentially indicating reversal to an early inflammatory state.
- PISA offers a promising clinical parameter for indicating whole-mouth clinical response.
- Exploratory analysis suggested high baseline salivary IL-17A was associated with an elevated response to treatment within the current cohort.

Subgingival plaque microbiome;

- The taxonomic diversity of subgingival plaque samples was reduced following treatment.
- Treatment induced consistent alterations in the relative abundance of 42 genera and 61 species, including a reduction in disease-associated microbial complexes.
- Exploratory analysis did not find any association between the baseline subgingival plaque microbiome and site specific pocket closure.
- Widespread microbial alterations were observed in some patients regardless of the degree of clinical improvement.

Chapter 4

The systemic inflammatory response following hand versus ultrasonic instrumentation – A randomised controlled trial

Work from this chapter has been presented at the following meetings;

'Analysing the immune response and plaque microbiome after periodontal treatment' by W. Johnston. Invited guest lecture presented at the Rajiv Gandhi University of Health Sciences, Bengaluru, India. Oral Microbiome Symposium, March 2021. Virtual lecture.

Work from this chapter has been published in the following journal;

Johnston, W., Paterson, M., Piela, K., Davison, E., Simpson, A., Goulding, M., Ramage, G., Sherriff, A. and Culshaw, S. 'The systemic inflammatory response following hand instrumentation versus ultrasonic instrumentation—A randomized controlled trial'. *Journal of Clinical Periodontology*. 2020; 47: 1087– 1097. https://doi.org/10.1111/jcpe.13342

Manuscript included as **appendix iii**

4.1. Introduction

The majority of longitudinal studies in periodontology focus on investigating clinical and biological parameters at a single follow-up timepoint such as 3, 6 or 12 months. Whilst such studies provide a crucial insight into lasting alterations induced by NSPT, they may miss important fluctuations in these variables between patient visits. Indeed, studies investigating the immediate impacts of NSPT have revealed an acute 'spike' in systemic inflammatory markers, notably CRP, 1 day following intensive/full mouth treatment (D'aiuto et al., 2004a, Tonetti et al., 2007, Graziani et al., 2010, Graziani et al., 2015, Morozumi et al., 2018). This response persists for up to one week and has been shown to induce an acute state of vascular impairment, with possible elevated risk of vascular events (Tonetti et al., 2007). Indeed, acute systemic inflammation as observed with urinary tract or respiratory infections may triple a patients risk of vascular events (Smeeth et al., 2004). In otherwise healthy patients this response is presumably self-limiting; however, in medically compromised patients, for example with pre-existing vascular disease, such perturbation to systemic homeostasis may carry a greater risk to overall health.

This response is believed to be driven by bacteraemia induced via treatment. In line with this theory, bacteraemia has been observed immediately following NSPT and persists for up to 30 minutes (Kinane et al., 2005, Forner et al., 2006, Lafaurie et al., 2007, Horliana et al., 2014). Dissemination of local microbes into circulation may therefore stimulate rapid increase in the level of serum CRP, IL-6 and TNFα at day 1 (Graziani et al., 2010, Graziani et al., 2015). Whilst this response is short lived and typically resolves within 7 days (Tonetti et al., 2007, Machado et al., 2021), a recent joint consensus statement from the EFP and AAP advised that for patients with any level of CVD, NSPT should be performed over several 30-45 minute sessions to specifically minimise this spike in systemic inflammation (Sanz et al., 2020).

Several variations of NSPT exist and there is evidence that the subsequent systemic inflammatory response can be modified according to the treatment protocol. For example, treatment may be performed using hand instruments, ultrasonic instruments, or a 'blended approach' using both. Likewise, treatment may be staged over several visits with a 'quadrant' approach, or with a 'full-

mouth debridement' (FMD) approach, also referred to as 'intensive treatment', that delivers complete debridement within a 24 hour period. The choice of instrumentation and visit scheduling falls largely down to patient and clinician preference, with no differences in clinical outcome (Kinane and Papageorgakopoulos, 2008, Ioannou et al., 2009, Suvan et al., 2019). However, it has been shown that there is a marked increase in the systemic inflammatory response following FMD. Specifically, FMD has been shown to trigger a significantly greater spike in serum CRP, IL-6 and TNFα at day 1 compared with quadrant scaling (Graziani et al., 2015). Furthermore, the extent of this response was positively correlated with treatment time, suggesting that the length of treatment sessions directly impacts the subsequent systemic response.

Studies investigating the systemic inflammatory response generally report use of both hand and ultrasonic instruments in combination. Although clinical outcomes are similar, several studies report lower treatment times using ultrasonic instruments in comparison with hand instruments (Copulos et al., 1993, Dragoo, 1992, Tunkel et al., 2002). Given the previous link between treatment time and the subsequent systemic inflammatory response, and the different features of each instrumentation technique including delivery of water irrigation and the reduction in cementum removal with ultrasonics (Ruhling et al., 2005, Bozbay et al., 2018), an RCT was designed to test whether hand and ultrasonic instruments may differ in the extent of systemic inflammation they induce following FMD. Secondary outcomes included assessing differences in the local inflammatory response and clinical outcomes between instrumentation groups. The research questions for this chapter are as follows;

- Is there a difference in the immediate systemic inflammatory response – as measured by elevations in serum CRP - following FMD performed with hand versus ultrasonic instrumentation?
- Is there a difference in clinical outcomes following FMD performed with hand versus ultrasonic instrumentation?
- Is the systemic inflammatory response following FMD associated with alterations in local inflammatory mediators?

4.2. Materials and methods

4.2.1. Study overview

The RCT analysed throughout this chapter; "The **I**mmune **R**esponse **A**fter **P**eriodontal **T**reatment (IRAPT)" was designed to measure systemic inflammatory changes following FMD conducted using hand instrumentation (HI) or ultrasonic instrumentation (UI). The study recruited patients between February 2018 and June 2019 and was a single-centre trial (Glasgow Dental Hospital).

The PICO question that the IRAPT trial aimed to answer was; 'For patients with periodontitis (problem/population), following full-mouth debridement (intervention), is there a difference in systemic inflammatory markers (outcome) comparing treatment provided exclusively by hand instruments or exclusively by ultrasonic instruments (comparison)?'

4.2.2. Patient selection and recruitment

4.2.2.1. Ethical considerations and registration

The study received ethical approval from the Office for Research Ethics Committees Northern Ireland (REC reference number: 18/NI/0059) and was conducted in accordance with the Declaration of Helsinki (7th revision, 2013) and the Research Governance Framework for Health and Community Care (2nd edition, 2006). The trial was registered with ClinicalTrials.gov (ID: NCT03501316) prior to patient recruitment.

4.2.2.2. Patient recruitment

Before attending Glasgow Dental Hospital, all patients received initial periodontal treatment and oral hygiene instruction from their general dental practitioner (GDP), as per local referral guidelines. Patients were recruited among referrals to Glasgow Dental Hospital Unit of Periodontics. Patients attending new patient clinics were screened by the chief investigator (Professor Shauna Culshaw) or other clinician responsible for the patient to establish eligibility. Eligible patients were informed of the study and supplied with a PIL. If patients indicated they would consider participation in the study, they were scheduled for a baseline visit in Glasgow Dental Hospital Clinical Research

Facility. In total, 42 patients were recruited to this study. Throughout the study, one patient was excluded due to a new diagnosis of systemic disease (n=1), and two were excluded at the discretion of the CI due to repeated fainting at the baseline visit venepuncture (n=2).

4.2.2.3. Inclusion and exclusion criteria

The inclusion criteria for this study were; male or females aged between 18 and 70 years old (inclusive) suffering from PD, defined as PPD ≥5 mm on 2 or more teeth at non-adjacent sites (excluding third molars) with cumulative pocket depths ≥40 mm. Cumulative pocket depth was calculated by evaluating 6-sites on each tooth, if the deepest site on each tooth was ≥5 mm, then this was counted towards the cumulative total. Each tooth was only counted once towards the final total. These criteria have been previously used to ensure an adequate baseline level of periodontal disease severity in clinical trials (Page and Eke, 2007, Dietrich et al., 2008, Serban et al., 2019, Lopez-Oliva Santa Cruz, 2018). Additional inclusion criteria included patients who required periodontal treatment at Glasgow Dental Hospital, were able to travel and attend all study visits and provide written informed consent.

Exclusion criteria were; known or suspected risk for tuberculosis, hepatitis B or HIV infections, require an interpreter or non-English language in order to provide written, informed consent, history of bleeding diathesis, pregnant or lactating females, reported diagnosis of any systemic illness (including cardiovascular, renal and liver diseases) and/or use of regular medication to control systemic illness, any pharmacological treatment within 1-month prior to beginning the study (including over the counter medications) and any specialist periodontal treatment in the previous 6 months.

4.2.2.4. Calibration of examiners

For this study, periodontal parameters were assessed by three trained clinicians. This included an experienced dental hygienist, a specialist trainee in restorative dentistry and a consultant periodontist. To calibrate examiners, pairs of clinicians completed pocket charts on the first twelve patients entering the study. Following completion, charts were assessed for agreement between all

three examiners and an unweighted Kappa (κ) coefficient was calculated equalling 0.66.

4.2.2.5. Measurement of clinical parameters

Clinical parameters were assessed at baseline and day 90. This included measurement of FMPS, FMBS and a 6-point pocket chart using a PCP-12 periodontal probe, as described previously in this thesis (**section 2.2.3**).

4.2.3. Randomisation

Patients were randomised to one of two groups; UI or HI, using a computerised random number generator with permuted blocks of 4 and 6. Patients were stratified according to smoking status prior to randomisation. Allocation concealment was achieved using opaque sequentially numbered envelopes containing the treatment group of each patient, which was opened immediately before commencing treatment.

4.2.4. Blinding

Both patients and clinicians remained blinded to the intervention until the treatment visit. Other personnel involved in the trial (laboratory and statistical staff) remained blinded to the group allocation throughout the entire study through the use of pseudo-anonymised patient barcodes. The key linking the codes to patients and treatment groups was only available to the chief investigator and were made available only once all analyses were complete.

4.2.5. Study outcomes

The primary outcome from this study was serum CRP levels at day 1 post treatment. Secondary outcomes included serum IL-6 and TNFα, clinical parameters, saliva and GCF cytokine analysis and microbiome analysis of subgingival plaque samples at baseline, day 1, 7 and 90 where appropriate.

4.2.6. Sample size calculation

The sample size calculation was based on data from a previous study evaluating changes in serum CRP 1 day following periodontal treatment (Graziani et al., 2015). From this previous study, a difference of 3.5 mg/L (standard deviation = 3 mg/L) in CRP was detected between two groups (quadrant vs. FMD) at day 1, which has been considered clinically relevant in recent guidelines (Sanz et al., 2020). Therefore, this was considered a reasonable estimate of the minimum clinically relevant difference. At 80% power and 5% significance level, a sample size of n=34 (17 in each group) was required to detect a minimum difference of at least 1 standard deviation between CRP levels at day 1 between HI and UI. To account for a 20% drop-out rate, 42 eligible participants were recruited to this study.

4.2.7. Visit scheduling

Patients participating in this study attended a baseline visit, treatment visit, day 1 post treatment visit, day 7 post treatment visit and a review visit (day 90 post treatment). A CONSORT flow diagram of the study design, sample size and visits is shown in **figure 4.1**.

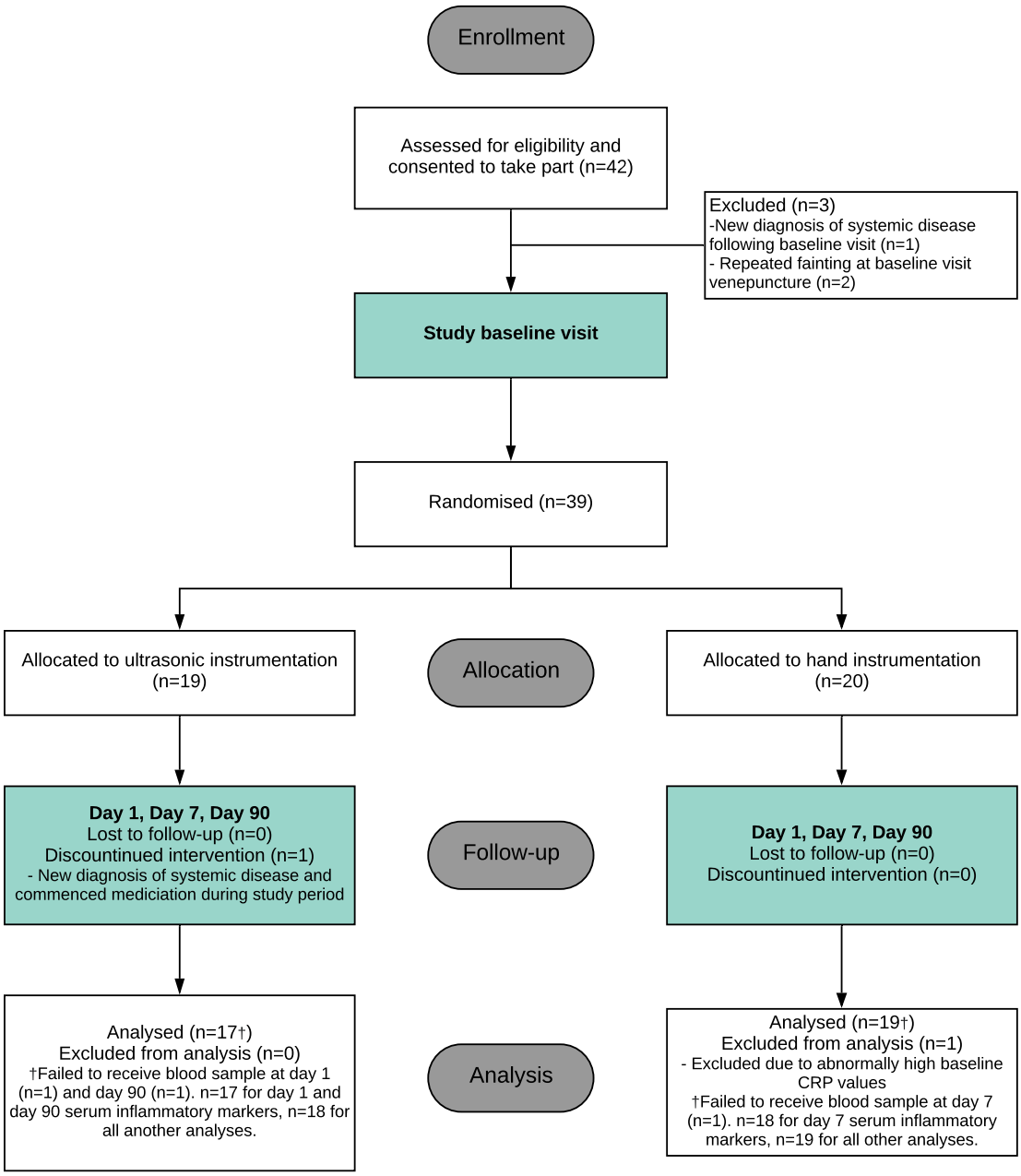

Figure 4.1. CONSORT flow diagram for the IRAPT study. Blood samples were not obtained from one patient at day 1 (UI group), one patient at day 7 (HI group) and one patient at day 90 (UI group). Therefore, for analysis of serum inflammatory markers; at day 1 (UI; n = 17, HI; n = 19), day 7 (UI; n = 18, HI; n = 18) and day 90 (UI; n = 17, HI; n = 19). For all other analyses (UI; n = 18, HI; n = 19).

4.2.7.1. Baseline visit

At the baseline visit, patients were asked if they had further considered participating in this study. If patients agreed to participate, consent forms were completed confirming consent to participate and for collection of clinical samples including whole blood, serum, saliva, GCF and subgingival plaque. In addition to sample collection, clinical parameters (FMPS, FMBS, periodontal charting) were assessed, blood pressure was measured using an automatic oscillometric unit, BMI was measured and smoking status was recorded. Following sample collection, patients were provided with detailed oral hygiene advice and superficial (supragingival) scaling of the teeth. Patients were randomised to one of two intervention groups; UI or HI, and appointments were made for treatment visits and subsequent follow-ups (day 1, day 7, day 90).

4.2.7.2. Treatment visit

Patients received full-mouth non-surgical periodontal treatment using either UI or HI as per randomisation, within a 24 hour period. For the UI group; treatment was provided using Cavitron ultrasonic inserts (Cavitron® Thinsert® 30K, Cavitron® Slimline® 10S 30K, Cavitron® Slimline® 10L 30K, Cavitron® Slimline® 10R 30K, Cavitron® Slimline 1000 30K, Cavitron® Powerline® 1000 30K, Dentsply Sirona). For the HI group; treatment was provided using Gracey and Universal curettes (Gracey 1/2, Gracey 7/8, Gracey 9/10, Gracey 11/12, Gracey 13/14, Columbia 4L-4R) and hoes (Hoe scaler-lateral, Hoe scaler-posterior, LM Dental). All instruments used throughout this study are shown in **figure 4.2**.

Treatment was performed until no supra or subgingival plaque or calculus deposits were detectable by visual examination with magnification or by tactile examination. All treatments were timed by digital stopwatch from first contact between instrument and tooth surface. No samples were collected during the treatment visits. All but one patient received treatment within the same day; a single patient completed debridement on consecutive days within a 24 hour period due to patient availability.

4.2.7.3. Day 1 visit

Patients returned 24 hours (± 6 hours) following treatment at which time clinical samples (whole blood, serum, saliva, GCF and subgingival plaque) were collected and blood pressure was measured. Following collection of samples, patients were provided with an electric toothbrush (Oral-B Pro 2000) in order to standardise self-performed oral hygiene over the remainder of the study, and oral hygiene advice was provided. Clinicians verbally enquired whether patients were happy to continue participation in the trial. If yes – a day 7 visit was scheduled (+/- 1 day).

4.2.7.4. Day 7 visit

Patients attended a subsequent visit 7 days (+/- 1 day) after treatment. At this visit, clinical samples (whole blood, serum, saliva, GCF and subgingival plaque) were collected and blood pressure was measured. Clinicians recorded any changes to medical history, plaque index and reinforced oral hygiene advice. Clinicians verbally enquired whether patients were happy to continue participation in the trial. If yes – a day 90 review visit was scheduled (+/- 10 days).

4.2.7.5. Day 90 visit

Patients returned 90 days (+/- 10 days) following treatment, where clinical parameters (FMPS, FMBS, periodontal charting) and samples (whole blood, serum, saliva, GCF and subgingival plaque) were collected. Following day 90 review, any further treatment need was evaluated by a Specialist in Periodontology and performed out with the current study.

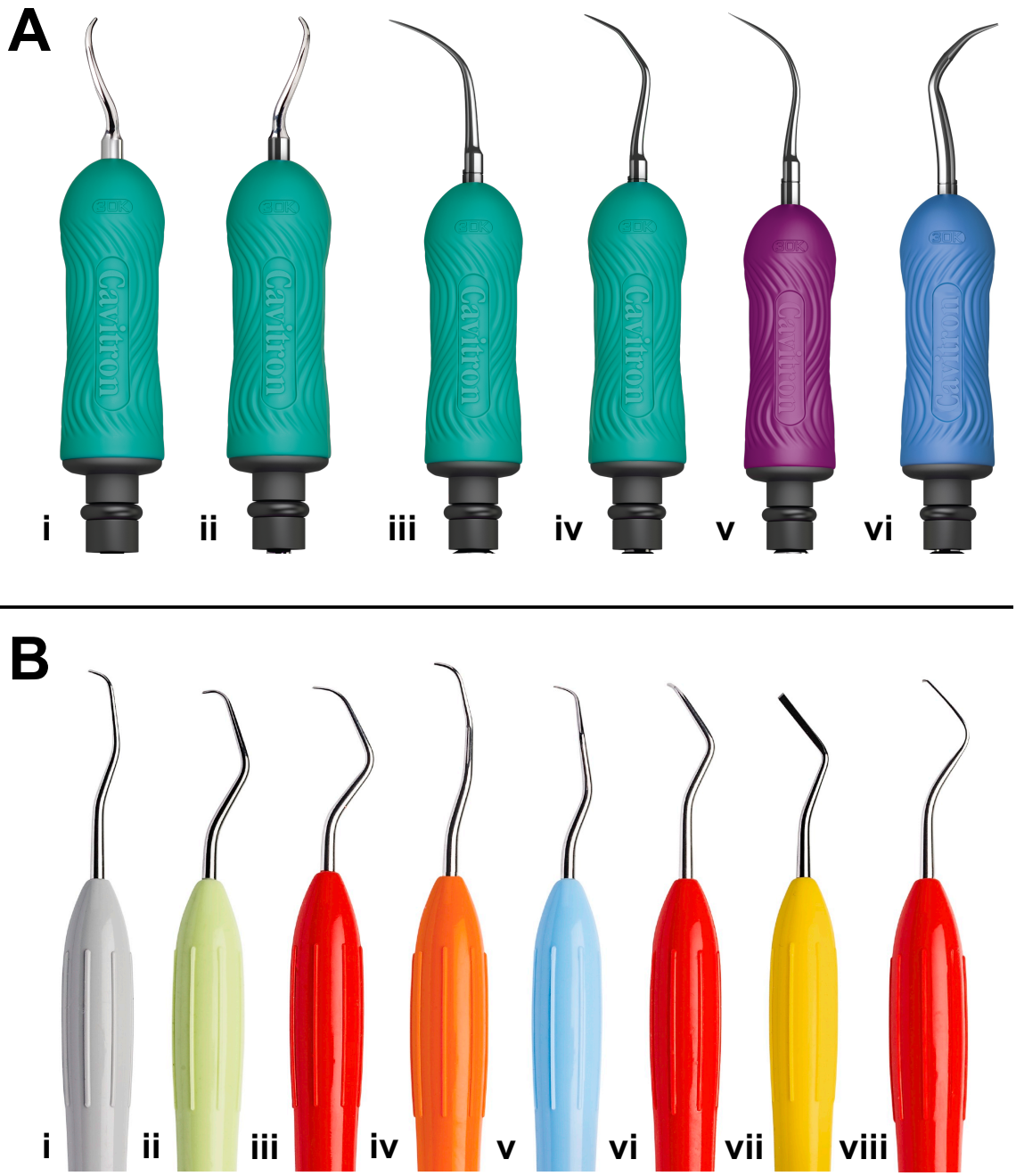

Figure 4.2. Instruments used to provide treatment in each group. (A) For UI, treatment was provided using; Cavitron® Thinsert® 30K (i), Cavitron® Slimline® 10S 30K (ii), Cavitron® Slimline® 10L 30K (iii), Cavitron® Slimline® 10R 30K (iv), Cavitron® Slimline 1000 30K (v), Cavitron® Powerline® 1000 30K (vi) from Dentsply Sirona. For HI; treatment was provided using; Gracey 1/2 (i), Gracey 7/8 (ii), Gracey 9/10 (iii), Gracey 11/12 (iv), Gracey 13/14 (v), Columbia 4L-4R (vi), Hoe scaler-lateral (vii) and Hoe scaler-posterior (viii) from LM-Dental (Copyright LM-Instruments Oy). Images reused with permission from Dentsply Sirona (A) and LM-dental (B).

4.2.8. Study population

In total, 42 patients were recruited to the study. Throughout the course of the study, four subjects were excluded. One patient was withdrawn due to developing new medical diagnoses following baseline visit and two patients were withdrawn at the request of the PI due to unexplained repeated fainting during baseline venepuncture. One patient completed all interventions but was excluded from analysis due to an unexplained pathologically high baseline serum CRP level (11.97 mg/L), as outlined in **figure 4.1**.

4.2.9. Sample collection and processing

Serum, saliva and GCF samples were collected at baseline, day 1, day 7 and day 90 using the same collection and processing techniques described previously in this thesis (**section 2.2.4** [serum], **3.2.1.1** [saliva], **3.2.1.2** [GCF]). Clinicians were unable to obtain blood samples from one patient in at day 1 (UI), another at day 90 (UI), and one patient at day 7 (HI) after repeated attempts. These patients were thus excluded from serum analysis only at these specific timepoints (**figure 4.1**).

4.2.10. Analysis of serum markers

4.2.10.1. CRP, IL-6 and TNFα

Serum CRP was quantified at the British Heart Foundation Glasgow Cardiovascular Research Centre. Levels of CRP were measured via immunoturbidometry using the Cobas C311 analyser (Cobas, Roche Diagnostic, Mannheim, Germany) as described previously (**section 2.2.5.4**). Serum IL-6 and TNFα were both quantified using high-sensitivity ProQuantum assays (Thermofisher, Loughborough, UK) measured on a StepOnePlus real-time PCR system (Applied Biosystems). Full experimental details are supplied previously (**section 2.2.5.5**). CRP and IL-6 were detected in all samples. TNFα was <LOD in seven samples (2x baseline, 3x day 1, 1x day 7, 1x day 90) which were assigned as LOD/2 for statistical analysis.

4.2.11. Multiplex assay

Serum and saliva samples from a subset of patients (n=12) were assayed using a Luminex™ assay. The six patients in each group with the largest CRP spike at day 1 were selected. For these experiments the 'Human Cytokine Magnetic 30-Plex Panel' was used (Catalogue #: LHC6003M, Thermofisher, Loughborough, UK), cytokines in this panel are provided in **table 4.1**. For the purposes of this exploratory analysis, only samples from baseline, day 1 and day 90 were included (no day 7 samples).

The assay was performed according to the manufacturer's instructions. Immediately prior to use, antibody beads were vortexed and sonicated for 30 seconds. A total of 25 µL antibody beads were then added to each well. Assay wells were then washed twice using a wash solution provided with the kit. For the washing procedure, 200 µL of wash solution was applied to wells for 30-60 seconds. Plates were attached to a magnetic 96-well separator, liquid was decanted, and plates were thoroughly blotted. After washing, 50 µL of incubation buffer was added to every well. This was followed by 100uL of diluted standards, 100 µL of assay diluent (blank control) or 100 µL diluted sample. Both serum and saliva samples were diluted 1:2 in assay diluent for these experiments, all samples were initially centrifuged at 10,000 RPM (8°C) prior to addition and had undergone a single freeze-thaw cycle. Seven standards were included using a 3-fold dilution. After addition of standards, blanks and samples, plates were incubated for 2 hours at room temperature under agitation on an orbital plate shaker.

After incubation, samples were decanted, and plates washed as described above. To detect analytes, 100 µL biotinylated antibody was added to each well and plates were incubated for 1 hour at room temperature on an orbital plate shaker. Plates were washed and 100 µL Streptavidin R-phycoerythrin (RPE) was added to each well for 30 minutes on an orbital plate shaker, before being washed three times. To read the assay results, 150 µL of wash solution was added to each well and plates were placed on an orbital shaker for 2-3 minutes. Results were generated on a Bio-Plex 200 analyser using 5 parameter-fit logistic curves (BioRad, Watford, UK). Experiment conducted with Robert Reilly.

Table 4.1. List of analytes included in the Luminex™ assay and the range of their standard curves. Experiment conducted with Robert Reilly.

Analyte (abbreviation)	Bead region	Standard curve (pg/mL)
Fibroblast growth factor basic (FGF-Basic)	12	8.56 – 6240
Interleukin-1ß (IL-1ß)	13	9.81 – 7150
Granulocyte colony-stimulating factor (G-CSF)	14	94.46 – 68,860
Interleukin-10 (IL-10)	15	5.42 – 3950
Interleukin-13 (IL-13)	18	16.46 – 12,000
Interleukin-6 (IL-6)	19	7.89 – 5750
Interleukin-12 (IL-12)	20	10.36 – 7550
RANTES	21	11.87 – 8650
Eotaxin	22	1.88 – 1370
Interleukin-17A (IL-17A)	25	26.95 – 19,650
Macrophage inflammatory protein 1α (MIP-1α)	26	17.85 – 13,015
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	27	6.86 – 5000
Macrophage inflammatory protein 1ß (MIP-1ß)	28	24.63 – 17,955
Monocyte chemoattractant protein 1 (MCP-1)	29	24.21 – 17,650
Interleukin-15 (IL-15)	30	59.12 – 43,100
Epidermal growth factor (EGF)	33	13.09 – 9545
Interleukin-5 (IL-5)	34	11.39 – 8300
Hepatocyte growth factor (HGF)	35	68.29 – 49,780
Vascular endothelial growth factor (VEGF)	36	0.8 – 585
Interferon-γ (IFN-γ)	38	7.13 - 5200
Interferon-α (IFN-α)	43	11.04 – 8050
Interleukin-1 receptor antagonist (IL-1RA)	51	57.46 – 41,890
Tumour necrosis factor α (TNFα)	52	8.57 – 6250
Interleukin-2 (IL-2)	54	13.31 – 9700
Interleukin-7 (IL-7)	55	13.58 - 9900
Interferon-γ induced protein 10 (IP-10)	56	0.52 – 380
Interleukin-2 receptor (IL-2R)	61	57.17 – 41,675
Monokine induced by γ (MIG)	63	25.92 – 18,895
Interleukin-4 (IL-4)	77	34.57 – 25,200
Interleukin-8 (IL-8)	78	12.21 - 8900

4.2.12. Analysis of saliva and GCF cytokines

Five cytokines were selected for further analysis across all patients in saliva and GCF samples: TNFα, IL-6, IL-8, IL-17A and IL-1ß. The methodology for these experiments is described in **section 3.2.2** and **3.2.3** and summarised in **table 3.1**. For saliva samples: TNFα was <LOD in 9 samples (4x baseline, 1x day 1, 1x day 7, 3x day 90), and IL-17A was <LOD in 15 samples (2x baseline, 6x day 1, 4x day 7, 3x day 90). Levels of IL-6, IL-8 and IL-1ß were detectable in all saliva samples. For GCF samples: TNFα was <LOD in 32 samples (15x baseline, 2x day 1, 4x day 7, 11x day 90), IL-6 was <LOD in 1 sample (1x baseline) and IL-17A was <LOD in 15 samples (11x baseline, 1x day 1, 1x day 7, 2x day 90). Levels of IL-8 and IL-1ß were detectable in all GCF samples. Samples <LOD were assigned as LOD/2 for statistical analysis. The level of GCF cytokines were standardised to the number of eluted strips as described in **section 3.2.6** of this thesis.

4.2.13. Statistical analysis

Raw study data were entered into SPSS (v26; IBM) using patient codes and then analysed using SPSS or PRISM (v8; GraphPad). Graphics were produced using PRISM. For tabulated data, outcomes are summarized using median (Q1, Q3) where appropriate. Longitudinal changes in clinical parameters, systemic and local analytes between baseline and the various follow-ups were assessed using Wilcoxon signed-rank tests. For between-group comparisons, GLMs were produced. The distribution of variables was assessed by visual inspection of histograms. Skewed variables were ln (natural logarithm) transformed and shown to follow a ln-normal distribution. GLMs were produced to test differences between the two groups (UI; HI), unadjusted (model 1), after adjusting for baseline levels (model 2), and after adjusting for baseline levels, sex, age, smoking status and BMI (model 3). Differences in clinical parameters between groups at day 90 were assessed using GLMs adjusting for baseline levels, number of teeth, age, gender and smoking status. Differences in treatment time were assessed by a GLM controlling for disease severity (PISA mm^2) and number of teeth at baseline. Correlation analysis was performed using Spearman-Rho or Pearson's depending on data distribution.

4.3. Results

4.3.1. Study population and clinical characteristics

In total, 42 patients recruited to this study and 37 were included in the analysis (reasons for exclusion in **section 4.2.9**). Among the included patients, 17 were diagnosed with Stage IV Grade C PD. The remaining patients were diagnosed with either Stage III Grade C (n=5) or Stage III Grade B (n=15) (Papapanou et al., 2018). For treatment, 18 patients were randomised to receive treatment using UI, and 19 patients were randomised to receive treatment using HI.

The baseline characteristics for patients in each group are outlined in **table 4.2**. Comparable demographics, clinical and biological variables were observed between groups. No statistical comparison of baseline characteristics were performed as per Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines (Schulz et al., 2010) under item number 15 'Baseline Data';

> *"Such significance tests assess the probability that observed baseline differences could have occurred by chance; however, we already know that any differences are caused by chance."*

4.3.2. Clinical outcomes

Clinical parameters were assessed at baseline and 90 days following treatment (**table 4.3)**. There were significant improvements in all parameters within both groups ($p<0.01$ for all parameters [Wilcoxon signed-rank tests]). This included reductions in the percentage of pockets ≥5 mm (UI: 26.73% to 10.88%, HI: 28.85% to 11.67%) and PISA (UI: 957.93 to 134.85 mm^2, HI: 1010.02 to 192.59 mm^2). Between-group comparisons were performed using GLMs adjusted for baseline levels of each parameter, number of teeth, age, sex and smoking status. This analysis suggested no significant difference in clinical improvement between UI or HI groups ($p[\text{treatment group}]>0.05$ for all clinical parameters) which was a secondary outcome of the current study.

Table 4.2. Baseline demographics and periodontal clinical parameters. Variables are presented as median (Q1, Q3), unless followed by '†' which are presented as n (%).

Variable	Ultrasonic instrumentation (n=18)		Hand instrumentation (n=19)	
	Median (Q1, Q3)	Min, max	Median (Q1, Q3)	Min, max
Age (years)	46.00 (36.75, 54.50)	32.00, 65.00.	41.00 (39.00, 49.00)	32.00, 59.00.
Sex, female (%)†	10 (56)	N/A	9 (47)	N/A
Smoking, current (%)†	5 (28)	N/A	6 (32)	N/A
BMI (kg/m^2)	27.80 (22.68, 30.15)	20.80, 32.90.	29.70 (23.30, 34.40)	20.40, 39.00.
Systolic BP (mmHg)	124.00 (114.50, 139.50)	105.00, 165.00.	123.00 (117.00, 134.00)	104.00, 159.00
Diastolic BP (mmHg)	79.50 (75.50, 89.00)	64.00, 100.00	81.00 (73.00, 84.00)	64.00, 86.00.
CRP (mg/L)	1.60 (0.62, 2.49)	0.20, 7.28.	1.21 (0.44, 2.03)	0.12, 9.89.
IL-6 (pg/mL)	2.61 (1.13, 3.54)	0.31, 6.91.	2.29 (1.52, 4.41)	0.51, 8.00.
TNFα (pg/mL)	0.22 (0.11, 1.09)	0.01, 1.64.	0.13 (0.09, 0.36)	0.01, 0.82.
Total teeth	27.50 (24.50, 30.00)	20.00, 32.00.	29.00 (27.00, 31.00)	24.00, 32.00.
FMPS (%)	45.92 (26.10, 63.33)	7.41, 100.00	60.48 (25.00, 67.74)	8.87, 86.46
FMBS (%)	38.11 (21.45, 61.49)	14.67, 100.00	45.00 (21.26, 69.44)	4.30, 90.28
PPD (mm)	3.70 (3.35, 4.12)	2.96, 5.83.	3.98 (3.11, 4.78)	2.32, 5.73.
CAL (mm)	4.14 (3.66, 4.44)	3.15, 7.53.	4.36 (3.29, 5.02)	2.35, 7.07.
Pockets ≥5mm (%)	26.73 (22.08, 36.71)	13.1, 68.89.	28.85 (18.33, 51.39)	10.71, 70.99
PISA (mm^2)	957.93 (385.55, 1759.57)	305.62, 3125.55	1010.02 (561.99, 2190.01)	105.77, 2914.90

BP; Blood pressure, BMI; Body mass index, CRP; C-Reactive protein, IL-6; Interleukin-6, TNFα; Tumour necrosis factor alpha, FMPS; Full-mouth plaque score, FMBS; Full-mouth bleeding score, PPD; Periodontal probing pocket depth, CAL; Clinical attachment loss, PISA; Periodontal inflamed surface area.

Table 4.3. Comparison of clinical parameters between treatment groups at baseline and day 90. Values are presented as median (Q1, Q3). Analysis of clinical variables conducted with Michael Paterson.

Variable	Timepoint	Ultrasonic instruments (n=18)	Hand instruments (n=19)	Between group p-value§
FMPS (%)	Baseline	45.92 (26.10, 63.33)	60.48 (25.00, 67.74)	
	Day 90	7.80 (3.50, 13.25)	8.33 (4.17, 14.06)	
	Within group p-value†	<0.001	<0.001	0.55
FMBS (%)	Baseline	38.11 (21.45, 61.49)	45.00 (21.26, 69.44)	
	Day 90	8.10 (4.12, 12.08)	8.33 (2.98, 13.10)	
	Within group p-value†	<0.001	<0.001	0.94
Pockets ≥5mm (%)	Baseline	26.73 (22.08, 36.71)	28.85 (18.33, 51.39)	
	Day 90	10.88 (3.87, 16.88)	11.67 (3.89, 30.95)	
	Within group p-value†	<0.001	<0.001	0.23
PISA (mm^2)	Baseline	957.93 (385.55, 1759.57)	1010.02 (561.99, 2190.01)	
	Day 90	134.85 (62.31, 219.72)	192.59 (59.78, 380.49)	
	Within group p-value†	<0.001	<0.001	0.68
PPD (mm)	Baseline	3.70 (3.35, 4.12)	3.98 (3.11, 4.78)	
	Day 90	2.68 (2.39, 3.09)	3.02 (2.52, 3.73)	
	Within group p-value†	<0.001	<0.001	0.08
CAL (mm)	Baseline	4.14 (3.66, 4.44)	4.36 (3.29, 5.02)	
	Day 90	3.64 (3.10, 4.12)	4.01 (3.03, 4.68)	
	Within group p-value†	<0.001	0.005	0.14

†Differences between baseline and day 90 within groups tested using Wilcoxon signed-rank test. §GLMs were used to test differences in clinical parameters between the groups at day 90 having adjusted for baseline levels, number of teeth, age, sex and smoking status.

4.3.3. Treatment time comparison

The degree of systemic inflammation (as measured by an increase in serum CRP at day 1) induced following FMD has been previously related to duration of treatment (Graziani et al., 2015). As such, the time taken to complete treatment was compared between UI and HI treatment groups (**figure 4.5**). Treatment time followed a normal distribution pattern and was therefore analysed using parametric statistics. The difference in treatment time between instrumentation groups (UI-HI) was assessed using a GLM controlling for baseline disease severity (PISA mm^2) and number of teeth. In this trial, treatment time for UI was significantly shorter than HI (β: −22.12, 95% CI: −35.19 to −9.06, p[treatment group]=0.002). The mean (±SD) treatment time for UI was 75.39 (±17.83) min, compared with 96.90 (±23.54) min for HI (**figure 4.5A**).

Treatment time for both instruments was also correlated against different periodontal parameters (**figure 4.5B**). This was performed using Pearson's (parametric) or Spearman-Rho (non-parametric) correlation coefficients depending on data distribution. For UI, this analysis suggested negligible or weak associations between treatment time and clinical parameters. In contrast, HI treatment time showed a moderate or strong positive association with all clinical parameters, but not the total number of teeth scaled. In particular, strong associations were observed with CAL (Pearson R=0.720, 95% CI: 0.392 to 0.884, p<0.001), full-mouth PPD (Pearson R=0.760, 95% CI: 0.468 to 0.903, p<0.001) and pockets ≥5mm (Spearman R=0.720, 95% CI: 0.384 to 0.888, p<0.001), suggesting that severe PD may influence HI treatment time more than UI treatment time. However, it should be noted that this is an exploratory analysis that the study was not powered to detect, and thus comes with inherent limitations.

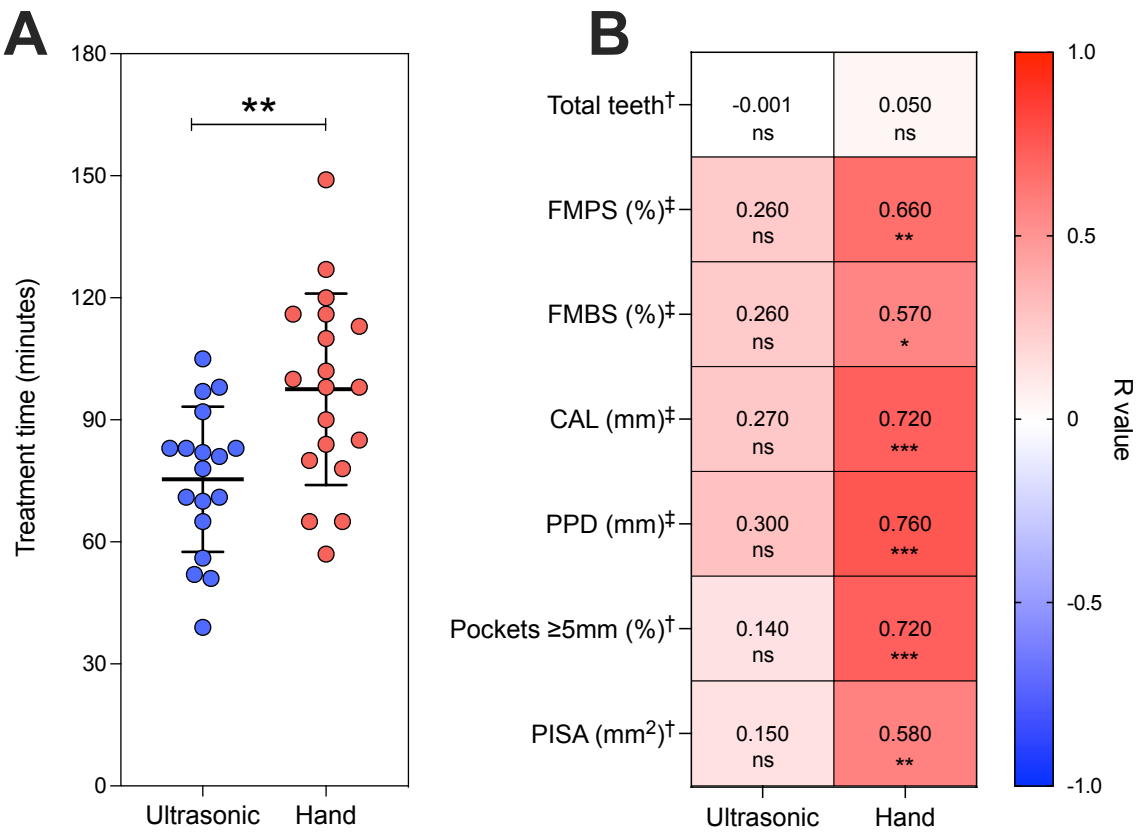

Figure 4.5. Treatment time comparison between ultrasonic (n=18) and hand (n=19) instrumentation groups. (A) Total treatment time controlled for number of teeth and disease severity (PISA mm^2) at baseline, **p<0.01, between-group GLM. Bars display mean ± standard deviation. (B) Correlation between total treatment time with ultrasonic instruments and hand instruments vs. clinical parameters. † refers to Pearson's and ‡ refers to Spearman-Rho correlation coefficients. 'ns' means no statistical significance, *p<0.05, **p<0.01, ***p<0.001. FMPS; full-mouth plaque score, FMBS; full-mouth bleeding score, CAL; clinical attachment level, PPD; periodontal probing pocket depth, PPD ≥5 mm; percentage of sites ≥5mm, PISA; periodontal inflamed surface area, all at baseline. Analysis conducted with Michael Paterson.

4.3.4. Systemic inflammation (primary outcome)

This RCT was designed to compare the immediate systemic inflammatory response following UI and HI. The primary outcome was serum CRP at day 1 post-treatment. Secondary outcomes included CRP at day 7 and day 90 post-treatment, serum IL-6 and TNFα at day 1, 7 and 90. The level of CRP, IL-6 and TNFα in UI and HI groups are displayed in **figure 4.6**, with between-group analyses presented in **table 4.4**. Upon visual inspection of histograms, all three cytokines followed non-normal distribution patterns and were ln-transformed prior to GLM analysis.

At day 1, the median level of serum CRP increased in both groups compared with baseline (UI; 1.60 to 2.57mg/L [p=0.019] and HI; 1.21 to 1.78mg/L [p=0.055], **figure 4.6A**). Differences between treatment groups were investigated using three GLM's. Model 1 was an unadjusted (direct) comparison, model 2 was adjusted only for baseline levels of CRP and model 3 was 'fully-adjusted' for baseline levels of CRP, smoking status, age, sex and BMI (**table 4.4**). Despite elevated CRP at day 1, there were no statistically significant difference between treatment groups for any GLM; p[treatment group]=0.69, p=0.60 and p=0.22 for model 1, 2 and 3 respectively. This remained consistent at day 7 and day 90 for CRP (all models p>0.05).

Longitudinal alterations in serum IL-6 appeared less consistent among treatment groups when comparing baseline and day 1 (UI; 2.61 to 3.90 pg/mL [p=0.03] and HI; 2.29 to 2.28 pg/mL [p=0.31], **figure 4.6B**). However, no significant differences were observed between groups for serum IL-6 at day 1 when assessed using GLMs (all models p[treatment group]>0.05, **table 4.4**). Similar data were observed at day 7 and day 90.

Across the entire cohort levels of serum TNFα were low, with seven samples below the assay detection limit (<0.01 pg/mL, given as LOD/2). Surprisingly within both groups, serum TNFα was lower at day 1 than baseline (UI; 0.22 to 0.12 pg/mL [p=0.003] and HI; 0.13 to 0.10 pg/mL [p=0.044], **figure 4.6C**). The level of TNFα did not significantly differ between UI or HI groups at any timepoint (all models p[treatment group]>0.05, **table 4.4**). Albeit the current study was not powered to detect changes in IL-6 or TNFα.

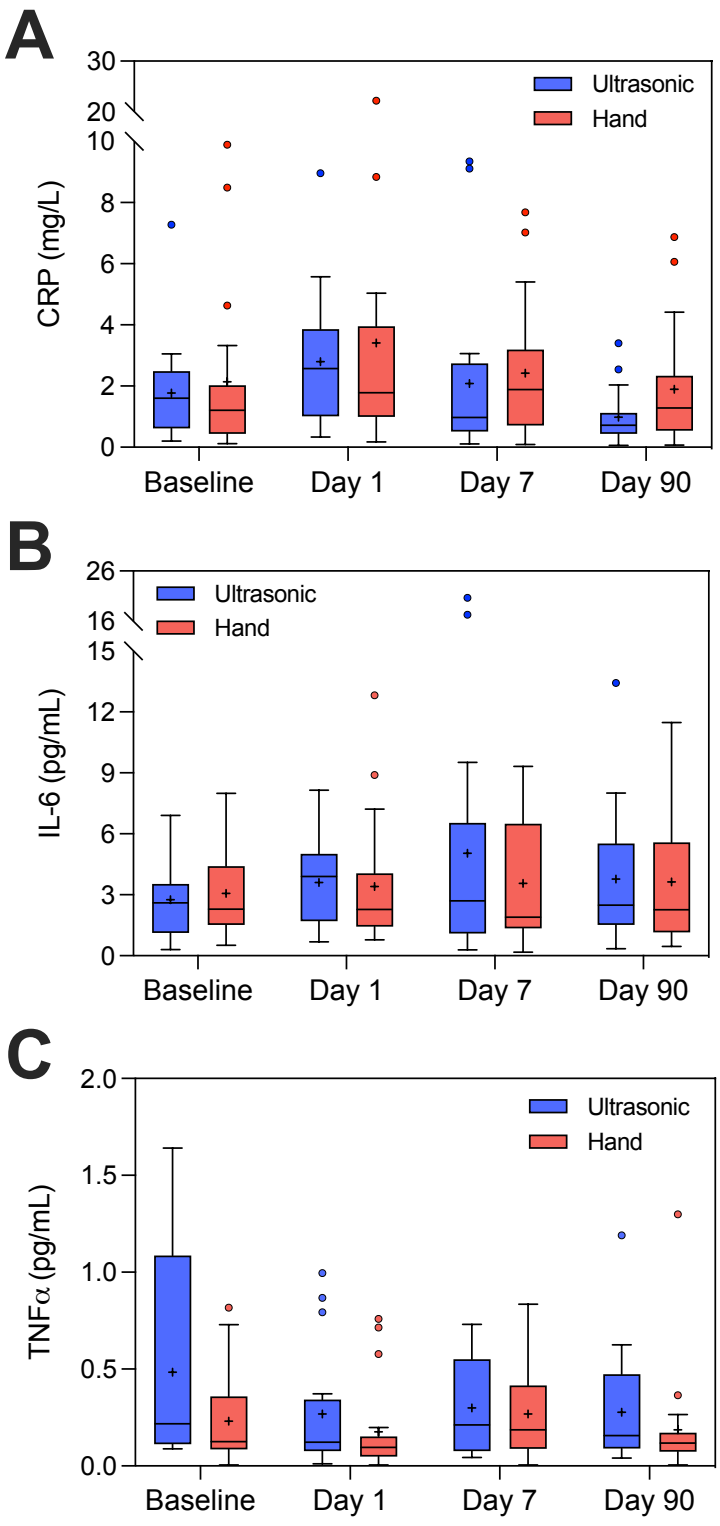

Figure 4.6. Raw data for serum inflammatory proteins in patients treated with ultrasonic and hand instruments at baseline, day 1, day 7 and day 90 follow-up. (A) CRP (B) IL-6 and (C) TNFα. Data are displayed as a Tukey boxplot, where the median is represented by the central horizontal line and mean displayed as '+'. As described previously, day 1: UI; n=17 HI; n=19, day 7: UI; n=18 HI; n=18, day 90: UI; n=17 HI; n=19. Between group statistical analysis displayed in **table 4.4** using ln-transformed data. Serum CRP analysis conducted with Michael Paterson.

Table 4.4. Parameter estimates with 95% confidence intervals for ultrasonic instrumentation compared with hand instrumentation (reference) for ln-transformed serum CRP, IL-6 and TNFα levels at day 1, day 7 and day 90. The primary outcome (serum CRP at day 1) is highlighted in yellow. Serum CRP analysis conducted with Michael Paterson.

	Day 1			Day 7			Day 90		
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
C-Reactive protein (CRP)									
Model 1†	0.143	-0.582 to 0.867	0.69	-0.271	-1.094 to 0.553	0.51	-0.518	-1.239 to 0.202	0.15
Model 2‡	0.130	-0.369 to 0.628	0.60	-0.311	-0.950 to 0.329	0.33	-0.482	-1.038 to 0.073	0.09
Model 3§	0.318	-0.196 to 0.832	0.22	-0.231	-0.972 to 0.510	0.53	-0.304	-0.867 to 0.259	0.28
Interleukin-6 (IL-6)									
Model 1†	0.133	-0.378 to 0.643	0.60	0.176	-0.620 to 0.972	0.66	-0.011	-0.671 to 0.650	0.97
Model 2‡	0.261	-0.093 to 0.614	0.14	0.233	-0.524 to 0.991	0.54	0.053	-0.546 to 0.653	0.86
Model 3§	0.193	-0.173 to 0.559	0.29	0.182	-0.634 to 0.999	0.65	-0.005	-0.645 to 0.636	0.99
Tumour necrosis factor α (TNFα)									
Model 1†	0.607	-0.307 to 1.521	0.19	0.189	-0.568 to 0.946	0.62	0.49	-0.192 to 1.171	0.15
Model 2‡	0.00003	-0.677 to 0.678	0.99	-0.087	-0.843 to 0.669	0.82	0.021	-0.512 to 0.555	0.94
Model 3§	-0.215	-0.902 to 0.472	0.53	-0.167	-1.022 to 0.688	0.69	-0.121	-0.687 to 0.445	0.67

Table displays between-group estimates only. †Model 1: Unadjusted, ‡Model 2: Adjusted for baseline levels of each serum marker, §Model 3: Adjusted for baseline levels of serum marker, sex, age, smoking status and BMI at baseline. Parameter estimates (β-values) are based on ln-transformed data. As described in **Figure 4.1**, day 1: UI; n=17 HI; n=19, day 7: UI; n=18 HI; n=18, day 90: UI; n=17 HI; n=19.

4.3.5. Evaluating factors influencing systemic inflammation

Although no difference was observed in the immediate systemic inflammatory response between treatment groups, our data does support this response occuring following FMD. Assessing all patients simultaneously (n=37), significant increases in serum CRP (p=0.002, **figure 4.7A**) and IL-6 (p=0.019, **figure 4.7B**) were observed at day 1 compared with baseline. In contrast, serum TNFα reduced at day 1 compared with baseline (p=0.002, **figure 4.7C**), albeit levels were low (<0.5 pg/mL) across the entire cohort. There also appeared to be a large heterogeneity in the extent of this response which was not related to instrumentation choice. For example, the absolute change in serum CRP across all patients ranged from a 3.45mg/L decrease to a 17.56 mg/L increase. Similarly, the change in serum IL-6 ranged from a 2.99 pg/mL decrease to a 5.30 pg/mL increase, with TNFα ranging from a -1.27 pg/mL decrease to a 0.09 pg/mL increase at day 1.

To evaluate whether this heterogeneity was associated with clinical factors (treatment time, number of teeth, disease severity), correlation analysis was performed between these variables and the absolute change in serum CRP, IL-6 and TNFα (**figure 4.8A-I**). For this analysis, PISA was used as an indicator of disease severity. Despite a previously reported link between treatment time and the immediate systemic inflammatory response (Graziani et al., 2015), there were negligible associations between the change in CRP, IL-6 or TNFα and time of treatment (**figure 4.8A,D,G**). Lastly, the current study did not find any evidence for an association between total teeth or baseline PISA with the change in any systemic marker. Thus, the heterogeneity in systemic response did not appear to be strongly associated with differences in treatment time, baseline clinical variables or number of teeth within the current study.

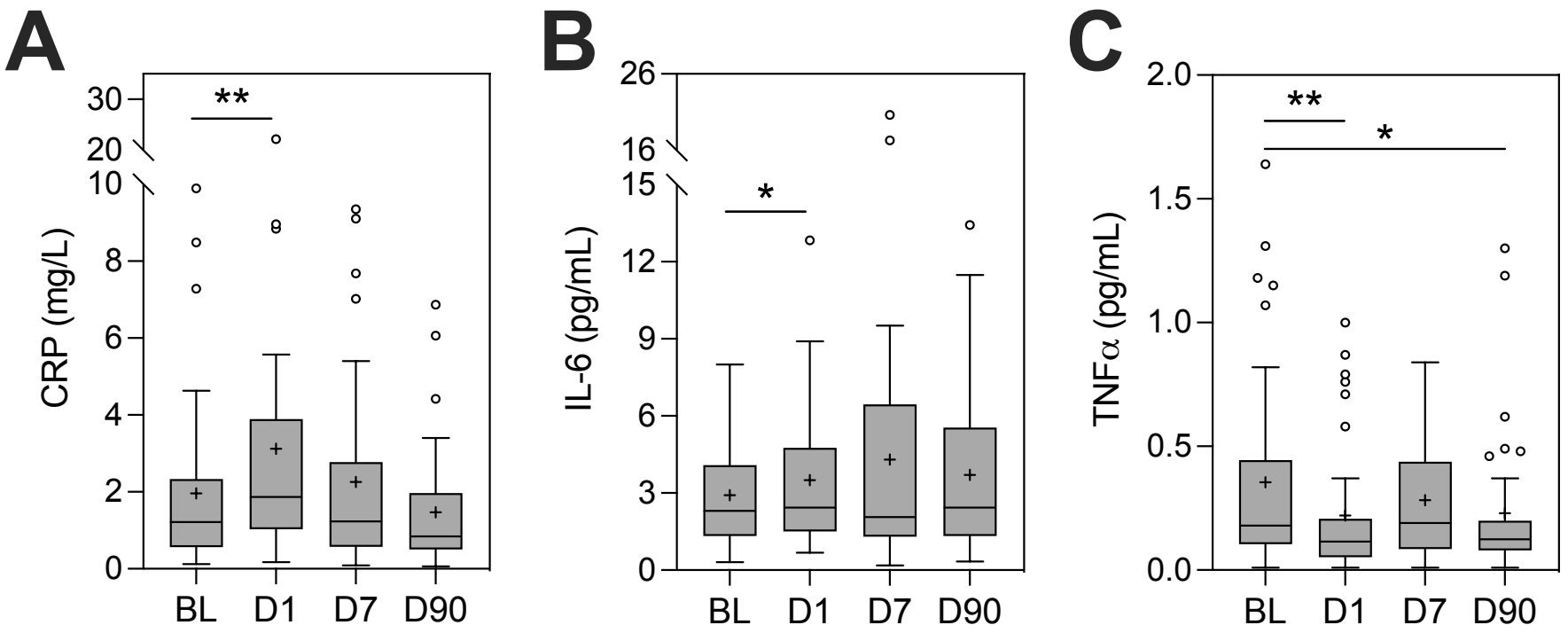

Figure 4.7. Serum inflammatory proteins across all patients following full-mouth debridement. (A) CRP, (B) IL-6 and (C) TNFα were compared across the entire cohort (n=37) at baseline (BL), day 1 (D1), day 7 (D7) and day 90 (D90). Data are displayed as a Tukey boxplot, where the median is represented by the central horizontal line and mean displayed as '+'. Statistics refer to Wilcoxon signed-rank tests were **$p<0.01$ and *$p<0.05$. No sign is displayed if $p>0.05$.

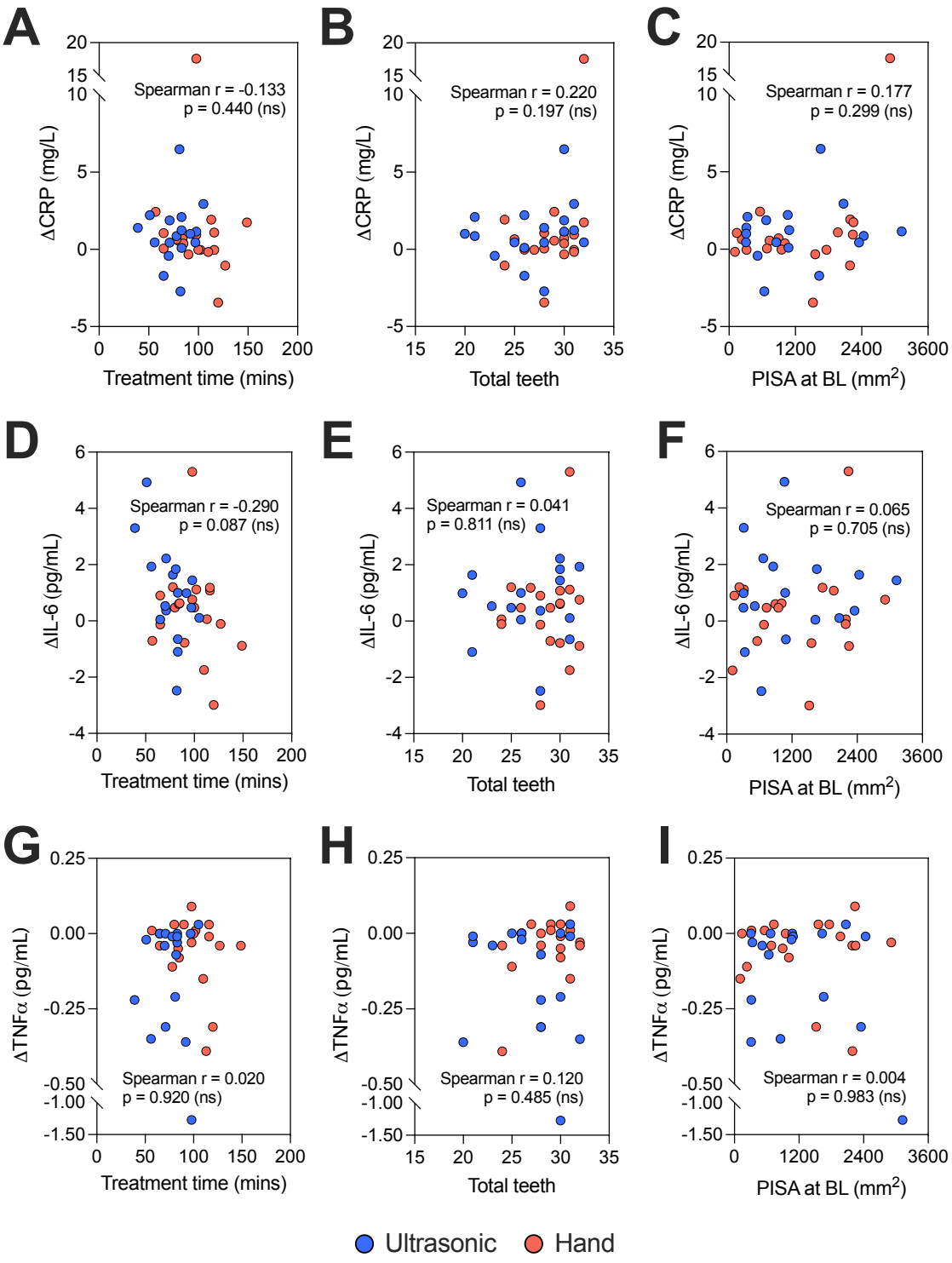

Figure 4.8. Investigating factors contributing to the systemic inflammatory response. Scatterplots showing the change (Δ) in serum CRP (A-C), IL-6 (D-F) and TNFα (G-I) against treatment time (A, D, G), total teeth (B, E, H) and PISA at baseline (C, F, I). Blue circles represent patients in ultrasonic treatment group, red represent hand treatment group. All correlations are Spearman-rho, ns indicates no significant difference. UI; n=18, HI; n=19.

4.3.6. Multiplex analysis of serum and saliva samples

Levels of serum CRP, IL-6 and TNFα were initially assessed given previous studies showing an elevation following FMD. To further analyse the systemic inflammatory response, serum samples from a subset of patients were selected for Luminex™ analysis. Saliva samples from the same patients were included to evaluate the local inflammatory response following treatment. Twelve patients (6x UI, 6x HI) were included in this analysis, selected based on the highest measured CRP elevation at day 1 within each group (**figure 4.9**).

Serum and saliva samples were evaluated using a Luminex™ assay containing 30 cytokines, chemokines and growth factors. For all analytes, the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) are displayed in **table 4.5**. In theory, any measurement above the blank control can be extrapolated off of the standard curve, however the LLOQ and ULOQ represent the range at which concentrations can be measured reliably. To avoid potential bias and imprecision, only analytes with at least 50% of samples between the LLOQ and ULOQ were analysed. This arbitrary threshold excluded analytes where the majority of samples were extrapolated off of the standard curve. Only 13 and 18 analytes met this threshold in serum and saliva samples respectively. The majority of undetectable analytes in both serum and saliva samples were cytokines.

Within serum samples, no longitudinal alterations were observed for any of the 13 analytes assessed, comparing day 1 and day 90 samples with baseline across all patients (n=12, **figure 4.10A**). In contrast, 7 of the 18 analytes assessed in saliva significantly increased at day 1 compared with baseline (**figure 4.10B**). This included salivary IL-1ß (medians; 24.63 to 49.20 pg/mL), IL-10 (2.39 to 3.40 pg/mL), IL-6 (20.67 to 138.10 pg/mL), MIP-1α (14.45 to 19.05 pg/mL), MIP-1ß (21.59 to 35.58 pg/mL), IL-7 (21.37 to 26.16 pg/mL) and IL-8 (770.00 to 2910.00 pg/mL). This analysis was conducted across all patients regardless of treatment group.

To compliment this analysis, the $\log_2$ fold-change between day 1 and baseline was visually compared between UI and HI groups (**figure 4.11**). In serum samples, the median $\log_2$ fold-change was low (>-1 and <1) with a large degree of variation observed within both groups. In saliva samples, the largest log2 fold-

change was observed for IL-6, IL-8 and IL-1β. Additionally, this value appeared larger for the UI group for all three analytes. Based on these data, IL-6, IL-8 and IL-1β were selected for further analysis and subsequently quantified across every patient. Additionally, salivary TNFα was included to compare with the serum analysis, as well as salivary IL-17A given the data observed previously in **chapter 3** of this thesis. Given that no longitudinal alterations were observed for any detectable serum analyte, despite clear CRP elevation in these patients, no serum analytes were investigated further.

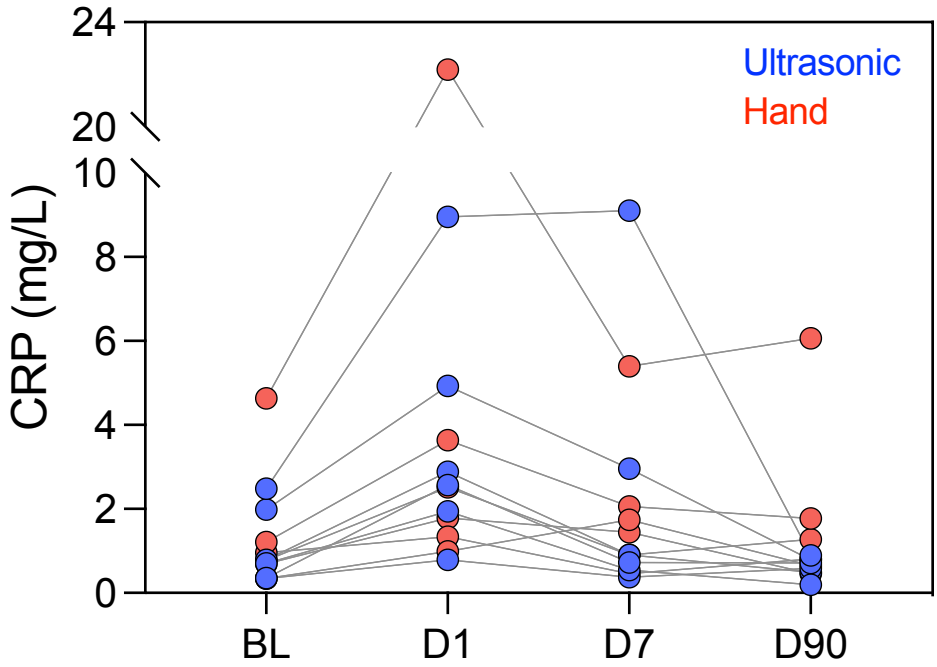

Figure 4.9. C-Reactive protein at each timepoint for the subset of patients selected for Luminex™ analysis. Equal numbers of patients from both the ultrasonic (n=6, blue) and hand group (n=6, red) were selected. Individual values are displayed with lines connecting each patient.

Table 4.5. Lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) for Luminex™ analytes. Analytes <LLOQ in >50% of samples (red text) were not further analysed. No samples were >ULOQ for any analyte.

Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)	Serum samples in range (n)	Saliva samples in range (n)
FGF-Basic	8.56	3924.77	18 (50.00)	35 (97.22)
IL-1ß	9.76	4222.22	7 (19.44)	35 (97.22)
G-CSF	94.10	64815.04	2 (5.56)	30 (83.33)
IL-10	5.41	2060.57	5 (13.89)	21 (58.33)
IL-13	16.16	6733.18	0 (0.00)	15 (41.67)
IL-6	7.78	5614.74	0 (0.00)	36 (100.00)
IL-12	10.33	3963.20	36 (100.00)	33 (91.67)
RANTES	11.79	3553.73	36 (100.00)	15 (41.67)
Eotaxin	1.84	696.04	36 (100.00)	15 (41.67)
IL-17A	26.78	11628.06	0 (0.00)	0 (0.00)
MIP-1α	17.82	12047.03	6 (16.67)	28 (77.78)
GM-CSF	6.81	2593.41	0 (0.00)	15 (41.67)
MIP-1ß	24.75	11398.27	24 (66.67)	32 (88.89)
MCP-1	24.00	10705.11	36 (100.00)	36 (100.00)
IL-15	58.68	10208.35	6 (16.67)	16 (44.44)
EGF	13.08	6390.40	25 (69.44)	36 (100.00)
IL-5	11.07	4481.81	0 (0.00)	15 (41.67)
HGF	68.16	46385.24	37 (52.78)	33 (91.67)
VEGF	0.80	311.97	10 (27.78)	36 (100)
INF-γ	7.21	2793.32	18 (50.00)	29 (80.56)
INF-α	10.81	5355.51	18 (50.00)	33 (91.67)
IL-1RA	55.84	28413.37	16 (44.44)	14 (38.89)
TNFα	8.54	7050.27	0 (0.00)	5 (13.89)
IL-2	13.25	5909.49	1 (2.78)	12 (33.33)
IL-7	13.18	6175.09	1 (2.78)	30 (83.33)
IP-10	0.52	219.75	36 (100.00)	35 (97.22)
IL-2R	57.41	22050.90	18 (50.00)	9 (25.00)
MIG	26.91	9738.56	20 (55.56)	33 (91.67)
IL-4	34.34	13192.38	1 (2.78)	12 (33.33)
IL-8	12.19	9705.19	3 (8.33)	36 (100)

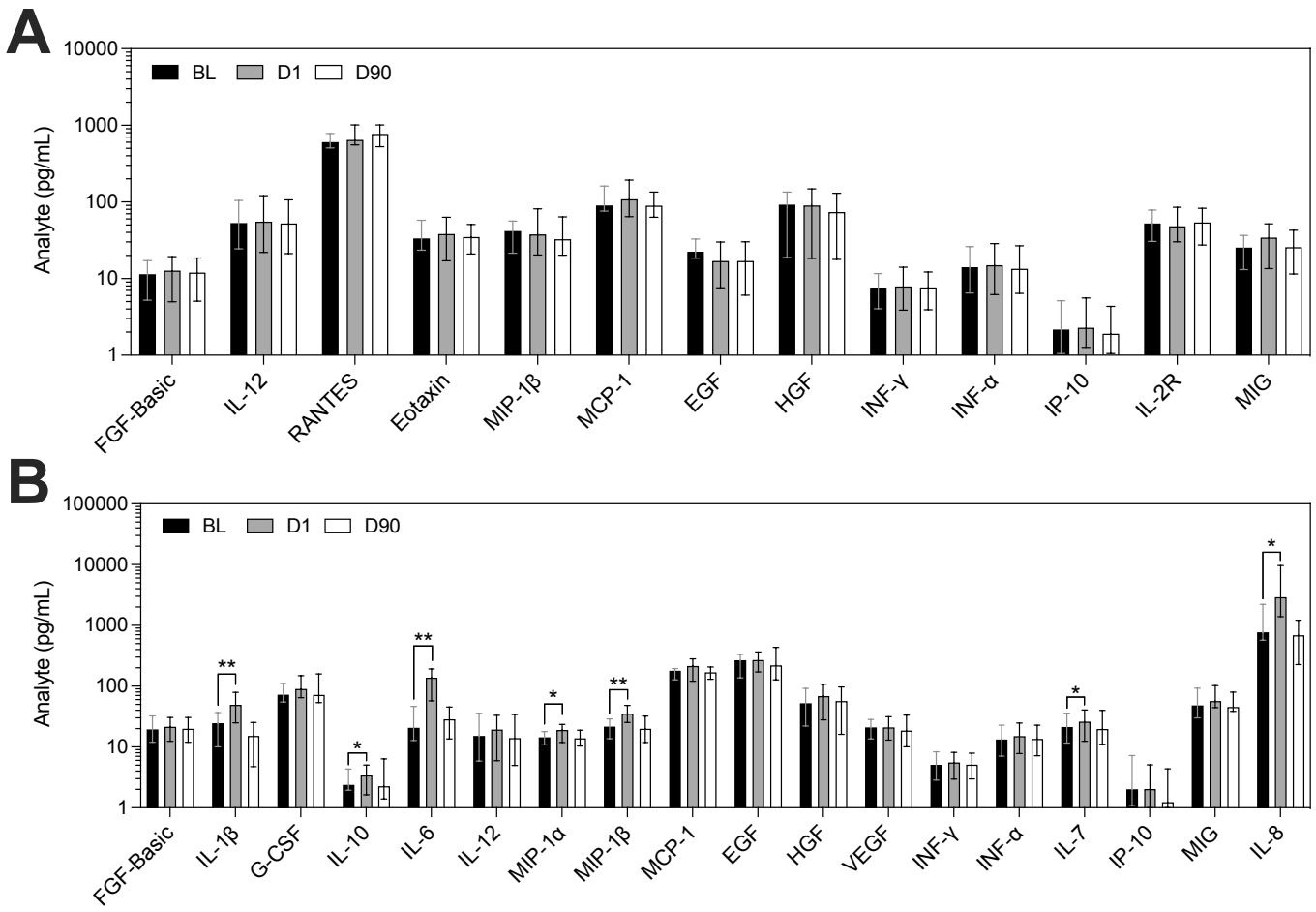

Figure 4.10. Longitudinal comparison of analytes in serum and saliva samples by Luminex™. Comparing the level of analytes with at least 50% of samples >LLOQ in serum (A) and saliva (B) samples. Bars display median ± 95% CI. Statistics are Wilcoxon signed-rank test corrected using the FDR (5%), *p<0.05, **p<0.01. N=12. Y-axis is $\log_{10}$-scaled.

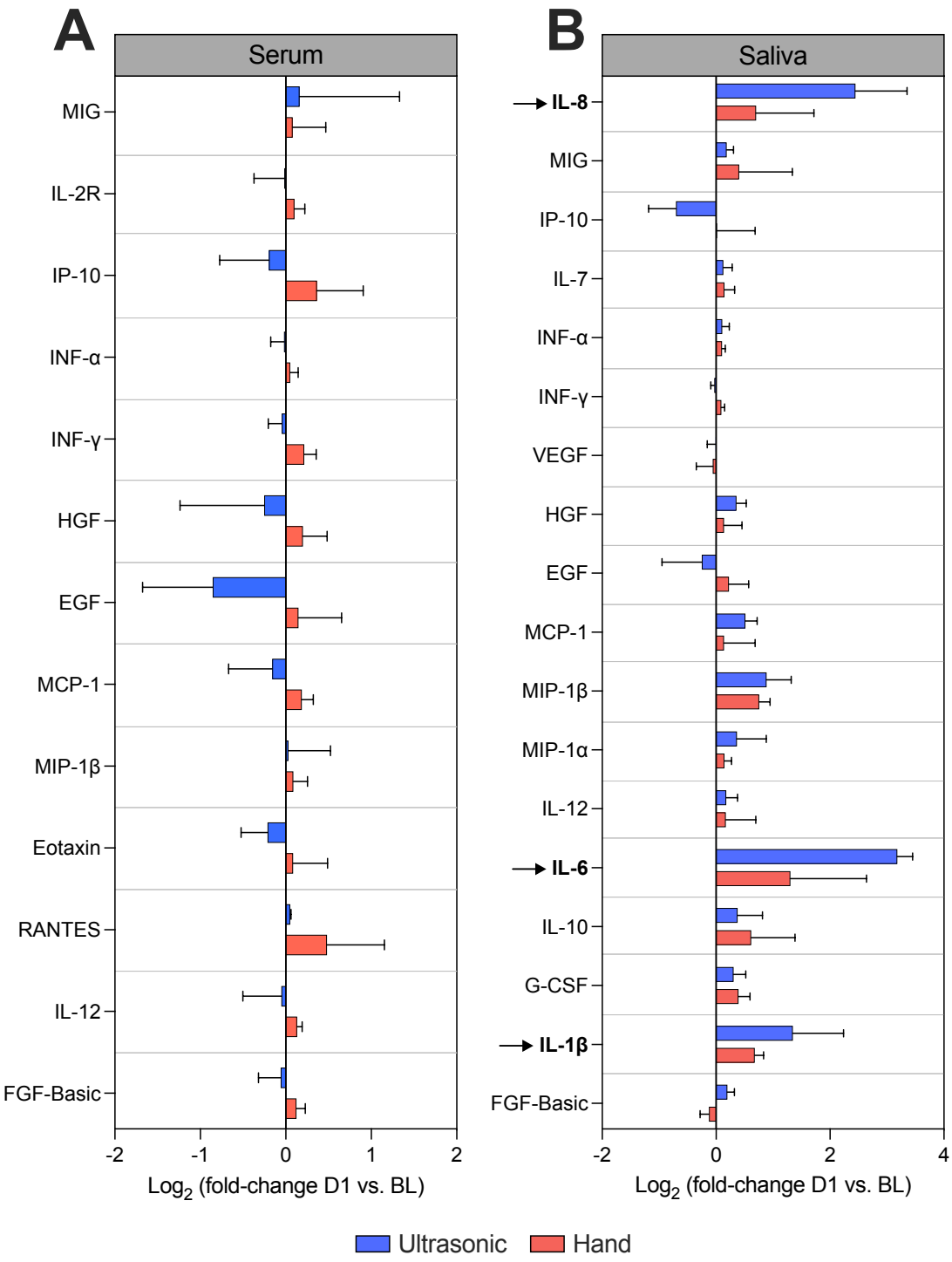

Figure 4.11. Fold change in analytes at day 1 compared with baseline. The log_2 fold-change in analytes from serum (A) and saliva (B) samples are compared between ultrasonic (blue) and hand (red) groups. Only analytes >LLOQ in at least 50% of samples are included. Bars display median ± 95% CI. Arrows indicate analytes selected for further analysis (salivary IL-1ß, IL-8 and IL-6). N=12.

4.3.7. Comparing Luminex™ and singleplex measurements

Levels of salivary IL-6, IL-8, IL-1ß, TNFα and IL-17A were assessed across all patients to assess the local inflammatory response between groups. To investigate inter-assay agreement between Luminex™ and conventional ELISA technologies, a correlation analysis was performed between the three detectable analytes assayed in the same samples (IL-1ß, IL-6, IL-8). Both TNFα and IL-17A were not included in this analysis as the nearly all Luminex™ measurements were extrapolated or undetectable. Reassuringly, levels of IL-1ß, IL-6 and IL-8 showed significant positive correlations between ELISA and Luminex™ measurements with Spearman R values of 0.772, 0.742 and 0.803 respectively (all $p<0.001$, **figure 4.12**). A rigorous coefficient threshold of $r>0.75$ has been previously employed to discern a 'significant' correlation between ELISA and Luminex™ measurements (Dupont et al., 2005). Coefficients within this study fell above (IL-1ß, IL-8) or within 5% (IL-6) of this threshold. Thus, measurements appeared relative regardless of assaying technique for these three markers.

Interestingly, although values did appear relative, there was a huge disparity in measured concentrations. For example, IL-1ß measurements were almost 10-fold higher for all samples when assayed using singleplex ELISAs compared with the Luminex™ assay (**figure 4.12A**). In contrast, IL-6 values were roughly 5-fold lower (**figure 4.12B**), and IL-8 values roughly 5-fold higher (**figure 4.12C**) when measured using ELISA compared with Luminex™ technology. These differences highlight the semi-quantitative nature of each assay and reinforce the point that comparing quantitative differences between different samples measured on different assays, or between different studies employing different detection techniques, can be misleading.

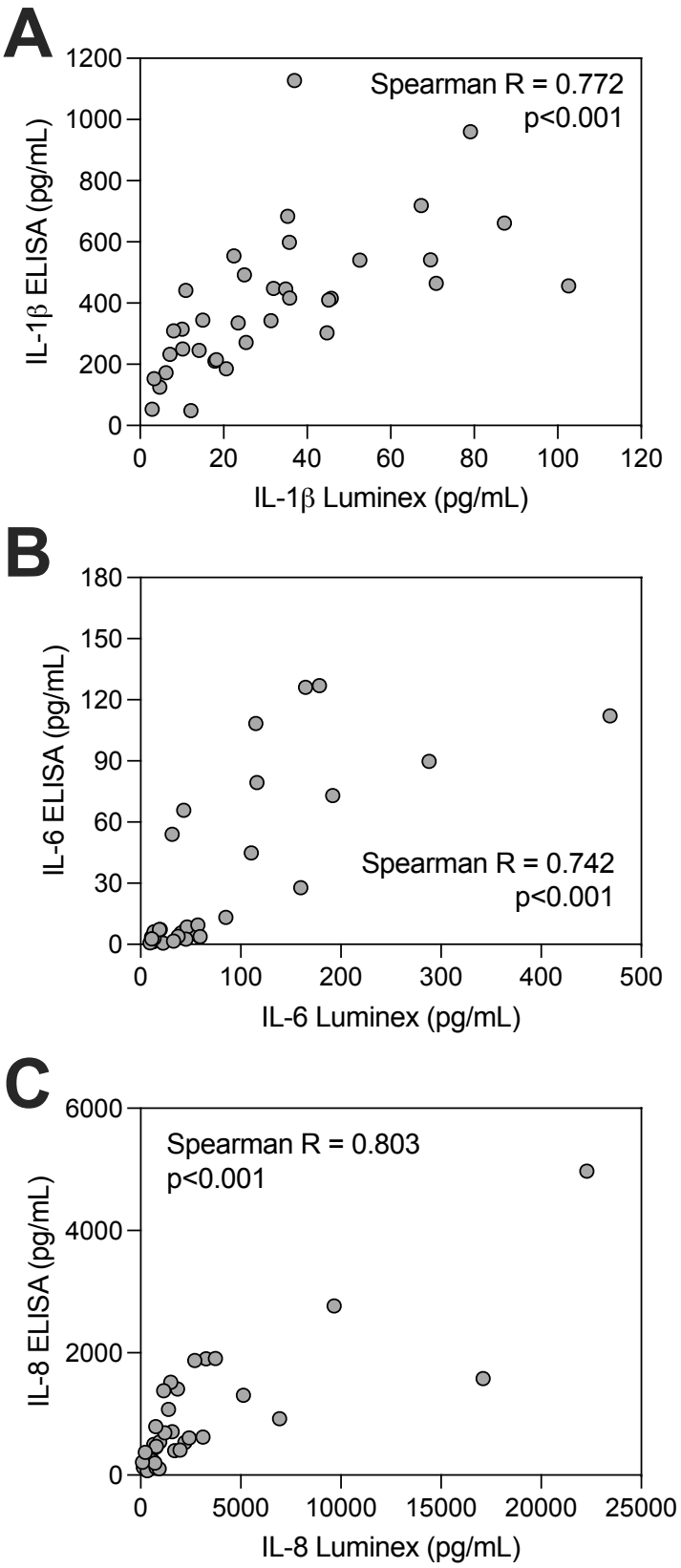

Figure 4.12. Scatterplot comparing salivary cytokine levels assayed using Luminex™ or ELISA assays. (A) IL-1ß, (B) IL-6 and (C) IL-8 were >LLOQ in at least 50% of samples when assayed on Luminex™ and could thus be compared between detection methods. Statistics are Spearman-Rho correlation coefficients, raw R and p-values are displayed.

4.3.8. Salivary analysis

An exploratory analysis was conducted to investigate the local inflammatory response following UI and HI. The five selected salivary cytokines were compared at day 1, 7 and 90 using GLMs, with p-values referring to fully-adjusted models herein (model 3). All cytokines showed a non-normal data distribution, and therefore values were ln-transformed prior to analysis.

Salivary TNFα increased in the UI (p=0.02, Wilcoxon signed-rank test) but not HI (p=0.39) group at day 1 compared with baseline (**figure 4.13A**). When comparing each group using a GLM, no significant difference between treatment groups was observed, likely attributed to the large variation in baseline measurements (ß: 0.485, 95% CI: -0.153 to 1.123, p[treatment group]=0.13). Likewise, this analysis suggested no difference in salivary TNFα between groups at days 7 and 90 (p>0.05 at both timepoints).

For salivary IL-6, a significant increase was observed at day 1 within both groups (p<0.001 for both, **figure 4.13B**), representing a median 3.8 and 2.5 $\log_2$ fold-change in UI and HI groups respectively (**figure 4.13F**). Between group analysis demonstrated that the level of IL-6 at day 1 was significantly higher following UI compared with HI (ß: 0.975, 95% CI: 0.420 to 1.531, p[treatment group]<0.001). Salivary IL-6 returned back to similar levels between groups at day 7 (p=0.69) and day 90 (p=0.49).

Similar data were observed for salivary IL-8; with significant increases observed within both groups at day 1 compared with baseline (p<0.001 for both, **figure 4.13C**). In line with IL-6, between-group analysis showed significantly greater levels of salivary IL-8 at day 1 following UI compared with HI (ß: 0.745, 95% CI: 0.285 to 1.205, p[treatment group]=0.002), which was not maintained at day 7 or day 90 (p>0.05 at both timepoints).

There was no increase in salivary IL-17A within either group at day 1. However, IL-17A increased significantly in the UI group at day 90 supporting results from **chapter 3** of this thesis. Additionally, between-group analysis suggested elevated IL-17A at day 90 within the UI group compared with HI (ß: 0.874, 95% CI: 0.184 to 1.563, p[treatment group]=0.015). No differences were observed between groups for salivary IL-17A at day 1 or day 7.

No alterations in salivary IL-1β were recorded within either group at day 1. However, significant reductions in this analyte were observed as early as day 7 in both groups ($p<0.05$ for both), which were maintained until day 90 ($p<0.01$ for both). Suggesting this analyte may be highly responsive to reductions in periodontal inflammation. Furthermore, the current analysis suggested no between-group differences at any timepoint for this cytokine (p[treatment group]>0.05 for all).

Collectively, these data suggest that both salivary IL-6 and IL-8 were higher following UI compared with HI. Whether this elevation represents a larger degree of local trauma induced via treatment, or simply 'flushing' of the pocket contents into saliva remains to be seen. Additionally, in contrast to serum CRP there is no published data defining a 'clinically meaningful' difference in this local inflammatory spike and such a conclusion cannot be drawn from the current study. Of note, these alterations did not appear to transition systemically. As mentioned throughout this chapter, salivary cytokine analysis was exploratory in nature and the study was not powered nor designed to detect these differences. This carries with it inherent limitations which should be considered when interpreting this analysis.

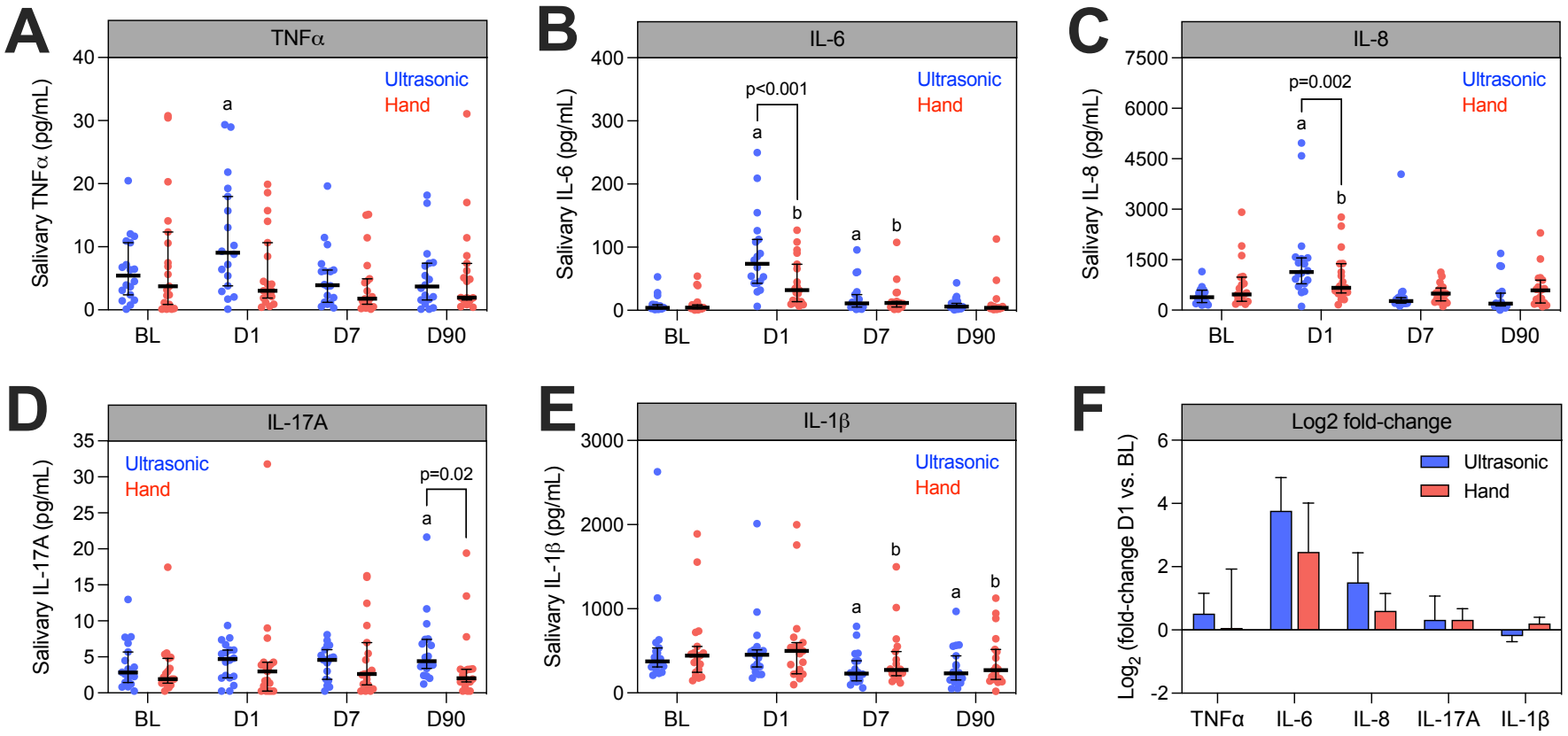

Figure 4.13. Levels of salivary cytokines in ultrasonic and hand groups at baseline, day 1, day 7 and day 90. Salivary (A) TNFα, (B) IL-6, (C) IL-8, (D) IL-17A, (E) IL-1ß and (F) log_2 fold-change at day 1 vs. baseline. Error bars display median ± 95% CI. Ultrasonic (blue): n=18, hand (red): n=19. Between-group comparisons refer to general linear models adjusted for baseline level of each salivary cytokine, sex, age, smoking status and BMI, significant differences are shown by connecting lines between groups. Longitudinal analysis represents Wilcoxon signed-rank test were a; significant difference in ultrasonic group and b; significant difference in hand group vs. baseline.

4.3.9. Gingival crevicular fluid (GCF) analysis

To investigate whether salivary alterations were consistent in GCF, the same five cytokines were measured in GCF samples. Any GCF strip with blood contamination was discarded prior to analysis. The number of eluted strips per patient at each timepoint is provided in **figure 4.14A-B**, with UI and HI groups displayed separately. As may be expected, blood contamination mainly impacted baseline samples within each group. At days 1, 7 and 90 the majority of patients had all 4 strips eluted. For subsequent GCF analysis, cytokines were standardised to the number of strips eluted, as described previously (**section 3.2.6.2**). Among the sampled sites, significant reductions in the average PPD (**figure 4.14C**) and CAL (**figure 4.14D**) were observed ($p<0.001$ for both, paired t-tests).

The current analysis suggested no differences in GCF TNFα between groups at any timepoint (**figure 4.15A**). GCF IL-6 increased in both groups at day 1 ($p<0.001$ vs. baseline, **figure 4.15B**), representing a 2.8 (UI) and 1.9 (HI) log_2 fold-change increase compared with baseline (**figure 4.15F**). In contrast to saliva, between-group analysis suggested no difference between UI and HI for IL-6 at day 1 (p[treatment group]=0.72). Similar data were observed for IL-8 at day 1, with increases observed within both groups compared with baseline ($p<0.001$, **figure 4.15C**). This elevation appeared comparable between groups with a 1.5 (UI) and 1.8 (HI) log_2 fold-change in each group (**figure 4.15F**). Accordingly, there was insufficient evidence to suggest a difference between groups at day 1 (p=0.89). A borderline difference in GCF IL-8 was observed between groups at day 7 (β: -0.406, 95% CI: -0.718 to -0.094, p=0.012), although this returned to similar levels by day 90 (p=0.66).

The level of IL-17A in GCF samples remained consistent throughout all timepoints and did not differ following UI or HI (**figure 4.15D**). No differences in IL-1β were observed between groups; however, significant reductions were observed after treatment - as early as day 7 in the UI group (p=0.003, day 7 vs. baseline) and day 1 in the HI group ($p<0.001$, day 1 vs. baseline), supporting comparable shifts observed in saliva from the same patients.

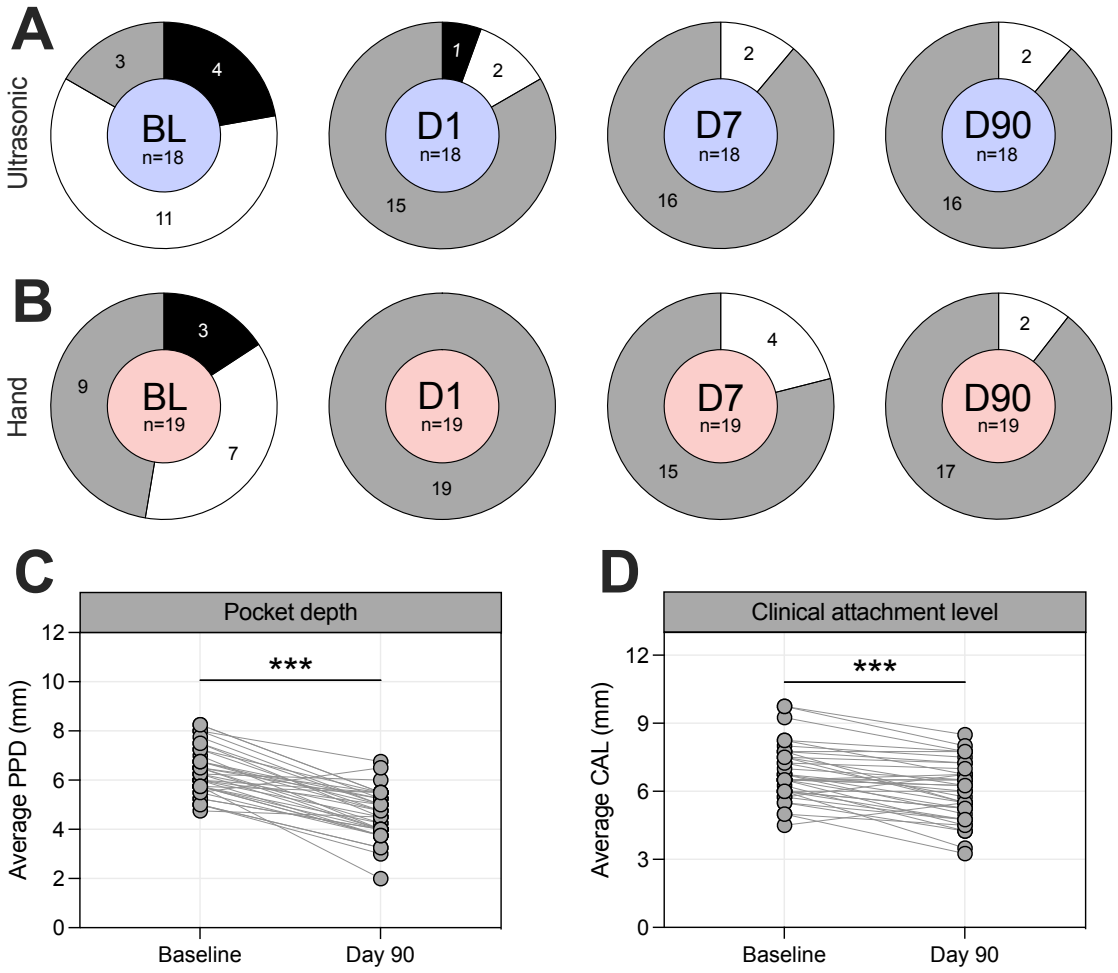

Figure 4.14. Eluted strips and clinical parameters for GCF sampled sites. Pie charts displaying the number of strips eluted per patient at each timepoint in the ultrasonic (A) and hand group (B). In pie charts, grey colouring represents four eluted strips, white represents three eluted strips and black represents two eluted strips. Average pocket depth (C) and CAL (D) of sampled sites at baseline and day 90. Statistics represent paired t-tests where ***p<0.001. BL; Baseline, D1; Day 1, D7; Day 7, D90; Day 90.

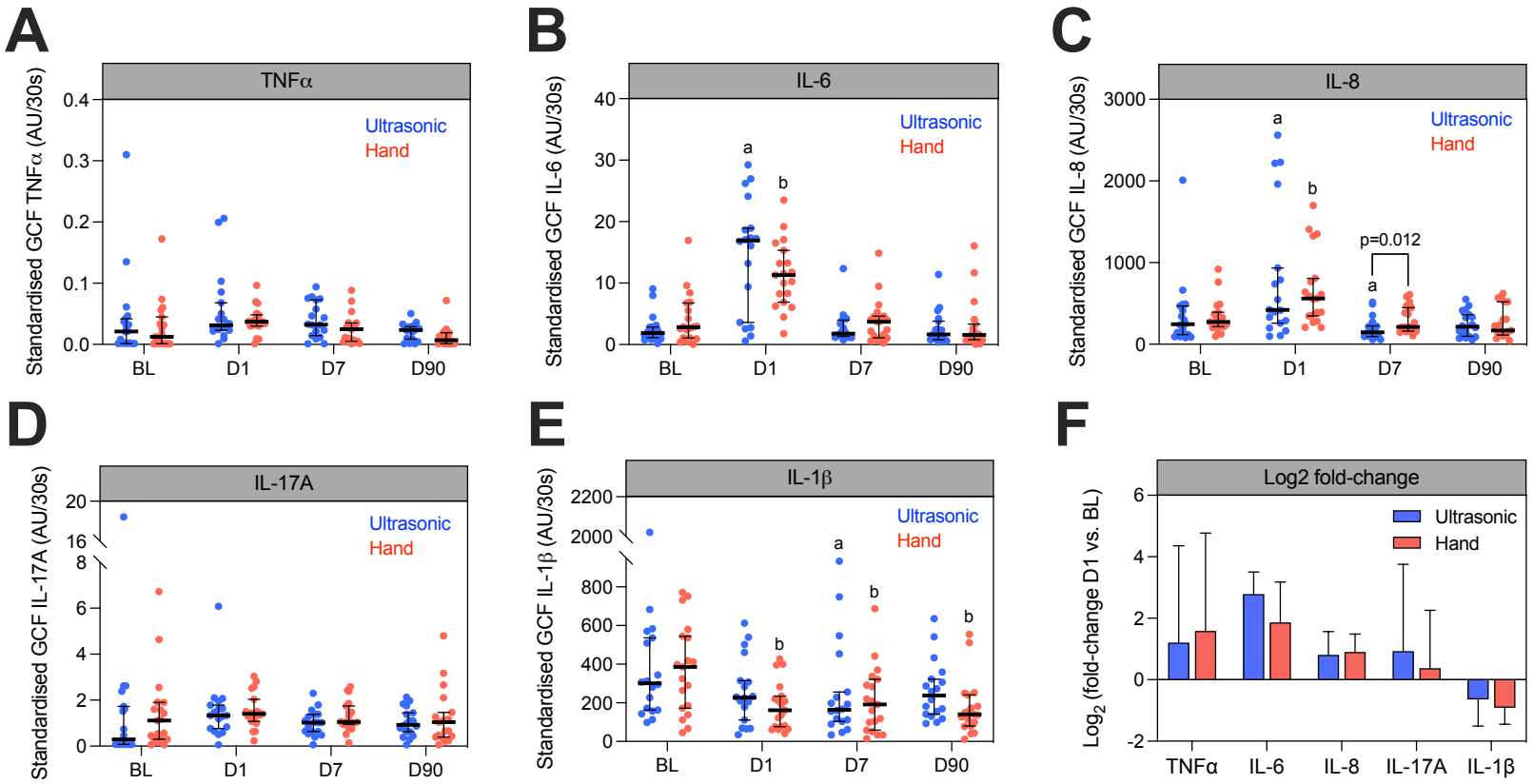

Figure 4.15. Standardised GCF cytokines in ultrasonic and hand groups at baseline, day 1, day 7 and day 90. Graph's display concentrations of TNFα (A), IL-6 (B), IL-8 (C), IL-17A (D), IL-1ß (E) and log_2 fold-change of all cytokines at day 1 vs. baseline (F). Error bars are median ± 95% CI. Ultrasonic (blue): n=18, hand (red): n=19. Between-group comparisons are general linear models adjusted for baseline level of each cytokine, sex, age, smoking status and BMI, significant differences are shown by connecting lines between groups. Longitudinal analysis represents Wilcoxon signed-rank test were a; significant difference in ultrasonic group and b; significant difference in hand group vs. baseline.

4.3.10. Association between day 1 'spikes' in serum, saliva, GCF

To evaluate whether elevated levels of local cytokines (saliva, GCF) were associated with an increased systemic response, Spearman-Rho correlations were performed using delta values from baseline to day 1. No strong associations were observed between the change in CRP at day 1 and the change in any local cytokine (**figure 4.16**). Weak positive associations existed between the change in serum CRP and salivary IL-6 (R=0.33, 95% CI: -0.01 to 0.60, p=0.048), IL-8 (R=0.39, 95% CI: 0.06 to 0.64, p=0.02), and TNFα (R=0.40, 95% CI: 0.07 to 0.65, p=0.02).

For serum IL-6, a weak positive association was observed with the change in GCF TNFα (R=0.37, 95% CI: 0.04 to 0.63, p=0.03), whilst a moderate negative association was observed with GCF IL-1ß (R=-0.54, 95% CI:-0.74 to -0.24, p=0.02). For serum TNFα, a weak negative association was observed with salivary IL-6 (R=-0.37, 95% CI: -0.63 to -0.04, p=0.03). Surprisingly, the change in salivary IL-6 and TNFα did not associate with the serum alterations in these same cytokines.

From this data it could be speculated that local trauma may play a role, along with bacteraemia, in driving the systemic inflammatory response to FMD. Although these associations were relatively weak with regard to CRP, the finding that local cytokines (IL-6, IL-8) were significantly elevated at day 1 is intriguing and should be further explored. Importantly, although UI appeared to induce larger increases in salivary levels of these markers at day 1, these trends were not maintained systemically with no difference between groups in serum CRP.

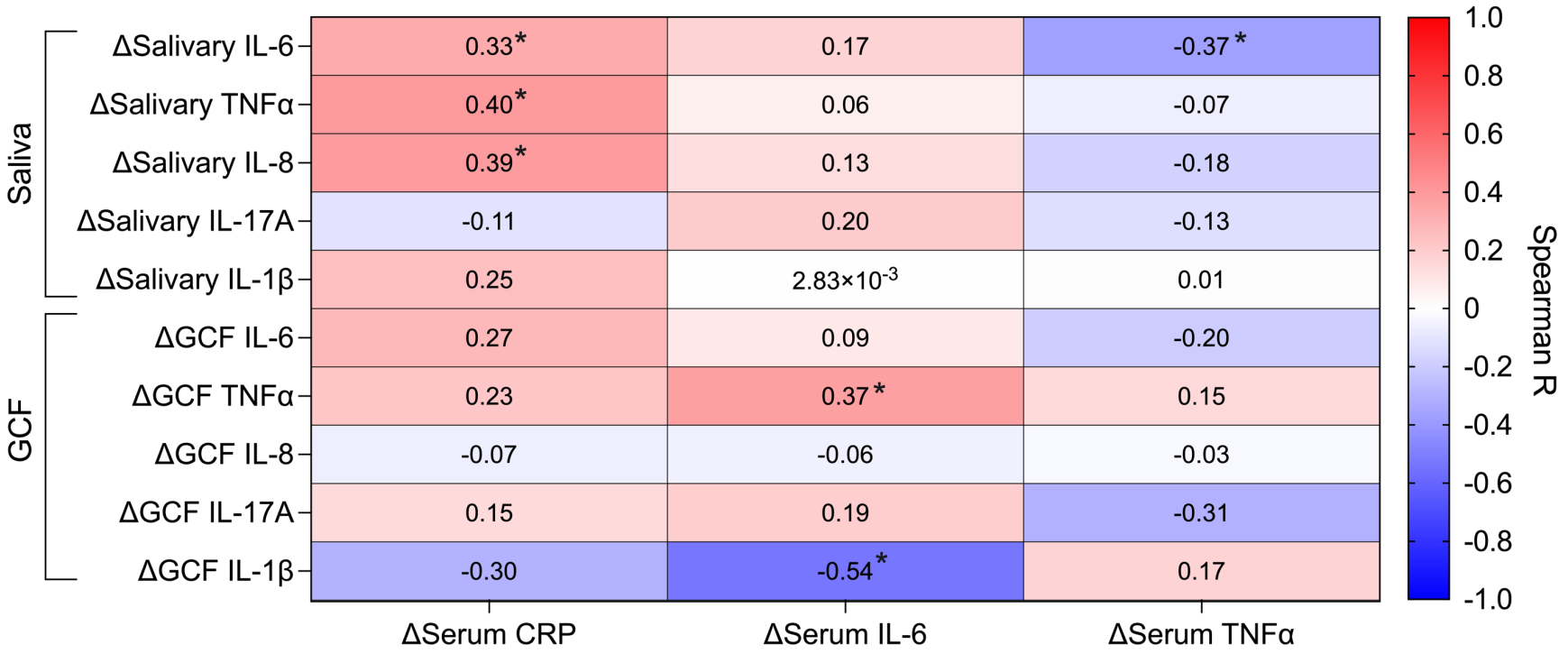

Figure 4.16. Heatmap displaying correlation coefficients between the change in CRP and cytokines at day 1 compared with baseline. The change in cytokines from saliva and GCF samples was correlated against the change in serum markers. Heatmap displays R values generated by Spearman-Rho correlation across all patients (n=37). *associated p-value <0.05.

4.4. Discussion

This chapter presents the first RCT to investigate the impact of different instrumentation techniques on systemic inflammation following FMD. A significant increase in serum CRP was observed one day after treatment, across all patients. However, there was no difference in CRP between hand or ultrasonic treatment groups at day 1 following adjustment, and the magnitude of this response was generally low across the majority of patients. Similar findings were observed for IL-6, whilst TNFα reduced at day 1 in contrast to previous studies. The local inflammatory response, as measured by an increase in salivary cytokines at day 1, appeared greater following ultrasonic compared with hand instrumentation. Clinical improvement was recorded irrespective of instrumentation group at day 90 follow-up, although lower treatment time was observed for the ultrasonic group.

The immediate increase in serum CRP following FMD is in line with several previous studies (D'aiuto et al., 2004a, Tonetti et al., 2007, Graziani et al., 2010, Graziani et al., 2015, Morozumi et al., 2018). However the magnitude of increase in the current study was notably lower than has been observed previously. Comparing baseline and day 1 serum CRP this study found a 1.67-fold increase across all patients, which is somewhat lower than Graziani et al. and Tonetti et al. with an approximately 3-fold and 8-fold increase respectively (Graziani et al., 2015, Tonetti et al., 2007). One difference which may explain this finding is the total treatment time within each study. Across all patients, mean treatment time was 86.6 minutes within the current study, compared with 123 minutes in the study by Graziani et al. for the FMD group (Graziani et al., 2015). Similarly, baseline plaque scores were lower in the current study (48.0%) than Graziani et al. (70.0%), which is likely due to all patients receiving basic oral hygiene instruction from GDPs prior to referral to the department. Patients also received an additional supragingival scale prior to treatment (after baseline plaque scoring) as per current clinical guidelines, which will have reduced scores even further (Scottish Dental Clinical Effectiveness Programme, 2014, Lang and Lindhe, 2015, Suvan et al., 2019).

A recent joint consensus statement between the EFP and AAP advised against the use of FMD in patients with any level of CVD (Sanz et al., 2020). In this

study, although an increase in serum CRP was recorded at day 1, there was large heterogeneity in the extent of this response. The average increase was 1.07 mg/L and ranged from a 3.45 mg/L decrease to a 17.56 mg/L increase, demonstrating large inter-patient variation which was not related to instrumentation choice, treatment time or baseline disease severity. Using a similar study design, a +1444C>T polymorphism in the CRP allele has been shown to influence day 1 CRP levels following FMD (D'aiuto et al., 2005). Individuals homozygous for the +1444T allele (TT) show a significantly higher CRP spike than individuals carrying the C-allele (CC or CT), with this finding being supported by an earlier study assessing CRP spikes following intensive exercise (Brull et al., 2003). A genetic predisposition may partially explain heterogeneity in CRP concentrations, although C-allele carriers still displayed far higher levels of CRP at day 1 following FMD compared with the current study (+10 to 15 mg/L). Differences in quantitative alterations therefore most likely lie in study design. To this extent, assessing whether supragingival debridement, prior to FMD, may help reduce this spike would be an important comparison within future studies.

Compared with CRP, the alterations in serum IL-6 and TNFα were less consistent. The latter decreased significantly at day 1 which is in contrast to previous studies (Graziani et al., 2015); however the biological significance of this is questionable given that only very low levels of TNFα were detected across all timepoints. There were no discernible differences between UI and HI with respect to CRP, IL-6 or TNFα at day 1, despite observing a significantly reduced treatment time with ultrasonics. Evaluating these results, it could be speculated that the difference in treatment time (22 minutes on average) may not be sufficient to translate into a noticeable difference in the systemic inflammatory response.

Assessing clinical outcomes between instrumentation groups there was no difference between UI and HI which is commensurate with previous studies (Ioannou et al., 2009). A pocket closure rate of 58.54% and mean PPD reduction of 0.98 mm were observed across all patients, which is similar to values reported from a systematic review with pocket closure rates of 57% and mean PPD reduction of 1 mm at 3-4 month follow-up (Suvan et al., 2019). Of particular interest may be the finding that UI was significantly quicker than HI. Additionally, treatment time for HI positively correlated with clinical disease

severity at baseline, and this trend was not observed for UI. Thus, it could be speculated that ultrasonics may offer a greater time saving capacity for the treatment of PD. However, it should be noted that this is an exploratory finding from the current study and more rigorous investigation is required.

In contrast to previous studies which have solely investigated systemic inflammation (D'aiuto et al., 2004a, D'aiuto et al., 2005, Tonetti et al., 2007, Graziani et al., 2010, Graziani et al., 2015), this study also assessed cytokines in GCF and saliva samples. There was an increase in certain salivary cytokines one day following FMD and this response was more pronounced than was observed in the serum. Of the five salivary cytokines assessed, there was a large elevation in salivary IL-6 and IL-8 at this timepoint. This response was significantly greater following UI compared with HI, and it could be hypothesised that ultrasonic instruments may induce more local trauma during FMD. However, this was an exploratory post-hoc analysis that the study was not powered nor designed to detect. These data do, however, suggest that local trauma may contribute to the observed spike in systemic inflammation following FMD. In contrast to saliva, there was no observed difference between groups for GCF IL-6 and IL-8 at day 1, with increases observed within each group. One factor which may explain this result is that GCF was selectively sampled from deep sites, where it could be speculated that elevated operative trauma may be more likely to arise. Future studies should further explore the relationship between local and systemic inflammation to confirm whether such a response dictates elevations in serum CRP.

Whilst robust randomisation and blinding were employed within this RCT, there remains inevitable limitations which must be considered. CRP may be influenced by numerous confounding variables that may be unevenly distributed between groups. To limit this potential bias, future studies may consider including specific levels of baseline CRP and BMI in inclusion criteria or stratification techniques. Additionally, it was not possible to blind clinicians to groups given the nature of the treatment which may potentially result in observation bias. As mentioned, analysis of the local cytokines (saliva, GCF) and treatment time were exploratory secondary outcomes, which additional studies will certainly be required to confirm. Another limitation is that bacteraemia analysis was not performed in the current study as blood sample timepoints were selected based

on assessing changes in serum CRP. It is believed that bacteraemia induced by scaling is rapidly cleared within 30 minutes (Lafaurie et al., 2007), meaning blood samples are required immediately post-intervention. Within future studies it would be useful to correlate the degree of bacteraemia immediately post-treatment with the change in serum CRP at day 1 and establish whether individual species may elicit a larger response. Finally, the current study found equivalence between the groups in terms CRP at day 1 (primary outcome). This study was designed to detect a difference between groups, rather than equivalence, and these results should therefore be interpreted with consideration of this caveat.

In conclusion, this chapter found that ultrasonic and hand instrumentation induced a comparable elevation in systemic inflammatory markers following FMD. Ultrasonic scalers were significantly quicker but appeared to induce a larger increase in local inflammatory cytokines which should be confirmed in future studies. A key finding from this study was that the magnitude of CRP increase at day 1 was generally low across most patients, which cannot be overlooked in the context of recent guidelines. Patients within this RCT presented at day 1 with only a minor elevation in serum CRP (+1.07 mg/L). Such an increase may also be observed following other dental procedures which elicit low-grade bacteraemia including dental flossing, toothbrushing or mastication (Forner et al., 2006, Crasta et al., 2009). Our data do support an elevated response occurring among a subset of patients, and future work is required to establish why there is a large disparity in the magnitude of reaction. With an ageing population and increased tooth retention, more medically compromised patients receive periodontal treatment. Establishing the risk of an elevated systemic inflammatory response to FMD will help ensure that that treatment can be performed safely in patients where repeated travel and attendance for treatment is a burden.

4.5. Chapter summary

The RCT presented within this chapter was the first of its kind, evaluating the impact of instrumentation techniques on the systemic inflammatory response to FMD. The study was powered to detect a difference in serum CRP between ultrasonic and hand instrumentation groups at day 1. Secondary outcomes included comparison of systemic cytokines, clinical outcomes and local cytokines between groups. The main findings from this chapter are highlighted below;

- There was no difference in the systemic inflammatory response following full-mouth debridement using hand or ultrasonic instruments.
- Notably, the magnitude of this systemic response was generally low within the current cohort, with large heterogeneity in the extent of response.
- There were no differences in clinical outcome following hand or ultrasonic instrumentation, although treatment was significantly quicker with ultrasonic instruments.
- Higher levels of salivary cytokines were observed following ultrasonic instrumentation, potentially indicating a larger degree of local trauma which future studies are needed to confirm.

Chapter 5

Subgingival biofilm reformation following hand and ultrasonic instrumentation

Work from this chapter has been presented at the following meetings;

'Periodontal biofilm reformation following mechanical disruption – A clinical study' by W. Johnston. Presented at the Glasgow University School of Medicine, Dentistry and Nursing PGR open day in May 2021. E-poster. Awarded first prize for best poster presentation.

'Periodontal biofilm reformation following hand and ultrasonic instrumentation' by W. Johnston. Presented at the 5th Penn Periodontal Conference in July 2021. E-poster.

Data from this chapter has been used as part of a marketing campaign by Dentsply Sirona.

5.1. Introduction

Hand and ultrasonic instruments are routinely used in isolation or in combination to provide NSPT, with selection largely guided by patient and clinician preference. Numerous studies, including **chapter 4** of this thesis, have demonstrated that both types of instrumentation improve the clinical status of patients with equal efficacy (Ioannou et al., 2009, Suvan et al., 2019). Similarly, studies employing targeted approaches to assess the level of certain bacterial species (*P. gingivalis, T. forsythia, T. denticola, A. actinomycetemcomitans*) have found comparable microbial outcomes following treatment with each type of instrument (Derdilopoulou et al., 2007, Christgau et al., 2007, Ioannou et al., 2009). The current chapter sought to further explore the microbial impacts of different instrumentation techniques, initially using an *in vitro* PD biofilm model and subsequently using 16S rRNA sequencing to investigate the subgingival plaque microbiota from the RCT presented within **chapter 4**.

As molecular techniques have progressed and allowed deeper understanding of the subgingival plaque microbiome, the theory of causative pathogens in PD has been superseded by the concept of community dysbiosis (Lamont et al., 2018, Abusleme et al., 2021). As such, profiling the subgingival plaque microbiota is required to capture the magnitude of microbial alterations following treatment. The impact of NSPT on the microbiome has been described within **chapter 3** of this thesis and by previous studies (Shi et al., 2015, Chen et al., 2018). However, the study in **chapter 3**, and all prior studies, have used a 'blended' approach with respect to instrumentation technique by employing the use of hand and ultrasonic scalers simultaneously. Additionally, most studies focus on a single follow-up timepoint between 1 and 3 months meaning that the immediate impact of NSPT on the subgingival plaque microbiome, and the steps involved in microbial community reformation remain poorly understood.

Whilst hand and ultrasonic scalers both aim to disrupt and remove subgingival plaque biofilms, there is evidence that instruments may differ in their direct impact both on the tooth surface and corresponding biofilm. Profilometry studies have highlighted that the surface roughness of specimens differ following use of hand and ultrasonics *in vitro*, although results are inconsistent. For example, some studies demonstrate that hand instruments may leave a

smoother surface in comparison with ultrasonics (Schlageter et al., 1996, Kocher et al., 2001, Zafar, 2016), whilst other studies suggest the opposite trend (De Mendonca et al., 2008, Hagi et al., 2015). Such varying results may be due to several factors including the specific instrument and technique used. However, if an effect is present in favour of either instrumentation technique, then this may influence biofilm removal and subsequent reformation (Arabaci et al., 2007). Likewise, early microbiology studies suggested that cavitation induced from ultrasonic scalers may have an antimicrobial effect against bacteria within the Spirochaetes phylum (Thilo and Baehni, 1987, Baehni et al., 1992). However, later studies demonstrated a lack of any antimicrobial effect against specific periodontal bacteria (Schenk et al., 2000) and others have suggested that heat generated from scaler tips may confound results (O'leary et al., 1997).

Defining whether the use of hand or ultrasonics have differing impacts on periodontal biofilms both *in vitro* and *in vivo* is important to guide appropriate instrumentation choice when performing NSPT. Furthermore, profiling the disrupted biofilm community at different timepoints is crucial to understand the steps involved in recovery and reformation of the biofilms following treatment, and may elucidate factors which help promote or accelerate a favorable clinical response. The current chapter sought to assess the impact of hand and ultrasonic instruments on an *in vitro* PD biofilm model and *in vivo* from the RCT presented within **chapter 4**. This study also sought to characterize the recovery of subgingival plaque communities following mechanical disruption, thereby providing a novel ecological insight into this microbial reassembling process *in vivo*. Specifically, the research questions for this chapter are as follows;

- Do hand and ultrasonic scalers differ in their impact on *in vitro* PD biofilms?
- Does the subgingival plaque microbiome differ following treatment with hand or ultrasonic instruments?
- How does the subgingival plaque microbiome reform following mechanical biofilm disruption?

5.2. Materials and methods

5.2.1. Part A: Pilot *in vitro* study

5.2.1.1. Bacterial culture conditions

Part A of this chapter aimed to explore the impacts of physical debridement on *in vitro* PD biofilms. Biofilms were prepared on 18 mm diameter round glass coverslips as previously described (Millhouse et al., 2014, Sherry et al., 2016, Brown et al., 2019a). Briefly, biofilms comprised 10 bacterial species which are standardised and sequentially added over a 1 week period. All strains used to prepare biofilms are shown in **table 5.1**. To grow bacteria, frozen stocks of three streptococci species (*S. mitis, S. oralis, S. intermedius*) and *A. actinomycetemcomitans* were cultured on CBA agar for 1-2 days, followed by TSB broth at 5% CO2, 37°C. All other bacteria (*F. nucleatum* ssp. *polymorphum*, *F. nucleatum* ssp. *vincentii*, *A. naeslundii, V. dispar, P. intermedia, P. gingivalis*) were grown on pre-reduced FAA agar for 2-3 days in an anaerobic cabinet. Both *A. naeslundii* and *V. dispar* were then cultured in pre-reduced BHI broth. *F. nucleatum spp. polymorphum, F. nucleatum spp. vincentii, P. gingivalis* and *P. intermedia* were cultured in pre-reduced SCH broth (Oxoid, Hampshire, UK). All protocols for FAA, BHI and SCH were performed in an anaerobic cabinet at 37°C (85 % N_2, 10 % CO_2 and 5 % H_2; Don Whitley Scientific, Shipley, UK).

5.2.1.2. Biofilm preparation

Following sub-culture, bacterial broths were centrifuged at 4,000 RPM for 10 minutes to obtain bacterial pellets (AWEL C20 model, Blain, France). Pellets were washed by resuspending in sterile PBS three times. Each species was standardised to $1x10^8$ CFU/mL in PBS using the OD_{550} reference values outlined in **table 5.1**. From here, species were diluted to $1x10^7$ CFU/mL (1:10) using artificial saliva (AS) media (Pratten et al., 1998). The AS media contains porcine stomach mucins (0.25% w/v; Sigma-Aldrich, Gillingham, UK), sodium chloride (0.35% w/v; VWR, Lutterworth, UK), potassium chloride (0.02% w/v; VWR), calcium chloride dihydrate (0.02% w/v; VWR), yeast extract (0.2% w/v; Formedium, Hunstanton, UK), lab lemco powder (0.1% w/v; Oxoid), protease peptone (0.5% w/v; Sigma-Aldrich) and urea (0.05% v/v; Sigma-Aldrich).

Sterile 18 mm round diameter glass coverslips were added to 12 well microtiter plates (Corning™ Costar™). A 500 µL cocktail of bacterial species in AS were sequentially added to coverslips over a 7 day maturation period. For example; 50 µL *S. mitis*, 50 µL *S. intermedius*, 50 µL *S. oralis* and 350 µL AS were added on day 0 (5% CO_2). This was followed by addition of *V. dispar*, *A. naeslundii*, *F. nucleatum* ssp. *polymorphum* and *F. nucleatum* ssp. *vincentii* on day 1 (anaerobic), and *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* on day 2 (anaerobic). After addition of all species, biofilms were incubated anaerobically for a further 4 days with daily media changing (**figure 5.1**).

5.2.1.3. Biofilm storage and reconstitution

On the 7th day, AS was removed and sealed plates containing biofilms on coverslips were immediately frozen at -80°C. Prior to treatment, biofilms were reconstituted adding 500 µL AS followed by overnight incubation at 37°C anaerobically. This protocol has been previously optimised for use on glass coverslips (Brown et al., 2019a), ensuring no discrepancies in biofilm composition or viability between fresh and frozen biofilms.

Table 5.1. Strains and culture conditions for species included in 10 species biofilms.

Bacterial strain	Agar	Broth	Culture conditions	OD_{550} $1x10^8$ CFU/ml
S. mitis NCTC 12261	CBA (1 day)	TSB (1 day)	5% CO_2	0.5
S. intermedius DSM 20573	CBA (1 day)	TSB (1 day)	5% CO_2	0.5
S. oralis NCTC 11427	CBA (1 day)	TSB (1 day)	5% CO_2	0.5
A. actinomycetemcomitans ATCC 43718	CBA (1-2 days)	TSB (2 days)	5% CO_2	0.2
V. dispar NCTC 11831	FAA (2-3 days)	BHI (2 days)	Anaerobic	0.5
A. naeslundii DSM 17233	FAA (2-3 days)	BHI (2 days)	Anaerobic	0.2
F. nucleatum ssp. polymorphum ATCC 10953	FAA (2-3 days)	SCH (2 days)	Anaerobic	0.2
F. nucleatum ssp. vincentii DSM 19507	FAA (2-3 days)	SCH (2 days)	Anaerobic	0.2
P. gingivalis W83	FAA (2-3 days)	SCH (2 days)	Anaerobic	0.2
P. intermedia DSM 20706	FAA (2-3 days)	SCH (2 days)	Anaerobic	0.2

CBA; Columbia Blood Agar, FAA; Fastidious Anaerobic Agar, TSB; Tryptic Soy Broth, BHI; Brain Heart Infusion broth, SCH; Schadler's anaerobic broth.

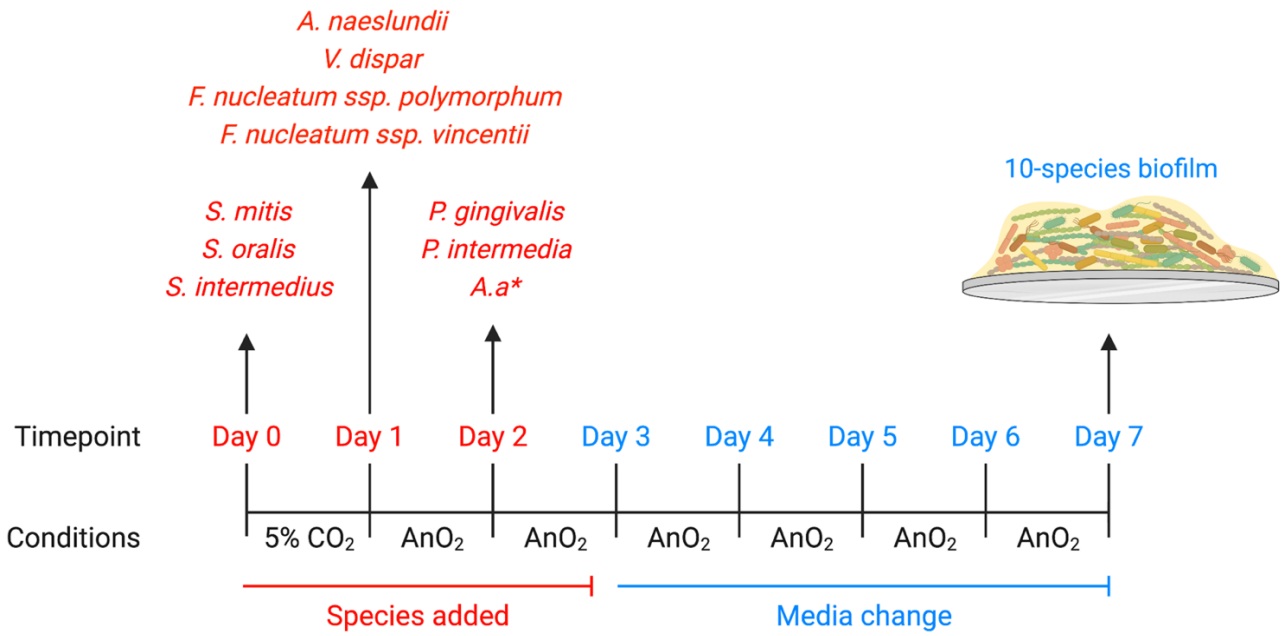

Figure 5.1. Growth of 'periodontitis' biofilms. Ten bacterial species were standardised to $1x10^7$ CFU/mL in AS and sequentially added to 18 mm round diameter glass coverslips. Biofilms were incubated at 5% CO_2 for 1 day, followed by anaerobic incubation (AnO_2) from days 1 to 7. Following species addition, media was changed daily for a total of 4 days. Once mature, biofilms were frozen at -80°C. *Aggregatibacter actinomycetemcomitans. Image created in BioRender.

5.2.1.4. Biofilm treatment

Biofilms were washed 3 times in sterile PBS prior to any subsequent treatment. For scaling treatments, coverslips were lifted upwards with sterile forceps and scaled using 10 single strokes of an ultrasonic scaler (Cavitron® Slimline® 10S 30K, Dentsply Sirona) or hand scaler (Gracey 1/2, LM Dental) into the well. Scaling motions were performed by a trained dental hygienist (Mrs Debbie McKenzie), using the same pressure and technique that would be applied in a clinical setting. Ultrasonic treatment was used at a power setting of 50% with water adjusted to form a fine mist with droplets as per manufacturer's instructions. For treatment, ultrasonic tips were held 45° to the coverslip surface. Hand treatment was performed by holding scalers at 90° to the coverslip surface and performing light sweeping strokes. For hand scaling, treatment was performed in 1 mL of sterile distilled water. For ultrasonic scaling, roughly 800-900 uL of sterile distilled water was consistently pulsed through the scaler during treatment and entered wells. This was topped up to 1 mL with sterile distilled water following treatment completion to ensure equal volumes in hand and ultrasonic wells.

Chlorhexidine was included as a positive control at 0.2% (v/v; Sigma-Aldrich). For chlorhexidine treatment, 1 mL was applied to biofilms and allowed to dwell for 2 minutes. After this period, chlorhexidine was removed and biofilms were washed with 1 mL sterile PBS prior to further assaying. Untreated biofilms were included in all assays as negative controls. These biofilms were washed with sterile PBS after AS removal but did not receive any further treatment. All experiments were performed on three separate occasions (n=3).

5.2.1.5. Resazurin metabolism assay

The metabolic activity of treated and untreated biofilms was assessed using resazurin sodium salt (Sigma-Aldrich). A resazurin stock was prepared at 1% by adding 0.1g to 10 mL sterile PBS (w/v), which was stored at 4°C for up to 1 month. The solution was diluted to 0.001% for use in biofilm metabolism assays as described previously (Delpech et al., 2018). To all wells, 500 µL of diluted resazurin was added and incubated for 30 minutes. After incubation, the

fluorescence was read at 544ex/590em (FLUOstar Omega Microplate Reader, BMG Labtech, Ortenberg, Germany).

5.2.1.6. Crystal violet biomass assay

Biofilm biomass was assessed using the crystal violet assay. This was performed on the same biofilms as the resazurin assay. Following assessment of metabolic activity, resazurin was removed and biofilms were gently washed in sterile PBS. After washing, biofilms were allowed to air dry for a further 48 hours. Five hundred microliters of 0.05% w/v crystal violet solution was added to each biofilm and incubated at room temperature for 30 minutes. No biofilm controls (glass coverslip only) were included in all assays to subtract background signal. After incubating, crystal violet stain was removed, and biofilms washed with tap water. Five hundred microliters of 100% ethanol was applied to de-stain biofilms, and five 75 µL aliquots from each well were transferred to a new 96 well flat-bottom microtiter plate. Well absorbance was read at 570 nm (FLUOstar Omega Microplate Reader, BMG Labtech, Ortenberg, Germany). The average of the 6 aliquots per well was averaged to give final biomass readings.

5.2.1.7. Analysis of Colony Forming Units (CFUs)

CFU analysis was performed on supernants following biofilm treatment. This was performed using the Miles and Misra technique (Miles et al., 1938), in which 20 µL of serially diluted supernants (ranging from 10^{-2} to 10^{-8}) were applied in triplicate to both CBA and FAA plates. CBA plates were incubated for 48 hours at 37°C in 5% CO_2, with FAA plates incubated for 48 hours at 37°C anaerobically. Colonies were counted to provide an aerobic and anaerobic CFU/mL estimation. The triplicate average was used per repeat as a final reading.

5.2.2. Part B: Subgingival plaque microbiota

5.2.2.1. Study outcomes and patient selection

Part B of this chapter was an exploratory secondary outcome from the RCT in chapter 4 of this thesis (Clinicaltrials.gov ID: NCT03501316). This analysis sought to investigate whether the subgingival plaque microbiome differs following hand and ultrasonic instrumentation at a range of timepoints (baseline, days 1, 7 and 90 post-treatment, **figure 5.2**). In total, 18 patients received full-mouth debridement using UI, and 20 patients received HI. For HI, this included one patient that was not included in **chapter 4** analysis due to pathologically high baseline serum CRP levels. Prior to inclusion, it was confirmed that this patient's baseline subgingival plaque microbiota did not substantially differ from the rest of the cohort.

5.2.2.2. Subgingival plaque collection

The protocol for subgingival plaque collection and processing was followed as described in **section 3.2.2.3**. Subgingival plaque was collected from the deepest pocket in each quadrant at baseline. At each subsequent timepoint (day 1, 7, 90), subgingival plaque was collected from the same pockets to ensure accurate single-site longitudinal analysis. Bacterial pellets were processed and stored immediately in adjacent laboratories at -80°C until analysis.

5.2.2.3. Subgingival site selection

This was a single-site comparison of the subgingival microbiota following UI and HI, and thus only 1 of the 4 sites collected at each timepoint was analysed. To ensure accurate comparison between groups, 'deep' sites (between 6 and 10 mm) which displayed clinical improvement were selected for analysis. The same site was analysed at each subsequent timepoint.

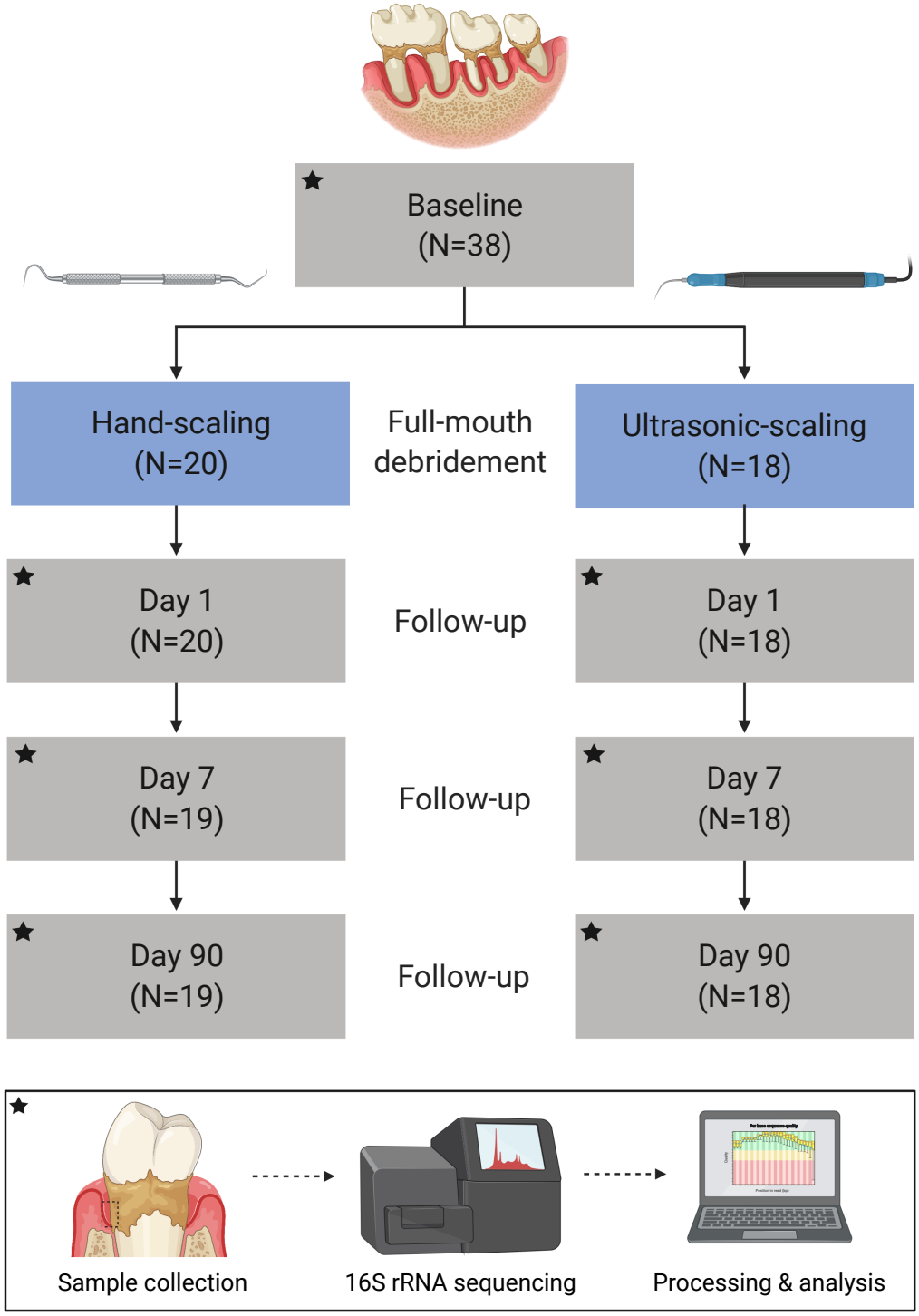

Figure 5.2. Flow chart of microbiota study design. Patients received full-mouth debridement using hand- or ultrasonic-scaling. Subgingival plaque was collected at baseline, day 1, day 7 and day 90 follow-up (black stars). DNA was extracted from samples at each timepoint and underwent 16S rRNA sequencing and analysis. Two samples from different patients in the hand-scaling group (1 at day 7, 1 at day 90) had <5000 reads and were not included in microbiome analysis (n=20 [baseline, day 1], n=19 [day 7, day 90]). Image created in BioRender.

5.2.2.4. DNA extraction

DNA was extracted from 152 samples (38 patients, 4 timepoints) using the same protocol outlined in **section 3.2.7.2** at the FISABIO institute (Valencia, Spain) with additional DNA concentration step. Following extraction into low-retention tubes, DNA was concentrated using Vivacon® 500 centrifugal concentrators (Sartorious, Germany). Final DNA concentrations were measured on a Qubit TM3 fluorometer (Thermo Scientific, Massachusetts, USA) prior to sequencing.

5.2.2.5. DNA sequencing and taxonomic classification

DNA was sequenced as described in **section 3.2.7.4**, using the 16S rRNA gene Metagenomic Sequencing library preparation protocol from Illumina (Part #15044223 Rev. A). Primers used in this study target the V3-V4 regions of the 16S rRNA gene (**table 3.2**). Taxonomic classification was performed using the same methodology outlined in **section 3.2.7.5**. ASVs tables were generated using the DADA2 pipeline (Callahan et al., 2016), with genus- and species-level assignment generated by comparison to the SILVA database (Quast et al., 2013). All raw sequencing reads from this study are publicly available in the NCBI SRA under BioProject PRJNA736618.

5.2.3. Data analysis

For *in vitro* data, differences in biomass, metabolic activity and colony counts were assessed using one-way ANOVA with Tukey's post-hoc for multiple comparisons. Paired t-tests were used to assess differences in aerobic and anaerobic colony counts from the same samples. All analysis was performed in GraphPad PRISM (v8).

For clinical analysis, GLMs were performed on clinical parameters using SPSS software (v26), with baseline levels included at covariates. Microbiota analysis was performed as described previously in this thesis (**section 3.2.8.2**) using a combination of R and the MicrobiomeAnalyst pipeline (Dhariwal et al., 2017, Chong et al., 2020). Longitudinal analysis was conducted using the wilcox.test function in R, adjusted for multiple statistical comparisons (FDR 5%). For the MicrobiomeAnalyst pipeline, default filtering was applied (prevalence = 4 counts in 20% of samples, variance = 10% IQR).

5.3. Results

5.3.1. Part A: *In vitro* biofilm treatment

The first section of this chapter sought to investigate the impact of hand and ultrasonic instruments on an *in vitro* PD biofilm model. The primary aim of this *in vitro* study was to assess whether hand and ultrasonic instruments have differing impacts on biofilms when controlling for the number of strokes. Additionally, the viability of removed organisms was assessed in supernants following treatment with each instrument.

5.3.1.1. Biomass and metabolic activity following scaling

To evaluate biomass removal following hand and ultrasonic scaling, biofilms were subjected to 10 strokes of each instrumentation type (carried out by an experienced dental hygienist). Biofilms were also treated for 2 minutes with chlorhexidine (0.2%) as a positive control, alongside untreated biofilms as negative controls. Following scaling, both hand and ultrasonic instrumentation resulted in significantly reduced biomass in comparison with untreated biofilms (**figure 5.3A-B**, $p<0.001$, one-way ANOVA). When directly comparing instruments, lower residual biomass was observed following 10 strokes using the ultrasonic instrument (17.02% ± 7.07) compared with 10 strokes using the hand instrument (66.97% ± 11.01), which was statistically significant ($p<0.001$). Residual biomass was visible in areas between hand scaler strokes; however this was not observed following ultrasonic treatment (**figure 5.3A**). Chlorhexidine treatment did not significantly alter biofilm biomass in comparison with untreated controls ($p=0.68$). As expected, similar results were observed when assessing the metabolic activity of biofilms (**figure 5.3C-D**), excluding chlorhexidine treatment. Chlorhexidine treated biofilms displayed low metabolic activity following treatment (14.91% ± 1.57, $p<0.001$ vs. untreated) with comparable biomass to untreated controls.

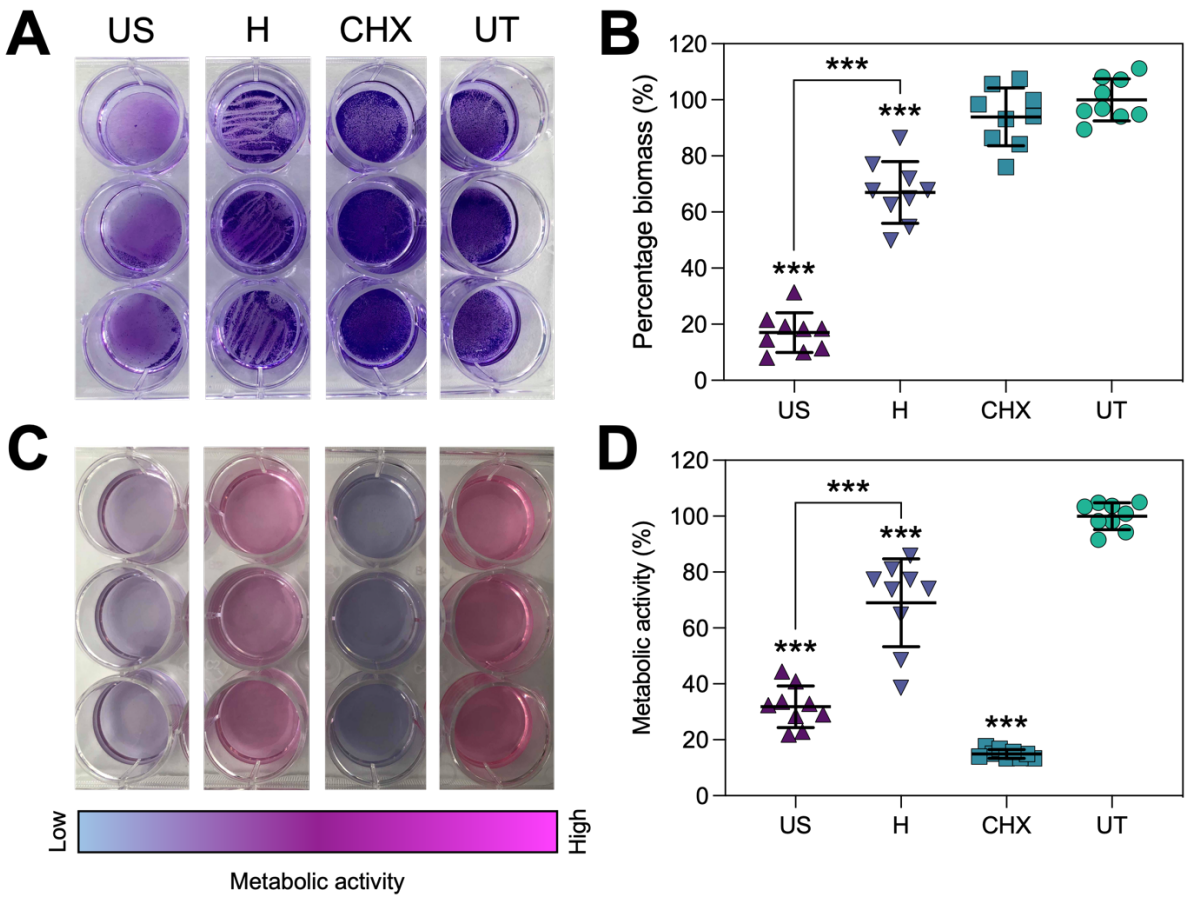

Figure 5.3. Biomass and metabolic activity of biofilms after treatment. Representative image of Crystal Violet assay (A), quantified as a percentage of untreated biofilms (B). Representative image of Resazurin assay (C), quantified as a percentage of untreated biofilms (D). Individual datapoints are shown with error bars displaying mean ± standard deviation, statistics are one-way ANOVA with Tukey's post-hoc, ***p<0.001. N=3. US; Ultrasonic instrumentation, H; Hand instrumentation, CHX; Chlorhexidine control, UT; Untreated control.

5.3.1.2. Microbial load in supernatant following treatment

The microbial load of supernants was next evaluated, given previous suggestions that cavitation from ultrasonics may have some antimicrobial effect (Baehni et al., 1992, Thilo and Baehni, 1987). One pitfall of previous studies of the effect of cavitation is that experiments were performed using planktonic bacteria. In the current study, we assessed the culturable microbial load in supernatants following biofilm disruption (experiment outline **figure 5.4A**). This model partially mimics the interface between bacteria and scalers *in vivo*, where subgingival planktonic microorganisms would be dispersed following mechanical biofilm disruption.

The supernatant microbial load was estimated by aerobic and anaerobic colony counts following treatment. Colony counts in untreated wells refer to a fourth wash with sterile water. There were significantly more viable bacteria in supernatant following 10 strokes using an ultrasonic instrument compared with 10 strokes using a hand instrument (**figure 5B-C**, $p<0.001$). No culturable bacteria were identified following chlorhexidine treatment, and no differences were observed between aerobic and anaerobic counts for any treatment (**figure 5.4D**). This is likely attributed to several included organisms being facultatively anaerobic and thus counted under both conditions. Collectively, ultrasonics removed larger amounts of biofilm per stroke, however removed organisms remain viable. Although this model cannot definitively rule out an ultrasonic antimicrobial effect, this data indicates that any effect is limited and unlikely to provide any additional clinical benefit when viewed in isolation.

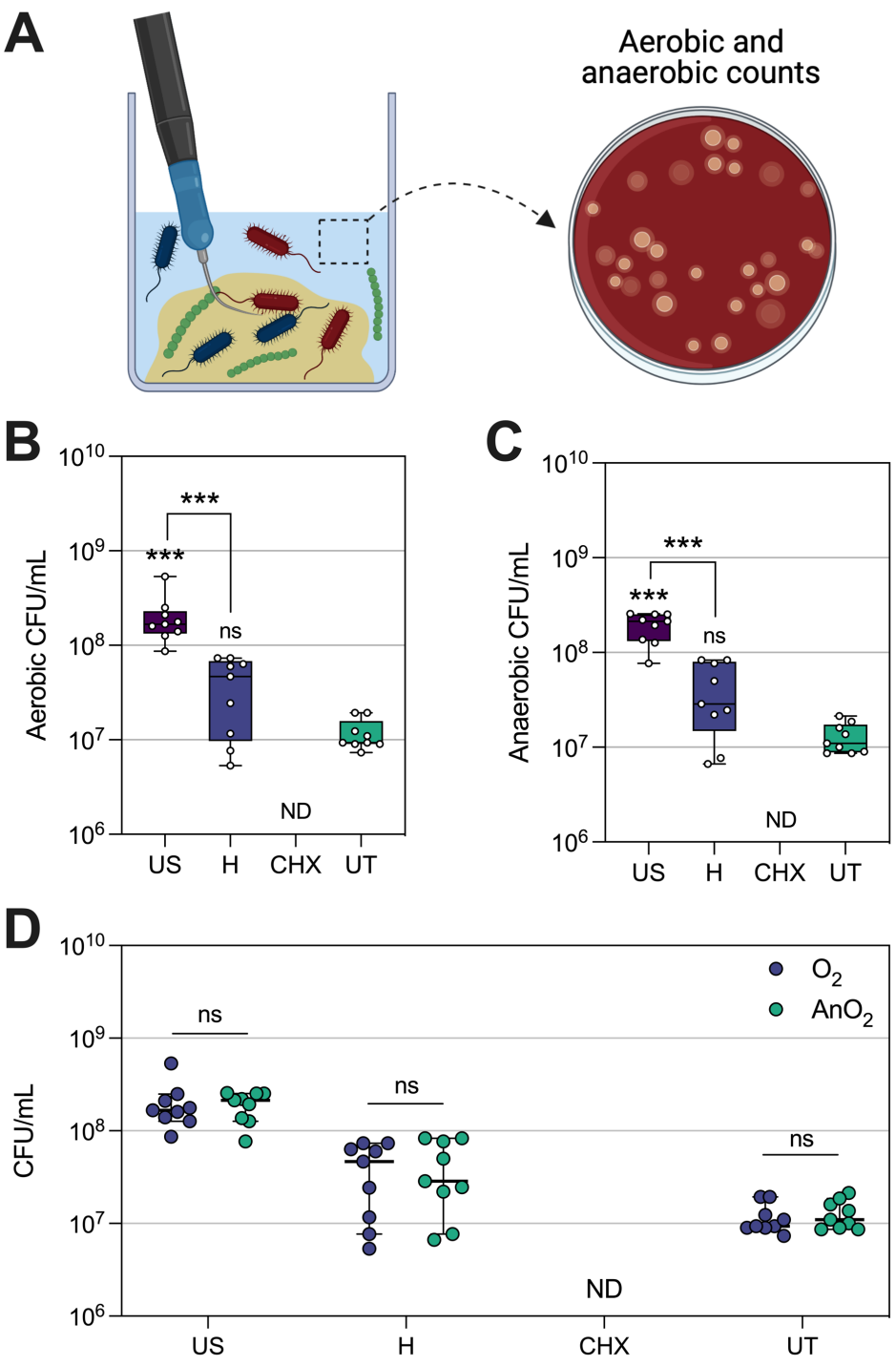

Figure 5.4. Quantifying dispersed organisms in supernatants following treatment. Experimental summary image (A), outlining collection and culture of supernatant (water) following treatment. Aerobic (A) and anaerobic (B) counts were assessed following ultrasonic-scaling (US), hand-scaling (H), chlorhexidine (30 seconds, CHX) and untreated biofilms (UT). Direct comparison between aerobic and anaerobic counts from each treatment (C). Results are represented as colony forming units per mL (CFU/mL), error bars show mean ± standard deviation. For B-C, statistics are one-way ANOVA with Tukey's post-hoc, ***p<0.001. For D, statistics are paired t-tests, ns means no significant difference observed. ND; not detected. N=3.

5.3.2. Part B: Subgingival plaque microbiota

Having evaluated the impact of hand and ultrasonic scalers *in vitro*, the second part of this chapter focussed on an *in vivo* microbiota comparison using 16S rRNA sequencing of subgingival plaque collected during the RCT from **chapter 4**.

5.3.2.1. Clinical treatment response within sampled sites

Clinical examination was performed at baseline and day 90 on sites from which subgingival plaque samples were collected (**table 5.2**). Comparing treatment groups, similar PPD and CAL were observed at baseline and were indicative of severe disease. Both PPD and CAL significantly reduced following treatment regardless of instrumentation choice ($p<0.001$ for all, paired t-test), and clinical improvement at sampled sites was comparable between instrumentation groups ($p=0.94$ [PPD], $p=0.66$ [CAL], GLM).

Table 5.2. Clinical characteristics of sites sampled for subgingival plaque. Data are played as mean (±SD) for each group.

Variable	Timepoint	Ultrasonic (n=18)	Hand (n=20)	Between group p-value[§]
Site PPD (nearest mm)	BL	7.67 (±1.28)	7.65 (±1.14)	
	D90	4.50 (±1.50)	4.60 (±1.35)	
	Within group p-value[†]	<0.001	<0.001	0.94
Site CAL (nearest mm)	BL	7.61 (±1.33)	7.95 (±1.91)	
	D90	5.23 (±1.67)	5.90 (±1.77)	
	Within group p-value[†]	<0.001	<0.001	0.66

[†]Differences within groups tested using paired t-test comparing baseline and day 90.
[§]Differences between groups tested using general linear models (GLMs) adjusted for baseline level of each variable, smoking status, sex and age.

5.3.2.2. Comparing α-diversity between treatment groups

Across the 152 subgingival plaque samples assessed during the current study, 4254 ASVs were detected with an average of 63,050 reads per sample. Two samples (1x day 7, 1x day 90, both HI) produced only a low number of total reads (1701 and 2244 respectively) compared with the rest of the samples. As such, these two samples were not included in any subsequent microbiome analysis. Rarefaction curves were generated at species-level prior to calculation of α-diversity indices at each timepoint (**figure 5.5A-D**). Across all samples, rarefaction curves suggested a reliable diversity estimate was possible at 14,000 reads per sample. After rarefying, Shannon and ACE indices were calculated to evaluate the diversity of subgingival plaque samples. Both indices were non-normally distributed and compared between-groups using Mann-Whitney U tests and within-groups using Wilcoxon signed-rank tests, with all p-values adjusted using the FDR (5%) approach (Benjamini and Hochberg, 1995).

Using these indices, significant reductions in the Shannon index were observed within both UI and HI groups as early as day 1 post-treatment (UI: $p<0.001$, HI: $p<0.001$, Wilcoxon signed-rank test, **figure 5.5E**). These reductions were maintained until day 90, with no significant differences between treatment groups at any timepoint ($p>0.05$ at all timepoints, Mann-Whitney U test). Similar longitudinal results were observed for the ACE index with the exception of UI at day 1 and HI at day 7 which both showed trends towards a reduction ($p=0.06$ for both, **figure 5.5F**). Importantly, no significant differences in the ACE index were observed between groups at any timepoint ($p>0.05$ at all timepoints, Mann-Whitney U test).

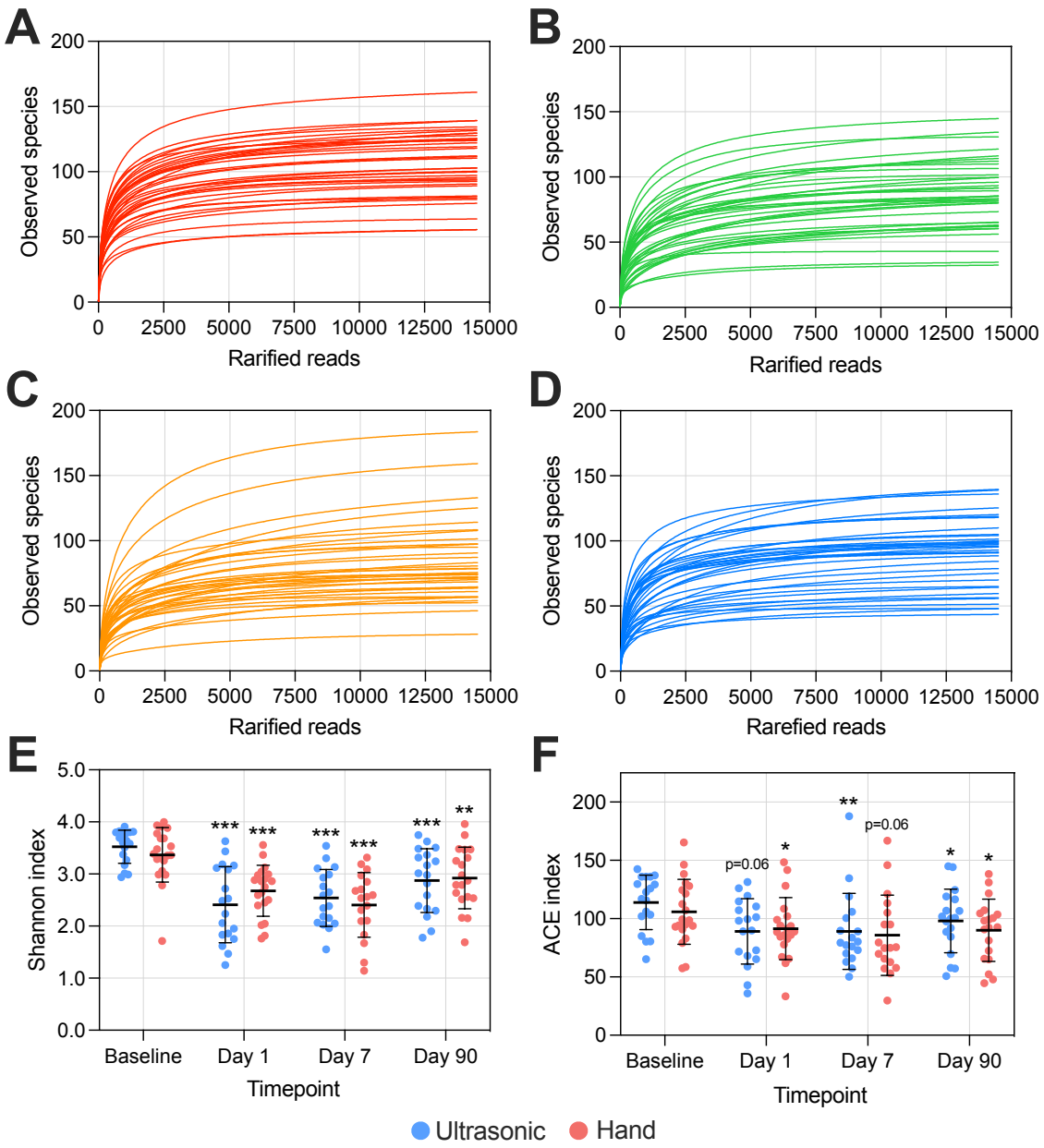

Figure 5.5. α-diversity of subgingival plaque samples. Rarefaction curves display the number of species observed when rarefying to different reads in baseline (A), day 1 (B), day 7 (C) and day 90 (D) samples. Comparing the Shannon (E) and ACE (F) indexes between ultrasonic (blue) and hand (red) treatment groups, calculated after rarefying to 14,000 reads. Individual sites are represented in each graph, error bars (E,F) display mean and standard deviation. Longitudinal statistics are Wilcoxon signed-rank test (E,F), where *p<0.05, **p<0.01 and ***p<0.001 and raw p-values displayed when p>0.05. All p-values were adjusted for multiple comparisons using the FDR (5%) approach. No between-group differences were observed at any timepoint. For baseline and day 1, n=38. For day 7 and day 90, n=37.

5.3.2.3. Community comparison between treatment groups

Alterations in the subgingival plaque composition were next assessed between groups. The top 20 most abundant genera, and top 30 most abundant species are displayed which equated to >60% of the total composition at all timepoints (**figure 5.6** and **5.7** respectively). Univariate analysis was performed across all genera and species with a minimum signal of detection, using Mann-Whitney U tests between UI and HI groups at each timepoint (**section 5.2.3**). The baseline microbiota was similar between instrumentation groups, with no alterations in the abundance of any genera or species (all p[adjusted]>0.05). Despite large-scale compositional changes observed longitudinally within groups, no significant difference in genus- or species-abundance was observed between UI and HI at day 1, 7 or 90 following treatment (all p[adjusted]>0.05).

These comparisons were supplemented with ß-diversity analysis, using Bray-Curtis based PCoA at each timepoint. Similar to the comparison of relative abundances, genus-level ANOSIM suggested no differences between HI at UI at baseline (p=0.86, **figure 5.8A**), day 1 (p=0.17, **figure 5.8B**), day 7 (p=0.09, **figure 5.8C**) or day 90 (p=0.83, **figure 5.8D**). Similar results were observed at species-level, with no differences between treatment groups at any timepoint (**figure 5.9A-D**). Thus, instrumentation choice did not appear to differentially influence the microbiome when comparing groups at each timepoint.

Direct assessment was also performed between disease-associated species. For this comparison, the sum of the red-complex, orange-complex and novel disease-associated species was utilised (outlined in **table 3.4**). These species were selected based on strong evidence from a systematic review, which did not consider red- and orange-complex members with existing rigorous evidence (Pérez-Chaparro et al., 2014). Analysis of these species demonstrated reductions within both groups at all post-treatment timepoints compared with baseline (**figure 5.10**). The only exception to this trend was HI at day 1, which showed a trend towards statistical significance (p=0.05, Wilcoxon signed-rank test). Notably, between-group comparisons suggested no significant difference in the abundance of these species at any timepoint (all p>0.05, Mann-Whitney U test).

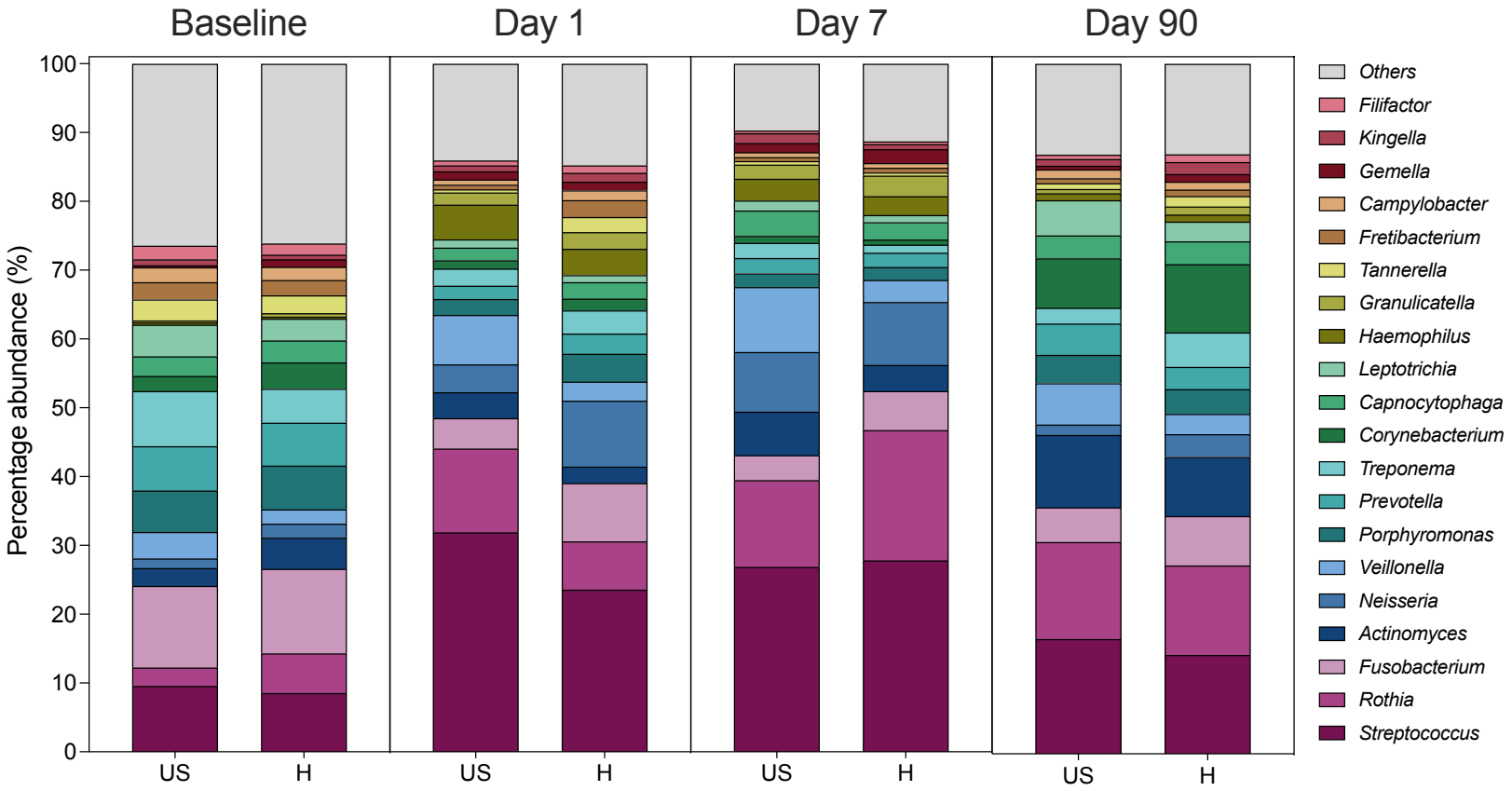

Figure 5.6. Genus composition of subgingival plaque samples. Graphs display the average abundance of genera within each group at baseline, day 1, day 7 and day 90. The top 20 most abundant genera are displayed, with all others grouped together (grey bars). No differentially abundant genera were identified between the US and H treatment groups in each of the time points (Mann-Whitney U tests adjusted for multiple comparisons using the FDR (5%) approach). US; Ultrasonic, H; Hand. For baseline and day 1, n=38. For day 7 and day 90, n=37.

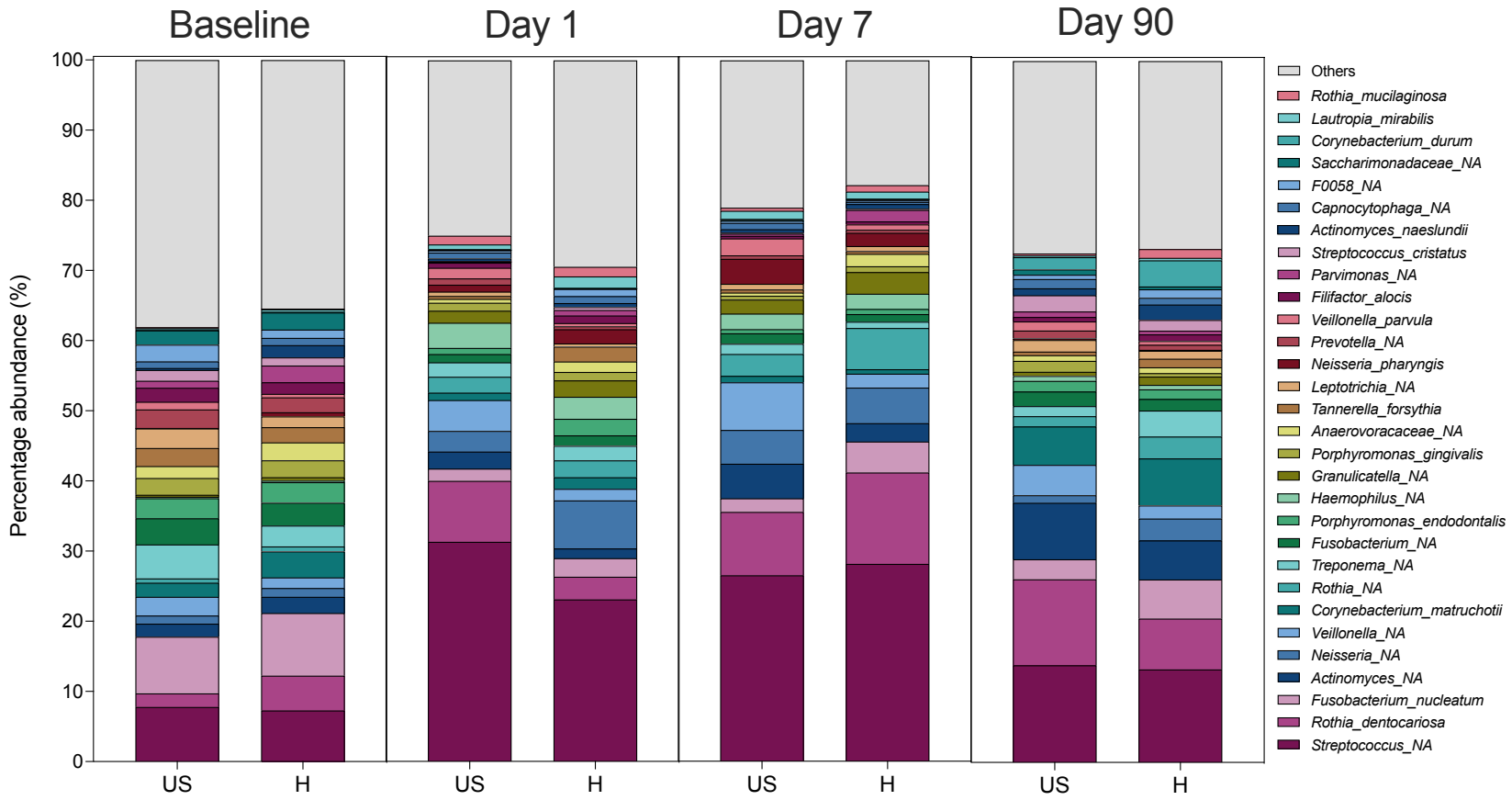

Figure 5.7. Species composition of subgingival plaque samples. Graphs display the average abundance of species within each group at baseline, day 1, day 7 and day 90. The top 30 most abundant species are displayed, with all others grouped together (grey bars). No differentially abundant species were identified between the US and H treatment groups in each of the time points (Mann-Whitney U tests adjusted for multiple comparisons using the FDR (5%) approach). US; Ultrasonic, H; Hand. For baseline and day 1, n=38. For day 7 and day 90, n=37.

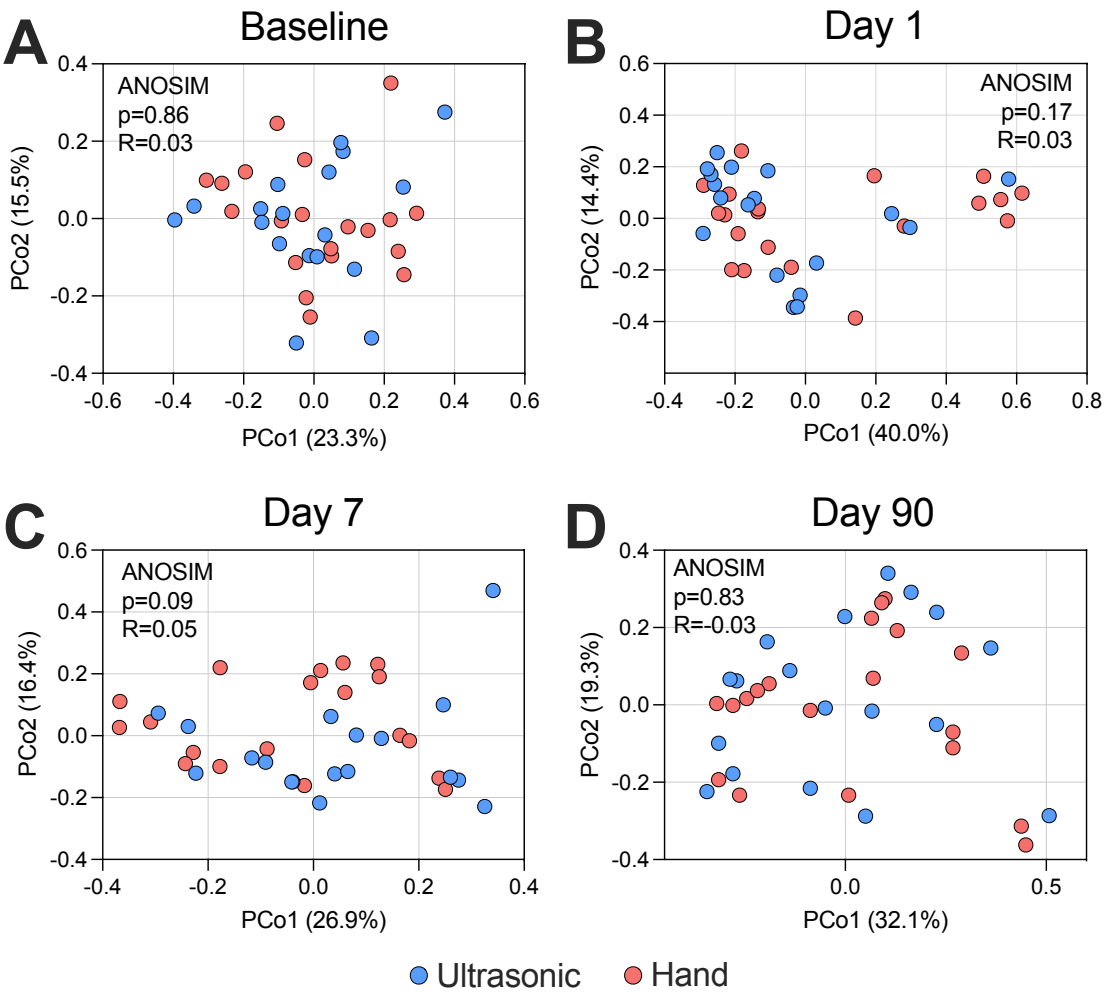

Figure 5.8. Comparing genus-level subgingival plaque communities between ultrasonic and hand treatment groups. Bray-Curtis based principal coordinate analysis (PCoA) was performed at genus-level across ultrasonic and hand-treated sites at baseline (A), day 1 (B), day 7 (C) and day 90 (D). Ultrasonic-treated sites are displayed in blue, hand-treated sites displayed in red. Statistics refer to ANOSIM between groups at each timepoint, all $p>0.05$. For baseline and day 1, n=38. For day 7 and day 90, n=37.

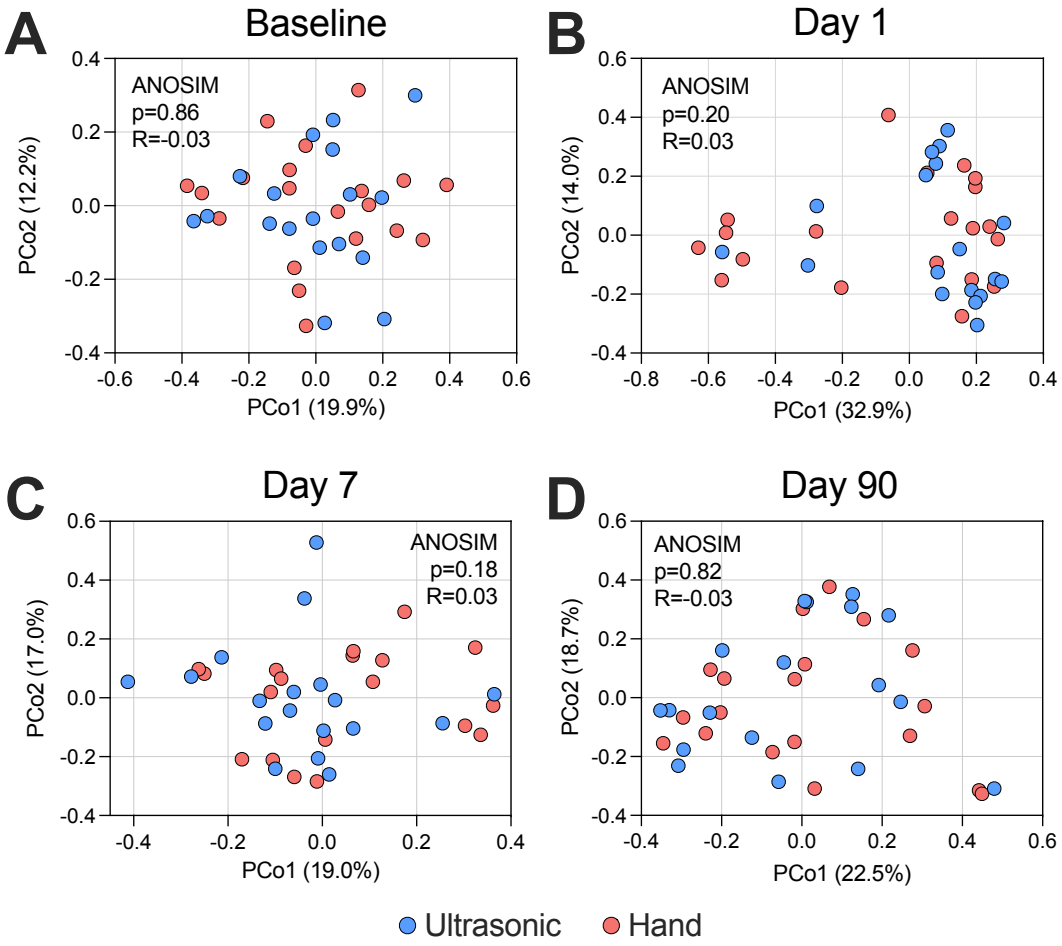

Figure 5.9. Comparing species-level subgingival plaque communities between ultrasonic and hand treatment groups. Bray-Curtis based principal coordinate analysis (PCoA) was performed at species-level across ultrasonic and hand-treated sites at baseline (A), day 1 (B), day 7 (C) and day 90 (D). Ultrasonic-treated sites are displayed in blue, hand-treated sites displayed in red. Statistics refer to ANOSIM between groups at each timepoint, all $p>0.05$. For baseline and day 1, n=38. For day 7 and day 90, n=37.

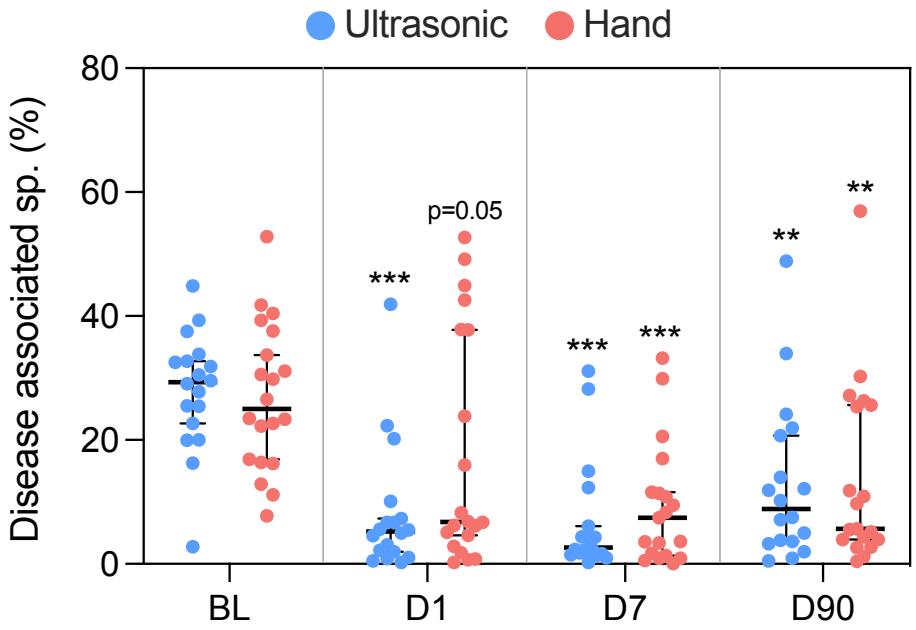

Figure 5.10: The abundance of disease-associated species between ultrasonic (blue) and hand (red) groups. Error bars display median and 95% confidence interval. Longitudinal within-group statistics are Wilcoxon signed-rank tests where **p<0.01 and ***p<0.001. No differences were observed between-groups (Mann-Whitney U test). For baseline and day 1, n=38. For day 7 and day 90, n=37. Data represents the sum of; *P. gingivalis, T. denticola, T. forsythia, P. intermedia, P. nigrescens, F. nucleatum, F. periodonticum, P. micra, C. gracilis, S. constellatus, C. showae, T. medium, P. stomatis, P. denticola, M. timidum, F. alocis, S. sputigena, A. tannerae, A. geminatus, F. fastidiosum, P. endodontalis, T. vincentii, T. lecithinolyticum, D. pneumosintes.*

5.3.2.4. Longitudinal alterations across timepoints

Given the lack of difference between instrumentation groups, longitudinal analysis was performed across the entire cohort to characterise immediate shifts and subsequent reformation of the subgingival plaque microbiota. Longitudinal abundance analysis was performed at genus- and species-level using Wilcoxon-signed rank tests, comparing each post-treatment timepoint with baseline. Only abundant genera (≥0.5% at any timepoint) and species (≥0.25% at any timepoint) were included in these heatmaps.

At genus-level, longitudinal analysis revealed shifts in the abundance of 32 genera following NSPT. Of these, 21 genera (65.6%) differed at all timepoints compared with baseline (**figure 5.11A**). This included several anaerobic genera such as *Porphyromonas, Tannerella, Treponema, Prevotella, Fusobacterium, Filifactor, Fretibacterium* and *Selenomonas* (**figure 5.11D**). It is noteworthy to

mention that these genera, whilst reduced, were detectable even at day 1 post-treatment. Several other genera including *Desulfobulbus, Dialister, Johnsonella, Lentimicrobium* and *Parvimonas* also followed a similar pattern. A surprising exception to this trend was *Corynebacterium*, which significantly reduced in abundance at days 1 and 7 but increased at day 90 compared with baseline. Other genera including *Campylobacter, Centipeda, Leptotrichia* and *Neisseria* significantly differed only at early timepoints (day 1, 7) and returned to baseline levels by day 90, whilst one; *Actinomyces*, significantly differed only at day 7 and 90. No genera was differentially abundant exclusively at days 1 or 90.

Similarly, 65 species significantly differed in abundance compared with baseline (**figure 5.11B**). Of which, 32 were significantly different at all timepoints, albeit many were unclassified (Genus_NA). Among those classified to species-level; *Filifactor alocis, Selenomonas sputigena, Tannerella forsythia, Treponema denticola, Treponema lecithinolyticum* and *Treponema socranskii,* all defined as disease-associated (Pérez-Chaparro et al., 2014), were significantly reduced at all subsequent timepoints compared with baseline (**figure 5.11C**). The majority (93.8%) of these species were significantly different at day 7, whilst a small number including *Porphyromonas catoniae* significantly differed only at day 1. Alternatively, a single species; *Actinomyces massiliensis,* significantly increased only at day 90.

Collectively, these data highlight immediate shifts in the subgingival plaque microbiome following FMD. This was generally characterised by a decrease in the abundance of disease-associated species which was maintained throughout the study period. With respect to longer term changes (day 90), there was a large degree of similarity between the results observed in the current study and the study presented within **chapter 3** of this thesis, suggesting similar changes occur consistently following various forms of NSPT. The majority of alterations were either common to all timepoints, or exclusively observed at early timepoints (day 1, day 7). Thus, shifts in composition were either induced rapidly and maintained until day 90, or recovered gradually over the course of the study.

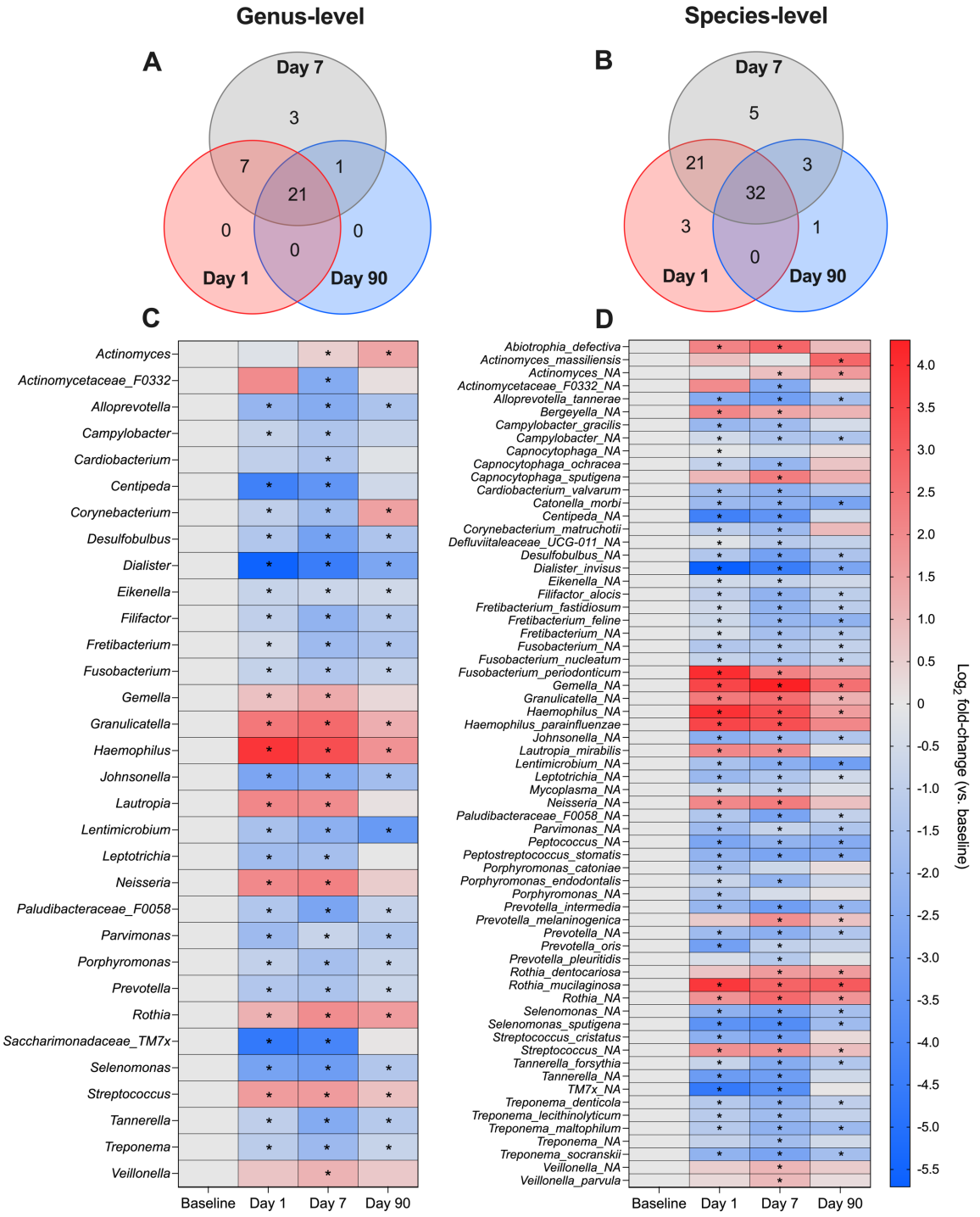

Figure 5.11. Differential abundance of genera and species following full-mouth debridement. Venn-diagram displaying genera (A) and species (B) which significantly differed at each timepoint. Heatmap displaying abundant genera (C) and species (D) which significantly differed compared with baseline. Data are mean log2 fold-change compared with baseline. Statistics are Wilcoxon signed-rank test versus baseline, adjusted for multiple comparisons using the FDR (5%) approach. *p[adjusted]<0.05. For baseline and day 1, n=38. For day 7 and day 90, n=37. Only abundant genera (≥0.5% at any timepoint) and species (≥0.25% at any timepoint) were plotted.

5.3.2.5. Visualising compositional shifts following FMD

To visualize the reformation of the subgingival plaque microbiota, a species-level PCoA was performed across timepoints with each timepoint added sequentially (**figure 5.12**). Near identical patterns were observed at genus-level and was thus not plotted separately. When viewing PCoAs, the baseline samples (red) clustered together on the right-hand side as expected. At day 1 (yellow) most samples distinctly clustered from those at baseline, albeit a small degree of overlap remained. This distinct clustering was maintained until day 7 (green), with a high degree of overlap between day 1 and 7 samples. Interestingly at day 90 (blue), no distinct clustering was observed, and samples appeared highly spread across each of the preceding timepoints. For example, a subset of day 90 samples appeared to reform a composition more similar to baseline, whilst others maintained a composition more comparable with days 1 and 7. Therefore, whilst the baseline composition appeared relatively consistent, the capacity for a similar composition to reform at day 90 was heterogenous across the study population.

In terms of reformation, genera and species which characterised the shift between the early (day 1, 7) and later (day 90) follow-up timepoints may be of particular interest despite this heterogeneity. At genus-level, these differences were largely characterised by reduced abundance of aerobes and facultative anaerobes such as *Haemophilus, Neisseria, Granulicatella Streptococcus* and *Lautropia* species (**figure 5.13A**). In contrast, 9 genera significantly increased in abundance at day 90 compared with either day 1 or day 7 (**figure 5.13B**). Although many of these genera were obligate anaerobes (*Selenomonas, Centipeda, Prevotella, Tannerella, Porphyromonas*), this trend was not unanimous. In fact, both *Actinomyces* and *Corynebacterium,* which have been previously associated with periodontal health (Abusleme et al., 2013, Cai et al., 2021), also showed a significantly increased abundance at day 90.

At species-level, many shifts were attributed to unclassified species (**figure 5.14**). Of note, one species which reduced in abundance at day 90 (*Fusobacterium periodonticum*) and one species which increased in abundance at day 90 (*Campylobacter gracilis*) belonged to the Socransky 'orange-complex' (Socransky et al., 1998). Likewise, Filifactor *alocis* and *Porphyromonas*

endodontalis (red boxes, **figure 5.14B**) are disease-associated and both significantly increased in abundance at day 90. Increased abundance of non-anaerobic species was also observed, including unclassified *Actinomyces*, *Corynebacterium matruchotii* and *Streptococcus cristatus*.

Collectively, these data map the reformation of the subgingival plaque microbiota following biofilm disruption. As expected, these shifts were largely governed by late colonisation of anaerobic species, which may feasibly require a longer period to grow and re-establish themselves within shrinking periodontal pockets. However, both *Actinomyces* and *Corynebacterium* may also be categorised 'late colonisers' under the conditions assessed here. This data may initially seem surprising, although a similar finding was described nearly 50 years ago using culture techniques; detailing the 'late colonisation' of *Actinomyces* and *Corynebacterium* in the absence of oral hygiene (Ritz, 1967). Thus, the paradigm of early colonisers being associated with health, and late colonisers being associated with disease may not relate to all organisms. The current study also suggests that the early post-treatment microbiome remains relatively consistent up to 1 week following debridement, whereas more heterogenous microbial compositions were observed at day 90. Therefore, whilst consistent shifts were observed, many more are likely masked by large differences in the day 90 microbiota between patients.

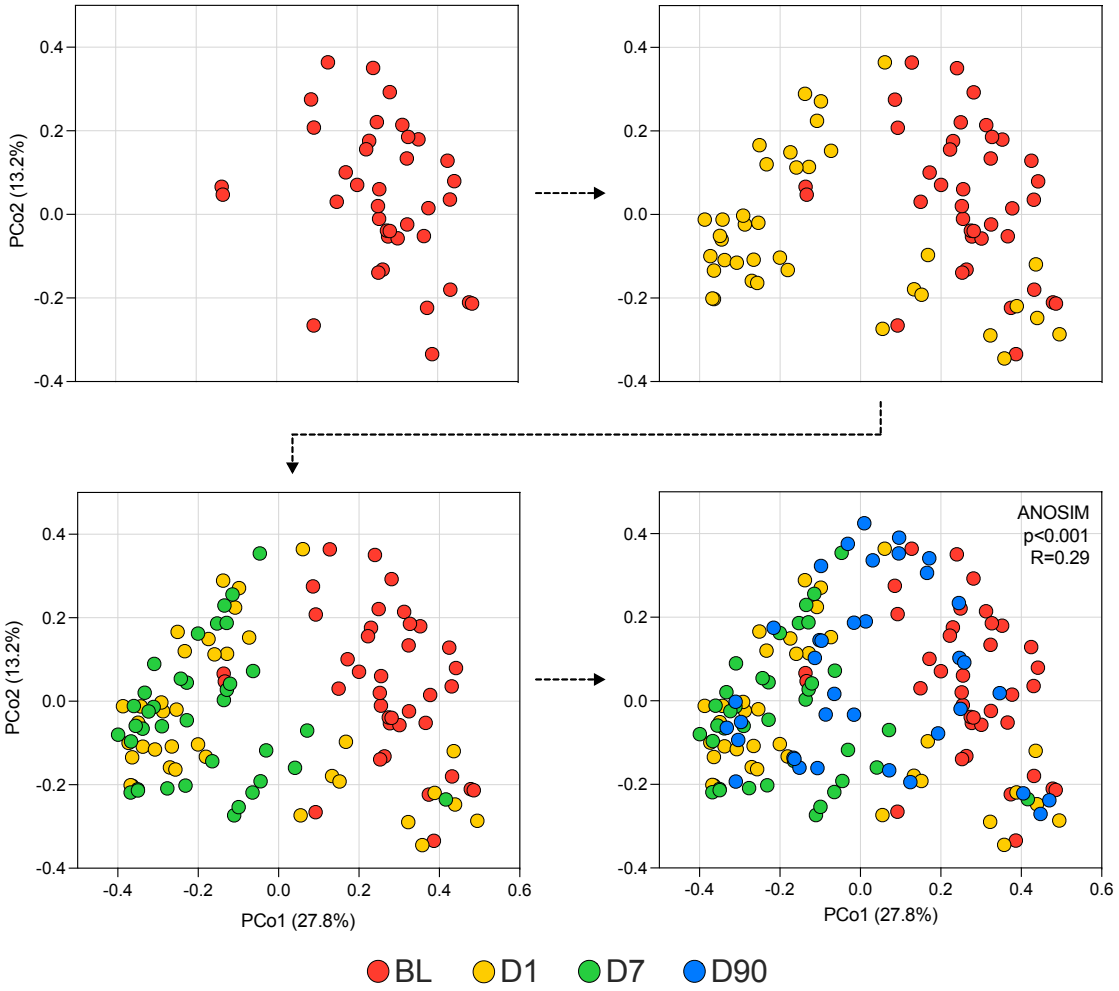

Figure 5.12. Community reformation following full-mouth debridement. A Bray-Curtis based PCoA was performed at species-level across all timepoints, with individual timepoints sequentially added on to the figure (indicated by arrows). For baseline and day 1, n=38. For day 7 and day 90, n=37. Statistics are ANOSIM between all timepoints.

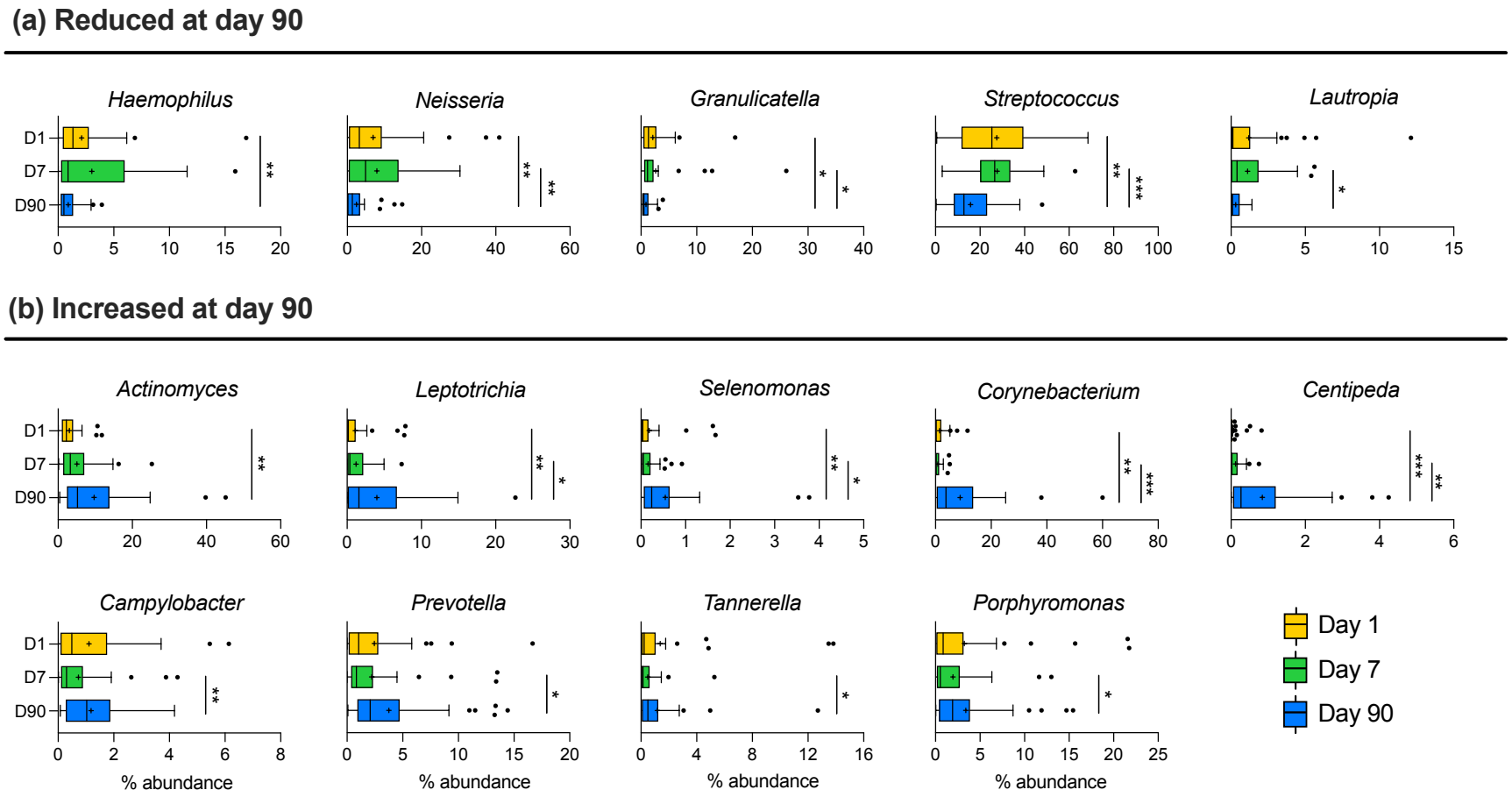

Figure 5.13. Differential abundance of genera between early (days 1, 7) and later (day 90) timepoints. Genera which reduced (A) and increased (B) in abundance at day 90. Graphs display Tukey boxplots of the abundance of each genera, where the median is represented by the central horizontal line and mean displayed as '+'. Statistics represent Wilcoxon signed-rank tests with p-values adjusted using FDR 5%, *p<0.05, **p<0.01, ***p<0.001.

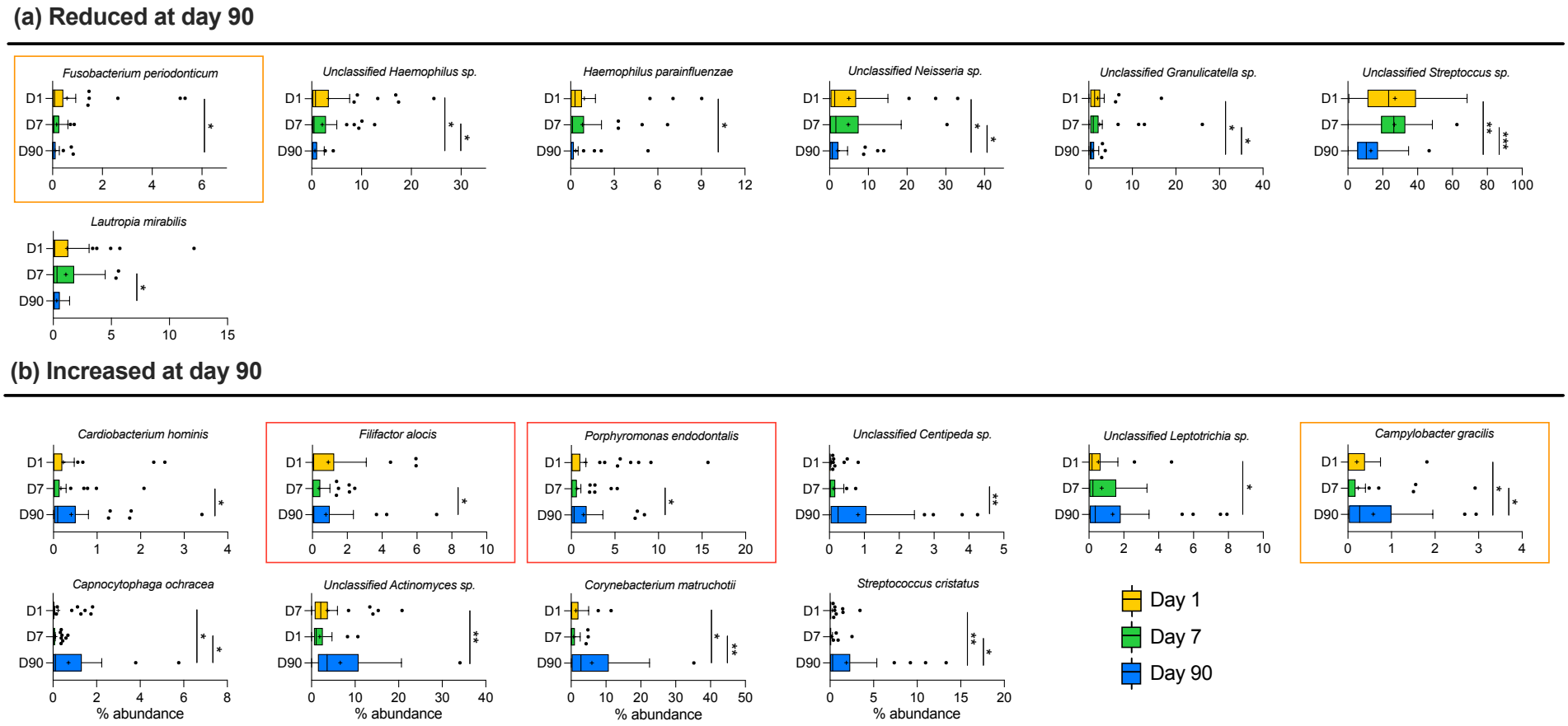

Figure 5.14. Differential abundance of species between early (days 1, 7) and later (day 90) timepoints. Species which reduced (A) and increased (B) in abundance at day 90. Graphs display Tukey boxplots of the abundance of each genera, where the median is represented by the central horizontal line and mean displayed as '+'. Statistics represent Wilcoxon signed-rank tests with p-values adjusted using FDR 5%, *p<0.05, **p<0.01, ***p<0.001.

5.3.2.6. Potential influences on day 90 community reformation

An interesting finding from this chapter was the apparent reformation of a 'baseline-like' microbiome in a subset of patients at day 90. The ability to predict this response would be of huge clinical benefit, potentially unravelling why some patients show an attenuated microbial response following NSPT. Furthermore, understanding this long-term microbial heterogeneity would be an important steppingstone in unravelling the complex factors which drive the bacterial composition of subgingival plaque samples, and may uncover aspects that drive dysbiosis of the community.

To investigate potential factors influencing the day 90 microbiota, various clinical, demographic and behavioral variables were assessed. To do this, the day 90 samples from the previous species-level PCoA analysis (**figure 5.12**) were coloured according to the abundance of disease-associated species to confirm the presence of a more disease-associated community (**figure 5.15**). As expected, this colour gradient revealed that samples clustering more similarly with baseline (right-hand side) had an elevated abundance of disease-associated species with a steady increase from left to right.

To establish whether microbial reformation was associated with residual clinical disease, samples were sized according to periodontal clinical variables using bubble plots. Given previous links between gingival inflammation and biofilm dysbiosis (Rosier et al., 2018, Van Dyke et al., 2020), it was hypothesised that the reformation of the microbiome following treatment may be at least partially associated with the level of residual inflammation at day 90. To test this, the PISA was employed as an estimate of local inflammatory burden (Nesse et al., 2008). Surprisingly, this analysis suggested no association between the subgingival plaque microbiome and PISA levels at day 90 (**figure 5.15A**). For example, some samples contained a low abundance of disease-associated species despite patients displaying higher levels of residual PISA, whilst the highest abundance of disease-associated species (56.9%) was found in a patient with a day 90 PISA of only 30.95 mm^2 – commensurate with periodontal health (Leira et al., 2018).

In addition to inflammation, patient's oral hygiene was evaluated as a potential driver of microbial reformation. Within the current cohort, FMPS was calculated

at baseline, day 7 and day 90. As expected, FMPS were generally low across the entire cohort at day 7 (≤20%). Despite standardising oral hygiene through administration of an electric toothbrush and detailed oral hygiene advice at each follow-up timepoint, FMPS increased at day 90 compared with day 7 with a large spread in day 90 values (ranging 0.86% to 52.34%). Thus, FMPS were employed as a crude estimate of patient's oral hygiene status. Similar to the PISA, this analysis did not reveal an association between the day 90 microbiota at FMPS (**figure 5.15B**).

Site-specific pocket depth was also considered as an indicator of the microenvironment from which subgingival plaque samples were harvested. Despite large heterogeneity in the PPD at day 90, there was no apparent association with the subgingival plaque microbiome in the current study (**figure 5.15C**). These data appear largely commensurate with those observed within **chapter 3**. Analysis of categorical variables was also considered and suggested no clear association between the day 90 microbiota and smoking status, treatment group or sampled site morphology (**figure 5.16A-C**), albeit the sample size of smokers was relatively low in this cohort. This visual assessment of bubble plots was statistically confirmed by negligible associations between disease-associated species and PISA (Spearman R=0.03, p=0.86), FMPS (R=0.27, p=0.11) or site-specific PPD (R=0.24, p=0.16). No differences were observed between categorical variables using Mann-Whitney (treatment group; p=0.94, site morphology; p=0.87) or Kruskall-Wallis analysis (smoking status; p=0.76). Therefore, whilst the baseline microbiota appeared to reform in a subset of patients, this could not be solely explained by differences in clinical disease, instrumentation choice, smoking status or site selection in the current study.

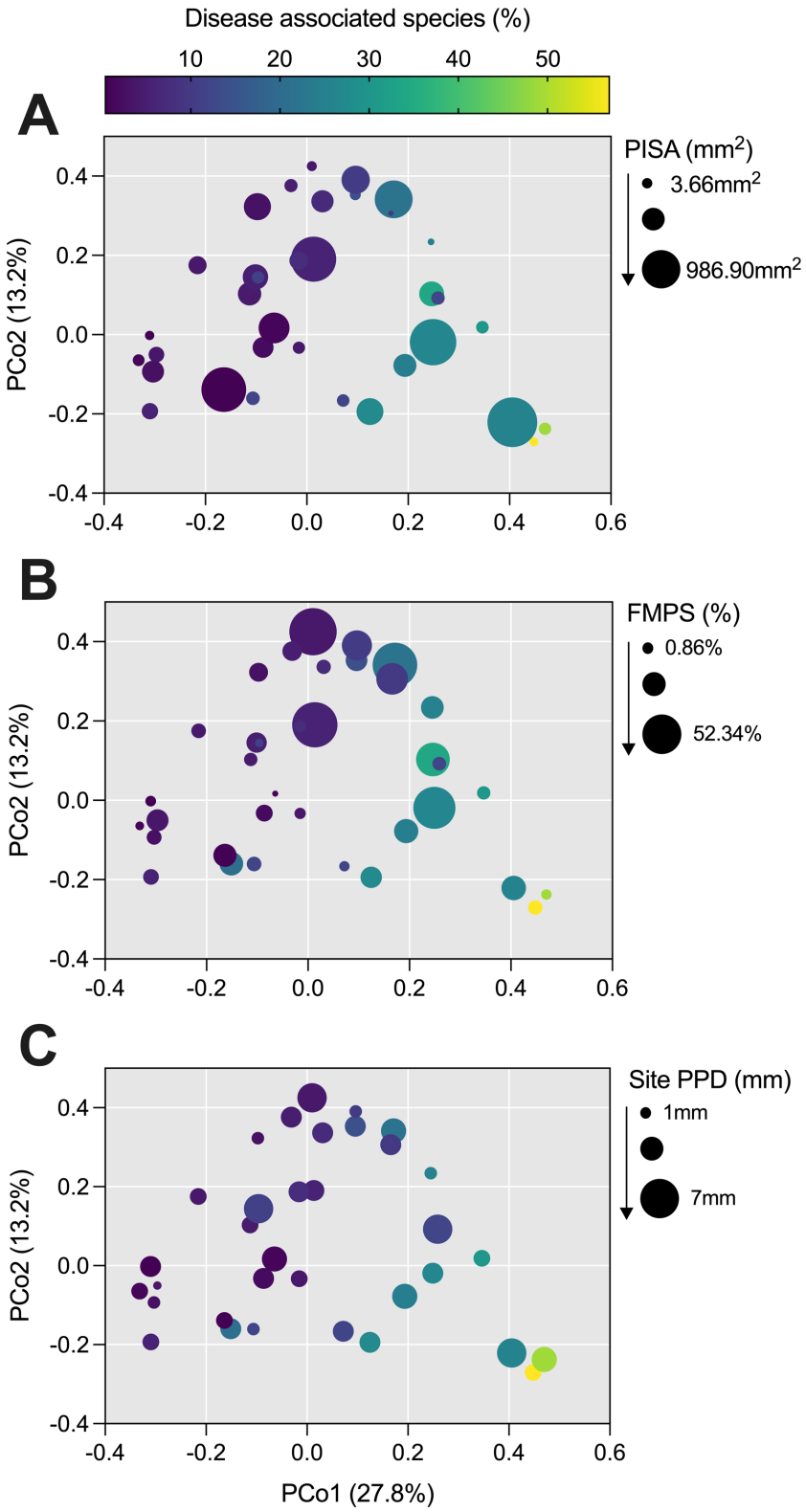

Figure 5.15. Relationship between the day 90 microbiota and periodontal clinical variables. Graphs are bubble plots displaying species-level PCoAs at day 90 (x/y axes), coloured according to the abundance of disease associated species and sized according to PISA (A), FMPS (B) and site PPD (C) at day 90. Disease associated species represent the sum of the 'red-complex', 'orange complex' (Socransky et al., 1998) and those identified in a recent systematic review (Pérez-Chaparro et al., 2014). N=37.

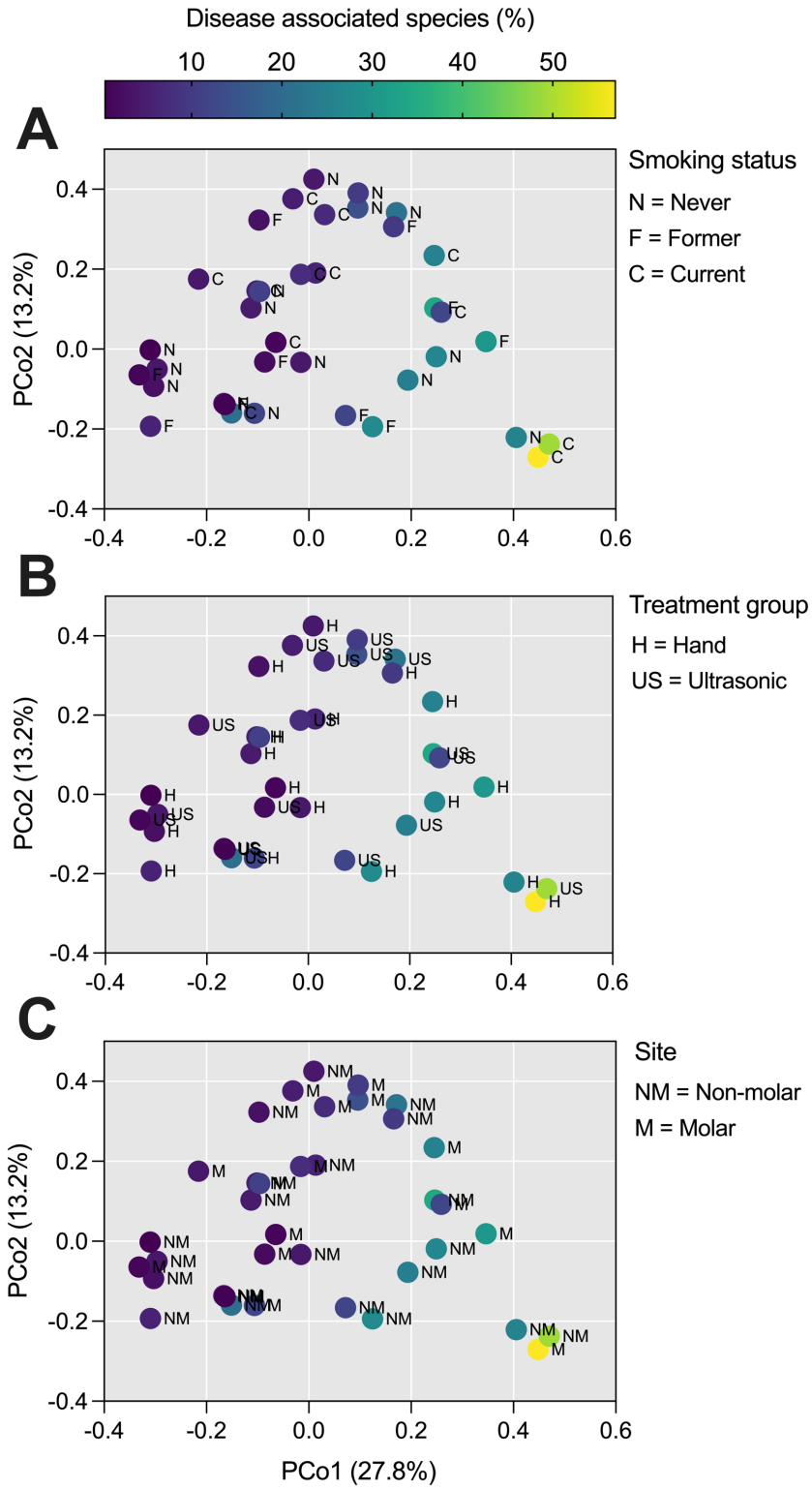

Figure 5.16. Relationship between the day 90 microbiota and categorical variables. Graphs are bubble plots displaying genus-level PCoAs at day 90 (x/y axes), coloured according to the abundance of disease associated species and labelled according to smoking status (A), treatment group (B) or molar/non-molar site selection (C). Disease associated species represent the sum of the 'red-complex', 'orange complex' (Socransky et al., 1998) and those identified in a recent systematic review (Pérez-Chaparro et al., 2014). N=37.

5.4. Discussion

Using an *in vitro* biofilm model, this chapter found that ultrasonic instruments removed more biomass than hand instruments when controlling for stroke number. Despite this, both hand and ultrasonic instruments appeared to induce comparable impacts on the microbiome *in vivo*, in terms of taxonomic diversity and overall composition at a range of follow-up timepoints. Within each group, instrumentation induced rapid compositional shifts in the subgingival plaque which was followed by a gradual recovery to baseline in a subset of patients. The importance of this community reformation, in the absence of elevated clinical disease is currently unknown and requires additional evaluation.

One previous study has compared hand and ultrasonic instruments on *in vitro* multispecies biofilms (Hagi et al., 2015). In this previous study, a 12-species biofilm model was treated using 10 strokes of a hand instrument or for 10 seconds using an ultrasonic instrument, within a complex periodontal pocket model. The authors observed comparable results with respect to an elevated reduction in biomass following ultrasonic compared with hand instrumentation. Ultrasonic scalers have also been recently assessed *in vitro* using a more advanced dental implant biofilm model (Vyas et al., 2020a). Here, the authors demonstrate that cavitation from ultrasonic scalers is capable of removing biofilms from implants in a contact-free treatment model. Considering the results of the current study, this cavitation effect may also explain the enhanced biofilm removal with ultrasonic compared with hand instruments.

The current chapter also incorporated analysis of supernatants, where it was established that disrupted organisms remained viable following removal from coverslips. As expected, this data suggests that instrumentation may give rise to the rapid dispersion of planktonic organisms in the oral cavity. In reality, clinicians do not control for strokes but aim for total disruption and removal of subgingival plaque biofilms. Therefore, targeting the viability of dispersed organisms (alongside 'residual' biofilms) may also be of benefit in preventing recolonisation of certain species in distal sites.

A limitation of the *in vitro* section of this chapter is that the model employed is not reflective of the oral cavity, as biofilms were treated on glass coverslips

within microtiter plates and treatment was performed aerobically. It is reassuring that the results here are commensurate with the previous study where an artificial periodontal pocket model was employed (Hagi et al., 2015). However, it is likely that organisms adhere differently to a glass coverslip compared with a more representative substrate such as hydroxyapatite or enamel. Additionally, some dispersed organisms may not have been captured within the current model, particularly following ultrasonic debridement where aerosol generation could not be fully contained. In light of these limitations, we are in the process of developing a new treatment model which will better collect aerosol and provide a more reflective setting for evaluating the impact of instrumentation on *in vitro* biofilms (**appendix iv**). Building upon the data presented in this chapter, future experiments should be performed in an anaerobic cabinet to minimise bias in supernatant viability. Additionally, the model developed by Vyas et al., for high-speed imaging biofilm removal could be considered to compare the effects of cavitation among different ultrasonic inserts (Vyas et al., 2020a, Vyas et al., 2020b).

Assessing the subgingival plaque microbiome from patients treated exclusively using hand or ultrasonic instruments, this chapter observed no compositional differences between groups at any follow-up timepoint (day 1, day 7, day 90). These data are in line with earlier studies using targeted approaches such as DNA-DNA hybridization and PCR for quantification of certain disease associated species (Christgau et al., 2007, Derdilopoulou et al., 2007, Ioannou et al., 2009), suggesting these effects likely extend across the entire microbiome.

Whilst no differences were observed between treatment groups, widespread compositional alterations were found longitudinally within each group (summarised in **figure 5.17**). Assessing all patients, *Streptococcus, Rothia* and *Neisseria* species were substantially elevated at day 1 and day 7 compared with baseline, indicating that these genera are able to rapidly recolonise the subgingival pockets and establish dominance within the early stages of biofilm reformation. This finding is in line with the spatiotemporal model of oral bacterial colonisation whereby streptococci species are primary colonisers and facilitate a base from which the mature biofilm can form (Kolenbrander et al., 2002). The rapid expansion of *Rothia* species has been previously documented at

2-week and 6-week timepoints indicating such alterations may be maintained even past the 7 day follow-up within the current study (Belstrom et al., 2018).

Surprisingly, although reduced in abundance there remained detectable levels of several disease-associated anaerobes even 1 day post-treatment (*P. gingivalis, T. forsythia, F. alocis*), implying that the polymicrobial nature of the subgingival plaque persists during treatment and recovery. This could be speculated to be the result of missing small areas of plaque during treatment, or as evidenced from *in vitro* data, dispersed species may rapidly recolonise at low levels. In agreement with the former, it has been widely documented that calculus is rarely – if ever – completely removed from deep pockets ≥5 mm (Brayer et al., 1989, Robertson, 1990). A similar finding has been documented using DNA-DNA hybridisation to assess subgingival biofilm development in the absence of oral hygiene (Teles et al., 2012). This previous study found that disease-associated species (red- and orange-complex) were reduced but detectable 1 day after NSPT, and persisted over a 1 week period in the absence of oral hygiene.

Within the current study, most alterations in the abundance of genera and species were either induced rapidly and maintained over the course of the trial, or recovered between days 7 and 90. The alterations observed at day 90 are largely similar to those observed within **chapter 3**, and as such it seems likely that different treatment plans induce comparable microbial outcomes. There are similar observations in healthy subjects; demonstrated in a study that collected the subgingival plaque at 10 timepoints following mechanical debridement ranging from 0 hours (immediately after) to 3 months (Wang et al., 2020). It was found that the largest compositional difference from baseline was observed between 7 hours and 3 days - rather than between 0 and 4 hours - with an almost entire recovery by 3 months. This community reformation may be expected within healthy subjects; however the current study also demonstrates a similar response within a subset of PD patients.

Compared with preceding timepoints, there was higher heterogeneity in the composition of day 90 samples. Indeed, it was confirmed that some samples at contained a high abundance of disease-associated species with no clear relationship with residual clinical disease, smoking status or treatment group when viewed in isolation. This is supported by previous studies where a clear disease associated microbial shift was observed in some patients despite clinical

improvement (Schwarzberg et al., 2014, Chen et al., 2018). The significance of these species in the absence of clinical disease is unknown, although it has been speculated that a high abundance of disease associated species, even without disease symptoms, may be prognostic of future disease progression (Griffen et al., 2012). Thus, the question remains as to why such a differing microbial response is observed among a cohort of patients who all improved clinically, received similar forms of therapy and contained a relatively comparable microbiota in preceding timepoints.

Several factors are known to alter the oral microbiome and may explain these results. It has recently been reported that children who have parents suffering from PD display an elevated abundance of disease-associated organisms (Monteiro et al., 2021), supporting earlier work that cohabitation can influence the composition of the oral microbiome (Song et al., 2013, Shaw et al., 2017). In addition, a genetic susceptibility to the colonisation of red-complex species has been reported by GWAS (Divaris et al., 2012). Evaluating genetic and environmental factors was out with the scope of the current study, however during reformation the composition subgingival plaque may be particularly sensitive to these variables.

Another recent study demonstrated that a nitrate rich diet substantially shifts the subgingival plaque microbiome in PD patients following treatment, with significantly lower abundances of *Fretibacterium, Prevotella* and *Treponema,* and higher abundances of *Rothia* and *Neisseria* (Jockel-Schneider et al., 2021). Similarly, *in vitro* nitrate supplementation can attenuate PD associated bacteria in biofilms grown from patient saliva (Rosier et al., 2020). Within the current study no data regarding patients diet was recorded and this may be another non-clinical factor which could contribute to differing compositions at day 90.

It is noteworthy to mention that a 'dysbiosis index' has recently been developed based on 49 discriminating species (Chen et al., 2021). This index is able to accurately discriminate between PD and healthy sites, and was validated by machine learning analysis across several previous studies. Of interest, the authors highlight that a small subset PD samples contained relatively low levels of dysbiosis, whilst a small subset healthy samples contained high levels of dysbiosis. It was suggested that PD samples with low dysbiosis may represent quiescent sites or patients hyperresponsive to dysbiosis, whereas healthy sites

with high dysbiosis may represent a form of tolerance or those at risk of disease progression. Such effects may also relate to the day 90 samples within the current study, although it remains unclear why and indeed how this dysbiosis arises. Future studies which identify this dysbiosis either in healthy patients or post-treatment, and subsequently monitor long-term clinical responses are of paramount importance moving forward in periodontology.

As previously mentioned, a limitation of the current microbiome analysis is that sequencing 16S rRNA fragments is not quantitative, as results are computed as relative abundances. Given the nature of the treatment performed, it is assumed that the total microbial load of day 1 and possibly day 7 samples will be less than that at baseline or day 90, and the results here merely reflect composition at each point in time. With respect to the disparity in day 90 microbial outcomes, it cannot be ruled out that the results throughout this chapter represent the pace of reformation rather than its presence or absence (**figure 5.17**). The current study was not designed to observe this trend and across a longer study period it is possible that the shift towards a 'baseline-like' microbial profile would be observed in a larger number of patients. Given that pockets were still present, disease associated organisms may eventually recolonise and establish themselves as biofilms continue to grow and mature. In future, the importance of this response must be established by investigating whether the presence or abundance of disease-associated species dictates the risk of subsequent disease development in specific sites.

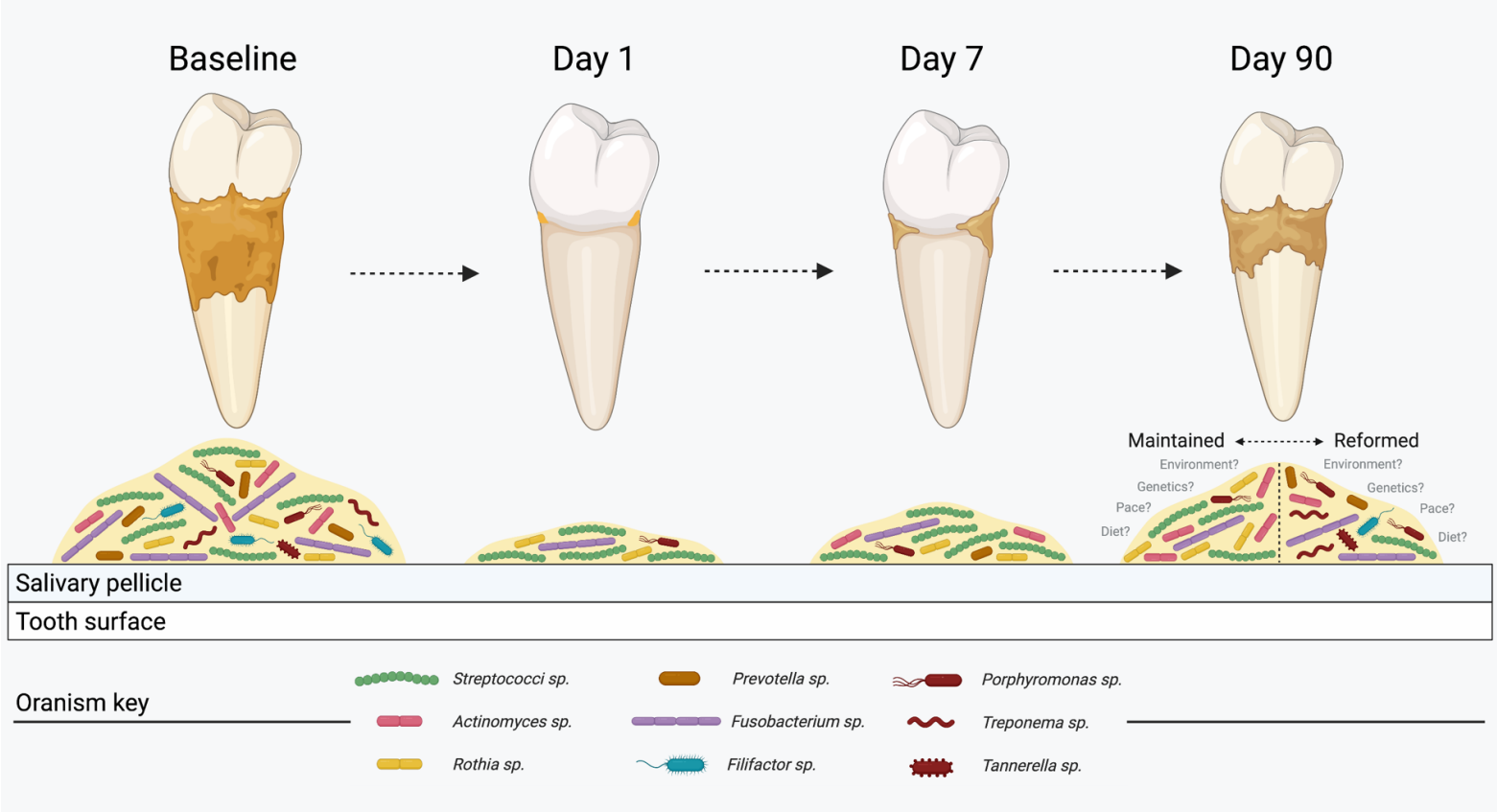

Figure 5.17. Summary schematic depicting the reformation of subgingival plaque biofilms following periodontal therapy. At days 1 and 7, biomass is presumed to be low, but composition is dominated by health associated genera (*Streptococcus, Actinomyces, Rothia*). By day 90, the composition was comparable to baseline in a subset of samples, whilst others were more similar to days 1 and 7. This was surprisingly independent of clinical disease and demographic risk factors in the current study. Variables which may influence this response that merit future research are environmental factors, genetic predispositions, diet and whether this effect may simply be the pace of reformation. Image created using BioRender.

5.5. Chapter summary

This chapter evaluated the impacts of different instrumentation techniques on periodontal biofilms both *in vitro* and *in vivo*. The main findings from this chapter are highlighted below;

- Ultrasonic instruments removed more biomass from *in vitro* biofilms than hand instruments when controlling for strokes.
- However, dispersed organisms following biofilm removal remained viable.
- The current study did not find a difference in the subgingival plaque microbiome of patients treated with ultrasonic or hand instruments.
- The composition of subgingival plaque samples was largely composed of *Streptococcus, Actinomyces* and *Rothia* species 1 day following treatment, which persisted for at least 7 days.
- By day 90, some samples displayed a composition highly similar to baseline irrespective of residual clinical disease, smoking status or instrumentation choice.

Chapter 6

Final discussion, future work and conclusions

6.1. Final discussion

The unifying goal of this PhD project was to investigate the host and microbial response to NSPT through the use of a longitudinal cohort study (**chapters 2-3**) and RCT (**chapters 4-5**). This is important given that NSPT is often only partially successful, and potentially detrimental off-target systemic effects have been reported following FMD. As such, there is a need to critically evaluate the efficacy of NSPT from a clinical, immunological and microbial viewpoint to form a holistic understanding of the response to treatment. Detailed individual discussions are provided at the end of each chapter, the current section seeks to provide context to the major findings of this thesis and highlight potential areas of future research.

6.1.1. The goal of periodontal treatment

In light of the reported epidemiological and mechanistic links between PD and RA, several questions have been posed such as whether NSPT may prevent the future development of RA (Bingham and Moni, 2013). Addressing this question can be particularly challenging given that an ideal comparator group would consist of PD patients who did not receive treatment, and hard-endpoints (RA development) would be measured over time. This design carries obvious ethical limitations, and demands an unrealistically long study duration. Instead, many researchers opt to measure surrogate markers of disease longitudinally following NSPT. **Chapter 2** of this thesis evaluated serum ACPAs, systemic inflammatory proteins and antibacterial antibody titres in 42 PD patients before and 3 months after receiving NSPT. As noted, there were no longitudinal alterations in either serum ACPAs or systemic inflammatory proteins – despite widespread reductions in periodontal disease severity. These findings are in contrast to two previous studies which found reductions in serum ACPAs following NSPT in only non-smokers (Lappin et al., 2013) or patients with generalised PD (Yang et al., 2018).

One limitation of all three studies is that both the sample sizes and ACPA titres of patients were low – and all analyses were exploratory in nature. Hence even when a reduction is observed, it can be difficult to delineate a clinically meaningful outcome when only a small magnitude of change is present. In hindsight it is perhaps unsurprising that no alterations were observed across all

patients in the current study. As evidenced by the microbiota analysis in both **chapters 3** and **5**, species such as *P. gingivalis* are reduced in abundance following treatment - but remain very much present in this community. If these bacteria are indeed the drivers of autoimmunity, then NSPT in isolation would appear unlikely to halt this reaction in the long term. Other factors such as smoking have also been shown to upregulate protein citrullination in the lungs by increasing the expression of host PAD2 (Makrygiannakis et al., 2008), and the bridge between these putative triggers and eventual RA development may be dependent on a genetic predisposition (HLA-DRB1 shared epitope). Animal models generally support a contributory role for PD in both RA initiation and progression (Bartold and Lopez-Oliva, 2020). Human studies – around which this thesis is centred – are far less conclusive. The development of RA is incredibly complex and remains incompletely understood. Thus, despite an association with PD it is perhaps unrealistic for NSPT to ameliorate any future risk of this extra-oral disease.

In a broader sense, it has been questioned whether oral health should be promoted based on its impact on systemic disease rather than being important in its own right (Pihlstrom et al., 2018), and a similar paradigm could be extended to periodontal therapy. The lack of reduction in serum ACPAs, systemic inflammatory proteins or failure to remove all disease-associated species from the subgingival plaque, are by no means a reflection on the success of treatment. Although many studies have assessed surrogate markers of systemic diseases following NSPT, the goal of this treatment is to preserve, improve and maintain the natural dentition in the mouth and induce a healthier periodontium with the absence of inflammation (American Academy of Periodontology, 2011). It is important not to lose sight of the fact that every patient within both studies in this thesis showed positive signs of clinical improvement after 90 days. Thus, NSPT was unequivocally beneficial in regard to improving oral health.

6.1.2. The blended treatment approach

NSPT is a versatile technique and while the main goal of treatment remains unchanged, it is applied in various forms throughout clinical practice. FMD was originally advocated to prevent the translocation of ‘pathogenic’ bacteria from untreated into treated sites during quadrant NSPT (Quirynen et al., 1995).

However, follow-up systemic reviews have highlighted only slight clinical improvements using FMD compared with quadrant NSPT (Lang et al., 2008). Instead of outright clinical superiority, FMD is preferred in some situations as it requires less time overall, reduces the need for repeated instrument sterilisation and may be more convenient for certain patients to avoid repeated travel (Koshy et al., 2005, Fang et al., 2016). Over the last 20 years, several studies have demonstrated a marked elevation in serum CRP following FMD (D'aiuto et al., 2004a, Tonetti et al., 2007, Graziani et al., 2010, Graziani et al., 2015, Morozumi et al., 2018) and recently it has been recommended that treatment be performed over several sessions for patients with CVD (Sanz et al., 2020).

This systemic response was further evaluated within the RCT of **chapter 4**, where no differences in serum CRP were observed between hand and ultrasonic treatment groups at day 1. This finding is directly applicable to clinical practice; demonstrating that the choice of instrumentation does not influence the systemic response to FMD. As mentioned previously, it is also of particular interest that the 'spike' in CRP following these treatments was markedly lower than has been previously observed. There remains some ambiguity around the treatment delivered in previous studies and for some patients the 'FMD' may have included additional treatment such as tooth extractions (Tonetti et al., 2007), supragingival scaling (Graziani et al., 2015) and root planing (Morozumi et al., 2018) during treatment visits. The current study, as per SDCEP and other current clinical guidelines provided supragingival scaling prior to subgingival instrumentation. The subgingival instrumentation was provided as 'FMD' in this context. From the previous studies, it is therefore difficult to distinguish how much of this spike is attributed solely to subgingival scaling and how much may be attributed to the other performed procedures.

From **chapter 4**, it could be speculated that subgingival scaling in isolation carries a relatively low risk of a marked elevation in systemic inflammation. Performing supragingival scaling or tooth extractions on alternative days may represent a simple modification to treatment plans which could attenuate such a response. However, the current study was not designed to reveal the clinical significance of this response, but simply investigate whether it differed following hand or ultrasonic instrumentation. As changes to treatment are being advocated, it is important that the impact of this response is further

investigated. Despite recent recommendations to minimise this spike in CVD patients, there remains only indirect evidence that such a response induces a clinically meaningful risk to patients overall health (Smeeth et al., 2004). These recommendations could perhaps be strengthened by a large retrospective analysis on the incidence of CVD events following FMD versus quadrant NSPT, so that the overall risk of this procedure can be firmly established. However, the data recording in most dental care systems, and lack of linkage to data regarding systemic disease, does not currently permit such an analysis.

With respect to clinical and microbial outcomes, **chapters 4** and **5** did not observe any differences between hand and ultrasonic instruments. This RCT was not powered nor designed to detect differences in these variables and they must therefore be interpreted with due caution. However, both results appear comparable with a range of previous studies (Derdilopoulou et al., 2007, Christgau et al., 2007, Ioannou et al., 2009), providing further support of the status quo (i.e. combined/'blended' use of instruments). The only difference was the time of treatment, which was 22 minutes longer for hand instruments on average. This finding may be of potential interest given the huge financial burden of periodontal treatment (**section 1.1.2**). Shorter treatments with ultrasonic instruments could allow more patients to be treated in a set timeframe, and save money on treatment associated expenses including clinician salaries and instrument sterilisation when compared with hand instruments. The cost of these variables is likely to differ per site, although could be assessed on a centre-to-centre basis. The time-saving capacity of ultrasonics may be somewhat explained by the in vitro results of **chapter 5** and those reported by Vyas et al. (Vyas et al., 2020a, Vyas et al., 2020b). Biofilm removal from ultrasonics appears larger *in vitro* which may be due to cavitation and/or effects from the coolant spray, and thus hand-scalers require more time to achieve comparable levels of biofilm removal. Notably, this time-saving appeared particularly pronounced in patients with more severe disease, supporting this hypothesis.

6.1.3. Dysbiosis, inflammation and disease

Evaluating the subgingival plaque microbiome provided an insight into how this polymicrobial biofilm recovers following mechanical debridement, which yielded

interesting results throughout **chapters 3** and **5**. Within both studies, treatment induced consistent alterations in the subgingival plaque composition including reductions in richness, diversity and abundance of disease-associated anaerobes (*Porphyromonas*, *Tannerella*, *Treponema*), coupled with increased abundance of health-associated genera (*Streptococcus*, *Actinomyces*, *Rothia*). These changes were not necessarily dramatic and disease-associated organisms, whilst reduced in abundance, remained present following treatment. This data is supportive of earlier research using DNA-DNA hybridisation to study over 4000 subgingival plaque samples before and after NSPT (Haffajee et al., 1997). In this study, the authors recorded modest reductions in disease-associated species, but highlight that radical shifts in the composition are not necessary for positive clinical outcomes. As discussed in **section 1.1.3**, these biofilms perform important functions for the host and it is therefore advantageous that a somewhat similar biofilm community reforms following NSPT.

In some cases, a high abundance of disease-associated species were observed following treatment which – surprisingly - appeared irrespective of clinical response. These results are intriguing as they indicate that the post-treatment composition may be highly individualised which has been previously suggested (Schwarzberg et al., 2014). Several non-clinical factors have been shown to influence the subgingival plaque microbiota (environment, genetics, diet, smoking), and may play a role in shaping this community reformation. This disparity was consistent over both studies in this thesis, with other reports documenting an increase in – or persistence of - the abundance of disease-associated genera (*Porphyromonas*, *Treponema*, *Filifactor*, *Tannerella*) despite major clinical improvement (Chen et al., 2018). The reason behind this response is unknown and cannot be conclusively drawn from the presented studies, but a key question moving forward is whether the path of biofilm reformation impacts on subsequent disease recurrence.

A number of well described organisms are undoubtedly associated with PD, but how these particular bacteria impact on disease progression is not entirely clear. As discussed in **chapter 1**, several theories have been elegantly posed to describe how such species directly cause PD (Hajishengallis and Lamont, 2012, Rosier et al., 2014). Yet, others have postulated that dysbiosis may arise only in the later stages of disease to further perpetuate existing inflammation (Van

Dyke et al., 2020). Therefore the belief that subgingival plaque dysbiosis causes disease, or occurs as a result of disease, is largely down to conjecture (Curtis et al., 2020). To date, studies demonstrating that heightened dysbiosis and/or inflammation in healthy patients precede future development of PD are notably absent in the literature.

It has been found that some periodontally healthy sites contain a high abundance of disease-associated species (Griffen et al., 2012, Chen et al., 2021). These findings lend further credence to the hypothesis that some patients may better tolerate these biofilms and dictate a form of resistance to clinical disease progression (Löe et al., 1986). Additional support comes from a recent experimental gingivitis study, where the authors observed three clinical responses in the absence of oral hygiene; “high”, “low” and “slow” (Bamashmous et al., 2021). The high group were characterised by rapid accumulation of plaque and clinical inflammation, the low group showed similar levels of plaque but attenuated inflammation and the slow group showed both delayed plaque accumulation and a minimal inflammatory response. Of note, the high and low groups exhibited “strikingly similar” patterns in the subgingival plaque microbiome, but displayed substantially different clinical responses and GCF cytokine profiles (particularly IL-1β).

Unfortunately, the studies in this thesis did not always sample GCF from the same site as subgingival plaque and this places a restriction on possible conclusions. Neither of the current studies were designed for these analyses and it was not possible at the time of design to foresee whether such comparisons would be required or even performed. It would have been of interest to evaluate whether the sites with a high abundance of disease-associated species also contained an elevated level of inflammatory cytokines in GCF (IL-1β, IL-17A) and this is a drawback that will be rectified within future studies from our group. The follow-up time (3 months/90 days) also limited the extent to which these findings could be interpreted as it was not possible to assess long-term clinical and microbial stability in these sites.

To this extent, it is important that the 16S rRNA sequencing data generated throughout this thesis be viewed as hypothesis-generating. This analysis was exploratory in nature, and the small sample size of some variables such as smoking mean they cannot be ruled out from future research. Based on the

findings from this thesis and those reported by Bamashmous et al., (2021), it would be of interest to assess whether 'reformation' of a microbiota more similar to baseline is accompanied by elevated GCF cytokine profiles, or whether patients instead show different responses to this composition. This analysis may reveal whether the immune response could dictate future disease regression, and start to uncover the complex associations between dysbiosis, inflammation and disease.

6.2. General limitations

A number of relevant limitations are discussed in each chapter. Some of the broader limitations of the studies presented in this thesis are inherent to clinical research and are largely unavoidable, whereas others remain in a state of continual improvement. A general limitation of these studies is that particularly motivated and enthusiastic patients may self-select for inclusion. This may limit the extent to which clinical, inflammatory and microbial outcomes may be generalised in the wider population, and this selection bias is likely be further exacerbated by selecting patients within a specialist setting. Similarly, all patients within both studies were recruited in the West of Scotland, with known geographic influences on the oral microbiome and presumably other factors such as diet and lifestyle (Li et al., 2014, Gupta et al., 2017).

The analysis of 16S rRNA sequencing data has inevitable limitations. For instance, sub-species taxonomic classification level is simply unobtainable by using a fragment of the 16S rRNA gene, and species-level is largely provisional. This is of importance in subgingival plaque where several sub-species of *F. nucleatum* are suggested to play differing roles in biofilm formation and architecture *in vitro* (Thurnheer et al., 2019). The analysis within this thesis may also be viewed as simplistic in nature, although was solely employed to assess global changes between groups or timepoints. More complex bioinformatic pipelines are constantly being developed to better handle this data and are becoming more frequently used in the field (Lin and Peddada, 2020). For example, future addition of UniFrac distances (which incorporate phylogenetic distance) alongside the Bray-Curtis metric, or applying a more sophisticated differential abundance technique such as ANCOM-II, could be useful upon re-analysis of this data. Ultimately there are limitations to any analysis pathway

and this is very much a constantly evolving field. Nonetheless, recently developed pipelines and breakthroughs in novel sequencing platforms such as the Nanopore MinION will undoubtedly continue to advance the scope and accuracy of microbiome profiling in coming years.

Much of the analysis in this thesis was exploratory in nature, and should be used to power and design future studies rather than draw firm conclusions. With exploratory analysis, non-significant results must also be interpretated tentatively. Such findings could indicate that no difference exists, that the sample size was not large enough to detect a significant change, or that the study was not designed to detect equivalence. The use of p-values implies the probability that such a difference could have occurred by chance. Hence, subtle differences may therefore become both statistically and clinically significant when applied to larger populations. These exploratory analyses have an important place in scientific research, being used to form new hypotheses and guide the design of future studies, but should be interpreted with extreme caution for the reasons outlined above.

6.3. Future work

Leading on from this project there are several unanswered questions which would be attractive focal points of future research. Some of these potential follow-up studies are noted in the discussions of each chapter, such as a multi-centre longitudinal treatment study of ACPA-positive PD patients; assessing the CRP spike following FMD with and without prior supragingival scaling; or a retrospective analysis on CVD events following FMD. However, if a step change in treatment strategy is to be initiated, arguably one of the most fundamentally important questions is whether a high abundance of disease-associated species ultimately leads to disease, both within healthy patients and following NSPT. If this is the case, then the data within this thesis demonstrate for the first time that microbiological monitoring may be of use at 3 months to predict this response and modify treatment plans accordingly. However, if this is not the case and disease is instead driven by host-specific factors, then it is of paramount importance that such features are established.

Defining the interplay between dysbiosis and dysregulated inflammation has long been a primary focus in periodontal research, and it is naïve to believe that a single clinical study would provide a definitive result. However, recent advancement of *in vitro* models of PD may help bridge this gap and be used to address the shortcomings of clinical research. For example, 2D and 3D co-culture systems have been developed to mimic aspects of the gingival epithelium (Brown et al., 2019b, Mountcastle et al., 2020). These systems could be coupled with protocols that provide more reflective *in vitro* biofilm models, such as those derived from patient saliva (Shang et al., 2019, Rosier et al., 2020) and subgingival plaque samples (Velsko and Shaddox, 2018).

Combining aspects of *in vitro* and *in vivo* research is therefore a feasible option to understand the relationship between dysbiosis, inflammation and disease. Building upon the data within this thesis, a suggestion for a pilot study is highlighted in **figure 6.1** to further explore these links. The concept of this proposed study is relatively simple, by recruiting healthy participants or treated PD patients and assessing the level of subgingival dysbiosis using the recently developed SMDI index (Chen et al., 2021). It is known that some healthy sites contain dysbiosis, and this thesis has demonstrated that a similar disparity exists in PD patients following NSPT. By measuring the corresponding GCF cytokine profiles, it would be possible to investigate whether dysbiosis is accompanied by elevated inflammatory responses *in vivo*. Subsequent long term follow-up of these patients would determine if these sites do eventually develop disease, and also establish the compositional stability of this dysbiosis (**figure 6.1B**). For example, if patients are predisposed to dysbiosis then repeated sampling should be reflective of this.

Simultaneously, the harvested samples could be grown *in vitro* and co-cultured with gingival tissue models (**figure 6.1C**). This would determine whether certain species dictate inflammation, or whether the inflammatory response is instead determined on a patient-by-patient basis. Development of technology such as Olink® proteomics allow comparison of up to 92 proteins in the inflammation panel, and could be applied to both GCF and co-culture supernatant to form a more holistic understanding of the immune response than was achieved during this thesis. In addition to assessing soluble mediators, ideally, local tissue biopsies could inform the cellular components of the local immune response.

However, this is far more invasive and demanding of participants. Therefore, whilst costly and difficult to manage, this pilot study could potentially provide significant new information and change the approach to managing PD. Targeting the right treatment, to the right patient at the right time remains an elusive goal in the treatment of many multifactorial inflammatory diseases. By building on our existing work, the proposed study may be a useful starting point towards eventually achieving this goal.

6.4. Conclusions

This thesis sought to evaluate the host and microbial response to NSPT with two notable findings that require recognition and further research. The first pertains to the observed spike in serum CRP following FMD. The RCT evaluated within this thesis found no significant difference following FMD performed exclusively with hand or ultrasonic instruments, and a somewhat attenuated CRP spike when compared with previous studies. These data suggest that FMD may be performed without a pronounced CRP spike under the settings of this RCT, and the impact of FMD on patients overall health must be further investigated. Additionally, the disparity in the microbial response to NSPT is intriguing not least because similar findings are observed in some periodontally healthy sites. Moving forward, it would be useful to establish whether this dysbiosis is prognostic of PD development and/or recurrence – or whether some patients are able to better tolerate these organisms without risk of future disease.

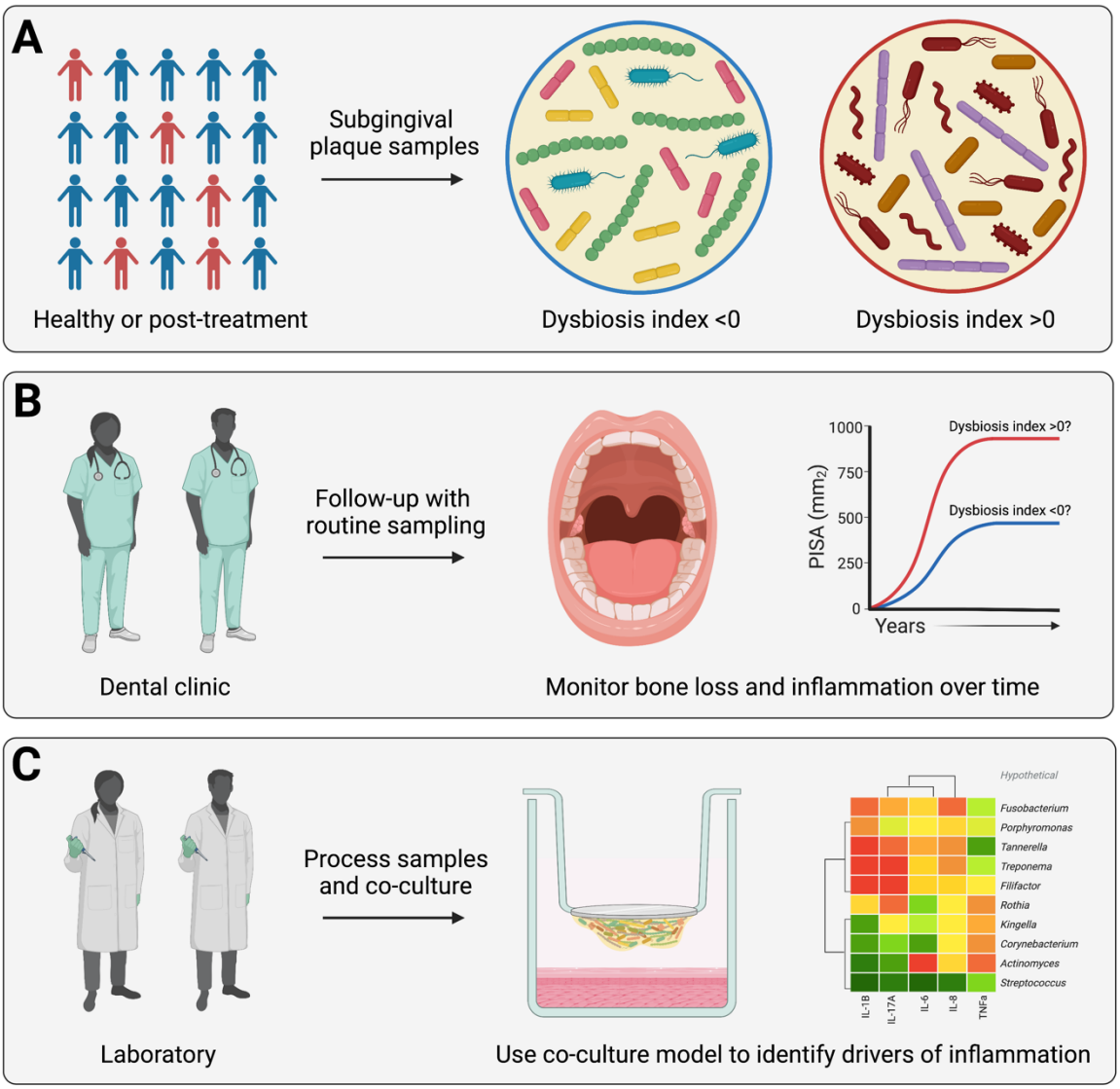

Figure 6.1. A suggestion for a proposed pilot combined *in vivo* and *in vitro* project to assess the relationship between dysbiosis, inflammation and disease. (A) Recruitment of periodontally healthy participants or treated PD patients whose subgingival plaque microbiota could be screened and designated as healthy (score <0) or dysbiotic (score >0) using the subgingival microbial dysbiosis index (Chen et al., 2021). (B) These participants are then followed-up over months or ideally years to measure both site specific and whole-mouth periodontal indices, with repeated subgingival plaque and GCF sampling to assess the interplay between host, microbial and clinical responses *in vivo*. (C) Simultaneously, healthy and dysbiotic subgingival plaque samples are grown *in vitro* and exposed to 2D or 3D gingival tissue models (Brown et al., 2019a, Mountcastle et al., 2020). This would identify possible drivers of inflammation and establish whether this response mimics those observed *in vivo*. Image created using BioRender.

7.1. References

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. & Dewhirst, F. E. 2005. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol,* 43**,** 5721-32.

Aberg, C. H., Kelk, P. & Johansson, A. 2015. Aggregatibacter actinomycetemcomitans: virulence of its leukotoxin and association with aggressive periodontitis. *Virulence,* 6**,** 188-95.

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., Gamonal, J. & Diaz, P. I. 2013. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME Journal,* 7**,** 1016-1025.

Abusleme, L., Hoare, A., Hong, B. Y. & Diaz, P. I. 2021. Microbial signatures of health, gingivitis, and periodontitis. *Periodontol 2000,* 86**,** 57-78.

Ainamo, J. & Bay, I. 1975. Problems and proposals for recording gingivitis and plaque. *International dental journal,* 25**,** 229-35.

Akobeng, A. K. 2007. Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta paediatrica,* 96**,** 644-647.

Al-Hashimi, I. & Levine, M. J. 1989. Characterization of in vivo salivary-derived enamel pellicle. *Arch Oral Biol,* 34**,** 289-95.

Albandar, J. M., Streckfus, C. F., Adesanya, M. R. & Winn, D. M. 2000. Cigar, pipe, and cigarette smoking as risk factors for periodontal disease and tooth loss. *J Periodontol,* 71**,** 1874-81.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J Mol Biol,* 215**,** 403-10.

American Academy of Periodontology 2011. Comprehensive periodontal therapy: a statement by the American Academy of Periodontology *. *J Periodontol,* 82**,** 943-9.

Ammons, W. F., Schectman, L. R. & Page, R. C. 1972. Host tissue response in chronic periodontal disease. 1. The normal periodontium and clinical manifestations of dental and periodontal disease in the marmoset. *J Periodontal Res,* 7**,** 131-43.

Anderson, J., Caplan, L., Yazdany, J., Robbins, M. L., Neogi, T., Michaud, K., Saag, K. G., O'dell, J. R. & Kazi, S. 2012. Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice. *Arthritis Care Res,* 64**,** 640-647.

Aoki, S., Hosomi, N., Nishi, H., Nakamori, M., Nezu, T., Shiga, Y., Kinoshita, N., Ueno, H., Ishikawa, K., Imamura, E., Shintani, T., Ohge, H., Kawaguchi, H., Kurihara, H. & Maruyama, H. 2020. Serum IgG titers to periodontal pathogens predict 3-month outcome in ischemic stroke patients. *PloS one,* 15.

Arabaci, T., Cicek, Y. & Canakci, C. F. 2007. Sonic and ultrasonic scalers in periodontal treatment: a review. *Int J Dent Hyg,* 5**,** 2-12.

Armitage, G. C. 1999. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol,* 4**,** 1-6.

Aruni, A. W., Dou, Y., Mishra, A. & Fletcher, H. M. 2015a. The Biofilm Community-Rebels with a Cause. *Curr Oral Health Rep,* 2**,** 48-56.

Aruni, A. W., Mishra, A., Dou, Y., Chioma, O., Hamilton, B. N. & Fletcher, H. M. 2015b. Filifactor alocis — a new emerging periodontal pathogen. *Microbes Infect,* 17**,** 517-30.

Awang, R., Lappin, D. F., Macpherson, A., Riggio, M., Robertson, D., Hodge, P., Ramage, G., Culshaw, S., Preshaw, P. M., Taylor, J. & Nile, C. 2014. Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. *Inflammation Research,* 63**,** 1001-1012.

Azuma, Y., Kaji, K., Katogi, R., Takeshita, S. & Kudo, A. 2000. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J Biol Chem,* 275**,** 4858-64.

Badersten, A., Nilveus, R. & Egelberg, J. 1984. Effect of nonsurgical periodontal therapy. II. Severely advanced periodontitis. *Journal of clinical periodontology,* 11.

Baehni, P., Thilo, B., Chapuis, B. & Pernet, D. 1992. Effects of ultrasonic and sonic sealers on dental plaque microflora in vitro and in vivo. *Journal of Clinical Periodontology,* 19**,** 455-459.

Baltacioglu, E., Kehribar, M. A., Yuva, P., Alver, A., Atagun, O. S., Karabulut, E. & Akalin, F. A. 2014. Total oxidant status and bone resorption biomarkers in serum and gingival crevicular fluid of patients with periodontitis. *J Periodontol,* 85**,** 317-26.

Bamashmous, S., Kotsakis, G. A., Kerns, K. A., Leroux, B. G., Zenobia, C., Chen, D., Trivedi, H. M., Mclean, J. S. & Darveau, R. P. 2021. Human variation in gingival inflammation. *Proc Natl Acad Sci U S A,* 118.

Barros, S. P., Williams, R., Offenbacher, S. & Morelli, T. 2016. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontol 2000,* 70**,** 53-64.

Bartold, P. M., Cantley, M. D. & Haynes, D. R. 2010. Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol 2000,* 53**,** 55-69.

Bartold, P. M. & Lopez-Oliva, I. 2020. Periodontitis and rheumatoid arthritis: An update 2012-2017. *Periodontol 2000,* 83**,** 189-212.

Baum, B. J., Yates, J. R., 3rd, Srivastava, S., Wong, D. T. & Melvin, J. E. 2011. Scientific frontiers: emerging technologies for salivary diagnostics. *Adv Dent Res,* 23**,** 360-8.

Beck, J. D., Koch, G. G., Rozier, R. G. & Tudor, G. E. 1990. Prevalence and risk indicators for periodontal attachment loss in a population of older community-dwelling blacks and whites. *J Periodontol,* 61**,** 521-8.

Beklen, A., Tuter, G., Sorsa, T., Hanemaaijer, R., Virtanen, I., Tervahartiala, T. & Konttinen, Y. T. 2006. Gingival tissue and crevicular fluid co-operation in adult periodontitis. *J Dent Res,* 85**,** 59-63.

Belibasakis, G. N. & Bostanci, N. 2012. The RANKL-OPG system in clinical periodontology. *J Clin Periodontol,* 39**,** 239-48.

Belstrom, D., Grande, M. A., Sembler-Moller, M. L., Kirkby, N., Cotton, S. L., Paster, B. J. & Holmstrup, P. 2018. Influence of periodontal treatment on subgingival and salivary microbiotas. *J Periodontol,* 89**,** 531-539.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological),* 57**,** 289-300.

Bernabe, E., Marcenes, W., Hernandez, C. R., Bailey, J., Abreu, L. G., Alipour, V., Amini, S., Arabloo, J., Arefi, Z., Arora, A., Ayanore, M. A., Barnighausen, T. W., Bijani, A., Cho, D. Y., Chu, D. T., Crowe, C. S., Demoz, G. T., Demsie, D. G., Dibaji Forooshani, Z. S., Du, M., El Tantawi, M., Fischer, F., Folayan, M. O., Futran, N. D., Geramo, Y. C. D., Haj-Mirzaian, A., Hariyani, N., Hasanzadeh, A., Hassanipour, S., Hay, S. I., Hole, M. K., Hostiuc, S., Ilic, M. D., James, S. L., Kalhor, R., Kemmer, L., Keramati, M., Khader, Y. S., Kisa, S., Kisa, A., Koyanagi, A., Lalloo, R., Le Nguyen, Q., London, S. D., Manohar, N. D., Massenburg, B. B., Mathur, M. R., Meles, H. G., Mestrovic, T., Mohammadian-Hafshejani, A., Mohammadpourhodki, R., Mokdad, A. H., Morrison, S. D., Nazari, J., Nguyen, T. H., Nguyen, C. T., Nixon, M. R., Olagunju, T. O., Pakshir, K., Pathak, M., Rabiee, N., Rafiei, A., Ramezanzadeh, K., Rios-Blancas, M. J., Roro, E. M., Sabour, S., Samy, A. M., Sawhney, M., Schwendicke, F., Shaahmadi, F., Shaikh, M. A., Stein, C., Tovani-Palone, M. R., Tran, B. X., Unnikrishnan, B., Vu, G. T., Vukovic, A., Warouw, T. S. S., Zaidi, Z., Zhang, Z. J. & Kassebaum, N. J. 2020. Global, Regional, and National Levels and

Trends in Burden of Oral Conditions from 1990 to 2017: A Systematic Analysis for the Global Burden of Disease 2017 Study. *J Dent Res*, 22034520908533.

Beukers, N. G., Van Der Heijden, G. J., Van Wijk, A. J. & Loos, B. G. 2017. Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands. *J Epidemiol Community Health,* 71, 37-42.

Bickel, M. & Cimasoni, G. 1985. The pH of human crevicular fluid measured by a new microanalytical technique. *J Periodontal Res,* 20, 35-40.

Bik, E. M., Long, C. D., Armitage, G. C., Loomer, P., Emerson, J., Mongodin, E. F., Nelson, K. E., Gill, S. R., Fraser-Liggett, C. M. & Relman, D. A. 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. *Isme j,* 4, 962-74.

Billings, F. 1930. Focal Infection as the Cause of General Disease. *Bull N Y Acad Med,* 6, 759-73.

Binshabaib, M., Alharthi, S. S., Akram, Z., Khan, J., Rahman, I., Romanos, G. E. & Javed, F. 2019. Clinical periodontal status and gingival crevicular fluid cytokine profile among cigarette-smokers, electronic-cigarette users and never-smokers. *Archives of oral biology,* 102.

Bizzaro, N., Bartoloni, E., Morozzi, G., Manganelli, S., Riccieri, V., Sabatini, P., Filippini, M., Tampoia, M., Afeltra, A., Sebastiani, G., Alpini, C., Bini, V., Bistoni, O., Alunno, A. & Gerli, R. 2013. Anti-cyclic citrullinated peptide antibody titer predicts time to rheumatoid arthritis onset in patients with undifferentiated arthritis: results from a 2-year prospective study. *Arthritis research & therapy,* 15.

Bizzarro, S., Laine, M. L., Buijs, M. J., Brandt, B. W., Crielaard, W., Loos, B. G. & Zaura, E. 2016. Microbial profiles at baseline and not the use of antibiotics determine the clinical outcome of the treatment of chronic periodontitis. *Scientific Reports,* 6.

Black, G. V. 1884. *The Formation of Poisons by Micro-organisms: A Biological Study of the Germ Theory of Disease*, P. Blakiston, son & Company.

Blicher, B., Joshipura, K. & Eke, P. 2005. Validation of self-reported periodontal disease: a systematic review. *J Dent Res,* 84, 881-90.

Bostanci, N. & Belibasakis, G. N. 2018. Gingival crevicular fluid and its immune mediators in the proteomic era. *Periodontol 2000,* 76, 68-84.

Boström, L., Linder, L. E. & Bergström, J. 1999. Smoking and crevicular fluid levels of IL-6 and TNF-alpha in periodontal disease. *Journal of clinical periodontology,* 26.

Bozbay, E., Dominici, F., Gokbuget, A. Y., Cintan, S., Guida, L., Aydin, M. S., Mariotti, A. & Pilloni, A. 2018. Preservation of root cementum: a comparative evaluation of power-driven versus hand instruments. *Int J Dent Hyg,* 16, 202-209.

Brayer, W. K., Mellonig, J. T., Dunlap, R. M., Marinak, K. W. & Carson, R. E. 1989. Scaling and root planing effectiveness: the effect of root surface access and operator experience. *J Periodontol,* 60, 67-72.

Brown, J. L., Johnston, W., Delaney, C., Rajendran, R., Butcher, J., Khan, S., Bradshaw, D., Ramage, G. & Culshaw, S. 2019a. Biofilm-stimulated epithelium modulates the inflammatory responses in co-cultured immune cells. *Scientific Reports,* 9, 15779-15779.

Brown, J. L., Johnston, W., Delaney, C., Short, B., Butcher, M. C., Young, T., Butcher, J., Riggio, M., Culshaw, S. & Ramage, G. 2019b. Polymicrobial oral biofilm models: simplifying the complex. *J Med Microbiol,* 68, 1573-1584.

Brull, D. J., Serrano, N., Zito, F., Jones, L., Montgomery, H. E., Rumley, A., Sharma, P., Lowe, G. D., World, M. J., Humphries, S. E. & Hingorani, A. D. 2003. Human CRP gene polymorphism influences CRP levels: implications for the prediction and pathogenesis of coronary heart disease. *Arterioscler Thromb Vasc Biol,* 23, 2063-9.

Buduneli, N., Biyikoğlu, B., Sherrabeh, S. & Lappin, D. F. 2008. Saliva concentrations of RANKL and osteoprotegerin in smoker versus non-smoker chronic periodontitis patients. *Journal of clinical periodontology,* 35.

Buduneli, N., Buduneli, E. & Kütükçüler, N. 2009. Interleukin-17, RANKL, and Osteoprotegerin Levels in Gingival Crevicular Fluid From Smoking and Non-Smoking Patients With Chronic Periodontitis During Initial Periodontal Treatment. *Journal of periodontology,* 80.

Buduneli, N. & Kinane, D. F. 2011. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *J Clin Periodontol,* 38 Suppl 11**,** 85-105.

Cai, Z., Lin, S., Hu, S. & Zhao, L. 2021. Structure and Function of Oral Microbial Community in Periodontitis Based on Integrated Data. *Front Cell Infect Microbiol,* 11**,** 663756.

Callahan, B. J., Mcmurdie, P. J. & Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J,* 11**,** 2639-2643.

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A. & Holmes, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods,* 13**,** 581-583.

Carda-Diéguez, M., Bravo-González, L. A., Morata, I. M., Vicente, A. & Mira, A. 2019. High-throughput DNA sequencing of microbiota at interproximal sites. *Journal of oral microbiology,* 12.

Caton, J., Armitage, G., Berglundh, T., Chapple, I., Jepsen, S., Kornman, K., Mealey, B., Papapanou, P., Sanz, M. & Tonetti, M. 2018. A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. *Journal of clinical periodontology,* 45 Suppl 20.

Cekici, A., Kantarci, A., Hasturk, H. & Van Dyke, T. E. 2014. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000,* 64**,** 57-80.

Chakravarti, A., Raquil, M. A., Tessier, P. & Poubelle, P. E. 2009. Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood,* 114**,** 1633-44.

Chakravorty, S., Helb, D., Burday, M., Connell, N. & Alland, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods,* 69**,** 330-9.

Chapple, I. L. C. 2014. Time to take periodontitis seriously. *BMJ,* 348**,** g2645.

Chapple, I. L. C., Mealey, B. L., Van Dyke, T. E., Bartold, P. M., Dommisch, H., Eickholz, P., Geisinger, M. L., Genco, R. J., Glogauer, M., Goldstein, M., Griffin, T. J., Holmstrup, P., Johnson, G. K., Kapila, Y., Lang, N. P., Meyle, J., Murakami, S., Plemons, J., Romito, G. A., Shapira, L., Tatakis, D. N., Teughels, W., Trombelli, L., Walter, C., Wimmer, G., Xenoudi, P. & Yoshie, H. 2018. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol,* 89 Suppl 1**,** S74-S84.

Chen, C., Hemme, C., Beleno, J., Shi, Z. J., Ning, D., Qin, Y., Tu, Q., Jorgensen, M., He, Z., Wu, L. & Zhou, J. 2018. Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME Journal,* 12**,** 1210-1224.

Chen, H. H., Huang, N., Chen, Y. M., Chen, T. J., Chou, P., Lee, Y. L., Chou, Y. J., Lan, J. L., Lai, K. L., Lin, C. H. & Chen, D. Y. 2013. Association between a history of periodontitis and the risk of rheumatoid arthritis: a nationwide, population-based, case-control study. *Ann Rheum Dis,* 72**,** 1206-11.

Chen, H. H., Liu, Y., Zhang, M., Wang, G., Qi, Z., Bridgewater, L., Zhao, L., Tang, Z. & Pang, X. 2015. A Filifactor alocis-centered co-occurrence group associates with periodontitis across different oral habitats. *Sci Rep,* 5.

Chen, T., Marsh, P. D. & Al-Hebshi, N. N. 2021. SMDI: An Index for Measuring Subgingival Microbial Dysbiosis. *J Dent Res*, 220345211035775.

Cheng, R., Wu, Z., Li, M., Shao, M. & Hu, T. 2020. Interleukin-1beta is a potential therapeutic target for periodontitis: a narrative review. *Int J Oral Sci,* 12**,** 2.

Cheng, Z., Do, T., Mankia, K., Meade, J., Hunt, L., Clerehugh, V., Speirs, A., Tugnait, A., Emery, P. & Devine, D. A. 2021. Dysbiosis in the oral microbiomes of anti-CCP positive individuals at risk of developing rheumatoid arthritis. *Ann Rheum Dis,* 80**,** 162-168.

Cheng, Z., Meade, J., Mankia, K., Emery, P. & Devine, D. A. 2017. Periodontal disease and periodontal bacteria as triggers for rheumatoid arthritis. *Best Pract Res Clin Rheumatol,* 31**,** 19-30.

Chong, J., Liu, P., Zhou, G. & Xia, J. 2020. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature protocols,* 15.

Christgau, M., Manner, T., Beuer, S., Hiller, K. A. & Schmalz, G. 2007. Periodontal healing after non-surgical therapy with a new ultrasonic device: a randomized controlled clinical trial. *J Clin Periodontol,* 34**,** 137-47.

Citterio, F., Gualini, G., Chang, M., Piccoli, G. M., Giraudi, M., Manavella, V., Baima, G., Mariani, G. M., Romano, F. & Aimetti, M. 2021. Pocket closure and residual pockets after non-surgical periodontal therapy. A systematic review and meta-analysis. *J Clin Periodontol*.

Claesson, M. J., Wang, Q., O'sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P. & O'toole, P. W. 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic acids research,* 38.

Claesson, R., Chiang, H. M., Lindholm, M., Hoglund Aberg, C., Haubek, D., Johansson, A. & Oscarsson, J. 2020. Characterization of Aggregatibacter actinomycetemcomitans Serotype b Strains with Five Different, Including Two Novel, Leukotoxin Promoter Structures. *Vaccines (Basel),* 8.

Copulos, T. A., Low, S. B., Walker, C. B., Trebilcock, Y. Y. & Hefti, A. F. 1993. Comparative Analysis Between a Modified Ultrasonic Tip and Hand Instruments on Clinical Parameters of Periodontal Disease. *Journal of Periodontology,* 64**,** 694-700.

Corbet, E. F., Ho, D. K. & Lai, S. M. 2009. Radiographs in periodontal disease diagnosis and management. *Aust Dent J,* 54 Suppl 1**,** S27-43.

Costerton, J. W., Irvin, R. T. & Cheng, K. J. 1981a. The bacterial glycocalyx in nature and disease. *Annu Rev Microbiol,* 35**,** 299-324.

Costerton, J. W., Irvin, R. T. & Cheng, K. J. 1981b. The role of bacterial surface structures in pathogenesis. *Crit Rev Microbiol,* 8**,** 303-38.

Crasta, K., Daly, C. G., Mitchell, D., Curtis, B., Stewart, D. & Heitz-Mayfield, L. J. A. 2009. Bacteraemia due to dental flossing. *Journal of Clinical Periodontology,* 36**,** 323-332.

Culshaw, S., Mcinnes, I. B. & Liew, F. Y. 2011. What can the periodontal community learn from the pathophysiology of rheumatoid arthritis? *J Clin Periodontol,* 38 Suppl 11**,** 106-13.

Curtis, J. & Bourne, F. J. 1973. Half-lives of immunoglobulins IgG, IgA and IgM in the serum of new-born pigs. *Immunology,* 24**,** 147-55.

Curtis, M. A., Diaz, P. I. & Van Dyke, T. E. 2020. The role of the microbiota in periodontal disease. *Periodontol 2000,* 83**,** 14-25.

Czesnikiewicz-Guzik, M., Osmenda, G., Siedlinski, M., Nosalski, R., Pelka, P., Nowakowski, D., Wilk, G., Mikolajczyk, T. P., Schramm-Luc, A., Furtak, A., Matusik, P., Koziol, J., Drozdz, M., Munoz-Aguilera, E., Tomaszewski, M.,

Evangelou, E., Caulfield, M., Grodzicki, T., D'aiuto, F. & Guzik, T. J. 2019. Causal association between periodontitis and hypertension: evidence from Mendelian randomization and a randomized controlled trial of non-surgical periodontal therapy. *Eur Heart J,* 40**,** 3459-3470.

D'aiuto, F., Casas, J. P., Shah, T., Humphries, S. E., Hingorani, A. D. & Tonetti, M. S. 2005. C-reactive protein (+1444C>T) polymorphism influences CRP response following a moderate inflammatory stimulus. *Atherosclerosis,* 179**,** 413-7.

D'aiuto, F., Gkranias, N., Bhowruth, D., Khan, T., Orlandi, M., Suvan, J., Masi, S., Tsakos, G., Hurel, S., Hingorani, A. D., Donos, N., Deanfield, J. E. & Group, T. 2018. Systemic effects of periodontitis treatment in patients with type 2 diabetes: a 12 month, single-centre, investigator-masked, randomised trial. *Lancet Diabetes Endocrinol,* 6**,** 954-965.

D'aiuto, F., Nibali, L., Mohamed-Ali, V., Vallance, P. & Tonetti, M. S. 2004a. Periodontal therapy: A novel non-drug-induced experimental model to study human inflammation. *Journal of Periodontal Research,* 39**,** 294-299.

D'aiuto, F., Parkar, M., Andreou, G., Suvan, J., Brett, P. M., Ready, D. & Tonetti, M. S. 2004b. Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *J Dent Res,* 83**,** 156-60.

Da Silva, M. K., De Carvalho, A. C. G., Alves, E. H. P., Da Silva, F. R. P., Pessoa, L. D. S. & Vasconcelos, D. F. P. 2017. Genetic Factors and the Risk of Periodontitis Development: Findings from a Systematic Review Composed of 13 Studies of Meta-Analysis with 71,531 Participants. *Int J Dent,* 2017**,** 1914073.

Dag, A., Firat, E. T., Arikan, S., Kadiroglu, A. K. & Kaplan, A. 2009. The effect of periodontal therapy on serum TNF-alpha and HbA1c levels in type 2 diabetic patients. *Aust Dent J,* 54**,** 17-22.

Damgaard, C., Danielsen, A. K., Enevold, C., Reinholdt, J., Holmstrup, P., Nielsen, C. H. & Massarenti, L. 2021. Circulating antibodies against leukotoxin A as marker of periodontitis grades B and C and oral infection with Aggregatibacter actinomycetemcomitans. *J Periodontol*.

Darcey, J. & Ashley, M. 2011. See you in three months! The rationale for the three monthly peridontal recall interval: a risk based approach. *British Dental Journal,* 211**,** 379-385.

Darrah, E. & Andrade, F. 2018. Rheumatoid arthritis and citrullination. *Curr Opin Rheumatol,* 30**,** 72-78.

Darveau, R. P. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol,* 8**,** 481-90.

De Cena, J. A., Zhang, J., Deng, D., Dame-Teixeira, N. & Do, T. 2021. Low-Abundant Microorganisms: The Human Microbiome's Dark Matter, a Scoping Review. *Front Cell Infect Microbiol,* 11**,** 689197.

De Mendonca, A. C., Maximo, M. B., Rodrigues, J. A., Arrais, C. A., De Freitas, P. M. & Duarte, P. M. 2008. Er:YAG Laser, ultrasonic system, and curette produce different profiles on dentine root surfaces: an in vitro study. *Photomed Laser Surg,* 26**,** 91-7.

De Oliveira, C., Watt, R. & Hamer, M. 2010. Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish Health Survey. *BMJ,* 340**,** c2451.

De Pablo, P., Dietrich, T. & Mcalindon, T. E. 2008. Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. *J Rheumatol,* 35**,** 70-6.

Deas, D. E., Mackey, S. A. & Mcdonnell, H. T. 2003. Systemic disease and periodontitis: manifestations of neutrophil dysfunction. *Periodontol 2000,* 32**,** 82-104.

Delpech, P., M, A. L., Jones, B., Gemmell, C. & Lang, S. 2018. An in vitro evaluation of the efficacy of tedizolid: implications for the treatment of skin and soft tissue infections. *Diagn Microbiol Infect Dis,* 91**,** 93-97.

Derdilopoulou, F. V., Nonhoff, J., Neumann, K. & Kielbassa, A. M. 2007. Microbiological findings after periodontal therapy using curettes, Er:YAG laser, sonic, and ultrasonic scalers. *J Clin Periodontol,* 34**,** 588-98.

Devine, D. A., Marsh, P. D. & Meade, J. 2015. Modulation of host responses by oral commensal bacteria. *J Oral Microbiol,* 7**,** 26941.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A. & Wade, W. G. 2010. The human oral microbiome. *J Bacteriol,* 192**,** 5002-17.

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B. & Xia, J. 2017. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research,* 45**,** 180-188.

Dietrich, T., Jimenez, M., Krall Kaye, E. A., Vokonas, P. S. & Garcia, R. I. 2008. Age-dependent associations between chronic periodontitis/edentulism and risk of coronary heart disease. *Circulation,* 117**,** 1668-74.

Dietrich, T., Ower, P., Tank, M., West, N. X., Walter, C., Needleman, I., Hughes, F. J., Wadia, R., Milward, M. R., Hodge, P. J., Chapple, I. L. C. & British Society of, P. 2019. Periodontal diagnosis in the context of the 2017 classification system of periodontal diseases and conditions – implementation in clinical practice. *BDJ,* 226**,** 16-22.

Dietrich, T., Sharma, P., Walter, C., Weston, P. & Beck, J. 2013. The epidemiological evidence behind the association between periodontitis and incident atherosclerotic cardiovascular disease. *J Periodontol,* 84**,** S70-84.

Divaris, K., Monda, K. L., North, K. E., Olshan, A. F., Lange, E. M., Moss, K., Barros, S. P., Beck, J. D. & Offenbacher, S. 2012. Genome-wide association study of periodontal pathogen colonization. *Journal of dental research,* 91.

Do, T., Devine, D. & Marsh, P. D. 2013. Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. *Clin Cosmet Investig Dent,* 5**,** 11-9.

Dominy, S. S., Lynch, C., Ermini, F., Benedyk, M., Marczyk, A., Konradi, A., Nguyen, M., Haditsch, U., Raha, D., Griffin, C., Holsinger, L. J., Arastu-Kapur, S., Kaba, S., Lee, A., Ryder, M. I., Potempa, B., Mydel, P., Hellvard, A., Adamowicz, K., Hasturk, H., Walker, G. D., Reynolds, E. C., Faull, R. L. M., Curtis, M. A., Dragunow, M. & Potempa, J. 2019. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci Adv,* 5.

Dommisch, H., Staufenbiel, I., Schulze, K., Stiesch, M., Winkel, A., Fimmers, R., Dommisch, J., Jepsen, S., Miosge, N., Adam, K. & Eberhard, J. 2015. Expression of antimicrobial peptides and interleukin-8 during early stages of inflammation: An experimental gingivitis study. *J Periodontal Res,* 50**,** 836-45.

Dragoo, M. R. 1992. A clinical evaluation of hand and ultrasonic instruments on subgingival debridement. Part I. With unmodified and modified ultrasonic inserts. *International Journal of Periodontics and Restorative Dentistry,* 12**,** 310-323.

Du Clos, T. W. 2000. Function of C-reactive protein. *Ann Med,* 32**,** 274-8.

Duarte, P. M., Feres, M., Yassine, L. L. S., Soares, G. M. S., Miranda, T. S., Faveri, M., Retamal-Valdes, B. & Figueiredo, L. C. 2018. Clinical and microbiological effects of scaling and root planing, metronidazole and amoxicillin in the treatment of diabetic and non-diabetic subjects with periodontitis: A cohort study. *Journal of clinical periodontology,* 45.

Dupont, N. C., Wang, K., Wadhwa, P. D., Culhane, J. F. & Nelson, E. L. 2005. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: determinations of a panel of nine cytokines in clinical sample culture supernatants. *Journal of reproductive immunology,* 66.

Dzidic, M., Collado, M. C., Abrahamsson, T., Artacho, A., Stensson, M., Jenmalm, M. C. & Mira, A. 2018. Oral microbiome development during childhood: an ecological succession influenced by postnatal factors and associated with tooth decay. *The ISME journal,* 12.

Ebersole, J. L., Machen, R. L., Steffen, M. J. & Willmann, D. E. 1997. Systemic acute-phase reactants, C-reactive protein and haptoglobin, in adult periodontitis. *Clinical and Experimental Immunology,* 107**,** 347-352.

Ebersole, J. L., Nagarajan, R., Akers, D. & Miller, C. S. 2015. Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Frontiers in Cellular and Infection Microbiology,* 5.

Eick, S., Mathey, A., Vollroth, K., Kramesberger, M., Bürgin, W., Sculean, A., Ramseier, C. & Jentsch, H. 2017. Persistence of Porphyromonas gingivalis is a negative predictor in patients with moderate to severe periodontitis after nonsurgical periodontal therapy. *Clinical oral investigations,* 21.

Erciyas, K., Sezer, U., Ustun, K., Pehlivan, Y., Kisacik, B., Senyurt, S. Z., Tarakcioglu, M. & Onat, A. M. 2013. Effects of periodontal therapy on disease activity and systemic inflammation in rheumatoid arthritis patients. *Oral Dis,* 19**,** 394-400.

European Federation of Periodontology 2021. Time to take gum disease seriously: The societal and economic impact of periodontitis. *In:* Bishop, C. (ed.) *Appendix I: Country Profiles.* The Economist.

Fang, H., Han, M., Li, Q. L., Cao, C. Y., Xia, R. & Zhang, Z. H. 2016. Comparison of full-mouth disinfection and quadrant-wise scaling in the treatment of adult chronic periodontitis: a systematic review and meta-analysis. *J Periodontal Res,* 51**,** 417-30.

Farquharson, D., Butcher, J. P. & Culshaw, S. 2012. Periodontitis, Porphyromonas, and the pathogenesis of rheumatoid arthritis. *Mucosal Immunology,* 5**,** 112-120.

Faust, K. & Raes, J. 2016. CoNet app: inference of biological association networks using Cytoscape. *F1000Research,* 5**,** 1519-1519.

Fawcett, T. 2006. An introduction to ROC analysis. *Pattern Recognition Letters,* 27**,** 861-874.

Feres, M., Louzoun, Y., Haber, S., Faveri, M., Figueiredo, L. C. & Levin, L. 2018. Support vector machine-based differentiation between aggressive and chronic periodontitis using microbial profiles. *International dental journal,* 68.

Feres, M., Retamal-Valdes, B., Faveri, M., Duarte, P., Shibli, J., Soares, G. M. S., Miranda, T., Teles, F., Goodson, M., Hasturk, H., Van Dyke, T., Ehmke, B., Eickholz, P., Schlagenhauf, U., Meyle, J., Koch, R., Kocher, T., Hoffmann, T., Kim, T. S., Kaner, D., Figueiredo, L. C. & Doyle, H. 2020. Proposal of a Clinical Endpoint for Periodontal Trials: The Treat-to-Target Approach. *Journal of the International Academy of Periodontology,* 22.

Ferrer, M. D., Lopez-Lopez, A., Nicolescu, T., Perez-Vilaplana, S., Boix-Amoros, A., Dzidic, M., Garcia, S., Artacho, A., Llena, C. & Mira, A. 2020. Topic Application of the Probiotic Streptococcus dentisani Improves Clinical and Microbiological Parameters Associated With Oral Health. *Front Cell Infect Microbiol,* 10**,** 465.

Fine, D. H., Morris, M. L., Tabak, L. & Cole, J. D. 1980. Preliminary characterization of material eluted from the roots of periodontally diseased teeth. *J Periodontal Res,* 15**,** 10-9.

Fisher, B. A., Cartwright, A. J., Quirke, A. M., De Pablo, P., Romaguera, D., Panico, S., Mattiello, A., Gavrila, D., Navarro, C., Sacerdote, C., Vineis, P., Tumino, R., Lappin, D. F., Apazidou, D., Culshaw, S., Potempa, J., Michaud, D. S., Riboli, E. & Venables, P. J. 2015. Smoking, Porphyromonas gingivalis and the immune response to citrullinated autoantigens before the clinical onset of rheumatoid arthritis in a Southern European nested case-control study Clinical rheumatology and osteoporosis. *BMC Musculoskeletal Disorders,* 16.

Fitzsimmons, T. R., Sanders, A. E., Bartold, P. M. & Slade, G. D. 2010. Local and systemic biomarkers in gingival crevicular fluid increase odds of periodontitis. *Journal of clinical periodontology,* 37.

Forner, L., Larsen, T., Kilian, M. & Holmstrup, P. 2006. Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *Journal of Clinical Periodontology,* 33**,** 401-407.

Fragkioudakis, I., Riggio, M. P. & Apatzidou, D. A. 2021. Understanding the microbial components of periodontal diseases and periodontal treatment-induced microbiological shifts. *J Med Microbiol,* 70.

Gadekar, N. B., Hosmani, J. V., Bhat, K. G., Kotrashetti, V. S., Nayak, R. S., Babji, D. V., Pattanshetty, S. M., Joshi, V. M. & Bansode, R. A. 2018. Detection of antibodies against Aggregatibacter actinomycetemcomitans in serum and saliva through ELISA in periodontally healthy individuals and individuals with chronic periodontitis. *Microbial Pathogenesis,* 125**,** 438-442.

Ganesan, S. M., Dabdoub, S. M., Nagaraja, H. N., Scott, M. L., Pamulapati, S., Berman, M. L., Shields, P. G., Wewers, M. E. & Kumar, P. S. 2020. Adverse effects of electronic cigarettes on the disease-naive oral microbiome. *Sci Adv,* 6**,** eaaz0108.

Gellen, L. S., Wall-Manning, G. M. & Sissons, C. H. 2007. Checkerboard DNA-DNA hybridization technology using digoxigenin detection. *Methods Mol Biol,* 353**,** 39-67.

Genco, R. J. & Van Dyke, T. E. 2010. Prevention: Reducing the risk of CVD in patients with periodontitis. *Nat Rev Cardiol,* 7**,** 479-80.

Global Burden of Disease. 2017. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet,* 390**,** 1211-1259.

Gonzalez-Febles, J., Rodriguez-Lozano, B., Sanchez-Piedra, C., Garnier-Rodriguez, J., Bustabad, S., Hernandez-Gonzalez, M., Gonzalez-Davila, E., Sanz, M. & Diaz-Gonzalez, F. 2020. Association between periodontitis and anti-citrullinated protein antibodies in rheumatoid arthritis patients: a cross-sectional study. *Arthritis Res Ther,* 22**,** 27.

González, I., Cao, K. A., Davis, M. J. & Déjean, S. 2012. Visualising Associations Between Paired 'Omics' Data Sets. *BioData mining,* 5.

Gorr, S. U. 2009. Antimicrobial peptides of the oral cavity. *Periodontol 2000,* 51**,** 152-80.

Grande, M. A., Belstrøm, D., Damgaard, C., Holmstrup, P., Thangaraj, S. S., Nielsen, C. H. & Palarasah, Y. 2020. Complement split product C3c in saliva as biomarker for periodontitis and response to periodontal treatment. *Journal of periodontal research*.

Granger, D. A., Kivlighan, K. T., Fortunato, C., Harmon, A. G., Hibel, L. C., Schwartz, E. B. & Whembolua, G. L. 2007. Integration of salivary biomarkers into developmental and behaviorally-oriented research: problems and solutions for collecting specimens. *Physiol Behav,* 92**,** 583-90.

Graves, D. T. & Cochran, D. 2003. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol,* 74**,** 391-401.

Graziani, F., Cei, S., Orlandi, M., Gennai, S., Gabriele, M., Filice, N., Nisi, M. & D'aiuto, F. 2015. Acute-phase response following full-mouth versus quadrant non-surgical periodontal treatment: A randomized clinical trial. *Journal of Clinical Periodontology,* 42**,** 843-852.

Graziani, F., Cei, S., Tonetti, M., Paolantonio, M., Serio, R., Sammartino, G., Gabriele, M. & D'aiuto, F. 2010. Systemic inflammation following non-surgical and surgical periodontal therapy. *Journal of Clinical Periodontology,* 37**,** 848-854.

Greenwood, D., Afacan, B., Emingil, G., Bostanci, N. & Belibasakis, G. N. 2020. Salivary Microbiome Shifts in Response to Periodontal Treatment Outcome. *Proteomics. Clinical applications,* 14.

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., Podar, M. & Leys, E. J. 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME Journal,* 6**,** 1176-1185.

Griffen, A. L., Becker, M. R., Lyons, S. R., Moeschberger, M. L. & Leys, E. J. 1998. Prevalence of Porphyromonas gingivalis and periodontal health status. *Journal of Clinical Microbiology,* 36**,** 3239-3242.

Grossi, S. G., Genco, R. J., Machtei, E. E., Ho, A. W., Koch, G., Dunford, R., Zambon, J. J. & Hausmann, E. 1995. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. *J Periodontol,* 66**,** 23-9.

Grossi, S. G., Zambon, J. J., Ho, A. W., Koch, G., Dunford, R. G., Machtei, E. E., Norderyd, O. M. & Genco, R. J. 1994. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol,* 65**,** 260-7.

Guentsch, A., Kramesberger, M., Sroka, A., Pfister, W., Potempa, J. & Eick, S. 2011. Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. *J Periodontol,* 82**,** 1051-60.

Guo, Q., Wang, Y., Xu, D., Nossent, J., Pavlos, N. J. & Xu, J. 2018. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. *Bone research,* 6.

Gupta, V. K., Paul, S. & Dutta, C. 2017. Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Front Microbiol,* 8**,** 1162.

Gursoy, U. K., Kononen, E., Uitto, V. J., Pussinen, P. J., Hyvarinen, K., Suominen-Taipale, L. & Knuuttila, M. 2009. Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. *J Clin Periodontol,* 36**,** 922-7.

Haffajee, A. D., Cugini, M. A., Dibart, S., Smith, C., Kent, R. L., Jr. & Socransky, S. S. 1997. The effect of SRP on the clinical and microbiological parameters of periodontal diseases. *J Clin Periodontol,* 24**,** 324-34.

Haffajee, A. D. & Socransky, S. S. 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000,* 5**,** 78-111.

Haffajee, A. D. & Socransky, S. S. 2001. Relationship of cigarette smoking to the subgingival microbiota. *J Clin Periodontol,* 28**,** 377-88.

Haffajee, A. D., Socransky, S. S., Patel, M. R. & Song, X. 2008. Microbial complexes in supragingival plaque. *Oral Microbiology and Immunology,* 23**,** 196-205.

Hagi, T. T., Klemensberger, S., Bereiter, R., Nietzsche, S., Cosgarea, R., Flury, S., Lussi, A., Sculean, A. & Eick, S. 2015. A Biofilm Pocket Model to Evaluate Different Non-Surgical Periodontal Treatment Modalities in Terms of Biofilm Removal and Reformation, Surface Alterations and Attachment of Periodontal Ligament Fibroblasts. *PLoS One,* 10**,** e0131056.

Hajishengallis, G. & Chavakis, T. 2021. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nat Rev Immunol,* 21**,** 426-440.

Hajishengallis, G., Darveau, R. P. & Curtis, M. A. 2012. The keystone-pathogen hypothesis. *Nature Reviews Microbiology,* 10**,** 717-725.

Hajishengallis, G. & Korostoff, J. M. 2017. Revisiting the Page & Schroeder model: the good, the bad and the unknowns in the periodontal host response 40 years later. *Periodontol 2000,* 75**,** 116-151.

Hajishengallis, G. & Lamont, R. J. 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol,* 27**,** 409-19.

Hajishengallis, G., Liang, S., Payne, M. A., Hashim, A., Jotwani, R., Eskan, M. A., Mcintosh, M. L., Alsam, A., Kirkwood, K. L., Lambris, J. D., Darveau, R. P. & Curtis, M. A. 2011. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host and Microbe,* 10**,** 497-506.

Hammer, O., Harper, D. A. T. & Ryan, P. D. 2001. PAST: Paleontological statistics software package for education and data analysis.

Hannig, C. & Hannig, M. 2009. The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man. *Clin Oral Investig,* 13**,** 123-39.

Hartenbach, F., Silva-Boghossian, C. M. & Colombo, A. P. V. 2018. The effect of supragingival biofilm re-development on the subgingival microbiota in chronic periodontitis. *Arch Oral Biol,* 85**,** 51-57.

Heitz-Mayfield, L. J. A., Trombelli, L., Heitz, F., Needleman, I. & Moles, D. 2003. A systematic review of the effect of surgical debridement vs. non-surgical debridement for the treatment of chronic periodontitis. *Journal of Clinical Periodontology,* 29**,** 92-102.

Herrera, B. S., Bastos, A. S., Coimbra, L. S., Teixeira, S. A., Rossa, C., Jr., Van Dyke, T. E., Muscara, M. N. & Spolidorio, L. C. 2014. Peripheral blood mononuclear phagocytes from patients with chronic periodontitis are primed for osteoclast formation. *J Periodontol,* 85**,** e72-81.

Hodzic, J., Gurbeta, L., Omanovic-Miklicanin, E. & Badnjevic, A. 2017. Overview of Next-generation Sequencing Platforms Used in Published Draft Plant Genomes in Light of Genotypization of Immortelle Plant (Helichrysium Arenarium). *Med Arch,* 71**,** 288-292.

Hong, B. Y., Furtado Araujo, M. V., Strausbaugh, L. D., Terzi, E., Ioannidou, E. & Diaz, P. I. 2015. Microbiome profiles in periodontitis in relation to host and disease characteristics. *PLoS One,* 10**,** e0127077.

Horibe, M., Watanabe, H. & Ishikawa, I. 1995. Effect of periodontal treatments on serum IgG antibody titers against periodontopathic bacteria. *J Clin Periodontol,* 22**,** 510-5.

Horliana, A. C. R. T., Chambrone, L., Foz, A. M., Artese, H. P. C., De Rabelo, M. S., Pannuti, C. M. & Romito, G. A. 2014. Dissemination of periodontal pathogens in the bloodstream after periodontal procedures: A systematic review.

Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H. & Matsuo, T. 2006. Cytokines differentially regulate ICAM-1 and VCAM-1 expression on human gingival fibroblasts. *Clin Exp Immunol,* 144**,** 494-502.

Huang, R., Li, M. & Gregory, R. L. 2011. Bacterial interactions in dental biofilm. *Virulence,* 2**,** 435-44.

Hughes, F. J., Syed, M., Koshy, B., Marinho, V., Bostanci, N., Mckay, I. J., Curtis, M. A., Croucher, R. E. & Marcenes, W. 2006. Prognostic factors in the treatment of generalized aggressive periodontitis: I. Clinical features and initial outcome. *Journal of clinical periodontology,* 33.

Hujoel, P. P. 1994. A meta-analysis of normal ranges for root surface areas of the permanent dentition. *Journal of clinical periodontology,* 21.

Hujoel, P. P., White, B. A., García, R. I. & Listgarten, M. A. 2001. The dentogingival epithelial surface area revisited. *Journal of periodontal research,* 36.

Human Oral Microbiome Database 2016. HOMD: Human Oral Microbiome Database.

Hunter, W. 1900. Oral Sepsis as a Cause of Disease. *Br Med J,* 2**,** 215-6.

Huynh, N. C., Everts, V., Pavasant, P. & Ampornaramveth, R. S. 2017. Interleukin-1beta induces human cementoblasts to support osteoclastogenesis. *Int J Oral Sci,* 9**,** e5.

Hyde, E. R., Andrade, F., Vaksman, Z., Parthasarathy, K., Jiang, H., Parthasarathy, D. K., Torregrossa, A. C., Tribble, G., Kaplan, H. B., Petrosino, J. F. & Bryan, N. S. 2014. Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: Implications for nitric oxide homeostasis. *PLoS ONE,* 9.

Illumina. 2013. *16S Metagenomic Sequencing Library Preparation* [Online]. Available: https://emea.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Ioannou, I., Dimitriadis, N., Papadimitriou, K., Sakellari, D., Vouros, I. & Konstantinidis, A. 2009. Hand instrumentation versus ultrasonic debridement in the treatment of

chronic periodontitis: A randomized clinical and microbiological trial. *Journal of Clinical Periodontology,* 36**,** 132-141.

Irwin, C. R. & Myrillas, T. T. 1998. The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis,* 4**,** 43-7.

Isola, G., Polizzi, A., Patini, R., Ferlito, S., Alibrandi, A. & Palazzo, G. 2020. Association among serum and salivary A. actinomycetemcomitans specific immunoglobulin antibodies and periodontitis. *BMC Oral Health,* 20**,** 283.

Jaedicke, K. M., Preshaw, P. M. & Taylor, J. J. 2016. Salivary cytokines as biomarkers of periodontal diseases. 70**,** 164-183.

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M. & Kamil, M. A. 2018. Bacterial biofilm and associated infections. *J Chin Med Assoc,* 81**,** 7-11.

Jiang, Y., Zhou, X., Cheng, L. & Li, M. 2020. The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease. *Front Microbiol,* 11**,** 66.

Jin, L. J., Lamster, I. B., Greenspan, J. S., Pitts, N. B., Scully, C. & Warnakulasuriya, S. 2016. Global burden of oral diseases: emerging concepts, management and interplay with systemic health. *Oral Dis,* 22**,** 609-19.

Jin, W. & Dong, C. 2013. IL-17 cytokines in immunity and inflammation. *Emerg Microbes Infect*.

Jockel-Schneider, Y., Schlagenhauf, U., Stolzel, P., Gossner, S., Carle, R., Ehmke, B., Prior, K. & Hagenfeld, D. 2021. Nitrate-rich diet alters the composition of the oral microbiota in periodontal recall patients. *J Periodontol*.

Johansson, L., Sherina, N., Kharlamova, N., Potempa, B., Larsson, B., Israelsson, L., Potempa, J., Rantapaa-Dahlqvist, S. & Lundberg, K. 2016. Concentration of antibodies against Porphyromonas gingivalis is increased before the onset of symptoms of rheumatoid arthritis. *Arthritis Res Ther,* 18**,** 201.

Johnson, R. B., Wood, N. & Serio, F. G. 2004. Interleukin-11 and IL-17 and the pathogenesis of periodontal disease. *J Periodontol,* 75**,** 37-43.

Kassebaum, N. J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C. J. L. & Marcenes, W. 2014. Global Burden of Severe Periodontitis in 1990-2010. *Journal of Dental Research,* 93**,** 1045-1053.

Kaushik, R., Yeltiwar, R. K. & Pushpanshu, K. 2011. Salivary interleukin-1beta levels in patients with chronic periodontitis before and after periodontal phase I therapy and healthy controls: a case-control study. *J Periodontol,* 82**,** 1353-9.

Kinane, D. F. & Papageorgakopoulos, G. 2008. Full mouth disinfection versus quadrant debridement: the clinician's choice. *J Int Acad Periodontol,* 10**,** 6-9.

Kinane, D. F., Preshaw, P. M., Loos, B. G. & Working Group 2 of Seventh European Workshop on, P. 2011. Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions--consensus of the Seventh European Workshop on Periodontology. *J Clin Periodontol,* 38 Suppl 11**,** 44-8.

Kinane, D. F., Riggio, M. P., Walker, K. F., Mackenzie, D. & Shearer, B. 2005. Bacteraemia following periodontal procedures. *Journal of Clinical Periodontology,* 32**,** 708-713.

Kinney, J. S., Morelli, T., Oh, M., Braun, T. M., Ramseier, C. A., Sugai, J. V. & Giannobile, W. V. 2014. Crevicular fluid biomarkers and periodontal disease progression. *Journal of clinical periodontology,* 41.

Kirst, M. E., Li, E. C., Alfant, B., Chi, Y. Y., Walker, C., Magnusson, I. & Wang, G. P. 2015. Dysbiosis and alterations in predicted functions of the subgingival microbiome in chronic periodontitis. *Appl Environ Microbiol,* 81**,** 783-93.

Kocher, T., Rosin, M., Langenbeck, N. & Bernhardt, O. 2001. Subgingival polishing with a teflon-coated sonic scaler insert in comparison to conventional instruments as assessed on extracted teeth (II). Subgingival roughness. *J Clin Periodontol,* 28**,** 723-9.

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S. & Palmer, R. J., Jr. 2002. Communication among oral bacteria. *Microbiol Mol Biol Rev,* 66**,** 486-505, table of contents.

Konig, M. F., Abusleme, L., Reinholdt, J., Palmer, R. J., Teles, R. P., Sampson, K., Rosen, A., Nigrovic, P. A., Sokolove, J., Giles, J. T., Moutsopoulos, N. M. & Andrade, F. 2016. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. *Science translational medicine,* 8.

Koshy, G., Kawashima, Y., Kiji, M., Nitta, H., Umeda, M., Nagasawa, T. & Ishikawa, I. 2005. Effects of single-visit full-mouth ultrasonic debridement versus quadrant-wise ultrasonic debridement. *J Clin Periodontol,* 32**,** 734-43.

Kotake, S., Udagawa, N., Takahashi, N., Matsuzaki, K., Itoh, K., Ishiyama, S., Saito, S., Inoue, K., Kamatani, N., Gillespie, M. T., Martin, T. J. & Suda, T. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest,* 103**,** 1345-52.

Kowashi, Y., Jaccard, F. & Cimasoni, G. 1980. Sulcular polymorphonuclear leucocytes and gingival exudate during experimental gingivitis in man. *J Periodontal Res,* 15**,** 151-8.

Krishna, R. & De Stefano, J. A. 2016. Ultrasonic vs. hand instrumentation in periodontal therapy: Clinical outcomes.

Kudo, C., Naruishi, K., Maeda, H., Abiko, Y., Hino, T., Iwata, M., Mitsuhashi, C., Murakami, S., Nagasawa, T., Nagata, T., Yoneda, S., Nomura, Y., Noguchi, T., Numabe, Y., Ogata, Y., Sato, T., Shimauchi, H., Yamazaki, K., Yoshimura, A. & Takashiba, S. 2012. Assessment of the plasma/serum IgG test to screen for periodontitis. *J Dent Res,* 91**,** 1190-5.

Kumar, P. S. 2017. From focal sepsis to periodontal medicine: a century of exploring the role of the oral microbiome in systemic disease. *J Physiol,* 595**,** 465-476.

Lafaurie, G. I., Mayorga-Fayad, I., Torres, M. F., Castillo, D. M., Aya, M. R., Barón, A. & Hurtado, P. A. 2007. Periodontopathic microorganisms in peripheric blood after scaling and root planing. *Journal of Clinical Periodontology,* 34**,** 873-879.

Lamont, R. J. & Hajishengallis, G. 2015. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med,* 21**,** 172-83.

Lamont, R. J., Koo, H. & Hajishengallis, G. 2018. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol,* 16**,** 745-759.

Lang, N. P. & Bartold, P. M. 2018. Periodontal health. *J Periodontol,* 89 Suppl 1**,** S9-S16.

Lang, N. P., Bartold, P. M., Cullinan, M., Jeffcoat, M., Mombelli, A., Murakami, S., Page, R., Papapanou, P., Tonetti, M. & Van Dyke, T. 1999. Consensus Report: Aggressive Periodontitis. *Annals of Periodontology,* 4**,** 53-53.

Lang, N. P. & Lindhe, J. 2015. *Clinical Periodontology and Implant Dentistry*, Wiley-Blackwell.

Lang, N. P., Tan, W. C., Krahenmann, M. A. & Zwahlen, M. 2008. A systematic review of the effects of full-mouth debridement with and without antiseptics in patients with chronic periodontitis. *J Clin Periodontol,* 35**,** 8-21.

Lang, N. P. & Tonetti, M. S. 2003. Periodontal risk assessment (PRA) for patients in supportive periodontal therapy (SPT). *Oral health & preventive dentistry,* 1.

Langmead, B. & Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods,* 9**,** 357-9.

Lappin, D. F., Apatzidou, D., Quirke, A. M., Oliver-Bell, J., Butcher, J. P., Kinane, D. F., Riggio, M. P., Venables, P., Mcinnes, I. B. & Culshaw, S. 2013. Influence of periodontal disease, Porphyromonas gingivalis and cigarette smoking on systemic anti-citrullinated peptide antibody titres. *Journal of Clinical Periodontology,* 40**,** 907-915.

Lee, C. H., Chen, Y. W., Tu, Y. K., Wu, Y. C. & Chang, P. C. 2018. The potential of salivary biomarkers for predicting the sensitivity and monitoring the response to nonsurgical periodontal therapy: A preliminary assessment. *Journal of periodontal research,* 53.

Lee, J., Taneja, V. & Vassallo, R. 2012. Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res,* 91**,** 142-9.

Lee, Y. H. & Wong, D. T. 2009. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent,* 22**,** 241-8.

Leira, Y., Martin-Lancharro, P. & Blanco, J. 2018. Periodontal inflamed surface area and periodontal case definition classification. *Acta Odontol Scand,* 76**,** 195-198.

Lew, P. H., Rahman, M. T., Safii, S. H., Baharuddin, N. A., Bartold, P. M., Sockalingam, S., Kassim, N. L. A. & Vaithilingam, R. D. 2021. Antibodies against citrullinated proteins in relation to periodontitis with or without rheumatoid arthritis: a cross-sectional study. *BMC Oral Health,* 21**,** 360.

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S. & Oppenheim, F. G. 2004. Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol,* 97**,** 1311-8.

Li, J., Quinque, D., Horz, H. P., Li, M., Rzhetskaya, M., Raff, J. A., Hayes, M. G. & Stoneking, M. 2014. Comparative analysis of the human saliva microbiome from different climate zones: Alaska, Germany, and Africa. *BMC Microbiol,* 14**,** 316.

Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. & Fouser, L. A. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med,* 203**,** 2271-9.

Lin, H. & Peddada, S. D. 2020. Analysis of microbial compositions: a review of normalization and differential abundance analysis. *NPJ Biofilms Microbiomes,* 6**,** 60.

Lindh, L., Aroonsang, W., Sotres, J. & Arnebrant, T. 2014. Salivary pellicles. *Monogr Oral Sci,* 24**,** 30-9.

Lindhe, J., Ranney, R., Lamster, I., Charles, A., Chung, C.-P., Flemmig, T., Kinane, D., Listgarten, M., Löe, H., Schoor, R., Seymour, G. & Somerman, M. 1999. Consensus Report: Chronic Periodontitis. *Annals of Periodontology,* 4**,** 38-38.

Lindsay, D. & Von Holy, A. 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. *J Hosp Infect,* 64**,** 313-25.

Listgarten, M. A. 1976. Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. *J Periodontol,* 47**,** 1-18.

Listl, S., Galloway, J., Mossey, P. A. & Marcenes, W. 2015. Global Economic Impact of Dental Diseases. *J Dent Res,* 94**,** 1355-61.

Liu, Y. C., Lerner, U. H. & Teng, Y. T. 2010. Cytokine responses against periodontal infection: protective and destructive roles. *Periodontol 2000,* 52**,** 163-206.

Liukkonen, J., Gürsoy, U. K., Pussinen, P. J., Suominen, A. L. & Könönen, E. 2016. Salivary Concentrations of Interleukin (IL)-1ß, IL-17A, and IL-23 Vary in Relation to Periodontal Status. *Journal of periodontology,* 87.

Loesche, W. J. 1976. Chemotherapy of dental plaque infections. *Oral Sci Rev,* 9**,** 65-107.

Loesche, W. J. 1996. Microbiology of Dental Decay and Periodontal Disease. *In:* Baron, S. (ed.) *Medical Microbiology*. 4th ed. Galveston: University of Texas Medical Branch at Galveston.

Loesche, W. J. & Nafe, D. 1973. Reduction of supragingival plaque accumulations in institutionalized Down's syndrome patients by periodic treatment with topical kanamycin. *Arch Oral Biol,* 18**,** 1131-43.

Loomer, P. M. 2004. Microbiological diagnostic testing in the treatment of periodontal diseases. *Periodontol 2000,* 34**,** 49-56.

Loos, B. G., Craandijk, J., Hoek, F. J., Wertheim-Van Dillen, P. M. & Van Der Velden, U. 2000. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *J Periodontol,* 71**,** 1528-34.

Lopez-Oliva Santa Cruz, I. 2018. *Rheumatoid arthritis and periodontitis: antibody response, oral microbiome, cytokine profile and effect of periodontal treatment*. Ph.D., University of Birmingham.

Lopez-Oliva Santa Cruz, I., Paropkari, A. D., Saraswat, S., Serban, S., Yonel, Z., Sharma, P., De Pablo, P., Raza, K., Filer, A., Chapple, I., Dietrich, T., Grant, M. M. & Kumar, P. S. 2018. Dysbiotic Subgingival Microbial Communities in Periodontally Healthy Patients With Rheumatoid Arthritis. *Arthritis Rheumatol,* 70**,** 1008-1013.

Loutan, L., Alpizar-Rodriguez, D., Courvoisier, D. S., Finckh, A., Mombelli, A. & Giannopoulou, C. 2019. Periodontal status correlates with anti-citrullinated protein antibodies in first-degree relatives of individuals with rheumatoid arthritis. *Journal of clinical periodontology,* 46.

Lundberg, K., Wegner, N., Yucel-Lindberg, T. & Venables, P. J. 2010. Periodontitis in RA-the citrullinated enolase connection. *Nat Rev Rheumatol,* 6**,** 727-30.

Löe, H., Anerud, A., Boysen, H. & Morrison, E. 1986. Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *J Clin Periodontol,* 13**,** 431-45.

Machado, V., Botelho, J., Escalda, C., Hussain, S. B., Luthra, S., Mascarenhas, P., Orlandi, M., Mendes, J. J. & D'aiuto, F. 2021. Serum C-Reactive Protein and Periodontitis: A Systematic Review and Meta-Analysis. *Frontiers in Immunology,* 12.

Machtei, E. E., Christersson, L. A., Grossi, S. G., Dunford, R., Zambon, J. J. & Genco, R. J. 1992. Clinical criteria for the definition of "established periodontitis". *J Periodontol,* 63**,** 206-14.

Mackenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A. & Surprenant, A. 2001. Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity,* 15**,** 825-35.

Makrygiannakis, D., Hermansson, M., Ulfgren, A. K., Nicholas, A. P., Zendman, A. J., Eklund, A., Grunewald, J., Skold, C. M., Klareskog, L. & Catrina, A. I. 2008. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann Rheum Dis,* 67**,** 1488-92.

Mankia, K., Cheng, Z., Do, T., Hunt, L., Meade, J., Kang, J., Clerehugh, V., Speirs, A., Tugnait, A., Hensor, E. M. A., Nam, J. L., Devine, D. A. & Emery, P. 2019. Prevalence of Periodontal Disease and Periodontopathic Bacteria in Anti-Cyclic Citrullinated Protein Antibody-Positive At-Risk Adults Without Arthritis. *JAMA Netw Open,* 2**,** e195394.

Marsh, P. D. 1994. Microbial ecology of dental plaque and its significance in health and disease. *Advances in dental research,* 8.

Marsh, P. D. 2000. Role of the Oral Microflora in Health. *Microbial Ecology in Health and Disease,* 12**,** 130-137.

Marsh, P. D. 2003. Are dental diseases examples of ecological catastrophes? *Microbiology,* 149**,** 279-294.

Marsh, P. D. 2004. Dental plaque as a microbial biofilm. *Caries Res,* 38**,** 204-11.

Marsh, P. D. & Bradshaw, D. J. 1995. Dental plaque as a biofilm. *J Ind Microbiol,* 15**,** 169-75.

Mcgraw, W. T., Potempa, J., Farley, D. & Travis, J. 1999. Purification, characterization, and sequence analysis of a potential virulence factor from Porphyromonas gingivalis, peptidylarginine deiminase. *Infect Immun,* 67**,** 3248-56.

Mcmurdie, P. J. & Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One,* 8**,** e61217.

Mercado, F., Marshall, R. I., Klestov, A. C. & Bartold, P. M. 2000. Is there a relationship between rheumatoid arthritis and periodontal disease? *J Clin Periodontol,* 27**,** 267-72.

Michalowicz, B. S., Diehl, S. R., Gunsolley, J. C., Sparks, B. S., Brooks, C. N., Koertge, T. E., Califano, J. V., Burmeister, J. A. & Schenkein, H. A. 2000. Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol,* 71**,** 1699-707.

Miles, A. A., Misra, S. S. & Irwin, J. O. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond),* 38**,** 732-49.

Miller, C. S., King, C. P., Langub, M. C., Kryscio, R. J. & Thomas, M. V. 2006. Salivary biomarkers of existing periodontal disease: A cross-sectional study. *Journal of the American Dental Association,* 137**,** 322-329.

Miller, W. D. 1890. The microorganisms of the human mouth. *The local and general disease which are caused by them***,** 274-341.

Millhouse, E., Jose, A., Sherry, L., Lappin, D. F., Patel, N., Middleton, A. M., Pratten, J., Culshaw, S. & Ramage, G. 2014. Development of an in vitro periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules. *BMC Oral Health,* 14**,** 80.

Mombelli, A., Almaghlouth, A., Cionca, N., Cancela, J., Courvoisier, D. S. & Giannopoulou, C. 2017. Microbiologic Response to Periodontal Therapy and Multivariable Prediction of Clinical Outcome. *Journal of periodontology,* 88.

Monteiro, M. F., Altabtbaei, K., Kumar, P. S., Casati, M. Z., Ruiz, K. G. S., Sallum, E. A., Nociti-Junior, F. H. & Casarin, R. C. V. 2021. Parents with periodontitis impact the subgingival colonization of their offspring. *Sci Rep,* 11**,** 1357.

Mooney, J., Adonogianaki, E. & Kinane, D. F. 1993. Relative avidity of serum antibodies to putative periodontopathogens in periodontal disease. *Journal of Periodontal Research,* 28**,** 444-450.

Morozumi, T., Yashima, A., Gomi, K., Ujiie, Y., Izumi, Y., Akizuki, T., Mizutani, K., Takamatsu, H., Minabe, M., Miyauchi, S., Yoshino, T., Tanaka, M., Tanaka, Y., Hokari, T. & Yoshie, H. 2018. Increased systemic levels of inflammatory mediators following one-stage full-mouth scaling and root planing. *Journal of Periodontal Research,* 53**,** 536-544.

Mountcastle, S. E., Cox, S. C., Sammons, R. L., Jabbari, S., Shelton, R. M. & Kuehne, S. A. 2020. A review of co-culture models to study the oral microenvironment and disease. *J Oral Microbiol,* 12**,** 1773122.

Mukaka, M. M. 2012. Statistics corner: A guide to appropriate use of correlation coefficient in medical research. *Malawi Med J,* 24**,** 69-71.

Mukherjee, A., Jantsch, V., Khan, R., Hartung, W., Fischer, R., Jantsch, J., Ehrenstein, B., Konig, M. F. & Andrade, F. 2018. Rheumatoid Arthritis-Associated Autoimmunity Due to Aggregatibacter actinomycetemcomitans and Its Resolution With Antibiotic Therapy. *Front Immunol,* 9**,** 2352.

Munz, M., Richter, G. M., Loos, B. G., Jepsen, S., Divaris, K., Offenbacher, S., Teumer, A., Holtfreter, B., Kocher, T., Bruckmann, C., Jockel-Schneider, Y., Graetz, C., Ahmad, I., Staufenbiel, I., Van Der Velde, N., Uitterlinden, A. G., De Groot, L., Wellmann, J., Berger, K., Krone, B., Hoffmann, P., Laudes, M., Lieb, W., Franke, A., Erdmann, J., Dommisch, H. & Schaefer, A. S. 2019. Meta-analysis of genome-wide association studies of aggressive and chronic periodontitis identifies two novel risk loci. *Eur J Hum Genet,* 27**,** 102-113.

Munz, M., Willenborg, C., Richter, G. M., Jockel-Schneider, Y., Graetz, C., Staufenbiel, I., Wellmann, J., Berger, K., Krone, B., Hoffmann, P., Van Der Velde, N., Uitterlinden, A. G., De Groot, L., Sawalha, A. H., Direskeneli, H., Saruhan-Direskeneli, G., Guzeldemir-Akcakanat, E., Keceli, H. G., Laudes, M., Noack, B., Teumer, A., Holtfreter, B., Kocher, T., Eickholz, P., Meyle, J., Doerfer, C., Bruckmann, C., Lieb, W., Franke, A., Schreiber, S., Nohutcu, R. M., Erdmann, J., Loos, B. G., Jepsen, S., Dommisch, H. & Schaefer, A. S. 2017. A genome-wide

association study identifies nucleotide variants at SIGLEC5 and DEFA1A3 as risk loci for periodontitis. *Hum Mol Genet,* 26**,** 2577-2588.

Murakami, S., Mealey, B. L., Mariotti, A. & Chapple, I. L. C. 2018. Dental plaque-induced gingival conditions. *J Periodontol,* 89 Suppl 1**,** S17-S27.

Nakib, N. M., Bissada, N. F., Simmelink, J. W. & Goldstine, S. N. 1982. Endotoxin penetration into root cementum of periodontally healthy and diseased human teeth. *J Periodontol,* 53**,** 368-78.

Nazir, M., Al-Ansari, A., Al-Khalifa, K., Alhareky, M., Gaffar, B. & Almas, K. 2020. Global Prevalence of Periodontal Disease and Lack of Its Surveillance. *ScientificWorldJournal,* 2020**,** 2146160.

Nesse, W., Abbas, F., Van Der Ploeg, I., Spijkervet, F. K. L., Dijkstra, P. U. & Vissink, A. 2008. Periodontal inflamed surface area: Quantifying inflammatory burden. *Journal of Clinical Periodontology*.

Nesse, W., Westra, J., Van Der Wal, J. E., Abbas, F., Nicholas, A. P., Vissink, A. & Brouwer, E. 2012. The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation. *Journal of clinical periodontology,* 39.

Newman, M. G., Socransky, S. S., Savitt, E. D., Propas, D. A. & Crawford, A. 1976. Studies of the microbiology of periodontosis. *J Periodontol,* 47**,** 373-9.

Nishi, H., Hosomi, N., Ohta, K., Aoki, S., Nakamori, M., Nezu, T., Shigeishi, H., Shintani, T., Obayashi, T., Ishikawa, K., Kinoshita, N., Shiga, Y., Sugiyama, M., Ohge, H., Maruyama, H., Kawaguchi, H. & Kurihara, H. 2020. Serum immunoglobulin G antibody titer to Fusobacterium nucleatum is associated with unfavorable outcome after stroke. *Clinical and experimental immunology,* 200.

Noack, B., Genco, R. J., Trevisan, M., Grossi, S., Zambon, J. J. & Nardin, E. D. 2001. Periodontal Infections Contribute to Elevated Systemic C-Reactive Protein Level. *Journal of Periodontology,* 72**,** 1221-1227.

Noble, J. M., Scarmeas, N., Celenti, R. S., Elkind, M. S., Wright, C. B., Schupf, N. & Papapanou, P. N. 2014. Serum IgG antibody levels to periodontal microbiota are associated with incident Alzheimer disease. *PloS one,* 9.

Nyvad, B. & Kilian, M. 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand J Dent Res,* 95**,** 369-80.

O'leary, R., Sved, A. M., Davies, E. H., Leighton, T. G., Wilson, M. & Kieser, J. B. 1997. The bactericidal effects of dental ultrasound on Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. An in vitro investigation. *J Clin Periodontol,* 24**,** 432-9.

O'leary, T. J., Drake, R. B. & Naylor, J. E. 1972. The Plaque Control Record. *Journal of Periodontology,* 43**,** 38-38.

Okada, M., Kobayashi, T., Ito, S., Yokoyama, T., Abe, A., Murasawa, A. & Yoshie, H. 2013. Periodontal treatment decreases levels of antibodies to Porphyromonas gingivalis and citrulline in patients with rheumatoid arthritis and periodontitis. *J Periodontol,* 84**,** e74-84.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., O'hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H., Szoecs, E. & Wagner, H. 2019. *vegan: Community Ecology Package. R package version 2.4-2* [Online]. Available: http://CRAN.R-project.org/package=vegan.

Oluwagbemigun, K., Yucel-Lindberg, T., Dietrich, T., Tour, G., Sherina, N., Hansson, M., Bergmann, M., Lundberg, K. & Boeing, H. 2019. A cross-sectional investigation into the association between Porphyromonas gingivalis and autoantibodies to citrullinated proteins in a German population. *Ther Adv Musculoskelet Dis,* 11**,** 1759720X19883152.

Ower, P. 2013. Minimally-invasive non-surgical periodontal therapy. *Dent Update,* 40**,** 289-90, 293-5.

Ozçaka, O., Nalbantsoy, A. & Buduneli, N. 2011. Interleukin-17 and interleukin-18 Levels in Saliva and Plasma of Patients With Chronic Periodontitis. *Journal of periodontal research,* 46.

Page, R. C. & Eke, P. I. 2007. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol,* 78**,** 1387-99.

Page, R. C. & Kornman, K. S. 1997. The pathogenesis of human periodontitis: an introduction. *Periodontology 2000,* 14**,** 9-11.

Page, R. C. & Schroeder, H. E. 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest,* 34**,** 235-49.

Pan, W., Wang, Q. & Chen, Q. 2019. The cytokine network involved in the host immune response to periodontitis. *Int J Oral Sci,* 11**,** 30.

Papapanou, P. N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D. H., Flemmig, T. F., Garcia, R., Giannobile, W. V., Graziani, F., Greenwell, H., Herrera, D., Kao, R. T., Kebschull, M., Kinane, D. F., Kirkwood, K. L., Kocher, T., Kornman, K. S., Kumar, P. S., Loos, B. G., Machtei, E., Meng, H., Mombelli, A., Needleman, I., Offenbacher, S., Seymour, G. J., Teles, R. & Tonetti, M. S. 2018. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol,* 89 Suppl 1**,** S173-s182.

Parachuru, V. P. B., Coates, D. E., Milne, T. J., Rich, A. M. & Seymour, G. J. 2018. FoxP3(+) regulatory T cells, interleukin 17 and mast cells in chronic inflammatory periodontal disease. *J Periodontal Res,* 53**,** 622-635.

Park, S. Y., Ahn, S., Lee, J. T., Yun, P. Y., Lee, Y. J., Lee, J. Y., Song, Y. W., Chang, Y. S. & Lee, H. J. 2017. Periodontal inflamed surface area as a novel numerical variable describing periodontal conditions. *Journal of Periodontal and Implant Science,* 47**,** 328-338.

Patel, R. 2005. Biofilms and antimicrobial resistance. *Clin Orthop Relat Res***,** 41-7.

Paul, O., Arora, P., Mayer, M. & Chatterjee, S. 2020. Inflammation in Periodontal Disease: Possible Link to Vascular Disease. *Front Physiol,* 11**,** 609614.

Pearson, T. A., Mensah, G. A., Alexander, R. W., Anderson, J. L., Cannon, R. O., 3rd, Criqui, M., Fadl, Y. Y., Fortmann, S. P., Hong, Y., Myers, G. L., Rifai, N., Smith, S. C., Jr., Taubert, K., Tracy, R. P., Vinicor, F., Centers for Disease, C., Prevention & American Heart, A. 2003. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation,* 107**,** 499-511.

Pedersen, T. L. 2020. *ggraph: An Implementation of Grammar of Graphics for Graphs and Networks* [Online]. Available: https://rdrr.io/cran/ggraph/ [Accessed 14th June 2020].

Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., Cosmi, L., Lunardi, C., Annunziato, F., Romagnani, S. & Cassatella, M. A. 2010. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood,* 115**,** 335-43.

Pepys, M. B. & Hirschfield, G. M. 2003. C-reactive protein: a critical update. *J Clin Invest,* 111**,** 1805-12.

Perez-Chaparro, P. J., Duarte, P. M., Pannuti, C. M., Figueiredo, L. C., Mestnik, M. J., Goncalves, C. P., Faveri, M. & Feres, M. 2015. Evaluation of human and microbial DNA content in subgingival plaque samples collected by paper points or curette. *J Microbiol Methods,* 111**,** 19-20.

Pihlstrom, B. L., Hodges, J. S., Michalowicz, B., Wohlfahrt, J. C. & Garcia, R. I. 2018. Promoting oral health care because of its possible effect on systemic disease is premature and may be misleading. *J Am Dent Assoc,* 149**,** 401-403.

Prakasam, S. & Srinivasan, M. 2014. Evaluation of Salivary Biomarker Profiles Following Non-Surgical Management of Chronic Periodontitis. *Oral diseases,* 20.

Pratten, J., Smith, A. W. & Wilson, M. 1998. Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. *J Antimicrob Chemother,* 42**,** 453-9.

Preshaw, P. M., Alba, A. L. & Herrera, D. 2011. Periodontitis and diabetes: a two-way relationship. *Diabetologia,* 55**,** 21-31.

Pussinen, P. J., Alfthan, G., Tuomilehto, J., Asikainen, S. & Jousilahti, P. 2004. High serum antibody levels to Porphyromonas gingivalis predict myocardial infarction. *European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology,* 11**,** 408-11.

Pussinen, P. J., Kononen, E., Paju, S., Hyvarinen, K., Gursoy, U. K., Huumonen, S., Knuuttila, M. & Suominen, A. L. 2011. Periodontal pathogen carriage, rather than periodontitis, determines the serum antibody levels. *J Clin Periodontol,* 38**,** 405-11.

Pérez-Chaparro, P. J., Gonçalves, C., Figueiredo, L. C., Faveri, M., Lobão, E., Tamashiro, N., Duarte, P. & Feres, M. 2014. Newly identified pathogens associated with periodontitis: A systematic review. *Journal of Dental Research,* 93**,** 846-858.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glöckner, F. O. 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research,* 41**,** D590-D596.

Quirynen, M., Bollen, C. M., Vandekerckhove, B. N., Dekeyser, C., Papaioannou, W. & Eyssen, H. 1995. Full- vs. partial-mouth disinfection in the treatment of periodontal infections: short-term clinical and microbiological observations. *J Dent Res,* 74**,** 1459-67.

R Core Team, R. 2014. *R: A language and environment for statistical computing*. [Online]. R Foundation for Statistical Computing, Vienna, Austria. Available: http://www.R-project.org/.

Ramage, G., Culshaw, S., Jones, B. & Williams, C. 2010. Are we any closer to beating the biofilm: novel methods of biofilm control. *Curr Opin Infect Dis,* 23**,** 560-6.

Ramfjord, S. P. 1959. Indices for Prevalence and Incidence of Periodontal Disease. *The Journal of Periodontology,* 30**,** 51-59.

Rang, F. J., Kloosterman, W. P. & De Ridder, J. 2018. From squiggle to basepair: computational approaches for improving nanopore sequencing read accuracy. *Genome Biol,* 19**,** 90.

Rantapää-Dahlqvist, S., De Jong, B. A., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., Sundin, U. & Van Venrooij, W. J. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis and rheumatism,* 48.

Raptopoulou, A., Sidiropoulos, P., Katsouraki, M. & Boumpas, D. T. 2007. Anti-citrulline antibodies in the diagnosis and prognosis of rheumatoid arthritis: evolving concepts. *Crit Rev Clin Lab Sci,* 44**,** 339-63.

Rathnayake, N., Akerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., Sorsa, T. & Gustafsson, A. 2013. Salivary biomarkers of oral health: a cross-sectional study. *J Clin Periodontol,* 40**,** 140-7.

Rebeis, E. S., Albuquerque-Souza, E., Paulino Da Silva, M., Giudicissi, M., Mayer, M. P. A. & Saraiva, L. 2019. Effect of periodontal treatment on Aggregatibacter actinomycetemcomitans colonization and serum IgG levels against A. actinomycetemcomitans serotypes and Omp29 of aggressive periodontitis patients. *Oral Dis,* 25**,** 569-579.

Ritz, H. L. 1967. Microbial population shifts in developing human dental plaque. *Arch Oral Biol,* 12**,** 1561-8.

Robertson, P. B. 1990. The residual calculus paradox. *J Periodontol,* 61**,** 65-6.

Rodríguez-Rabassa, M., López, P., Rodríguez-Santiago, R. E., Cases, A., Felici, M., Sánchez, R., Yamamura, Y. & Rivera-Amill, V. 2018. Cigarette Smoking Modulation

of Saliva Microbial Composition and Cytokine Levels. *International journal of environmental research and public health,* 15.

Rohart, F., Gautier, B., Singh, A. & Lê Cao, K. A. 2017. mixOmics: An R Package for 'Omics Feature Selection and Multiple Data Integration. *PLoS computational biology,* 13.

Rosier, B. T., Buetas, E., Moya-Gonzalvez, E. M., Artacho, A. & Mira, A. 2020. Nitrate as a potential prebiotic for the oral microbiome. *Scientific Reports,* 10**,** 1-15.

Rosier, B. T., De Jager, M., Zaura, E. & Krom, B. P. 2014. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol,* 4**,** 92.

Rosier, B. T., Marsh, P. D. & Mira, A. 2018. Resilience of the Oral Microbiota in Health: Mechanisms That Prevent Dysbiosis. *Journal of dental research,* 97.

Rosier, B. T., Palazon, C., Garcia-Esteban, S., Artacho, A., Galiana, A. & Mira, A. 2021. A Single Dose of Nitrate Increases Resilience Against Acidification Derived From Sugar Fermentation by the Oral Microbiome. *Front Cell Infect Microbiol,* 11**,** 692883.

Ruhl, S., Hamberger, S., Betz, R., Sukkar, T., Schmalz, G., Seymour, R. A., Hiller, K. A. & Thomason, J. M. 2004. Salivary proteins and cytokines in drug-induced gingival overgrowth. *J Dent Res,* 83**,** 322-6.

Ruhling, A., Bernhardt, O. & Kocher, T. 2005. Subgingival debridement with a teflon-coated sonic scaler insert in comparison to conventional instruments and assessment of substance removal on extracted teeth. *Quintessence Int,* 36**,** 446-52.

Ruppert, M., Cadosch, J., Guindy, J., Case, D. & Zappa, U. 2002. In vivo ultrasonic debridement forces in bicuspids: a pilot study. *J Periodontol,* 73**,** 418-22.

Russell, A. L. 1956. A system of classification and scoring for prevalence surveys of periodontal disease. *J Dent Res,* 35**,** 350-9.

Ryder, M. I. 2007. The influence of smoking on host responses in periodontal infections. *Periodontol 2000,* 43**,** 267-77.

Sakai, Y., Shimauchi, H., Ito, H. O., Kitamura, M. & Okada, H. 2001. Porphyromonas gingivalis-specific IgG subclass antibody levels as immunological risk indicators of periodontal bone loss. *J Clin Periodontol,* 28**,** 853-9.

Sakanaka, A., Kuboniwa, M., Hashino, E., Bamba, T., Fukusaki, E. & Amano, A. 2017. Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status. *Scientific reports,* 7.

Sanchez, G. A., Miozza, V. A., Delgado, A. & Busch, L. 2013. Salivary IL-1beta and PGE2 as biomarkers of periodontal status, before and after periodontal treatment. *J Clin Periodontol,* 40**,** 1112-7.

Sanz, M., D'aiuto, F., Deanfield, J. & Fernandez-Avilés, F. 2010. European workshop in periodontal health and cardiovascular disease—scientific evidence on the association between periodontal and cardiovascular diseases: a review of the literature. *European Heart Journal Supplements,* 12**,** B3-B12.

Sanz, M., Marco Del Castillo, A., Jepsen, S., Gonzalez-Juanatey, J. R., D'aiuto, F., Bouchard, P., Chapple, I., Dietrich, T., Gotsman, I., Graziani, F., Herrera, D., Loos, B., Madianos, P., Michel, J. B., Perel, P., Pieske, B., Shapira, L., Shechter, M., Tonetti, M., Vlachopoulos, C. & Wimmer, G. 2020. Periodontitis and cardiovascular diseases: Consensus report. *J Clin Periodontol*.

Sato, K., Suematsu, A., Okamoto, K., Yamaguchi, A., Morishita, Y., Kadono, Y., Tanaka, S., Kodama, T., Akira, S., Iwakura, Y., Cua, D. J. & Takayanagi, H. 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med,* 203**,** 2673-82.

Schenk, G., Flemmig, T. F., Lob, S., Ruckdeschel, G. & Hickel, R. 2000. Lack of antimicrobial effect on periodontopathic bacteria by ultrasonic and sonic scalers in vitro. *J Clin Periodontol,* 27**,** 116-9.

Scher, J. U., Bretz, W. A. & Abramson, S. B. 2014. Periodontal disease and subgingival microbiota as contributors for rheumatoid arthritis pathogenesis: modifiable risk factors? *Curr Opin Rheumatol,* 26**,** 424-9.

Scher, J. U., Ubeda, C., Equinda, M., Khanin, R., Buischi, Y., Viale, A., Lipuma, L., Attur, M., Pillinger, M. H., Weissmann, G., Littman, D. R., Pamer, E. G., Bretz, W. A. & Abramson, S. B. 2012. Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. *Arthritis Rheum,* 64**,** 3083-94.

Schlageter, L., Rateitschak-Pluss, E. M. & Schwarz, J. P. 1996. Root surface smoothness or roughness following open debridement. An in vivo study. *J Clin Periodontol,* 23**,** 460-4.

Schulz, K. F., Altman, D. G., Moher, D. & Consort Group 2010. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ (Clinical research ed.),* 340.

Schwarzberg, K., Lindsay, S., Caporaso, J. G., Casaburi, G., Saber, M. H., Le, R., Slots, J., Kelley, S. T., Bharti, B., Gottlieb, R. A., Salvatore, F. & Alonaizan, F. 2014. The Personal Human Oral Microbiome Obscures the Effects of Treatment on Periodontal Disease. *PLoS ONE,* 9**,** e86708-e86708.

Scottish Dental Clinical Effectiveness Programme, S. 2014. Prevention and Treatment of Periodontal Diseases in Primary Care.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. & Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol,* 12**,** R60.

Sell, A. M., Bazzo De Alencar, J., Visentainer, J. E. L. & De Oliveira E Silva, C. 2017. Immunopathogenesis of Chronic Periodontitis. *In:* Pachiappan, A. (ed.) *Periodontitis.* Rijeka: IntechOpen.

Seneviratne, C. J., Balan, P., Suriyanarayanan, T., Lakshmanan, M., Lee, D. Y., Rho, M., Jakubovics, N., Brandt, B., Crielaard, W. & Zaura, E. 2020. Oral microbiome-systemic link studies: perspectives on current limitations and future artificial intelligence-based approaches. *Crit Rev Microbiol,* 46**,** 288-299.

Seneviratne, C. J., Zhang, C. F. & Samaranayake, L. P. 2011. Dental plaque biofilm in oral health and disease. *Chin J Dent Res,* 14**,** 87-94.

Serban, S., Dietrich, T., Lopez-Oliva, I., De Pablo, P., Raza, K., Filer, A., Chapple, I. L. C. & Hill, K. 2019. Attitudes towards Oral Health in Patients with Rheumatoid Arthritis: A Qualitative Study Nested within a Randomized Controlled Trial. *JDR Clin Trans Res,* 4**,** 360-370.

Seror, R., Le Gall-David, S., Bonnaure-Mallet, M., Schaeverbeke, T., Cantagrel, A., Minet, J., Gottenberg, J. E., Chanson, P., Ravaud, P. & Mariette, X. 2015. Association of Anti-Porphyromonas gingivalis Antibody Titers With Nonsmoking Status in Early Rheumatoid Arthritis: Results From the Prospective French Cohort of Patients With Early Rheumatoid Arthritis. *Arthritis & rheumatology (Hoboken, N.J.),* 67.

Sexton, W. M., Lin, Y., Kryscio, R. J., Dawson, D. R., Ebersole, J. L. & Miller, C. S. 2011. Salivary biomarkers of periodontal disease in response to treatment. *Journal of clinical periodontology,* 38.

Shaddox, L. M. & Walker, C. B. 2010. Treating chronic periodontitis: current status, challenges, and future directions. *Clin Cosmet Investig Dent,* 2**,** 79-91.

Shang, L., Deng, D., Buskermolen, J. K., Roffel, S., Janus, M. M., Krom, B. P., Crielaard, W. & Gibbs, S. 2019. Commensal and Pathogenic Biofilms Alter Toll-Like Receptor Signaling in Reconstructed Human Gingiva. *Front Cell Infect Microbiol,* 9**,** 282.

Shaw, L., Ribeiro, A. L. R., Levine, A. P., Pontikos, N., Balloux, F., Segal, A. W., Roberts, A. P. & Smith, A. M. 2017. The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals. *mBio,* 8.

Shelburne, C. E., Shelburne, P. S., Dhople, V. M., Sweier, D. G., Giannobile, W. V., Kinney, J. S., Coulter, W. A., Mullally, B. H. & Lopatin, D. E. 2008. Serum antibodies to Porphyromonas gingivalis chaperone HtpG predict health in periodontitis susceptible patients. *PloS one,* 3.

Sherry, L., Lappin, G., O'donnell, L. E., Millhouse, E., Millington, O. R., Bradshaw, D. J., Axe, A. S., Williams, C., Nile, C. J. & Ramage, G. 2016. Viable compositional analysis of an eleven species oral polymicrobial biofilm. *Frontiers in Microbiology,* 7.

Shi, B., Chang, M., Martin, J., Mitreva, M., Lux, R., Klokkevold, P., Sodergren, E., Weinstock, G. M., Haake, S. K. & Li, H. 2015. Dynamic Changes in the Subgingival Microbiome and Their Potential for Diagnosis and Prognosis of Periodontitis. *mBio,* 6.

Shi, M., Wei, Y., Hu, W., Nie, Y., Wu, X. & Lu, R. 2018. The Subgingival Microbiome of Periodontal Pockets With Different Probing Depths in Chronic and Aggressive Periodontitis: A Pilot Study. *Frontiers in Cellular and Infection Microbiology,* 8.

Silva, N., Dutzan, N., Hernandez, M., Dezerega, A., Rivera, O., Aguillon, J. C., Aravena, O., Lastres, P., Pozo, P., Vernal, R. & Gamonal, J. 2008. Characterization of progressive periodontal lesions in chronic periodontitis patients: levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells. *J Clin Periodontol,* 35**,** 206-14.

Siqueira, J. F., Jr. & Rocas, I. N. 2013. As-yet-uncultivated oral bacteria: breadth and association with oral and extra-oral diseases. *J Oral Microbiol,* 5.

Slade, G. D., Offenbacher, S., Beck, J. D., Heiss, G. & Pankow, J. S. 2000. Acute-phase inflammatory response to periodontal disease in the US population. *Journal of Dental Research,* 79**,** 49-57.

Slots, J. 1977. The predominant cultivable microflora of advanced periodontitis. *Scandinavian journal of dental research,* 85 2**,** 114-21.

Smeeth, L., Thomas, S. L., Hall, A. J., Hubbard, R., Farrington, P. & Vallance, P. 2004. Risk of myocardial infarction and stroke after acute infection or vaccination. *N Engl J Med,* 351**,** 2611-8.

Socransky, S. S. & Haffajee, A. D. 2002. Dental biofilms: difficult therapeutic targets. *Periodontol 2000,* 28**,** 12-55.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. 1998. Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology,* 25**,** 134-144.

Socransky, S. S., Smith, C., Martin, L., Paster, B. J., Dewhirst, F. E. & Levin, A. E. 1994. "Checkerboard" DNA-DNA hybridization. *Biotechniques,* 17**,** 788-92.

Song, S. J., Lauber, C., Costello, E. K., Lozupone, C. A., Humphrey, G., Berg-Lyons, D., Caporaso, J. G., Knights, D., Clemente, J. C., Nakielny, S., Gordon, J. I., Fierer, N. & Knight, R. 2013. Cohabiting family members share microbiota with one another and with their dogs. *Elife,* 2**,** e00458.

Sproston, N. R. & Ashworth, J. J. 2018. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol,* 9**,** 754.

Stadler, A. F., Angst, P. D. M., Arce, R. M., Gomes, S. C., Oppermann, R. V. & Susin, C. 2016. Gingival crevicular fluid levels of cytokines/chemokines in chronic periodontitis: a meta-analysis.

Stashenko, P., Dewhirst, F. E., Peros, W. J., Kent, R. L. & Ago, J. M. 1987. Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. *J Immunol,* 138**,** 1464-8.

Stathopoulou, P. G., Benakanakere, M. R., Galicia, J. C. & Kinane, D. F. 2009. The host cytokine response to Porphyromonas gingivalis is modified by gingipains. *Oral Microbiol Immunol,* 24**,** 11-7.

Subbarao, K. C., Nattuthurai, G. S., Sundararajan, S. K., Sujith, I., Joseph, J. & Syedshah, Y. P. 2019. Gingival Crevicular Fluid: An Overview. *J Pharm Bioallied Sci,* 11**,** S135-S139.

Suvan, J., Leira, Y., Moreno, F., Graziani, F., Derks, J. & Tomasi, C. 2019. Subgingival Instrumentation for Treatment of Periodontitis. A Systematic Review. *Journal of Clinical Periodontology***,** jcpe.13245-jcpe.13245.

Sweier, D. G., Shelburne, P. S., Giannobile, W. V., Kinney, J. S., Lopatin, D. E. & Shelburne, C. E. 2009. Immunoglobulin G (IgG) class, but Not IgA or IgM, antibodies to peptides of the Porphyromonas gingivalis chaperone HtpG predict health in subjects with periodontitis by a fluorescence enzyme-linked immunosorbent assay. *Clin Vaccine Immunol,* 16**,** 1766-73.

Syed, S. A. & Loesche, W. J. 1978. Bacteriology of human experimental gingivitis: effect of plaque age. *Infect Immun,* 21**,** 821-9.

Tanaka, M., Hanioka, T., Takaya, K. & Shizukuishi, S. 1998. Association of Oxygen Tension in Human Periodontal Pockets With Gingival Inflammation. *Journal of periodontology,* 69.

Taylor, J. J. 2014. Protein Biomarkers of Periodontitis in Saliva. *ISRN Inflammation,* 2014.

Taylor, J. J., Preshaw, P. M. & Lalla, E. 2013. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *J Clin Periodontol,* 40 Suppl 14**,** S113-34.

Techatanawat, S., Surarit, R., Chairatvit, K., Khovidhunkit, W., Roytrakul, S., Thanakun, S., Kobayashi, H., Khovidhunkit Son, P. & Izumi, Y. 2020. Salivary and serum interleukin-17A and interleukin-18 levels in patients with type 2 diabetes mellitus with and without periodontitis. *PLoS One,* 15.

Teeuw, W. J., Slot, D. E., Susanto, H., Gerdes, V. E., Abbas, F., D'aiuto, F., Kastelein, J. J. & Loos, B. G. 2014. Treatment of periodontitis improves the atherosclerotic profile: a systematic review and meta-analysis. *J Clin Periodontol,* 41**,** 70-9.

Teles, F. R., Lynch, M. C., Patel, M., Torresyap, G. & Martin, L. 2021. Bacterial resistance to minocycline after adjunctive minocycline microspheres during periodontal maintenance: Randomized clinical trial. *J Periodontol*.

Teles, F. R., Teles, R. P., Uzel, N. G., Song, X. Q., Torresyap, G., Socransky, S. S. & Haffajee, A. D. 2012. Early microbial succession in redeveloping dental biofilms in periodontal health and disease. *Journal of periodontal research,* 47.

Teles, R. P., Gursky, L. C., Faveri, M., Rosa, E. A., Teles, F. R., Feres, M., Socransky, S. S. & Haffajee, A. D. 2010. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. *J Clin Periodontol,* 37**,** 313-23.

Teles, R. P., Haffajee, A. D. & Socransky, S. S. 2006. Microbiological goals of periodontal therapy. *Periodontol 2000,* 42**,** 180-218.

Teles, R. P., Likhari, V., Socransky, S. S. & Haffajee, A. D. 2009. Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: A cross-sectional study. *Journal of Periodontal Research,* 44**,** 411-417.

Teng, F., Darveekaran Nair, S. S., Zhu, P., Li, S., Huang, S., Li, X., Xu, J. & Yang, F. 2018. Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. *Sci Rep,* 8**,** 16321.

Theilade, E. 1986. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol,* 13**,** 905-11.

Thilo, B. E. & Baehni, P. C. 1987. Effect of ultrasonic instrumentation on dental plaque microflora in vitro. *J Periodontal Res,* 22**,** 518-21.

Thurnheer, T., Karygianni, L., Flury, M. & Belibasakis, G. N. 2019. Fusobacterium Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models. *Front Microbiol,* 10**,** 1716.

Tobon-Arroyave, S. I., Jaramillo-Gonzalez, P. E. & Isaza-Guzman, D. M. 2008. Correlation between salivary IL-1beta levels and periodontal clinical status. *Arch Oral Biol,* 53**,** 346-52.

Tomar, S. L. & Asma, S. 2000. Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *J Periodontol,* 71**,** 743-51.

Tonetti, M. S., Chapple, I. L., Jepsen, S. & Sanz, M. 2015. Primary and secondary prevention of periodontal and peri-implant diseases: Introduction to, and objectives of the 11th European Workshop on Periodontology consensus conference. *J Clin Periodontol,* 42 Suppl 16**,** S1-4.

Tonetti, M. S., D'aiuto, F., Nibali, L., Donald, A., Storry, C., Parkar, M., Suvan, J., Hingorani, A. D., Vallance, P. & Deanfield, J. 2007. Treatment of Periodontitis and Endothelial Function. *New England Journal of Medicine,* 356**,** 911-920.

Tonetti, M. S., Greenwell, H. & Kornman, K. S. 2018. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J Periodontol,* 89 Suppl 1**,** S159-S172.

Tonetti, M. S., Jepsen, S., Jin, L. & Otomo-Corgel, J. 2017. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *Journal of Clinical Periodontology,* 44**,** 456-462.

Torres De Heens, G. L., Loos, B. G. & Van Der Velden, U. 2010. Monozygotic twins are discordant for chronic periodontitis: clinical and bacteriological findings. *J Clin Periodontol,* 37**,** 120-8.

Trombelli, L., Farina, R., Silva, C. O. & Tatakis, D. N. 2018. Plaque-induced gingivitis: Case definition and diagnostic considerations. *J Clin Periodontol,* 45 Suppl 20**,** S44-S67.

Tsai, C., Hayes, C. & Taylor, G. W. 2002. Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population. *Community Dent Oral Epidemiol,* 30**,** 182-92.

Tugnait, A., Clerehugh, V. & Hirschmann, P. N. 2000. The usefulness of radiographs in diagnosis and management of periodontal diseases: a review. *J Dent,* 28**,** 219-26.

Tunkel, J., Heinecke, A. & Flemmig, T. F. 2002. A systematic review of efficacy of machine-driven and manual subgingival debridement in the treatment of chronic periodontitis.

Tymkiw, K. D., Thunell, D. H., Johnson, G. K., Joly, S., Burnell, K. K., Cavanaugh, J. E., Brogden, K. A. & Guthmiller, J. M. 2011. Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. *Journal of clinical periodontology,* 38.

Vaizey, J. M. & Clark-Kennedy, A. E. 1939. Dental Sepsis: Anaemia, Dyspepsia, and Rheumatism. *Br Med J,* 1**,** 1269-73.

Van Den Hoek, J., Boshuizen, H. C., Roorda, L. D., Tijhuis, G. J., Nurmohamed, M. T., Van Den Bos, G. A. & Dekker, J. 2017. Mortality in patients with rheumatoid arthritis: a 15-year prospective cohort study. *Rheumatol Int,* 37**,** 487-493.

Van Der Weijden, G. A. F., Dekkers, G. J. & Slot, D. E. 2019. Success of non-surgical periodontal therapy in adult periodontitis patients: A retrospective analysis. *Int J Dent Hyg,* 17**,** 309-317.

Van Dyke, T. E., Bartold, P. M. & Reynolds, E. C. 2020. The Nexus Between Periodontal Inflammation and Dysbiosis. *Frontiers in immunology,* 11.

Van Dyke, T. E. & Sheilesh, D. 2005. Risk factors for periodontitis. *J Int Acad Periodontol,* 7**,** 3-7.

Van Palenstein Helderman, W. H. 1981. Microbial etiology of periodontal disease. *J Clin Periodontol,* 8**,** 261-80.

Vartoukian, S. R., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. E. & Wade, W. G. 2016. In Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by

Community Culture and Media Supplementation with Siderophores. *PLoS One,* 11**,** e0146926.

Velsko, I. M. & Shaddox, L. M. 2018. Consistent and reproducible long-term in vitro growth of health and disease-associated oral subgingival biofilms. *BMC Microbiol,* 18**,** 70.

Vyas, N., Grewal, M., Kuehne, S. A., Sammons, R. L. & Walmsley, A. D. 2020a. High speed imaging of biofilm removal from a dental implant model using ultrasonic cavitation. *Dent Mater,* 36**,** 733-743.

Vyas, N., Sammons, R. L., Kuehne, S. A., Johansson, C., Stenport, V., Wang, Q. X. & Walmsley, A. D. 2020b. The effect of standoff distance and surface roughness on biofilm disruption using cavitation. *PLoS One,* 15**,** e0236428.

Wade, W. G. & Prosdocimi, E. M. 2020. Profiling of Oral Bacterial Communities. *J Dent Res,* 99**,** 621-629.

Wang, D., Koshy, G., Nagasawa, T., Kawashima, Y., Kiji, M., Nitta, H., Oda, S. & Ishikawa, I. 2006. Antibody response after single-visit full-mouth ultrasonic debridement versus quadrant-wise therapy. *J Clin Periodontol,* 33**,** 632-8.

Wang, J., Jia, Z., Zhang, B., Peng, L. & Zhao, F. 2020. Tracing the accumulation of in vivo human oral microbiota elucidates microbial community dynamics at the gateway to the GI tract. *Gut,* 69**,** 1355-1356.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol,* 73**,** 5261-7.

Wassall, R. R. & Preshaw, P. M. 2016. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontology 2000,* 70.

Wegner, N., Wait, R., Sroka, A., Eick, S., Nguyen, K. A., Lundberg, K., Kinloch, A., Culshaw, S., Potempa, J. & Venables, P. J. 2010. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: Implications for autoimmunity in rheumatoid Arthritis. *Arthritis and Rheumatism,* 62**,** 2662-2672.

Welch, J. L. M., Rossetti, B. J., Rieken, C. W., Dewhirst, F. E. & Borisy, G. G. 2016. Biogeography of a human oral microbiome at the micron scale. *Proc Natl Acad Sci U S A,* 113**,** E791-800.

White, D. A., Tsakos, G., Pitts, N. B., Fuller, E., Douglas, G. V., Murray, J. J. & Steele, J. G. 2012. Adult Dental Health Survey 2009: common oral health conditions and their impact on the population. *Br Dent J,* 213**,** 567-72.

Wozniak, K. L., Arribas, A., Leigh, J. E. & Fidel, P. L., Jr. 2002. Inhibitory effects of whole and parotid saliva on immunomodulators. *Oral Microbiol Immunol,* 17**,** 100-7.

Yamada, R., Suzuki, A., Chang, X. & Yamamoto, K. 2005. Citrullinated proteins in rheumatoid arthritis. *Front Biosci,* 10**,** 54-64.

Yang, H.-W., Huang, Y.-F. & Chou, M.-Y. 2004. Occurrence of Porphyromonas gingivalis and Tannerella forsythensis in Periodontally Diseased and Healthy Subjects. *Journal of Periodontology,* 75**,** 1077-1083.

Yang, N. Y., Wang, C. Y., Chyuan, I. T., Wu, K. J., Tu, Y. K., Chang, C. W., Hsu, P. N., Kuo, M. Y. & Chen, Y. W. 2018. Significant association of rheumatoid arthritis-related inflammatory markers with non-surgical periodontal therapy. *J Formos Med Assoc,* 117**,** 1003-1010.

Yu, J. J., Ruddy, M. J., Wong, G. C., Sfintescu, C., Baker, P. J., Smith, J. B., Evans, R. T. & Gaffen, S. L. 2007. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood,* 109**,** 3794-802.

Zafar, M. S. 2016. Comparing the effects of manual and ultrasonic instrumentation on root surface mechanical properties. *Eur J Dent,* 10**,** 517-521.

Zein Elabdeen, H. R., Mustafa, M., Ali, R. & Bolstad, A. I. 2017. Cytokine profile in gingival crevicular fluid and plasma of patients with aggressive periodontitis. *Acta odontologica Scandinavica,* 75.

Zenobia, C. & Hajishengallis, G. 2015. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000,* 69**,** 142-59.

Zhao, Y., Li, Z., Su, L., Ballesteros-Tato, A., Katz, J., Michalek, S. M., Feng, X. & Zhang, P. 2020. Frontline Science: Characterization and regulation of osteoclast precursors following chronic Porphyromonas gingivalis infection. *J Leukoc Biol,* 108**,** 1037-1050.

Zheng, W., Tsompana, M., Ruscitto, A., Sharma, A., Genco, R., Sun, Y. & Buck, M. J. 2015. An accurate and efficient experimental approach for characterization of the complex oral microbiota. *Microbiome,* 3**,** 48.

Zhou, Y. H. & Gallins, P. 2019. A Review and Tutorial of Machine Learning Methods for Microbiome Host Trait Prediction. *Front Genet,* 10**,** 579.

8.1. Appendices

Appendix i (chapter 2 manuscript)

pathogens

MDPI

Article

The Subgingival Plaque Microbiome, Systemic Antibodies against Bacteria and Citrullinated Proteins following Periodontal Therapy

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Abstract: Periodontitis (PD) shows an association with rheumatoid arthritis (RA) and systemic inflammation. Periodontal pathogens, namely *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, are proposed to be capable of inducing citrullination of peptides in the gingiva, inducing the formation of anti-citrullinated protein antibodies (ACPAs) within susceptible hosts. Here, we sought to investigate whether periodontal treatment influenced systemic inflammation and antibody titres to *P. gingivalis*, *A. actinomycetemcomitans*, *Prevotella intermedia* and ACPA in 42 systemically health patients with periodontal disease. Subgingival plaque and serum samples were collected from study participants before (baseline) and 90 days after treatment to analyse the abundance of specific bacteria and evaluate anti-bacterial antibodies, C-reactive protein (CRP), tumour necrosis factor α (TNF-α), interleukin 6 (IL-6) and ACPA in serum. Following treatment, all patients showed reduced periodontal inflammation. Despite observing a weak positive correlation between CRP and IL-6 with periodontal inflammation at baseline, we observed no significant reductions in any indicators of systemic inflammation 90 days after treatment. In contrast, anti-*P. gingivalis* IgG significantly reduced post-treatment ($p < 0.001$, Wilcoxon signed rank test), although no changes were observed for other antibody titres. Patients who had detectable *P. gingivalis* in subgingival plaques had significantly higher anti-*P. gingivalis* IgG and ACPA titres, suggesting a potential association between *P. gingivalis* colonisation and systemic antibody titres.

Keywords: periodontitis; periodontal therapy; *Porphyromonas gingivalis*; *Aggregatibacter actinomycetemcomitans*; anti-citrullinated protein antibodies; subgingival plaque; inflammation

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Citation: Davison, E.; Johnston, W.; Piela, K.; Rosier, B.T.; Paterson, M.; Mira, A.; Culshaw, S. The Subgingival Plaque Microbiome, Systemic Antibodies against Bacteria and Citrullinated Proteins following Periodontal Therapy. *Pathogens* **2021**, *10*, 193. https://doi.org/10.3390/pathogens10020193

Academic Editors: Jan Potempa and Anna Maria Łasica
Received: 30 December 2020
Accepted: 4 February 2021
Published: 10 February 2021

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1. Introduction

Periodontitis (PD) is a chronic inflammatory disease characterised by diverse dysbiotic microbial communities and an aberrant immune response, resulting in irreversible destruction of the tooth supporting apparatus and ultimately tooth loss [1]. Although previously thought to be limited to the oral cavity, PD has been linked to systemic diseases with complex pathogenesis involving inflammation and immune dysregulation, such as diabetes, cardiovascular disease and rheumatoid arthritis (RA) [2–4]. However, the biological mechanisms underlying these links remain incompletely understood.

In PD, there is a marked shift in the composition of the subgingival plaque microbiome compared with the microbial community found in periodontal health. This shift likely reflects the inflammatory milieu that changes the environmental conditions, and the

presence of key periodontitis-associated bacteria that directly affect the behaviour of neighboring organisms [5]. Such species include *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. These key pathogens cluster to form discrete bacterial complexes that can remodel the healthy microbial population into dysbiosis [6].

RA is a debilitating autoimmune disease resulting in joint destruction, with systemic effects that ultimately results in a 54% higher observed mortality rate compared to that of the general population [7]. Both RA and PD share genetic and environmental risk factors, as well as exhibiting similar pathophysiological processes. Both diseases involve cellular infiltration at an inflammatory focus, express similar cytokine profiles, involve activation of matrix metalloproteinase and result in irreversible destruction of bone and connective tissue [8]. In RA, cytokines including interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) play a pivotal role in driving systemic inflammation and acute phase proteins, such as C-reactive protein (CRP), become increased and can be used diagnostically [9]. Autoantibodies known as anti-citrullinated peptide antibodies (ACPAs) target peptides in the joints, which have been modified to contain citrulline [10]. ACPAs are an important predictive factor for the development of RA, as well as the disease progression and prognosis of the disease [11]. ACPAs can be detected several years prior to the clinical onset of RA, suggesting that the joints may not be the triggering site for autoantibody generation [12–14]. ACPAs have been detected in PD patients and studies have suggested an association between PD and citrullination of host and bacterial peptides in the gingiva [15,16]. *P. gingivalis* and *A. actinomycetemcomitans* have been identified as possible bacterial triggers for the formation of ACPAs as both bacteria have been shown to expose the immune system to citrullinated epitopes. It has been hypothesised that in genetically predisposed patients, exposure to citrullination in the gingival tissues could induce a breach of immunological tolerance, resulting in the development of autoantibodies [16–18].

The presence of serum anti-bacterial IgG antibodies directed against periodontal pathogens has been suggested as a potential serological diagnostic tool for PD [19] Several studies have shown elevated levels of these antibodies in patients with PD compared with healthy controls, especially directed against *P. gingivalis* [20–22]. In contrast, others suggest anti-bacterial antibodies directed against periodontal pathogens are only modestly associated with PD [20]. Furthermore, the true sensitivity and specificity of the assays employed to detect such antibodies remains an area of controversy, and it is currently unclear whether these antibodies indicate periodontal disease status, current exposure, previous exposure or possibly cross-reaction with other bacterial epitopes, and thus their diagnostic use remains limited [19–22].

Non-surgical periodontal therapy (NST) involves the mechanical debridement of supra- and subgingival plaque and aims to disrupt microbial biofilms and reduce inflammation. NST has been shown to be an effective method of improving clinical periodontal parameters [23], and there is a noted increase in presence of health-associated bacteria following treatment [24,25]. The complex relationships between the oral microbiome, local inflammation in the mouth, systemic inflammatory and antibody responses remain poorly understood. Understanding how these factors interact could help understand the link between periodontitis and systemic diseases and potentially identify novel preventative or therapeutic opportunities. We sought to investigate whether—alongside changing clinical and microbiological changes following periodontal treatment —there are changes in systemic inflammation, anti-bacterial antibody titres, and anti-citrullinated protein antibody titres. The aim of this longitudinal study was to investigate the effect of routine NST on systemic anti-bacterial titre, markers of systemic inflammation, ACPA titre and the subgingival plaque microbiome, and explore any associations between these parameters in a cohort of patients with PD. Patients with PD only, rather than PD and RA, were chosen to avoid the confounding factors associated with RA-related treatments.

2. Results

2.1. Study Cohort and Clinical Improvement

Thirty females and 12 males received NST with varying numbers of treatment visits (ranging from one to six). Across all patients, NST resulted in significant improvement in all periodontal parameters. This included reductions in periodontal pocket depth (median (Q1, Q3): 3.59 mm (3.08–4.16) to 2.70 mm (2.56–3.02), $p < 0.001$ Wilcoxon Signed Rank test) and percentage of pockets ≥5 mm (27.50% (18.75–41.75) to 9.00% (5.75–12.25), $p < 0.001$ Wilcoxon Signed Rank test), with a treatment response commensurate with a recently published systematic review [23]. Similar improvements were observed for the periodontal inflamed surface area (PISA) (1224.60 mm^2 (754.80–1687.08) to 138.85 mm^2 (65.52–293.88), $p < 0.001$ Wilcoxon Signed Rank test), which was subsequently used as an indicator of clinical inflammation.

2.2. Association between Clinical Parameters and Systemic Markers

Prior to treatment, we observed a large spread in the baseline disease severity of included patients. For example, the percentage of pockets ≥5 mm ranged from 7.00% to 65.00%, and PISA values ranged from 45.60 mm^2 to 3655.90 mm^2 across all patients. Given this heterogeneity, we investigated whether systemic inflammatory markers and antibody titres were associated with periodontitis severity (Figure 1). There were weak positive associations between CRP and several periodontal indices. This included full-mouth plaque index (r = 0.368, 95% CI: 0.063 to 0.612, $p = 0.016$), percentage of pockets ≥5 mm (r = 0.357, 95% CI: 0.050 to 0.602, $p = 0.020$) and PISA (r = 0.346, 95% CI: 0.038 to 0.594, $p = 0.025$). Similarly, a weak association was observed between IL-6 and PISA (r = 0.344, 95% CI: 0.036 to 0.593, $p = 0.025$). Interestingly, a weak negative correlation was observed between anti-*A. actinomycetemcomitans* IgG and PISA (r = −0.315, 95% CI: −0.574 to 0.001, $p = 0.045$), while a weak positive correlation was observed between ACPA and full-mouth plaque score (FMPS) (r = 0.328, 95% CI: 0.017 to 0.581, $p = 0.034$). However, following correction and adjustment for multiple comparisons, no association remained statistically significant (all p (adjusted) > 0.05). Notably, there were no significant associations between IgG antibodies directed against *P. gingivalis* and clinical indices.

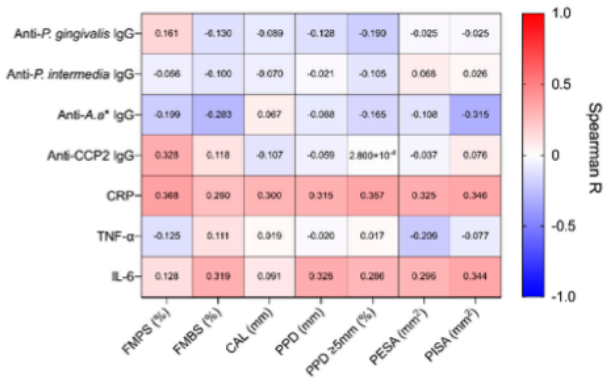

Figure 1. Correlation between systemic markers and periodontal clinical parameters at baseline. Correlations were performed using Spearman–Rho method, with individual R values shown above. P-values were adjusted for multiple comparisons using the False Discovery Rate (5%) method, no significant correlations were found following this adjustment. FMPS, full-mouth plaque score; FMBS, full-mouth bleeding score; CAL, clinical attachment level; PPD, periodontal probing depth, PPD ≥ 5 mm; percentage of pockets ≥ 5 mm; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area.

2.3. Longitudinal Alterations in Systemic Inflammatory Markers

Having observed a weak relationship between periodontal and systemic inflammation, we next sought to assess whether NST impacted systemic inflammatory markers (CRP, IL-6, TNF-α). However, despite consistent reductions in periodontal inflammation, we observed no significant change in any systemic marker following treatment (Figure 2A–C). Given that patients within this study displayed varying degrees of disease severity at baseline, and as such, the reduction in periodontal inflammation varied following treatment, we hypothesized that these results may be skewed by patients with only a small change in their periodontal inflammation. To evaluate whether this impacted alterations in systemic inflammatory markers, we split patients into mild/moderate and severe disease based on PISA [26]. Patients with severe disease at baseline showed significantly greater reduction in PISA after treatment, indicating a larger reduction in local inflammatory load (Figure 2D). Consistent with previous results, those with severe disease had significantly higher levels of CRP at baseline (Figure 2E, $p = 0.008$ Mann–Whitney test). However, to our surprise, there were no significant changes in CRP following periodontal treatment in either the severe disease group or in the mild/moderate disease group (Figure 2E–G).

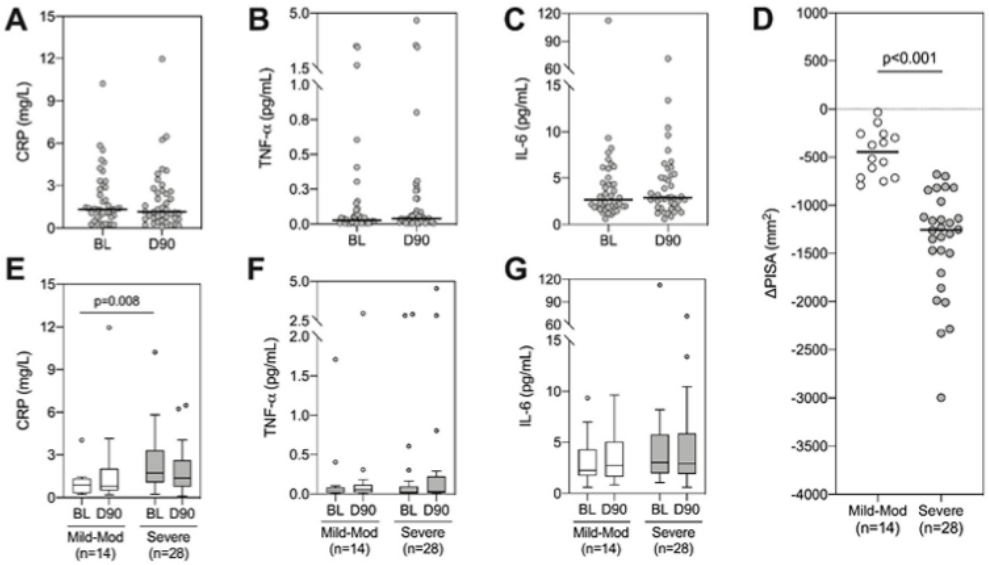

Figure 2. Longitudinal analysis of systemic inflammatory markers following periodontal therapy. (**A**) CRP, (**B**) TNF-α and (**C**) IL-6 were quantified at baseline (BL) and day 90 (D90). Wilcoxon signed rank tests were performed, all $p > 0.05$. (**D**) Patients were split into mild-moderate or severe based on their baseline PISA value [26] and the change in PISA following treatment is displayed, $p < 0.001$ using Mann–Whitney test. Longitudinal analysis of CRP (**E**), TNF-α (**F**) and IL-6 (**G**) was analysed within each group using Wilcoxon signed rank tests (all $p > 0.05$), between-group comparisons refer to Mann–Whitney tests (raw p-values shown when $p < 0.05$).

2.4. Subgingival Plaque Colonisation by *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia*

We next assessed the relative abundance of three periodontal pathogens (*P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia*) in the subgingival plaque using 16S rRNA sequencing (Figure 3). Although all patients in this study had periodontitis, our data suggested varying colonization patterns by *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia*. At

baseline, 13 patients had detectable levels of *P. gingivalis* (31%), four had detectable levels of *A. actinomycetemcomitans* (9.5%) and 24 had detectable levels of *P. intermedia* (57%). In general, the proportions of these bacteria decreased 90 days after treatment, although this reduction was only statistically significant for *P. intermedia* (Figure 3C).

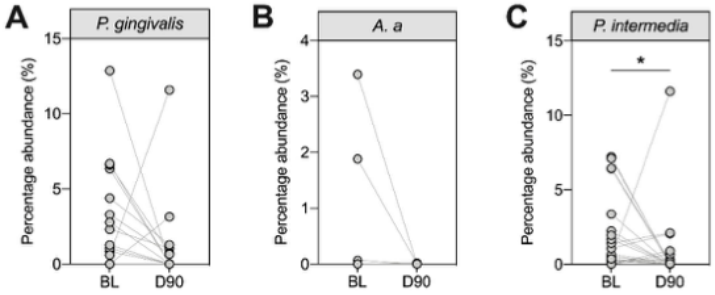

Figure 3. Longitudinal assessment of selected periodontal pathogens in subgingival plaque samples at baseline (BL) and day 90 (D90). Subgingival plaque samples from all patients were analysed at baseline and day 90 following non-surgical periodontal therapy (NST) for the proportion of *P. gingivalis* (**A**), *A. a* (**B**) and *P. intermedia* (**C**) using 16S rRNA sequencing (n = 42). Each dot shows one patient. Patients who were negative at BL and D90 were not included. Statistics refer to Wilcoxon signed rank tests where * $p < 0.05$. A. a: *Aggregatibacter actinomycetemcomitans*.

2.5. Longitudinal Alterations in Antibody Titres

Having identified varying colonization patterns of *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia*, we next sought to establish whether antibody titres directed against these organisms, and ACPAs, changed following treatment. We observed a significant reduction in anti-*P. gingivalis* IgG at day 90 (Figure 4A, $p < 0.001$ Wilcoxon Signed Rank test). In contrast, there were no significant changes in antibody titres for anti-*P. intermedia*, anti-*A. actinomycetemcomitans* (Figure 4B,C), $p > 0.05$ for all, Wilcoxon Signed Rank test). Using the ACPA assay diagnostic positivity threshold (25 AU/mL), we found that three patients were 'ACPA-positive' at baseline (7.14%), and six patients were positive at day 90 (14.29%) (Figure 4D). Only anti-*P. gingivalis* antibodies showed a reduction following treatment (Figure 4E).

2.6. Anti-Bacterial Antibodies and ACPAs according to Periodontal Disease Severity

Similar to analysis of systemic inflammatory markers, we evaluated whether baseline disease severity and the degree of inflammation reduction following treatment impacted anti-bacterial antibodies and ACPAs. Using the PISA grouping method described previously, levels of antibodies directed against *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans* and ACPAs were compared between 'mild-moderate' and 'severe' groups at baseline and day 90, and longitudinally within each group following treatment (Figure 5). Matching with analysis of the entire cohort, reductions in anti-*P. gingivalis* IgG were observed following treatment in both the mild-moderate ($p = 0.006$, Wilcoxon signed rank test) and severe groups ($p < 0.001$, Figure 5A). In contrast, no significant difference in anti-*P. gingivalis* IgG was observed between groups at baseline ($p = 0.35$, Mann–Whitney test) or day 90 ($p = 0.41$, Figure 5A). Interestingly, no longitudinal alterations were observed within groups for anti-*P. intermedia*, anti-*A. actinomycetemcomitans* or ACPAs, suggesting that a larger reduction in periodontal inflammation did not impact changes in antibody titres (Figure 5B–D). In line with the weak negative correlation between PISA and anti-*A. actinomycetemcomitans* IgG, a trend was observed whereby the severe PISA group showed lower anti-*A. actinomycetemcomitans* IgG, albeit this did not reach statistical significance at either timepoint ($p = 0.07$ at baseline, $p = 0.06$ at day 90, Mann–Whitney test, Figure 5C).

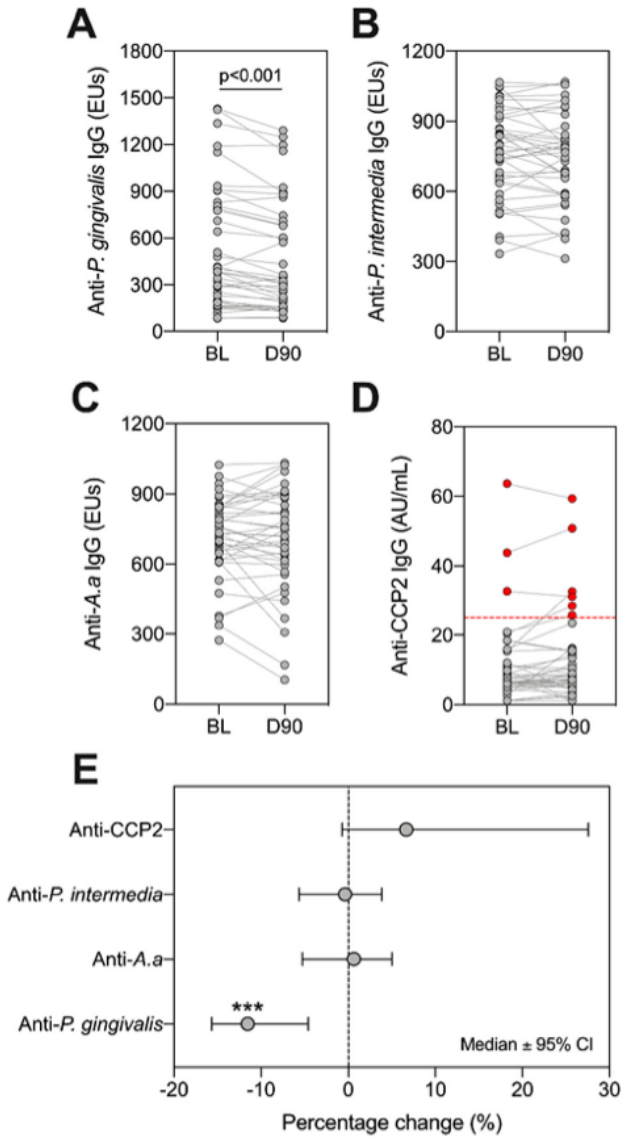

Figure 4. Assessing changes in serum IgG against ACPA and periodontal bacteria at baseline (BL) and day 90 (D90). Antibodies in serum reactive against *P. gingivalis* (**A**), *P. intermedia* (**B**), A.a (**C**) and CCP2 (**D**) were investigated. 'ACPA-positive' patients are highlighted as red dots, with the positivity threshold (25 AU/mL) indicated by dotted red line (**D**). Lines between points indicate the same patient. Statistics are Wilcoxon signed rank test, where *** $p < 0.001$. The percentage change across all patients is highlighted (**E**), where dots indicate medians and error bars are 95% confidence intervals. A and D: $n = 42$, B and C: $n = 41$. A. a: *Aggregatibacter actinomycetemcomitans*.

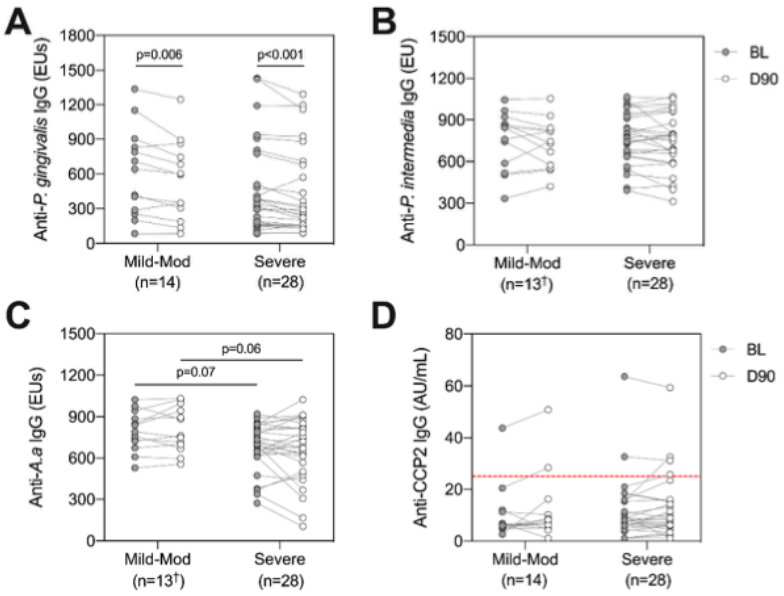

Figure 5. Investigating anti-bacterial antibodies and ACPAs at baseline (BL, grey circles) and day 90 (D90, white circles) according to PISA groups. Patients were split into mild-moderate (n = 14[†]) or severe (n = 28) based on their baseline PISA value [26]. Anti-bacterial antibodies directed against *P. gingivalis* (**A**), *P. intermedia* (**B**), *A. a* (**C**) and ACPAs (**D**) were compared between groups at each timepoint, and longitudinally within groups following NST. The ACPA positivity threshold (25 AU/mL) is indicated by dotted red line (D). Lines connect individual patients at each timepoint, between group comparisons refer to Mann–Whitney test, within group comparisons refer to Wilcoxon signed rank test. Raw p-values are displayed. A.a: *Aggregatibacter actinomycetemcomitans*. [†] One patient had limited serum volume and was not included in analysis of anti-*P. intermedia* or anti-*A.a* IgG, n = 13.

2.7. *P. gingivalis* Colonisation and Antibody Titres

Given differences in *P. gingivalis* colonization, we compared ACPA and anti-*P. gingivalis* IgG titre with subgingival *P. gingivalis* plaque status (positive or negative) and assessed whether levels of serum anti-*P. gingivalis* IgG titre were associated with ACPA titre at baseline (Figure 6). We found that patients positive for *P. gingivalis* in subgingival plaques had significantly higher anti-*P. gingivalis* (Figure 6A, p = <0.001, Mann–Whitney test), and ACPA titres (Figure 6B, p = 0.031, Mann-Whitney test). However, this was not observed in patients with *A. actinomycetemcomitans* and *P. intermedia* positivity in subgingival plaque (data not shown). When correlated among all patients, we observed no direct association between anti-*P. gingivalis* IgG and ACPA (Figure 6C, black lines). When assessing only *P. gingivalis* "seropositive" patients, this trend appeared stronger but remained non-significant (Figure 6C, red lines).

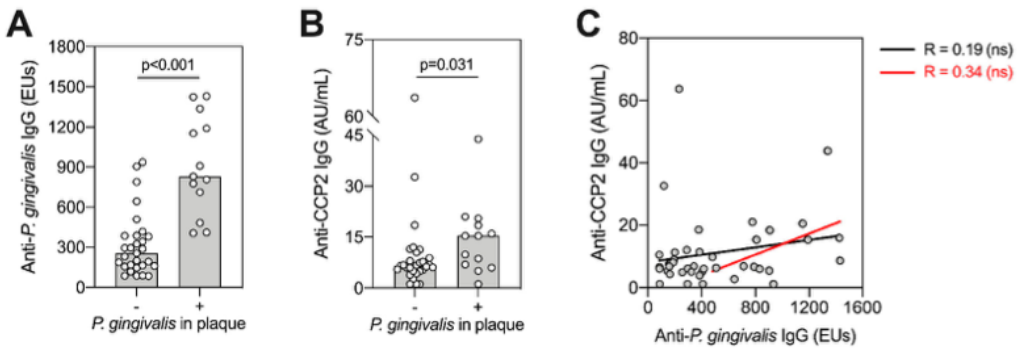

Figure 6. Association between ACPA and *P. gingivalis*. Patients were split according to whether *P. gingivalis* was detectable (+) or undetectable (-) using 16S rRNA sequencing (**A**,**B**). Bars display medians with individual values ($n = 42$). Comparisons between groups were performed using Mann–Whitney U-tests, with individual p-values shown. (**C**) Anti-*P. gingivalis* IgG antibodies were correlated against anti-CCP2 IgG antibodies using Spearman–Rho. This was performed across all patients (black line, $n = 42$), or including only seropositive patients (red line, $n = 16$), ns means no significant difference observed ($p > 0.05$). Seropositivity was determined as antibody titres at least two standard deviations above the mean antibody titre from an independent periodontally healthy control group, set at 435.07 ELISA units (EUs).

2.8. ACPA Association with Subgingival Plaque Microbiome

Finally, taking a non-bias approach, we investigated whether ACPA titres correlated with the relative abundance of any bacterial genera or species in the subgingival plaque at baseline or day 90 (Figure 7A,B). Assessing correlations among abundant species (see methods section), no positive or negative correlation was observed with serum ACPA titre (Figure 7B). A similar finding was obtained at genus-level (Figure 7A). In addition, no association was found between the abundance of species of interest in this study (*P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*) or with the taxonomic diversity of the microbiome at baseline or day 90.

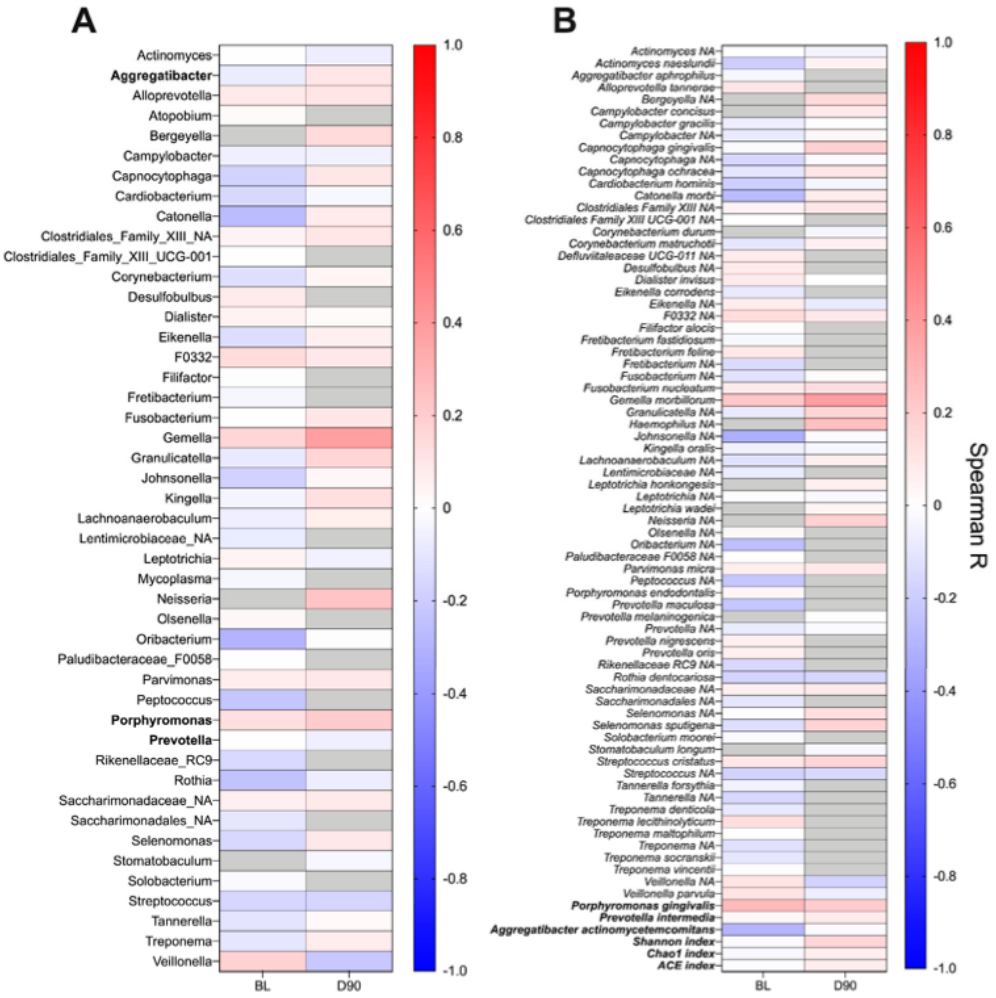

Figure 7. Heatmap presenting correlations between the relative abundance of genera (**A**) and species (**B**) in the subgingival plaque and serum ACPA titre. Correlation analysis was performed at baseline (BL) and day 90 (D90) using Spearman–Rho. Genera and species were included if they were present in ≥50% of samples at each timepoint. Grey boxes indicate organisms not meeting this abundance threshold and were thus not included in the correlation analysis. In addition, post-hoc correlation analysis was performed using species of interest in this study and alpha-diversity indexes (bold). No significant correlations were observed at either timepoint.

3. Discussion

This study uniquely investigated the relationship of the ACPA serum response with anti-bacterial serum antibodies and the subgingival plaque microbiome in a periodontitis cohort before and after NST. Our data suggest that *P. gingivalis* carriage may be associated with anti-*P. gingivalis* IgG antibodies and ACPAs; findings that support previous studies of

RA patients and their first-degree relatives (FDR-RA) cohorts, and supporting previously proposed links between *P. gingivalis* and ACPA [27–31].

Limited studies exist investigating ACPA in periodontitis patients following NST, (two to the authors' knowledge), and none have evaluated the entire subgingival plaque microbiota [27,32]. In our cohort, three patients were ACPA positive at baseline, and six patients were ACPA positive at day 90, representing 7.14% and 14.29% of patients respectively. This value is higher than the general population, where it is believed that ACPA positivity is roughly 2.8% using the same CCP2 assay [33]. However, in our cohort, the anti-CCP2 titre in ACPA-positive individuals is considerably lower compared with the average titre in RA-positive patients. It is recognised that the higher the ACPA titre, the more likely the individual is to be RA-positive or develop RA. The meaning of our low-titre ACPA-positive individuals therefore remains uncertain, and further follow up is needed to ascertain whether these ACPA-positive individuals may eventually go on to develop RA and how their ACPA titre varies over time. Longer term effect of NST on ACPA titre has never been documented. Previous studies have assessed ACPA titre at baseline and 6 months or 8 weeks only [27,32]. Periodontitis is a chronic lifelong condition with many patients relapsing. Further follow up could address this gap in the literature to investigate the long-term effects of periodontal therapy and maintenance on ACPA titre.

In contrast to previous studies, we did not observe a significant decrease in ACPA titre post-NST. Both Yang et al. and Lappin et al. conducted similar studies investigating the effect of NST on ACPA in PD patients following NST [27,32]. Both found ACPA titre significantly reduced; Yang in all patients and Lappin only in non-smokers. We also did not see a significant decrease in CRP, IL-6 or TNF-α, in contrast with Yang et al. where a decrease in TNF-α was observed. The follow up period for these studies was 8 weeks and 6 months compared with our timeframe of 90 days, and this could account for some differences between study results. Interestingly, we did observe a weak association between CRP/IL-6 and PISA prior to correction for multiple comparisons. It would be useful in further studies to increase the study population to discern whether this finding is significant. Similarly, for patients with high baseline PISA scores, CRP levels did not change despite substantial reductions in periodontal inflammation. It has been previously shown that changes in CRP post-treatment are most impactful in patients with co-morbidities [34]. Thus, it is possible that since our patients were generally fit and well, we were less likely to observe changes in serum CRP.

One limitation of our study is that we did not have a control group with periodontitis that did not have treatment, as this study focused on the effect on antibody titre and microbiome before and after NST. Furthermore, ACPA positivity has been previously related to the presence and severity of periodontitis [35]. It is possible that the association seen in literature reflects *P. gingivalis* carriage, rather than the severity of periodontal disease. To account for this, future studies should consider swabbing in areas such as tongue and tonsil for *P. gingivalis* carriage.

Our data suggest that *P. gingivalis* presence in subgingival plaques may influence ACPA titre. Although whole microbiome analysis did not identify a correlation between any particular species with ACPA, there appeared to be a weak correlation between ACPA titre and anti-*P. gingivalis* IgG, albeit not statistically significant. A recent meta-analysis showed that RA patients have an increased immune response to *P. gingivalis* reflected through increased anti-*P. gingivalis* IgG titre compared with healthy and PD controls [36]. *P. gingivalis* expresses the enzyme peptidylarginine deiminase (PPAD), a bacterial virulence factor unique to *P. gingivalis* that citrullinates epitopes [18]. PPAD is hypothesised to create a citrullinated antigen that acts as a systemic immunogen responsible for ACPA formation in PD patients. Further investigations should identify if PPAD activity correlates with ACPA in PD patients. As different *P. gingivalis* strains have different PPAD activity, there may be variation between patients that could influence ACPA titre [27]. ACPA-positive PD patients could also be tested for ACPA specificities, e.g., CEP, citFib, and a non-citrullinated native protein control, which our study lacks. This will help further determine whether

the ACPA-positive patients found in our PD cohort are specific for bacterial citrullinated epitopes, or are cross-reactive with human citrullinated peptide.

Our results indicate that the presence, rather than the relative abundance of *P. gingivalis* in subgingival plaques is associated with systemic antibody titres. Whilst the results from this current study are based on 16S rRNA analysis of a single site, the presence of *P. gingivalis* in subgingival plaque has been shown to associate with serum IgG antibodies directed against *P. gingivalis* and gingipains (RgpB) in several previous studies [37,38].

Our second key finding is that periodontal inflammation and anti-*P. gingivalis* IgG significantly decrease post-NST, and these findings are commensurate with previous results [30,39,40]. The literature on changes in anti-*P. intermedia* IgG and anti *A. actinomycetemcomitans* IgG are less consistent, and our data showed no significant change for either [19,39,41–43]. One possible reason for this is that anti-*A. actinomycetemcomitans* and anti-*P. intermedia* IgG titre reflects history of colonisation rather than active infection: only four patients tested positive for *A. actinomycetemcomitans* in subgingival plaques, but all patients had detectable levels of anti-*A. actinomycetemcomitans* IgG. This raises the question of the usefulness and accuracy of using serum antibodies to identify periodontal disease-associated bacteria. We observed no correlation between antibody titres and clinical disease severity, which is in contrast with previous studies [44]. There is not a single well-defined protocol for assays to detect anti-bacterial antibodies, with studies using single strains, multiple strains or bacterial virulence factors, such as PPAD, or LtxA for detection [45–47]. These different approaches present a challenge when comparing studies investigating ACPAs and serum antibodies. Until the clinical usefulness of anti-bacterial IgG is discerned and standardised, clinical examination remains the most accurate way to classify and identify periodontal disease.

In conclusion, the data presented show evidence for a relationship between *P. gingivalis* colonisation and ACPA titre. If ACPA positivity in periodontitis patients precedes the development of RA, this is an important clinical finding as patients could be identified to be treated earlier.

4. Materials and Methods

4.1. Study Population, Treatment and Clinical Examination

This study received ethical approval (REC reference: 14/LO/2064) and was conducted in accordance with the Declaration of Helsinki 2013. Forty-five patients were recruited for this study. This was based upon a pilot study, showing that the minimum detectable change in antibody titres pre- and post-treatment in a study population of 34 would be 0.5 SD (based on 80% power at the 5% significance level) [27]. Forty-two patients were included in analysis after excluding 3 individuals who were diagnosed with a systemic disease throughout the trial period. Patients suffering from PD, defined as probing pocket depths $\geq$5 mm on 2 or more teeth at non-adjacent sites, were selected for inclusion. Additional inclusion criteria included providing written informed consent and males or females aged $\geq$18 years, generally fit and well. Exclusion criteria included patients with known or suspected risk for arthritis, diabetes, tuberculosis, hepatitis B, HIV infections, history of bleeding diathesis, systemic antibiotics in last 3 months, requiring interpreter or non-English language written material to provide written informed consent.

The number of treatment visits varied and depended on patient preference for treatment schedule, and baseline disease severity. Periodontal parameters were assessed at baseline (BL) and 90 days following periodontal treatment (D90). Periodontal parameters were assessed at six sites per tooth, including full-mouth plaque scores (FMPS), full-mouth bleeding scores (FMPS), periodontal probing depths (PPD) and clinical attachment levels (CAL). Following measurements, the periodontal epithelial surface area (PESA) and periodontal inflamed surface area (PISA) were calculated as previously described [48] and used as indicators of periodontal inflammation. We classified 14 patients as having moderate periodontal disease and 22 having severe disease using PISA values based off a classification

method described by Leira [27]. Briefly, patients with PISA scores of >934.71 mm^2 were classed as severe, 521.56–934.71 mm^2 as moderate and <531.56 mm^2 as mild.

4.2. Sample Collection and Processing

Subgingival plaque and serum samples were collected prior to periodontal measurements at baseline and day 90 as previously described [49]. Plaque was collected from the deepest pocket in each quadrant using a curette. For serum samples, blood was collected by venepuncture into sterile collection tubes (Vacuette, Greiner bio-one, Gloucestershire, UK) and centrifuged at 2500 RPM for 10 min. All samples were stored at −80 °C until analysis.

4.3. CCP2 ELISA

ACPAs in serum was tested at baseline and day 90 using the commercially available CCP2 ELISA kit Immunoscan CCPlus® (EuroDiagnostica AB, Malmö, Sweden) in accordance with manufacturer's instructions. All samples were assayed in duplicate, and the average value was taken to calculate ACPA titres. The CCP2 assay has a diagnostic cut off for positivity at >25 U/mL. The limit of detection (LOD) for this assay was 2.295 AU/mL, calculated using the blank mean + SD*5. ACPA was <LOD in 7 samples (4 at baseline, 3 at day 90), which were given as LOD/2 (1.14743923) for statistical analysis.

4.4. Anti-Bacterial Antibody Titres

Serum IgG antibody titres against periodontal bacteria (*P. gingivalis* W83, *P. intermedia* ATCC 25611, and *A. actinomycetemcomitans* ATCC 43718) were determined as outlined previously with minor modifications [27,50]. In brief, Immulon 1B low-binding microtitre plates (Thermofisher, Loughborough, UK) were coated overnight with heat-killed organisms in carbonate bicarbonate buffer pH 9.6 (Sigma Aldrich, Gillingham, UK). Coating concentrations of each organism were determined using healthy and periodontitis control serum and were selected based on maximising signal-to-noise ratio. After coating, wells were washed and blocked using 0.1% foetal bovine serum in PBS (Gibco, Thermofisher, Loughborough, UK). Serum dilutions were then added to wells ranging from 1/50 to 1/400 for patient and control samples, with each sample being assayed in duplicate. Bound antibody was detected using biotin conjugated anti-human IgG antibodies, extravidin peroxidase and TMB substrate (Sigma Aldrich, Gillingham, UK). Antibody titres are presented as ELISA units calculated as previously described [27,50].

4.5. Analysis of Inflammatory Markers

Levels of serum CRP were determined by immunoturbidometry using the Cobas C311 analyser (Cobas, Roche Diagnostic, Mannheim, Germany). Serum IL-6 and TNF-α were determined using high-sensitivity ProQuantum immunoassays, performed using a StepOne plus real-time PCR analyser (Thermofisher, Loughborough, UK). Each sample was assayed in duplicate. Limit of detection (LOD) for ProQuantum assays were: IL-6 (0.119 pg/mL) and TNF-α (0.012 pg/mL). IL-6 and CRP were detected in all samples (n = 84). TNF-α was <LOD in 17 samples (10 at baseline, 7 at day 90). For statistical analysis, these values were given as LOD/2.

4.6. Bacterial 16S rRNA Sequencing

One site from each patient was selected for 16S rRNA sequencing, with subgingival plaque the same site being analysed at baseline and day 90. DNA from subgingival plaque collected from all patients at baseline and day 90 was extracted using the MagNA Pure LC DNA isolation kit (Roche Diagnostics, Mannheim, Germany), and measured with the QubitTM 3 Fluorometer (Thermofisher, Loughborough, UK), and then sequenced using an Illumina MiSeq sequencer and taxonomy assigned as reported elsewhere [49,51].

4.7. Data Analysis

Statistical analyses of clinical characteristics, serum inflammatory markers and anti-bacterial antibody titres were performed using GraphPad PRISM version 8.0 (La Jolla, California, CA, USA). For comparison of clinical parameters at baseline and day 90, Wilcoxon sign rank test was used for non-normally distributed data. For comparison of ACPA and anti-bacterial antibody titres, Wilcoxon Signed Rank tests were used. For correlation analysis between clinical characteristics, ACPA and anti-bacterial antibody titres, Spearman–Rho correlation coefficient was used. For analysis of ACPA and anti-bacterial antibody titres with subgingival plaque positivity and ACPA with anti-bacterial antibody positivity, Mann–Whitney U-test was used. Correlations between abundant genera and species and ACPA titres were performed in R (version 3.4) using Spearman–Rho correlation coefficient. Abundant genera and species were defined as those present in at least 50% of samples at baseline (relevant for baseline correlations) or day 90 (relevant for day 90 correlations), with an abundance greater than five times the smallest percentage above zero.

Author Contributions: E.D., W.J., K.P. and M.P. performed sample processing and laboratory experimental work and aided in manuscript preparation. E.D., W.J., K.P. and M.P. performed clinical data acquisition and data analysis. W.J., B.T.R., A.M. and S.C. were responsible for 16S rRNA sequencing data acquisition and analysis. S.C. and A.M. conceived the study, participated in study design and produced the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by grants from EU Marie Curie ITN RAPID (grant number 290246) and Versus Arthritis (grant number 20823).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, with ethical approval (London-Stanmore Research Ethics Committee, Reference: 14/LO/2064).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is available upon reasonable request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest for this manuscript.

References

1. Hajishengallis, G. Periodontitis: From microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* **2015**, *15*, 30–44. [CrossRef] [PubMed]
2. Sanz, M.; Del Castillo, A.M.; Jepsen, S.; Gonzalez-Juanatey, J.R.; D'Aiuto, F.; Bouchard, P.; Chapple, I.; Dietrich, T.; Gotsman, I.; Graziani, F.; et al. Periodontitis and cardiovascular diseases: Consensus report. *J. Clin. Periodontol.* **2020**, *47*, 268–288. [CrossRef] [PubMed]
3. Potempa, J.; Mydel, P.; Koziel, J. The case for periodontitis in the pathogenesis of rheumatoid arthritis. *Nat. Rev. Rheumatol.* **2017**, *13*, 606–620. [CrossRef]
4. Ide, R.; Hoshuyama, T.; Wilson, D.; Takahashi, K.; Higashi, T. Periodontal Disease and Incident Diabetes. *J. Dent. Res.* **2010**, *90*, 41–46. [CrossRef] [PubMed]
5. Mira, A.; Simon-Soro, A.; Curtis, M.A. Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J. Clin. Periodontol.* **2017**, *44*, S23–S38. [CrossRef]
6. Hajishengallis, G.; Darveau, R.P.; Curtis, M.A. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* **2012**, *10*, 717–725. [CrossRef]
7. Hoek, J.V.D.; Boshuizen, H.C.; Roorda, L.D.; Tijhuis, G.J.; Nurmohamed, M.T.; Bos, G.A.M.V.D.; Dekker, J. Mortality in patients with rheumatoid arthritis: A 15-year prospective cohort study. *Rheumatol. Int.* **2017**, *37*, 487–493. [CrossRef]
8. Culshaw, S.; McInnes, I.; Liew, F.Y. What can the periodontal community learn from the pathophysiology of rheumatoid arthritis? *J. Clin. Periodontol.* **2011**, *38*, 106–113. [CrossRef]
9. Anderson, J.; Caplan, L.; Yazdany, J.; Robbins, M.L.; Neogi, T.; Michaud, K.; Saag, K.G.; O'Dell, J.R.; Kazi, S. Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice. *Arthritis Rheumatol.* **2012**, *64*, 640–647. [CrossRef]
10. Firestein, G.S.; McInnes, I.B. Immunopathogenesis of Rheumatoid Arthritis. *Immunity* **2017**, *46*, 183–196. [CrossRef]
11. Rocha, S.D.B.; Baldo, D.C.; Andrade, L.E.C. Clinical and pathophysiologic relevance of autoantibodies in rheumatoid arthritis. *Adv. Rheumatol.* **2019**, *59*, 2. [CrossRef]

12. Bizzaro, N.; Bartoloni, E.; Morozzi, G.; Manganelli, S.; Riccieri, V.; Sabatini, P.; Filippini, M.; Tampoia, M.; Afeltra, A.; Sebastiani, G.D.; et al. Anti-cyclic citrullinated peptide antibody titer predicts time to rheumatoid arthritis onset in patients with undifferentiated arthritis: Results from a 2-year prospective study. *Arthritis Res. Ther.* **2013**, *15*, R16. [CrossRef]
13. Dahlqvist, S.R.; De Jong, B.A.W.; Berglin, E.; Hallmans, G.; Wadell, G.; Stenlund, H.; Sundin, U.; Van Venrooij, W.J. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheumatol.* **2003**, *48*, 2741–2749. [CrossRef]
14. Guo, Q.; Wang, Y.; Xu, D.; Nossent, J.; Pavlos, N.J.; Xu, J. Rheumatoid arthritis: Pathological mechanisms and modern pharmacologic therapies. *Bone Res.* **2018**, *6*, 1–14. [CrossRef]
15. Nesse, W.; Westra, J.; Van Der Wal, J.E.; Abbas, F.; Nicholas, A.P.; Vissink, A.; Brouwer, E. The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation. *J. Clin. Periodontol.* **2012**, *39*, 599–607. [CrossRef] [PubMed]
16. Wegner, N.; Wait, R.; Sroka, A.; Eick, S.; Nguyen, K.-A.; Lundberg, K.; Kinloch, A.; Culshaw, S.; Potempa, J.; Venables, P.J. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: Implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheumatol.* **2010**, *62*, 2662–2672. [CrossRef] [PubMed]
17. Konig, M.F.; Abusleme, L.; Reinholdt, J.; Palmer, R.J.; Teles, R.P.; Sampson, K.; Rosen, A.; Nigrovic, P.A.; Sokolove, J.; Giles, J.T.; et al. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. *Sci. Transl. Med.* **2016**, *8*, 369ra176. [CrossRef] [PubMed]
18. McGraw, W.T.; Potempa, J.; Farley, D.; Travis, J. Purification, Characterization, and Sequence Analysis of a Potential Virulence Factor from Porphyromonas gingivalis, Peptidylarginine Deiminase. *Infect. Immun.* **1999**, *67*, 3248–3256. [CrossRef] [PubMed]
19. Papapanou, P.N.; Neiderud, A.-M.; Papadimitriou, A.; Sandros, J.; Dahlén, G. "Checkerboard" Assessments of Periodontal Microbiota and Serum Antibody Responses: A Case-Control Study. *J. Periodontol.* **2000**, *71*, 885–897. [CrossRef]
20. Pussinen, P.J.; Könönen, E.; Paju, S.; Hyvärinen, K.; Gursoy, U.K.; Huumonen, S.; Knuuttila, M.; Suominen, A.L. Periodontal pathogen carriage, rather than periodontitis, determines the serum antibody levels. *J. Clin. Periodontol.* **2011**, *38*, 405–411. [CrossRef]
21. Vlachojannis, C.; Dye, B.A.; Herrera-Abreu, M.; Pikdöken, L.; Lerche-Sehm, J.; Pretzl, B.; Celenti, R.; Papapanou, P.N. Determinants of serum IgG responses to periodontal bacteria in a nationally representative sample of US adults. *J. Clin. Periodontol.* **2010**, *37*, 685–696. [CrossRef]
22. Dye, B.A.; Herrera-Abreu, M.; Lerche-Sehm, J.; Vlachojannis, C.; Pikdöken, L.; Pretzl, B.; Schwartz, A.; Papapanou, P.N. Serum Antibodies to Periodontal Bacteria as Diagnostic Markers of Periodontitis. *J. Periodontol.* **2009**, *80*, 634–647. [CrossRef]
23. Suvan, J.; Leira, Y.; Moreno, F.; Graziani, F.; Derks, J.; Tomasi, C. Subgingival instrumentation for treatment of periodontitis. A systematic review. *J. Clin. Periodontol.* **2019**, *47*, 155–175. [CrossRef]
24. Chen, C.; Hemme, C.; Beleno, J.; Shi, Z.J.; Ning, D.; Qin, Y.; Tu, Q.; Jorgensen, M.; He, Z.; Wu, L.; et al. Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J.* **2018**, *12*, 1210–1224. [CrossRef] [PubMed]
25. Shi, B.; Chang, M.; Martin, J.; Mitreva, M.; Lux, R.; Klokkevold, P.; Sodergren, E.; Weinstock, G.M.; Haake, S.K.; Li, H. Dynamic Changes in the Subgingival Microbiome and Their Potential for Diagnosis and Prognosis of Periodontitis. *MBio* **2015**, *6*. [CrossRef]
26. Leira, Y.; Martín-Lancharro, P.; Blanco, J. Periodontal inflamed surface area and periodontal case definition classification. *Acta Odontol. Scand.* **2017**, *76*, 195–198. [CrossRef]
27. Lappin, D.F.; Apatzidou, D.; Quirke, A.-M.; Oliver-Bell, J.; Butcher, J.P.; Kinane, D.F.; Riggio, M.P.; Venables, P.; McInnes, I.B.; Culshaw, S. Influence of periodontal disease, Porphyromonas gingivalis and cigarette smoking on systemic anti-citrullinated peptide antibody titres. *J. Clin. Periodontol.* **2013**, *40*, 907–915. [CrossRef]
28. Mikuls, T.R.; Payne, J.B.; Yu, F.; Thiele, G.M.; Reynolds, R.J.; Cannon, G.W.; Markt, J.; McGowan, D.; Kerr, G.S.; Redman, R.S.; et al. Periodontitis andPorphyromonas gingivalisin Patients with Rheumatoid Arthritis. *Arthritis Rheumatol.* **2014**, *66*, 1090–1100. [CrossRef]
29. Mikuls, T.R.; Thiele, G.M.; Deane, K.D.; Payne, J.B.; O'Dell, J.R.; Yu, F.; Sayles, H.; Weisman, M.H.; Gregersen, P.K.; Buckner, J.H.; et al. Porphyromonas gingivalisand disease-related autoantibodies in individuals at increased risk of rheumatoid arthritis. *Arthritis Rheumatol.* **2012**, *64*, 3522–3530. [CrossRef]
30. Okada, M.; Kobayashi, T.; Ito, S.; Yokoyama, T.; Abe, A.; Murasawa, A.; Yoshie, H. Periodontal Treatment Decreases Levels of Antibodies toPorphyromonas gingivalisand Citrulline in Patients with Rheumatoid Arthritis and Periodontitis. *J. Periodontol.* **2013**, *84*, e74–e84. [CrossRef]
31. Shimada, A.; Kobayashi, T.; Ito, S.; Okada, M.; Murasawa, A.; Nakazono, K.; Yoshie, H. Expression of anti-Porphyromonas gingivalis peptidylarginine deiminase immunoglobulin G and peptidylarginine deiminase-4 in patients with rheumatoid arthritis and periodontitis. *J. Periodontal Res.* **2016**, *51*, 103–111. [CrossRef] [PubMed]
32. Yang, N.-Y.; Wang, C.; Chyuan, I.-T.; Wu, K.-J.; Tu, Y.-K.; Chang, C.-W.; Hsu, P.N.; Kuo, M.Y.-P.; Chen-Ying, W. Significant association of rheumatoid arthritis-related inflammatory markers with non-surgical periodontal therapy. *J. Formos. Med. Assoc.* **2018**, *117*, 1003–1010. [CrossRef] [PubMed]
33. Hensvold, A.H.; Frisell, T.; Magnusson, P.K.E.; Holmdahl, R.; Askling, J.; Catrina, A.I. How well do ACPA discriminate and predict RA in the general population: A study based on 12 590 population-representative Swedish twins. *Ann. Rheum. Dis.* **2016**, *76*, 119–125. [CrossRef]

34. Teeuw, W.J.; Slot, D.E.; Susanto, H.; Gerdes, V.E.A.; Abbas, F.; D'Aiuto, F.; Kastelein, J.J.P.; Loos, B.G. Treatment of periodontitis improves the atherosclerotic profile: A systematic review and meta-analysis. *J. Clin. Periodontol.* **2014**, *41*, 70–79. [CrossRef]
35. Loutan, L.; Alpízar-Rodríguez, D.; Courvoisier, D.S.; Finckh, A.; Mombelli, A.; Giannopoulou, C. Periodontal status correlates with anti-citrullinated protein antibodies in first-degree relatives of individuals with rheumatoid arthritis. *J. Clin. Periodontol.* **2019**, *46*, 690–698. [CrossRef]
36. Bender, P.; Bürgin, W.B.; Sculean, A.; Eick, S. Serum antibody levels against Porphyromonas gingivalis in patients with and without rheumatoid arthritis—A systematic review and meta-analysis. *Clin. Oral Investig.* **2017**, *21*, 33–42. [CrossRef]
37. Seror, R.; Le Gall-David, S.; Bonnaure-Mallet, M.; Schaeverbeke, T.; Cantagrel, A.; Minet, J.; Gottenberg, J.-E.; Chanson, P.; Ravaud, P.; Mariette, X. Association of Anti-Porphyromonas gingivalisAntibody Titers with Nonsmoking Status in Early Rheumatoid Arthritis: Results from the Prospective French Cohort of Patients with Early Rheumatoid Arthritis. *Arthritis Rheumatol.* **2015**, *67*, 1729–1737. [CrossRef]
38. Fisher, A.B.; Cartwright, A.; Quirke, A.-M.; De Pablo, P.; Romaguera, D.; Panico, S.; Mattiello, A.; Gavrila, D.; Navarro, C.; Sacerdote, C.; et al. Smoking, Porphyromonas gingivalis and the immune response to citrullinated autoantigens before the clinical onset of rheumatoid arthritis in a Southern European nested case–control study. *BMC Musculoskelet. Disord.* **2015**, *16*, 1–11. [CrossRef]
39. Horibe, M.; Watanabe, H.; Ishikawa, I. Effect of periodontal treatments on serum IgG antibody titers against periodontopathic bacteria. *J. Clin. Periodontol.* **1995**, *22*, 510–515. [CrossRef]
40. Kudo, C.; Naruishi, K.; Maeda, H.; Abiko, Y.; Hino, T.; Iwata, M.; Mitsuhashi, C.; Murakami, S.; Nagasawa, T.; Nagata, T.; et al. Assessment of the Plasma/Serum IgG Test to Screen for Periodontitis. *J. Dent. Res.* **2012**, *91*, 1190–1195. [CrossRef]
41. Darby, I.B.; Mooney, J.; Kinane, D.F. Changes in subgingival microflora and humoral immune response following periodontal therapy. *J. Clin. Periodontol.* **2001**, *28*, 796–805. [CrossRef] [PubMed]
42. Lange, L.; Thiele, G.M.; McCracken, C.E.; Wang, G.; Ponder, L.A.; Angeles-Han, S.T.; Rouster-Stevens, K.A.; Hersh, A.O.; Vogler, L.B.; Bohnsack, J.F.; et al. Symptoms of periodontitis and antibody responses to Porphyromonas gingivalis in juvenile idiopathic arthritis. *Pediatr. Rheumatol.* **2016**, *14*, 8. [CrossRef]
43. Lakio, L.; Antinheimo, J.; Paju, S.; Buhlin, K.; Pussinen, P.J.; Alfthan, G. Tracking of plasma antibodies againstAggregatibacter actinomycetemcomitansandPorphyromonas gingivalisduring 15 years. *J. Oral Microbiol.* **2009**, *1*, 1. [CrossRef] [PubMed]
44. Naito, Y.; Okuda, K.; Takazoe, I.; Watanabe, H.; Ishikawa, I. The Relationship between Serum IgG Levels to Subgingival Gram-negative Bacteria and Degree of Periodontal Destruction. *J. Dent. Res.* **1985**, *64*, 1306–1310. [CrossRef]
45. Damgaard, C.; Reinholdt, J.; Enevold, C.; Fiehn, N.-E.; Nielsen, C.H.; Holmstrup, P. Immunoglobulin G antibodies against Porphyromonas gingivalis or Aggregatibacter actinomycetemcomitans in cardiovascular disease and periodontitis. *J. Oral Microbiol.* **2017**, *9*, 1374154. [CrossRef]
46. Kharlamova, N.; Jiang, X.; Sherina, N.; Potempa, B.; Israelsson, L.; Quirke, A.-M.; Eriksson, K.; Yucel-Lindberg, T.; Venables, P.J.; Potempa, J.; et al. Antibodies toPorphyromonas gingivalisIndicate Interaction Between Oral Infection, Smoking, and Risk Genes in Rheumatoid Arthritis Etiology. *Arthritis Rheumatol.* **2016**, *68*, 604–613. [CrossRef]
47. Sjöström, K.; Ou, J.; Whitney, C.; Johnson, B.; Darveau, R.; Engel, D.; Page, R.C.; Myers, B.; Pharmaceutical, S. Effect of Treatment on Titer, Function, and Antigen Recognition of Serum Antibodies to Actinobacillus Actinomycetemcomitans in Patients with Rapidly Progressive Periodontitis. *Inject. Immun.* **1994**, *62*, 145–151. [CrossRef]
48. Nesse, W.; Abbas, F.; Van Der Ploeg, I.; Spijkervet, F.K.L.; Dijkstra, P.U.; Vissink, A. Periodontal inflamed surface area: Quantifying inflammatory burden. *J. Clin. Periodontol.* **2008**, *35*, 668–673. [CrossRef]
49. Johnston, W.; Rosier, B.T.; Artacho, A.; Paterson, M.; Piela, K.; Delaney, C.; Brown, J.L.; Ramage, G.; Mira, A.; Culshaw, S. Mechanical biofilm disruption causes microbial and immunological shifts in periodontitis patients. *Sci. Rep.* **2021**. Under Review.
50. Mooney, J.; Adonogianaki, E.; Kinane, D.F. Relative avidity of serum antibodies to putative periodontopathogens in periodontal disease. *J. Periodontal Res.* **1993**, *28*, 444–450. [CrossRef]
51. Rosier, B.T.; Buetas, E.; Moya-Gonzalvez, E.M.; Artacho, A.; Mira, A. Nitrate as a Potential Prebiotic for the Oral Microbiome. *Sci. Rep.* **2020**, *10*, 12895. [CrossRef]

Appendix ii (chapter 3 manuscript)

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Mechanical biofilm disruption causes microbial and immunological shifts in periodontitis patients

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Periodontitis is characterized by subgingival biofilm dysbiosis, inflammation and tissue destruction. Current treatment involves mechanical biofilm disruption known as non-surgical periodontal therapy (NSPT). This study sought to characterise the impact of treatment on microbial diversity and overall community, and the parallel impact on host inflammation in the oral cavity. Fourty-two periodontitis patients were included in this study, with periodontal clinical parameters, subgingival plaque and saliva samples collected at baseline and 90 days after treatment. Salivary cytokines were quantified, and subgingival plaque was analysed using 16S rRNA sequencing. After treatment, there were marked health-associated alterations in microbial composition and diversity, including differential abundance of 42 genera and 61 species. These changes were accompanied by substantial clinical improvement (pockets ≥ 5 mm, 27.50% to 9.00%, $p < 0.001$) and a decrease in salivary IL-1β ($p < 0.001$)—a putative marker of periodontal inflammation. Despite significant reductions in disease associated anaerobes, several genera (*Fusobacterium*, *Prevotella*, *Tanenerella*, *Treponema*) remained present and formed a distinct subnetwork associated with residual disease. Collectively, this study shows that current periodontal treatment results in partial restoration of a healthy microbial ecosystem, but features of biofilm dysbiosis and host inflammation remain in some patients, which were surprisingly independent of clinical response.

Periodontitis (PD) is a highly prevalent, biofilm-mediated chronic oral inflammatory condition affecting 50% of the population, with 10% suffering from severe disease[1,2]. PD arises via the formation of dysbiotic subgingival plaque biofilms, which perpetuate an aggravated immune response within the gingival tissue[3]. In turn, this leads to a continuous cycle of host-bacterial interplay, whereby inflammation facilitates greater dysbiosis of the microbiome, and vice-versa[4]. If left untreated, this immune response leads to irreversible damage to the periodontal ligament and alveolar bone, ultimately resulting in tooth loss in susceptible individuals. Emerging evidence suggests that the consequences of PD may extend beyond the oral cavity, with previous work suggesting an association between periodontitis and risk of diabetes[5,6], rheumatoid arthritis[7–9], atherosclerosis[10,11], hypertension[12,13] and Alzheimer's disease[14]. Given the nutritional and psychosocial consequences of tooth loss and the links between PD and systemic disease, restoring periodontal health (PH) is essential to both oral and general health. Current treatments are resource intensive, time consuming and often only partially successful. Therefore, there is a need to understand the microbial and host immune response to further optimise treatment outcomes.

Several studies have consistently documented a unique bacterial signature of the subgingival plaque in PD, with Socransky's seminal findings of distinct complexes—each designated a colour—desbribing related groups of bacteria based on their association with health and disease (red, orange, green, purple, blue, yellow)[15]. Of note, the 'red complex' consisting of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* were

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found to strongly relate to PD severity. More recently, advances in molecular approaches have revealed that the diversity of the subgingival plaque is higher than previously assumed[16–20]. Whilst the 'red complex' relationship with disease remains[21], large-scale analyses have revealed dysbiosis of the entire plaque community, associated with increased microbial diversity and anaerobic bacteria[16,17]. In line with these findings, a recent systematic review documented several new species associated with PD such as *Filifactor alocis, Fretibacterium fastidiosum* and *Treponema vincentii*[22].

Chronic inflammation in response to dysbiotic subgingival plaque biofilms is a consistent feature of PD and escape of locally generated inflammatory mediators into the circulation has been proposed as a putative link between oral and systemic health[23]. Pro-inflammatory cytokines in saliva have been implicated as potential biomarkers for PD, with independent cross-sectional studies showing higher levels of several cytokines in PD compared with health[24,25]. Yet, this does not appear to be consistent across independent studies and patient cohorts. For example, Ebersole et al., found that salivary Interleukin-1β and Interleukin-6 were elevated in 101 PD patients compared with 65 healthy controls[24]. In contrast, Teles et al., found no significant differences when assessing both cytokines in the saliva of 74 PD patients and 44 healthy controls[26]. Hence, characterising how cytokines respond to improvements in clinical status may provide a critical insight into their role in PD.

Given the previous identification of host and microbial markers associated with PD, we sought to evaluate how these markers reflect disease severity and change following treatment and improvements in clinical status. Non-surgical periodontal therapy (NSPT) represents the first line of treatment for the majority of PD patients and involves physical debridement of biofilms from beneath the gum-line using hand or ultrasonic scaling instruments, inducing widespread—although not total—clinical improvement[27,28]. To prevent disease recurrence and potential systemic complications, it is crucial to understand whether physical biofilm disruption is sufficient to reduce local inflammation and microbial dysbiosis. Salivary cytokines and microbiome have been well characterised in cross sectional studies comparing health and disease[16,17,24,26]; however, there is limited understanding of changes in response to treatment. In this study, we aimed to investigate the subgingival plaque microbiome and salivary inflammatory cytokines—in relation to baseline disease severity, and longitudinally following NSPT.

Methodology

Study population, treatment and clinical examination. This longitudinal study was conducted in accordance with the Declaration of Helsinki (2013), with ethical approval (London-Stanmore Research Ethics Committee, Reference: 14/LO/2064). Eligible patients were recruited from referrals to Glasgow Dental Hospital. Periodontitis was defined as probing pocket depths ≥ 5 mm on 2 or more teeth at non-adjacent sites excluding third molars[29]. Additional inclusion criteria included written informed consent, and male or female ≥ 18 years of age. Exclusion criteria included patients with diabetes, rheumatoid arthritis, known or suspected risk of tuberculosis, hepatitis B or HIV infections, or history of bleeding diathesis. In total, 45 patients were recruited and provided written consent to take part in the study. Three patients were subsequently excluded due to a new diagnosis of a systemic disease during the trial period. Therefore, 42 patients were included for analysis.

NSPT was delivered by a single experienced dental hygienist, who was calibrated with experienced periodontists. Patients returned for varying numbers of treatment visits (between 1–6) depending on disease severity and preference for treatment scheduling. Periodontal parameters were assessed before any treatment visits (baseline) and 90-days following the last treatment visit (day 90) (Fig. 1A). Periodontal parameters were assessed at six sites per tooth, including full-mouth plaque scores[30] and full-mouth bleeding scores[31] (Fig. 1B). Periodontal pocket depths (PPD), location of the gingival margin (LGM) and clinical attachment level (CAL) were recorded to the nearest millimetre per site and divided by the total number of sites to give average full-mouth scores. The periodontal inflamed surface area (PISA) was calculated as described previously[32].

Sample collection and processing. Subgingival plaque and saliva were collected at baseline and day 90. At baseline, subgingival plaque was collected from the deepest pocket in each quadrant using a curette and placed into an Eppendorf tube containing 500uL sterile phosphate buffered saline (Sigma Aldrich, Gillingham, UK). Bacterial cells were harvested via centrifugation (13,500 RPM for 10-min) and supernatant was discarded. One site was selected from each patient for sequencing, and samples from the same site were sequenced before and after treatment to ensure accurate longitudinal analysis. Samples collected from the upper right quadrant were preferentially selected (n = 35), unless unable to sample the same site at day 90. In this case, pre- and post-treatment samples from a different quadrant were selected; upper left (n = 1), lower right (n = 3), lower left (n = 3). Saliva was collected using the passive drool method and was clarified via centrifugation (13,000 RPM for 5-min) as described previously[33]. All samples were transferred to adjacent laboratories and processed immediately. Samples were stored at -80 °C until analysis.

Analysis of salivary cytokines. Concentrations of salivary tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were determined using enzyme-linked immunosorbent assay (ELISA) kits (Thermofisher, Loughborough, UK). Salivary interleukin-8 (IL-8) was quantified using DuoSet ELISA kit (Biotechne—R&D Systems, Abingdon, UK). Salivary interleukin-17A (IL-17A) was quantified using high-sensitivity ProQuantum immunoassays, performed using a StepOne plus real-time PCR analyser (Thermofisher, Loughborough, UK). Samples were diluted 1:2 for IL-1β, TNFα and IL-6 assays, 1:3 for IL-8 assays and 1:5 for IL-17A assays, as per manufacturers instructions. One patient had limited sample volume and was not included in any salivary analysis (n = 41 for salivary cytokines). The limit of detection (LOD) for cytokines was IL-6: 0.04 pg/mL, IL-1β: 1.35 pg/mL, IL-8: 6.78 pg/mL, TNFα: 0.14 pg/mL and IL-17A: 2.75 pg/mL. IL-6, IL-1β and IL-8 were detected in all samples. TNFα was < LOD in 3 samples (1 at baseline, 2 at day 90) and IL-17A was < LOD in 12 samples (10 at baseline, 2 at day 90). For statistical analysis, samples < LOD were assigned LOD/2.

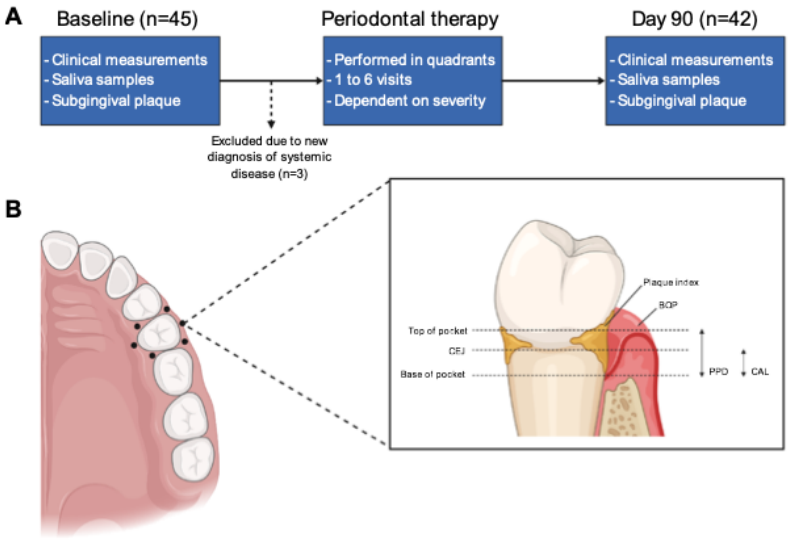

Figure 1. Outlining study protocol (**A**) and collection of periodontal clinical measurements (**B**). Six sites around each tooth were measured for plaque index, BOP, PPD and CAL. CEJ; Cementoenamel junction, BOP; Bleeding on probing, PPD; Probing pocket depth, CAL; Clinical attachment loss. Image created using BioRender.

Bacterial 16S rRNA sequencing. DNA was extracted from subgingival samples (baseline and day 90) using the MagNA Pure LC DNA isolation kit (Roche Diagnostics, Mannheim, Germany) with the addition of a chemical lysis step with an enzymatic cocktail containing lysozyme, mutanolysin and lysostaphin, following Rosier et al. 2020[34]. DNA concentrations were measured using a QubitTM 3 Fluorometer (Thermofisher, Waltham, Massachusetts, USA), and an Illumina amplicon library was prepared following the 16S rRNA gene Metagenomic Sequencing library preparation Illumina protocol (Part #15,044,223 Rev. A). The primer sequences used in this protocol were; *Illumina_16S_341F* (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG) and *Illumina_16S_805R* (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATCC) which target the 16S V3 and V4 region. Following amplification, DNA was sequenced with an Illumina MiSeq Sequencer according to manufacturer's instructions using the 2 × 300 base paired-ends protocol. For taxonomic classification, an amplicon sequence variant (ASV) table was obtained using the DADA2 pipeline in R[35]. Taxonomy was assigned by comparison to the SILVA database[36], where the naive Bayesian classifier was used to assign sequences at the genus- and species-level.

Data analysis. Data was initially collected into SPSS (v26, IBM). Following collection, longitudinal analysis of clinical characteristics and salivary cytokines was performed using GraphPad PRISM (v8) using Wilcoxon signed rank tests. Correlation analysis was conducted using Spearman-rho (non-parametric) or Pearson's (parametric) depending on data distribution, which was determined by visual inspection of histograms.

For analysis of the subgingival plaque microbiome, R programming language (v3.4 +) was used for statistical computing[37]. Alpha-diversity indexes (Shannon, ACE, Chao1) were calculated at species-level rarefying to 9000 reads using the Vegan library in R[38], and longitudinal differences were assessed using paired t-tests in GraphPad PRISM (v.8).

Relative abundances were calculated using the total sum scaling (TSS) method. Only genera and species with a minimal signal of detection were included in microbiome statistical analysis. Specifically, a species or genus was included if it was present in 50% of the samples from at least one of the two groups (relevant for Wilcoxon tests) or from the given group under study (relevant for association networks and Spearman's correlations) with an abundance superior to five times the smallest percentage above zero. For univariate analyses, Wilcoxon signed rank tests (i.e., wilcox.test function of stats library of R) were performed to test the differences in genera and species between baseline and day 90, corrected for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) of 5%[39]. Thus, only adjusted p-values are reported for taxonomic comparisons. To compare the abundance of known health and disease-associated organisms, species were grouped according the

Variable	Baseline (n = 42)	Day 90 (n = 42)	*p*-value[§]
Full-mouth			
FMPS (%)	71.00 (46.75, 76.50)	12.0 (8.75, 29.25)	< 0.001
FMBS (%)	61.00 (32.75, 76.25)	8.00 (3.75, 15.25)	< 0.001
PPD (mm)	3.59 (3.08, 4.16)	2.78 (2.56, 3.02)	< 0.001
PPD ≥ 5 mm (%)	27.50 (18.75, 41.75)	9.00 (5.75, 12.25)	< 0.001
CAL (mm)	4.43 (3.73, 5.33)	3.72 (3.18, 4.31)	< 0.001
PISA (mm^2)	1224.60 (754.80, 1687.08)	138.85 (65.52, 293.88)	< 0.001
Sites from which plaque sample collected			
PPD (mm)	7.00 (6.00, 8.00)	4.00 (4.00, 5.00)	< 0.001
CAL (mm)	8.00 (7.00, 9.00)	6.00 (5.00, 7.00)	< 0.001

Table 1. Comparison of periodontal parameters at baseline and day 90 (n = 42). Data represent full-mouth and the site from which subgingival plaque was harvested. Data are displayed as medians (Q1, Q3). FMPS; full-mouth plaque score, FMBS; full-mouth bleeding score, PPD; probing pocket depth, CAL; clinical attachment level, PISA; periodontal inflamed surface area. [§]*p*-values refer to Wilcoxon signed rank test.

colour complexes outlined by Socransky et al[15]. The grouping represents the sum of relevant species, outlined in Supplementary Table 2.

For association networks, pairwise associations were computed between genera and species based on a multivariant approach described by González et al.[40], which was implemented in the 'mixOmics' R package[41]. In short, association between genera and species are obtained based on their projection onto a correlation circle plot derived from a principal component analysis. In the association networks, only negative associations below -0.4 and positive associations above 0.4 between genera are shown. The network graphs were obtained with the R 'ggraph' package[42]. To complement the association networks, correlations between genera and species were determined with Spearman's rho, along with associated p-value using the cor.test function.

For multivariant analysis, an Adonis test (Permutational Multivariate Analysis of Variance Using Distance Matrices), provided by the Vegan library of R[38], was used to compare the overall composition between groups. To visualize groups and their differences in a two-dimensional map, we computed constrained principal components via constrained correspondence analysis (CCA) which is also part of Vegan library[38]. Principal coordinate analysis (PCoA) was conducted using MicrobiomeAnalyst[43], with Bray–Curtis similarity calculated using PAST software (v4.01)[44]. All graphs were assembled using GraphPad PRISM (v8) or R[37].

Results

Study population and clinical parameters. Patients with periodontitis who required specialist periodontal treatment were recruited (Fig. 1A). The average age of patients included in the analysis was 50 years old, with 12 males and 30 females (Supplementary Table 1). Following treatment, there was a marked improvement in all periodontal clinical parameters ($p < 0.001$ for all, Table 1). Compared with full-mouth parameters (average of all teeth), there was higher disease severity at baseline and greater clinical improvement following treatment in sites from which subgingival plaque samples were collected (PPD and CAL, both $p < 0.001$, Table 1).

The microbiome following periodontal treatment. Sequencing of subgingival plaque samples yielded 4745 ASVs across 84 samples, with an average of 90,830 reads per sample (ranging from 35,372 to 343,259 reads). After removing singletons, these ASVs were classified into 206 genera and 379 species. Rarefaction curves suggested a reliable estimate of taxonomic diversity was feasible rarefying to 9,000 reads per sample (Fig. 2A). Following treatment, there was a marked reduction in subgingival plaque taxonomic diversity, independent of method of evaluation ($p < 0.001$ for all, Fig. 2B).

Along with shifts in microbial diversity, treatment resulted in clear alterations to the microbiota composition, identified using CCA analysis at genus—(Fig. 3A) and species-level (Fig. 3B). Evaluation of genera and species which differed significantly revealed changes in 42 genera and 61 species comparing baseline and day 90 samples (Supplementary Fig. 1). Of the 42 genera, 34 decreased whilst 8 increased in abundance; at species level 46 species decreased and 15 species increased in abundance following treatment (Supplementary dataset 1). To identify genera and species contributing most to the compositional alterations, a further abundance filter (average abundance > 0.15% at either timepoint) was applied, and demonstrated differential abundance of 31 genera and 44 species. Of the 31 genera, 25 decreased whilst 6 increased in abundance (Fig. 3C). A similar trend was observed at species-level, with 32 species decreasing and 12 species increasing in abundance following treatment (Fig. 3D).

Overall there was a clear trend whereby the abundance of strict anaerobic species decreased following NSPT, and facultative anaerobic or aerobic bacteria increased. Interestingly at species-level the largest fold-increase was found for *Porphyromonas pasteri* (p[adjusted] = 0.02) belonging to a conventionally disease-associated genus, though it remained at relatively low abundance (0.6% of total, Supplementary Fig. 1).

Changes in health and disease-associated organisms. Historically, Socransky et al.[15] identified distinct complexes and assigned each a colour (red, orange, green, purple, blue and yellow), depending on their strength of association with disease. Red and orange complexes contain groups of disease-associated organ-

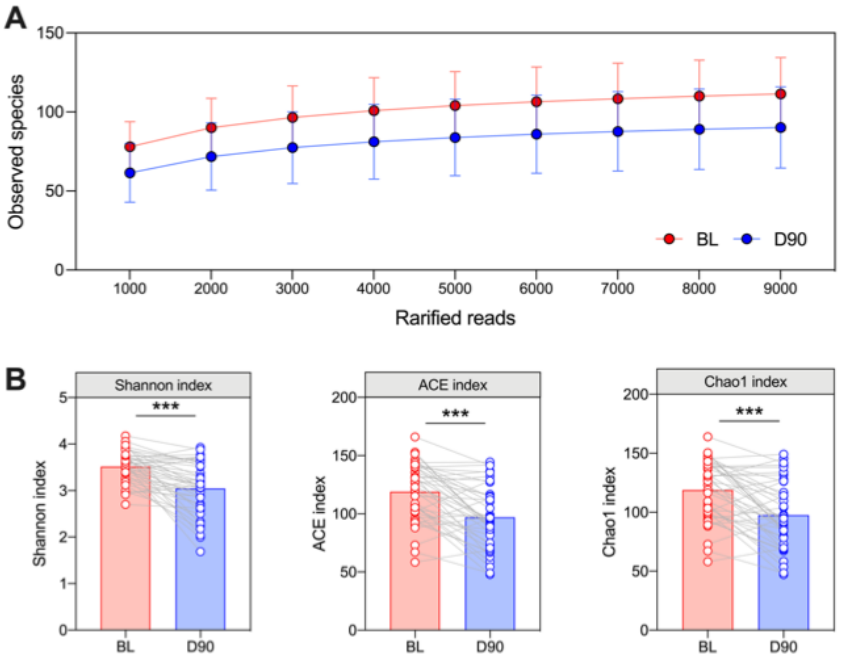

Figure 2. Alpha-diversity of subgingival plaque samples. Rarefaction curves for observed species at baseline (red line) and day-90 (blue line) (**A**). Comparing Shannon, ACE and Chao1 indexes before and after treatment at species-level rarefied to 9000 reads (**B**). Statistics are paired t-test, ***$p < 0.001$. n = 42. Bars represent mean with individual values shown.

isms, whilst the green, purple, blue and yellow complexes contain species more associated with PH (Supplementary Table 2). Following treatment, there were significant reductions in the abundance of the red and orange complexes ($p < 0.001$ for both). No difference was observed in green and purple complexes, whilst significant increases were found in the blue ($p < 0.01$) and yellow ($p < 0.001$) complexes at day 90 (Fig. 4A). A recent review by Perez-Chaparro et al., identified a more complex community of disease associated bacteria[22], and there was a similar reduction in the abundance of these species following treatment when grouped together ($p < 0.001$, Fig. 4B). Significant decreases were found in the majority of individual species within this group ($p < 0.05$, Wilcoxon signed rank tests), with the exception of *Treponema medium* and *Mogibacterium timidum*, which were present only at very low abundance.

Genera abundance and association networks. Along with compositional changes (Supplementary Fig. 2), analysis of bacterial association networks revealed a shift in the overall community structure. Network analysis was performed using correlation circle plots (Supplementary Fig. 3), and highlighted distinct clustering of individual genera and species at each timepoint. Most associations shown between genera (93.2% at baseline, 90.0% at day 90) and species (88.3% at baseline, 90.3% at day 90) were supported by significant Spearman-Rho correlations (p(adjusted) < 0.05, Supplementary dataset 1).

At baseline there were two disease-associated clusters consisting of anaerobic genera (Fig. 5A). The first pre-treatment network consisted of *Prevotella*, *Selenomonas*, *Dialister*, *Solobacterium*, *Olsenella* and *Atopobium*, whilst *Eikenella* and *Aggregatibacter* showed negative associations with some of these genera (Fig. 5A, red lines). Within the second cluster, *Filifactor* and *Fretibacterium* were central players, both correlating positively with all three red-complex genera (i.e., *Porphyromonas*, *Treponema*, *Tannerella*). After treatment (Fig. 5B), a new disease-associated network was observed consisting of *Treponema*, *Prevotella*, *Parvimonas*, *Fusobacterium*, *Alloprevotella*, *Selenomonas*, *Dialister*, and *Catonella*. Importantly, *Rothia* showed negative associations with *Selenomonas*, *Fusobacterium*, and *Prevotella* of this cluster, whilst a positive correlation was found between *Rothia* and *Actinomyces*.

Similar results were observed at species-level, where *Filifactor alocis*, *Fretibacterium fastidiusum* and *Fretibacterium feline* were central in a closely associated network of anaerobic bacteria, forming strong connections to various PD-associated organisms at baseline (Fig. 5C). Following treatment (Fig. 5D), *Rothia dentocariosa* negatively associated with unclassified *Selenomonas* sp. and *Lachnoanaerobaculum* sp., whilst *Corynebacterium*

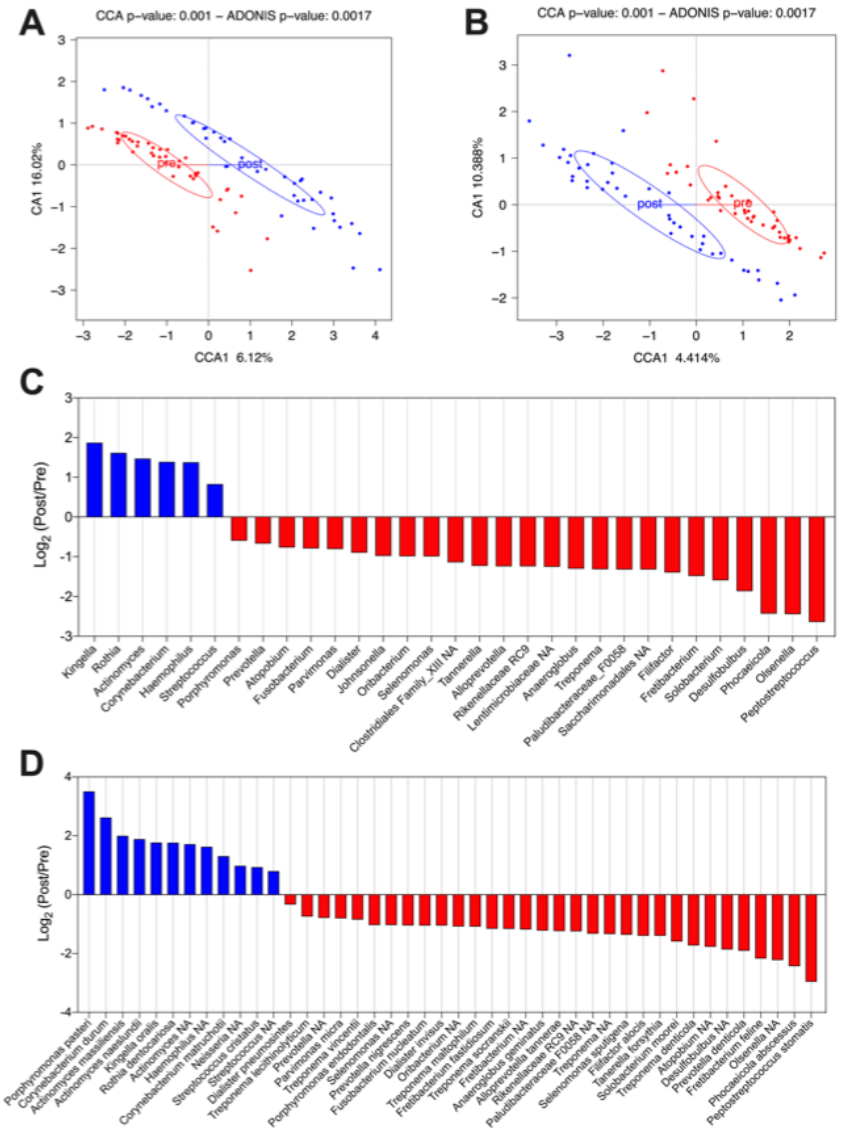

Figure 3. Assessing longitudinal changes in the composition of sub-gingival plaque samples following treatment. Canonical Correspondence Analysis (CCA) comparing baseline (red) and day-90 (blue) samples at genus- and species-level (**A** and **B** respectively). Log_2 fold change of all significant genera (**C**) and species (**D**) with average abundance > 0.15% at either timepoint. Positive values (blue bars) indicate organisms which increased significantly following treatment, negative values (red bars) indicate organisms which decreased significantly following treatment. Wilcoxon signed rank test, corrected using Benjamini–Hochberg false discovery rate (5%). All plotted organisms p(adjusted) < 0.05. n = 42. NA indicates no classification was obtained at genus- or species-level.

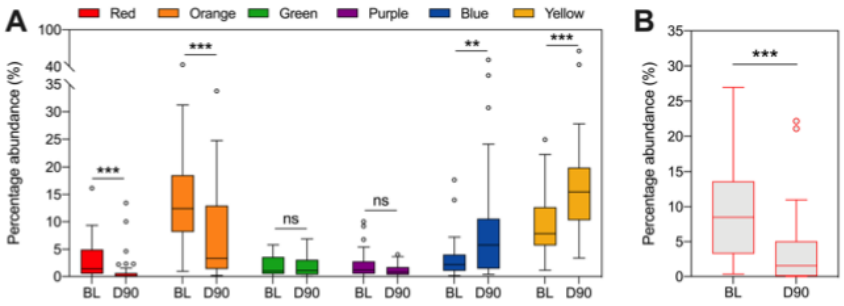

Figure 4. Comparing the abundance of health and disease associated species in the subgingival plaque. Species detected by 16S rRNA sequencing were grouped in the Socransky (1998) red, orange, green, purple, blue and yellow complexes (**A**). Comparing the sum of 'novel' species associated with disease identified by Perez-Chaparro (2014) (**B**). Species included in figure B are; *Treponema medium, Peptostreptococcus stomatis, Prevotella denticola, Mogibacterium timidum, Filifactor alocis, Selenomonas sputigena, Alloprevotella tannerae, Anaeroglobus geminatus, Fretibacterium fastidiosum, Porphyromonas endodontalis, Treponema vincentii, Treponema lecithinolyticum and Dialister pneumosintes.* Tukey box plots of all individuals (N = 42) are shown. Wilcoxon signed rank test where ***$p < 0.001$, *$p < 0.05$, ns means no significant difference. *P*-values were adjusted using FDR (5%).

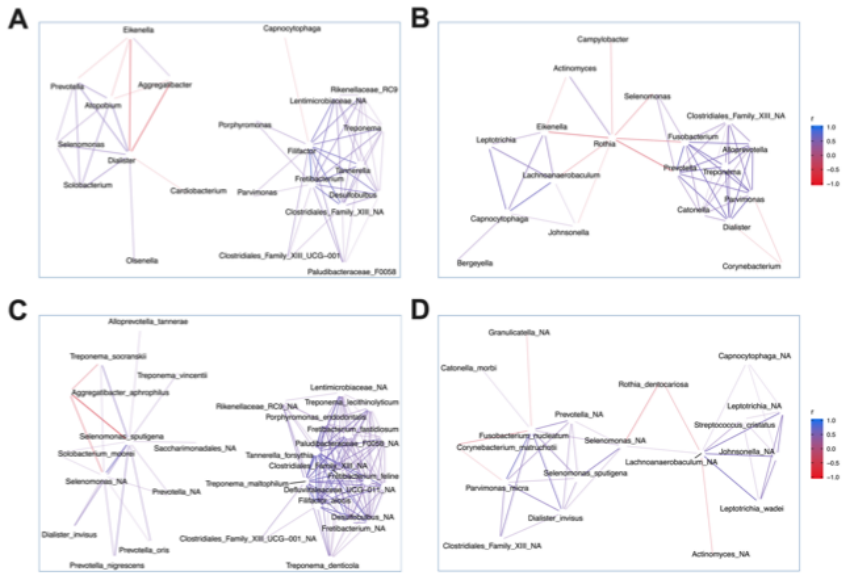

Figure 5. Bacterial association networks. Association networks were constructed between abundant genera and species based on correlation circle plots derived from principal component analysis at each timepoint. (**A**) Genus-level network at baseline, (**B**) genus-level network at day 90, (**C**) species-level network at baseline, (**D**) species-level network at day 90. Only associations < −0.4 (negative associations) and > 0.4 (positive associations) are displayed.

matruchottii showed negative associations with *Fusobacterium nulceatum* and *Parvimonas micra*, two orange-complex species.

Microbiome, disease severity and response to treatment. To investigate the relationship between the microbiota and clinical disease severity, Spearman-Rho correlations were performed between the pocket depth of sampled teeth, individual genera/species abundance and alpha-diversity indexes. No associations were observed between the site pocket depth and the abundance of any genera or species. However, when considering the disease severity of the entire tooth (average of the 6 sites), several positive and negative associations were noted. At baseline, there was a weak positive correlation between tooth pocket depth and alpha-diversity indexes, particularly the Shannon index ($r = 0.403$, $p = 0.008$), whilst no association was found with the abundance of any individual genera or species. At day 90, there was no association with the Shannon index (Supplementary Table 3). Instead, weak positive correlations were observed between tooth PPD and strict anaerobic genera (*Treponema*, *Prevotella*, *Parvimonas*, *Alloprevotella*, *Dialister* and unclassified *Clostridiales Family XIII*), whilst negative correlations were observed for facultative anaerobes (*Corynebacterium*, *Granulicatella* and *Kingella*). Interestingly, all of the genera which positively correlated with tooth pocket depth were also key members of the remaining disease-associated network identified at day 90 (Fig. 5C). In contrast, *Corynebacterium*, which negatively correlated with pocket depth, showed negative associations with two of these genera (*Dialister* and *Parvimonas*).

To establish whether microbial profiles impacted treatment response, an exploratory analysis was conducted and patients were grouped depending on whether 'pocket closure' was achieved in the sampled sites (defined as the conversion of a site measuring ≥ 5 mm to a site measuring ≤ 4 mm)[27,45]. Sites < 5 mm were sampled in two patients at baseline, and these patients were excluded from this analysis (Supplementary dataset 1). Evaluating pocket closure rates within the remaining sites ($n = 40$), 21 sites achieved pocket closure at day 90, whilst 19 sites did not. When comparing pocket depths at baseline between these two groups, no significant difference was observed indicating similar levels of starting disease severity (Fig. 6A). There were statistically significant reductions in pocket depth in both groups, albeit less pronounced in sites which did not achieve pocket closure ($p < 0.001$). To investigate the difference in the baseline microbiota of each group, a Bray–Curtis based principal coordinate analysis was performed (Fig. 6B). Despite differences in clinical outcomes, there was no significant difference in baseline microbial profiles of each group. Likewise, no difference was observed in Bray–Curtis similarity profiles (BL vs. D90), indicating similar microbial responses to NST (Fig. 6C). Comparing the relative abundance of species revealed no significant differences between groups at either timepoint (Fig. 6D), which was consistent for alpha-diversity indexes (data not shown). Additional exploratory analysis revealed no differences in baseline microbial profile or response among different demographic (sex, age) and behavioural subgroups (smoking status) within the current cohort (Supplementary Fig. 4).

Inflammatory response, disease severity and response to treatment. Given the key role of inflammation in periodontal pathogenesis, we next sought to investigate whether clinical and microbial improvements were accompanied by alterations in the local inflammatory response. At baseline, salivary IL-1β showed a positive association with all clinical parameters, including the PISA ($r = 0.534$, $p < 0.001$), which reflects the inflammatory burden of PD (Fig. 7A). A weak positive association was observed between salivary IL-8 and full-mouth pocket depth, proportion of deep periodontal pockets and the PISA ($r = 0.423, 0.424, 0.372$ respectfully), whilst a weak negative association was observed between IL-17A and plaque index ($r = -0.375$, $p = 0.049$). Salivary TNFα and IL-6 showed no significant association with any periodontal clinical parameter. Additionally, no significant positive or negative association between genera or species abundance and salivary cytokines was observed at either timepoint following adjustment for multiple comparisons (Supplementary Table 4).

Following treatment (Fig. 7B), there was a notable reduction in salivary IL-1β ($p < 0.001$), and, somewhat surprisingly, an increase in the level of salivary IL-17A, albeit a quantitatively small change ($p < 0.001$). No significant longitudinal changes were observed for TNFα, IL-6 or IL-8. To further evaluate the relationship between clinical disease and local inflammation, we assessed correlations in the change in each cytokine against the change in PISA (Fig. 7C). The change in salivary IL-1β showed a positive correlation with the change in the PISA, matching its association with disease severity at baseline and reductions following treatment. Despite the lack of change at day 90, salivary IL-8 also showed a similar relationship ($r = 0.45$, $p = 0.003$), whereby those with the greatest reduction in PISA tended to show a reduction in salivary IL-8 following treatment, whilst patients with less pronounced changes appeared to show similar or increased levels compared with baseline.

Discussion

Although previous studies have evaluated the microbiome following NSPT[46–48], to our knowledge, this is the first study to simultaneously investigate longitudinal alterations in the subgingival plaque microbiome, local inflammatory markers and periodontal clinical parameters in the same patient cohort. Furthermore, this study has one of the largest cohorts for studying alterations in the subgingival plaque microbiome. In our study, we show a clear decrease in disease associated organisms in the subgingival microbiota after NSPT, which was accompanied by alterations in salivary cytokines. Although patients all showed positive clinical changes, only 47% obtained a PISA commensurate with periodontal health ($130.33 mm^2$)[49]—which is comparable with a myriad of published studies. Thus, defining how patients respond to non-surgical periodontal treatment—encompassing clinical, microbial and inflammatory markers is critical to rationally test and implement much needed NSPT adjuncts and novel therapeutics.

Previous studies comparing PH and PD have shown higher microbial diversity in the subgingival plaque of PD patients, suggesting that increased pocket depths, nutrient availability and/or immune impairment allow

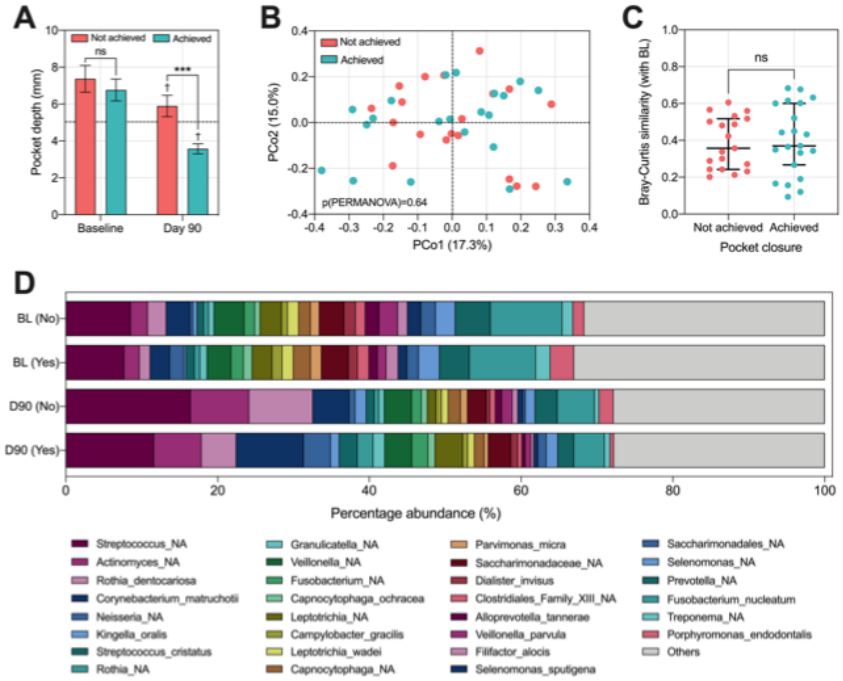

Figure 6. Investigating the subgingival plaque microbiome in relation to treatment response. (**A**) Comparing the pocket depth of sampled sites at baseline (BL) and day 90 (D90) depending on whether pocket closure was achieved (n = 21) or not achieved (n = 19) post-treatment. Dotted black line indicates 5 mm. Between group comparisons refer to unpaired t-tests (ns: non-significant, ***$p < 0.001$), within-group comparisons refer to paired t-tests (†$p < 0.001$), graphs display mean ± 95% confidence interval. (**B**) Species-level Bray–Curtis based PCoA analysis of each group at baseline, statistics are PERMANOVA with raw p-value displayed. (**C**) Comparing Bray–Curtis similarity with baseline between groups, statistics refer to Mann–Whitney test, ns: non-significant. (**D**) Plotting the top 30 most abundant species at BL and D90 between groups, No; pocket closure not achieved, Yes; pocket closure achieved.

for growth of a more diverse biofilm community[16,50]. In line with this theory, we observed consistent reductions in diversity following treatment, matching with reduced pocket depths and clinical inflammation. Along with shifts in taxonomic diversity, we observed differential abundance of 42 genera and 61 species following NSPT, representing large-scale alterations in the microbiota composition. The majority of organisms that decreased following treatment were strict anaerobes (e.g. *Fusobacterium, Prevotella, Porphyromonas*), with increases found typically in facultative anaerobes or aerobes (e.g. *Kingella, Rothia, Actinomyces*). This trend indicates that in addition to physical biofilm disruption, clinical improvement likely drives environmental shifts which contribute to biofilm composition, such as increased oxygen availability (from decreased pocket depths) and the reduction of inflammation-related nutrient availability[51,52].

Interestingly, we observed no significant microbiota differences between sites that did and did not achieve pocket closure, despite similar baseline disease severity. This study uniquely investigated the entire subgingival plaque microbiome factoring in pocket closure as a clinically relevant treatment outcome for patients receiving NSPT. We selected this outcome as the most clinically meaningful threshold which determines the need for further active treatment[27]. Similar data has been reported previously in a smaller cohort when PPD, CAL and BoP were used as indicators of treatment success[47]. An important observation to consider when interpreting the analysis from the current study is that even sites which did not achieve pocket closure did display significant, albeit less pronounced, clinical improvement following treatment. Future studies incorporating true 'non-responders' to NSPT would be useful to establish any causation between the baseline subgingival plaque microbiota and response to treatment.

Assessing longitudinal changes within the entire cohort, we observed a significant decrease in the levels of disease associated species following treatment, including *Fretibacterium fastidiosum*, *Fretibacterium feline* and *Filifactor alocis*. *Filifactor alocis* has been suggested as a biomarker for periodontitis and was proposed as

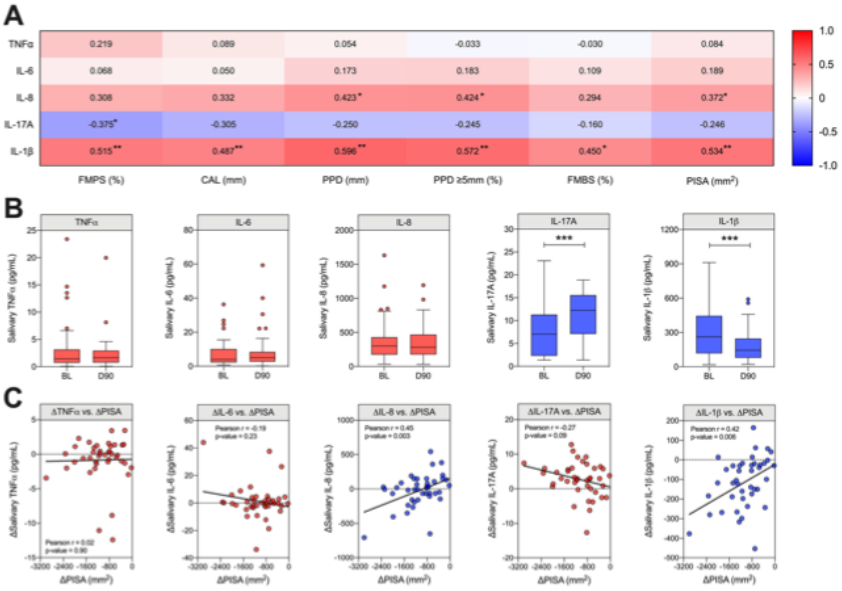

Figure 7. Levels of local inflammatory markers in saliva. (**A**) Correlating the levels of salivary inflammatory markers at baseline with clinical periodontal parameters, values represent Spearman-Rho correlation. P-values were corrected using FDR (5%) where **p*(adjusted) < 0.05 and ***p*(adjusted) < 0.01. Confidence intervals are supplied in supplementary dataset 1 (**B**) Comparing changes in the levels of TNFα, IL-6, IL-8, IL-17A and IL-1β at baseline (BL) and day-90 (D90). Graphs are Tukey boxplots where horizontal line indicates the median and individual points indicate potential outliers. Statistics are Wilcoxon signed rank test, where ****p* < 0.001. n = 41. (**C**) Pearson's correlation between change in PISA and change in salivary cytokines, raw p-values are shown.

a diagnostic marker organism[53,54]. In addition, *Fretibacterium* spp., including *F. fastidiosum*, have been associated with PD in different independent studies. Interestingly, red complex genera (*Porphyromonas*, *Treponema*, *Tannerella*) correlated positively with *Filifactor* and *Fretibacterium* at baseline in our study, which led to the formation of a clearly disease-associated cluster. After treatment, these associations were lost and a newly formed disease-associated network was formed, including genera that are abundant in health but increase in PD (e.g., *Fusobacterium* and *Prevotella*)[16], along with *Treponema*, *Alloprevotella*, *Selenomonas*, *Parvimonas*, *Dialister* and *Catonella*. Whether groups of disease-associated organisms may persist to drive future disease recurrence remains unclear, and requires a longer study period with microbiological monitoring.

Positive associations provide a useful indicator of key interactions within the subgingival plaque that drive dysbiosis; although investigating negative associations between organisms is an often-overlooked concept in PD. Such associations may elucidate interactions which help maintain the biofilm in a eubiotic state and could be a useful starting point for identifying potential probiotics. In our study at day 90, we observed negative associations between *Rothia* and several PD-associated genera (*Selenomonas*, *Fusobacterium* and *Prevotella*), and a positive correlation between *Rothia* and *Actinomyces*. Both *Rothia* and *Actinoymces* are confirmed nitrate reducing genera[55,56], and several strictly anaerobic PD-associated species have shown to be sensitive to oxidative stress and nitric oxide[57]. Thus, the possible role of nitric oxide and other antimicrobial components produced by these genera in the inhibition of PD-associated organisms and maintenance of biofilm eubiosis warrants further investigation.

In previous smaller studies assessing the subgingival plaque microbiome following NSPT, ungrouped genera such as *Dialister* and *Olsenella* decreased post-treatment, whilst *Rothia* and *Corynebacterium* increased [47,48], commensurate with our results. However, a greater number of microbial alterations were observed in the current study. For example, Shi et al., demonstrated an increase in 4 genera and decrease in 8 genera across 12 patients following NSPT, whilst Chen et al., included 19 patients and observed large patient-to-patient variation in microbial response. In our study of 42 patients, we identified consistent shifts in 42 genera and 61 species. One reason for these findings may be that in previous studies[48], patients returned at varying timepoints (4–19 weeks) which may impact the stage of biofilm redevelopment and thus composition. In contrast, all patients returned at 90 days following treatment in this study, which is a recommended clinical follow-up period[58]. Taken together, a larger sample size and standardised follow-up period may help explain the greater number of microbial alterations found in the current study following treatment.

From an inflammatory perspective, this study highlights a clear association between salivary IL-1β and periodontal inflammation, supporting previous cross-sectional and longitudinal studies[25,59,60]. However, this result was not observed for other pro-inflammatory markers (TNFα, IL-6, IL-8). In general, salivary levels of TNFα and IL-6 are low[61,62], and, similar to our findings, previous studies have failed to demonstrate a consistent relationship between salivary IL-8 and PD[63]. Saliva is a harsh environment containing mucins, bacteria and various proteolytic enzymes. Therefore, it is possible that low values of TNFα and IL-6 merely represents their ability to survive in this setting, rather than lack of production within gingival tissue.

To our surprise, we observed a significant increase in the levels of salivary IL-17A after treatment. Primarily produced by Th17 cells, IL-17A plays an important role in the host defence against external pathogens at mucosal sites[64]. Despite this function being co-aligned with the pathophysiology of PD (biofilm challenge in the periodontal pocket), in reality, the relationship between IL-17A and PD remains complex and unclear, with evidence to suggest both protective and destructive effects[65]. Previous cross-sectional studies assessing local production of IL-17A in the gingival crevicular fluid (GCF) and saliva have been largely incolclusive, with some studies reporting higher levels in PD[66,67], whilst others find higher levels in PH[68,69]. Another study reported elevated levels in localised PD compared with generalised PD and PH, suggesting that IL-17A may peak in early gingival inflammation rather than established periodontitis[70]. In relation to our study, day 90 samples are reflective of a post-treatment state of stability, rather than total periodontal health. Given the findings by Liukkonen et al., it is possible that treatment induces a shift back to an early inflammatory state, where IL-17A is a driving force. To further investigate this hypothesis, it would be beneficial for future studies to compare salivary IL-17A across a range of clinical states, including post-treatment PD, gingivitis and healthy patients.

Whilst the overall goal of this study was to identify longitudinal changes in clinical, microbial and immunological parameters post-treatment—certain limitations remain, and results should be interpreted in consideration of these factors. One such limitation is the lack of healthy control samples for a comparison with post treatment clinical health. Despite observing consistent shifts in known disease associated microbial and immunological markers[15,22,61,62,71], future studies should investigate whether these resort back to a state comparable with periodontally healthy subjects. Another limitation is that patients were only followed-up at a single timepoint. Patients are evaluated three months following non-surgical treatment, and decisions made about future treatment plans[72]. Future studies could consider the impact of supportive treatment, repeated treatment, or adjunctive treatments on the microbiome, and whether the microbiome post NSPT could be used to guide decision making. Evaluating the microbiome at earlier timepoints would provide a much-needed indication of the early microbial recolonization pattern following biofilm disruption. Longer follow up evaluation (6 months—1 year) would establish whether shifts are maintained, and which (if any) could be used to predict disease recurrence.

In conclusion, we present the first longitudinal study of NSPT to evaluate local inflammatory cytokines and microbiome sequencing in the same patient cohort. Our data highlight large-scale subgingival community shifts following NSPT, consistent with the reformation of an ecosystem more compatible with PH. However, a tightly clustered network of disease-associated genera remained at day 90, underlining the resilience of a microbial core that may be partly responsible for the chronicity of the disease. From an inflammatory perspective, our study confirms the relationship between salivary IL-1β and PD, showing a close association with periodontal inflammation, and a proportional reduction following treatment. Collectively, this study shows that current treatment results in partial restoration of health but features of biofilm dysbiosis and inflammation remain. Means of modifying these parameters to improve clinical outcomes and reduce disease recurrence—that are not antibiotic dependent—are very much needed to improve periodontal treatment outcomes.

Data availability

A supplementary dataset containing figure source data accompanies this manuscript. All sequencing reads are deposited in the NCBI Sequencing Read Archive (SRA) under BioProject PRJNA725103. Any further data is available upon reasonable request from the corresponding author.

Received: 30 November 2020; Accepted: 5 April 2021
Published online: 07 May 2021

References

1. Albandar, J. M. & Rams, T. E. Global epidemiology of periodontal diseases: An overview. *Periodontol.* **2000**(29), 7–10. https://doi.org/10.1034/j.1600-0757.2002.290101.x (2002).
2. Sanz, M. *et al.* Periodontitis and cardiovascular diseases: Consensus report. *J. Clin. Periodontol.* **47**, 268–288. https://doi.org/10.1111/jcpe.13189 (2020).
3. Hajishengallis, G., Darveau, R. P. & Curtis, M. A. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* **10**, 717–725. https://doi.org/10.1038/nrmicro2873 (2012).
4. Rosier, B. T., Marsh, P. D. & Mira, A. Resilience of the oral microbiota in health: mechanisms that prevent dysbiosis. *J. Dental Res.* **9**, 7. https://doi.org/10.1177/0022034517742139 (2018).
5. Ide, R., Hoshuyama, T., Wilson, D., Takahashi, K. & Higashi, T. Periodontal disease and incident diabetes: A seven-year study. *J. Dent. Res.* **90**, 41–46. https://doi.org/10.1177/0022034510381902 (2011).
6. Preshaw, P. M., Alba, A. L. & Herrera, D. Periodontitis and diabetes: a two-way relationship. *Diabetologia* **55**, 21–31. https://doi.org/10.1007/s00125-011-2342-y (2011).
7. Chou, Y. Y., Lai, K. L., Chen, D. Y., Lin, C. H. & Chen, H. H. Rheumatoid arthritis risk associated with periodontitis exposure: A nationwide, population-based cohort study. *PLoS ONE* https://doi.org/10.1371/journal.pone.0139693 (2015).
8. Lundberg, K., Wegner, N., Yucel-Lindberg, T. & Venables, P. J. Periodontitis in RA-the citrullinated enolase connection. *Nat. Rev. Rheumatol.* **6**, 727–730. https://doi.org/10.1038/nrrheum.2010.139 (2010).
9. Reilly, R. J. R., Johnston, W. & Culshaw, S. Autoimmunity and the oral cavity. *Current Oral Health Rep.* **6**, 1–8. https://doi.org/10.1007/s40496-019-0203-9 (2019).

10. Joshipura, K., Zevallos, J. C. & Ritchie, C. S. Strength of evidence relating periodontal disease and atherosclerotic disease. *Compendium Continuing Educat. Dentistry* **30**, 430–430. https://doi.org/10.1016/j.bbi.2008.05.010 (2009).
11. Kebschull, M., Demmer, R. T. & Papapanou, P. N. Gum bug, leave my heart alone!-epidemiologic and mechanistic evidence linking periodontal infections and atherosclerosis. *J. Dent. Res.* **89**, 879–902. https://doi.org/10.1177/0022034510375281 (2010).
12. Czesnikiewicz-Guzik, M. *et al.* Causal association between periodontitis and hypertension: evidence from Mendelian randomization and a randomized controlled trial of non-surgical periodontal therapy. *Eur. Heart J.* **40**, 3459–3470. https://doi.org/10.1093/eurheartj/ehz646 (2019).
13. Munoz, A. E. *et al.* Periodontitis is associated with hypertension: a systematic review and meta-analysis. *Cardiovasc Res.* **116**, 28–39. https://doi.org/10.1093/cvr/cvz201 (2020).
14. Dominy, S. S. *et al.* Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci. Adv.* **5**, 1. https://doi.org/10.1126/sciadv.aau3333 (2019).
15. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* **25**, 134–144. https://doi.org/10.1111/j.1600-051X.1998.tb02419.x (1998).
16. Abusleme, L. *et al.* The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* **7**, 1016–1025. https://doi.org/10.1038/ismej.2012.174 (2013).
17. Griffen, A. L. *et al.* Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* **6**, 1176–1185. https://doi.org/10.1038/ismej.2011.191 (2012).
18. Kumar, P. S., Griffen, A. L., Moeschberger, M. L. & Leys, E. J. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J. Clin. Microbiol.* **43**, 3944–3955. https://doi.org/10.1128/JCM.43.8.3944-3955.2005 (2005).
19. Paster, B. J. *et al.* Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**, 3770–3783. https://doi.org/10.1128/JB.183.12.3770-3783.2001 (2001).
20. Bik, E. M. *et al.* Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J* **4**, 962–974. https://doi.org/10.1038/ismej.2010.30 (2010).
21. Teles, R., Teles, F., Frias-Lopez, J., Paster, B. & Haffajee, A. Lessons learned and unlearned in periodontal microbiology. *Periodontol.* **2000**(62), 95–162. https://doi.org/10.1111/prd.12010 (2013).
22. Pérez-Chaparro, P. J. *et al.* Newly identified pathogens associated with periodontitis: A systematic review. *J. Dent. Res.* **93**, 846–858. https://doi.org/10.1177/0022034514542468 (2014).
23. Hajishengallis, G. Periodontitis: From microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* **15**, 30–44. https://doi.org/10.1038/nri3785 (2015).
24. Ebersole, J. L., Nagarajan, R., Akers, D. & Miller, C. S. Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Front. Cellular Infect. Microbiol.* https://doi.org/10.3389/fcimb.2015.00062 (2015).
25. Miller, C. S., King, C. P., Langub, M. C., Kryscio, R. J. & Thomas, M. V. Salivary biomarkers of existing periodontal disease: A cross-sectional study. *J. Am. Dent. Assoc.* **137**, 322–329. https://doi.org/10.14219/jada.archive.2006.0181 (2006).
26. Teles, R. P., Likhari, V., Socransky, S. S. & Haffajee, A. D. Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: A cross-sectional study. *J. Periodontal Res.* **44**, 411–417. https://doi.org/10.1111/j.1600-0765.2008.01119.x (2009).
27. Suvan, J. *et al.* Subgingival instrumentation for treatment of periodontitis. A systematic review. *J. Clin. Periodontol.* https://doi.org/10.1111/jcpe.13245 (2019).
28. Heitz-Mayfield, L. J. A., Trombelli, L., Heitz, F., Needleman, I. & Moles, D. A systematic review of the effect of surgical debridement vs. non-surgical debridement for the treatment of chronic periodontitis. *J. Clin. Periodontol.* **29**, 92–102. https://doi.org/10.1034/j.1600-051x.29.s3.5.x (2003).
29. Page, R. C. & Eke, P. I. Case definitions for use in population-based surveillance of periodontitis. *J. Periodontol.* https://doi.org/10.1902/jop.2007.060264 (2007).
30. O'Leary, T. J., Drake, R. B. & Naylor, J. E. The plaque control record. *J. Periodontol.* **43**, 38–38. https://doi.org/10.1902/jop.1972.43.1.38 (1972).
31. Ainamo, J. & Bay, I. Problems and proposals for recording gingivitis and plaque. *Int. Dent. J.* **25**, 229–235 (1975).
32. Nesse, W. *et al.* Periodontal inflamed surface area: Quantifying inflammatory burden. *J. Clin. Periodontol.* https://doi.org/10.1111/j.1600-051X.2008.01249.x (2008).
33. Simon-Soro, A. *et al.* Combined analysis of the salivary microbiome and host defence peptides predicts dental disease. *Sci. Rep.* **8**, 1. https://doi.org/10.1038/s41598-018-20085-x (2018).
34. Rosier, B. T., Buetas, E., Moya-Gonzalvez, E. M., Artacho, A. & Mira, A. Nitrate as a potential prebiotic for the oral microbiome. *Sci. Rep.* **10**, 1–15. https://doi.org/10.1038/s41598-020-69931-x (2020).
35. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583. https://doi.org/10.1038/nmeth.3869 (2016).
36. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590–D596. https://doi.org/10.1093/nar/gks1219 (2013).
37. Team, R. C. *R: A language and environment for statistical computing.*, http://www.R-project.org/. (2014).
38. Oksanen, J. *et al. vegan: Community Ecology Package. R package version 2.4–2* http://CRAN.R-project.org/package=vegan (2019).
39. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Royal Stat. Soc. Ser B Methodol* **57**, 289–300 (1995).
40. González, I., Cao, K. A., Davis, M. J. & Déjean, S. Visualising associations between paired "omics" data sets. *Biodata Min.* https://doi.org/10.1186/1756-0381-5-19 (2012).
41. Rohart, F., Gautier, B., Singh, A. & Lê Cao, K. A. mixOmics: an r package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* **13**, 1. https://doi.org/10.1371/journal.pcbi.1005752 (2017).
42. Pedersen,T. L. *ggraph: An Implementation of Grammar of Graphics for Graphs and Networks*, https://rdrr.io/cran/ggraph/ (2020).
43. Dhariwal, A. *et al.* MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **45**, 180–188. https://doi.org/10.1093/nar/gkx295 (2017).
44. Hammer, O., Harper, D. A. T. & Ryan, P. D. PAST: Paleontological statistics software package for education and data analysis. (2001).
45. Johnston, W. *et al.* The systemic inflammatory response following hand instrumentation vs ultrasonic instrumentation—a randomised controlled trial. *J. Clin. Periodontol.* https://doi.org/10.1111/jcpe.13342 (2020).
46. Feres, M. *et al.* Microbiome changes in young periodontitis patients treated with adjunctive metronidazole and amoxicillin. *J. Periodontol.* https://doi.org/10.1002/JPER.20-0128 (2020).
47. Chen, C. *et al.* Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J.* **12**, 1210–1224. https://doi.org/10.1038/s41396-017-0037-1 (2018).
48. Shi, B. *et al.* Dynamic changes in the subgingival microbiome and their potential for diagnosis and prognosis of periodontitis. *MBio* **6**, 1. https://doi.org/10.1128/mbio.01926-14 (2015).
49. Leira, Y., Martin-Lancharro, P. & Blanco, J. Periodontal inflamed surface area and periodontal case definition classification. *Acta Odontol Scand* **76**, 195–198. https://doi.org/10.1080/00016357.2017.1401659 (2018).

50. Shi, M. *et al.* The subgingival microbiome of periodontal pockets with different probing depths in chronic and aggressive periodontitis: a pilot study. *Front. Cellular Infect. Microbiol.* https://doi.org/10.3389/fcimb.2018.00124 (2018).
51. Tanaka, M., Hanioka, T., Takaya, K. & Shizukuishi, S. Association of oxygen tension in human periodontal pockets with gingival inflammation. *J. Periodontol* https://doi.org/10.1902/jop.1998.69.10.1127 (1998).
52. Loesche, W. J. in *Medical Microbiology* (ed S. Baron) Ch. 99, (University of Texas Medical Branch at Galveston, 1996).
53. Aruni, A. W. *et al.* Filifactor alocis—a new emerging periodontal pathogen. *Microbes Infect* **17**, 517–530. https://doi.org/10.1016/j.micinf.2015.03.011 (2015).
54. Chen, H. *et al.* A Filifactor alocis-centered co-occurrence group associates with periodontitis across different oral habitats. *Sci Rep* https://doi.org/10.1038/srep09053 (2015).
55. Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M. & Allaker, R. P. Evaluation of bacterial nitrate reduction in the human oral cavity. *Europ. J. Oral Sci.* https://doi.org/10.1111/j.1600-0722.2004.00184.x (2005).
56. Rosier, B., Moya-Gonzalvez, E., Corell-Escuin, P. & Mira, A. Isolation and characterization of nitrate-reducing bacteria as potential probiotics for oral and systemic health. *Front. Microbiol.* https://doi.org/10.3389/fmicb.2020.555465 (2020).
57. Backlund, C. J., Sergesketter, A. R., Offenbacher, S. & Schoenfisch, M. H. Antibacterial efficacy of exogenous nitric oxide on periodontal pathogens. *J. Dental Res.* https://doi.org/10.1177/0022034514529974 (2014).
58. Darcey, J. & Ashley, M. See you in three months! The rationale for the three monthly peridontal recall interval: a risk based approach. *Nat. Publ. Group* https://doi.org/10.1038/sj.bdj.2011.868 (2011).
59. Gursoy, U. K. *et al.* Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. *J. Clin. Periodontol.* **36**, 922–927. https://doi.org/10.1111/j.1600-051X.2009.01480.x (2009).
60. Kaushik, R., Yeltiwar, R. K. & Pushpanshu, K. Salivary interleukin-1beta levels in patients with chronic periodontitis before and after periodontal phase I therapy and healthy controls: a case-control study. *J. Periodontol.* **82**, 1353–1359. https://doi.org/10.1902/jop.2011.100472 (2011).
61. Jaedicke, K. M., Preshaw, P. M. & Taylor, J. J. Salivary cytokines as biomarkers of periodontal diseases. *Periodontol* **2000**(70), 164–183. https://doi.org/10.1111/prd.12117 (2016).
62. Taylor, J. J. Protein biomarkers of periodontitis in Saliva. *ISRN Inflammation.* https://doi.org/10.1155/2014/593151 (2014).
63. Rathnayake, N. *et al.* Salivary biomarkers of oral health: a cross-sectional study. *J. Clin. Periodontol.* **40**, 140–147. https://doi.org/10.1111/jcpe.12038 (2013).
64. Jin, W. & Dong, C. in *Emerg Microbes Infect* Vol. 2 e60- (2013).
65. Zenobia, C. & Hajishengallis, G. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol* **2000**(69), 142–159. https://doi.org/10.1111/prd.12083 (2015).
66. Awang, R. *et al.* Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. *Inflamm. Res.* **63**, 1001–1012. https://doi.org/10.1007/s00011-014-0776-7 (2014).
67. Techatanawat, S. *et al.* Salivary and serum interleukin-17A and interleukin-18 levels in patients with type 2 diabetes mellitus with and without periodontitis. *PLoS ONE* **15**, 1. https://doi.org/10.1371/journal.pone.0228921 (2020).
68. Ozçaka, O., Nalbantsoy, A. & Buduneli, N. Interleukin-17 and interleukin-18 levels in saliva and plasma of patients with chronic periodontitis. *J. Periodontal Res.* https://doi.org/10.1111/j.1600-0765.2011.01377.x (2011).
69. Prakasam, S. & Srinivasan, M. Evaluation of salivary biomarker profiles following non-surgical management of chronic periodontitis. *Oral Dis.* https://doi.org/10.1111/odi.12085 (2014).
70. Liukkonen, J., Gürsoy, U. K., Pussinen, P. J., Suominen, A. L. & Könönen, E. salivary concentrations of interleukin (IL)-1β, IL-17A, and IL-23 vary in relation to periodontal status. *J. Periodontol.* **87**, 1. https://doi.org/10.1902/jop.2016.160146 (2016).
71. Feres, M., Retamal-Valdes, B., Gonçalves, C., Cristina Figueiredo, L. & Teles, F. Did Omics change periodontal therapy?. *Periodontol.* **2000**, 85. https://doi.org/10.1111/prd.12358 (2021).
72. Badersten, A., Nilveus, R. & Egelberg, J. Effect of nonsurgical periodontal therapy II Severely advanced periodontitis. *J. Clin. Periodontol.* https://doi.org/10.1111/j.1600-051x.1984.tb01309.x (1984).

Acknowledgements
The authors are very grateful for the help provided from Glasgow Dental Hospital Clinical Research Facility, in particular Clare Brown and Debbie McKenzie. We would also like to thank Dr. Miguel Carda Diéguez at FISABIO for his help with bioinformatics.

Author contributions
SC, AM, GR conceived the study, participated in study design and produced the final manuscript. WJ, BTR, JLB, MP, KP performed laboratory experimental work and manuscript preparation. WJ, MP, KP, SC performed sample processing, clinical data acquisition and analysis. WJ, BTR, AA, CD, GR, AM, SC were responsible for 16S rRNA sequencing data acquisition, bioinformatic analysis and manuscript preparation. All authors have read and approved the final manuscript.

Funding
The current study was funded by grants from EU Marie Curie ITN RAPID (grant number 290246), Versus Arthritis (Grant Number 20823) and the BBSRC (BB/P504567/1). William Johnston is supported by a student stipend from the University of Glasgow and Dentsply Sirona (Project Number 300881). Alex Mira is supported by grant RTI2018-102032-B-I00 and Bob T. Rosier by a FPI fellowship with reference Bio2015-68711-R, both from the Spanish Ministry of Science, Innovation and Universities.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-89002-z.

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Appendix iii (chapter 4 manuscript)

Received: 1 April 2020 | Revised: 22 June 2020 | Accepted: 28 June 2020

DOI: 10.1111/jcpe.13342

CLINICAL PERIODONTOLOGY

Journal of Clinical Periodontology WILEY

The systemic inflammatory response following hand instrumentation versus ultrasonic instrumentation—A randomized controlled trial

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Funding information
The current study was jointly funded by the University of Glasgow's PhD programme and Dentsply Sirona. The trial protocol was designed by AS and SC.

Abstract

Objective: This study sought to investigate whether the immediate systemic inflammatory response following full-mouth debridement differs following use of hand compared with ultrasonic instruments.

Methods: Thirty-nine periodontitis patients were randomized to treatment with full-mouth debridement using either hand or ultrasonic instrumentation completed within 24 hr. Serum and periodontal clinical parameters were collected at baseline, day 1, day 7 and day 90 post-treatment. Differences in systemic inflammatory markers were assessed using general linear models at each timepoint, corrected for age, gender, smoking status, body mass index and baseline levels of each marker.

Results: Across all patients, serum C-reactive protein increased at day 1, with no differences between hand and ultrasonic groups (p(adjusted) = .22). There was no difference between groups in interleukin-6 (p(adjusted) = .29) or tumour necrosis factor α (p(adjusted) = .53) at day 1. Inflammatory markers returned to baseline levels by day 7. Treatment resulted in equal and marked improvements in clinical parameters in both groups; however, total treatment time was on average shorter for ultrasonic instruments (p(adjusted) = .002).

Conclusions: Ultrasonic instrumentation resulted in shorter treatment time with comparable clinical outcomes. Levels of serum C-reactive protein at day 1 were similar following debridement with hand or ultrasonic instruments.

KEYWORDS

hand instruments, periodontal treatment, randomized controlled trial, systemic inflammation, ultrasonic instruments

William Johnston and Michael Paterson have equal contributions.

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1 | INTRODUCTION

Periodontitis (PD) affects 20%–50% of the total global population, with severe disease occurring in 9.8% of individuals (Albandar & Rams, 2002; Bernabe et al., 2020). For the majority of patients, non-surgical periodontal treatment (NSPT) represents the first line of treatment and involves the physical debridement of subgingival plaque biofilms. There is significant evidence of clinical improvements in PD patients following NSPT, including gains in clinical attachment level, reductions in gingival inflammation and reduced periodontal pocket depths (Graziani et al., 2010; Heitz-Mayfield, Trombelli, Heitz, Needleman, & Moles, 2003; Suvan et al., 2019).

Non-surgical periodontal treatment may be carried out with hand instruments, ultrasonic instruments or a "blended approach" using both. Similarly, treatment may be staged over several visits with a "quadrant" approach, or with a "full-mouth debridement" approach, also referred to as an "intensive treatment" approach, that delivers complete debridement within 24 hr. Although the clinical outcomes for quadrant and full-mouth treatment appear similar, there is a measurable difference in the inflammatory response following full-mouth compared with quadrant debridement (Graziani et al., 2015). Intensive/full-mouth NSPT has been consistently shown to trigger a larger systemic inflammatory response, demonstrated by significant increases in serum levels of C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) 24 hr following treatment (Graziani et al., 2015; Morozumi et al., 2018; Tonetti et al., 2007). Whilst this increase in systemic inflammation is short lived and typically resolves within 7 days (Graziani et al., 2015; Graziani et al., 2010; Tonetti et al., 2007), a recent joint consensus statement advised against full-mouth debridement for some medically compromised patients (Sanz et al., 2020).

Studies investigating the systemic inflammatory response generally report use of both hand and ultrasonic instruments in combination. When used individually, both types of instrument have been shown to significantly improve the clinical status of patients with equal efficacy (Ioannou et al., 2009; Suvan et al., 2019). Although the clinical outcomes are similar, several studies report lower treatment times using ultrasonic instruments in comparison with hand instruments (Copulos, Low, Walker, Trebilcock, & Hefti, 1993; Dragoo, 1992; Tunkel, Heinecke, & Flemmig, 2002; Yukna, Scott, Aichelmann-Reidy, LeBlanc, & Mayer, 1997). Interestingly, a positive correlation between treatment time and CRP levels 24 hr after treatment has been reported (Graziani et al., 2015).

Given this previous link between treatment time and the subsequent systemic inflammatory response, and the different features of each instrumentation technique including delivery of water irrigation and the reduction in cementum removal with ultrasonics (Bozbay et al., 2018; Ruhling, Bernhardt, & Kocher, 2005), we hypothesized that hand and ultrasonic instruments may differ in the extent of systemic inflammation they induce. Thus, the aim of this study was to investigate changes in systemic markers of inflammation 24 hr following full-mouth debridement, comparing hand instrumentation (HI) and ultrasonic instrumentation (UI). Secondary outcomes included comparing clinical parameters and treatment time between each group.

Clinical Relevance

Scientific rationale for the study: Intensive full-mouth debridement has been consistently demonstrated to induce a systemic inflammatory response 24 hr after treatment, which has previously been linked to duration of treatment. In medically compromised patients, this inflammatory response is hypothesized to potentially aggravate pre-existing conditions.

Principle findings: We have demonstrated that this systemic inflammatory response is comparable following full-mouth debridement with hand or ultrasonic instruments, with faster treatment time with ultrasonic instruments. Improvements in clinical outcomes were comparable between groups.

Practical implications: The choice of hand or ultrasonic instruments does not appear to impact the systemic inflammatory response 1 day following full-mouth debridement.

2 | METHODS

2.1 | Study design and patient selection

This was a single-centre randomized controlled trial with two intervention arms, with patients returning at day 1, day 7 and day 90 post-treatment. The study received approval from the Research Ethics Committee (18/NI/0059) and was registered with ClinicalTrials.gov (ID: NCT03501316). The principles in the Declaration of Helsinki were adhered to throughout the trial.

Patients were referred by their General Dental Practitioner (GDP) to Unit of Periodontics at Glasgow Dental Hospital for specialist management of periodontal disease. The specialist service is provided by the National Health Service, and prior to referral patients attend their GDP for assessment and initial periodontal treatment including oral hygiene instruction. Patients were approached during new patient assessment clinics in the Unit of Periodontics. All participants gave informed, written consent. The inclusion criteria were as follows: male or female patients aged 18–70 years inclusive with probing depths ≥5 mm on two or more teeth at non-adjacent sites with cumulative probing depths of ≥40 mm. Cumulative probing depth was calculated by examining six sites on each tooth. The deepest site on each tooth was recorded and if the value was >4 mm, this contributed to the cumulative total, with each tooth being only counted once towards the total to ensure extent of disease. The use of cumulative probing pocket depth ensured a minimum level of periodontal disease (≥2 sites with probing depths with ≥5 mm) (Page & Eke, 2007; Tonetti & Claffey, 2005) and has recently been adopted as a means of including patients

with a disease burden that is potentially relevant to systemic inflammation (Lopez-Oliva Santa Cruz 2018; Serban et al., 2019). Exclusion criteria included the following: known or suspected high risk for tuberculosis, hepatitis B or HIV infections; required interpreter/non-English language written material to understand and provide written, informed consent or any other reason for being unable to provide written, informed consent; history of bleeding diathesis; pregnant or lactating females; self-reported diagnosis of any systemic illnesses including cardiovascular, renal and liver diseases, and/or regular use of medication to control systemic illness; any pharmacological treatment within 1 month before the beginning of the study, including routine use of any over the counter medications and specialist periodontal treatment in the previous 6 months.

All visits were carried out within the clinical research facility of the Glasgow Dental Hospital. Baseline data were gathered including height, weight, blood pressure (using an automatic oscillometric unit) and smoking status was recorded as "never," "current" or "previous." Samples taken at baseline included serum, whole blood, saliva, subgingival plaque and gingival crevicular fluid.

2.2 | Study procedures

At the baseline visit, patients were provided with detailed oral hygiene instruction, dental health education and a full-mouth supragingival scale (using a Cavitron® Powerline® 1000 30K insert). All interventions and clinical data collection were carried out by an experienced dental hygienist (DM) and specialist trainee in restorative dentistry (MP). For calibration, both examiners completed pocket charts on the first twelve patients entering the study. Charts were assessed for agreement and a kappa score was calculated (0.66). Following collection of blood samples at day 1 post-treatment, patients were provided with an electric toothbrush (Oral-B Pro 2000) to standardize self-performed plaque control prior to day 90 follow-up.

At baseline and day 90, clinical parameters (full-mouth plaque, bleeding scores and detailed 6-point periodontal pocket charting) were assessed using a PCP-12 periodontal probe at six sites per tooth, excluding third molars (unless other molar units missing), with measurements rounded to the nearest millimetre. Following collection of clinical data, the periodontal inflamed surface area (PISA) was calculated as previously described (Nesse et al., 2008).

Patients were randomized to one of two treatments (HI or UI) (Figure 1). Randomization was performed using a computerized random number generator (using permuted blocks of 4 and 6). Patients were stratified according to smoking status prior to randomization.

For allocation concealment and blinding, patients were allocated to each arm of the study by a member of the research team not involved with the clinical delivery of the experimental interventions. Upon attending their treatment appointment, an opaque sequentially numbered envelope contained the allocated intervention arm for the patient. This was opened immediately before treatment was commenced.

Patients and clinicians both remained blinded to the intervention until the intervention visit. Statistical and laboratory personnel remained blinded to specific patient allocation throughout the entire process via patient barcodes. The key linking barcodes to patients was available only to the chief investigator. Intervention codes were only available once all analyses took place.

Full-mouth debridement was carried out within a 24-hr period. All but one patient completed treatment within the same day; a single patient completed debridement on consecutive days, within 24 hr, due to patient availability. Debridement was completed using Gracey and Universal curettes (Gracey 1/2, Gracey 7/8, Gracey 9/10, Gracey 11/12, Gracey 13/14, Columbia 4L-4R) and hoes (Hoe Scaler-lateral, Hoe Scaler-posterior, LM Dental) for the hand instrumentation (HI) group; or Cavitron Ultrasonic inserts (Cavitron® Thinsert® 30K, Cavitron® Slimline® 10S 30K, Cavitron® Slimline® 10L 30K, Cavitron® Slimline® 10R 30K, Cavitron® Slimline® 1000 30K, Cavitron® Powerline® 1000 30K; Dentsply Sirona) for the ultrasonic instrumentation (UI) group. Treatment was provided using local anaesthetic and timed by digital stopwatch from the point of first contact between instrument and tooth surface. Debridement was carried out until no supra or subgingival plaque or calculus deposits were detectable by visual examination with magnification or by tactile examination. Patients were recalled following periodontal treatment at day 1, day 7 and day 90. Samples were collected as per baseline visit (serum, whole blood, saliva, subgingival plaque, GCF) at each timepoint, with clinical parameters measured at day 90 only. Following day 90 review, any further treatment need was evaluated by a Specialist in Periodontology.

2.3 | Systemic inflammatory markers

Systemic inflammatory markers CRP (primary outcome), IL-6 and TNFα (secondary outcomes) were measured at all timepoints (baseline, day 1, day 7 and day 90). Levels of serum CRP were determined by high-sensitivity immunoturbidometry using the Cobas C311 analyser (Cobas; Roche Diagnostic). Serum IL-6 and TNFα were determined using high-sensitivity ProQuantum qPCR immunoassays (Thermo Fisher) measured on a StepOnePlus real-time PCR system. The limit of detection (LOD) for IL-6 and TNFα was 0.12 and 0.01 pg/ml, respectively. CRP and IL-6 were detected in all samples; TNFα was below LOD in seven samples which were assigned as LOD/2 for statistical analysis. All laboratory assays were conducted following study completion by laboratory staff masked to treatment groups. Analysis of serum CRP was performed at the British Heart Foundation Glasgow Cardiovascular Research Centre, with IL-6 and TNFα measurements performed at Glasgow Dental School. Intra- and inter-assay coefficients of variations were <5%.

2.4 | Sample size calculation

The primary outcome for this study was serum CRP levels at day 1 post-treatment. Secondary outcomes were CRP at day 7 and day

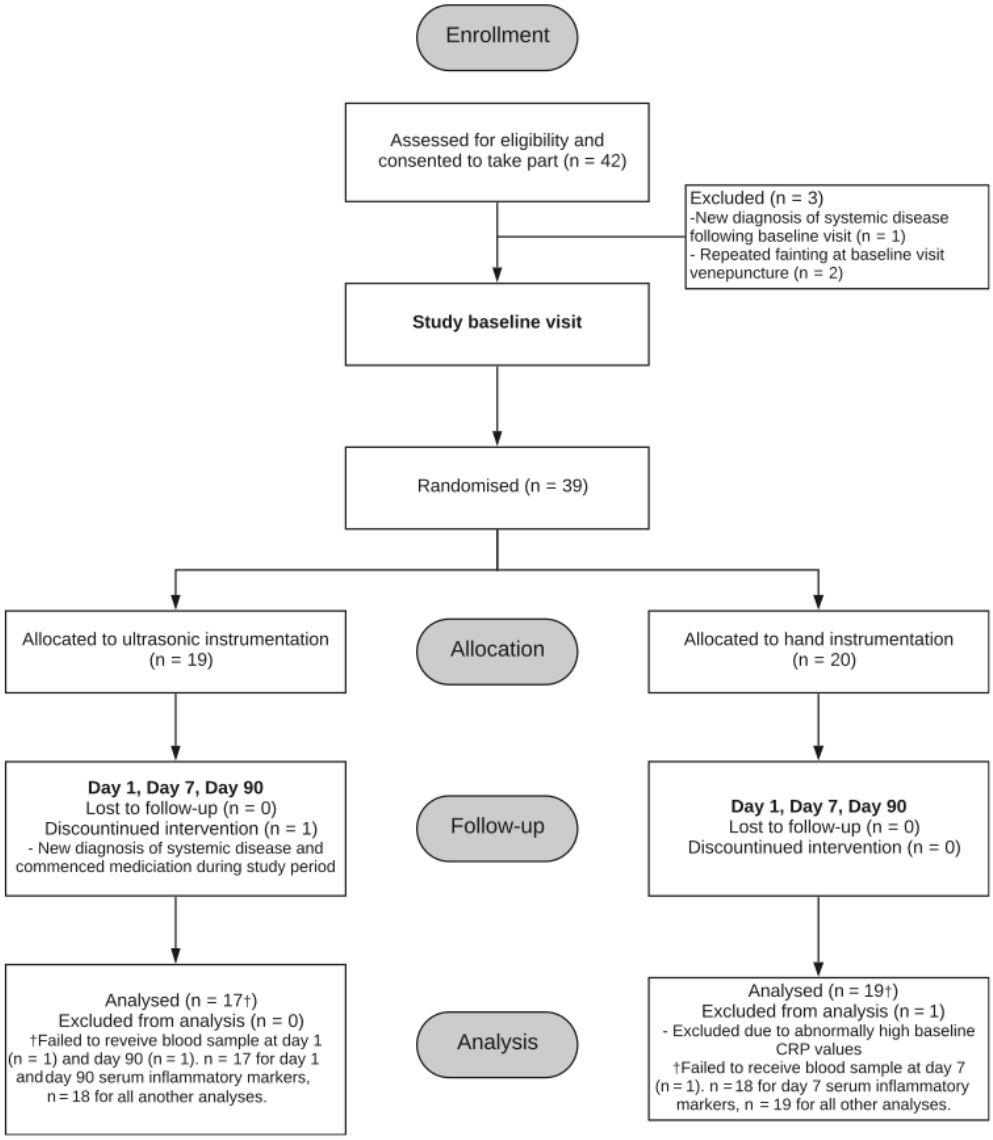

FIGURE 1 CONSORT flow diagram for study. Blood samples were not obtained from one patient at day 1 (UI group), one patient at day 7 (HI group) and one patient at day 90 (UI group). Therefore, for analysis of serum inflammatory markers; at day 1 (UI; $n = 17$, HI; $n = 19$), day 7 (UI; $n = 18$, HI; $n = 18$) and day 90 (UI; $n = 17$, HI; $n = 19$). For analysis of clinical parameters and treatment time (UI; $n = 18$, HI; $n = 19$)

90, other systemic inflammatory markers (IL-6, TNFα) at day 1, day 7 and day 90, clinical parameters at day 90 and treatment time. The sample size calculation was based on data from a previous study that measured changes in CRP following periodontal treatment (Graziani et al., 2015). From this study, a difference of 3.5 mg/L (SD = 3 mg/L) in CRP was detected between the two groups receiving different schedules of periodontal treatment (quadrant versus full-mouth debridement) at primary endpoint, and this has been considered

clinically relevant in recent guidelines (Sanz et al., 2020); therefore, this was considered a reasonable estimate of the minimum clinically relevant difference. At 80% power and a 5% significance level, a sample size of $n = 34$ (17 in each group) was required to detect a minimum difference of at least 1 standard deviation between CRP levels at primary endpoint (day 1) between the two groups (HI versus UI). To account for potential drop-out of 20%, 42 eligible patients were recruited.

2.5 | Statistical analysis

Study data were entered into SPSS (v26; IBM) using anonymous patient codes and then analysed using Stata (v21; Statacorp). Graphics were produced using PRISM (v8; GraphPad). All outcome data are summarized using median (Q1, Q3). Changes in clinical parameters and serum inflammatory markers between baseline and the various follow-ups were assessed using Wilcoxon signed rank tests. For between-group comparisons, general linear models (GLMs) were produced. Serum CRP, IL-6 and TNFα were skewed on visual inspection of histograms; therefore these were ln-transformed and shown to follow a ln-normal (symmetrical) distribution. GLMs were produced to test differences in systemic inflammatory markers (CRP, IL-6 and TNFα all ln-transformed) between the two groups (HI; US), unadjusted (model 1), after adjusting for baseline levels (model 2), and after adjusting for baseline levels, gender, age, smoking status and BMI (model 3). Differences in clinical parameters between groups at day 90 were assessed using GLMs adjusting for baseline levels, number of teeth, age, gender and smoking status. For skewed variables (FMPS, FMBS, Pockets ≥5 mm and PISA), ln-transformation resulted in normal distribution and these values were used. Differences in treatment time were assessed by a GLM controlling for disease severity (PISA mm^2) and number of teeth at baseline. Correlations between treatment time and disease severity (PISA mm^2) and change in inflammatory markers (day 1 minus baseline values) were conducted using Spearman's Rho. The correlations were ancillary post hoc analyses and were not pre-specified in the trial protocol.

3 | RESULTS

3.1 | Study population and clinical parameters

In total, 42 patients were recruited to the study. Throughout the course of the study, four subjects were excluded. One patient was withdrawn due to developing new medical diagnoses following baseline visit and two patients were withdrawn due to unexplained repeated fainting during venepuncture. One patient completed all interventions but was excluded from analysis due to an unexplained pathologically high baseline serum CRP level (11.97 mg/L), as shown in Figure 1.

TABLE 1 Baseline demographics and periodontal clinical parameters

Variable	Ultrasonic instruments ($n = 18$)	Hand instruments ($n = 19$)
Age, years	46.00 (36.75, 54.50)	41.00 (39.00, 49.00)
Gender, female (%)†	10 (56)	9 (47)
Smoking, current (%)†	5 (28)	6 (32)
BMI, kg/m^2	27.80 (22.68, 30.15)	29.70 (23.30, 34.40)
Systolic BP, mm Hg	124.00 (114.50, 139.50)	123.00 (117.00, 134.00)
Diastolic BP, mm Hg	79.50 (75.50, 89.00)	81.00 (73.00, 84.00)
CRP, mg/L	1.60 (0.62, 2.49)	1.21 (0.44, 2.03)
IL-6, pg/ml	2.61 (1.13, 3.54)	2.29 (1.52, 4.41)
TNFα, pg/ml	0.22 (0.11, 1.09)	0.13 (0.09, 0.36)
Number of teeth	27.50 (24.50, 30.00)	29.00 (27.00, 31.00)
FMPS (%)	45.92 (26.10, 63.33)	60.48 (25.00, 67.74)
FMBS (%)	38.11 (21.45, 61.49)	45.00 (21.26, 69.44)
PPD (mm)	3.70 (3.35, 4.12)	3.98 (3.11, 4.78)
CAL (mm)	4.14 (3.66, 4.44)	4.36 (3.29, 5.02)
Pockets ≥5 mm (%)	26.73 (22.08, 36.71)	28.85 (18.33, 51.39)
PISA (mm^2)	957.93 (385.55, 1,759.57)	1,010.02 (561.99, 2,190.01)

Note: Variables are presented as median (Q1, Q3), unless followed by "†" which are presented as *n* (%).

Abbreviations: BMI, body mass index; BP, blood pressure; CAL, clinical attachment loss; CRP, C-reactive protein; FMBS, full-mouth bleeding score; FMPS, full-mouth plaque score; IL-6, interleukin-6; PISA, periodontal inflamed surface area; PPD, probing pocket depth; TNFα, tumour necrosis factor alpha.

Baseline characteristics were comparable between treatment groups, including demographic, clinical and biological variables (Table 1). According to the 2017 Classification, 45% of the patients presented with Generalized Stage 4 Grade C periodontitis, 42% with Generalized Stage 3 Grade B periodontitis and 13% Generalized Stage 3 Grade C periodontitis (Papapanou et al., 2018).

There were significant improvements in clinical parameters across all patients following treatment ($p < .01$ for all clinical parameters comparing baseline and day 90 values, irrespective of treatment group). This included reductions in the percentage of pockets ≥5 mm (HI: 28.85%–11.67%, UI: 26.73%–10.88%) and PISA (HI: 1010.02–192.59 mm^2, UI: 957.93–134.85 mm^2). The improvement in clinical parameters was comparable between treatment groups ($p > .05$ for all; Table 2).

TABLE 2 Comparison of clinical parameters between groups at baseline (BL) and day 90 (D90)

Variable	Timepoint	Ultrasonic instruments $n = 18$	Hand instruments $n = 19$	Between group p-value[b]
FMPS (%)	Baseline	45.92 (26.10, 63.33)	60.48 (25.00, 67.74)	
	Day 90	7.80 (3.50, 13.25)	8.33 (4.17, 14.06)	
	Within group p-value[a]	<.001	<.001	.55
FMBS (%)	Baseline	38.11 (21.45, 61.49)	45.00 (21.26, 69.44)	
	Day 90	8.10 (4.12, 12.08)	8.33 (2.98, 13.10)	
	Within group p-value[a]	<.001	<.001	.94
Pockets ≥5 mm (%)	Baseline	26.73 (22.08, 36.71)	28.85 (18.33, 51.39)	
	Day 90	10.88 (3.87, 16.88)	11.67 (3.89, 30.95)	
	Within group p-value[a]	<.001	<.001	.23
PISA (mm^2)	Baseline	957.93 (385.55, 1,759.57)	1,010.02 (561.99, 2,190.01)	
	Day 90	134.85 (62.31, 219.72)	192.59 (59.78, 380.49)	
	Within group p-value[a]	<.001	<.001	.68
PPD (mm)	Baseline	3.70 (3.35, 4.12)	3.98 (3.11, 4.78)	
	Day 90	2.68 (2.39, 3.09)	3.02 (2.52, 3.73)	
	Within group p-value[a]	<.001	<.001	.08
CAL (mm)	Baseline	4.14 (3.66, 4.44)	4.36 (3.29, 5.02)	
	Day 90	3.64 (3.10, 4.12)	4.01 (3.03, 4.68)	
	Within group p-value[a]	<.001	.005	.14

Note: Values are presented as median (Q1, Q3).

[a]Differences between baseline and day 90 within groups tested using Wilcoxon signed rank test.

[b]GLMs were used to test differences in clinical parameters between the groups at day 90 having adjusted for baseline levels, number of teeth, age, gender and smoking status. For skewed variables (FMPS, FMBS, Pockets ≥5 mm and PISA) ln-transformed values were used.

3.2 | Systemic inflammation

When combining all patients, there were significant increases in the levels of serum CRP at day 1 ($p = .002$ day 1 versus baseline) which returned to baseline levels at day 7 ($p = .215$ day 7 versus baseline) and day 90 ($p = .255$ day 90 versus baseline; Figure 2a). Similarly, levels of serum IL-6 in all patients increased at day 1 ($p = .019$ day 1 versus baseline) and returned to baseline levels at day 7 ($p = .765$ day 7 versus baseline) and day 90 ($p = .671$ day 90 versus baseline; Figure 2b). There were low levels of serum TNFα in the majority of patients, with seven samples below the assay detection limit. Serum TNFα was significantly reduced at day 1 ($p = .002$ versus baseline). Interestingly, TNFα returned to baseline levels at day 7 ($p = .765$ versus baseline) but reduced again at day 90 ($p = .013$ versus baseline; Figure 2c).

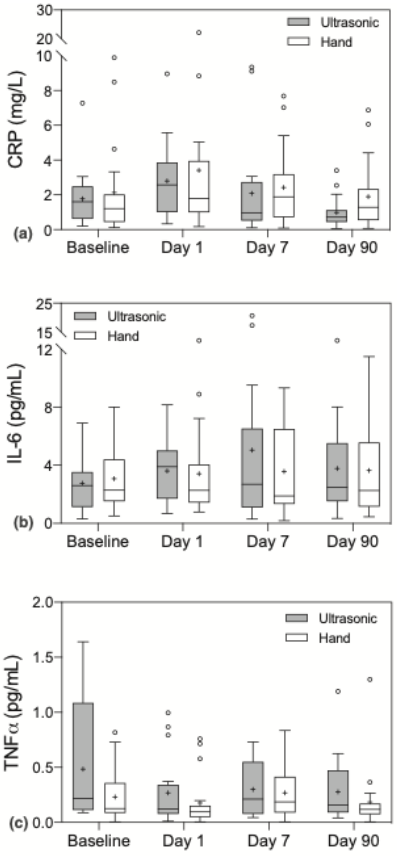

FIGURE 2 Levels of serum inflammatory markers in patients treated with ultrasonic (grey boxes) and hand instruments (clear boxes) at baseline, day 1, day 7 and day 90 follow-up. (a) CRP (b) IL-6 and (c) TNFα. Data are displayed as a Tukey boxplot, where the median is represented by the central horizontal line and mean displayed as "+". As described previously, day 1: UI; $n = 17$ HI; $n = 19$, day 7: UI; $n = 18$ HI; $n = 18$, day 90: UI; $n = 17$ HI; $n = 19$. For statistical analysis, see Table 3

The differences between treatment groups for serum inflammatory markers at day 1 (primary outcome), day 7 and day 90 were then investigated using a GLM controlling for baseline levels of each marker, smoking status, age, gender and BMI (Table 3). Despite significant increases in serum CRP at day 1, there were no statistically significant differences between treatment groups (p(adjusted) = .22). This remained consistent for CRP levels at day 7 (p(adjusted) = .53) and day 90 (p(adjusted) = .28). Similarly, there was no difference in levels of serum IL-6 and TNFα between treatment groups at day 1, day 7 and day 90 ($p > .05$ for both cytokines at all timepoints).

3.3 | Treatment time

We next sought to assess differences in treatment time between HI and UI groups. For total treatment time, the mean (SD) for UI was 75.39 (17.83) min, compared with 96.90 (23.54) min for HI (Figure 3a). The difference in treatment time between instruments (UI-HI) was assessed using a GLM controlling for disease severity (PISA mm^2) and number of teeth at baseline (β: −22.12, 95% CI: −35.19 to −9.06, $p = .002$). Following post hoc analysis, there was no correlation between treatment time and disease severity at baseline (PISA mm^2) for UI (Spearman $r = .152$, $p = .547$; Figure 3b), whilst there was a positive correlation for HI (Spearman $r = .598$, $p = .007$; Figure 3c). We found no significant positive or negative correlation between the change in serum inflammatory markers (day 1 minus baseline values) and the time of treatment (Figure 3d,f).

4 | DISCUSSION

To the authors' knowledge, this is the first randomized controlled trial to investigate the impact of different periodontal instrumentation techniques on systemic inflammation following full-mouth debridement. As expected, a significant increase in CRP was observed one day following treatment across all patients; however, the level of CRP at day 1 did not differ following hand or ultrasonic instrumentation.

The observed short-term CRP increase is consistent with previous studies conducted with similar inclusion and exclusion criteria (D'Aiuto, Nibali, Mohamed-Ali, Vallance, & Tonetti, 2004; Graziani et al., 2015; Graziani et al., 2010; Morozumi et al., 2018; Tonetti et al., 2007). However, the average magnitude of the increase in immediate post-treatment CRP was 1.67-fold in this study; somewhat less than that reported in previous studies (Graziani et al., 2015; Tonetti et al., 2007), which showed an approximately 3-fold and 8-fold increase in CRP 24-hr post-treatment respectively. An explanation for this may lie in the differential in mean treatment times between studies (Graziani et al., 2015; Tonetti et al., 2007). Mean treatment time for all patients in this study was 86.8 ± 23.5 min, which was less than Graziani *et al.* where the reported mean treatment time was 123 ± 18 min for their full-mouth debridement group. Similarly, the mean baseline plaque score for this study was 44.68 ± 25.68% (UI group) whereas Graziani et al. reported 70 ± 26% in their full-mouth debridement group. This difference may be due to patients in this study having received basic oral hygiene instruction prior to referral to the department. Furthermore, study patients received a full-mouth supragingival scale prior to treatment (Lang & Lindhe, 2015; Scottish Dental Clinical Effectiveness Programme 2014; Suvan et al., 2019) (following baseline plaque scoring), which will likely have reduced plaque scores even further prior to full-mouth debridement. These factors may help explain relative differences in mean treatment time or CRP response between studies.

A recently released joint consensus statement between the European Federation of Periodontology and American Academy

TABLE 3 Parameter estimates with 95% confidence intervals for ultrasonic instrumentation compared with hand instrumentation for ln-transformed serum CRP, IL-6 and TNFα levels at day 1, day 7 and day 90

	Day 1			Day 7			Day 90		
	β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value
C-reactive protein									
Model 1[a]	0.143	−0.582 to 0.867	.69	−0.271	−1.094 to 0.553	.51	−0.518	−1.239 to 0.202	.15
Model 2[b]	0.130	−0.369 to 0.628	.60	−0.311	−0.950 to 0.329	.33	−0.482	−1.038 to 0.073	.09
Model 3[c]	0.318	−0.196 to 0.832	.22	−0.231	−0.972 to 0.510	.53	−0.304	−0.867 to 0.259	.28
Interleukin-6									
Model 1[a]	0.133	−0.378 to 0.643	.60	0.176	−0.620 to 0.972	.66	−0.011	−0.671 to 0.650	.97
Model 2[b]	0.261	−0.093 to 0.614	.14	0.233	−0.524 to 0.991	.54	0.053	−0.546 to 0.653	.86
Model 3[c]	0.193	−0.173 to 0.559	.29	0.182	−0.634 to 0.999	.65	−0.005	−0.645 to 0.636	.99
Tumour necrosis factor α									
Model 1[a]	0.607	−0.307 to 1.521	.19	0.189	−0.568 to 0.946	.62	0.49	−0.192 to 1.171	.15
Model 2[b]	0.00003	−0.677 to 0.678	.99	−0.087	−0.843 to 0.669	.82	0.021	−0.512 to 0.555	.94
Model 3[c]	−0.215	−0.902 to 0.472	.53	−0.167	−1.022 to 0.688	.69	−0.121	−0.687 to 0.445	.67

Note: Parameter estimates (β-values) are based on ln-transformed data.

As described in Figure 1, day 1: UI; $n = 17$ HI; $n = 19$, day 7: UI; $n = 18$ HI; $n = 18$, day 90: UI; $n = 17$ HI; $n = 19$.

[a]Model 1: Unadjusted.

[b]Model 2: Adjusted for baseline levels of each serum marker.

[c]Model 3: Adjusted for baseline levels of serum marker, gender, age, smoking status and BMI at baseline.

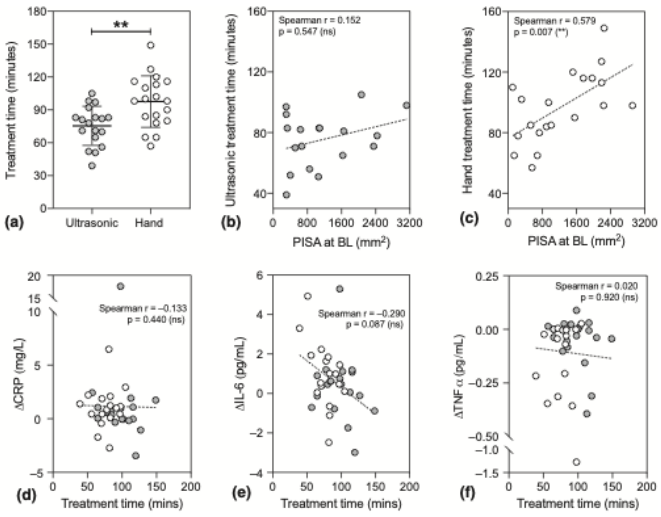

FIGURE 3 Treatment time comparison between ultrasonic ($n = 18$) and hand ($n = 19$) instrumentation. (a) Total treatment time controlled for number of teeth and disease severity (PISA mm^2) at baseline, $^{**}p < .01$, GLM. Correlation between total treatment time with (b) ultrasonic instruments and (c) hand instruments versus disease severity. Correlations between the change (Δ) in serum CRP (d), IL-6 (e) and TNFα (f) and treatment time. Grey circles represent patients in ultrasonic treatment group, white represent hand treatment group. All correlations are Spearman-rho, $^{**}p < .01$, ns indicates no significant difference. UI; $n = 18$, HI; $n = 19$

of Periodontology advised against the use of full-mouth debridement in patients with any level of cardiovascular disease (Sanz et al., 2020). In this study, an increase in serum CRP was observed across all patients (average increase of 1.07 mg/L), although we observed heterogeneity in the extent of this response. The absolute change in serum CRP from baseline to day 1 ranged from a 3.45 mg/L decrease to a 17.56 mg/L increase, suggesting large inter-patient variation, which did not relate to instrumentation choice. Therefore, establishing whether demographic, clinical or biological factors at baseline influence or predict this response is important in order to establish which patients may be more at risk of a significant CRP spike. As noted, there are differences in plaque indexes between

our study and those conducted previously. Thus, assessing whether supragingival debridement, prior to full-mouth debridement, may help reduce this spike would be an important addition to future research.

In this study, with respect to long-term changes in CRP, at day 90 follow-up serum CRP was similar to baseline levels. There were no changes in serum IL-6, although a reduction was found in TNFα at day 90 compared with baseline. However, the significance of this is questionable given that only very low levels of TNFα were detected at all time points. In relation to CRP and IL-6, 90-days following treatment may be too early to detect a net reduction in these circulating inflammatory markers. Moreover, the current study was not powered to detect this long-term difference. Some studies report a significant decrease in serum CRP (and IL-6) following intensive periodontal therapy at a follow-up of 2 months (D'Aiuto, Nibali, Parkar, Suvan, & Tonetti, 2005), whilst others report no change after 6 weeks (Ide et al., 2003). One explanation for these contrasting results may be patient comorbidities. A previous systematic review concluded that patients with comorbidities demonstrate significantly greater reductions in CRP following NSPT compared with systemically healthy patients (Teeuw et al., 2014). As the patients in the current study were systemically healthy and did not display elevated CRP levels (43% patients <1 mg/L, 41% patients 1–3 mg/L, 16% patients >3 mg/L at baseline), it is perhaps unsurprising that there was no notable reduction in serum CRP following treatment.

There were no discernible differences in clinical outcome, comparing instrumentation techniques, albeit this finding should be viewed as a secondary outcome measure that the study was not powered to investigate. Encouragingly, all patients' clinical parameters showed marked improvement following therapy. A pocket closure rate (defined as conversion of a periodontal site measuring ≥5 mm to a site measuring ≤4 mm) of 58.54%, mean periodontal probing depth reduction of 0.98 mm and mean PISA reduction of 985.92 mm^2 (80.83% reduction) was recorded on average across all patients at day 90. These findings are similar to a recent rigorous systematic review that identified pocket closure rates of 57% and mean periodontal probing depth reductions of 1.0 mm at 3/4 month follow-up (Suvan et al., 2019).

The reduced treatment time for use of ultrasonic compared with hand instruments is commensurate with previous studies (Badersten, Nilveus, & Egelberg, 1981; Breininger, O'Leary, & Blumenshine, 1987; Dragoo, 1992; Laurell, 1990; Tunkel et al., 2002; Yukna et al., 1997). Interestingly, treatment time for hand instrumentation correlated with disease severity, whist ultrasonic showed no such relationship; it could be speculated that ultrasonics may offer greater time saving for the treatment of severe PD. However, it should be noted that this is an incidental finding of this study and confirmation would require more rigorous investigation.

The current study was designed from the outset to investigate CRP levels at day 1 following two methods of periodontal instrumentation. Robust randomization, concealed allocation and appropriate blinding were implemented. Inevitably, potential for bias and limitations remain in certain aspects of this clinical trial. For example, there may be uneven distribution of unidentified confounding variables between interventional arms. Inclusion of factors such as BMI and baseline CRP in stratification techniques could be considered for future studies. In addition, patients with good motivation and compliance with dental care may self-select for inclusion within trials and this has implications for external validity as patients within the larger population may not adhere to oral hygiene instruction to the same extent—thus influencing their periodontal disease outcome. In this study, it was impossible to blind operators to treatment modality which may potentially result in observation bias. The current study found equivalence between the groups in terms of the primary outcome measure of change in CRP post-treatment. The findings from the current study can be considered robust; however, the study was designed to detect a meaningful difference, and not designed to detect equivalence, thus the data should be interpreted with due consideration to this caveat.

In conclusion, the data presented show that systemic inflammation, as measured by serum CRP, showed a significant increase 1-day following full-mouth debridement. There was no difference in CRP at day 1 between hand instrumentation and ultrasonic instrumentation techniques. Treatment efficacy was similar in both groups; however, treatment was significantly quicker with ultrasonic instruments regardless of disease severity.

ACKNOWLEDGEMENTS

The authors are very grateful for all the help provided from Glasgow Dental Hospital Clinical Research Facility, in particular Clare Brown and Debbie McKenzie. We would also like to thank Dr. Paul Welsh and Josephine Cooney at the University of Glasgow Institute of Cardiovascular and Medical Sciences for their support.

CONFLICT OF INTEREST

William Johnston received grant funding from Dentsply Sirona. Marilyn Goulding is the Manager of Clinical Research, Global Clinical Affairs, Dentsply Sirona. Shauna Culshaw received honoraria from Dentsply Sirona. Michael Paterson, Krystyna Piela, Emily Davison, Annabel Simpson, Gordon Ramage and Andrea Sherriff declare no conflicts of interest.

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REFERENCES

Albandar, J. M., & Rams, T. E. (2002). Global epidemiology of periodontal diseases: An overview. *Periodontology 2000*, *29*(1), 7–10.

Badersten, A., Nilveus, R., & Egelberg, J. (1981). Effect of nonsurgical periodontal therapy. I. Moderately advanced periodontitis. *Journal of Clinical Periodontology*, *8*(1), 57–72.

Bernabe, E., Marcenes, W., Hernandez, C. R., Bailey, J., Abreu, L. G., Alipour, V., … Kassebaum, N. J. (2020). Global, regional, and national levels and trends in burden of oral conditions from 1990 to 2017: A systematic analysis for the global burden of disease 2017 study. *Journal of Dental Research*, *99*(4), 362–373. https://doi.org/10.1177/0022034520908533

Bozbay, E., Dominici, F., Gokbuget, A. Y., Cintan, S., Guida, L., Aydin, M. S., … Pilloni, A. (2018). Preservation of root cementum: a comparative evaluation of power-driven versus hand instruments. *International Journal of Dental Hygiene*, *16*(2), 202–209. https://doi.org/10.1111/idh.12249.

Breininger, D. R., O'Leary, T. J., & Blumenshine, R. V. (1987). Comparative effectiveness of ultrasonic and hand scaling for the removal of subgingival plaque and calculus. *Journal of Periodontology*, *58*(1), 9–18. https://doi.org/10.1902/jop.1987.58.1.9.

Copulos, T. A., Low, S. B., Walker, C. B., Trebilcock, Y. Y., & Hefti, A. F. (1993). Comparative analysis between a modified ultrasonic tip and hand instruments on clinical parameters of periodontal disease. *Journal of Periodontology*, *64*(8), 694–700. https://doi.org/10.1902/jop.1993.64.8.694.

D'Aiuto, F., Nibali, L., Mohamed-Ali, V., Vallance, P., & Tonetti, M. S. (2004). Periodontal therapy: A novel non-drug-induced experimental model to study human inflammation. *Journal of Periodontal Research*, *39*(5), 294–299. https://doi.org/10.1111/j.1600-0765.2004.00741.x.

D'Aiuto, F., Nibali, L., Parkar, M., Suvan, J., & Tonetti, M. S. (2005). Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. *Journal of Dental Research*, *84*(3), 269–273. https://doi.org/10.1177/154405910508400312.

Dragoo, M. R. (1992). A clinical evaluation of hand and ultrasonic instruments on subgingival debridement. Part I. With unmodified and modified ultrasonic inserts. *International Journal of Periodontics and Restorative Dentistry*, *12*(4), 310–323. https://doi.org/10.11607/prd.00.0020.

Graziani, F., Cei, S., Orlandi, M., Gennai, S., Gabriele, M., Filice, N., … D'Aiuto, F. (2015). Acute-phase response following full-mouth versus quadrant non-surgical periodontal treatment: A randomized clinical trial. *Journal of Clinical Periodontology*, *42*(9), 843–852. https://doi.org/10.1111/jcpe.12451.

Graziani, F., Cei, S., Tonetti, M., Paolantonio, M., Serio, R., Sammartino, G., … D'Aiuto, F. (2010). Systemic inflammation following non-surgical and surgical periodontal therapy. *Journal of Clinical Periodontology*, *37*(9), 848–854. https://doi.org/10.1111/j.1600-051X.2010.01585.x.

Heitz-Mayfield, L. J. A., Trombelli, L., Heitz, F., Needleman, I., & Moles, D. (2003). A systematic review of the effect of surgical debridement vs. non-surgical debridement for the treatment of chronic periodontitis. *Journal of Clinical Periodontology*, *29*(s3), 92–102. https://doi.org/10.1034/j.1600-051x.29.s3.5.x

Ide, M., McPartlin, D., Coward, P. Y., Crook, M., Lumb, P., & Wilson, R. F. (2003). Effect of treatment of chronic periodontitis on levels of serum markers of acute-phase inflammatory and vascular responses. *Journal of Clinical Periodontology*, *30*(4), 334–340.

Ioannou, I., Dimitriadis, N., Papadimitriou, K., Sakellari, D., Vouros, I., & Konstantinidis, A. (2009). Hand instrumentation versus ultrasonic debridement in the treatment of chronic periodontitis: A randomized clinical and microbiological trial. *Journal of Clinical Periodontology*, *36*(2), 132–141. https://doi.org/10.1111/j.1600-051X.2008.01347.x

Lang, N. P., & Lindhe, J. (2015). *Clinical periodontology and implant dentistry*. Chichester, UK: Wiley-Blackwell.

Laurell, L. (1990). Periodontal healing after scaling and root planing with the Kavo Sonicflex and Titan-S sonic scalers. *Swedish Dental Journal*, *14*(4), 171–177.

Lopez-Oliva Santa Cruz, I. (2018). *Rheumatoid arthritis and periodontitis: antibody response, oral microbiome, cytokine profile and effect of periodontal treatment*. PhD, University of Birmingham.

Morozumi, T., Yashima, A., Gomi, K., Ujiie, Y., Izumi, Y., Akizuki, T., … Yoshie, H. (2018). Increased systemic levels of inflammatory mediators following one-stage full-mouth scaling and root planing. *Journal of Periodontal Research*, *53*(4), 536–544. https://doi.org/10.1111/jre.12543.

Nesse, W., Abbas, F., Van Der Ploeg, I., Spijkervet, F. K. L., Dijkstra, P. U., & Vissink, A. (2008). Periodontal inflamed surface area: Quantifying inflammatory burden. *Journal of Clinical Periodontology*, *35*(8), 668–673. https://doi.org/10.1111/j.1600-051X.2008.01249.x

Page, R. C., & Eke, P. I. (2007). Case definitions for use in population-based surveillance of periodontitis. *Journal of Periodontology*, *78*(7 Suppl), 1387–1399. https://doi.org/10.1902/jop.2007.060264

Papapanou, P. N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D. H., … Tonetti, M. S. (2018). Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Periodontology*, *89*(Suppl 1), S173–S182. https://doi.org/10.1002/jper.17-0721

Ruhling, A., Bernhardt, O., & Kocher, T. (2005). Subgingival debridement with a teflon-coated sonic scaler insert in comparison to conventional instruments and assessment of substance removal on extracted teeth. *Quintessence International*, *36*(6), 446–452.

Sanz, M., Marco Del Castillo, A., Jepsen, S., Gonzalez-Juanatey, J. R., D'Aiuto, F., Bouchard, P., … Wimmer, G. (2020). Periodontitis and cardiovascular diseases: Consensus report. *Journal of Clinical Periodontology*, *47*(3), 268–288. https://doi.org/10.1111/jcpe.13189

Scottish Dental Clinical Effectiveness Programme (2014). *Prevention and treatment of periodontal diseases in primary care*. Retrieved from https://www.sdcep.org.uk/published-guidance/periodontal-management/

Serban, S., Dietrich, T., Lopez-Oliva, I., de Pablo, P., Raza, K., Filer, A., … Hill, K. (2019). Attitudes towards oral health in patients with rheumatoid arthritis: A qualitative study nested within a randomized controlled trial. *JDR Clinical & Translational Research*, *4*(4), 360–370. https://doi.org/10.1177/2380084419833694

Suvan, J., Leira, Y., Moreno, F., Graziani, F., Derks, J., & Tomasi, C. (2019). Subgingival instrumentation for treatment of periodontitis. A systematic review. *Journal of Clinical Periodontology*. https://doi.org/10.1111/jcpe.13245

Teeuw, W. J., Slot, D. E., Susanto, H., Gerdes, V. E., Abbas, F., D'Aiuto, F., … Loos, B. G. (2014). Treatment of periodontitis improves the atherosclerotic profile: A systematic review and meta-analysis. *Journal of Clinical Periodontology*, *41*(1), 70–79. https://doi.org/10.1111/jcpe.12171

Tonetti, M. S., & Claffey, N. (2005). Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C Consensus report of the 5th European workshop in periodontology. *Journal of Clinical Periodontology*, *32*(s6), 210–213. https://doi.org/10.1111/j.1600-051X.2005.00822.x

Tonetti, M. S., D'Aiuto, F., Nibali, L., Donald, A., Storry, C., Parkar, M., … Deanfield, J. (2007). Treatment of periodontitis and endothelial function. *New England Journal of Medicine*, *356*(9), 911–920. https://doi.org/10.1056/NEJMoa063186

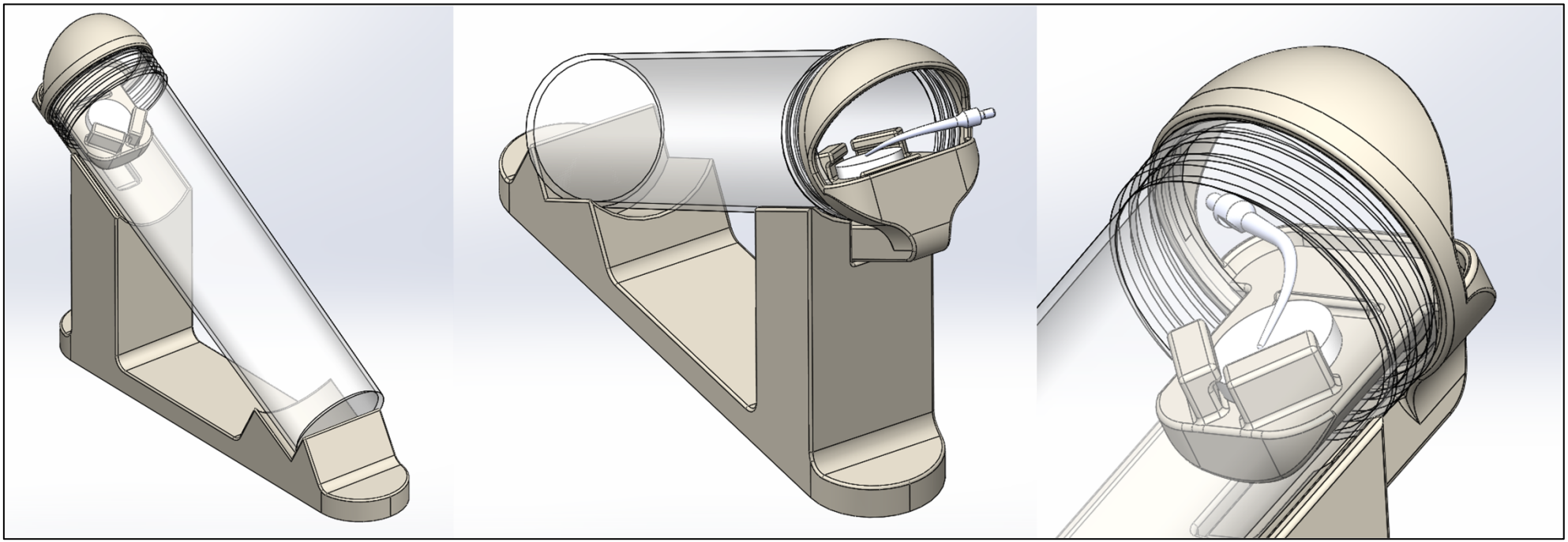
Tunkel, J., Heinecke, A., & Flemmig, T. F. (2002). A systematic review of efficacy of machine-driven and manual subgingival debridement in the

treatment of chronic periodontitis. *Journal of Clinical Periodontology*, *29*(s3), 72–81.

Yukna, R. A., Scott, J. B., Aichelmann-Reidy, M. E., LeBlanc, D. M., & Mayer, E. T. (1997). Clinical evaluation of the speed and effectiveness of subgingival calculus removal on single-rooted teeth with diamond-coated ultrasonic tips. *Journal of Periodontology*, *68*(5), 436–442. https://doi.org/10.1902/jop.1997.68.5.436

How to cite this article: Johnston W, Paterson M, Piela K, et al. The systemic inflammatory response following hand instrumentation versus ultrasonic instrumentation—A randomized controlled trial. *J Clin Periodontol*. 2020;00:1–11. https://doi.org/10.1111/jcpe.13342

Appendix iv (treatment model for ultrasonic adjuncts)



Development of a novel testing apparatus for evaluating the efficacy of ultrasonic adjuncts. Biofilms can be grown on hydroxyapatite coverslips which are fixed in the designed holder. Ultrasonic scalers can be applied within this system, allowing collection of supernatants (flow-through) for further viability analysis. Residual biofilms can also be retained for downstream analysis. Developed in collaboration with Marylin Goulding and Ken Guaragno at Dentsply Sirona.