The role of the oral microbiome in the immunobullous diseases pemphigus vulgaris and mucous membrane pemphigoid and oral lichen planus

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The role of the oral microbiome in the immunobullous diseases pemphigus vulgaris and mucous membrane pemphigoid and oral lichen planus

A thesis submitted for the degree of Doctor of Philosophy

By

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"Science and everyday life cannot and should not be separated."-R. Franklin to her father Ellis Franklin

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ABSTRACT

Saliva is formed from contributions of salivary glands and the serum exudates principally from gingival margins or damaged mucosa combined with components derived from the environment, including a community of microorganisms - the microbiome. I postulate that changes in microbial diversity and population structure play key roles in the modulation of host-microbial interactions which influence both the hypersensitive autoimmune responses and inflammation seen in these inflammatory mucocutaneous disorders. For my research, a total of 186 participants were recruited: 48 mucous membrane pemphigoid (MMP), 48 pemphigus vulgaris (PV), 50 oral lichen planus (OLP) patients, and 40 healthy controls. Unstimulated whole saliva, subgingival plaque, serum, and plasma samples were collected from 186 participants. In addition, metadata were collected on the following covariates: age, gender, ethnicity, type of the diet, disease history and therapeutic intervention in the preceding six months. Oral disease severity scores (ODSS) were assessed, and periodontal status was examined using a periodontal six pocket chart.

To characterise microbiome profiles, saliva and subgingival plaque were processed for sequencing genomic DNA using the NGS Shotgun metagenomics sequencing technique. Inflammatory cytokines and proteases were investigated in saliva and serum using Human Magnetic Luminex Screening Assay (R&D Systems). Selected cytokines were analysed by enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems) to determine host inflammatory responses in saliva and serum samples. Additionally, saliva and plasma samples were analysed for metabolites by nuclear magnetic resonance (NMR).

Significant increases in periodontal score (PISA) in all three groups of disease were identified compared to healthy control group with significant positive correlation between oral disease severity (ODSS) and PISA in OLP and PV groups.

All three groups of diseases had significantly higher levels of inflammatory Th2/Th17 cytokines (IL-6, IL-13 and IL-17 in saliva samples), as well as higher levels of MMP-3 matrixins in saliva. In addition, there were positive correlations between ODSS and salivary IL-6, IL-13 and MMP-3 in saliva of OLP, salivary and serum levels of IL-6 and MMP-3 in MMP group, and significant association of salivary IL-6, IL-1β and MMP-3 in PV group.

Metabolomic data showed that saliva is a better biofluid for correlation of the metabolomic profile with oral disease severity than plasma. Salivary ethanol was corelated with disease severity in the OLP group, whereas in PV was a strong correlation of ODSS with choline. Finally, a unique microbial community was found in each group of diseases. In the MMP group, ODSS was significantly correlated with *L. hofstadii, C. sputigena, N. meningitidis, N. cinerea and P. saccharOlytica.* In PV, a positive correlation was found with *F. nucleatum, G. morbillorum, and E. corrodens, G. elegans, H. sapiens and T. vincentii.* In OLP, the disease tends to worsen when there was reduced abundance of *X. cellulosilytica, Actinomyces ICM 47, S. parasanguinis, S. salivarius, L. mirabilis and O. sinus.* Lower microbial diversity was correlated with ODSS in saliva and plaque of the OLP group.

In conclusion, this study provides strong evidence of the complex interplay between the oral microbiome, immunological factors, and metabolites in the context of immunobullous diseases and OLP. The findings highlight the integral role of oral bacteria in disease progression, the significance of immune dysregulation, and the potential impact of specific microbial species and metabolic pathways. These insights give the way for further research and clinical applications, offering the promise of personalized approaches for diagnosis, and management of OLP, MMP and PV. Future investigations should focus on discovering the mechanistic details underlying these associations and validating the identified biomarkers in larger patient cohorts, ultimately contributing to a deeper understanding of the pathogenesis of these conditions.

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List of Abbreviations

AB- Antibiotics

AIBD- Autoimmune Bullous Diseases

ATP- Adenosine Triphosphate

AZA- Azathioprine

BCFA - Branched-Chain Fatty Acid

BP- Bullous Pemphigoid

CM- Cutaneous Microbiome

CS- Corticosteroids

DAMP- Damage-Associated Molecular Pattern

Dsc- Desmocollin

Dsg- Desmoglein

EDTA- Ethylene Diamine Tetra Acetic Acid

ELISA-Enzyme-Linked Immunosorbent Assay

EMC- Extracellular Matrix

FDR- False Discovery Rate

HC- Healthy Controls

HCQ- Hydroxychloroquine

HLA -Human Leukocyte Antigen

HSP- Heat Shock Protein

HPV- Human Papillomavirus

HCV – Hepatitis C Virus

IL- Interleukin

IS- Immunosuppressant

LC- Langerhans cells

LOAx- Loss of Attachment

LP- Lichen Planus

LPS- Lipopolysaccharide

MMF- Mycophenolate Mofetil

MMP Mucous Membrane Pemphigoid

MMPs -Matrix Metalloproteinases

MSEA- Metabolite Set Enrichment Analysis.

NGS- Next Generation Sequencing

NMR- Nuclear magnetic resonance

NO- Nitric oxide

ODSS -Oral Disease Severity Score

OLP-Oral Lichen Planus

OM- Oral Microbiome

PAMP-Pathogen-associated molecular patterns

PAD- Peptidyl Arginine Deiminase

PBS- Phosphate Buffered Saline

PCA- Principal Component Analysis

PCoA -Principal Coordinates Analysis

PCR -Polymerase chain reaction

PD -Periodontitis

PIS- Participant Information Sheet

PISA - Periodontal Inflamed Surface Area

PLS-DA- Partial Least Squares Discriminant Analysis

PUFAs- Polyunsaturated fatty acids

PV- Pemphigus vulgaris

RA- Rheumatoid Arthritis

SBP- Subgingival Plaque

SCFA- Short Chain Fatty Acids

SFR- Salivary Flow Rate

TMAO- trimethylamine N-oxide

TMA-trimethylamine

TNF- Tumour Necrosis Factor

VIP-Variable Importance in Projection

Declaration.

The procedures listed below reflect the contributions made by Dr. Malika Karimova and the instances where assistance was provided by other individuals as indicated.

Procedure	Done by Dr. Malika Karimova	With Assistance
Clinical Assessments:	Explaining the project and consent participants	
	Questioning and recording medical history	
	Reporting dietary habits	
ODSS score assessment	Recording and calculating ODSS scores	Prof. J. Setterfield, Dr. B. Carey, Dr. M. Ormond, Dr. Yogarajah
	Organising patient transfers from the examination room to the blood collection point	
	Collecting saliva samples	
	Performing clinical statistical analysis	
6 pocket periodontal examination, plaque collection	Instrument preparation and plaque collection	Prof. M. Ide, Dr. S. Zaric, Dr. V. Neves, Dr. S. Yogarajah
	Recording pocket measurements	
	Calculating periodontal scores	

Lab work: Metagenome Sequencing -	DNA extraction	
	Quality control (Nanodrop, qubit, Gel electrophoresis)	
	Sending samples and inserting the data into the sequencing platform	
Shotgun metagenomics library preparation and sequencing		SZA Omics lab, Turkey External support
Bioinformatics data analysis. - Dr. Shoaie group, Dr. F. Classen	Statistical analysis of diversity, species, phylum relative abundance, Spearman's rank correlation coefficient	Data analysis - Dr. Frederick Classen
Luminex and ELISA	Performing assays and statistical analysis	
Metabolomics analysis. NMR - King's College London NMR Core Facility	Statistical analysis of the raw data (peak areas)	NMR spectroscopy analysis Dr. Adrien Le Guennec
Manuscript Writing	Manuscript writing	

Chapter I. Introduction

1.1 Clinical and immunopathological aspects of AIBD, OLP and periodontitis

Autoimmunity occurs when the immune system mistakenly attacks and destroys healthy body tissues (Nikitakis et al., 2017). Autoimmune Disease (AD) can be mediated by T cells or B cells. It can involve a loss of regulatory mechanisms that normally help control and limit immune responses. This loss of regulation allows the immune system to continue attacking healthy tissues (Rosenblum et al, 2015). Normally, the immune system has mechanisms in place to recognize and tolerate the body's own cells and proteins, preventing it from attacking them. In AD, there is a breakdown in this self-tolerance whereby the immune system becomes unable to distinguish between "self" and "non-self."(Sakaguchi et al., 2008).

In many autoimmune diseases, the immune system produces autoantibodies that target and attack the body's own tissues or proteins causing inflammation and damage to affected tissues. In addition to autoantibodies, certain immune cells, such as T cells, can become activated and play a role in autoimmune diseases. These cells can directly attack healthy tissues or release inflammatory molecules that contribute to tissue damage (Rosenblum et al., 2015). It is not known what triggers autoimmune diseases (AD), but the most significant roles are assigned to genetic predisposition and environmental factors. Various environmental factors, such as infections, certain medications, and exposure to toxins, can trigger or exacerbate autoimmune responses in genetically susceptible individuals. These triggers may cause the immune system to react abnormally by promoting autoreactive lymphocyte release, activation, and proliferation, individually or in combination, leading to tissue damage and clinical disease (Chervonsky, 2013) .

For the past two decades, scientists have studied microbiome's composition in different ADs, including psoriasis, rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus (Dréno, 2019; Santoro et al., 2020; Zeeuwen et al., 2012). These studies have shown that it is not only the altered composition of microbiome but also some specific bacterial taxa and their metabolites that are associated with clinical course of patients with AD. The current paradigm of the impact of oral microbiome on development of other autoimmune diseases is related to the hypothesis that dysbiosis and chronic inflammation can trigger autoimmunity through

several different mechanisms, including autoimmunity enhancement by cytokines, epitope spreading, microbial translocation or molecular mimicry. In this chapter, I will discuss from a descriptive point of view, the autoimmune mechanisms associated with pathogenesis of B-cell mediated diseases acquired immunobullous diseases (AIBD), T-cell-mediated oral lichen planus (OLP) and involvement of oral dysbiosis in local autoimmune reactions.

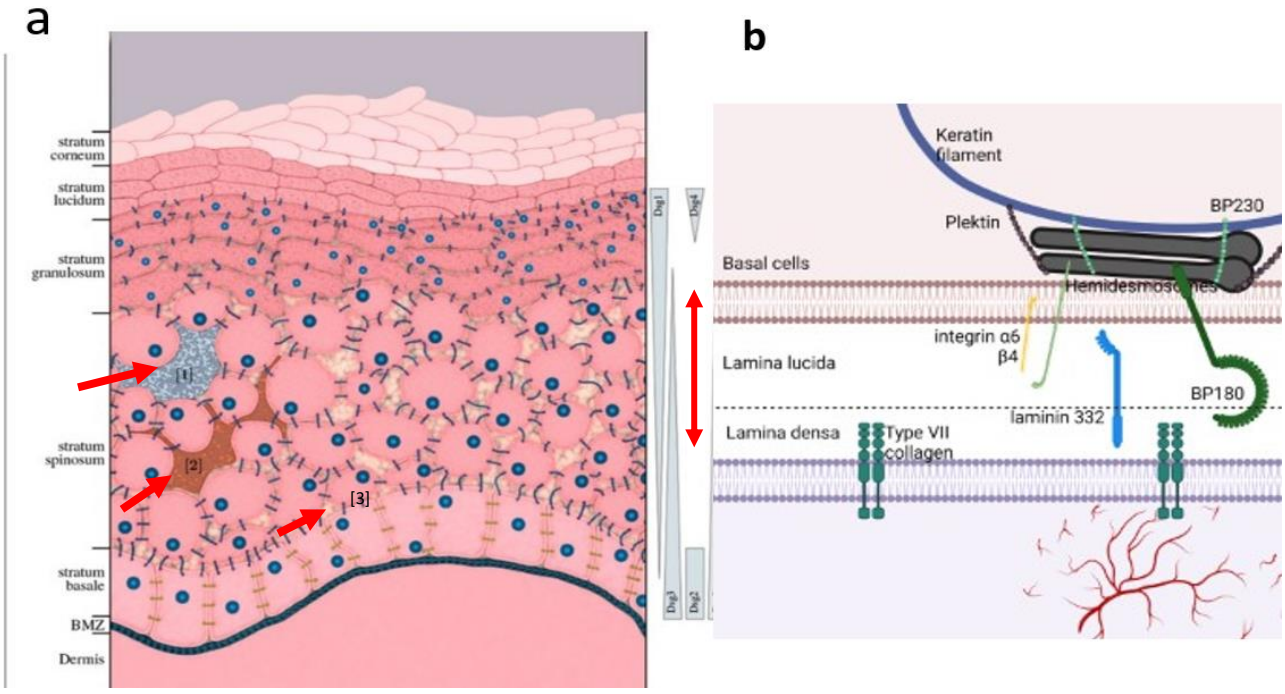


Figure 1.1 Structure of human epidermis and basement membrane zone (BMZ). *a) Shows the structure of the human epidermis/epithelia, including [1] melanocytes and [2] Langerhans cells (arrows). The distribution of PV antigens is within the layers of the epithelia [3]. While the components of desmosomes (epithelial intercellular junctions)- desmogleins 1 and 4 are expressed in the upper layers of the epithelia, desmogleins 2 and 3 are expressed in the lower layers. b) the BMZ with its hemidesmosomes (junctions connecting keratinocytes with BMZ) and its cellular adhesion proteins (BP180, BP230, Laminin 332, type VII collagen, integrin α6β4), connecting the epithelial layer with dermal layers, relevant for the subepidermal disorders, including MMP. Modified on* ***Biorender*** *from* (Olbrich et al., 2019b)

1.1.1 Acquired immunobullous diseases

Autoimmune blistering diseases (AIBD) comprises a rare group of mucocutaneous disorders caused by autoantibodies directed against proteins expressed in the epidermis and at the dermal-epidermal junction (DEJ) of skin and/or mucous membranes. Depending on the target proteins of the autoimmune response and their location, they are classified as intraepidermal/intraepithelial (pemphigus disorders) and subepidermal/subepithelial (pemphigoid group, epidermolysis bullosa, linear IgA dermatosis **Figure 1.1** (Olbrich et al., 2019a). Proteins of the autoimmune response and their location, they are classified as

intraepidermal/intraepithelial (pemphigus disorders) and subepidermal/subepithelial (pemphigoid group, epidermolysis bullosa, linear IgA dermatosis **Figure 1.1** (Olbrich et al., 2019a).

Despite the multi-site involvement of all AIBD, Pemphigus Vulgaris (PV) and Mucous Membrane Pemphigoid (MMP) both clinically manifest in oral mucosa in 50-80% and 90% of cases respectively (Buonavoglia et al., 2019a). Because of the similarity in clinical features (presentation) with oral blisters transforming into erosions and ulcers very quickly, a diagnosis of Pemphigus Vulgaris (PV) and Mucous Membrane Pemphigoid (MMP) can be challenging. Moreover, because both these diseases are rare, they are often misdiagnosed by dental specialists as more common conditions such as aphthae, erosive lichen planus or oral candidiasis (Patel et al., 2016).

1.1.1.1 Epidemiology and histopathology of PV

Pemphigus Vulgaris (PV) is the most common subtype within the pemphigus group of diseases. Onset of this disease is often between 40 and 60 years of age, with an equal distribution between men and women (Hofmann et al., 2018). The overall incidence is 0.1 to0.5/100,000th of population in certain ethnic groups. Among the Ashkenazi Jewish population, Asian and Mediterranean countries, a higher prevalence is reported (Alpsoy et al., 2015; Kneisel & Hertl, 2011).

The specific autoantibody profile varies between different pemphigus subtypes. Patients with pemphigus foliaceus (PF) have anti-Dsg1 antibodies, mucosal-dominant-type PV patients have anti-Dsg3 antibodies exclusively, and mucocutaneous-type PV patients have both anti-Dsg3 and anti-Dsg1 antibodies (Egami, Yamagami, & Amagai, 2020). Each subtype of pemphigus can be distinguished by having a specific clinical presentation and target antigen in each subtype, as shown in **Table 1.1.**

Table 1.1 Classification of Pemphigus diseases with their specific target autoantigens (*modified from* (Egami et al., 2020)*)*

Intraepidermal/intraepithelial Disease	**Target autoantigen**
Pemphigus vulgaris(mucosaltype)	Dsg 3 (Dc3)
Pemphigus vulgaris(mucocutaneoustype)	Dsg3, Dsg1
Pemphigus foliaceus	Dsg1
Paraneoplasticpemphigus	Dsg3,Dsg1, Dsc-3, BP180, BP 230, desmoplakin, envoplakin, plectin, epiplakin, periplakin
Pemphigus vegetans	Dsg3, Dsg1, Dsc3
Pemphigus erythematosus	Dsg1
Drug-induced pemphigus	Dsg1, Dsg3
Pemphigus herpetiformis	Dsg1, Dsg 3 (Dsc1, Dsc3)
IgA pemphigus(intracellularIgA dermatosis)	Subcorneal pustular dermatosis : Dsc1

1.1.1.2 Clinical presentation of PV

PV is clinically manifested by the formation of painful flaccid blisters and erosions on either the skin and/or mucous membranes (Schmidt & Zillikens, 2013 a) **Figure 1.2.** In the oral cavity, PV lesions are usually localized on the buccal mucosa, palate, lips, and gingivae. The oral mucosa may be the only site leading to late diagnosis or misdiagnosis. Rarely, laryngeal, oesophageal, conjunctival and genital lesions may occur. Because of bleeding and pain in oral lesions, patients struggle to eat and maintain oral hygiene, which leads to connection of secondary infection in the lesional area and accumulation of plaque (Jascholt et al., 2017).

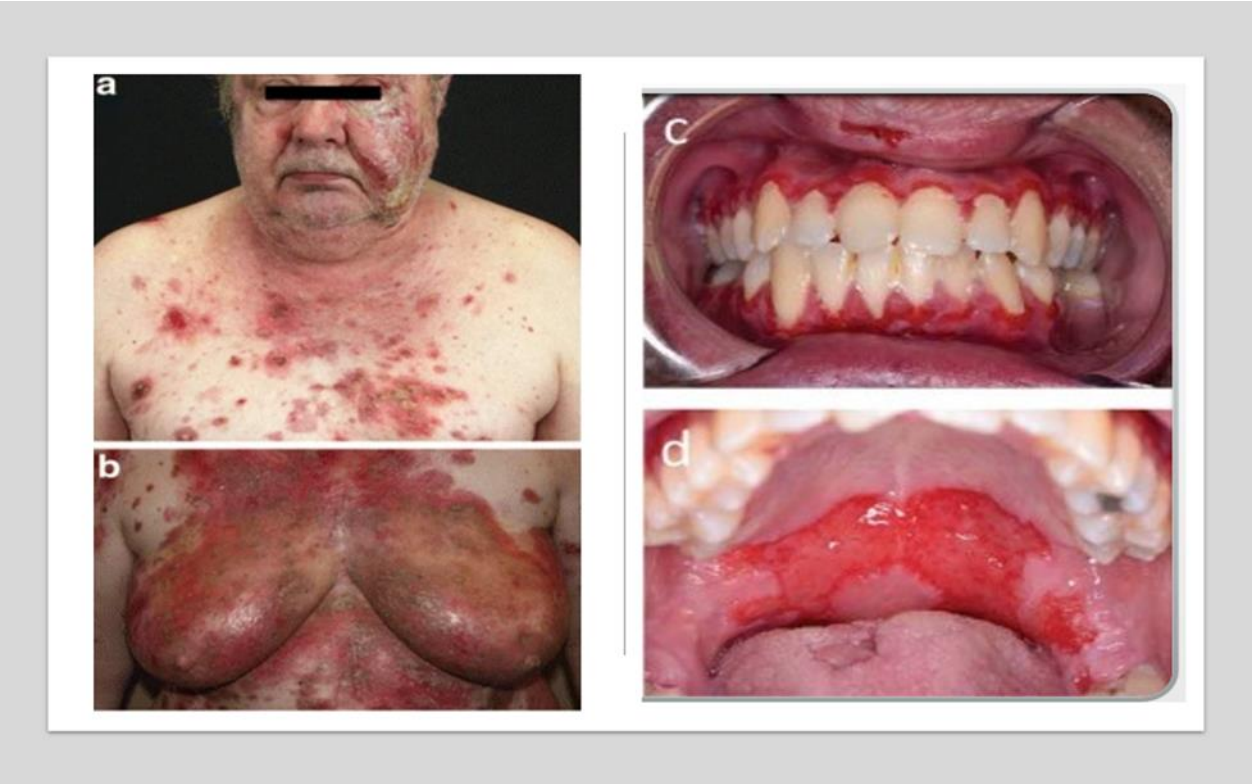
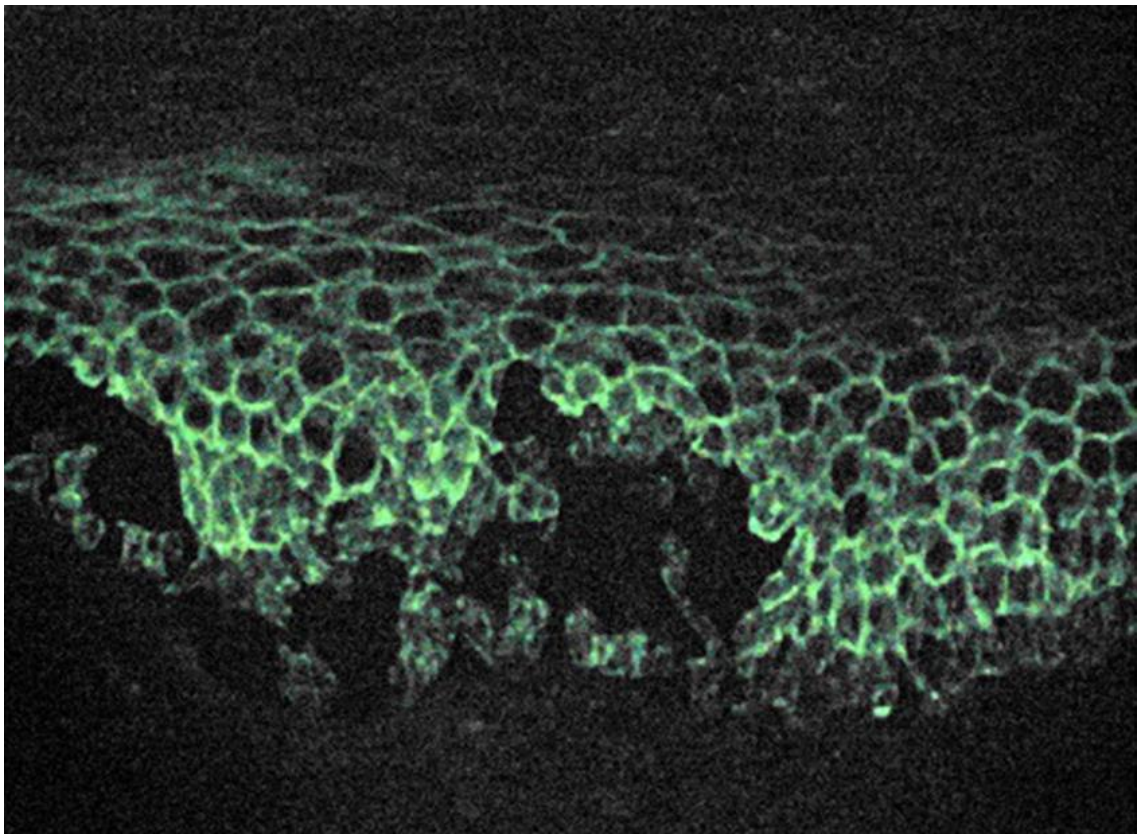


Figure 1.2 Clinical characteristics of Pemphigus vulgaris*. a) b) Widespread flaccid blisters and erosions on the trunk of a patient with PV. c) lip and gingival involvement of the mouth d) erosions on the hand and soft palate. (Pollman et al., 2018; Setterfield et.al., 2000).*

1.1.1.3 Immunopathogenesis of PV

Pathogenesis of PV is characterized as a Type II hypersensitivity reaction, with direct binding of IgG/IgA antibodies targeting desmosomal proteins. Binding of these autoantibodies ultimately leads to loss of cell-cell adhesion in skin and/or mucous membranes, which results in erosions and/or blister formation (Porro et al., 2019). **Figure 1.3**

In general, there are 3 distinct steps leading to onset of diseases, these are: loss of tolerance, T and B lymphocyte-dependent production of autoantibodies and a tissue damage phase.



1.3. Direct Immunofluorescence findings pemphigus vulgaris. *Intraepithelial/intraepidermal deposition of IgG in PV. (Porro et al., 2019)*

AIBD development is considered multifactorial and they are driven by both genetic and environmental factors. Environmental factors may include infection, drugs and stress. Antigens are presented as a fragment by the major histocompatibility complex (MHC) class II, also known as human leukocyte antigen (HLA) region in humans. A recent study identified the presence of allele associations in HLA-DQB1 for both diseases, with DQB1*05:03 having the strongest association with PV diseases (J. Setterfield et al., 2001; Y. Sun et al., 2019; J. Zhang & Wang, 2020). Genome-wide association studies (GWAS) on 100 Jewish PV patients found the region 8q11.23 on gene ST18 closely associated with PV. ST18 is a gene regulating apoptosis and inflammation and is overexpressed on skin of PV patients compared to healthy individuals (Sarig et al., 2012).

Even though the exact mechanism of AIBD is unknown, it is known that the antibodies are predominantly IgG4 and bind autoantigens causing a loss of adhesion between cells in PV. Several studies indicate a link between rotavirus infection and PV with the rotavirus capsid protein V1-H6 found in B cells. Interestingly, antibodies against this protein are responsible for protection against rotavirus as well as disrupting keratinocyte adhesion (M. J. Cho et al.,

2014). Another study on endemic form of pemphigus, fogo selvagem, found that antibodies against Dsg1 cross-react with a component of salivary glands from sandfly, LJM11. This suggests that autoantibodies are produced because of a molecular mimicry between two antigens (Qian et al., 2012). Various mechanisms have been proposed to explain the connection between bacteria and autoimmunity. These include pathogen persistence, changes in gene function, molecular mimicry, bystander activation or epitope spreading, with oral bacteria triggering autoimmune pathology (Chervonsky, 2013; Nikitakis et al., 2017).

Interestingly, circulating Dsg-3 autoreactive CD4+T cells in PV can be found in healthy individuals as well. The only difference is that T cells from healthy individuals produce IFN-γ, while those from PV patients produce both IFN-γ and IL-4 (Hertl & Riechers, 1999). This suggests that autoreactive Th2 cells contribute to production of antibodies in PV. A study in a mouse model of PV identified that antigen-specific T cells promote production of autoantibodies by B cells, and the T cell-derived IL-4 is required for antibody production. Moreover, Dsg3-reactive T cells from healthy individuals and PV can produce IL-10, a cytokine, which supresses inflammation and proliferation of Th2 cells in vitro, providing evidence that autoreactive T cells can be critical in regulating autoimmunity in this disease (Veldman et al., 2003, 2004). Skin and oral mucosa Langerhans cells function as antigen presenting cells, taking up and presenting autoantigen to CD4+ T cells. Th1 cells promote a proinflammatory immune response while Th2 cells produce IL-4 and regulate pathogenic antigen-specific IgG antibody production. Th17 cells migrate into skin tissues, utilising IL-17 production to trigger neutrophil activation, intensify inflammatory reaction, and damage tissues (Fang et al., 2021). Th17 cells offer protection against extracellular bacterial and fungal infections and have been implicated in autoimmunity (Park et al., 2005). Previous studies showed elevated levels of IL-17 in PV and BP in serum (Timoteo et al., 2017; Żebrowska et al., 2017), emphasizing the significance of Th17 cells and IL-17 in development of both PV and other AIBD.

1.1.1.4 Management of PV

Standard management of PV begins with optimising oral hygiene, use of anti-inflammatory therapies e.g., topical corticosteroids +/- systemic corticosteroids. Adjuvant systemic immunosuppressive therapy with azathioprine, mycophenolate mofetil (MMF) are the first

line treatments for PV in the UK with Rituximab used as second line agent (Harman et al., 2017; Taurone et al., 2019b).

1.1.1.5 Epidemiology and histopathology of MMP

Mucous membrane pemphigoid (MMP) is an uncommon blistering disorder from pemphigoid group of AIBD, primarily affecting mucous membranes such as the oral cavity, conjunctiva, anogenital areas, and the upper aerodigestive tract, with occasional skin involvement. The most common disease in pemphigoid group is bullous pemphigoid (BP).

The incidence of MMP is estimated to be 0.5– 2 new cases per million. Women are predisposed to the condition more frequently than men, with an average onset of disease between 55 to 65 years old (Schmidt & Zillikens, 2013b).

MMP is characterized by a linear deposition of IgG, IgA, and/or C3 along the epidermal basement membrane zone **Figure 1.4**. The target antigens are macromolecules within the

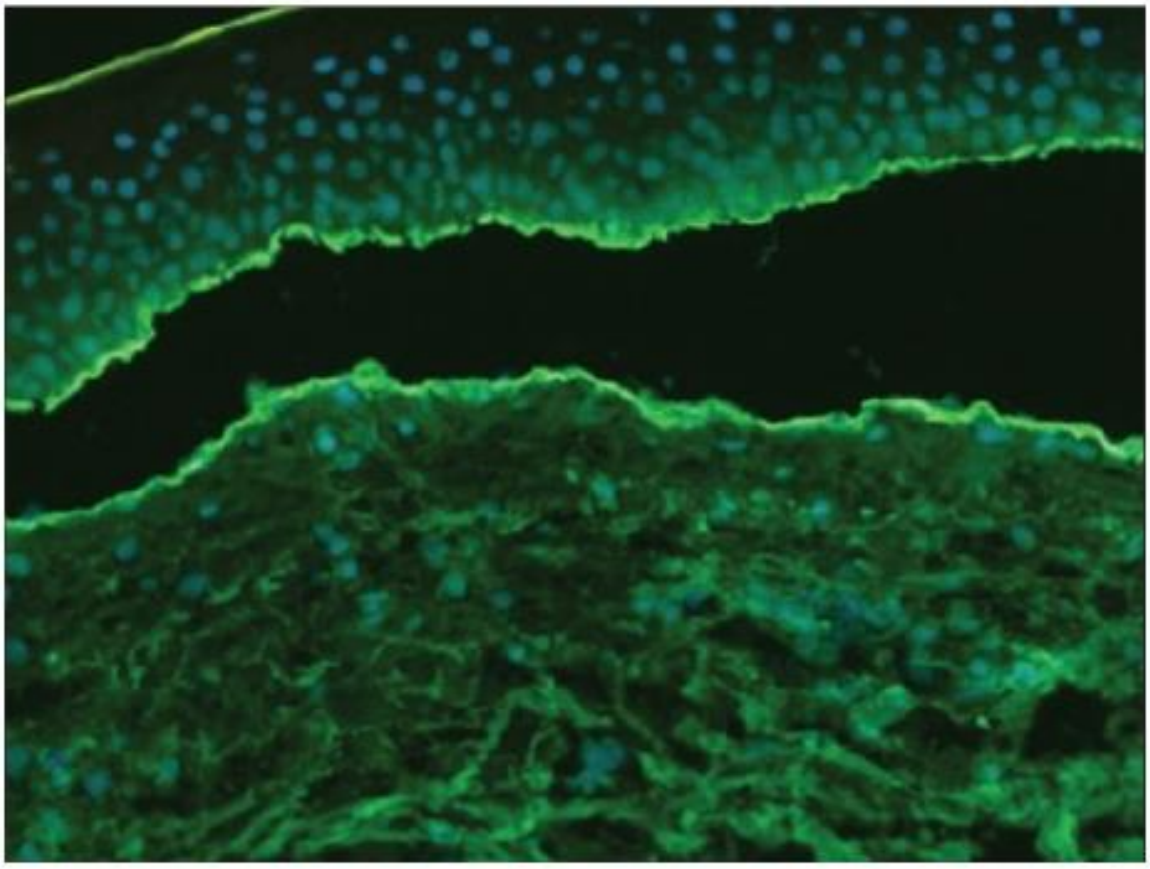


Figure 1.4 Direct Immunofluorescence findings in bullous pemphigoid linear deposition of IgG/IgA on BMZ in MMP (Porro et al., 2019)

hemidesmosome (junctions between keratinocytes and BMZ) and include BP180, BP230, laminin 332, α6β4 integrin, and type VII collagen. **Table 1.2**

Table 1.2 Classification of Pemphigoid group disease according to the target antigen in BMZ (*modified from* (Egami et al., 2020)

Subepidermal/subepithelial disease	Target autoangiten
Bullous pemphigoid	BP180, BP230
Mucous membrane pemphigoid	BP180, BP230, a6b4 integrin, laminin 332, type VII collagen
Epidermolysis bullosa acquisita	type VII collagen
Dermatitis herpetiformis (Duhring disease)	Epidermal transglutaminase
Pemphigoid gestationis	BP180, BP230
Linear IgA bullous dermatosis	LAD-1, LABD-97 (fragments of BP180), type VII collagen
Antilaminin gamma -1 (p200) pemphigoid	Laminin y1 (subunit of laminin 311)

1.1.1.6 Clinical presentation of MMP

The pemphigoid group of AIBD is clinically manifested by an appearance of tense blisters or erosions and ulcers on the skin and/or mucous membranes. The most common disease in the group is bullous pemphigoid (BP) while mucous membrane pemphigoid (MMP) is very rare (Carey & Setterfield, 2019). In contrast to BP, a common characteristic of MMP is ulceration and scarring of various mucous membranes, which may cause significant functional limitation of the affected tissue: the most common are in the mouth (86%) and eye (70%). Lesions are localized on intraoral sites include the gingiva, buccal mucosa, palate, alveolar ridge, tongue, and lower lip. Desquamative gingivitis (DG) presents as gingival erythema or generalized inflammation with blistering or ulceration. **Figure 1.5** Most cases of DG are associated with mucous membrane pemphigoid (MMP) or oral lichen planus (OLP). There is a question as to whether the impact and the potential influence of DG in these diseases on the onset and progression of plaque-related periodontitis is related to the possible shared pathogenetic

mechanisms/mediators, or whether the indirect accumulation of the plaque in these diseases causes the onset and progression of PD.

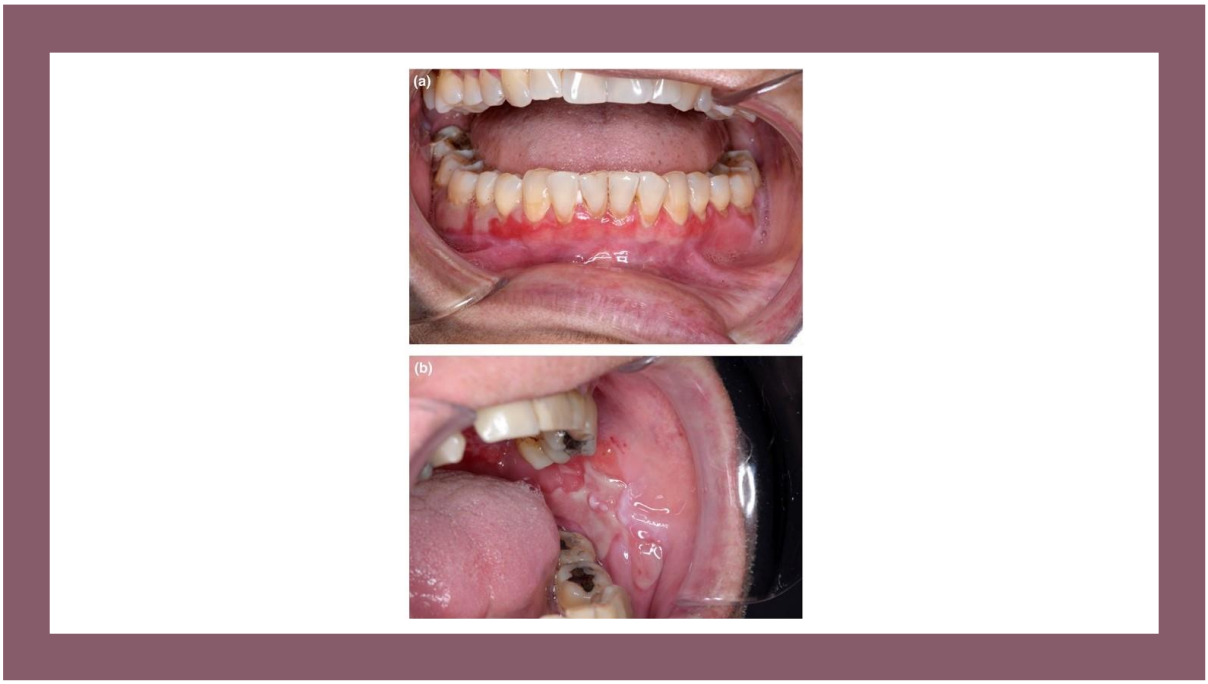


Figure 1.5 Clinical characteristics of mucous membrane pemphigoid *a) desquamative gingivitis b) Ulceration of the buccal mucosa* (Carey & Setterfield, 2019b).

1.1.1.7 Immunopathogenesis of MMP

Pathogenesis of MMP is characterized with the direct binding of IgG/IgA antibodies targeting hemidesmosomal proteins. Binding of these autoantibodies ultimately leads to loss of cell-matrix adhesion in the skin and/or mucous membranes, which results in erosions and/or blister formation (Porro et al., 2019).

There has been significant research that supports a genetic predisposition. Researchers identified susceptibility alleles of MHC II gene HLA-DQβ1*03:01 associated with pemphigoid group in several studies (J. Setterfield et al., 2001; Y. Sun et al., 2019; Chagury et al., 2017).

GWAS study on MMP patients found an association of MMP with single nucleotide polymorphisms (SNPs) in region rs17203398, located in the β-galactocerebrosidase gene (GALC), indicating that β-galactocerebrosidase may be involved in the pathogenesis of MMP (Sadik et al., 2017).

However, the specific mechanism responsible for blister formation in the oral mucosa in MMP remains unidentified. In BP studies, researchers have used murine models, particularly one where BP-like blistering occurs when anti-murine IgG BP180 antibodies are introduced into the skin (Zhao et al., 2006). These antibodies appear to trigger communication between the innate and adaptive immune systems. In this model, when the pathogenic IgG antibodies interact with skin-infiltrating neutrophils through FcγRIII, it prompts the release of substances like neutrophil elastase and matrix-metalloprotease-9 (MMP-9), which contribute to blister formation (Zhao et al., 2006). Similarly, Sitaru et al. described buildup of cells beneath the epidermal layer, primarily comprising neutrophils during the acute phases (Sitaru et al., 2002). Neutrophils play a key role in the first line of immune defence against invading pathogens, as well as being the first immune cells to migrate to a site of inflammation (Kolaczkowska & Kubes, 2013). Further stimulation by IL-17 or IL-23, lead neutrophils to release other proteases, including metalloproteinase MMP-9 and neutrophil elastase, which are responsible for dermal-epidermal junction (DEJ) disruption, thus providing a potential source of autoantigens (Giusti et al., 2019). Further understanding of the interaction of microbes and the host immune system may allow a better understanding of the pathogenesis of MMP.

1.1.1.8 Management of MMP.

For individuals with mild to moderate MMP, topical mouthwashes e.g., betamethasone 0.5 mg can be applied directly to the affected areas (Carey & Setterfield, 2019). It is crucial to maintain good oral hygiene as plaque buildup can exacerbate gingival inflammation. While not generally helpful in PV or OLP, systemic antibiotics (dapsone and tetracyclines) in MMP are used. This suggests empirically a potential pathogenic role for oral bacteria in disease activity (Williams et al., 2017). However, both dapsone and tetracyclines have anti-inflammatory effects alongside antibiotic and therefore their mechanism of action in MMP is unclear. For MMP, the antibiotics dapsone or a tetracycline are often tried before prednisolone or other immunosuppressive agents (Rashid et al., 2021). However, addition of second line immunosuppressive agents is required for more severe cases.

1.1.2 OLP

1.1.2.1 Epidemiology and histopathology of OLP

Oral lichen planus (OLP) is a chronic T cell-mediated mucocutaneous condition with to date unknown antigens, triggering an inflammatory response. (H. Wang et al., 2016). As with other autoimmune diseases, females are more affected than males, with the onset of disease between 30 and 60 years. The prevalence of OLP ranges from 0.5-2.2% in the general population (Setterfield et al., 2000).

Histopathological features of OLP include damage to basal keratinocytes and infiltration of T-lymphocytes in the superficial lamina propria (Müller, 2017). **Figure 1.6**

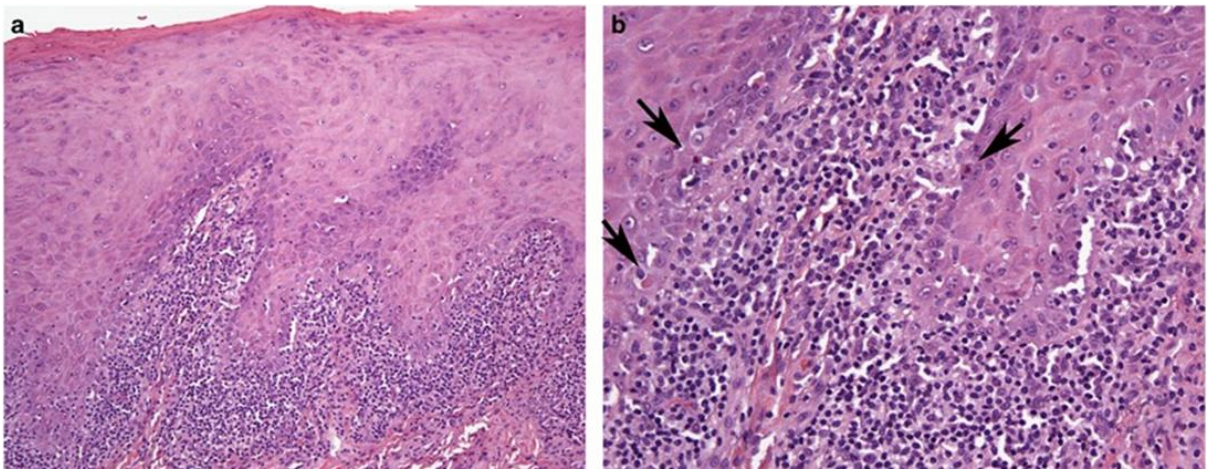


Figure 1.6 Histological characteristics of lichen planus of the oral cavity. *(a) A thickened surface of oral stratified squamous epithelium and a dense infiltrate of inflammatory cells in superficial lamina propria. (b) Lymphocyte-mediated damage to stratified squamous epithelium of oral mucosa with keratinocyte apoptosis represented by a colloidal (civatte) body (arrows)* (Müller, 2017)

1.1.2.2 Clinical presentation of OLP.

Clinically OLP presents with reticular, papular, plaque-like, atrophic/erythematous, ulcerative, or bullous lesions. **Figure 1.7** Depending on the type of OLP, lesions vary from plaque and white patchy striae to blisters and ulcers on buccal mucosa, tongue and gums. (Gingival lesions may be indistinguishable from those seen in PV and MMP). In addition to the oral cavity, other mucosal sites may be affected including the oesophagus, conjunctivae and urogenital tracts. Apart from the mucosal lesions, the skin and nails can also be involved in the process.

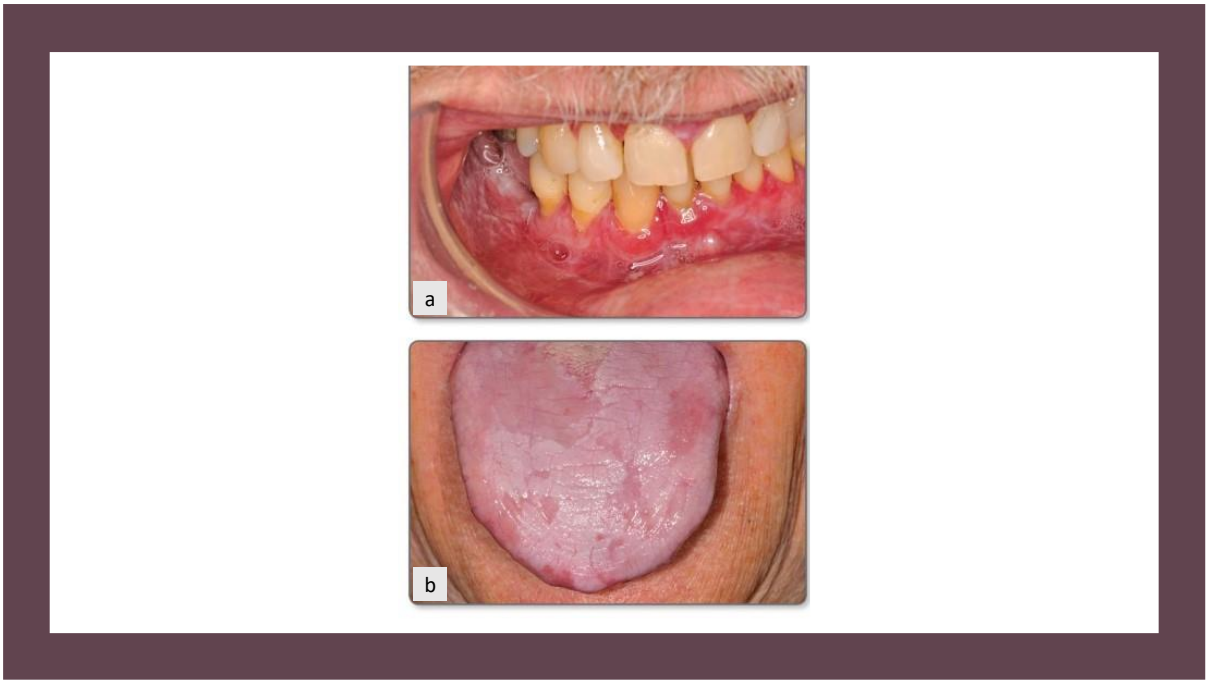


Figure1.7 Clinical characteristics of oral lichen planus a*) desquamative gingivitis and reticular striae and in buccal mucosa b) papular lesions in plaque-type lichen planus on dorsum of the tongue* (*Recent Advances in Dermatology: 1 - Shirin Zaheri, Iaisha Ali - Google Books*, n.d.)

1.1.2.3 Immunopathogenesis of OLP.

OLP is a T-cell mediated immune disease with unknown aetiology (H. Wang et al., 2016).

One of the initial steps in formation of OLP lesions might involve an expression or exposure of keratinocyte antigens at a future lesion site. This expression or exposure can be induced by various factors, including systemic medications, resulting in a lichenoid drug reaction, contact allergens found in dental restorative materials or toothpaste, leading to a contact hypersensitivity reaction, bacterial or viral infections, or potentially an as-yet-unknown agent (Gueiros et al., 2012). Internal factors including the role of stress, dysregulation of the gene responsible for the expression of heat shock proteins (HSP) and genetic predisposition have been studied. Sugerman et al. associated the polymorphism of HSP70 genes with the OLP development (Sugerman, Savage, Xu, Walsh, & Seymour, 1995).

Two distinct mechanisms are proposed in the pathogenesis of autoimmunity in OLP: antigen-specific and non-specific mechanisms. Over the years, numerous studies have explored the potential role of various microorganisms in OLP, including Epstein-Barr (EBV), human papilloma virus (HPV), and hepatitis C viruses (HCV), which have been closely linked to the

disease. Investigations have focused on HPV as a potential causative factor in OLP. Research consistently indicates a high prevalence of HPV in OLP lesions, with a stronger association observed in severe OLP cases, suggesting its possible involvement in both the onset and progression of OLP (Schiller et al., 2010; Baek et al., 2018). However, it's crucial to acknowledge that a presence of HPV cannot solely account for origin of OLP. It's possible that recurrent ulcerative lesions in OLP can increase susceptibility to HPV infection, and a prolonged use of steroids might facilitate HPV replication (Alpagot et al., 2001). A more robust link has been established between OLP and HCV. Findings by Lodi et al. strongly support an association between OLP and HCV (Giovanni Lodi et al., 2005). Subsequent meta-analyses conducted years later confirmed that individuals OLP are significantly more likely to have HCV (G. Lodi et al., 2010).

Furthermore, there are reports suggesting a relationship between OLP and bacteria associated with PD (Baek & Choi, 2018; Jung & Jang, 2022). A presence of PD or any other chronic inflammation can trigger the secretion of chemokines, which in turn attract hematopoietic LC precursors to the tissue, fostering an ongoing immune response in OLP. This immune response may lead to destruction of keratinocytes by cytotoxic CD 8+ T cells. Supporting this theory is the discovery that *Candida albicans*, an opportunistic fungal pathogen, can invade oral epithelium and is found in increased abundance in the oral cavity of roughly 48% of patients with erosive OLP (Y. Li et al., 2019).

While OLP is typically associated with Th1 cells cytokines, there is evidence suggesting a Th1/Th2 cells imbalance towards a Th2 response, as indicated by a decrease in IL-2 and an increase in IL-10 levels in saliva (Carvalho et al., 2019; Piccinni et al., 2014). Furthermore, another study has identified elevated concentrations of IL-17 in both saliva and serum samples from OLP patients, implying a potential role for microbial pathogens in the inflammatory response associated with OLP (K. Wang et al., 2015a). IL-17 is a pro-inflammatory cytokine produced by immune cells in response to microbial pathogens, particularly extracellular bacteria, fungi, and other inflammatory stimuli, and it is driven by antigen-specific Th17 cells. Notably, Th17 cells possess immunological memory, which may enhance the host's response upon encountering the same pathogen again (Chen et al., 2011).

Non-specific mechanisms include mast cell degranulation and matrix metalloproteinases (MMPs) activation in OLP lesions. Both mechanisms may be present together, causing accumulation of T cells in the superficial lamina propria, destroying the basement membrane, and inducing T cell migration and keratinocyte apoptosis (DeAngelis et al., 2019).

MMPs have been strongly implicated as a key contributor in destruction of the basal lamina zone in OLP (Mazzarella et al., 2006), specifically, MMP-2, MMP-3 and MMP-9. Generally, MMPs are involved in cell migration, angiogenesis and proteolytic activation of growth factors, events needed in normal tissue remodelling as well as wound healing, and tumour invasion. These proteinases cleave collagen type IV and laminin, the major constituent proteins of basement membrane zone. Mazzarella et al. reported that expression of MMP-3 was higher in erosive OLP, suggesting that a permanent or prolonged presence of high MMP-3 in serum may contribute to malignant transformation of OLP lesions. Zhou et al. found that *in vitro* activation rate of MMP-9 was significantly higher ($p < 0.05$) in OLP lesions. (X. J. Zhou et al., 2001). Antigen-specific CD8+ cytotoxic T cells were shown to secrete MMP-9, concluding that the overexpression of MMP-9 may result in basement membrane disruption, facilitated by migration of cytotoxic T-cells and keratinocyte apoptosis.

1.1.2.4 Management of OLP

In OLP, the treatment is aimed primarily at achieving symptomatic improvement and extending periods of remission. Corticosteroids (CS) are used as first line therapy. Administration can be topical, intralesional or systemic. Hydroxychloroquine (HCQ) followed by immunosuppressive agents is sometimes required (Platais et al., 2023).

Because gingival involvement is present in all three above-described conditions (PV, MMP and OLP), as evidence by studies (Akman et al., 2008; Arduino et al., 2011; Ramón-Fluixá et al., 1999), and since maintaining good oral hygiene tends to affect the progress of these autoimmune conditions positively, an important query arises. This question revolves around whether these long-lasting autoimmune diseases increase the likelihood of developing PD or if it's the other way around i.e., whether the PD and associated inflammatory markers/mediators contribute to triggering autoimmune conditions.

1.1.3 Periodontitis

1.1.3.1 Epidemiology of PD.

Periodontal disease (PD) is a widespread condition, affecting up to 50% of the global population. It is a multifactorial chronic inflammatory bacteria-associated condition with a variety of risk factors, including smoking, hormonal changes, poor oral hygiene, diabetes, age, and genetics. The development of periodontitis is influenced by various factors, including age, gender, inadequate oral hygiene, and smoking (Kinane & Chestnutt, 2000; Grover et al., 2016; De Angelis et al., 2018). Within the same adult demographic, there is a high prevalence of systemic diseases such as cardiovascular disease (CVD), hypertension, diabetes, and inflammatory conditions (Borgnakke et al., 2013; Aguilera et al., 2019; Larvin et al., 2020a). This association may be attributed to shared risk factors among these conditions, or alternatively, periodontitis could act as a contributing factor in the development of systemic diseases (Esteves-Lima et al., 2020). Nevertheless, a comprehensive understanding of the entire disease progression of PD and its interactions with subsequent coexisting health issues remains to be fully understood.

1.1.3.2 Clinical presentation of PD.

Clinically, PD can be divided into two subcategories depending on the nature of inflammation and its location - gingivitis and periodontitis. PD is clinically characterized by inflammation of gums and underlying tissue and the damage is directly related to an activation of host inflammatory mechanisms by the presence of bacterial plaque **Figure 1.8a.** Gingivitis is a reversible condition with inflammation of marginal soft tissues surrounding the teeth. If not treated, gingivitis can progress to PD (Lang et al., 2009).

Clinically, gingivitis is characterized by symptoms such oedema, erythema and bleeding during a periodontal examination. These symptoms are also observed in PD, accompanied by additional indicators like an increase in periodontal pocket depth, attachment loss, and alveolar bone resorption **Figure 1.8d.** PD often presents with gingival recession and related

issues such as tooth hypersensitivity, tooth mobility, drifting, and the potential development of periodontal abscesses, which can ultimately result in tooth loss (Coates et al., 2017).

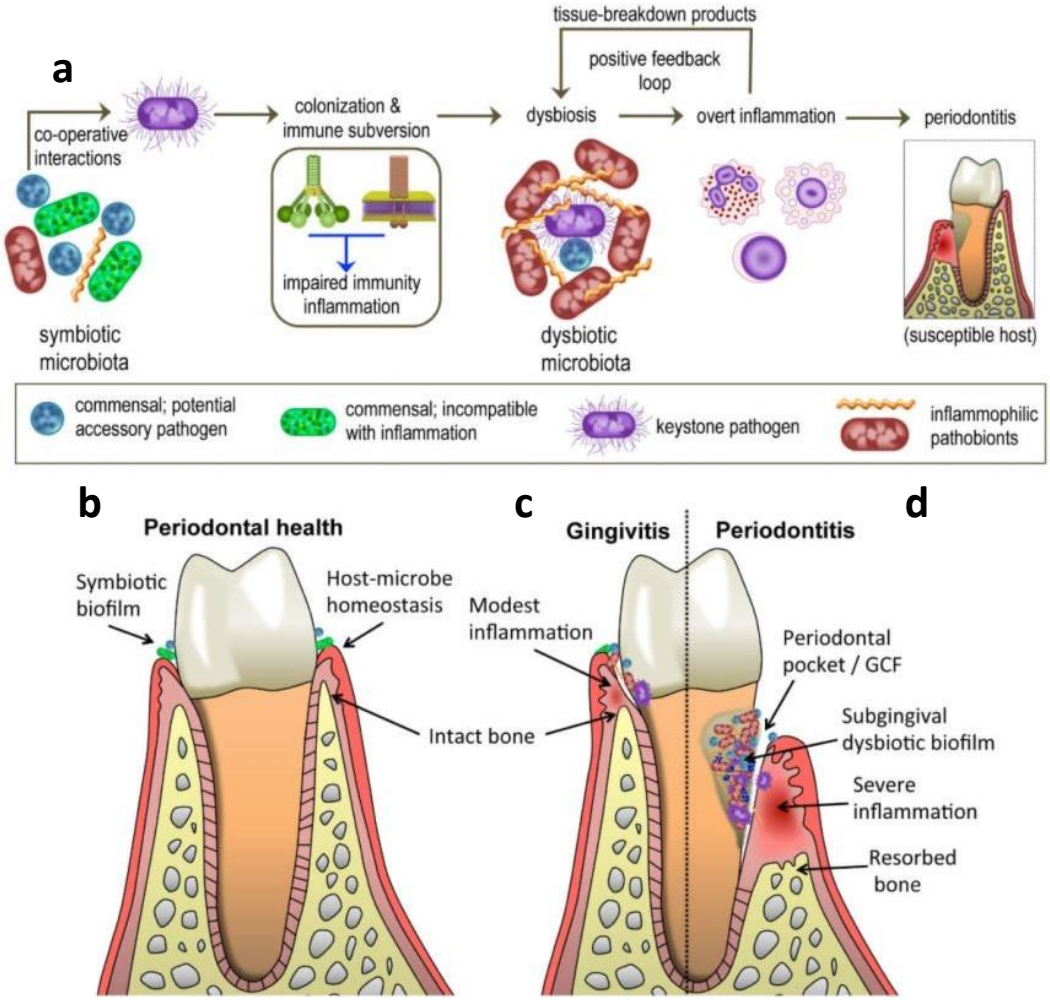


Figure 1.8 Oral microbiome dysbiosis and periodontitis *a) The different stages and interactions within the biofilm leading to periodontitis. (b) Healthy gingiva (c) Gingivitis caused by built-up bacterial plaque at and below the gum line (d) Severe periodontitis, with deep pockets and bone loss.* (Coates, 2017)

1.1.3.3 Dysbiosis in PD

With advent of new technologies in particular Next generation sequencing (NGS), the science of periodontology has made significant advances. The main periopathogenic bacteria have now been described, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia and Fusobacterium nucleatum,* also known as "keystone pathogens"(Torrungruang et al., 2015). These microorganisms cause dysbiosis in the subgingival and oral microbiome, triggering immunological and inflammatory processes

along with production of inflammatory mediators such as cytokines, prostaglandins, C-reactive protein, and interleukins. These can spread systemically through bloodstream and have an impact on systemic inflammation, which in turn may injure other extra-oral organs such as joints, the heart, kidneys or the liver (Hajishengallis, 2015a). One of those well-known discoveries was the presence of a periodontal keystone pathogen *P. gingivalis* in synovial fluid of patients with RA (Araújo et al., 2015). The understanding that various immune-related skin conditions, like OLP and AIBD, frequently show distinct symptoms in the oral mucosa, and the identification of similar biomarkers seen in autoimmune diseases present in PD, indicates a potential strong link between PD, AIBD, and OLP through an imbalance in the oral microbiome. It starts from colonization of keystone pathogens, which release tissue-breakdown product, such as degraded collagen peptides and haem-containing compounds, starting an inflammatory process (Hajishengallis, 2014). Periodontal bacteria can adapt their interactions with a host's immune responses, such as neutrophils and complement, to enhance their survival and adaptability. Neutrophils are the primary leukocytes recruited to the subgingival crevice or periodontal pockets, responsible for considerable tissue damage (Sima & Glogauer et al., 2014). Higher numbers of local neutrophils correlate with the severity of chronic PD. An excessive number of neutrophils may be attracted to the infected gum pockets. This can happen because these neutrophils are unable to effectively deal with bacteria causing the infection, despite being capable of mounting an immune response. As a result, these bacteria not only evade being killed by neutrophils but also promote inflammation, contributing to an imbalance in oral microbial community (Landzberg et et al., 2014). This dysregulation can lead to local and systemic autoimmune responses mediated by mechanisms like molecular mimicry, bystander activation, and epitope spreading, as shown in **Figure 1.9** (Suárez, Garzón, Arboleda, & Rodríguez et al., 2020). Furthermore, certain periodontal bacteria, like *P. gingivalis, T. forsythia, and Prevotella intermedia*, have evolved ways to evade the complement immune response. Complement proteins are part of the immune system that help in identifying and destroying harmful bacteria. These bacteria can block complement activation and even degrade key components of the complement system, protecting themselves and other nearby bacteria from being targeted and killed. These bacteria can also manipulate neutrophils by causing crosstalk between different signalling

pathways, leading to a suppression of antimicrobial effects, inhibition of bacterial engulfment, and a promotion of inflammation (Hajishengallis et al., 2015).

1.1.3.4 Management of PD.

Treatment of PD is primarily focused on stopping progression of disease (Ide et al., 2022). The management of PD is based on daily careful oral hygiene and removal of plaque. Additionally, risk factors such as smoking, or diabetes directly affect disease susceptibility by altering a host's inflammatory response, which makes a healthy lifestyle an important factor for a healthy oral gum and microbiome. If not treated-on time, PD can lead to tooth loss.

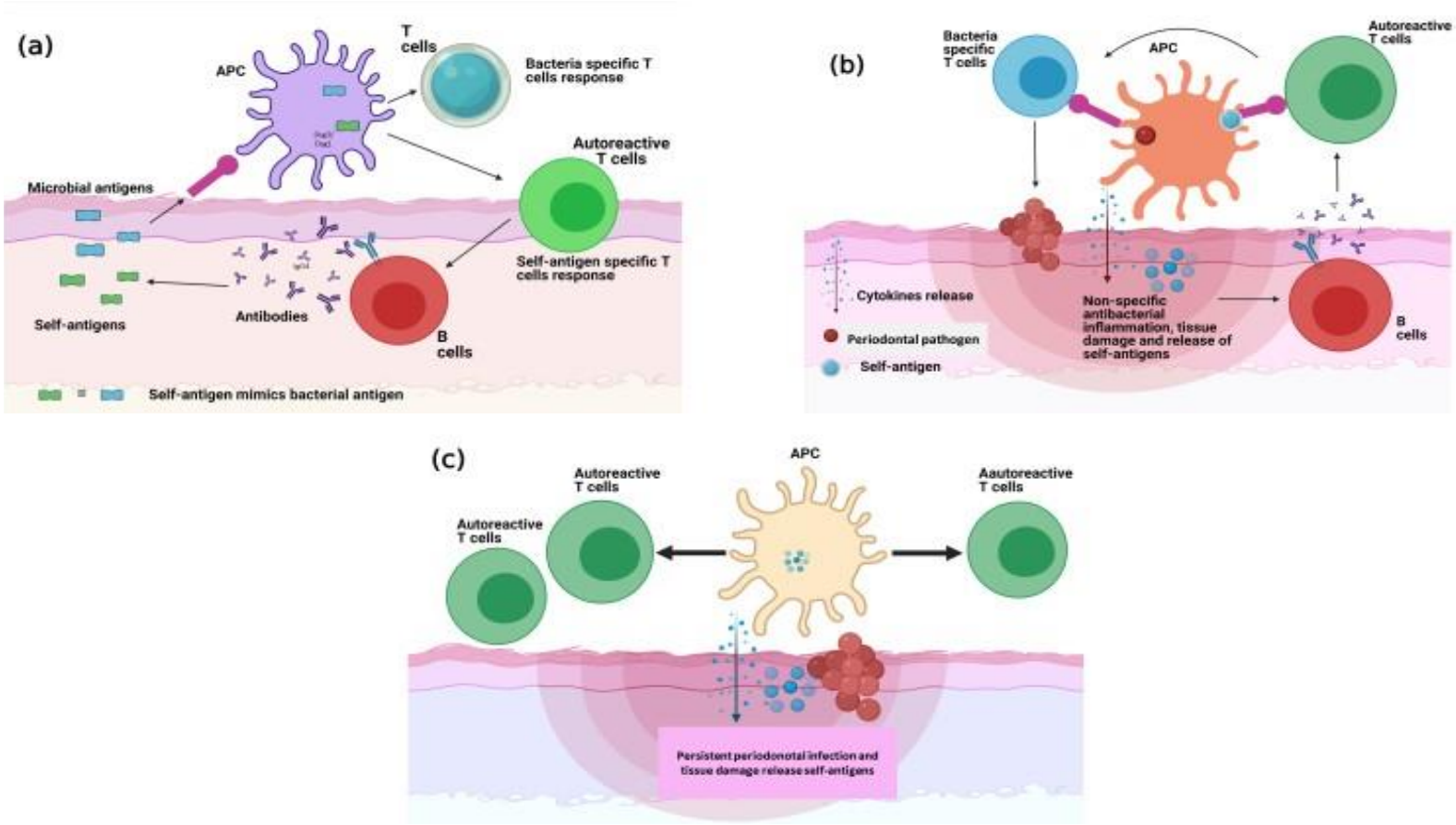


Figure 1.9 Mechanisms of bacteria-induced autoimmunity *(a) Molecular mimicry model: Bacteria carry epitopes structurally similar to self-epitopes. Presentation of bacterial epitopes by antigen presenting cells (APCs) activate autoreactive T cells that bind to both, self and non-self-antigens, and induce tissue damage. (b) Bystander activation model: Non-specific and over reactive antibacterial immune responses lead to the liberation of self-antigens and release of inflammatory cytokines from the damaged tissue. Self-antigen is taken up and presented by APCs. Autoreactive T cells activated by APCs, leading to tissue destruction. (c) Epitope spreading model: Persistent bacterial infection causes tissue damage and release of new self-antigens. Self-antigens are taken up and presented by APCs. Nonspecific activation of more autoreactive T cells leading to autoimmunity.* ***(Created with BioRender.com)***

1.2 Human microbiome

Scientists have known about microbes for centuries. Since Anthony van Leeuwenhoek first described plaque-dwelling microbes in the late 1600s, scientists have cultivated many bacteria and linked microbes to diseases. The advent of high throughput sequencing and bioinformatics technologies to detect microorganisms has allowed researchers to identify an ever-increasing number of microorganisms that could not otherwise be cultured in a lab. This has led to an increase in our knowledge about microorganisms and lead to the emergence of a new field - the microbiome (Gao et al., 2018a). The human microbiome is a sum of all microorganisms and their genes that live in a particular habitat in the human body, such as on the skin, mouth, or gut (I. Cho & Blaser, 2012). The new concept of studying microbiome prefers to separate the terms "microbiota" and "microbiome", where the microbiota refers to physical microorganisms in a microbial community, the microbiome includes bacteria, viruses, archaea, fungi, small protozoa and all their genes (Berg et al., 2020). Since bacteria constitute the largest percentage of microbiome and thus are the most studied to date, fungi and viruses also make up a significant proportion of both microbiota and microbiome. The latest estimates put the number of bacterial cells living in our body as slightly exceeding the number of human cells in a ratio of 1.3:1(Sender et al., 2016). However, the situation is much more one-sided when looking at genes. Whereas the number of human genes is about 22,000, the number of microbial genes exceeds the number of human genes by at least 150 times at around 3.3 million. This has given rise to the idea of a person as being a “superorganism” or “holobiont”, i.e., a human with its microorganisms and their genes that are mutually dependent on each other (Simon et al., 2019).

To investigate the role of microbiome in the human body, The Human Microbiome Project was launched in 2008 (Moon & Lee, 2016). The main aim of this project was to generate resources to help to study the human microbiome by characterizing the microbiome associated with human health, as well as disease. So far, it has been found that every human microbiome is as unique as a fingerprint. Despite the differences in composition of the microbiome in individuals, the functions of the microbiota are relatively similar. These include differentiation and maturation of the host mucosa and its immune system, food digestion and nutrition, metabolic regulation, vitamin production, processing, and detoxification of chemicals from the environment, maintenance of the skin and mucosa barrier function, and

finally prevention of invasion of pathogens (Rackaityte & Lynch, 2020). Numerous factors, including the method of delivery at birth, early use of antibiotics, and type of feeding method all shape the core microbiome from early days of a newborn and are associated with long-lasting effect on the human organism (Casterline & Paller, 2020; Rothschild et al., 2018).

1.2.1 Oral Microbiome

Recent data indicate that an imbalance in the oral microbiome may be involved in pathogenesis of autoimmune diseases or affect the course of disease (Acharya et al., 2017; Costalonga & Herzberg, 2014; Wade, 2013a; Zheng et al., 2021). Since 2010, the Human Oral Microbiome Database (HOMD) has been updating with cultivable and non-cultivable bacteria in the mouth (HOMD: Human Oral Microbiome Database, n.d.). These microorganisms have been investigated for their interactions with the host cells and direct influence on human physiology, metabolism and immune responses (Verma et al., 2018a; J. C. Yu et al., 2019; X. Zhang et al., 2015). The various microbial communities in different body niches have several beneficial roles, such as preventing the establishment of harmful organisms, aiding host defense functions, and contributing to metabolic activities in both health and disease (Huttenhower et al., 2012; Kilian et al., 2016b; Nagpal, Shively, Register, Craft, & Yadav, 2019). Therefore, identifying factors that contribute to any imbalance between the host immune system and microbiota leading to dysbiosis, loss of tolerance, and subsequent progression of autoimmune diseases are important questions for researchers (Deo & Deshmukh, 2019b; Gao et al., 2018a).

The oral cavity is the second largest human microbial community after the gut. Bacteria are the largest population among microorganisms (Gao et al., 2018b). The current database contains information on 775 taxa and over 770 prokaryote species from 15 different phyla (http://www.homd.org). However, only 54% of these microorganisms have been named to date (Xiao et al., 2020). The “core microbiome” is common to all the individuals. In the mouth, this core microbiome comprises Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and Fusobacteria at the phylum level (Risely, 2020). **Table 1.4**

Table 1.4. The composition of the core microbiome in different body sites.

Body site	Predominant Phyla
Oral microbiome	Firmicutes (e.g., Streptococcus); Bacteroidetes (e.g, Prevotella); Proteobacteria (e.g., Haemophilus); Actinobacteria (e.g, Actinomyces); Spirochaetes (e.g, Treponema); Fusobacteria (e.g. Fusobacterium)
Gut microbiome	Firmcutes (e.g., Lachnospiraceae, Ruminococcaceae), Bacteroidetes (e.g. Bacteroides and/or Prevotella), Actinobacteria (Bifidobacterium)
Skin microbiome	Actinobacteria (Propionibacterium, Corynebacterium) and Firmicutes (Staphylococcus)
Vaginal microbiome	Firmicutes (Lactobacillus)

Oral microorganisms are highly organized and form structured communities. Polymicrobial association in oral cavity follows a unique pattern, where bacteria produce various glycoproteins and polysaccharides for adherence of other microbes sequentially (Darrene and Cecile 2016). Microbes colonize the oral cavity within a few hours after childbirth. Physiological factors such as the method of a child's delivery, early use of antibiotics, method of feeding a child (breast feeding or formula feeding), postnatal development and eruption of primary teeth, and replacement of primary teeth with permanent teeth significantly influence the microbial composition (Xu et al., 2015).

The oral cavity has two types of surfaces on which bacteria can colonize: the hard surface of the teeth, including supra- and subgingival plaque, and the surface of the oral mucosa. (Willis & Gabaldón, 2020). Microorganisms maintain equilibrium to keep an oral ecosystem healthy. Healthy individuals harbour personalized microbiota with the different microbial composition on the different sites of a mouth (Zaura et al., 2009). Variations in the microbiome composition across different areas of an oral cavity are attributed to differences in the growth environment and nutrient availability within those regions (Ximénez-Fyvie, Haffajee, & Socransky, 2000). Subgingival sites, compared to supragingival ones, exhibit a higher

prevalence of anaerobic species like *Prevotella, Fusobacterium, Campylobacter,* and *Treponema*. Conversely, the tongue microbiome differs from gingival microbiomes by having a greater presence of facultatively anaerobic and aerobic species, including *Streptococcus, Neisseria,* and *Rothia* (Wilbert, Mark Welch, & Borisy, 2020). Saliva is considered a reservoir of the oral microbiota and a fingerprint of the entire oral microbiome (Humphrey & Williamson, 2001). This biological fluid, containing nutrients for microbial growth and protective enzymes and proteins, plays a vital role in maintaining a healthy microbiome (Artico et al., 2014).

Various exogenous and endogenous factors such as food habits, smoking, alcohol consumption, stress, hormonal imbalance, poor oral hygiene, diabetes, periodontal inflammation and ageing perturb the bacterial community (Janiak et al., 2021; L. Zhang et al., 2020).

1.2.2 Oral microbiome and ageing

Ageing is a complex process that brings various changes in an oral cavity's tissues and functions, as well as a decrease in physiological functions of all body systems. At molecular level, ageing is influenced by factors like genome instability, telomere depletion, epigenetic changes, mitochondrial dysfunction, cellular senescence, and stem cell depletion (J. P. Liu, 2014). Several factors like genetic predisposition, lack of exercise, poor sleep, diet, nutritional deficiencies, susceptibility to chronic infections, and an imbalance in the oral microbial community can trigger ageing and result in both local and systemic inflammation (Hayflick et al., 2000).

Ageing has physiological effects on various tissues and functions in an oral cavity. For example, bone density in jaws decreases due to loss of calcified tissue and changes in collagen structure. Dental pulp becomes more calcified and smaller, leading to reduced blood and nerve supply (Ebersole et al., 2016). Sensory functions, including taste perception can decline due to changes in taste buds, receptors, the central nervous system. Salivary glands also undergo histopathological changes, causing reduced salivary production and altered saliva composition. This can result in xerostomia, affecting over 30% of people over 65, leading to problems like taste disturbances, and difficulty swallowing. Finally, thinning of oral mucosa

compromises mucosal immunity, making it less effective against microbes (Schwartz et al., 2021).

The immune system experiences changes, potentially weakening a host's defences. The concept of “immunosenescence” refers to a gradual alteration of immune system, making it more vulnerable to infections, cancer, and autoimmune diseases (Santoro et al., 2020). Immune cells like neutrophils, monocytes, and dendritic cells may have impaired chemotaxis, affecting their ability to migrate to infection sites. These cells may also have reduced capacity to kill invading microbes. The pool of naive and regulatory T- and B-lymphocytes declines and there is an increase in memory and cytotoxic phenotypes, creating an inflammatory environment (Morrisette-Thomas et al., 2014).

The data indicates that amongst elderly population, the occurrence of oral conditions like dental caries, soft tissue ailments, and PD are more prevalent (Nassar, Hiraishi, Islam, Otsuki, & Tagami, 2014; Perez-Lopez, Behnsen, Nuccio, & Raffatellu, 2016). However, there is still lack of understanding of the role of oral microbiota in ageing, despite of the known local and systemic health functions of bacteria, such as immunomodulation, pathogen resistance, and cross-colonization (Calder et al., 2017). Growing evidence suggests that the oral microbiome composition, species identity, density and distribution of bacteria may profoundly affect frailty. For example, Liu et al. conducted a study on microbial composition in three oral sites (gingival crevicular fluid, tongue, and saliva), revealing that bacterial alpha diversity tends to decrease with age (S. Liu, Wang, Zhao, Sun, & Feng et al., 2020). However, Willis et al. found that individuals in older age groups displayed more varied microbiomes characterized by the presence of taxa that are usually found in low abundance (Willis et al., 2022). One of the factors associating microbiome with frailty is changes in the microbiota linked with diet (Ghosh et al., 2020). Modifying dietary patterns, for example, adopting a Mediterranean diet, has been suggested as an effective therapeutic strategy to address the ageing microbiome (Nagpal et al., 2019).

1.2.3 The oral microbiome and diet

Life in Western countries has significantly changed the diet of people. In particular, eating habits referred to as the "Western diet" or post-industrial diet have resulted in consumption of foods high in fat and cholesterol, refined carbohydrates and highly processed foods (Woelber & Tennert, 2020). Numerous studies showed that this diet contributes to chronic inflammation and a risk of obesity and cardiovascular disease(Vinke et al., 2017). Dietary changes from the Neolithic to the post-industrial diet may also be a possible promoter of autoimmune diseases (Adler et al., 2013).

While comparing calculus (calcified dental plaque samples) of ancient, medieval, and western people, scientists found that the composition of oral microbiome changed only after the industrial revolution. For example, *S. mutans* is found only in post-industrial samples. The modern diet has also produced an oral microbiome characterized by fewer (beneficial) *Ruminococcaceae* bacteria (Lassalle et al., 2018). Wright et al. explored the link between diet and periodontitis and found that a dietary pattern emphasizing fruits, vegetables, salads, water, and tea while minimizing the consumption of fermentable carbohydrates, fatty acids, protein, and sugary beverages, corresponded to a reduced extent of periodontal disease. This reduction was attributed to the diminished presence of periodontal bacteria within the oral environment (Wright et al., 2020). In another study, dietary habits to align with a Mediterranean Diet (MedDiet) has a potential to decrease levels of periodontopathogenic bacteria in the saliva of overweight individuals with cardiometabolic risk resulting from an unhealthy lifestyle (Laiola et al., 2020).

Nagpal et al. described that MedDiet associated with a prevention of cardiovascular, metabolic diseases, autoimmune diseases, and age-related Alzheimer's disease (Sleiman et al., 2015). MedDiet is characterized by the dietary patterns found in Greece and Southern Italy. It represents foods rich in vegetables, fruits, legumes, nuts, beans, cereals, grains, fish and unsaturated fats such as olive oil (Davis et al., 2015). Ntemiri et al. showed changes in gut microbiota composition of the elderly across five European countries after 1 year of MedDiet improving the health status of aged people and reducing frailty (Ntemiri et al., 2020). Scientists explain positive effects of this eating pattern with a high fibre content, mono- and polyunsaturated fatty acids, and polyphenols (Nagpal et al., 2019). In contrast to the gut

microbiome, current knowledge of effects of diet on oral microbiota are limited. Microbes and their products of metabolism were analysed in one study on 161 healthy individuals who followed an omnivorous, ovo-lacto-vegetarian, or vegan diet and found no significant differences between groups. (De Filippis et al., 2014).

A high-fat diet contributes to obesity, leading to excess White Adipose Tissue (WAT) accumulation and systemic inflammation (Balistreri et al., 2010). WAT is currently considered an "endocrine organ" producing various pro-inflammatory mediators such as TNF-α, IL-6, leptin, and C-reactive protein, which are responsible for chronic mild systemic inflammation in obese people (D. Zheng et al., 2020). These chronic inflammatory signals have demonstrated a significant effect on CD4+ T cells, and studies in mice have shown that diet-induced obesity can affect specific fat regulatory T cells (Treg). On the other hand, a plant-based diet rich in low-glycaemic complex carbohydrates (e.g., found in fruits, vegetables, legumes), omega-3 fatty acids, micronutrients (such as vitamins, minerals), phytochemicals, plant nitrates, and fibre seems to bring benefits in both periodontal inflammation, caries, and overall health (Woelber & Tennert, 2020).

However, there is no current indication that a choice of an omnivore, ovo-lacto-vegetarian or vegan diet can lead to a specific composition of the oral microbiome with consequences on oral homeostasis, supporting the paradigm of resilience of the oral microbiome (Wade, 2021a). Conversely, another study noted minor variations in salivary metabolomic profiles about dietary practices such as omnivorous, ovo-lacto-vegetarian, or vegetarian diets. This could be attributed to the impact of diet on the gut microbiota, which subsequently affects the oral microbiota (De Filippis et al., 2014).

1.2.4 The oral microbiome and metabolites

Beyond identifying the specific groups of microorganisms that inhabit different mucosal sites in a host, researchers are investigating how these microorganisms communicate and interact to coexist and maintain host well-being (Foratori-Junior et al., 2022). The human oral microbiome holds a rich reservoir of metabolites. Specific microbiome-derived metabolites play significant roles as mediators of health and disease. For example, recently discovered antibiotic lugdunin, produced by commensal *Staphylococcus lugdunensis* found in the nasal cavity exhibited robust inhibitory effects against antibiotic-resistant Staphylococcus aureus

(Zipperer et al., 2016). In the context of oral cancer, introducing oral alkaline-producing species like *Streptococcus gordonii* and *Actinomyces spp.* with strong arginine metabolic capabilities can counterbalance acidity and restrain the growth of acid-producing bacteria, thus inhibiting their cancer-promoting effects (Chattopadhyay et al., 2019). Alternatively, some species within the oral cavity, like *Neisseria spp*., produce toxic bacterial metabolite acetaldehyde, which promote tumours by either inducing mutations in host cells or impairing normal antitumor immune responses (Yokoyama et al., 2018).

On average, 108 metabolites are found in saliva (Maruyama et al., 2022). The primary metabolites are nucleic acids, vitamins, lipids, organic acids, carbohydrates, thiols, and amino acids. One of the essential metabolic function of bacteria in the mouth is maintaining homeostasis by preventing an acidic environment in this place. Saliva flushing neutralizes saliva acids and minerals and prevents tooth surface demineralization resulting from carbohydrate fermentation (Takahashi et al., 2014). Bacteria break down carbohydrates and peptides into smaller and simpler molecules or create more complex molecules from them (Barbour et al., 2022). For example, the metabolite urea is the primary alkaline metabolite of saliva. Urealytic bacteria, organisms that produce urease enzymes, catalyse a breakdown of urea into ammonia. Ammonia neutralizes acids, protecting acid-sensitive bacteria.

Oral bacteria ferment most carbohydrates and proteins in the mouth to form adenosine triphosphate (ATP) for their activity (Ohshima et al., 2017). |The end products of further fermentation of this process are short chain fatty acids (SCFAs), including lactate, acetate, butyrate, propionate and isobutyrate (Takahashi 2015). SCFAs are well-studied in the gut microbiome (Corrêa et al., 2017a), however, their role in the oral cavity is less studied. It is known that on the supragingival surface, SCFAs have an antimicrobial property, but on the subgingival plaque, SCFAs have a cytotoxic effect (N. Takahashi et al., 2010). These metabolites can damage epithelial cells and cause inflammation. Moreover, they can regulate the host's immune response (Correa et al. 2017). Butyrate has been found to impair neutrophil effector functions by altering neutrophil production of inflammatory mediators and suppressing their phagocytic capacity. As other bacteria continue to populate the biofilm during the pathogenic process, butyrate producers such as *P. gingivalis, T. forsythia, P. intermedia, F. nucleatum and T. denticola* continue to expand (Takeshita et al., 2016).

Considering these findings in oral metabolomics, comparative analysis of metabolites of healthy individuals and individuals with AIBD and OLP may have a high potential for identifying metabolic biomarkers for early diagnosis and treatment of these diseases.

1.2.5 Oral microbiome dynamic communities and host interactions

The oral cavity is an ecosystem for microorganisms and their genes collectively known as the oral microbiome. This ecosystem is characterized by its remarkable diversity and adaptability, influenced by factors such as diet, oral hygiene, and genetic factors. The oral microbiome comprises bacteria, viruses, fungi, and other microorganisms, coexisting in complex communities within different niches of the oral cavity (Lamont, Koo, & Hajishengallis, 2018).These communities are predominantly bacterial and can vary significantly in composition and diversity among individuals. Advances in high-throughput sequencing technologies have enabled comprehensive profiling of the oral microbiome, revealing the presence of previously unculturable microorganisms and expanding our understanding of its complexity (Mcdermott & Huffnagle, 2014).

Host-microbiome interactions in the oral cavity are bidirectional. The host provides a suitable environment and nutrients for microbial growth while also employing various mechanisms to maintain microbial homeostasis. Microbial populations within the oral cavity can rapidly respond to changes in environmental conditions, such as variations in pH, oxygen levels, and nutrient availability. This adaptability allows for the formation of polymicrobial biofilms, which play a critical role in dental plaque formation and oral diseases, including dental caries and PD (Wade, 2013). Furthermore, microbial colonization of oral surfaces is influenced by host factors, such as salivary proteins, mucins, and the immune system, which can shape the composition and stability of oral microbial communities (Radaic & Kapila, 2021). For example, commensal oral bacteria contribute to the host's defence by competing with pathogens for resources and by stimulating the immune system. Host saliva plays a role in maintaining ecosystem stability by buffering the oral environment, providing nutrients to the community, and delivering antimicrobial substances that counteract external species. Therefore, while gingivitis often occurs due to the prolonged presence of biofilms on tooth surfaces, it

represents a controlled immune-inflammatory state that doesn't permanently harm the supporting tissues of teeth.

Conversely, dysbiosis, characterized by an imbalance in the oral microbiome, can lead to the overgrowth of pathogenic species and the development of oral diseases. Shifts in the composition and function of microbial communities can disrupt host-microbiome interactions, leading to chronic inflammation and tissue damage (Rosier, Marsh, & Mira, 2018).

The shift from a healthy microbial community to a dysbiotic state involves various processes. Changes in host immune function or diet can impact community composition and metatranscription, leading to increased production of virulence factors (Bostanci & Belibasakis, 2012). As the community evolves, microbial metabolism and host immune responses can alter the local environment, promoting the growth of microorganisms associated with dysbiosis. For instance, in PD polymicrobial communities trigger an unregulated and destructive host response, a phenomenon known as polymicrobial synergy, which is characterised with microorganisms often interacting synergistically to enhance colonization, persistence or pathogenicity (Murray, Connell, Stacy, Turner, & Whiteley, 2014). PD is associated with increased microbial diversity, likely resulting from additional nutrients derived from host tissue damage and expanding physical space as the gingival crevice deepens (Hajishengallis, 2015b). This shift is characterised by an increased dominance of pre-existing species rather than the introduction of entirely new ones. The main host factor here is inflammation which occurs as a consequence of tissue damage, which releases various nutrients such as degraded collagen, haem-containing compounds, amino acids, and sources of iron. These nutrients are transported into the gingival crevice, where they nourish the growth of subgingival bacteria with proteolytic properties. Detailed analysis of PD-associated subgingival biofilms has revealed elevated expression of genes related to proteolysis, peptide transport, iron acquisition, and lipopolysaccharide synthesis, thereby increasing the pro-inflammatory potential of the microbial community. Consequently, a subset of species, known as inflammophilic pathobionts, tends to expand selectively, creating an imbalanced dysbiotic community (Kilian et al., 2016a). Supporting this idea, an introduction of serum, haemoglobin, or hemin into in vitro generated multispecies oral biofilms induces a selective

overgrowth of pathobionts. These pathobionts upregulate virulence-related genes, including those responsible for producing proteases, hemolysins, and proteins involved in hemin transport. This transformation of the original balanced biofilm into a dysbiotic one significantly enhances its ability to stimulate pro-inflammatory cytokine production by host cells, mimicking the real-life scenario where dysbiosis leads to inflammation.

While human studies may not fully elucidate disease mechanisms, the integration of metagenomic and metatranscriptomic data suggests that instead of a discrete pathogenic group of organisms, a specific set of gene functions is necessary to induce dysbiosis.

1.2.6 Methods in microbiome research

For many years, the cultivation approach was the only method for identifying microbes. The advantage of this traditional approach was the low-cost and simplicity of the procedure. However, it was unable to grow all microorganisms, from which only 50% are cultivated to date (Deo & Deshmukh, 2019a). In addition, due to individual growth requirements, it is necessary to know what to grow in advance, making this method inconvenient and limited in the information it provides (Nikitakis et al., 2017). The new molecular approach introduced by Sanger in the 1980s advanced the microbiome field as it identified organisms by sequencing their genes in microbial DNA extracted from samples (Paul et al., 2018). However, being more expensive and only able to give short pieces of DNA about 300 to 1000 base pairs, this technique was not practical for whole genomes or complex communities. The more recent advent of high throughput DNA sequencing as applied to targeted gene sequencing techniques rapidly became a major method of identification of the microbial species and their relative abundance in a community by targeting specific conserved areas of the genome. The most frequent target for a molecular approach is the 16S rDNA gene, which is the gene that encodes the 16S ribosomal RNA in prokaryotic DNA. The 16S rRNA molecule is part of the 30S ribosomal subunit of bacterial rRNA molecule. This molecule been widely used for microbial identification, characterisation and classification. The 16S rRNA gene includes both conserved and variable regions. The conserved regions are identical in all members of a prokaryotic domain while the variable regions carry specific information about genus and species and differ substantially among bacterial taxa, hence those variable regions are used for organism identification. This gene pioneered by Carl Woese in 1977 is known as “molecular clock”, due

to the slow rates of evolution of this region of the gene (Woese & Fox, 1977). While it is recognized that cultivation of bacteria allows traceability of samples retesting, unfortunately, not all bacteria can be cultured. It is thought that only about 20% of the intestinal microbiota can be cultured (Rizal et al., 2020). For example, anaerobic pathogens can be killed when exposed to oxygen during transport or culture. Using 16S RNA next generation sequencing (NGS) for bacterial identification avoids bacterial culture, as this method requires only the DNA of the pathogen under investigation. This will allow the identification of pathogens with simple and streamlined protocols from sequencing library creation to bioinformatics analysis. Additionally, the relatively low cost of the analysis and the minimal amount of the sample required, makes this method widely used in numerous projects throughout the world. Other types of amplicon sequencing can identify other microorganisms, such as 18S rRNA gene, primarily employed for detailed taxonomic examinations of fungi, and its ITS region, which serves as a fungal barcode marker and is predominantly applied for exploring fungal diversity (J. Liu, Yu, Cai, Bartlam, & Wang, 2015). In contrast to the 18S rRNA gene, the ITS region exhibits greater variability, making it a more appropriate genetic marker for assessing within-species genetic variations.

The 16S rRNA sequencing provides DNA sequences that can then be analysed using several basic bioinformatic steps, which when combined are known as “pipelines”. These bioinformatic pipelines remove sequencing errors or dubious reads, binning sequences into operational taxonomic unites (OTUs) and identify the bacteria (and archaea) that were present in the samples (Johnson et al., 2019). Nevertheless, the 16S RNA gene also exhibits drawbacks, including limited resolution in analysis that restricts bacterial identification to phylum and genus levels. Furthermore, it is prone to bias due to primer preference in the gene amplification step (Rizal et al., 2020). These short-read sequencing technologies have limitations in the length of DNA fragments they can analyse, resulting in the use of different primer sets targeting various parts of genes like the bacterial 16S rRNA gene. However, these primer sets can amplify different bacterial groups unequally due to factors like hybridization rates and inhibitory flanking DNA, causing over- or under-detection of certain bacteria. This variation in primer efficiency leads to differences in detected community composition and taxonomic discrepancies across studies (Kennedy, Hall, Lynch, Moreno-Hagelsieb, & Neufeld, 2014).

Further advances in bioinformatics and the reducing cost of DNA sequencing have subsequently led to the advent of shotgun metagenomic sequencing as a way around this (Quince et al., 2017). This method is characterized by randomly breaking ("fragmenting") DNA into many small pieces. These fragmented pieces of DNA are then sequenced, and their DNA sequences are stitched back together (assembled) using bioinformatics approaches to create longer sequences (contigs) that can then be used to identify the genes and species present in the sample (Durazzi et al., 2021). Unlike 16S rRNA sequencing, shotgun metagenomic sequencing can read all genomic DNA in a sample, rather than just one specific region of DNA. For microbiome studies, this means that shotgun sequencing can identify and profile bacteria, fungi, viruses and many other types of microorganisms at the same time. Additionally, it is also possible to identify and profile microbial genes that are present in the sample, providing additional information about microbiome functional potential. Shotgun sequencing reads require more complex bioinformatics methods to analyse results (Balaji et al., 2019). **Table 1.3**

Even though metagenomics can determine potential functions of bacteria at the gene level, it is difficult to determine with this method what micro-organisms are doing in an environment that is influenced by environmental factors such as host and diet and where microorganisms influence each other in biofilm growth patterns. The use of additional omics simulations can help shed light on this area (Knight et al., 2018).

Table1.3 Difference between 16S rRNA sequencing and Shotgun metagenomics.

Characteristics	16S rRNA SEQUENCING	SHOTGUN METAGENOMIC SEQUENCING
Functional profiling (profile microbial genes)	n/a	it reveals information on functional potential
Taxonomic resolution	Bacterial genus or species	Species of bacteria (sometimes strains and single nucleotide variants, if sequencing is deep enough)
Taxonomic coverage	Bacteria and archaea	Bacteria, fungi, archaea, viruses
Bioinformatics requirements	Expertise ranging from beginner to intermediate	Intermediate to advanced level of knowledge
Databases	Established	Comparatively new, still improving
Bias	High-to-medium (acquired taxonomic composition is dependent on selected primers and targeted variable area)	Lower (while metagenomics is "untargeted," experimental and analytical biases can occur at any point)

1.3 Dysbiosis in AIBD

To date, studies characterising the microbiome of patients with AIBD are lacking, and those that have been carried out are contradictory. Several different microbiomes have been proposed to have an impact on AIBD e.g., the skin microbiome and the gut microbiome.

The skin microbiome was most investigated in a relationship of the microbiome to AIBD. This can be due to the common involvement of the skin in these group of diseases (Ellebrecht et al., 2016; Künstner et al., 2018a; Miodovnik et al., 2017). Ellebrecht et.al. studied epidermolysis bullosa equisita (EBA), a type of pemphigoid disease (**Table 1.2.),** in immunized mice. Researchers found a large infiltration of neutrophils in mice with the disease and a higher microbial diversity in healthy mice, suggesting that the dominance of certain types of bacteria protects against manifestation of the disease (Ellebrecht et al., 2016). However, Miodovnik et al. and Kunstner et.al. did not find any difference in the sin microbiome of patients with BP and PV, when compare with HC. All these studies used low resolution NGS 16S rRNA sequencing technique.

Other studies suggest the gut microbiome may have an impact on AIBD diseases. The gut microbiome in PV, showed enrichment in the *Flavonifractor* genus which correlated with plasma levels of inflammatory cytokines IL -6, IL-8, IL-7, IL-1β, IL17A, IL-21, and complement factor C5a. Flavonifractor has ability to breakdown a flavonoid quercetin, known for its anti-inflammatory, anti-tumour, anti-mutagenic and antioxidant activities (Huang et al., 2019a). De Scaglione et.al. investigated the gut, skin and mouth microbiomes using 16s rRNA technique in PV and BP patients and showed similarities in the intestinal microbiota between the disease's groups. Moreover, the data did not demonstrate a difference between diseased and HC groups. The authors state that gut microbiome composition most likely does not play a particular role in PV and BP. However, in both diseases, a significant decrease of Bacteroidetes phylum was found in the oral microbiome. Additionally, one of the latest studies of the gut microbiome of 40 MMP patients was analysed using targeted 16S rRNA, where MMP was found to be associated with lower abundances and Shannon diversity, and a significant correlation of those with conjunctival bulbar inflammation and use of systemic immunotherapy. Notably, no significant differences in alpha diversity were found between MMP patients who had never used, had previously used, or were currently receiving antimicrobial treatment with oral tetracycline, which is known for its anti-inflammatory effects (in addition to its antimicrobial effect) due to its matrix metalloproteinase inhibitor properties (Low et al., 2022).

Finally, only two studies to date have shown the relationship between oral microbiome and AIBD. In a study by Arduino et.al. of subgingival plaque, there was a higher prevalence of periodontal pathogens *A. actinomycetemcomitans, E. corrodens, and F. nucleatum/periodonticum* in MMP patients (Arduino et al., 2017). However, they used PCR technique. Later, Zorba et al. using 16S rRNA sequencing technique, found significantly higher α-diversity in the oral smear of PV patients compared to HC (Zorba et al., 2021a). **Table 1.5**

Periodontitis and autoimmune bullous diseases, such as pemphigus vulgaris and mucous membrane pemphigoid, are inflammatory conditions causing oral tissue damage due to microbial plaque and autoantibody responses, respectively. Although evidence suggests a potential link between these autoimmune disorders and periodontitis, a comprehensive evaluation is lacking. The earlier findings from this systematic review showed that majority of

the studies demonstrated a connection between periodontal health and AIBD (Jascholt et al., 2017). The review revealed that most studies indicated a correlation between poor periodontal health and these bullous diseases. Some studies also suggested that oral hygiene measures could improve periodontal parameters and disease severity of the blistering disorders (Arduino et al., 2011; Giacometti et al, 2013). However, inconsistencies existed among the results due to methodological limitations. Dermatologists should advise these patients to collaborate with dentists for professional periodontal follow-up. Further comprehensive prospective studies are needed to thoroughly examine the relationship and interaction between these diseases.

PD and AIBD are inflammatory conditions causing oral tissue damage due to microbial plaque and immune responses, respectively. Although evidence suggests a potential link between these autoimmune disorders and PD, a comprehensive evaluation is lacking (Tricamo et al., 2006; Zorba et al., 2021). Moreover, factors related to ageing should be kept in mind because patients with PV or MMP are typically older and have an increased likelihood of developing periodontal disease (Jascholt, Lai, Zillikens, & Kasperkiewicz, 2017). Due to painful lesions of the oral cavity, they avoid daily routine oral hygiene, which leads to an accumulation of periopathogenic microorganisms. Further studies with a powered sample size are required to determine the exact relationship between AIBD and oral microbiome.

In summary, previous microbiome studies in AIBD have some clear limitations. Firstly, the studies had relatively small sample sizes and lack of statistical power. Secondly, because they used lower resolution techniques, many of the microorganisms identified couldn't be classified down to the species and strain levels, which is a significant drawback. Another important limitation is the lack of information about what these identified bacteria are doing metabolically. Additionally, these studies missed an opportunity to investigate the role of functional genes in AIBD. These data gaps make it difficult to understand how these microorganisms' function in the context of AIBD. Lastly, most of these studies didn't explore fungi, viruses, or other microorganisms. To gain a more complete understanding of the microbiome's role in AIBD, it's essential to conduct more comprehensive investigations that not only examine what types of micro-organisms are present but also their function.

Table 1.5 Summary of the microbiome studies in AIBD.

	Author and year	Number of samples	Sample type	Method	Key microbial feature related to disease	Study limitations
1	Low et al., 2022	49=MMP 40=HC	Stool	16S rRNA gene sequencing	MMP is associated with lower number of operational taxonomic units (OTUs) and Shannon diversity. OTU numbers are correlated with inflammation score and systemic treatment	1. Lower resolution sequencing (primer bias)
2	Li et al., 2021	10=PV 10=HC	Skin swab	16S rRNA gene sequencing	Staphylococcus was the most abundant in the PV; Shannon and Simpson indexes were significantly lower in the PV group; high relative abundance was observed in the class Bacilli	1. Lack of statistical power 2. Lower resolution sequencing (primer bias)
3	Zorba et.al. 2021	15=PV 15=HC	Oral smear	16S rRNA gene sequencing	α - diversity was significantly decreased. *F. nucleatum, G. haemolysans and P. micra* were statistically abundant; lower levels of *S. salivarius and R. mucilaginosa*	1. Lack of statistical power 2. Lower resolution sequencing (primer bias)
4	Scaglione et al., 2020)	12=PV 8 =BP	Stool swab Skin swab Oral swab	16S rRNA gene sequencing	Similar composition of gut microbiome in BP and PV. Significant decrease in Bacteroidetes phylum abundance in skin microbiome	1. Lack of statistical power 2. Lower resolution sequencing (primer bias) 3. No control group
5	Huang et al., 2019	18 =PV 14=HC	stool	16S rRNA gene sequencing	At the genera level, *Lachnospiracea incertae sedis and Coprococcus decreased, while Granulicatella, Flavonifractor* enriched in PV.	1. Lack of statistical power 2. Lower resolution sequencing (primer bias)

6	Künstner et al., 2018)	47 =PV	Skin swab	16S rRNA gene sequencing	No significant difference in α and β- diversity	1. Lack of statistical power2. Lower resolution sequencing (primer bias) 3. No data about control group
7	Arduino et al., 2017	33 =MMP 33 =OLP	Subgingival plaque	PCR	Prevalence and higher levels of *A. actinomycetemcomitans, E. corrodens, and F. nucleatum/periodonticum* were statistically higher	1. Lack of statistical power 2. Lower resolution sequencing (primer bias) 3. No healthy control group
8	Miodovnik et.al.2017	12= BP 12=HC	Skin swabs	16S rRNA gene sequencing	No difference in α diversity of the skin microbiome. Abundances at the phylum level were found to different between BP and HC. Significant decrease in Actinobacteria in back, elbow and perilesional samples from BP patients, compared to site- matched control samples.	1. Lack of statistical power 2. Lower resolution sequencing (primer bias)
9	(Ellebrecht et al., 2016	n/a EBA	mice Immunization	16S rRNA 454 pyrosequencing	Greater richness protects from the disease development	1. Lack of statistical power 2. Lower resolution sequencing (primer bias) 3. Animal experimental study

1.4 Dysbiosis in OLP

In early studies of the association of OLP with oral microbiome, many microorganisms, such as *Helicobacter pylori, Mycoplasma salivarium, Candida albicans, Human papillomavirus, Epstein-Barr virus, and Hepatitis C virus*, have been proposed to be related to OLP development (Chainani-Wu et al., 2001; Lodi et al., 2005). However, none of the hypotheses could prove a causal relationship with the development of OLP.

Later, two studies by Ertugrul et al. and Ruso et al. emphasised the role of dysbiosis in OLP, Dysbiosis refers to a disruption of the microbiome, "imbalance" in the microbial community that can be associated with disease. This imbalance could be due to the gain or loss of community members or changes in relative abundance of microbes (Verma, Garg, & Dubey, 2018). Periodontal pathogens, *P. gingivalis, A. actinomycetemcomitans, T. denticola, P. intermedia, and T. forsythia* were found in subgingival plaque samples from OLP patients without periodontal disease in the first study (Seckin Ertugrul et al., 2013a). Authors stated that plaque plays a local irritant role and slows the healing of OLP lesions. In a second study, *A. actinomycetemcomitans* was correlated with the gingival lesions in OLP (Lo Russo et al., 2013). However, both studies used the PCR technique to detect specific micro-organisms given in a kit, and with, both studies were constrained by a small sample size.

Wang et al., using the PCR-denaturing gradient gel electrophoresis (DGGE), observed a decrease in microbial diversity in saliva samples of 45 OLP patients compared to healthy controls. In addition, the study identified a significant correlation between the cytokine IL-17 and the altered microbial community composition (K. Wang et al., 2015b). However, a limitation of this approach was that it could only detect bacterial species with a relative abundance exceeding 1%, neglecting the presence of rare taxa.

The same authors soon after described the salivary microbiome of 19 reticular and 18 erosive OLP patients using the molecular 16S RNA sequencing technique. Surprisingly they found that the structure of the salivary microbiome was not significantly affected by disease status (K. Wang et al., 2016). However, they found that *Porphyromonas and Solobacterium* showed significantly higher relative abundances, whereas *Haemophilus, Corynebacterium,*

Cellulosimicrobium and Campylobacter were in lower abundances in OLP compared to HC. The authors described the phenomena of polymicrobial synergy and dysbiosis, finding *Porphyromonas* to be correlated significantly with *Eubacterium.* Because scientists used 16sRNA gene sequencing, the microbiome was analysed only at genus level.

Choi et al., using the 16S RNA gene sequencing, found an intracellular bacterial invasion of the epithelium and lamina propria in the biopsy of OLP patients. Intracellular bacteria were assumed as an important triggering factor of T cell infiltration (Choi et al., 2016). The limitation of this study was a small sample size.

One of the latest studies of the oral microbiome in OLP found a decrease in microbial alpha diversity in OLP tissue (Baek et al., 2020). Earlier, He et.al. investigated the buccal microbiome of OLP patients, where they found a decrease of Streptococcus and an increase of *Fusobacterium, Leptotrichia and Lautropia* compared to HC (He et al., 2017). Interestingly, the gut microbiome studies show that loss of microbial diversity causes an inflammatory disease, such as Crohn's disease (Chaudhari et al., 2020; Du Teil Espina et al., 2019). However, in PD, the accumulation of plaque and aggravation of PD is associated with increased microbial diversity. The summary of findings in the microbiome and OLP can be found in **Table 1.6**

In contrast to AIBD, which are rare and have limited microbiome research, OLP has seen more extensive studies, including those investigating metabolomics. However, there hasn't been a prior attempt to conduct higher-resolution microbiome studies and integrate clinical data, metabolomic and the microbiome analysis to gain a comprehensive understanding of this condition. By examining all these aspects together, potential links can be found in the underlying mechanisms of the disease. In turn, this holistic approach can ultimately lead to more effective diagnostic and treatment strategies.

Table 1.6 A summary of the microbiome studies in OLP.

	AUTHORS	DISEASE	SAMPLE TYPE	STUDY METHOD	FINDINGS	LIMITATIONS
1.	H. Liu et al., 2021	20 CPD 19 CPD with erosive OLP	Subgingival plaque	16S rRNA sequencing	Decreased microbial diversity. The dominant genera were *Pseudomonas and Granulicatella* compared to PD.	1.Lack of statistical power 2.Lower resolution sequencing (primer bias) 3.No control group
2.	Baek et al., 2020	22 OLP 12 HC	**0.** Buccal mucosa Buccal tissue	16S rRNA sequencing	Decreased alpha diversity *and E. coli* enrichment within OLP tissue. *Escherichia, Acinetobacter, Sphingomonas*	1. Lack of statistical power 2.Lower resolution sequencing (primer bias) 3.No clinical data
3.	Du et al., 2020	10 HC 20 OLP (eOLP/rOLP)	Buccal mucosa	16S rRNA sequencing	*eOLP has more diverse microbiome than HC, distinct community structure.* *Streptococcus, Neisseria overrepresented in HC* *Fusobacterium, Granulicatella-overrepresented in OLP group compared to HC.*	1. Lack of statistical power 2.Lower resolution sequencing 3. No clinical data
4.	F. Y. Yu et al., 2020	10 HC 10 r OLP 10 e OLP	Unstimulated saliva	16S rRNA sequencing	*Alpha-diversity was similar in all 3 groups.* *The same number of OUT in eOLP and rOLP.* *Microbial community in OLP is distinct from HC;* *Relative abundance of Lautropia and Gemella were higher in eOLP;* *Abiotrophia and Oribacterium were higher in OLP (combining eOLP and rOLP);*	1. Lack of statistical power 2. Lower resolution sequencing (primer bias)

		10 RAU			*decreased relative abundance of genera Streptococcus and Sphingomonas in RAU.*	
5.	Kragelund & Keller, 2019	22 OLP	1)lesional cytobrush (CB) 2) mouthwash (MW)	16SrRNA sequencing ITS1 sequencing	Microbial composition of MW and CB differs significantly. Initial antimycotic treatment seems to influence the bacteriome. Mycobiome was stable even after 1-year *Malassezia restricta* is the most abundant fungus.	1. Lack of statistical power 2.Lower resolution sequencing (primer bias) 3.No control group 4. no clinical data
6.	Li, Yan et.al. 2019	18 HC 17 r OLP 18 e OLP	Unstimulated saliva	ITS2 sequencing	*Ascomycota*_unidentified_*1_1, Trichosporon Candida, Aspergillus Alternaria, Sclerotiniaceae*_unidentified	1. Lack of statistical power 2.Lower resolution sequencing (primer bias)
7.	Y He et.al. 2017	21 HC 43 (21 erosive and 22 non-erosive)	Buccal mucosa	16S rRNA gene	Higher microbial diversity in OLP compared to HC, distinct community structure in OLP compared to HC. Decrease o*f Streptococcus and* Increase *of Fusobacterium, Leptotrichia and Lautropia in OLP group*	1. Lower resolution sequencing (primer bias)
8.	Arduino et al., 2017	33 MMP 33 OLP	Subgingival plaque	PCR	Prevalence of *A. actinomycetemcomitans, E. corrodens, and F. nucleatum/periodonticum* were statistically higher in samples from patients with desquamative gingivitis. No	1.Method detects only selected bacteria in a kit 2.No information about control group

					statistical association of these bacteria with DG severity	
9.	Choi Y. Et.al. 2016	11 HC 13 OLP	Oral mucosal tissue	16S rRNA gene Pyrosequencing	decrease in *Streptococcus* and increases in gingivitis/periodontitis-associated bacteria in OLP lesions.	1. Lack of statistical power 2.Lower resolution sequencing (primer bias)
10.	Wang et al. (2016)	18 HC 19 r OLP 18 e OLP	Unstimulated saliva	16S rRNA gene	*Haemophilus, Corynebacterium, Cellulosimicrobium, Campylobacter were increased in HC.* *Porphyromonas increase in eOLP*	1. Lack of statistical power 2.Lower resolution sequencing (primer bias)
11.	Wang K. et.al. 2015	30 OLP 15 HC	Unstimulated saliva	16 S rRNA PCR-DGGE	Significantly less microbial diversity than in HC	Method detects bacteria more than 1% abundance.
12.	Seckin Ertugrul et al., 2013b)	27 (18CPD and 9 OLP with gingivitis (OLPG))	Subgingival plaque	PCR	Higher levels of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia and T. denticola*	1.Method detects only selected bacteria in a kit 2.no negative controls
13	Lo Russo et al., 2013)	8 (OLP) and 4 MMP	Sub-gingival plaque	real-time PCR	Significant correlations of gingival OLP lesions and *Aggregatibacter actinomycetemcomitans (AA)*	1.Method detects only selected bacteria in a kit. 2.No positive and negative controls

Rationale for the present study

In AIBD, the specific causes and pathogenesis remain unclear, hampering the development of effective treatments. Similarly, in OLP, the exact mechanisms behind the condition are not well-defined, making it challenging to create precise interventions.

Summary of Research Gaps.

After analysing previous studies, the current research gaps in the context of AIBD and OLP are summarised as follows:

1. Previous AIBD studies have often been investigated with small sample sizes due to the diseases' rarity. These limited samples restrict the generalizability of findings, weaken the ability to detect true effects or differences, and make it challenging to control for confounding factors. In the case of OLP, research has primarily focused on PCR and 16S rRNA techniques, which offer limited taxonomic resolution, making it difficult to distinguish closely related species or strains, and may introduce bias during the amplification process.

2. The previous techniques primarily focus on taxonomic information, failing to offer insights into the functional capabilities of the microbial community. Metabolomics in microbiome studies helps bridge this gap by examining the end products of various metabolic processes, contributing to a more holistic understanding of the diseases.

3. While newer techniques like shotgun metagenomics and metabolomics have proven valuable in profiling bacteria and metabolites, they have not been widely applied in AIBD and OLP studies. These advanced methods provide a more comprehensive and unbiased approach, offering high taxonomic and functional resolution compared to the older, lower-resolution techniques.

4. Another notable research gap in the existing studies is the lack of a multi-omics approach that connects clinical, periodontal, immunological, metabolomic and metagenomic data together in the context of AIBD and OLP.

Specific aims of this study therefore are:

1. Evaluate the role of periodontal status in these diseases and to investigate the relationship between periodontal status and the composition of the oral microbiome in large disease groups, while also assessing its correlation with disease severity.

2. Explore the oral microbiome: examine the role of the oral microbiome in the inflammatory response observed in individuals with MMP and PV compared to both healthy individuals and those with OLP.

3. Correlate cytokine responses: analyse and correlate cytokine responses in saliva and serum with the oral microbiome.

4. Investigate the oral metabolome: observe the oral metabolome and its association with the microbial composition of the oral microbiome, while also exploring its connection with disease severity in the respective groups.

This study aimed to pioneer a multi-omics approach that combines clinical, periodontal, immunological, metabolomic, and metagenomic data. This integrated framework will allow for a more holistic examination of AIBD and OLP, providing a deeper understanding of the complex interactions and mechanisms underlying these diseases and uncover new insights into the pathogenesis of AIBD and OLP, potentially identifying novel biomarkers and therapeutic approaches.

Chapter II. Materials and methods

This chapter describes the materials and methods used in this project. Consecutive patients were recruited from patients referred to the Guy's and St. Thomas' Hospital Oral Medicine Department (GSTT NHS Foundation Trust) and St John's Institute of Dermatology. Recruitment of all patients was systematic and consecutive, accepting all who were willing participate and who satisfied recruitment inclusion and exclusion criteria. Age- and gender-matched healthy subjects were enrolled as controls. The clinical part of the study included recruiting patients and healthy participants, following with the assessment of their general health by collecting the data at baseline such as age, gender, ethnicity, smoking habits, alcohol consumption, comorbidities, medication, dietary habits and BMI. Consent taking and recording the clinical metadata of the patients were done during consultation clinic by questioning the participants and extracting the data by consultants from electronic patient's records. After written informed consent was obtained from all participants, sample collection and clinical examination were performed. All samples were transported on ice and stored in the microbiome laboratory at King's College London, Guy's Campus.

2.1.1 Ethical approval

Ethical approval for the clinical study was obtained through the Health Research Authority (HRA)REC reference # 17/LO/0898 and NIHR IRAS ID: 20779. (**Appendix 1**)

2.1.2 Inclusion and Exclusion Criteria

Candidates were recruited considering the inclusion and exclusion criteria listed in **Appendix 2.** In summary, all participants were over 18 years old. From a dental perspective, all had at least one tooth. Pregnant women, patients, who had taken antibiotics within one month and patients without teeth were excluded. The diagnosis was based on clinical manifestations, histopathological evaluation, and direct and immunofluorescence. A subgroup of MMP patients was later included, in whom treatment with the antibiotic dapsone was commenced.

2.1.3 Participant recruitment

I was responsible for facilitating participants recruitment and the consent process. A copy of the consent was given to every participant. (**Appendix 3**). Participants were given verbal and written explanations (PIS) of the study (**Appendix 4**), given time to consider participating in the study and an opportunity to ask any questions. In addition, an explanation was provided of the study's aims, methods, objectives and potential risks. Participants were given 24 hours to consider consent. In cases where immediate consent was provided, all procedures, including sample collection, height and weight measurements recording (for BMI calculation), and oral and dental health assessment, were carried out during the same clinical session.

Patient diet information was collected for future research studies. Dietary questionnaire was intended to integrate this data in future research, involving systems biology and modelling-based approaches to predict microbiome responses to dietary patterns, identify causal relationships, and analyse longitudinal data, respectively, providing a deeper understanding of how diet influences the microbiome **(Appendix 5).**

All information was pseudonymised, using encrypted systems following the Data Protection Act (DPA). Details were kept on a password-protected computer in a swipe card-accessed department. Consent forms and hard copies of participant information were kept in a locked cupboard.

Disease groups comprised patients with PV (n=48) and MMP (n=48), which are predominantly mucosal diseases with linear basement membrane zone (BMZ) IgG, IgA and or C3 on direct immunofluorescence (DIF) for MMP, oral erosions and intercellular IgG+/- CC3on DIF in PV. OLP (n=50) diagnosis was based on clinical presentation and confirmatory histology. HC participants (n=40) were recruited predominantly from the KCL Dentistry faculty department staff or the chaperons of the patients. In our study, the number of patients in HC groups is not consistent due to objective reasons (COVID-19), when clinical research had a significant impact on patient and age-matched healthy participants recruitment due to the challenges posed by lockdowns and social distancing policies. The peak of the crisis resulted in clinical research programs diverting from their planned schedules to prioritize front-line care and crisis management. This situation led to the cancellation or restriction of personal interactions

with healthy elderly study participants. These factors collectively contributed to the disruption and slowdown of healthy age-matched participants recruitment.

2.2 Sample collection

The clinical part should come first so move that section here please. You mentioned BMI – that needs to be fitted in somewhere too

No statistical methods were used to predetermine sample size as this was an exploratory pilot study. The aim was to recruit 50 participants into each disease group and healthy control. Recruitment was limited by two lockdowns and a limited number of patients attending in person during this time.

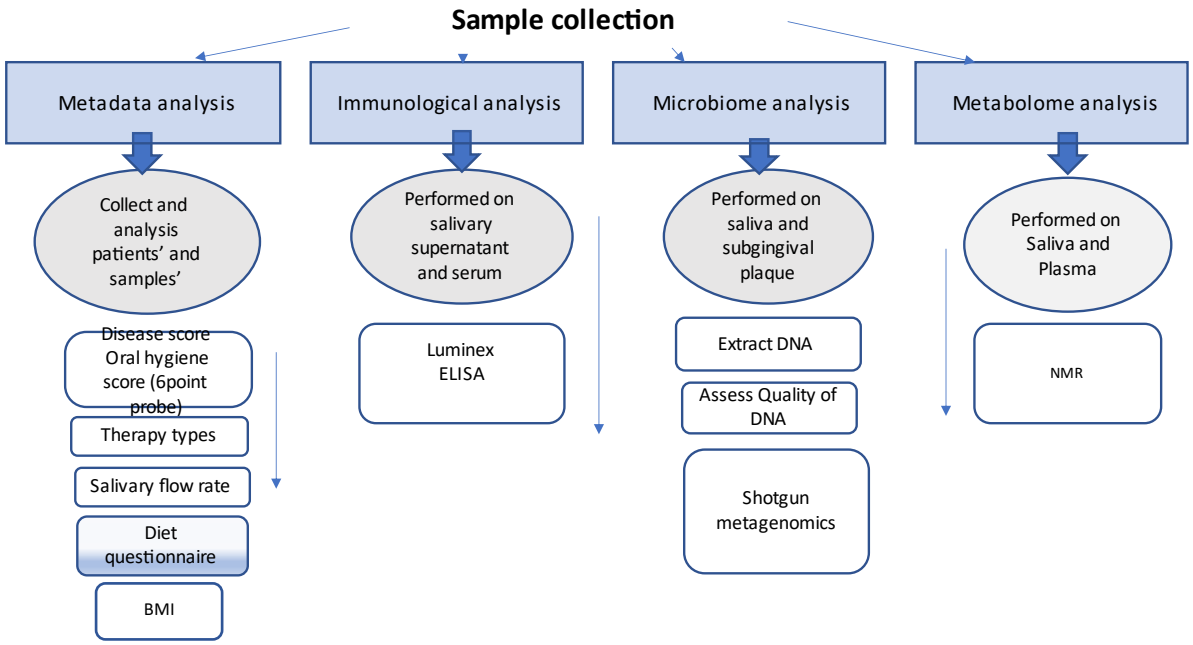


Figure 2.1. Study workflow. *A summary of the clinical data and samples collection from Mucous membrane pemphigoid (MMP), pemphigus vulgaris (PV), disease control oral lichen planus (OLP) and healthy control (HC) groups.*

2.2.1 Clinical assessment and use of validated scoring methodologies.

A detailed medical history, including age, gender, ethnicity, salivary flow rate, smoking habits, alcohol consumption, comorbidities, dietary habits was undertaken for both patients and healthy controls. Current and past medication was recorded.

2.2.1.1 ODSS

The examination of subjects involved the assessment of the Oral Disease Severity Score (ODSS), where 17 mucosal sites of involvement were observed, and activity at each site was recorded to calculate an overall ODSS using a published scoring methodology (Escudier et al., 2007; Ormond et al., 2018, 2019). The scoring process was performed by trained clinicians who were aligned in their approach. The findings from ODSS scoring were actively recorded by me during the assessments conducted and reported by a trained specialist.

The 17 sites within the oral mucosa include the outer lip, inner lips, right/left buccal mucosa, right/left soft palate, right/left hard palate, right/left dorsum of the tongue, right/left ventrolateral tongue, the floor of the mouth on the right/left oropharynx and six defined areas of the gingivae. The presence of a lesion is evaluated by 1, and the absence by 0; in the buccal mucosa involvement of ≤50%, = 1 and > 50% =2. The dorsum of the tongue, the floor of the mouth, the hard or soft palate and the oropharynx are scored according to the involvement of one or both sides and scored 1 and 2, respectively. Each site unit is evaluated according to the level of activity of the lesion: 1= mild erythema; 2 =marked erythema without ulceration; 3=presence of erosion or ulcers. In asymptomatic lesions without signs of inflammation, i.e., erythema, activity score is 0.

Pain from the mouth is also assessed on a scale of 0-10, by asking a patient, the degree of pain on average over the preceding week. A maximum ODSS score is 106. A score of over 30 generally indicates moderate disease with scores of 50 or more indicating severe disease. **(Appendix 7)**

This validation study involved ten clinicians from four UK oral medicine centres, who assessed patients using the ODSS and the PGA (Physician Global Assessment) scores (Ormond et al., 2022). To ensure consistency, clinicians underwent training, calibration, and discussion of methodologies. There was a strong correlation between ODSS and PGA scores. The advantages of ODSS are superiority to other methods in published validation studies, its sensitivity, reproducibility and ease of measurement, taking an average of 90 seconds per patient.

2.2.1.2 6-point pocket periodontal chart

After mucosal examination of disease activity, a periodontist or oral medicine specialist evaluated the number of teeth present, and a 6-point probing chart was recorded in mm using a UNC 15 manual periodontal probe according to standard practice (Rams et al., 1993), as well as recording bleeding on probing at each site and tooth attachment. In addition, a standardized digital sheet was used for recording periodontal parameters (**Appendix 7**). During the examination, the recording and calculation of the periodontal activity of the disease in each participant were carried out with my active participation in data collection and analysis. Clinical assessment was carried out by experienced clinicians. We found that it would not have been appropriate to carry out a reproducibility assessment for these participants since they were attending a clinic requiring multiple other assessments at the same visit, and it was apparent that participants / patients were largely unable to stay on site long enough for repeated clinical assessments. The same examiners, in other projects on patients without mucosal disease have shown good levels of reproducibility. Finally, these patients were often travelling long distances and were difficult to recruit.

2.2.1.3 BMI

Body Mass Index (BMI) data has been systematically collected from study participants. BMI, a widely used metric for assessing body weight in relation to height. (BMI) defined as weight (kg) divided by the square of height (m), and the waist-hip ratio (WHR) defined as waist circumference (cm) divided by hip circumference (cm).

2.3.1 Saliva and plaque collection

Once consent was received, subjects were asked to refrain from consuming food or drink and perform oral hygiene for at least one hour before saliva or dental plaque collection. The collection date and a study label were written on a sterile white capped pre-weighed 20 mL universal tube (Sterillin Polypropylene Universal Tube SKU: 128BBAC/P). All participants were asked to spit saliva into the collection tube for 10 minutes. All collected samples were transported on ice within four hours to the microbiome laboratory on floor 17, Tower Wing at King's College London, Guy's Campus. The labelled collection tubes were weighed, and post-collection weight was recorded and stored in a -80 °C freezer for further analysis (Sanyo Electric Co, Japan).

Dental professionals collected sub-gingival plaque from the Oral Medicine or Periodontology departments. First, target dental surfaces were identified, and the supragingival plaque was gently removed with a sterile curette. The subgingival plaque was then inserted by a sterile curette for 15 seconds and placed into a pre-labelled 1.5 mL microcentrifuge tube with 200 µl RNA-Later and held on ice. The total collection time for each sample was 10 min. All collected samples were transported on ice within two hours to the microbiome laboratory on floor 17, Tower Wing at King's College London, Guy's Campus, for further analysis and stored in a -80 °C freezer (Sanyo Electric Co, Japan).

A total of 365 samples, 186 saliva samples and 179 subgingival plaque samples were collected.

The fluctuation in the quantity of subgingival plaque samples is a result of some participants who, due to gum hypersensitivity, were unable to complete the entire procedure.

The labelling and weighing of the tubes, saliva collection, transportation, salivary flow rate measurement and sample aliquoting were carried out under my responsibility. Furthermore, assistance was provided by me to the periodontist for plaque samples collection.

2.3.2 Serum and plasma sample collection.

Serum and plasma samples were collected by venepuncture into yellow and purple lid tubes by a trained medical practitioner in the Guy's and St. Thomas' Hospital Oral Medicine Department (GSTT NHS Foundation Trust), St John's Institute of Dermatology. Whole blood was collected in the 4 mL Vacuette K3 EDTA tube (Greiner Bio-One), transported on ice within four hours to the microbiome laboratory on floor 17 and was centrifuged for 10 min at 2000 x g and 4°C in an Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany), and plasma collected and separated into aliquots of 0.5 mL in 1.5 mL microcentrifuge tubes (Fisher Scientific), then stored in a -80 °C freezer for further metabolomics analysis. In addition, blood samples collected in the BD Vacutainer SST II Advance 8.5 ml tube (Becton Dickinson) clotting tubes were centrifuged for 10 min at 2000 x g and 4 °C in an Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany). The separated serum was then stored in a -80 °C freezer in aliquots of 0.5 mL in 1.5 mL microcentrifuge tubes (Fisher Scientific) for further processing.
In total there were 181 serum, and 181 plasma samples were collected. Discrepancies of smaller number of samples resulted from challenges in blood collection, resulted from poor vein accessibility or patient discomfort during venipuncture.

2.4 DNA Extraction

Saliva and subgingival plaque samples were processed for DNA extraction, quantification and sequencing.

The extraction of microbial DNA from saliva and plaque samples used an optimized extraction protocol based on the DNeasy PowerSoil Pro kit (Qiagen) **Figure 2.2.**

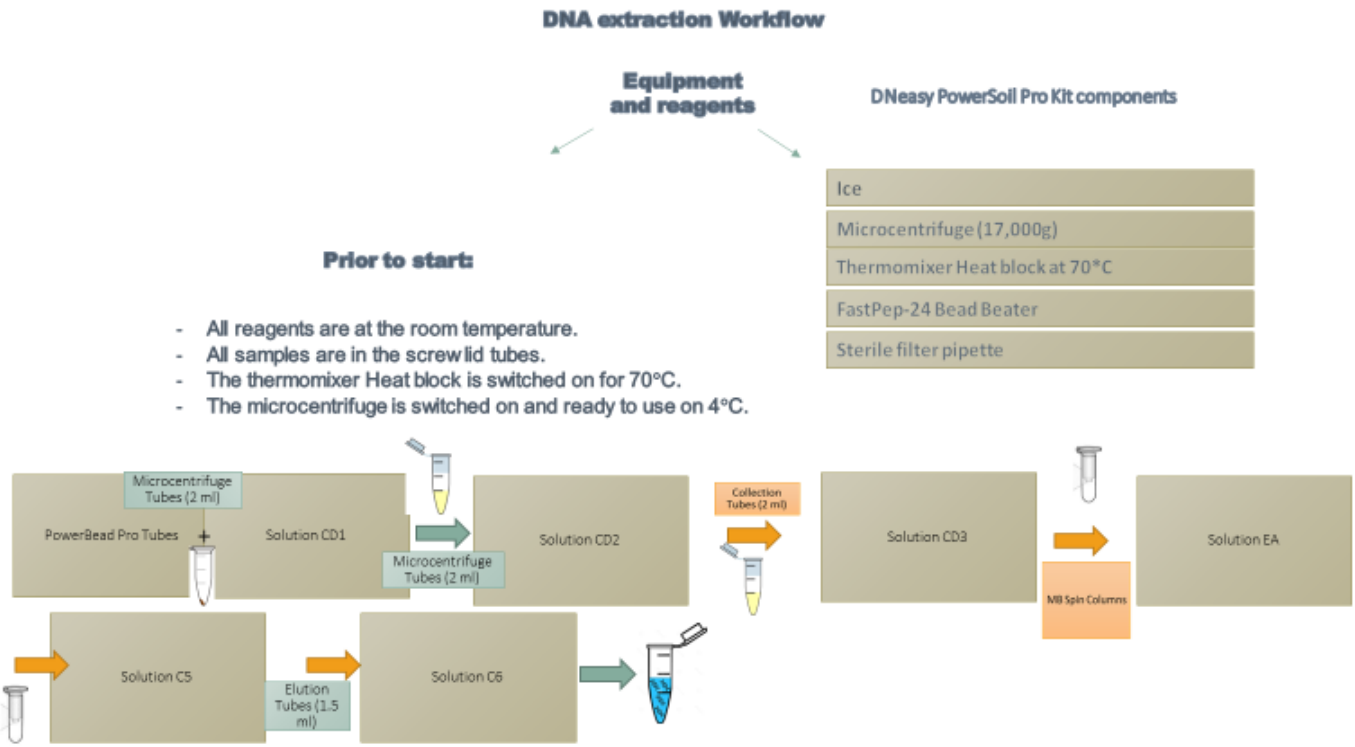


Figure 2.2. Microbial DNA extraction workflow. *(Created on Biorender)*

2.4.1 DNA extraction of saliva and plaque samples.

DNA was extracted from samples using the DNA Powersoil Pro extraction kit (Qiagen) according to manufacturer's instructions. Saliva (1 ml) or plaque sample in 200 µl of RNA-Later from -80 °C storage was thawed to room temperature for 10 min. The sample was spun in a microcentrifuge at 17,000 x g for 15 minutes, and supernatant was collected without touching the pellet. The powder from bead tube was added to the pellet, and 800 µL of Solution CD1 (lysis buffer) was added. Modifications were applied to manufacturer's

instruction, by placing the tube into a heat block at 70 °C for 10 min prior vortexing it for 5 seconds. Additionally, the FastPrep-24 bead-beater was set up to homogenize every sample for 4 min for four cycles at 6.5m/s while ensuring the tubes were placed on ice between cycles (1 min cycle : 1 min on ice) so the sample does not overheat. Post-bead-beating, the tube was centrifuged at 15,000 x g (4°C) for 1 min. Up to 500-600 µL of supernatant was carefully transferred to a clean 2 mL microcentrifuge tube. Next, 200 µL of solution CD2 (inhibitor removal technology) was added and the tube vortexed for 5 s before centrifuging at 15,000 x g (room temperature) for 1 min. Up to 700 µl of supernatant was transferred to a clean 2 mL microcentrifuge tube. Next, 600 µL of solution CD3 (a high-concentration salt solution) was added and vortexed for 5 s. A total of 650 µL of lysate was added to an MB Spin Column and centrifuged at 15,000 x g (Room temperature) for 1 min. The flow-through was discarded. This step was repeated until all of the sample was passed through MB Spin Column. Next, the MB Spin Column was again placed into a clean 2 mL collection tube, and 500 µl of solution EA (a wash buffer) was added to the MB Spin Column and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and placed into the new 2 ml collection tube. Next, 500 µL of Solution CD5 (an ethanol-based wash solution) was added to the MB Spin Column and centrifuged at 15,000 x g for 1 min before discarding the flow-through and placing the MB Spin Column into a new 2 ml collection tube. The tube was then centrifuged at 16,000 x g for 2 min. Next, the MB Spin Column was placed into a new 1.5 ml microfuge tube, and 50–100 µL of solution CD6 (Tris buffer) was added to the centre of a white filter membrane keeping the pipette vertical, parallel to walls of the tube, and centrifuged at 15,000 x g for 1 min. The eluted DNA was then stored in a –80°C freezer. DNA extraction process was undertaken by me.

2.4.2 Quality and quantity control of DNA

The quantity and quality of the isolated DNA were measured initially with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and then quality was further checked by performing agarose gel electrophoresis or using an Agilent bioanalyzer.
DNA purity was assessed to ensure that samples did not contain > 1 mM EDTA and were free of organic contaminants, such as phenol and ethanol. These substances can interfere with the fragmentation reaction and result in assay failure. The DNA is generally accepted as “pure”

when absorbance ratios at A260/A280 and A260/A230 vary between 1.8-2.2. Purity ratios that are significantly different may require further optimization.

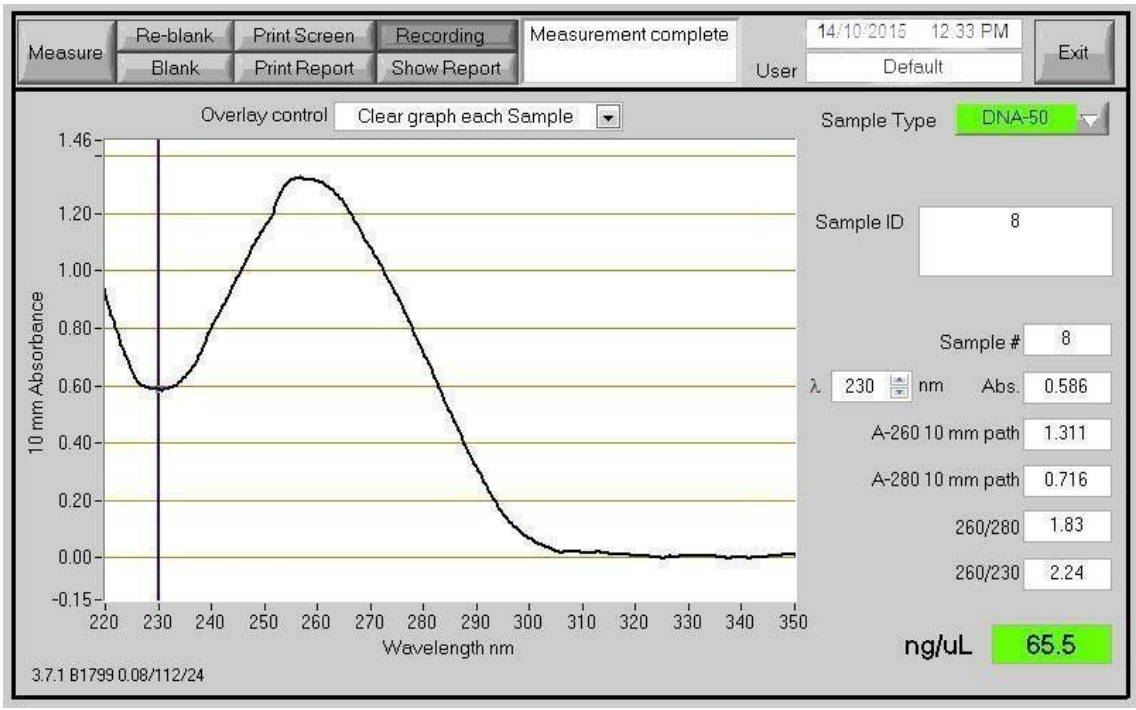


Figure 2.3 Nanodrop spectrophotometer measurements of DNA from saliva sample. *The DNA quality was assessed based on the absorbance ratios at A260/A280 and A260/A230 using spectrophotometry.*

Since the concentration estimation based on UV absorbance is not reliable, more precise concentration estimation of the samples was also done fluorometrically with a Qubit (Thermo Fisher), using a recommended protocol (Mullegama et al., 2019).

For accurate calculation of concentrations, Qubit analysis was performed mixing 1 µL of Qubit dsDNA HS/ BR concentrated assay reagent with 199 µL of Qubit buffer to make a working solution. 190 µL of this working solution was mixed with 10 µL of standard 1 and the same for standard 2 in a 0.1 mL microcentrifuge tube. Then, 1 µl of the sample was mixed with 199 µL of the working solution in a 0.1 mL microcentrifuge tube and vortexed for 2 – 3 s. The tubes were left to incubate at room temperature for 2 min and then inserted in the Qubit fluorometer to take the reading.

Finally, the integrity of extracted DNA was assessed by gel electrophoresis. This was done in 1% agarose gel by running the extracted products at 100V for approximate 30 minutes. Staining was carried out using the intercalating dye GelRed Nucleic Acid Gel Stain (Biotium,

CA, USA) at 0.01%. Visualisation of the bands was performed under a UV transilluminator and image acquisition system ChemiDoc MP Imaging System (BioRad, Watford, UK).

2.5 Human Magnetic Luminex Screening Assay

Circulating cytokine levels in the blood were assessed through a magnetic bead-based immunoassay kit (Luminex 200; Luminex Corp., Austin, TX, USA) for identification of inflammatory biomarkers levels IL-6, TNF-α, IL-13, IL-1β, IL-4, IL-8, IL-17A, IL-23, and matrix metalloproteinase, including MMP-3, MMP-9 and MMP-13 using the Bio-Rad Bio-Plex 200 analysers (Bio-Rad, CA, USA). The assay was carried out in accordance with manufacturer's instructions, with the adjustment of halving reagents to allow for two usages of the kit and reducing the sample amount suggested for the analysis.

In brief, saliva and serum samples were diluted using calibrator diluent RD6-52 in a 1:1 dilution before being centrifuged for 4 min at 16,000 x g. Wash buffer was prepared by adding 20 mL of 25x wash buffer concentrate to 480 mL of deionized water. Standards were then prepared. Each standard cocktail provided with the kits (LXSAHM-02, LXSAHM-08, LXSAHM-17) was reconstituted with calibrator diluent RD6-52 according to the manufacturer's stated volume. They were then left to sit for 15 min. Standard 1 was generated by adding 100 µL of each standard cocktail and then made up with a calibrator diluent to a final volume of 1 mL. Next, 200 µL of calibrator diluent RD6-52 was added into seven tubes, and 100 µL of standard 1 was added to the first tube, which was mixed thoroughly before carrying on a 1:3 serial dilution series to generate a standard curve.

A diluted microparticle cocktail was prepared by adding 250 µL of the provided microparticle cocktail to 2.5 mL diluent RD2-1. Next, 25 µL of this diluted microparticle cocktail was added to each well of a thin-bottomed 96-well plate, followed by 25 µL of standards or sample in duplicate. The plate was then covered securely using a foil plate sealer and incubated for 2 h at room temperature on a horizontal orbital microplate shaker set at 800 rpm.

The beads were washed three times using a magnetic device to accommodate a microplate. Briefly, the plate was secured in the magnetic plate washer and left for 1 min before removing the liquid by flicking it out. Each well was washed with 200 µL of wash buffer and placed onto the magnetic device for 1 min before removing the liquid. A diluted biotin-antibody cocktail was prepared by adding 250 µL of the provided biotin-antibody cocktail to 2.5 mL diluent RD2-

1. After washing, 25 µL of a diluted biotin-antibody cocktail was added to each well. The plate was then incubated for 1 hour at room temperature on the horizontal orbital microplate shaker as before. Post-incubation, the wells were washed three times with wash buffer as before. A streptavidin-PE mix was prepared by adding 110 µL of the provided Strep-PE concentrate to 2.7 mL wash buffer. After washing, 25 µL of this diluted streptavidin-phycoerythrin (PE) was added to each well. The plate was secured with a foil plate sealer and incubated for 30 min on the horizontal orbital microplate shaker as before. Following incubation, the microparticles were washed three times, as mentioned previously. Finally, a 75 µL of wash buffer was added to each well, and the plate was incubated for 2 min on the horizontal orbital microplate shaker. The plate was read using a Bio-Plex immune-analyser Bioplex 200 machine (Bio-Rad Laboratories).

2.6 Sandwich Enzyme-linked immunosorbent Assay (ELISA)

Due to the unavailability of complement of MMP-8 analyte in magnetic multiplex microbead assay, saliva and serum samples for this analyte were analysed using Human ELISA Duoset kits (DY1965, DY908). The list of reagents and their components is given in **Table 2.1.**

Likewise, a similar to Luminex modifications were introduced in ELISA protocol, to optimize cost-efficiency of the study and to ensure a greater quantity of samples for other analyses of this study, half amount of the samples and reagents were used.

Table 2.1 The reagents for Sandwich ELISA and their components.

REAGENT	REAGENT COMPONENTS
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4,
Wash Buffer	25 mL of 5% Tween 20 in 475 mL of PBS pH 7.2-7.4
Reagent Diluent	1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered.
Substrate solution	1:1 mixture of Colour Reagent A (H2O2) and Colour Reagent B (Tetramethylbenzidine)
Stop solution	2 N H2SO4

First, the capture antibody (CA) was diluted to the working concentration indicated in manufacturer's protocol in phosphate buffered saline (PBS). Next, 50 µL of CA was reconstituted in PBS according to the manufacturer's protocol and was added to each well of a 96-well plate (Thermo Scientific), following incubation overnight at 4 °C. The next day, the plate was aspirated and washed with wash buffer (**Table 2.1**) three times, then blotted against a clean paper towel and blocked with Reagent Diluent (RD) for 1 hour. First, standards were diluted in RD according to the manufacturer's protocol. Next, standards or samples diluted in RD were added to the wells of the plate. Next, the plate was covered with an adhesive film and incubated at room temperature for 2 hours. The plate was then aspirated and washed 3 times, and a detection antibody (DA) was added. After 2 hours incubation at room temperature, the plate was aspirated again and washed as before. Next, streptavidin-HRP (STR) was diluted to the working concentration in RD according to the manufacturer's protocol and added to each well before the plate was incubated at room temperature for 20 minutes. The plate was then aspirated and washed as before. After this, the plate was rewashed with wash buffer and one more time with PBS. Next, 100 µL of substrate solution was added, leaving the plate in the dark. Next, 100 µL of stop solution was added to each well,

and the absorbance on the plate was read using an iMark microplate absorbance reader (Bio-Rad) set at a wavelength of 450 nm. **Table 2.1**

2.7 Metagenomic shotgun sequencing analysis.

2.7.1 Samples Quality Control (QC)

After sending the samples to SZAOmics laboratory (Genomics Production Istanbul), the sample quality control (QC) was performed repeatedly. The quality and quantity of samples were assessed spectrophotometrically with NanoDrop (Thermo Fisher, USA). The gDNA samples with A260/280:1.8-2.0 and A260/A230:2.0-2.2 passed QC.

2.7.2 Library Prep Methods and Sequencing

Fully automated library preparation was performed with Hamilton NGS Star robot in SZAOmics laboratory. DNA samples for metagenomics were prepared for 150 bp and 100 bp paired-end sequencing using Novaseq 6000 (Illumina, USA) platform. Numerically coded aliquots of approximately 1 µg DNA per sample were used to create sequencing libraries. First, genomic DNA was fragmented to approximately 300 base pairs (bp). Fragmented DNA was used to synthesize indexed sequencing libraries using the Illumina DNA Sample Prep Kit (Illumina, Inc., San Diego, CA), according to manufacturer's recommended protocol. Libraries were sequenced with an Illumina NovaSeq 6000 with read lengths of 100 base pairs (bps) and at for 150 base pairs (bps) single read (Illumina). Only those reads with a quality score ≥17 for at least 80% of the read length (i.e., probability of correct base call ~98%) were retained.

2.7.3 Data analysis

Raw reads were mapped against an in-house gene catalogue of human oral microbiome to calculate gene abundances using the METEOR software suite (available at https://forgemia.inra.fr/metagenopolis/meteor) that includes trimming of raw reads using Alien Trimmer. The gene count table was used for the quantification of MSP abundance using the MetaOMineR (momr) package in R. This was done by calculating the mean abundance of 100 'marker' genes of each MSP per sample.

2.8 Metabolomics analysis. Nuclear magnetic resonance (NMR) technique

2.8.1 Sample preparation

Samples of both saliva and plasma were transported to the NMR Centre of King's College London for further processing. A 540 µL aliquot of each saliva sample was mixed with 60 µL of potassium phosphate buffer (pH 7.4) containing D2O. Trimethylsilyl propionic--d4 acid, sodium salt (TSP 0.1% in D2O) was used as an internal reference for alignment and quantification of NMR signals. A 90 µL of each plasma sample was mixed with 90 µL of NMR buffer, the buffer containing 90% H2O, 10% D2O, 2 mM NaN3, 5 mM TSP-d4 and around 100 mM phosphate buffer. In addition to individual analysis of the samples, a pooled saliva sample (15 µL of each saliva sample) and pooled plasma sample (4 µL of each plasma sample) were analysed to ensure unambiguous assignment and as an external standard to verify the stability of automation in the NMR analysis.

2.8.2 1H-NMR Spectroscopy Analysis

All spectra were acquired on a Bruker Avance NEO 600 MHz equipped with a TCI Cryoprobe Prodigy (Bruker Biospin, Karlsruhe, Germany), operating at a proton frequency of 600.2 MHz at 298 K. For saliva samples, 1H spectra were acquired using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with water suppression by pre-saturation, with a spectral window of 20.8 ppm, a total spin-echo time of 77 ms, a relaxation time of 4 seconds, an acquisition of 2.62 seconds and 64 scans. The PROJECT pulse sequence was used for plasma samples with the same parameters as the CPMG pulse sequence. Additionally, a diffusion-ordered spectrum was applied to look at lipoproteins, using a gradient of 80% for the diffusion, a spectral width of 29.75 ppm, a relaxation time of 4 s, an acquisition time of 1.84 s, and 64 scans. TSP peak (0 ppm) was used as an internal reference/standard.

2.8.3 NMR spectroscopy and processing

After processing the spectra on TopSpin, the spectra were exported into MATLAB software (version 2018b; MathWorks, Natick, MA, USA), where post-processing (alignment with PAFFT3 and normalisation using PQN4) was handled. The assignment was performed based on the human metabolome database (HMDB, http://www.hmdb.ca). The resulting list of peak

areas for each metabolite was converted to a .csv file to be exported to MetaboAnalyst 5.0 software (www.metaboanalyst.ca), which was used for data analysis.

2.9 Statistical analysis

For clinical and immunological parameters, a Kolmogorov-Smirnov normality test was applied to analyse distribution of the data. Statistical comparisons were conducted using the nonparametric Kruskal-Wallis multiple comparisons test, followed by a post-hoc Dunn's test to account for the numerous pairwise comparisons necessary to analyse the data effectively. It helps avoid the issue of multiple testing and controls the familywise error rate.

The normally distributed variables were described using mean and standard deviation (SD), while the median and interquartile range (IQR) were used to describe nonnormally distributed data. Finally, correlation analysis was performed using Spearman nonparametric, correlation test to evaluate the strength of association between variables, and results are presented together with the rank correlation coefficient (r). Differences were considered significant when $P < 0.05$.

To study the risks associated with diabetes (dependent variable), simple logistic regression analysis was performed to examine the association between diabetes and ODSS and thyroid disease and ODSS in diseases groups.

For multivariate data analysis of metabolites Principal Component Analysis (PCA) was used to define a homogeneous cluster of samples. PCA indicated the outliers outside the 95% confidence interval that could substantially affect the supervised models. Only those metabolites with variable importance to projection (VIP) > 1 were considered for the discriminant classification using partial least squares-discriminant analysis (PLS-DA). Accordingly, excluding the outliers, PLS-DA models were developed to identify the separation between the MMP, PV, OLP and control groups. Statistical comparisons for metabolites different between groups were performed with the nonparametric Kruskal-Wallis test, following Dunn's multiple comparison test and the p values were adjusted for multiple testing by Benjamini Hochberg corrections to analyse the variables that were not normally distributed. Metaboanalyst 5.0 software was used for the statistical analyses. The Benjamini-Hochberg method for multiple hypothesis correction was used with the false discovery rate set to 0.05. The Benjamini-Hochberg test, also known as False Discovery Rate (FDR) correction. The purpose of the Benjamini-Hochberg test is to control the FDR, which is the

expected proportion of falsely rejected null hypotheses among all the hypotheses that are rejected (Benjamini & Hochberg et al., 1995).

For metagenomics analysis, MSP abundances were mapped to their corresponding species taxonomy for diversity analysis using Python 3.10. Alpha diversity was calculated using the Shannon diversity metric from the Scikit-bio, and beta diversity was calculated using the Bray-Curtis distance measurement from Scipy, which was used for principal coordinate analysis (PCoA) in the Scikit-bio package. PCoA plot was visualised using the Plotly package in Python 3.10. The differentially abundant species were identified by Kruskal–Wallis followed by Dunns' test. Species abundances were correlated with various clinical metadata using Spearman's rank correlation coefficient. Linear regression analysis was used to determine the association between bacterial richness and ODSS on GraphPad Prism. The Benjamini-Hochberg method for multiple hypothesis correction was used with the false discovery rate set to 0.05.

GraphPad Prism 10.0.3 was used for conducting all multiple comparison tests and multiple correlation analysis.

Chapter III. Results: characteristics of study population

In this study, 48 MMP, 48 PV, 50 OLP and 40 age-matched HC were included. First, the demographic profile in each group was assessed, including age, sex, ethnicity. Participants' eating habits were collected through the dietary questionnaire **(Appendix 5),** consisting of daily or weekly consumption of typical (i.e., fruit, vegetables, cereals, legumes, and fish) and non-typical (i.e., meat, meat products, and dairy products) foods. The patients' comorbidities and medication history were recorded. Clinical examination for Oral disease severity was documented for all sites of the involvement as well as PISA scores. The BMI was also recorded.

Periodontal disease has been associated with numerous autoimmune diseases in the literature, including AIBD and OLP (Jascholt et al., 2017a; Nunes et al., 2022; Thorat et al., 2010). However, there was lack of studies related to microbiological aspects in patients with these diseases. In the current study a detailed periodontal assessment was undertaken in all of the three disease groups and healthy controls.

Although we aimed initially for all MMP patients to not be taking an antibiotic, due to difficulty with recruitment during the Covid-19 pandemic, 12/48 MMP patients were on dapsone or sulfapyridine as these are first line therapeutic agents in the management of this condition. Having two subgroups within MMP did however enable a preliminary analysis of the effect of the low-dose AB on the oral microbiome in comparison to other treatments. MMP was subgrouped into topical/treatment-naïve (n=28), immunosuppressive therapy (azathioprine, MMF, prednisolone and rituximab) (n=8), and the group on AB only/AB+IS (n=12).

3.1 Descriptive results

The summary of demographic data and summarised clinical parameters have been shown in **Table 3.1.**

Table 3.1. Demographics of the participants.

CHARACTERISTIC	MMP (N=48)	PV (N=48)	OLP (N=50)	HC (N=40)
(Median (Min-Max))				
Age	65 (26-90)	56.50 (22-78)	61 (31-82)	59 (35-85)
Sex Female/Male	31/17	29/19	41/9	20/20
Ethnicity				
Caucasian/White	42	21	34	29
Asian	5	24	10	8
Black/Afro Caribbean		1	1	
Other	1	2	5	3
Smoking	5	2	1	1
BMI (Median (Min-Max))	25 (20.2-32.4)	26.2 (19.5-36.4)	25.5 (16.4-36)	25.45 (18.7-32.3)
Diet type				
Mixed	36	29	35	32
Vegetarian	5	8	4	2
Mediter/Red Meat free	7	11	6	6
Diabetes	10	4	12	3
Thyroid disease	7	3	8	1

3.1.1 Age and sex

There was no statistically significant difference in age comparing PV, MMP and OLP groups. However, there was a significant difference between MMP and HC groups because of the difficulties recruiting healthy participants the same age as those with MMP during Covid-19 (p=0.02). The distribution of females in disease groups was higher than in HC, where the females and males were distributed evenly. **Figure 3.1**

The number of female participants was significantly higher in all three groups of diseases with the ratio of 1.82:1 in MMP, 1.5:1 in PV and 4.5:1 in OLP, 1:1 in HC.

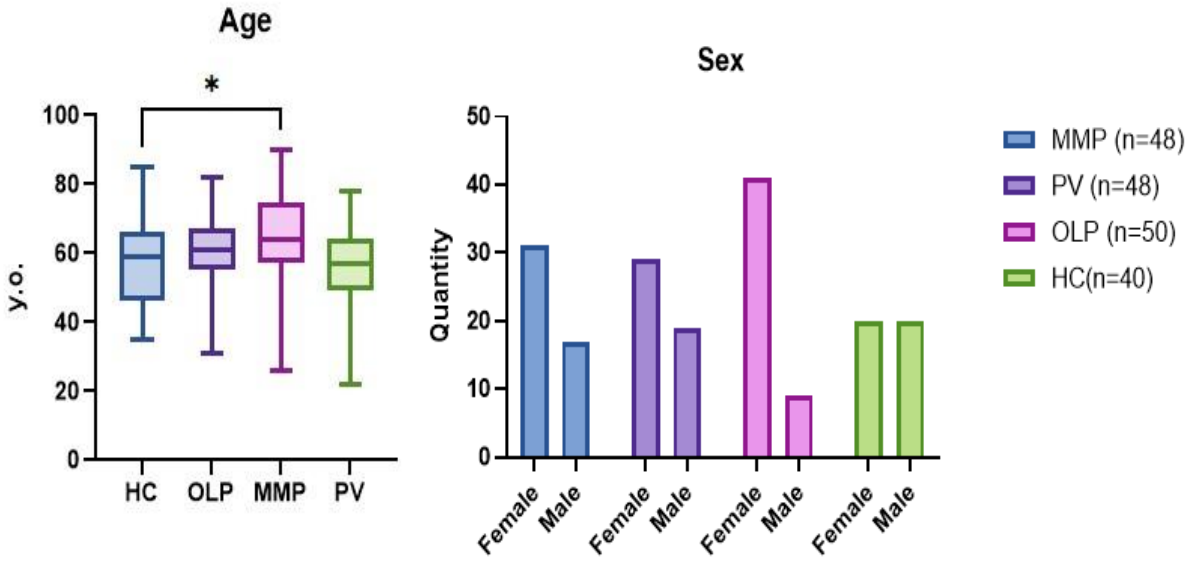


Figure 3.1. Differences in age and sex in disease groups and HC. *(p<0.05).

3.1.2 Ethnicity

Ethnic demographic analysis showed that participants in MMP, OLP and HC groups belonged to white European ethnicity, while in PV the higher ethnic group was Asians. **Figure 3.2**

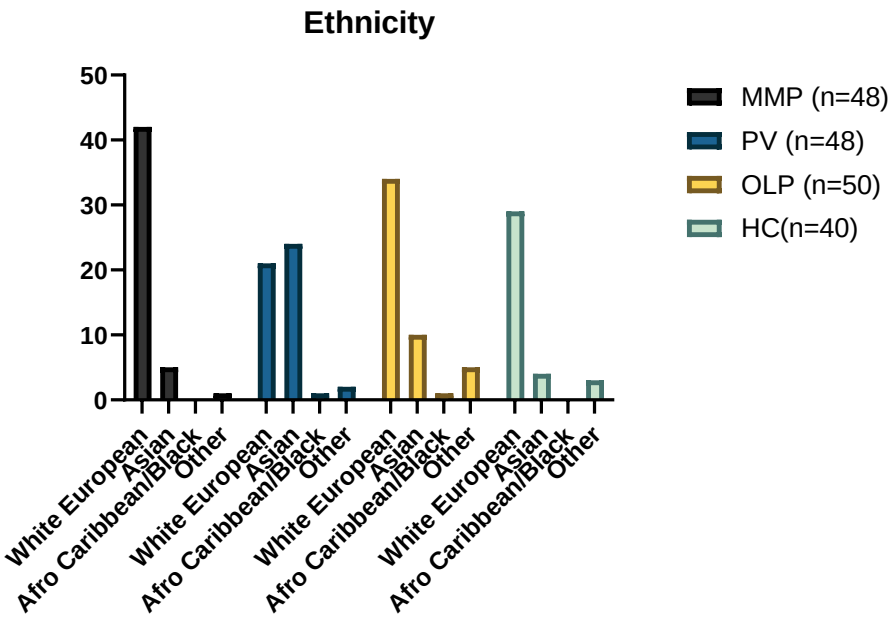


Figure 3.2 Ethnic distribution between groups.

3.1.3 BMI

The values of body mass indexes (BMI) were higher in the OLP group, but the results of the statistics were non-significant (p value> 0.99). **Figure 3.3**

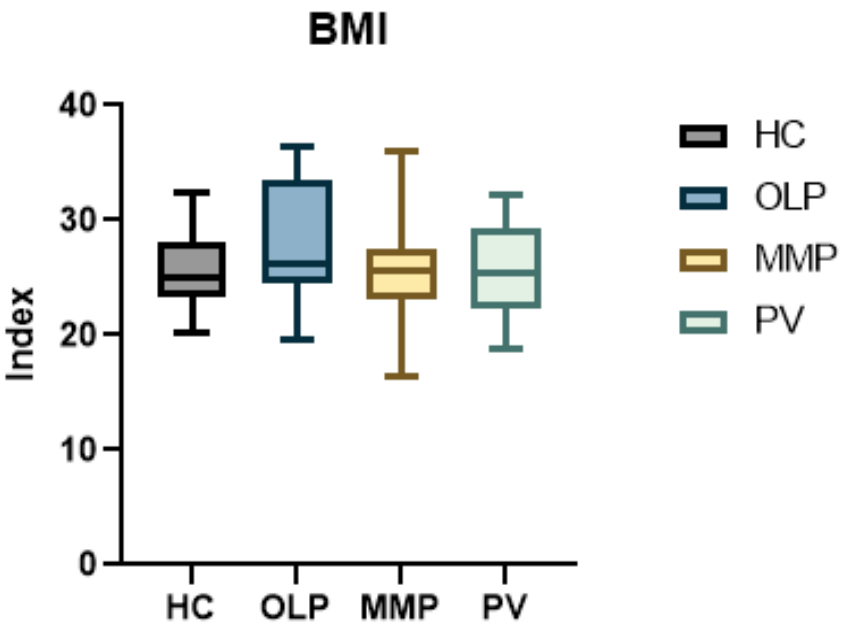


Figure 3.3 Body mass index among the groups.

3.1.4 Salivary flow rate

There was no difference in salivary flow rate (SFR) between groups, with the values of the SFR within the normal flow (Iorgulescu, 2009).**Table 3.2**

Table 3.2 Differences in salivary flow rate between MMP, PV, OLP and HC groups.

Salivary flow rate	HC	MMP	PV	OLP
Median	0.5 (0.1-1)	0.4 (0.2-1.2)	0.5 (0.17-1.4)	0.4 (0.15-1.3)

3.1.5 Treatment type

Among PV patients (2 were either treatment naive or on topical CS. Forty-six were taking a combination of Pred with Aza/MMF and some had additonally been treated with Rituximab. In the MMP group, 28 patients were either treatment naïve or on topical CS therapy only. In addition, 8 patients had combination or one systemic IS therapy and 12 participants were taking dapsone or sulphapyridine with or without IS agent. Finally, in the OLP group, 30 patients were either treatment-naive or on topical treatment. Another 20 were on HCQ with or without IS. **Table 3.3**

Table 3.3. Treatment type among the groups of diseases. *T. naive- Treatment naïve; Topical- on topical corticosteroids; Pred-Prednisolone, Aza- azathioprine, MMF- Mycophenolate, Ritux- Rituximab, AB- antibiotics; HCQ- Hydroxychloroquine*

Treatment type	MMP (n=48)	PV (n=48)	OLP (n=50)
T.naive/Topical	28	2	30
Pred/Aza/MMP/Ritux	8	46	10 (2 were on HCQ+IS)
AB alone or +/-IS	12		
HCQ alone			10

3.1.6 Oral disease severity score

The ODSS showed significant difference between groups of disease with the higher score in OLP group, following by MMP and PV (p<0.0001) **Tab 3.4.**

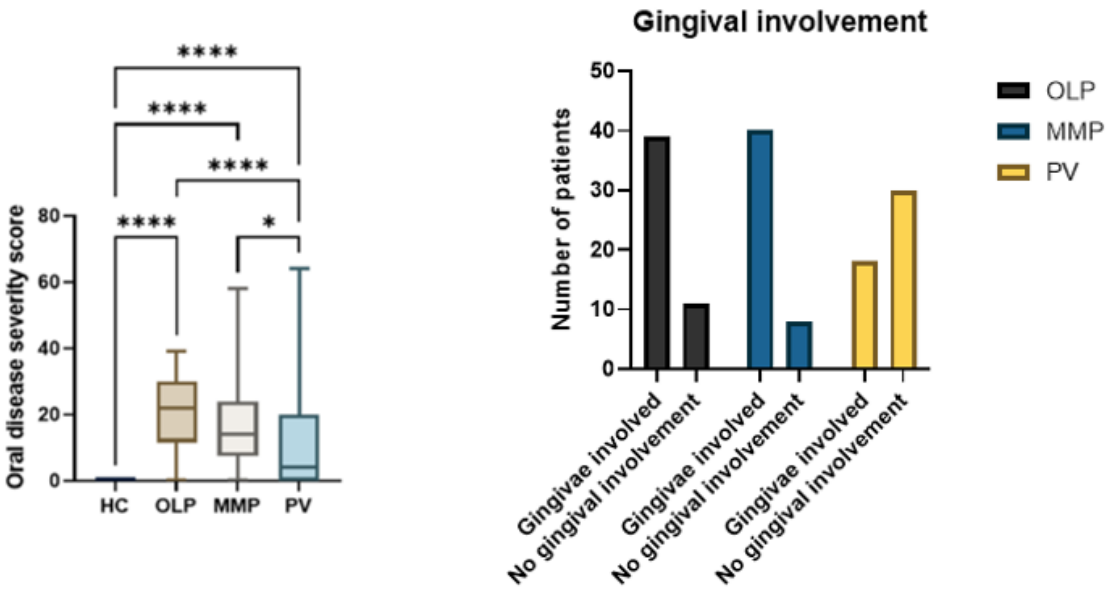


Figure 3.4. a) Oral disease severity score in 3 groups of disease in comparison with HC*. Statistical analysis done using Kruskal-Wallis test; b) Gingival involvement graph shows the number of patients in the groups with presence of mucosal diseases lesions on gingivae. *(p<0.05); ****(p<0.0001).*

There were a higher number of patients in the OLP and MMP group with thyroid disease and type 2 diabetes than among HC. The Bivariate logistic regression analysis was used to examine the association between these two most frequent comorbidities, diabetes and thyroid disease and oral disease severity score. T2D was found to be significantly associated with ODSS in the PV group (CI 95%) 0.9813 to 1.113. (p=0.03). No associations were found in other groups, as well as no associations between ODSS with thyroid diseases in all groups**. Table 3.5**

The gingival involvement, presented with lesions and/or local inflammation on the gums and was higher in MMP and OLP subgroups than in PV group. **Table 3.4**

Table 3.4. Mucosal disease severity assessment. *ODSS- Oral disease severity score; Involvement sites indicates the number of participants who have lesions on different sites of the body. For MMP it usually involves oral and ocular involvement; in PV- oral and skin involvement, in OLP- oral, skin and genital involvement.*

MUCOSAL DISEASE SEVERITY ASSESSMENT	MMP (N=48)	PV (N=48)	OLP (N=50)	HC (N=40)
ODSS (Mean)	16.71±12.36	9.98±13.41	20.90±11.40	
ODSS (Median)	14 (0-58)	4.5. (0-64)	22 (0-39)	
Gingival involvement (n= yes/no)	40/8	18/30	39/11	
Ulcers (n= yes/no)	25/23	22/26	22/28	
Involvement sites				
Oral only	23	22	11	
Two sites	14	19	26	
Multi sites (<3)	11	7	13	

Table 3.5. The relationship between Type 2 Diabetes and ODSS. *Dependent variable: diabetes, Bivariate simple logistic regression analysis was used to examine the association between diabetes and ODSS in diseases groups.*

OLP	Coefficient (CI 95%)	P value	R squared
Intercept	0.03925 to 0.7605	0.00	
ODSS	0.9701 to 1.092	0.3675	0.014
MMP	**Coefficient (CI 95%)**	**P value**	R squared
Intercept	0.1270 to 1.196	0.00	
ODSS	0.9269 to 1.038	0.6026	0.006
PV	**Coefficient (CI 95%)**	**P value**	R squared
Intercept	0.007820 to 0.1636	0.00	
ODSS	1.001 to 1.127	**0.048***	0.137

Table 3.6. The relationship between Thyroid diseases and ODSS. *Dependent variable: Thyroid disease, Bivariate simple logistic regression analysis was used to examine the association between diabetes and ODSS in diseases groups.*

OLP	Coefficient (CI 95%)	P value	R squared
Intercept	0.01190 to 0.4948	0.00	
ODSS	0.9617 to 1.111	0.4204	0.012
MMP	**Coefficient (CI 95%)**	**P value**	R squared
Intercept	0.04034 to 0.5066	0.00	
ODSS	0.9704 to 1.082	0.3544	0.02
PV	**Coefficient (CI 95%)**	**P value**	R squared

Intercept	0.01123 to 0.2040	0.00	
ODSS	0.9578 to 1.091	0.3652	0.014

3.1.7 Periodontal status

The periodontal parameters PISA and bleeding score were significantly greater in all 3 diseases compared with healthy subjects at p value < 0.05. **Figure 3.5.** Furthermore, tooth loss was significantly greater in the MMP group only, with no significant difference in the number of teeth between the patient groups and the healthy control group.

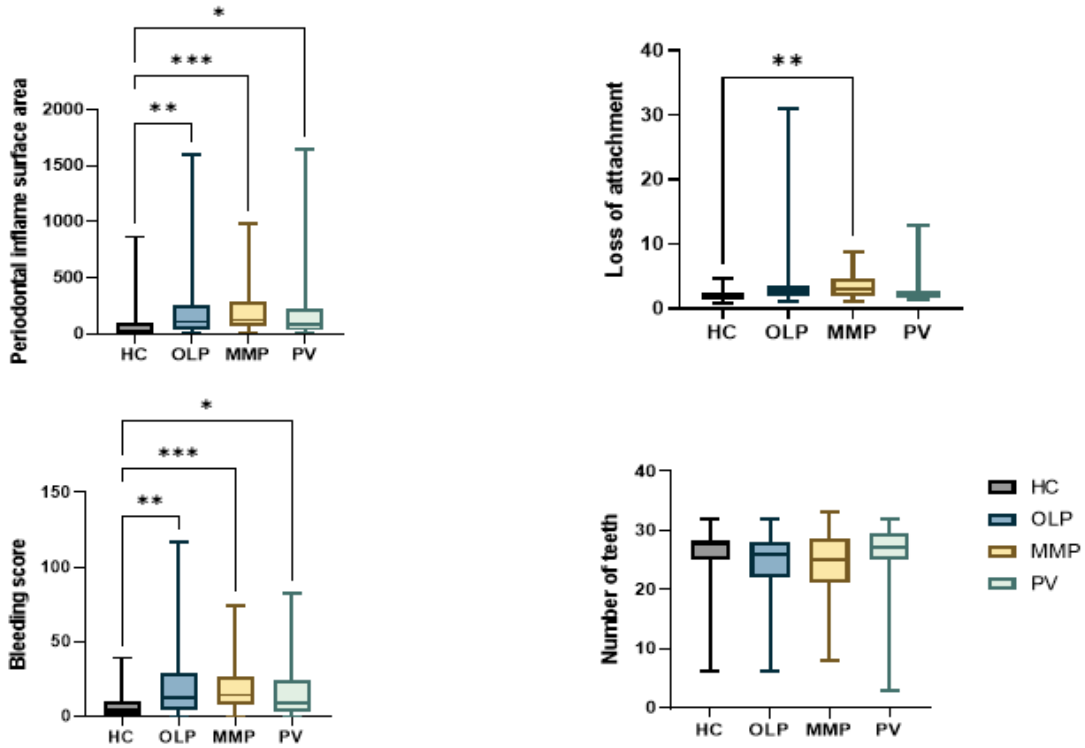


Figure 3.5. Comparison of periodontal status indexes in all 3 disease groups and healthy control group: a) periodontal inflame surface area (PISA); b) loss of tooth attachment; c) bleeding score; d) number of teeth. *Statistical analysis done using Kruskal-Wallis test. *(p<0.05); **(p<0.01); ***(p<0.001); ****(p<0.0001).*

3.1.8 Relationship of ODSS with periodontal status.

Spearman rank correlation showed significant correlation among clinical variables i.e between ODSS and PISA, as well as bleeding scores in OLP (p=0.036) and PV (p=0.038). The positive correlation was found also between ODSS and bleeding score in the same groups (p=0.015 in OLP and p=0.016 in PV).

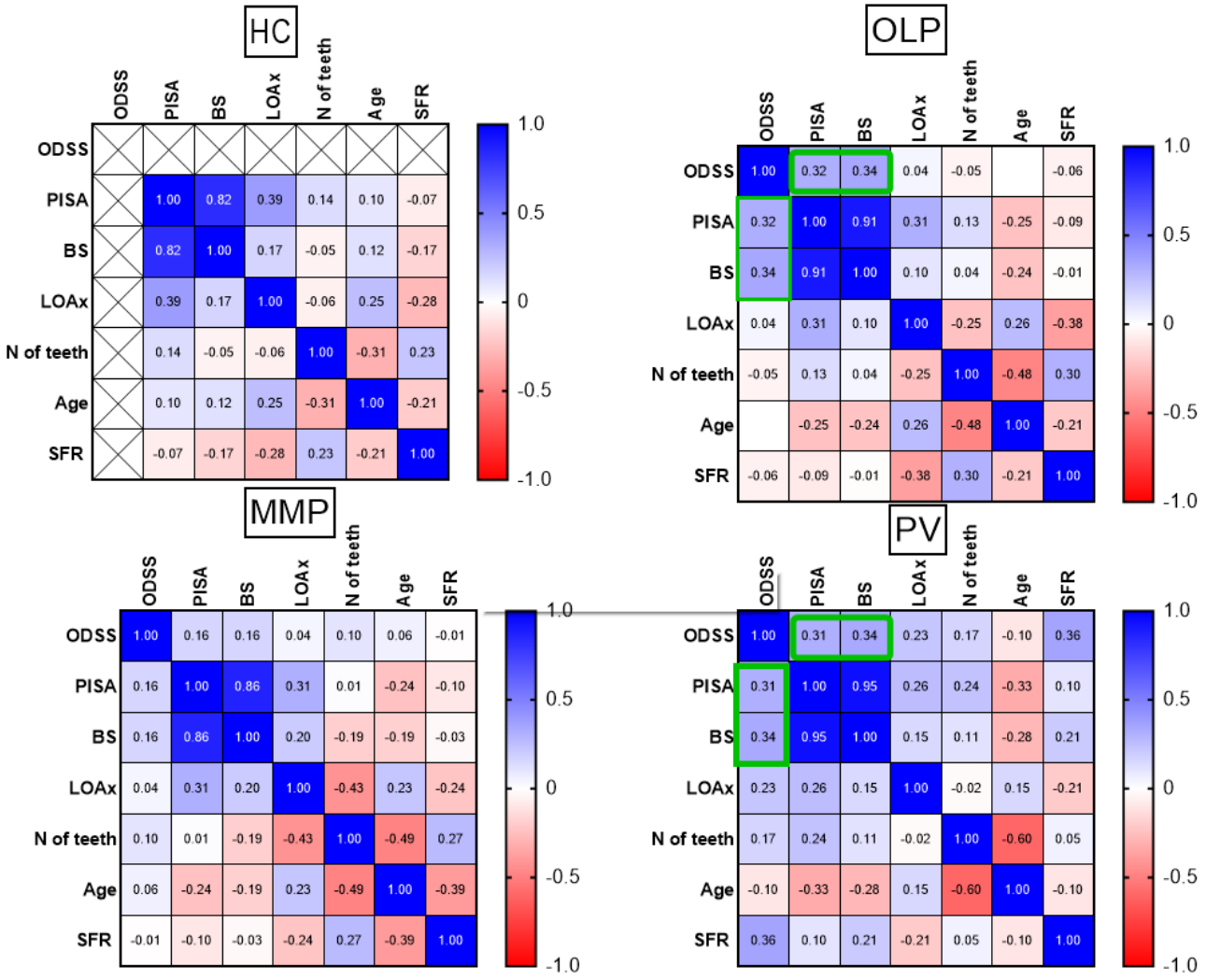


Figure 3.6. Spearman correlation matrix of clinical variables among groups. *The green square shows the positive significant correlation between variables. (p<0.05)*

To investigate whether AB treatment therapy type may influence the disease severity parameters, MMP groups was subdivided and analysed in three subgroups: 1) on topical/treatment naïve, 2) IS and 3) AB/AB+IS groups. A correlation matrix rank was compared in the 3 subgroups (**Figure 3.7).** The analysis showed that in the IS therapy subgroup there was significant correlation with disease severity. The group on AB showed a negative association i.e., reduced bleeding scores, PISA and loss of attachment.

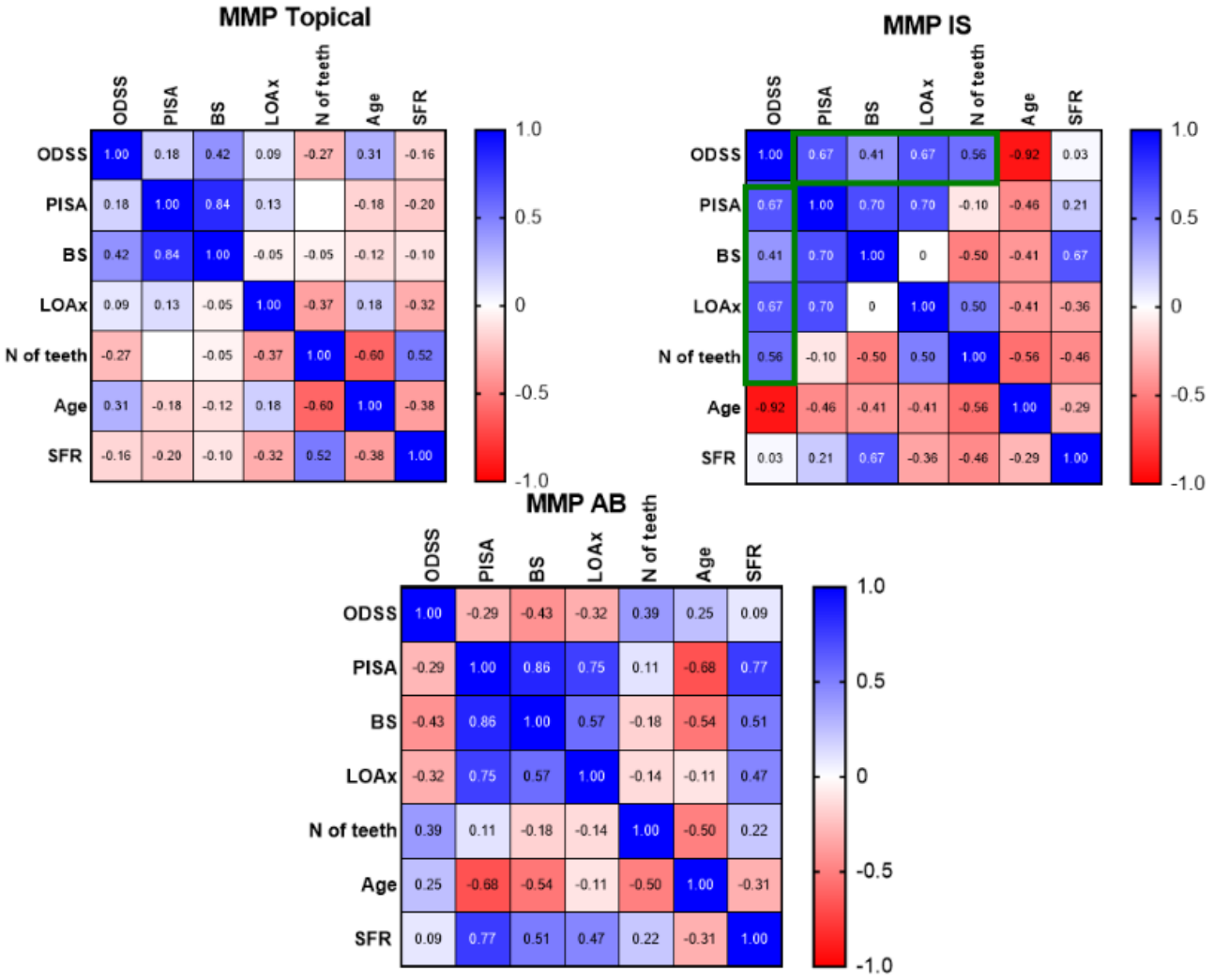


Figure 3.7. Correlation of the clinical parameters. *MMP Topical - in MMP group in patients on Topical; MMP IS- Immunosuppressants; MMP AB - AB/AB+IS treatment (AB- antibiotics). The significant positive correlations between ODSS and PISA were boxed in green. P>0.05. Topical (n=26), IS (n=5), AB/AB+IS (n=12)*

3.2 Discussion

This study aimed to describe the clinical profiles of OLP, MMP, and PV and to look for potential associations of each disease with age, sex, diet, BMI and comorbidities, therapies and then to relate these to disease severity score including PISA, bleeding, loss of attachment and ODSS scores and periodontal status.

Firstly, it was found that patients with MMP had the highest age among disease groups, with a mean age of 65.19 ± 14.41 years (Rauz et al., 2005), corresponding to the average age of patients with MMP previously described in the literature. Further, the women in the cohort were more predisposed than men, corresponding to the knowledge that autoimmune diseases occur more often in women than men. Overall, no association was identified between age and clinical severity scores in the three disease subgroups. The relationship between ageing and autoimmune diseases is complex and not fully understood. The ageing process affects immune system function and lead to the immune response changes, which is known as "inflammageing" (Franceschi et al., 2018). It can contribute to the development or exacerbation of autoimmune diseases. However, AIBD and OLP are complex autoimmune disorders that are influenced by multiple factors, such as genetic predisposition, environmental triggers, and immune system function (Kayani & Aslam, 2017; Van Der Waal, 2009). Therefore, the severity of these diseases may be influenced by a combination of factors beyond just age. Instead, other factors such as the duration of the disease, type of lesions, and treatment history may also play a role in determining disease severity (Nikitakis et al., 2017). Therefore, it is essential to consider multiple factors when assessing the relationship between ageing and autoimmune diseases, including the individual characteristics of the participants, that may influence disease severity.

Regarding ethnicity, while in MMP and OLP the larger proportion were white European, among the PV group the higher proportion were Asian patients. This contrasts with some studies that report that PV has a higher incidence among Ashkenazi Jews (Olbrich et al., 2019). Moreover, an endemic form of pemphigus – pemphigus foliaceus (PF) have been described in Brazil and Tunisia (Olbrich et al., 2019). Even though no endemic forms of PV, previous studies reported relatively higher frequency of PV in the Mediterranean population (Kyriakis et al.,

1989). For example, in Germany the incidence PV among southern Europeans living in Germany significantly higher compared to native Germans (Zillikens et al., 2002).

In our patient cohort, the most common comorbidities were diabetes and thyroid diseases. There was an association found between the PV group and the development of diabetes(p=0.048). Furthermore, bivariate logistic regression analysis T2D was found to be significantly associated with ODSS in the PV group (CI 95%) 0.9813 to 1.113. This might reflect the clinical need for prednisolone in patients with a more severe disease subsequently leading to diabetes. However, the primary type of diet in all these groups, including the healthy participants, was a Western mixed diet. This dietary pattern may account for the higher BMI values observed in all groups, including HC, with the highest BMI being in the OLP group, followed by PV, HC, and MMP. It's essential to note that a normal BMI typically falls within the range of 18.5 to 24.9 (Oliveros et al., 2014). Previous studies have also reported an increase in the incidence rates of inflammatory bowel disease, hypothyroidism, and type 1 diabetes mellitus, which were significantly higher among PV patients (Kridin, 2018). There was an increased number of patients in the MMP and OLP groups with thyroid disease, but this was not associated with ODSS and reflects only the higher risk of additional autoimmune disease in patients with one autoimmune condition.

It is recognised that normal salivary flow is important for maintaining a healthy periodontium and therefore alteration be reflected in periodontal status and potentially ODSS. There were no significant changes found in salivary flow rate in the groups when compared to HC. It is known that aging can affect the quality and composition of saliva, and volume of saliva produced (Percival et al., 1994). Ageing can influence the salivary flow rate, as the function of salivary glands can decline with age. However, it is crucial to recognize that a salivary flow rate within the reference range does not necessarily indicate the absence of an ageing effect. While a normal salivary flow rate within the normal range may indicate of good oral health, the quality and composition of saliva can still be affected by ageing (Humphrey & Williamson, 2001).

With regards to ODSS within the three disease subgroups, the highest mean score was found in the OLP group. Specifically gingival involvement (inflammation and gingival erosions/ulcers in diseases) was more frequent in MMP and OLP compared to PV. As mentioned, PV often requires immediate systemic immunosuppressive therapy to manage the autoimmune

response effectively. This systemic approach can lead to better control of the disease, potentially resulting in lower ODSS scores for PV patients. MMP and OLP being primarily treated with topical agents, which may not provide the same level of immune suppression. Several authors have suggested that mucosal diseases (Arduino et al., 2011; Azizi & Rezaee, 2012a; Giacometti et al., 2013; Ramón-Fluixá et al., 1999) may contribute to the development and progression of PD. The presence of persistent and painful oral lesions results in ineffective oral hygiene, leading to the buildup of plaque—a contributing factor to PD (Newman et al., 2019). OLP studies also have shown that periodontal health in gingival OLP is worse than in healthy controls (Azizi & Rezaee, 2012). Our results showed that periodontal parameters such as PISA score, which measures the amount of clinical gingival inflammation, erythema, and oedema (Ohlrich et al., 2010) and bleeding scores were significantly higher in all three disease groups than HC, with the highest scores in MMP and OLP, followed by PV. Loss of clinical attachment of teeth was significantly higher only in MMP group when compared to HC. However, Spearman correlation analysis between ODSS of diseases and periodontal parameters found significant correlation between PISA and bleeding scores in OLP ($p=0.027$) and PV ($p=0.037$) groups. The number of teeth and the attachment of teeth index exhibited a positive trend for association with ODSS only in the PV group. MMP did not show any association with PD, albeit MMP had the highest association with gingivitis. Our results are in concordance with other studies. Schellinck et al. and Tricamo et al. reported that patients diagnosed with MMP experienced a statistically significant increase in gingival inflammation compared with controls. However, all other periodontal parameters, including plaque index, bleeding index, tooth mobility, furcation involvement, loss of clinical attachment, recession, and periodontal status, did not change (Schellinck et al., 2009; Tricamo et al., 2006). Similarly, Lo Russo et al. did not find correlation between DG in OLP and MMP patients and their periodontal status (Lo Russo et al., 2010).

To investigate whether treatment might be related to disease severity outcomes / periodontal status, the MMP group was subdivided according to the treatment type. MMP on topical/treatment naive therapy showed similar results to the previous analysis with overall MMP. The IS group showed a significant correlation with all periodontal parameters. The data suggest that either periodontal disease is adversely affected by IS or that patients with the most severe gingival /periodontal disease are the ones requiring systemic

immunosuppression i.e., it is unclear what is cause and what is effect. The AB group showed a negative correlation with ODSS. This might suggest a beneficial effect of antibiotics on gingival health and indeed the majority of patients are prescribed this to treat and prevent gingival inflammation. However, it is difficult to trust the statistical validity of the results due to the lack of statistical power in each group. To date, the minimal sample size prevented from drawing a definitive conclusion. The results of our study are partly consistent with previous findings. The increase in gingival inflammation seen in patients with MMP and OLP, however, further research is needed to better understand the factors that contribute to the severity of PV and to identify effective treatments for the disease. (Lo Russo et al., 2010).

The strength of the study included collection of a wide range of data, including clinical profiles of patients with OLP, MMP and PV as well as factors such as age, sex, diet, BMI, comorbidities, therapies, and disease severity scores. Moreover, this study conducted analyses to explore relationships between oral disease severity score and various factors, such as age, periodontal parameters, and comorbidities. The statistical analyses conducted to explore relationships between oral disease severity scores and various factors add depth to the analysis by identifying potential connections.

However, the study acknowledges its limitations, including the sample size, which can affect the statistical power of the analysis (Charan & Saxena, 2012). Larger sample sizes would provide more robust findings and enable subgroup analyses. Furthermore, the cross-sectional design of the study offers only a snapshot of the patient cohort at a specific point in time.

Longitudinal data would be valuable for tracking changes in disease severity and understanding disease progression over time (Schellinck, 2009; J. Wang & Jia, 2016). Lastly, the study did not extensively address potential confounding factors that could influence disease severity. It is crucial to examine patients with nutrient deficiencies (iron, vitamin B12, folic acid) as they can lead to poor oral epithelial cell turnover (Challacombe, 1977). Additionally, the use of systemic immunosuppression in treating mucosal diseases should be considered as a confounding factor, affecting the disease activity, and pathophysiology. Considering and controlling for these factors, such as treatment duration and disease subtype, could strengthen the methodology.

In conclusion, this study sheds light on the relationship between periodontal disease and autoimmune diseases, highlighting a stronger association between PV and PD than with MMP and OLP. However, due to the paucity of available literature, the cross-sectional design, limited sample sizes in the disease groups, and confounding factors associated with different therapeutic interventions, drawing definitive conclusions regarding the relationships between these diseases and periodontal status remains challenging. Additionally, there is a lack of high-resolution microbiological studies in the published literature showing the relationship between periopathogens with mucosal diseases. I will attempt to address this in **Chapter 6.**

Chapter IV. Immunological profile in OLP, MMP and PV

4.1 Cytokines and matrix metalloproteinase response in saliva and serum

AIBD and OLP are two different types of autoimmune diseases with different pathomechanisms. The T cell-mediated autoimmune disease seen in OLP is caused by the activation of T cells that attack healthy cells and tissues in the body (Nayee et al., 2021). On the other hand, B-cell-mediated autoimmune diseases such as PV and MMP are caused by the production of autoantibodies by B cells, which also attack healthy cells and tissues in the body. This occurs when B cells produce antibodies that target self-antigens or proteins located in the intraepidermal/intraepithelial layer in PV and in the subepidermal/subepithelial layer of the skin or mucosa in MMP (Hammers & Stanley, 2016).

To gain insights into the complex immune responses and inflammatory processes underlying AIBD and OLP, potentially identifying key factors that contribute to disease pathogenesis or serve as potential therapeutic targets. Th2 cell cytokines are crucial in enhancing humoral immunity, through Th2 cells, which secrete cytokines like IL-4, and IL-13 (Buonavoglia et al., 2019b). Th2 cells are known to be primarily involved in defence against extracellular parasites and allergens. There is strong evidence indicating that AIBD are predominantly influenced by Th2-mediated processes, with an elevation in Th2 cell cytokines. Recently, attention has shifted to exploring the involvement of the Th17 subset in autoimmune conditions, and existing evidence indicates that this inflammatory pathway might also contribute to the pathogenesis of AIBD and OLP. Cytokines from Th17 cells promote the recruitment and activation of neutrophils in inflamed tissues, critical for the immune response against extracellular pathogens such as bacteria and fungi (Betelli et al., 2008). Th17 cells play a role in autoimmune diseases and chronic inflammation. Meanwhile, matrix metalloproteinases (MMPs) are a family of enzymes that play a critical role in the breakdown and remodelling of extracellular matrix components (EMC) such as collagen, elastin, and laminin. However, they can also contribute to pathological conditions, including autoimmune diseases. It is known that MMPs are involved in losing tolerance to self-antigens and developing chronic inflammation (Ram et al., 2006). MMPs are also involved in the activation and recruitment of immune cells, including T and B cells, macrophages, and dendritic cells, to the site of inflammation. Lastly, MMPs contribute to the destructive process by destroying extracellular

matrix proteins in target tissues, including joints, skin, and blood vessels, resulting in tissue damage (Stamenkovich et al., 2003).

This study assessed the levels of selected inflammatory cytokines and matrixins in saliva and serum samples. Inflammatory marker levels including levels of IL-6, TNF-α, IL-13, IL-1β, IL-4, IL-8, IL-17A, IL-23, and matrix metalloproteinase, including MMP-3, MMP-9 and MMP-13 were examined by multiplex immunoassay and MMP-8 was performed with ELISA assays. Cytokine levels in disease groups were compared with HC. In addition, correlations between selected inflammatory markers, ODSS, and periodontal scores were assessed.

This study analysed 169 saliva and 181 serum samples to investigate an inflammatory response in these autoimmune conditions compared to HC (HC = 35, OLP = 46, MMP = 44, PV = 44 in saliva, and HC = 38, OLP = 47, MMP = 48, PV =48 in serum). Discrepancies between the number of serum and saliva samples arose from the insufficient availability of saliva samples for analysis, as well as challenges related to poor vein accessibility or patient discomfort during venipuncture for serum.

4.1.1 Inflammatory response

There were no significant differences observed for local circulating levels of pro-inflammatory cytokines TNF- α, IL-1 β and IL-8 between all groups of disease and HC **Figure 4.1**. There was a trend for higher levels of TNF- α in all three disease groups. The higher levels of salivary IL-8 (p=0.7) when compared to HC. Additionally, no significant changes were found in serum samples in all disease groups when compared to HC.

Analysis for Th-2 cell cytokines showed significant elevation of levels of IL-13 in MMP and PV groups in both saliva and serum in comparison to HC; in MMP (p=0.04 in saliva, p=0.006 in serum) and PV (p=0.01 in saliva, p=0.025 in serum). IL-4 was raised in serum in PV compared to OLP patients (p=0.001).

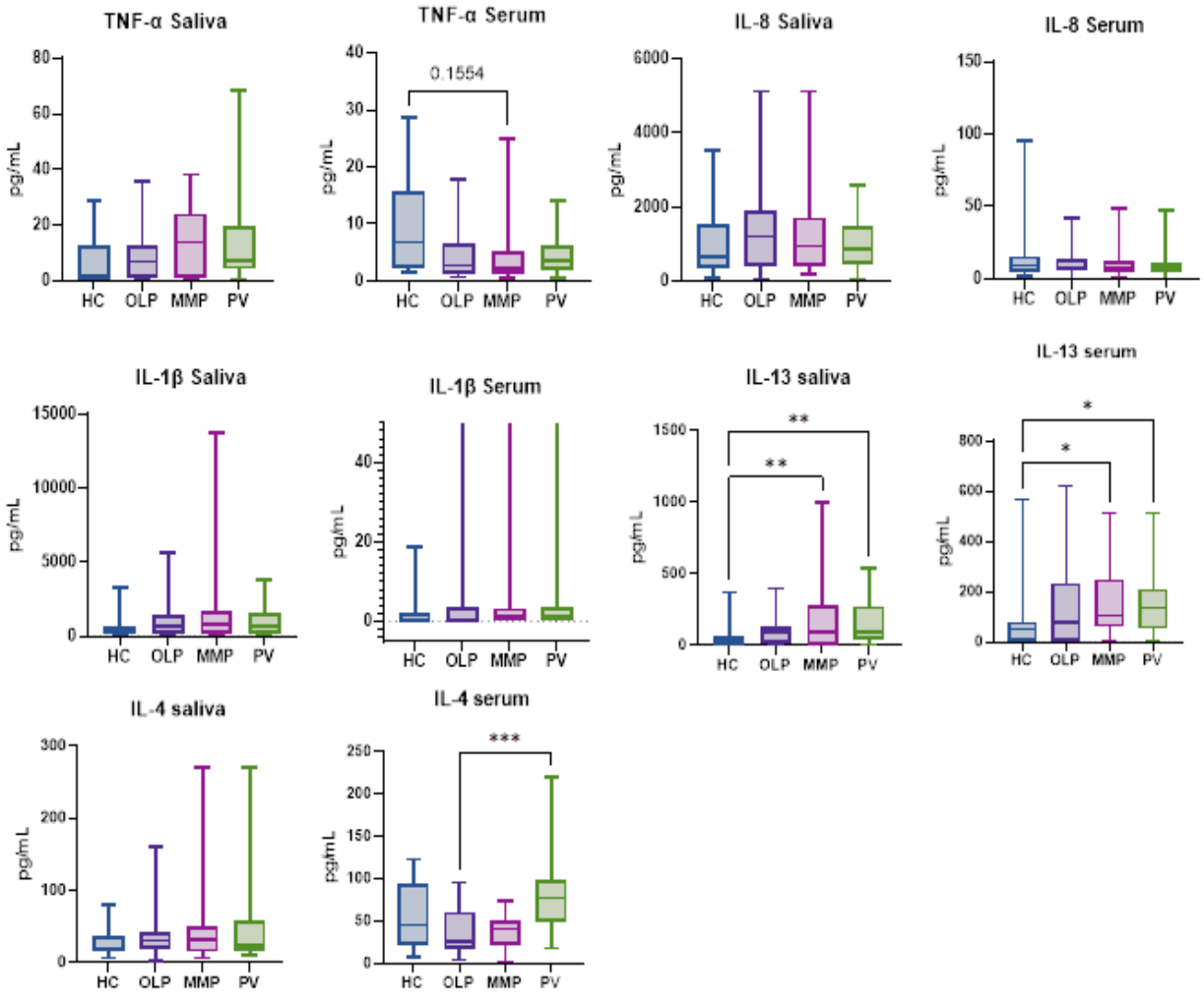


Figure 4.1 Comparison of the proportion of detectable pro-inflammatory cytokines levels between patient groups and HC in saliva and serum. *Kruskal-Wallis test, with Dunn's post-hoc multiple comparison test has been applied. *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant.*

The levels of IL-17A in saliva were significantly elevated in PV and MMP groups (p=0.001 in MMP; p=0.028 in PV) compared with HC. Moreover, the levels of IL-6 in saliva were significantly higher in all 3 groups of diseases compared to HC. (p=0.03, in OLP; p=0.002 in MMP p=0.02 s; p=0.08 in PV) **Figure 4.2.** The levels of IL-6 in serum were elevated significantly only in OLP group (p=0.02).

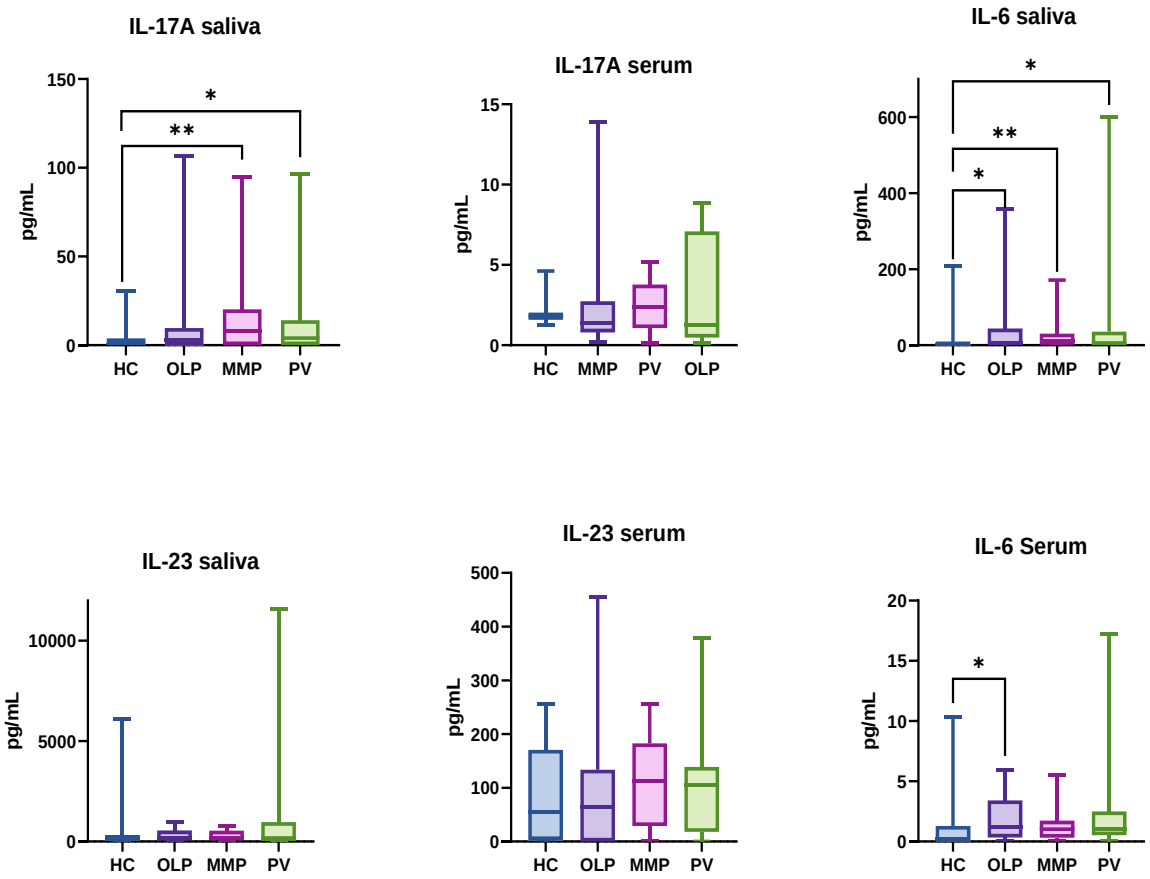


Figure 4.2 Comparison of the proportion of detectable Th-17 cells cytokine and matrix metalloproteinase levels between patient groups and HC in saliva and serum. *Kruskal-Wallis test, with Dunn's post-hoc multiple comparison test has been applied. *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant.*

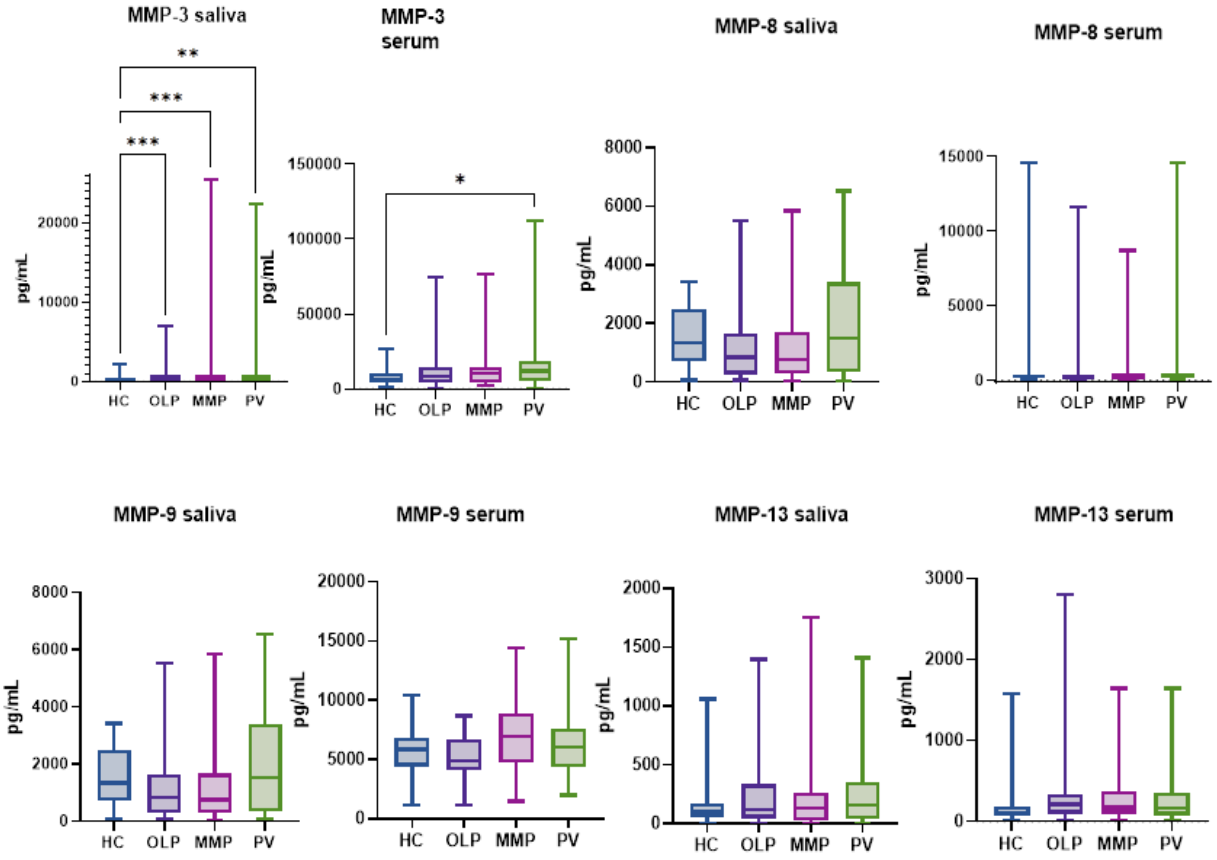


Figure 4.3 Comparison of the proportion of detectable matrixins levels between patient groups and HC in saliva and serum. *Kruskal-Wallis test, with Dunn's post-hoc multiple comparison test has been applied. *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant.*

Among matrixins, salivary MMP-3 was elevated in all three groups of disease compared to HC (p=0.0002 in OLP; p=0.0001 in MMP; p=0.002 in PV). Moreover, MMP-3 in serum showed the significant increase only in the PV group (p=0.004). **Figure 4.3.**

4.2 Correlation of the inflammatory response with the clinical parameters

The relationship between clinical profile and selected cytokines and matrixins was assessed using Spearman rank correlation coefficient analysis. In the OLP group, the significant positive correlation was found between ODSS and both salivary IL-6 (p=0.002) and IL-13 (p=0.041) (**Figure 4.4).** No association was found between cytokine or matrixins response and periodontal disease scores. Moreover, no significant correlations of inflammatory response

with age in this group.

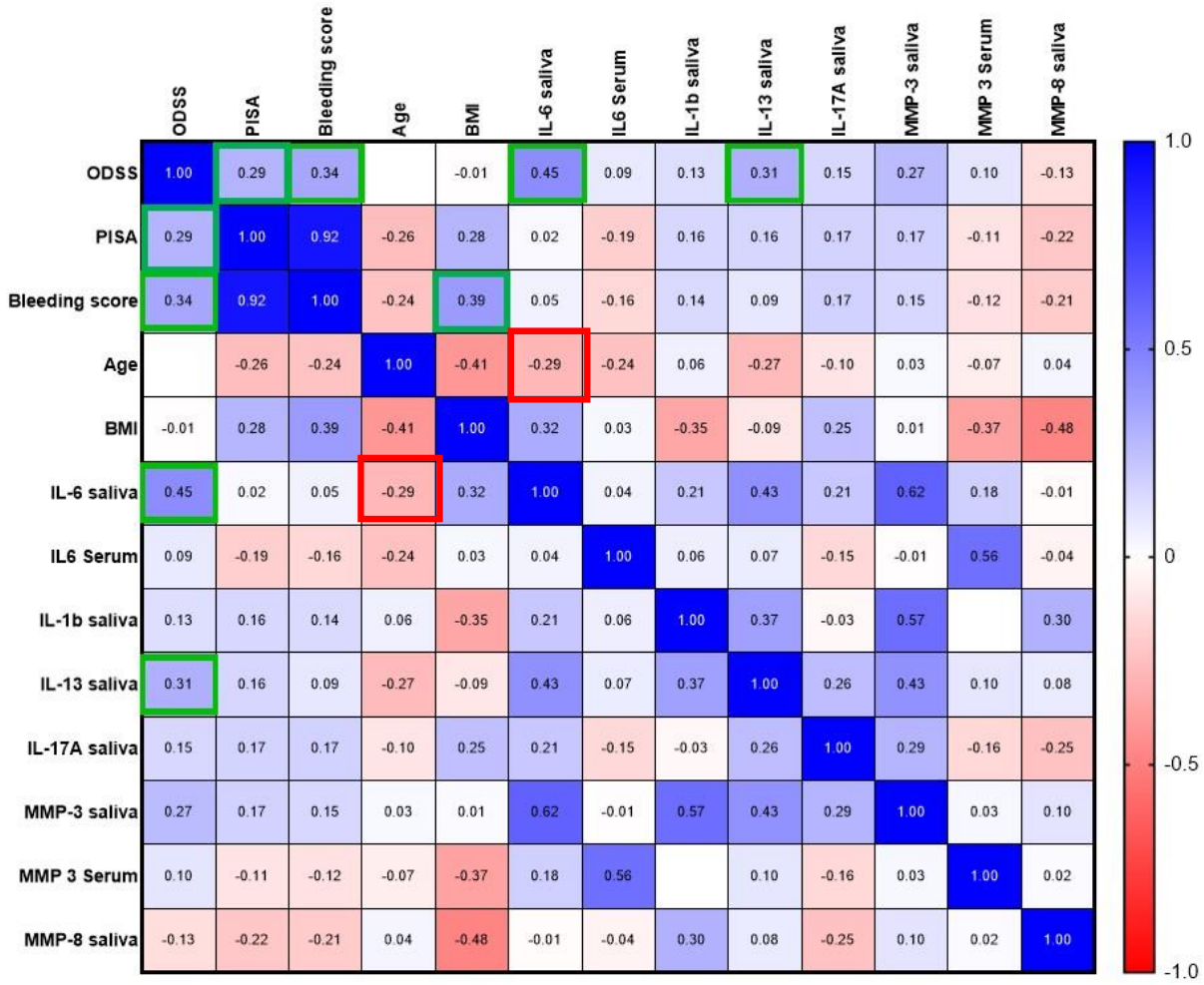


Figure 4.4 Correlation of the selected salivary and serum cytokines and matrixins with clinical parameters in OLP group, Spearman correlation analysis was performed. *The numbers represent the Spearman r values. The boxes pointed out in green boxes represent the significant positive correlations (p<0.05).*

In MMP, ODSS showed positive correlation with salivary IL-6 (p=0.01), salivary MMP-3 (p=0.001) and serum MMP-3 (p=0.048) levels. PISA score showed significant positive associations with IL-1 β (p=0.01), IL-17A (p=0.043), IL-13 (p=0.031) and MMP-3 (p=0.020) only in saliva samples. Age showed inverse correlation with salivary IL-6 (p=0.05) **Figure 4.5.**

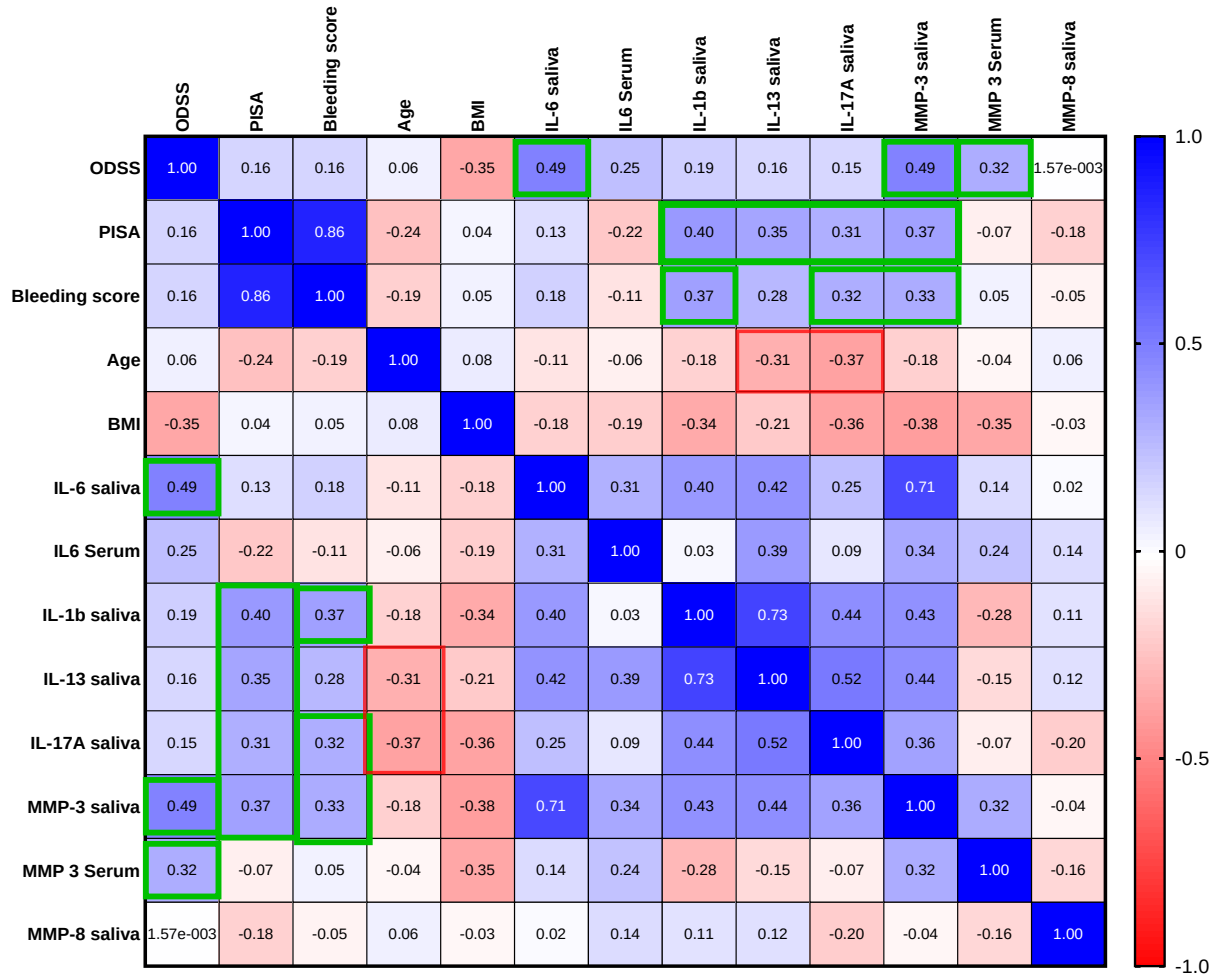


Figure 4.5 Correlation of the selected salivary and serum cytokines and matrixins with clinical parameters in MMP, Spearman correlation analysis was performed. The numbers represent the Spearman r values. *The boxes pointed out in green boxes represent the significant positive correlations (p<0.05), the boxes in red are significant negative.*

The correlation analysis of immunological variables was undertaken for the MMP subgroups on AB and without **Figure 4.6.** The results were similar to the overall MMP group analysis with the significant correlation of ODSS with salivary IL-6 (p=0.05) and MMP-3 (0.007). ODSS was significantly associated only with levels of salivary MMP-8 (p=0.025). Age was correlated negatively with salivary IL-13 and IL-17A (p=0.044 and p=0.014).

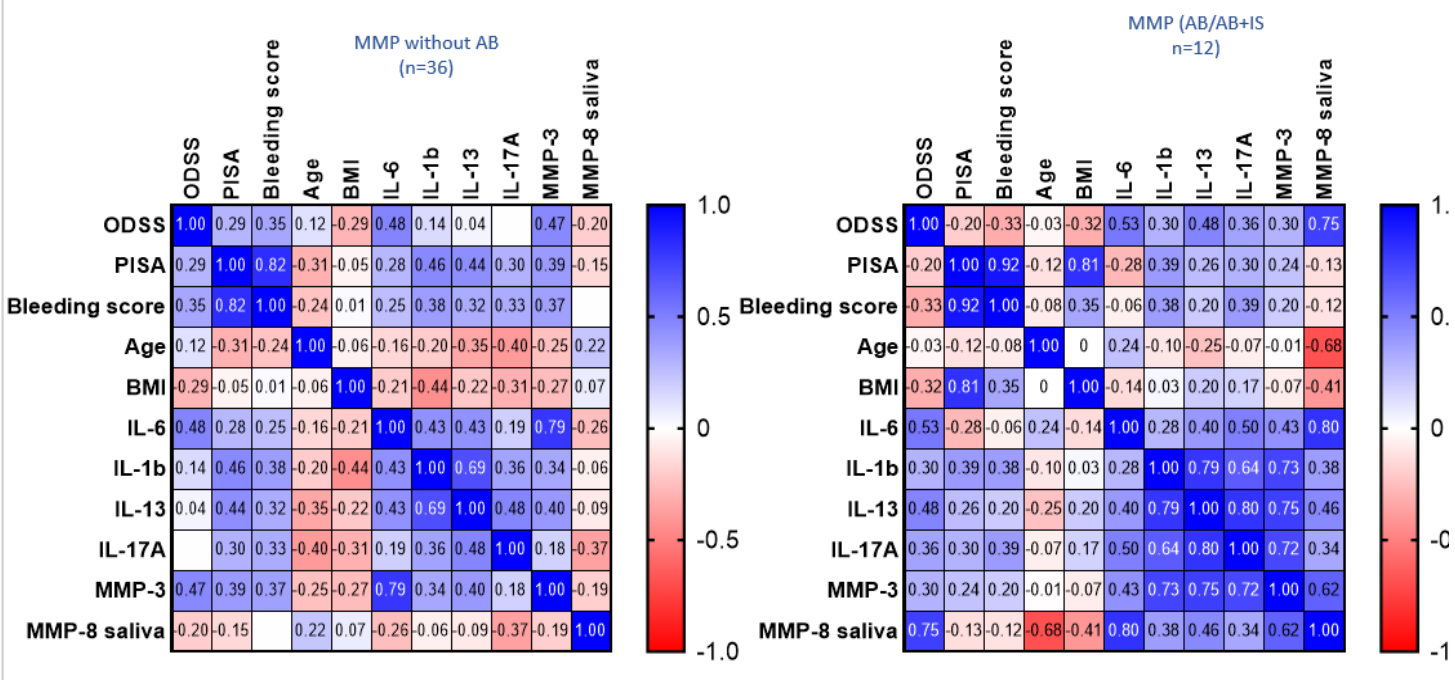


Figure 4.6 Correlation of the selected salivary and serum cytokines and matrixins with clinical parameters in MMP on AB treatment and without. *Spearman correlation analysis was performed. The numbers represent the Spearman r values. (p<0.05).*

Finally, in the PV group we found an abundance of associations. A higher ODSS was associated with elevation of salivary IL-6, IL-1β and MMP-3 (p=0.0001, p=0.023, p=0.0001, respectively). PISA score was associated only with salivary MMP-3 (p=0.044). BMI was found significantly associated with the levels of salivary and serum IL-6 (p=0.01; p=0.013). **Figure 4.7**

4.3 Discussion

In this study, immunological responses were observed in two different groups of immune-mediated diseases.

Cytokines play a crucial role in the immune system as they act as mediators and regulators of immune and inflammatory responses. While the precise causes of AIBD and OLP remain elusive, there is a consensus that an imbalance of selected pro-inflammatory, including Th2 and Th17 cells plays a significant role in the pathogenesis of these conditions (Alrashdan et al., 2016; Kourosh & Yancey, 2011; Porro et al., 2019).

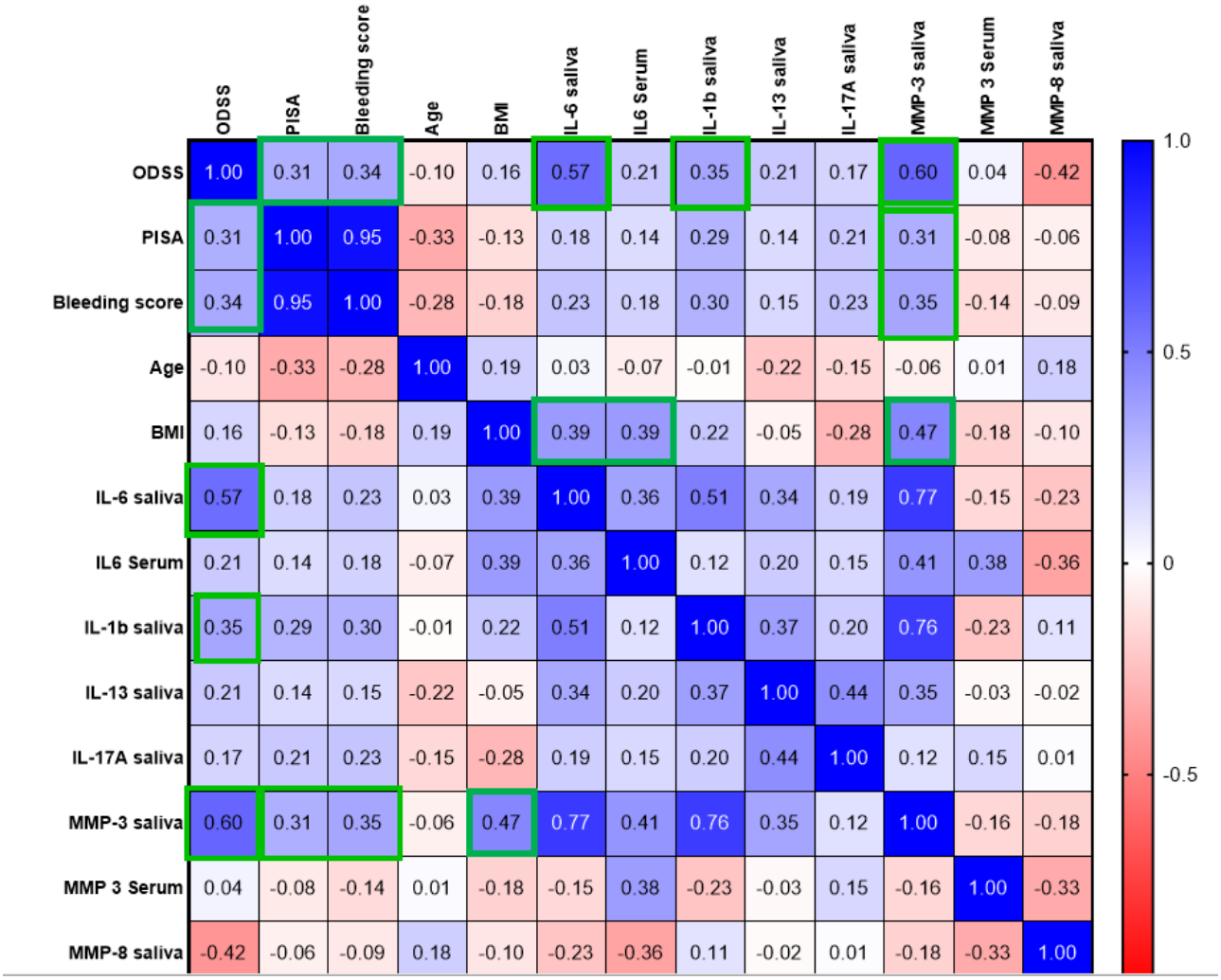


Figure 4.7 Correlation of the selected salivary and serum cytokines and matrixins with clinical parameters in PV, *Spearman correlation analysis was performed. The numbers represent the Spearman r values. The boxes pointed out in green boxes represent the significant correlations (p<0.05).*

In the context of OLP pathogenesis, some studies have proposed a Th1 cytokine bias in OLP, citing the expression of Th1 cytokines such as IFN-γ, and pro-inflammatory TNF-α within the lymphocytic infiltrate of OLP lesions (Carvalho et al., 2019; Zhu et al., 2022). Previous studies have identified IL-1β-expressing cells, particularly in the monocyte/macrophage subset, within these lesions (Yamomoto et al, 1994). Importantly, IL-1β is known to exert its pro-inflammatory effects by stimulating the production of other cytokines and chemokines, thereby amplifying the immune response (Lu, Zhang et al., 2015a). Conversely, other research has detected concurrent expression of both Th1 and Th2 cytokines in OLP lesions and tissue transudates (Rhodus, Cheng, & Ondrey, 2007; Y. Wang, Zhou, Fu, Wang, & Zhou, 2015). Additionally, certain investigators have reported elevated levels of IL-4 cytokine in the saliva

and serum of OLP patients (W. Z. Liu et al., 2013; Malekzadeh, Robati, Yousefimanesh, Boroujerdnia, & Nadripour, 2015). Notably, the study conducted by Zhang et al. indicated that disease-related cytokine production might be more sensitively reflected in saliva compared to serum (Zhang, Yuanyuan, et al., 2008). Our findings align with the latter observations, as we detected inflammatory cytokine expression primarily in saliva samples rather than in serum. The reason may be that saliva is in direct contact with the affected tissue, and changes in salivary biomarkers can occur more rapidly and consequently may provide information about the oral disease status (Martina et al., 2020; Streckfus, 2015). Specifically, I observed a trend towards higher levels of TNF-α and IL-8 cytokines in saliva of OLP though this did not reach significance compared with HC.

Liu et al. demonstrated that IL-4 levels were significantly elevated in OLP patients with more severe disease (W. Z. Liu et al., 2013). In our study IL-13 in saliva showed a positive correlation with a severity of OLP, which suggests that Th2 cells, which are known to produce IL-13 among other cytokines, may have a role in driving the immune response and inflammation associated with OLP. Th2 cells are involved in regulating immune responses, and their presence in OLP lesions may modulate the local immune environment (Lu, Zhang et al., 2015b). This could potentially influence the balance between pro-inflammatory and anti-inflammatory responses in the oral mucosa, contributing to the chronic inflammation observed in OLP. However, potential explanations for discrepancies with previous studies can arise from the disease heterogeneity, which could mask significant differences in cytokine levels. Moreover, smaller sample sizes can limit the statistical power of the study. Finally, cytokine levels can fluctuate over time, especially in response to disease activity or treatment. The timing of sample collection relative to disease progression or treatment initiation can influence cytokine measurements.

There was the significant increase of IL-13 in saliva and serum in the PV and MMP groups, which is consistent with other studies. Earlier studies have shown that IL-13 is involved in pathogenesis of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, systemic scleroderma, ulcerative colitis, type 1 diabetes, and Sjogren's syndrome by regulating several T-helper (Th) cell subtypes (Mao et al., 2019). IL-13 has been found to be upregulated in the serum and saliva of PV patients. This cytokine was implicated in keratinocyte apoptosis and activation of B cells to produce autoantibodies (Cocks et al., 1993).

Increased IL-13 levels may contribute to the tissue damage seen in the oral mucosa, leading to the characteristic lesions associated with these diseases.

Levels of IL-6 were elevated in saliva and serum of OLP group, and only in saliva of MMP and PV patients. Salivary IL-6 was associated with oral disease severity in all three groups of diseases IL-6 exhibits a wide range of biological functions, influencing immune regulation, inflammation, haematopoiesis, and oncogenesis. It is known that IL-6 is produced by various immune and non-immune cells, such as T and B cells, keratinocytes, and eosinophils (Choy & Rose-John, 2017). One of the most important functions of IL-6 is activating Th17 cells (Kimura & Kishimoto, 2010), influencing neutrophil recruitment and is critical in transitioning from acute to chronic inflammation (Gabay, 2006). The increase in IL-6 has been described in many AIBD and OLP (Kasperkiewicz et al., 2017; Lee et al., 2017; Lu et al., 2015). Additionally, in OLP the role of IL-6 has been associated with malignant transformation of OLP into oral cancer by the immune cell-mediated pathways, such as cytokine signalling (Giannetti et al., 2018). In particular, the cytokine IL-6 has been shown to promote the growth and survival of cancer cells, and elevated levels of this cytokine have been found in both OLP and OSCC. Initially IL-6 was recognized for its role in promoting plasma cell differentiation and B cell antibody production. Additionally, IL-6 is essential for the differentiation of Th17 cells from naïve CD4+ T cells and can overcome Treg-mediated immune suppression (Dienz et al., 2009). These multifaceted functions highlight a pivotal role for IL-6 in shifting the immune response from a state of tolerance to active inflammation.

There were also elevated levels of IL-17 A in saliva samples of PV and MMP patients. IL-17 A, as a cytokine of Th17 cells, plays an essential role in protection against extracellular bacteria and fungi, inflammation, and the development of autoimmune diseases (Harrington et al, 2005). IL-17 elicits the production of chemokines, which in turn mobilizes neutrophils and macrophages to eliminate invading pathogens. Additionally, it enhances the production of antimicrobial peptides, contributing to the immune response against these pathogens. Moreover, IL-17 has pleiotropic effects on various tissue and immune cells. It stimulates the generation of a multitude of inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8 and MMPs in cell monocytes, epithelial cells, keratinocytes, and fibroblasts (Onishi et al., 2010). Consequently, IL-17, along with its downstream molecules, actively contributes to the creation and perpetuation of a local inflammatory microenvironment.

IL-17A has been implicated in the development of autoimmune diseases. It can promote the production of autoantibodies that target specific proteins or tissues in the body. In the context of PV, these autoantibodies may target desmosomal proteins like desmogleins, which are essential for cell adhesion in the epidermis (Timoteo et al., 2017). The presence of elevated IL-17A levels in the saliva of PV and MMP patients suggests that there may be a link between the immune response, bacterial activity, and the development of these diseases. It's possible that dysregulation of the Th17 cell pathway and excessive IL-17A production could contribute to the autoimmune response, observed in these conditions.

Lastly, matrixins have been known playing a critical role in the pathomechanisms of AIBD and OLP (Farzin et al., 2012;). Niimi et al. described the role of MMP-9 and MMP-13 in the pathogenesis of bullous pemphigoid by degrading type IV collagen, which is the major constitutive component of the basement membrane zone (Niimi, 2004). Cirillo et al. suggested that MMP-9 activity is necessary for blister formation in experimental models of PV and BP (Cirillo & Prime, 2021). However, in our study, MMP-9 did not significantly differ across the disease groups. Instead, the results showed that salivary MMP-3 levels were significantly elevated in all three diseases (and in serum in PV). The literature describes that MMP-3 plays a role in degrading ECM substrates, including proteoglycans, fibronectin, laminin, and certain types of collagens. MMP-3 has been shown to contribute to the degradation of the basement membrane and extracellular matrix components in OLP (S. Sun et al., 2014). Kim et al. identified increased expressions of MMP-3 in the epithelium of OLP, particularly in cases with erosive changes, suggesting its involvement in the destructive process of the basal epithelium membrane (Kim et al., 2006). These findings align with Mazzarella et al., who observed higher levels of MMP-3 mRNA in erosive OLP compared to the reticular form (Mazarella et al., 2006). The prolonged presence of elevated MMP-3 in the serum may contribute to the malignant transformation of OLP lesions, as MMP-3 is associated with oncogenesis and is expressed in OSCC (Kusukawa et al., 2006).

Similarly, in PV and MMP, MMP-3 may play a role in the degradation of the basement membrane that separates the epidermis from the dermis. The degradation of this barrier leads to the formation of blisters and erosions on the skin. Discrepancies in our study's

findings regarding matrixins specifically the elevation of salivary MMP-3 but with no significant difference in MMP-9 levels in diseases groups compared to healthy controls, in contrast to to previous studies, may be attributed to the limited number of papers published on the role of matrixins in AIBD. To address these discrepancies, a larger study with a more diverse patient cohort, but stratifying patients based on disease subtype and severity may be informative.

The next aim was to investigate the relationship between AIBD, OLP and PD. Auto-reactive T cells, natural killer cells, autoantibodies, and genetic factors are also essential in the immune component of PD (Obando-Pereda, 2018), accompanied by a production of inflammatory cytokines, chemokines and MMPs, which participate in morphogenesis, physiological tissue turnover, and pathological tissue destruction in PD (Bunte & Beikler, 2019). The cytokines IL-17, IL-6, IL-1, TNF-α, macrophage colony-stimulating factor, and prostaglandin E 2 triggering osteoclast activation cause tissue destruction and bone resorption in PD (Ramadan et al., 2020). Our study showed no positive correlation between periodontal scores and inflammatory markers in OLP. However, in the MMP group I observed significant positive correlations between PISA and the levels of salivary IL-1β, IL-13, IL-17A, and MMP-3. These findings suggest a potential association between these salivary biomarkers and periodontal inflammation in the context of MMP. However, it's important to note that correlation does not necessarily imply causation, and further research is needed to elucidate the complex interplay between these biomarkers and the development or progression of periodontal diseases in MMP patients.

In the PV group, only MMP-3 in saliva correlated with the periodontal score. The different and unique profile of correlations between inflammatory response and periodontal scores in all these three diseases suggest that the pathogenesis of periodontal diseases with oral autoimmune diseases may not be straightforward and requires a more nuanced understanding of the underlying mechanisms.

In this study I also looked for age related changes such as low-grade inflammation or inflammageing (Kirkwood, 2018). Ageing-associated changes in the immune system include senescence of immune cells represented by the slower immune cell division and proliferation, absence of more robust response to the antigens and production of pro-inflammatory cytokines, I.e., IL-6, TNF-α, IL-1β, IL-8 and IFN-y (Fransen et al., 2017). Additionally, increasing

and accumulating damage-associated molecules (DAMPs) can also activate immune cells and promote chronic inflammation (Rodrigues et al., 2021). Pathogen-associated molecular patterns (PAMPs), conversely, are molecules associated with pathogens and can activate the immune system to fight off infections. With age, the function of immune cells that respond to DAMPs and PAMPs can become impaired, leading to reduced immune responses to infections (Calder et al., 2017). In addition, dysbiosis of the gut microbiome can lead to increased levels of pathogenic bacteria that release PAMPs, leading to chronic inflammation and age-related diseases. All these factors lead to immune system impairment, making the elderly more susceptible to infections and developing age-related diseases (Franceschi et al., 2018). In this study, there was no positive correlation between age with cytokines in OLP and PV groups. Conversely, in MMP, a significant inverse correlation was observed between age and salivary IL-17 and IL-13 cytokines and an inverse correlation of salivary IL-6 levels and age in OLP. The descriptive analysis showed an increase of IL-6 in saliva in all three groups of diseases, IL-13 and IL-17 increase in MMP and PV groups. All these cytokines have been suggested to play a role in Inflammaging (J. Sun et al., 2015). This chronic inflammation can contribute to the development of various diseases, including autoimmune diseases.

Finally, some of the specific immunological changes noted might be useful biomarkers to monitor disease progression and treatment response. From a clinical point, the correlation of elevated biomarkers salivary IL-6 and MMP-3 with ODSS in all three diseases implies their potential utility as diagnostic, prognostic and treatment tools. According to Kobayashi et al., employing IL-6 receptor inhibition therapy, such as tocilizumab, with IL-6 inhibitors, could help managing and suppressing rheumatoid arthritis activity, and benefit periodontal health by improving the clinical condition of the periodontium (Kobayashi et al, 2014).

Additionally, non-invasive approach through saliva-based assays offers convenience and reduced patient discomfort compared to traditional diagnostic methods. The quantification of IL-6 and MMP-3 levels may offer insights into disease severity and prognosis. Monitoring these cytokines during the course of treatment can help assess therapeutic response and guide adjustments in management strategies.

In this study Spearman correlation analysis was used, over linear regression for its suitability to our dataset, characterized by non-normal distribution and susceptibility to outliers. Non-parametric nature of this test aligns with our exploratory focus on understanding variable relationships, prioritizing strength and direction over predicting future outcomes. However,

for future research, using a regression model, which is powerful for understanding the quantitative impact of one variable on another, allowing for prediction and assessing causality (Lindley, 1990). Limitation of this analysis is that it can be sensitive to outliers. Furthermore, longitudinal studies may shed light on how inflammation evolves in relation to disease progression. Expanding biomarker profiling beyond pro-inflammatory cytokines is crucial to identifying additional factors contributing to these diseases' pathogenesis. Investigating treatment response can further evaluate the impact of various therapies on disease outcomes, while patient stratification based on disease characteristics will help to understand the study heterogeneity.

In conclusion, in our study, salivary IL-6 and MMP-3 showed a significant difference in all groups of diseases, which correlates with ODSS in these diseases. Clinically, the identification of IL-6 and MMP-3 as significant markers in AIBD and OLP holds diagnostic and prognostic relevance. Elevated levels of these cytokines can serve as diagnostic biomarkers, guiding clinicians toward appropriate therapeutic interventions.

Chapter V. Results: Metabolomic profile in OLP, MMP and PV.

Bacterial metabolites are small molecules produced by various bacterial species in the microbiota (Takeda et al., 2009). These molecules can interact with the host immune system and play a crucial role in maintaining immune homeostasis. Emerging evidence suggests that metabolite imbalance or overproduction can lead to autoimmune diseases by promoting inflammation or altering immune cell function (Kang et al., 2015). Therefore, if bacterial metabolites play a role in maintaining immune homeostasis, their imbalance can contribute to developing autoimmune diseases.

To date, short-chain fatty acids (SCFAs) are among most investigated metabolites. They are produced by gut bacteria fermentation of dietary fibre (Mcdermott & Huffnagle, 2014). SCFAs are essential in regulating the host immune system by promoting the differentiation of regulatory T cells, which control inflammation and immune responses (Corrêa et al., 2017b). SCFAs can also suppress the production of pro-inflammatory cytokines. Metabolomic pathways may also play a critical role in metabolic reprogramming, which is the reconfiguration of metabolic networks in response to disease (Tan et al., 2014). For example, in cancer, metabolic reprogramming can lead to changes in glucose, amino acid, and lipid metabolism to provide energy and building blocks for tumour growth (O'Keefe, 2016).

For this study, the untargeted 1 H-nuclear magnetic resonance (1H-NMR) technique was used to detect and quantify salivary and plasma metabolites. This technique helps to identify and quantify the metabolites present in biological fluids and can provide insight into the metabolic pathways involved in developing the diseases (Schripsema, 2010). 1H-NMR technique is based on the on the magnetic characteristics of metabolites, resulting in the generation of spectra (peaks) that contribute to the determination of the metabolite's structure and, consequently, its identity (Holmes et al., 2006).The advantage of NMR metabolomics at this stage is that it does not require prior knowledge of the metabolites present in the sample, which helps to discover new biomarkers or metabolic pathways in exploratory studies, providing a broad overview of the metabolites present in the sample (Gardner et al., 2020).

Therefore, the aims of the study were to describe the metabolic profile associated with MMP, PV and OLP in saliva and plasma. Thus, 181 plasma samples were divided into: OLP (n=47),

MMP (n=48), PV (n=48) and HC (n=38), and 170 saliva samples were divided into: OLP (n= 42), MMP (n = 44), PV (n = 45), and HC (n = 39). Discrepancies of smaller number of saliva samples were associated with insufficient amount of the sample for NMR analysis, while the reduced number of plasma samples resulted from challenges in blood collection, resulted from poor vein accessibility or patient discomfort during venipuncture.

Along with conventional statistical tests, including Shapiro-Wilk's test for normality and if normally distributed, parametric tests (one-way ANOVA with multiple comparisons) or, non-parametric tests (Kruskal Wallis with multiple comparisons) were used. The Benjamini-Hochberg method for multiple hypothesis correction was used with the false discovery rate set to 0.05. Correlation analysis was performed using Spearman nonparametric, correlation test. Specific metabolomic tests were applied, including:

1.Principal Component Analysis (PCA) was for identifying patterns in the data by exploring the variance and structure of the data set and identifying outliers and clusters of samples with similar or distinct metabolic profiles.

2. Partial Least Squares Discriminatory Analysis (PLS-DA) was used for identifying the metabolic differences between groups. PLS-DA identifies latent variables that explain the covariance between the data and the group classification. Variables with the highest correlation to the group classification are used to create a predictive model. Within this analysis Variable Prediction (VIP) method was applied for selection of variables that identifies metabolites important for distinguishing between groups in PLS-DA. VIP scores are calculated for each metabolite in the model and reflect how much each metabolite contributes to group separation. Metabolites with high VIP scores are considered important for distinguishing between groups.

3. Metabolic Set Enrichment Analysis (MSEA) is a pathway enrichment analysis method that identifies the metabolic pathways responsible for group differences in a PLS-DA model (Szymańska et al., 2012).

5.1 Metabolomic profile in saliva and plasma

The PCA plot showed the distribution of data in a two-dimensional and three-dimensional space based on the first two principal components. It was observed that there was no significant separation of samples in the groups for both saliva and plasma samples, as the ellipses of these groups overlapped. This suggests that metabolic profiles of the disease groups were similar to those of the control group. **Figure 5.1.a**

However, the PLS-DA analysis identified a set of metabolites important for distinguishing between the disease groups and the control group in both saliva and plasma samples. This was represented by the VIP plot, where metabolites with high VIP scores were considered significant in explaining group differences. It was observed that several metabolites had high VIP scores, indicating their importance in differentiating between the groups. The plots demonstrated positive higher relative concentrations of propionic acid, acetic acid, succinic acid, 2,3-butanediol, lactate and putrescine in saliva, and total fatty acids, apolipoprotein, glucose, saturated fatty acids, and choline in plasma. **Figure 5.1. b**

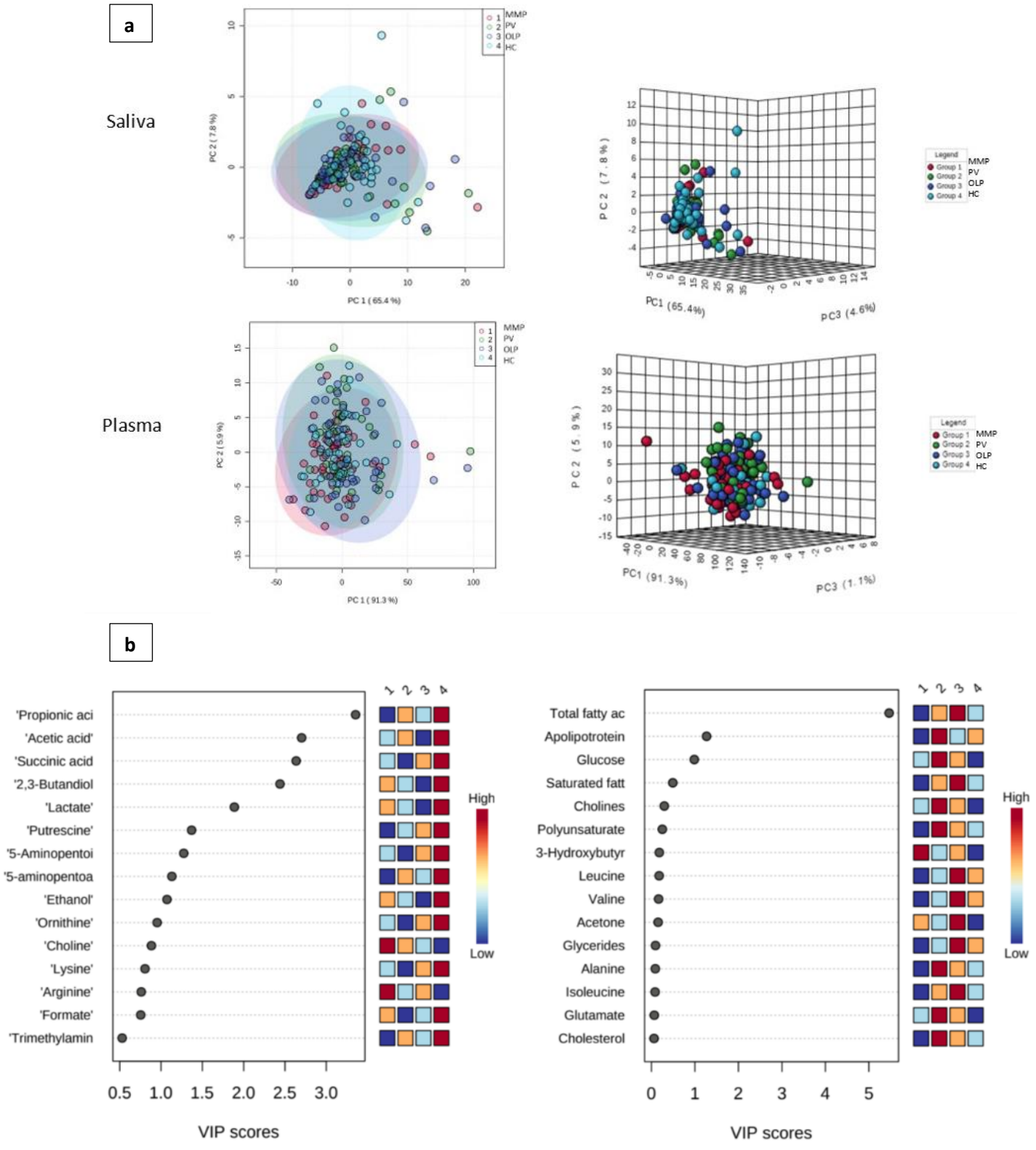


Figure 5.1. PCA and PL-SDA plots for saliva and plasma samples*. a) The subjects in all groups are shown 2D and 3D plots. Ellipses around groups of samples indicate the extent of variation within each group and the degree of overlap between groups. The size and shape of ellipse reflect the variance and correlation structure of the data, and the centre of the ellipse represents the mean of the scores for the group. Overlapping ellipses suggest that the groups may have similar metabolic profiles. b) Variable Prediction (VIP) method was applied for selection of variables that identifies metabolites important for distinguishing between groups in PL-SDA: 1=MMP, 2=PV, 3= OLP, 4 = HC*

However, the VIP plot can only identify metabolites that contribute most to the separation between the groups and may miss important metabolites that are not as strongly associated with the disease state. If a metabolite shows a significant increase in the Kruskal-Wallis test, it suggests that it is associated with the disease state and may be a potential biomarker. The results in saliva samples indicated few metabolites with significantly changes in the OLP and MMP groups. The salivary urea was significantly higher, and formate was lower in OLP ($p<0.05$).

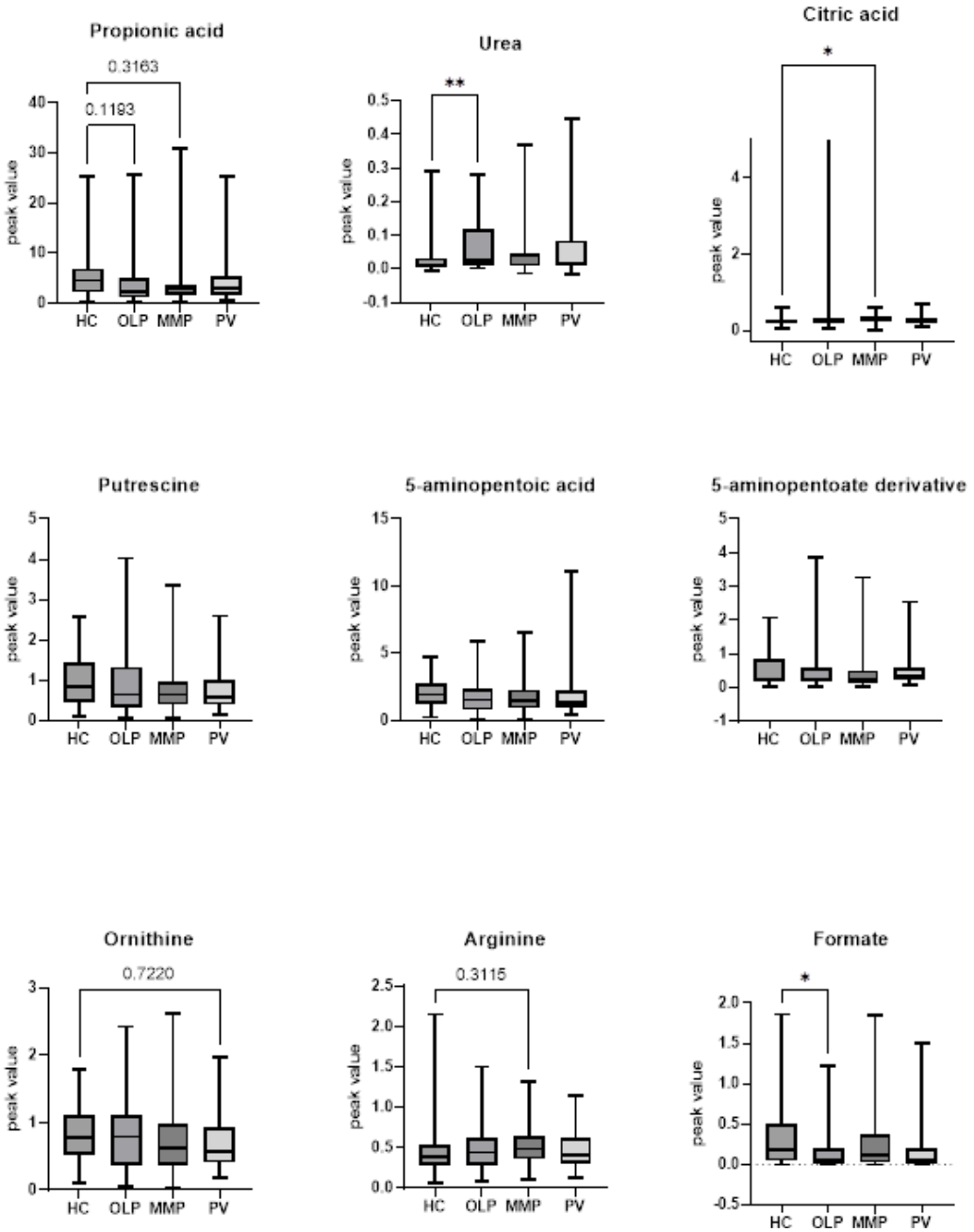


Figure 5.2. Comparison of the saliva metabolites between OLP, MMP, PV and HC. *Selected metabolites are displayed. The stars indicate * p<0.05, ** p<0.01. Kruskal–Wallis followed by Dunn's multiple comparisons post-hoc analysis. p values generated were corrected for false discovery rate (FDR) set to 0.05 (Benjamini-Hochberg test) to generate q (FDR) values shown in Appendix 16.*

And salivary citric acid was significantly elevated in MMP. These data remained significant following correction for multiple comparisons **Figure 5.2 and Appendix 16.**

In plasma samples of OLP patients the levels of cholesterol, polyunsaturated fatty acids and glucose were increased significantly compared to HC. In PV, a significant increase of choline, polyunsaturated acids and glucose was observed. In MMP group, the levels of glucose were found to have been increased significantly. **Figure 5.3**

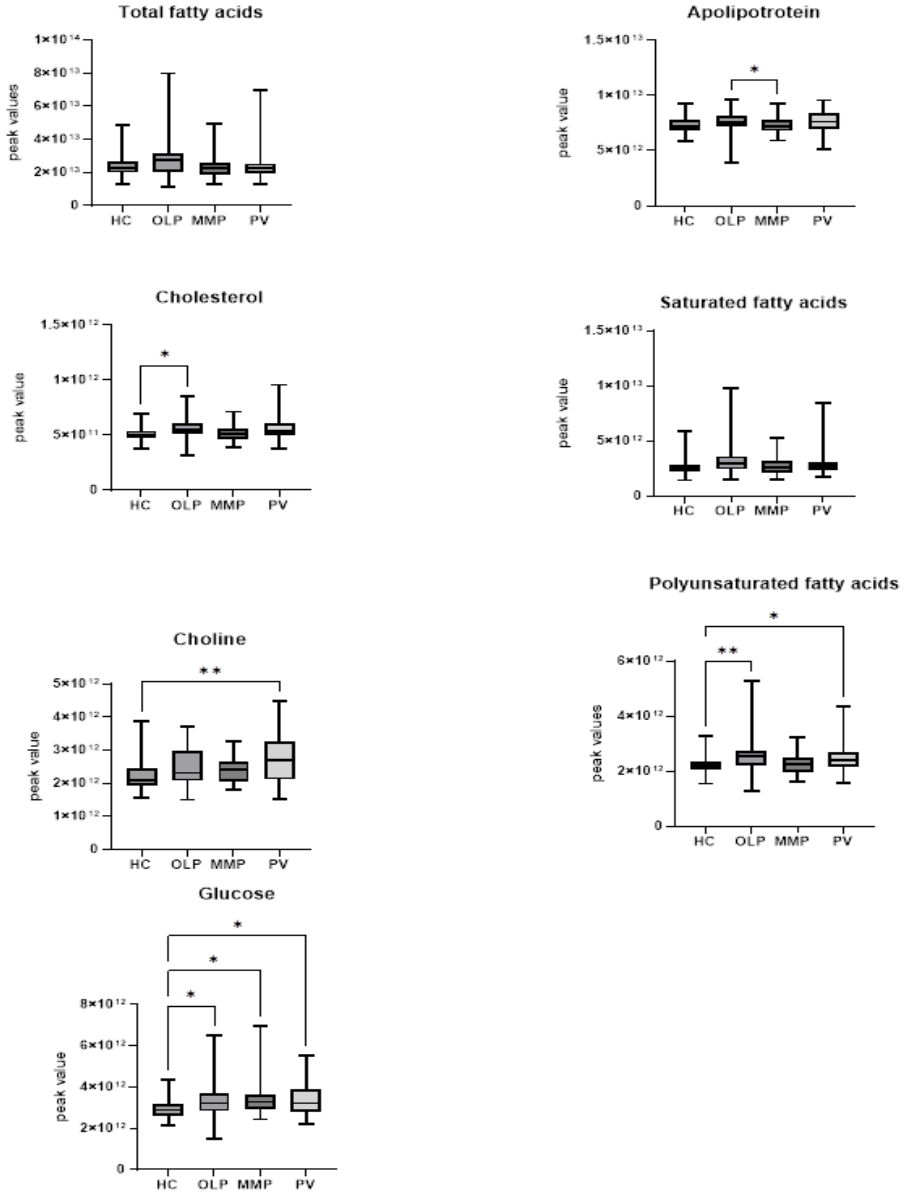


Figure 5.3. Comparison of the plasma metabolites between OLP, MMP, PV and HC. *Selected metabolites are displayed. The stars indicate * P<0.05, ** P<0.01. Kruskal–Wallis followed by Dunn's multiple comparisons post-hoc analysis. p values generated were corrected for FDR set to 0.05 (Benjamini-Hochberg test) to generate q (FDR) values shown in* ***Appendix 17.***

Subsequently, the metabolic pathways were analysed, and metabolite set enrichment analysis (MSEA) analysis showed that in saliva samples, the identified pathways in all three disease groups were distinct. In OLP, the top significant metabolomic pathways were 1) glyoxylate and carboxylate metabolism, 2) glycolysis, gluconeogenesis and 3) pyruvate metabolism, whereas for MMP they were 1) propanoate metabolism, 2) arginine and proline metabolism and 3) arginine biosynthesis. In PV, there were no significant pathways observed, but the closest pathway was arginine biosynthesis (p=0.06). Interestingly, for both MMP and PV, arginine biosynthesis was a critical pathway. **Table 5.1** For plasma samples, no significant differences were found between pathways.

Table 5.1. The pathways associated with the disease groups in saliva samples.

OLP (p<0.05)	MMP (p<0.05)	PV (p=0.06)
Glyoxylate and dicarboxylate metabolism	Propionate metabolism	Arginine biosynthesis
Glycolysis/Gluconeogenesis	Arginine and proline metabolism	
Pyruvate metabolism	Arginine biosynthesis	
Glycine, serine and threonine metabolism	Pyruvate metabolism	
Porphyrin and chlorophyll metabolism	Glycolysis/Gluconeogenesis	
Primary bile acid biosynthesis	Glutathione metabolism	

To investigate the relationship of salivary metabolites with clinical scores, Spearman correlation rank analysis was performed. Ethanol showed a significant positive correlation with ODSS in OLP (p=0.048). No correlations with ODSS were found in the MMP group. Inverse correlations were observed between ODSS and putrescine (p<0.0001), acetic acid (p=0.006), 5-aminopentoic acid (p=0.002), 5 aminopentoate derivatives (p=0.002), lysine (p=0.01), and ornithine (p=0.006) in saliva of the PV group. Choline correlated with ODSS positively in the

PV group (p=0.029). PISA score correlated positively with butyric and isovaleric acid in MMP (p=0.059 and p=0.002) and OLP (p=0.1 p=0.038) and HC groups (p=0.026 and p=0.059) groups. In PV, PISA score was significantly associated with succinic acid (p=0.008) **Figure 5.4**

There were fewer correlations of clinical scores with plasma metabolites. A significant positive correlation between ODSS with glucose (p=0.01) and polyunsaturated fatty acids(p=0.05) was observed in plasma of PV patients. **Figure 5.5**

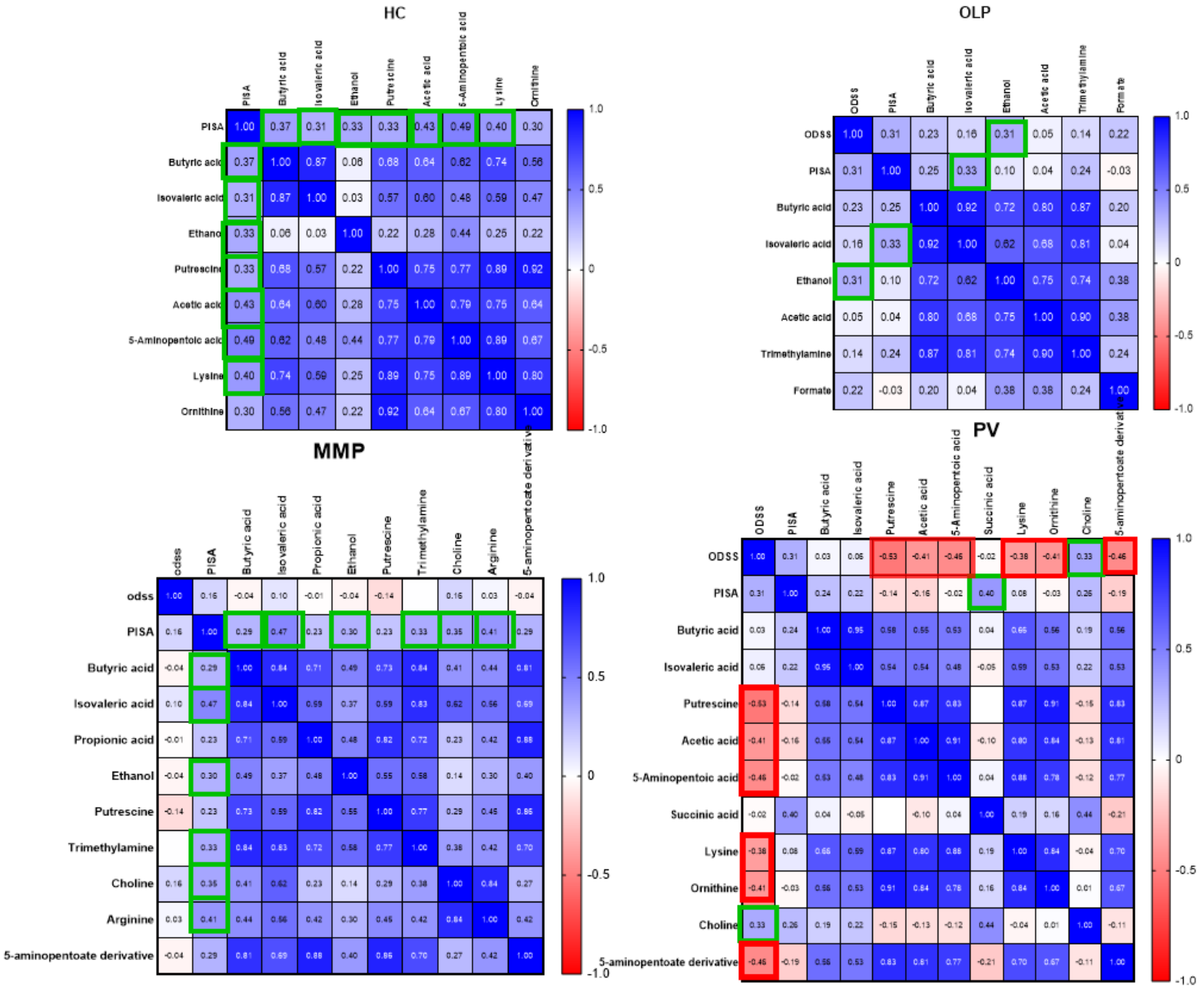


Figure 5.4. Correlation of clinical scores with plasma metabolites in HC, OLP, MMP and PV groups. *Significant associations p<0.05 were circled in the graph. Green circles show the significant positive correlation, whereas red circles show significant negative association, where p<0.05.*

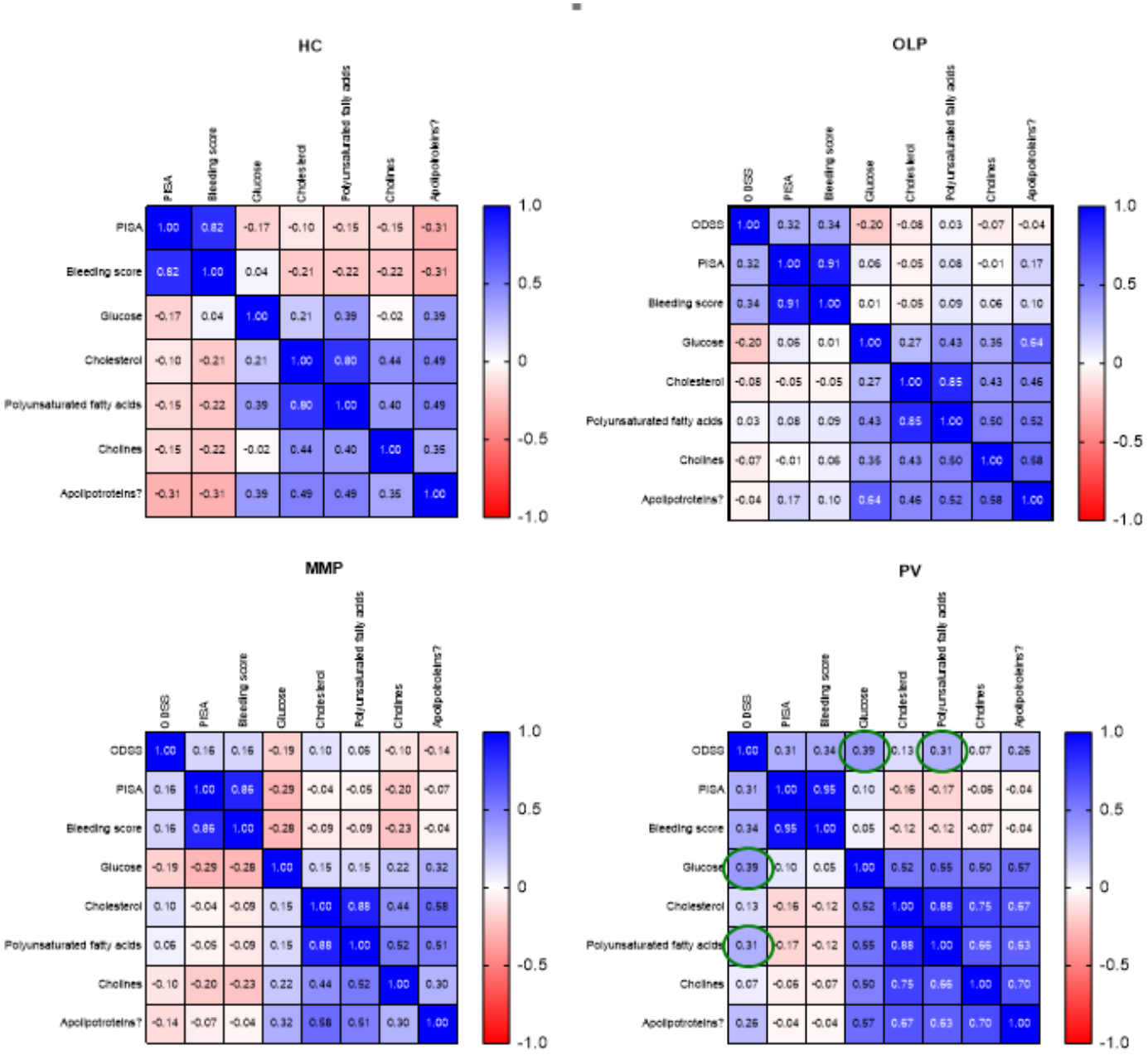


Figure 5.5 Correlation of clinical scores with plasma metabolites in HC, OLP, MMP and PV groups*. Significant associations p<0.05 were circled in the graph. Green circles show the significant positive correlation, whereas red circles show significant negative association, where p<0.05.*

5.2 Discussion

To date, there is a lack of literature investigating metabolic analysis with a focus on understanding the mechanisms related to immunobullous diseases and OLP. For the first time, this study describes metabolomic profile of saliva and plasma samples associated with immunobullous diseases PV and MMP and OLP taking into consideration the severity of oral mucosal disease and periodontal status. Despite slight differences in the metabolic profiles between the groups, the PCA plot results demonstrated a more considerable difference and differentiation of metabolites in saliva samples compared with plasma (Y. Zhang et al., 2016). A rational explanation for this is that OLP, MMP and PV are diseases that primarily affect the oral cavity, therefore, the metabolomic changes occurring in the oral cavity will be better reflected in saliva samples than in plasma (Foratori-Junior et al., 2022). Unlike plasma, the saliva contains numerous bacteria, and its composition changes according to diet, hygiene, and oral health status (Humphrey & Williamson, 2001), which directly result in changes in the oral microbiome.

The results for saliva samples showed increased urea in the OLP group. Urea is a source of nitrogen that can be utilized by some bacteria in the oral cavity, including those associated with periodontal disease. The increase in salivary urea levels in OLP patients could be related to a shift in the oral microbiome, with an increase in urease-producing bacteria that use urea as a nutrient source. Urease-producing bacteria have been shown to play an essential role in maintaining the oral microbiome and developing oral diseases, including PD (Gaál Kovalčíková et al., 2019). Moreover, the salivary formate levels in OLP patients were significantly underrepresented. Formate is an organic acid produced by the microorganisms and known to have anti-inflammatory properties (De Filippis et al., 2014). It is possible that dysbiosis in the oral microbiome associated with OLP could lead to alterations in formate production or utilization and these two metabolites could be markers of the OLP diseases in saliva samples.

Moreover, one of the most significant metabolic pathways in the saliva of OLP patients was glycolysis and gluconeogenesis. The regulation of glycolysis is critical in modulation of immune responses of T cells and B cells, which undergo a metabolic shift during activation to support their energy and metabolic needs (Malaisse et al., 1991). The literature (Ohshima et al., 2017) described that glycolysis is essential for oral cancer metabolism and periodontal

diseases. Glycolysis is the first step in hexose metabolism pathway that converts glucose into pyruvate or lactate, producing energy as ATP (Ishikawa et al., 2020). The association of OLP with ethanol could be a biological explanation for the observed carcinogenic action of alcohol in the mouth (Ishikawa et al., 2020).

To determine whether salivary metabolites differ with changes in the oral disease severity, we undertook Spearman rank correlation analysis. Results showed that ODSS were positively correlated with salivary metabolites, showing distinct metabolomic correlations in disease groups. A significant correlation was observed between ethanol and ODSS in the OLP group. This can be related to alcohol consumption habits among the groups. Alcohol consumption is one of the factors increasing microbial toxic acetaldehyde production (Salaspuro & Mezey, 2008). Studies have suggested that acetaldehyde can trigger autoimmunity in susceptible individuals and increase the risk of developing autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and autoimmune hepatitis (Bazewicz et al., 2019). Chronic alcohol consumption can lead to alterations in the balance of immune cells and increase production of pro-inflammatory cytokines, leading to tissue damage and promoting development of autoimmune disorders (Viitala et al., 2000a). Bacterial examination indicated that, in a study conducted by Moritani et al. it was found that *Neisseria* species, *Rothia mucilaginosa, Streptococcus mitis*, and *Prevotella histicola*, exhibited the capability to generate acetaldehyde from ethanol (Moritani et al., 2015). Acetaldehyde can modify proteins and render them immunogenic, leading to the production of autoantibodies and the activation of the immune system. Acetaldehyde produced by alcohol consumption can also affect the gut microbiome, leading to alterations in microbial community and an increase in gut permeability (Meijnikman et al., 2022; Viitala et al., 2000b). These changes can worsen autoimmune disorders by allowing entry of pathogenic microbes and antigens into the systemic circulation, leading to autoimmune reactions (Caslin, Mohler, Thiagarajan, & Melamed, 2021).

Next, this study showed that choline was elevated in PV and MMP groups in the PL-SDA plot. It showed a significant correlation with ODSS in the PV group, with a tendency (nonsignificant) towards a correlation with PISA score. Moreover, the ANOVA showed a significant increase in this metabolite in plasma of PV patients. Choline and its metabolites have been shown to correlate with markers of inflammation, oxidative stress, and endothelial function (Glunde et

al., 2011). They are also associated with age-related diseases like Alzheimer's, cardiovascular, and cancer (Glunde, Bhujwalla, & Ronen, 2011; Shoemark & Allen, 2015; Z. Wang et al., 2011). Choline is an essential nutrient obtained from the diet or synthesized endogenously (Dave et al., 2023). In the oral cavity, choline can be metabolized by oral microbes, including certain species of bacteria that possess choline-utilizing genes (Martínez-del Campo et al., 2015). These bacteria can convert choline to trimethylamine (TMA) and subsequently to trimethylamine N-oxide (TMAO), which can affect host-microbe homeostasis in several ways (Ufnal et al., 2015). Studies have reported that TMAO may impact bacterial metabolism and virulence by altering the expression of microbial genes and pathways involved in adaptation, adherence, and biofilm formation (Awan et al., 2018). Additionally, TMAO can influence the host immune response to oral microbes by modulating the production of pro-inflammatory cytokines and chemokines in the gingiva, which may contribute to developing or progressing oral diseases, such as periodontitis (J. Zhou et al., 2022). Furthermore, some studies have suggested that the choline-TMAO pathway may be involved in interkingdom signalling between oral bacteria and the host and may contribute to the development of systemic diseases, such as cardiovascular or metabolic disorders, by affecting the gut microbiome and metabolome (Nagpal et al., 2019).

A metabolic pathway analysis in saliva showed similar metabolic pathways in MMP and PV, including arginine biosynthesis and arginine metabolism as the crucial pathways. Additionally, in PL-SDA analysis, arginine was higher in MMP and PV groups than HC. Arginine is an essential amino acid that plays a role in many physiological processes, including nitric oxide synthesis and immune function (Wu & Morris, 1998). Arginine can also regulate inflammation, which can contribute to development or progression of oral diseases including periodontitis and dental caries (Xiong et al., 2022). Studies have reported that arginine can impact expression of cytokines and other inflammatory molecules in the oral cavity. For instance, arginine could be catabolised by local arginase secreted from the bacterial cells, such as *P. gingivalis*, increasing the production of polyamines, such as putrescine, spermidine, and spermine, as part of their metabolic activity (Charlier & Glansdorff, 2004). *P. gingivalis*--produced polyamines modulate a host immune response by enhancing production of pro-inflammatory cytokines, such as IL-6 and TNF-α, and suppressing production of anti-inflammatory cytokines, such as IL-10. This can lead to chronic inflammation and tissue damage. Earlier, it was

described that *P. gingivalis*-produced polyamines may contribute to autoimmune disease by initiating citrullination in RA (Maresz et al., 2013). *P. gingivalis* has been shown to produce an enzyme called peptidyl arginine deiminase (PAD), which can citrullinate proteins. Citrullination is the process by which arginine residues in proteins are converted to citrulline, which can lead to changes in protein function and antigenicity. *P. gingivalis*-induced citrullination, particularly in the presence of polyamines, can produce autoantibodies that recognize citrullinated proteins. These autoantibodies are commonly found in patients with RA (Rooney et al., 2020).

Periodontal PISA score showed a positive correlation with branched-chain fatty acid (BCFA)-isovaleric acid, and short-chain fatty acid (SCFA) - butyric acid in all disease groups. BCFA and SCFA are two types of fatty acids produced by the metabolism of dietary fibre and other carbohydrates by bacteria in the oral cavity (Cook & Sellin, 1998). BCFAs include isobutyric and isovaleric acids. Isovaleric acid is usually absorbed in the colon as a source of energy and enters the bloodstream, where it supports regulation of fatty acid, glucose, and cholesterol metabolism (Zarling & Ruchim, 1987). SCFAs such as acetate, propionate, and butyrate have been suggested to have anti-inflammatory effects and may help maintain periodontal health by inhibiting the growth of pathogenic bacteria and promoting growth of beneficial bacteria in the oral cavity (Corrêa et al., 2017b). Butyrate is important in regulating immune function, inhibiting inflammation, and promoting osteogenesis in periodontal tissues (Canani et al., 2011). However, it was observed that succinic acid, acetic acid, lactic acid, propionic acid, butyric acid and isovaleric acids in gingival crevicular fluid were significantly higher in aggressive PD (Seymour et al., 2007), where they induce apoptosis of gingival fibroblasts and contribute to the destruction of gingival tissues, enhancing inflammation and promoting the growth of pathogenic bacteria (Takahashi et al., 2010).

While PCA and PLS-DA provide a broader perspective on the overall structure and discriminatory power within the metabolomic dataset, Kruskal-Wallis test of metabolites is crucial for identifying specific metabolites, showing significant changes. Using these methods in combination enhances the depth of understanding in metabolomics studies. (**Figure 5.2, 5.3**) (Broadhurst and Kell, 2006). To address the issue of Type I errors, where in a statistical test erroneously rejects a true null hypothesis, the Kruskal-Wallis test is a test of choice. To mitigate the risk of Type I errors, FDR analysis was selected with a specific preference for the

Benjamini-Hochberg approach (Benjamini & Hochberg, 2000). This methodology offers the capacity to manage false positives while still enabling detection of potentially significant differences among metabolite peaks.

NMR technique offers several distinct advantages in metabolite measurement. It allows for direct sample analysis, eliminating the need for extensive sample preparation, such as extraction or chromatographic separation (Schripsema, 2010). The technique's ability to provide a holistic metabolic fingerprint, even though it may lack specific metabolite information, is another strength. Additionally, NMR is known for its relatively rapid and cheap analysis, often producing metabolite measurements within minutes, making it a practical tool for high-throughput studies. However, NMR does have its limitations. One significant limitation is its sensitivity compared to mass spectrometry (MS). NMR may not be as effective in detecting low-abundance metabolites, such as lipids. Additionally, NMR often produces multiple signals during metabolite detection, which can complicate the differentiation of individual metabolites without prior chromatographic separation. This signal multiplicity can present challenges in the accurate identification and quantification of metabolites, potentially impacting the precision of the results. Further integrating a targeted metabolomics technique, such as MS after untargeted metabolomics provides a synergistic approach that strengthens both techniques.

Alternatively, replicating results in different datasets or over time in longitudinal study can provide strong evidence for the validity of the identified metabolites. Finally, implementing machine learning algorithms to filter and prioritize metabolites based on their predictive power and stability, reducing the likelihood of selecting false positives (Akobeng, 2016). Even though no significant changes were found in metabolic pathways of OLP, MMP and PV in plasma samples, the Kruskal-Wallis test showed a significant increase of glucose in all three disease groups compared to HC, as well as elevated polysaturated fatty acids and cholesterol in OLP and PV. These results are consistent with PL-SDA results. Elevated levels of cholesterol and glucose are often associated with an increased risk of cardiovascular disease and diabetes. (Drew et al., 2009; Grosso et al., 2022). Considering that diabetes was associated with PV in our study, and BMI was above the normal range in all three groups of the diseases, the increase of these two metabolites in plasma is associated with patients' metabolic status. PUFAs are found in plant and animal foods, such as salmon, vegetable oils, and some nuts and

seeds, and elevated levels of these metabolites can be associated with the dietary habits between groups (Czernichow et al., 2010).

To conclude, this study provides an overview of salivary and plasma metabolites in OLP, MMP and PV identifying markers of metabolomic alterations in the context of these diseases. It is crucial to underscore that the findings presented here serve as a first step for further investigations, which will involve gene expression patterns, targeted metabolomics analysis, validation studies, longitudinal analysis, and the adoption of advanced machine learning methodologies which promise to enhance understanding of the metabolomic dynamics in PV, MMP and OLP.

Chapter VI. The oral microbiome in OLP, MMP and PV

There is a significant literature supporting the role of commensal bacteria in maintaining immune homeostasis (Belkaid & Naik, 2013; Mcdermott & Huffnagle, 2014; Walker, 2017). Recent studies indicate that changes in the oral microbiome may lead to imbalance in this homeostasis which may contribute to the pathogenesis of autoimmune disease (Acharya et al., 2017; Costalonga & Herzberg, 2014; Guo et al., 2018; Wade, 2013). Reports indicate that dental plaque is associated with more active gingival inflammation in PV, MMP and OLP; that plaque control improves symptoms of oral disease, and that treatment with antibiotics (doxycycline, sulfapyridine, dapsone) improves immunobullous diseases (Akman et al., 2008; Lo Russo et al., n.d.; Thorat et al., 2010; Tricamo et al., 2006). These findings suggest a possible association between the oral bacteria and these mucosal diseases. Therefore, this chapter aimed to identify the oral microbial composition in saliva and subgingival plaque samples derived from patients and controls and to identify the microorganisms associated with the diseases.

Earlier microbiome studies on AIBD and OLP were designed using the 16S rRNA gene amplicon sequencing technique (de Paolis et al., 2019; S. Li et al., 2021; H. Liu et al., 2021; Zorba et al., 2021b). However, due to the lower resolution and potential inaccuracies caused by primer bias in 16S rRNA sequencing, where certain primers tend to favour specific regions of the 16S rRNA gene over others, it can result in an uneven representation of microbial species in the sequencing data. To address these limitations, shotgun metagenomic sequencing approach was chosen, which offers a greater read depth as it sequences the entire genetic material of the microbial community without targeting specific gene regions.(Quince et al., 2017).

To gain further insight into the composition and structure of oral microbial communities in these diseases, this study for the first time integrated metagenomic sequence data to profile the microbial community of salivary and subgingival plaque samples, comparing differentially represented microbial species between OLP, MMP, PV and healthy controls. A comparison of the microbial diversity, the relative abundance of the species between groups, and the correlation of the microbiome with the main clinical, periodontal, metabolomic and immunologic parameters were performed. In total 365 DNA extracts were sent for sequencing: 186 saliva samples and 179 subgingival plaque samples. The discrepancies in the

number of subgingival plaque samples are related to the fact that some participants, due to the hypersensitivity of their gums, did not allow the procedure to be completed. From those total amount of samples, 326 passed quality control checks and were used in further sequencing analysis (38 HC, 49 OLP, 46 MMP and 45 PV of saliva; 35 HC, 40 OLP, 34 PV and 39 MMP plaque samples) **Appendix 15**. The Benjamini-Hochberg method for multiple hypothesis correction was used with the false discovery rate set (FDR) to 0.05. A detailed set of adjusted p values (q values) from these analyses is available in the Appendix 18-20.

6.1. Microbial diversity

6.1.1 Alpha diversity

Microbial richness and Shannon diversity are two measures of biodiversity in microbial communities (Huttenhower et al., 2012). Microbial richness refers to the number of different species present in a community, while Shannon diversity considers both the number of species and their evenness. The microbial richness of all saliva samples did not show a significant difference when compared to plaque samples. However, Shannon diversity was significantly higher in plaque samples (p=0.05). **Figure 6.1**

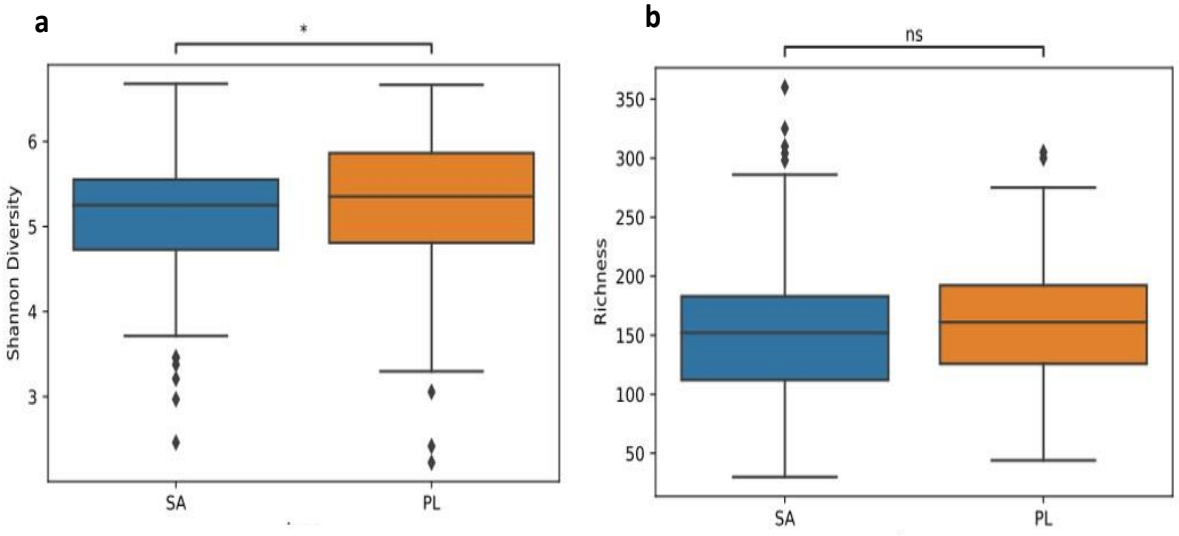


Figure 6.1. Alpha diversity for saliva and subgingival plaque samples. ***a.*** *Shannon diversity for saliva (SA) and subgingival plaque (PL) samples.* ***b.*** *Microbial richness. Statistical analyses were done using Mann Whitney test *(p<0.05).*

Comparison of the microbial diversity between disease groups and HC showed that there were no significant differences in Shannon diversity either in saliva or plaque samples. The microbial richness between disease groups and HC did not show significant differences in

saliva samples. However, the plaque samples showed a significant decrease in richness in all three groups of diseases compared to HC, with the lowest richness in the MMP group (p=0.0036) followed by OLP (p<0.01) and PV (p=0.1). **Figure 6.2**

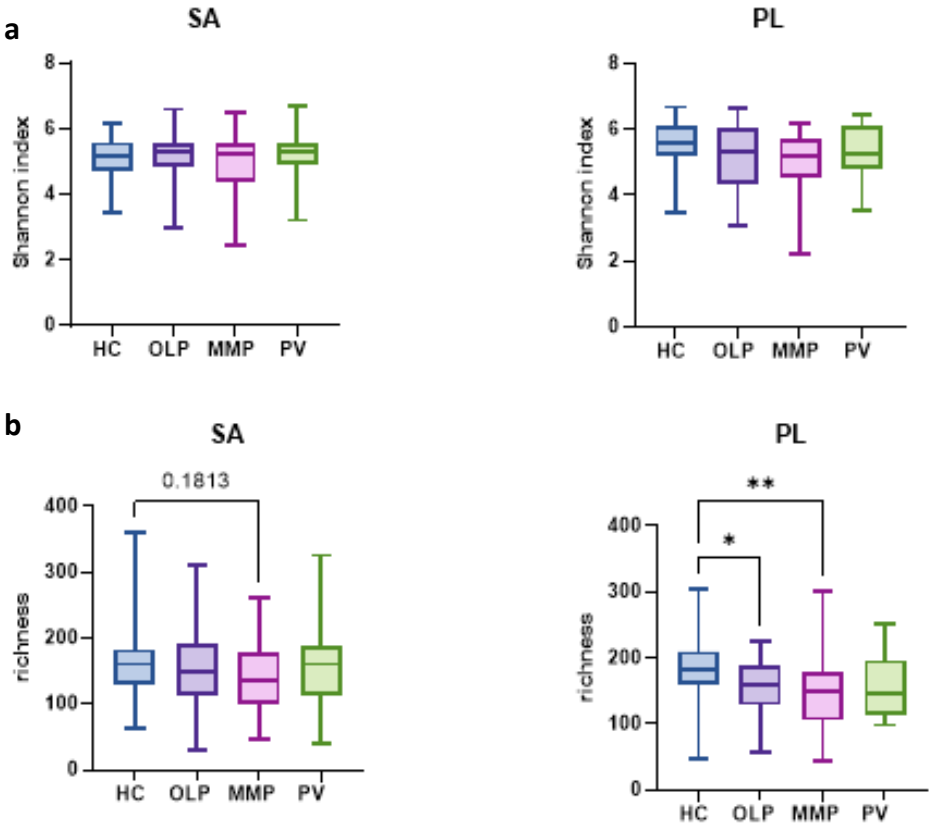


Figure 6.2 Alpha diversity in OLP, MMP, PV and HC groups. a. *Shannon diversity for saliva (SA) and subgingival plaque (PL) samples between OLP, MMP, PV and HC groups.* ***b.*** *Microbial richness in diseases groups and HC. Statistical significance when *(p<0.05), **(p<0.01). Kruskal–Wallis followed by Dunn's multiple comparisons post-hoc analysis.*

6.1.2 Beta diversity

To analyse beta diversity, a Principal Coordinate Analysis (PCoA) was performed. Beta diversity is a measure of the differences in microbial community composition between different samples or environments(Consortium et al., 2012). Beta diversity comparison of all saliva and plaque suggested a significant community differences between two sample sources. **Figure 6.3**

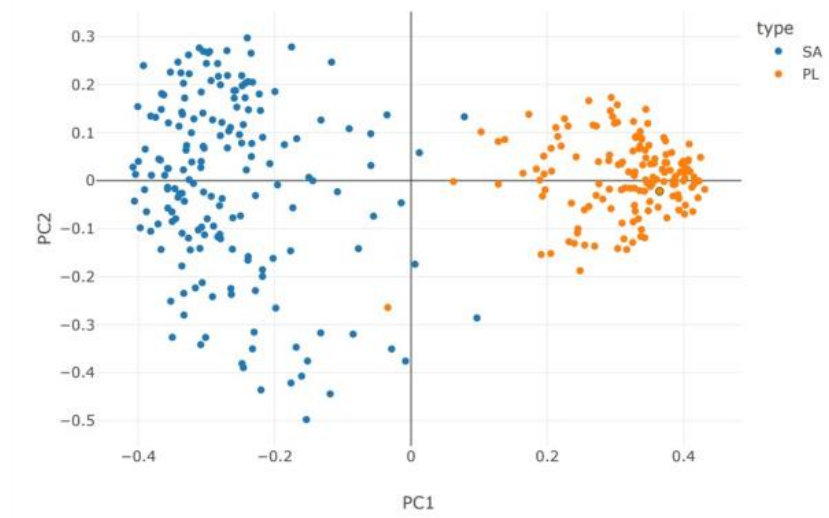


Figure 6.3 Principal coordinate analysis (PCoA) of total saliva and plaque samples, *based on Jaccard distance metrics dissimilarity in the bacterial compositions between the plaque and saliva samples. The dots represent each saliva (SA) or plaque (PL) sample.*

Further clustering of samples according to disease groups showed that saliva in the MMP group overlaps with the plaque samples in the same group. Notably, there is a slight overlap of saliva and plaque samples in the OLP group. Beta diversity was calculated using the Bray-Curtis distance measurement from Scipy which was used for principal coordinate analysis (PCoA) in the Scikit-bio package. **Figure 6.4**

6.1.3 Changes of the oral microbiome at the phylum level.

Overall, 16 phyla were identified from all samples. In saliva samples, Bacillota (100%) were the most prevalent phylum; this was followed by Actinobacteria (100%), Bacteroidetes (100%), Proteobacteria (99.7%), Fusobacteria (99%), Candidatus Saccharibacteria (TM7) (92%), Spirochaetes (84%), Synergistetes (55%), unclassified Bacteria (44%), Candidatus Gracilibacteria (29%), Tenericutes (18%), Chloroflexi (12%), Ascomycota (7%), Chlamydia (5.8%) and Euryarchaeota (0.61%) **Figure 6.5.** The most abundant phylum in plaque samples

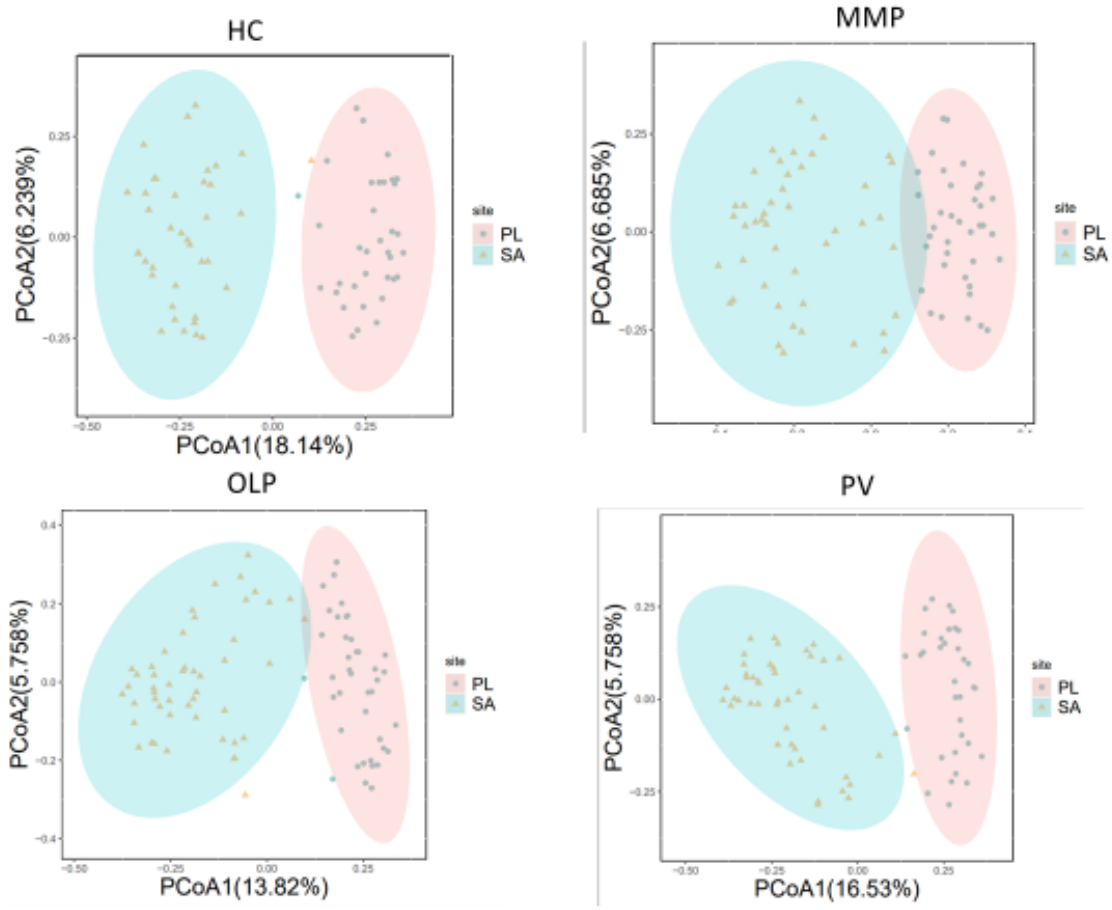


Figure 6.4 Principal composition analysis (PCoA) comparing saliva (SA) and plaque (PL) samples in HC, OLP, MMP and PV*. Samples from the same source are wrapped with an eclipse indicating a 95% confidence interval.*

was Actinobacteria (100%), followed by Bacillota (100%), Bacteroidetes, (100%) Proteobacteria (100%), Fusobacteria (99.7%), Candidatus Saccharibacteria (93.8%) and an unclassified group of microorganisms. **Figure 6.5** Ranking phyla at 100% means that a particular phylum has been detected in every single sample within the study, which helps to understand the core microbial composition of the oral microbiome.

Comparing the median relative abundance between diseases groups in saliva samples for each phylum **Figure 6.6**, Bacillota was significantly underrepresented in the saliva of OLP when compared to HC (q=0.047). Actinobacteria in saliva was statistically lower in PV compared to MMP (q=0.02). Finally, Spirochaetes were significantly increased in OLP than in HC (q=0.0002).

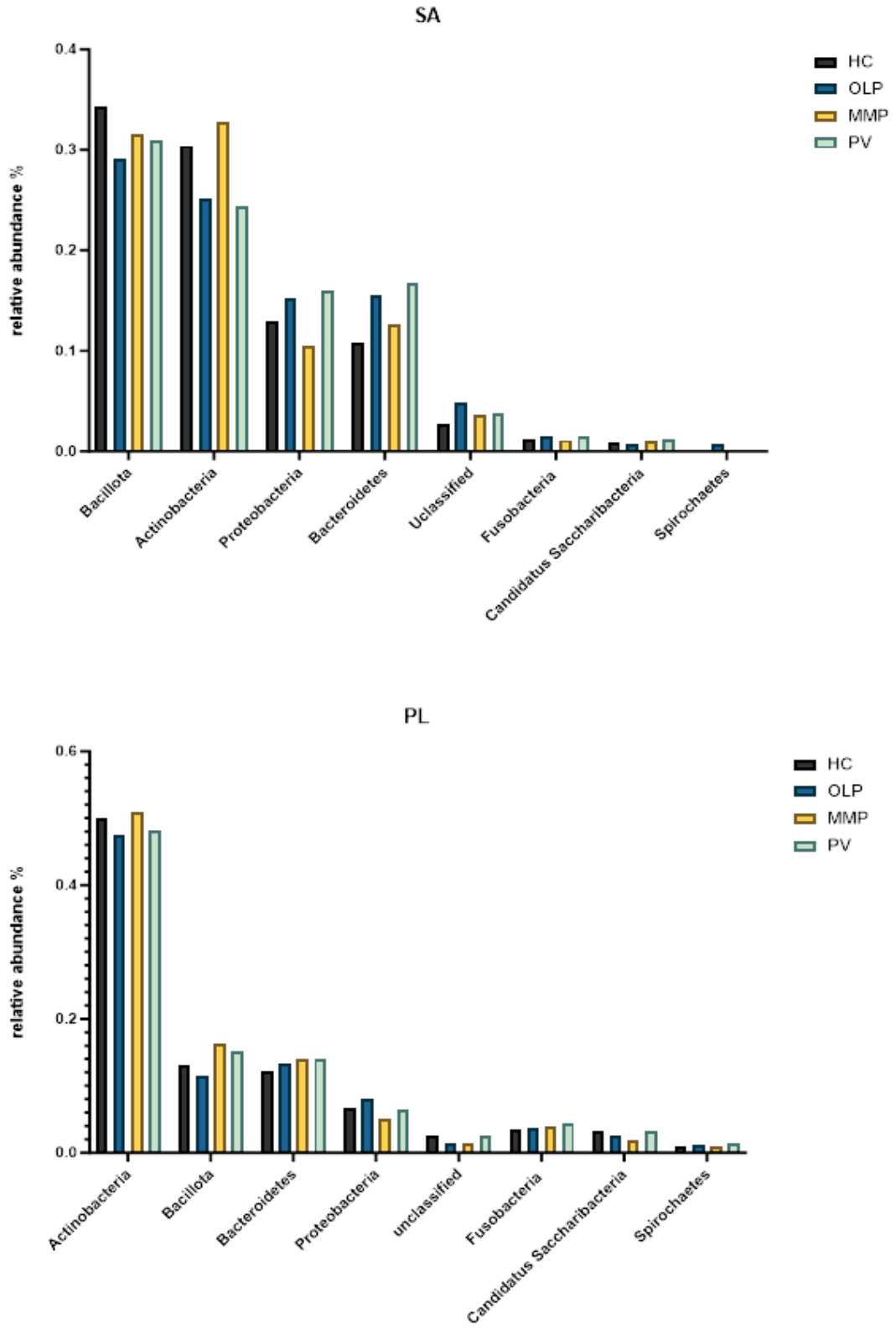


Figure 6.5. Differences between the most abundant phylum in diseases groups and healthy control *in saliva (SA) and subgingival plaque (PL) samples.*

In plaque samples, there were no significant differences between groups; however, the Proteobacteria phylum in MMP was found to be relatively lower than in HC, OLP and PV. F**igure 6.6**

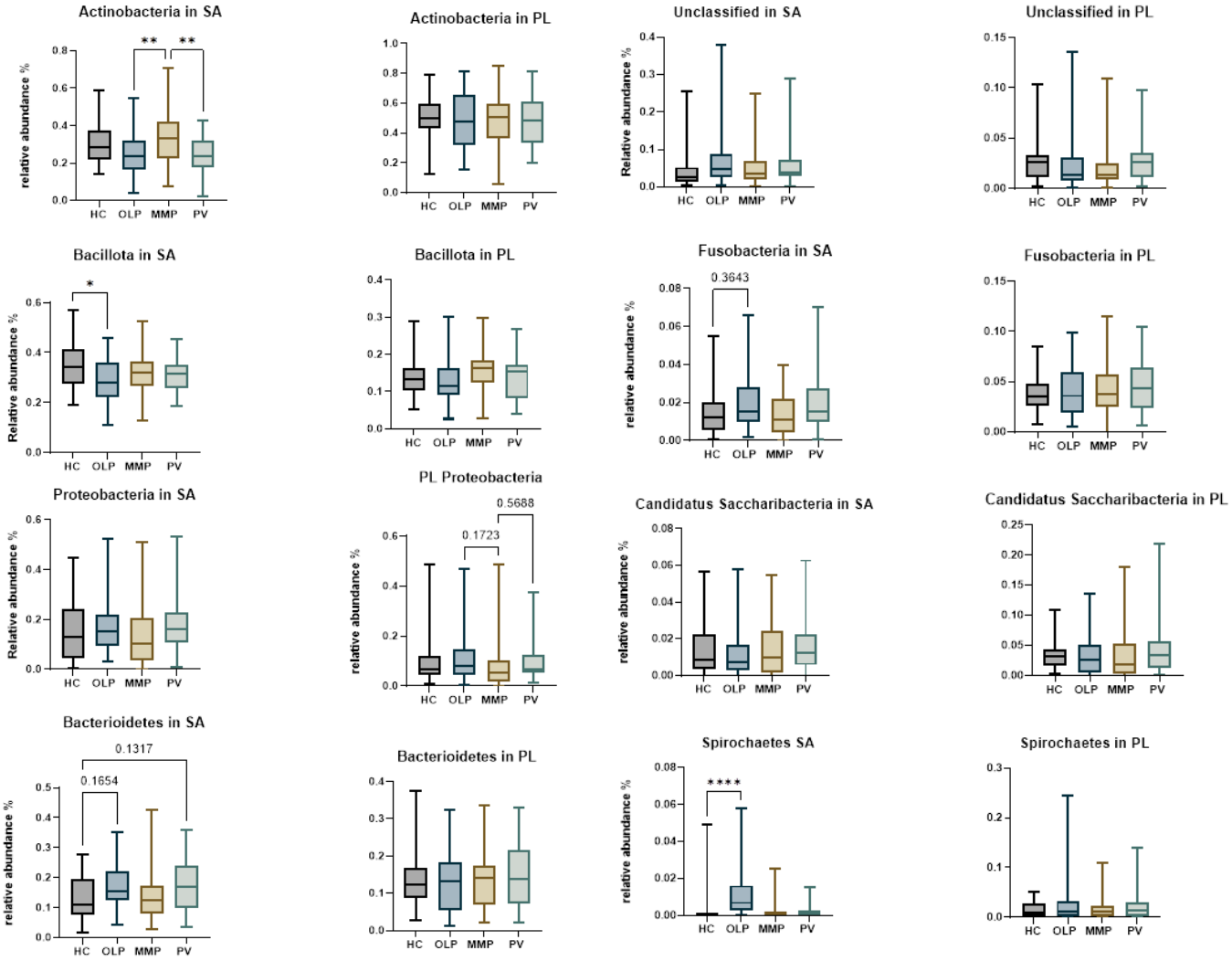


Figure 6.6 Comparison between the median relative abundance of different phyla in saliva (SA) and plaque (PL) samples between groups. *Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post-hoc analysis *(p<0.05); **(p<0.01); ***(p<0.001); ****(p<0.0001). p values generated were corrected for false discovery rate (FDR) set to 0.05 (Benjamini-Hochberg test) to generate q (FDR) values shown in* ***Appendix 18.***

6.1.4 Impact of the therapy type on the oral microbiome in MMP and OLP

The interaction between systemic medications used in autoimmune diseases and microbe composition is essential for understanding the resilience of oral microbiome.

A comparison of the relative composition of microbiome in saliva and plaque samples on phylum level in MMP and OLP groups was performed using the Kruskal–Wallis analysis followed by Dunn's multiple comparisons post-hoc analysis. Most of the PV patients were on systemic immunosuppressants (IS), therefore they were not included in the analysis.

Next, I compared the MMP group, subdividing it into patients on topical or systemic immunosuppressant (IS) and low-dose antibiotic (doxycycline/dapsone) treatment groups. The antibiotic group included some patients taking additional systemic immunosuppressants (IS). Overall, the relative abundance of MMP and HC was included in the graphs for general comparison. **Figure 6.7**

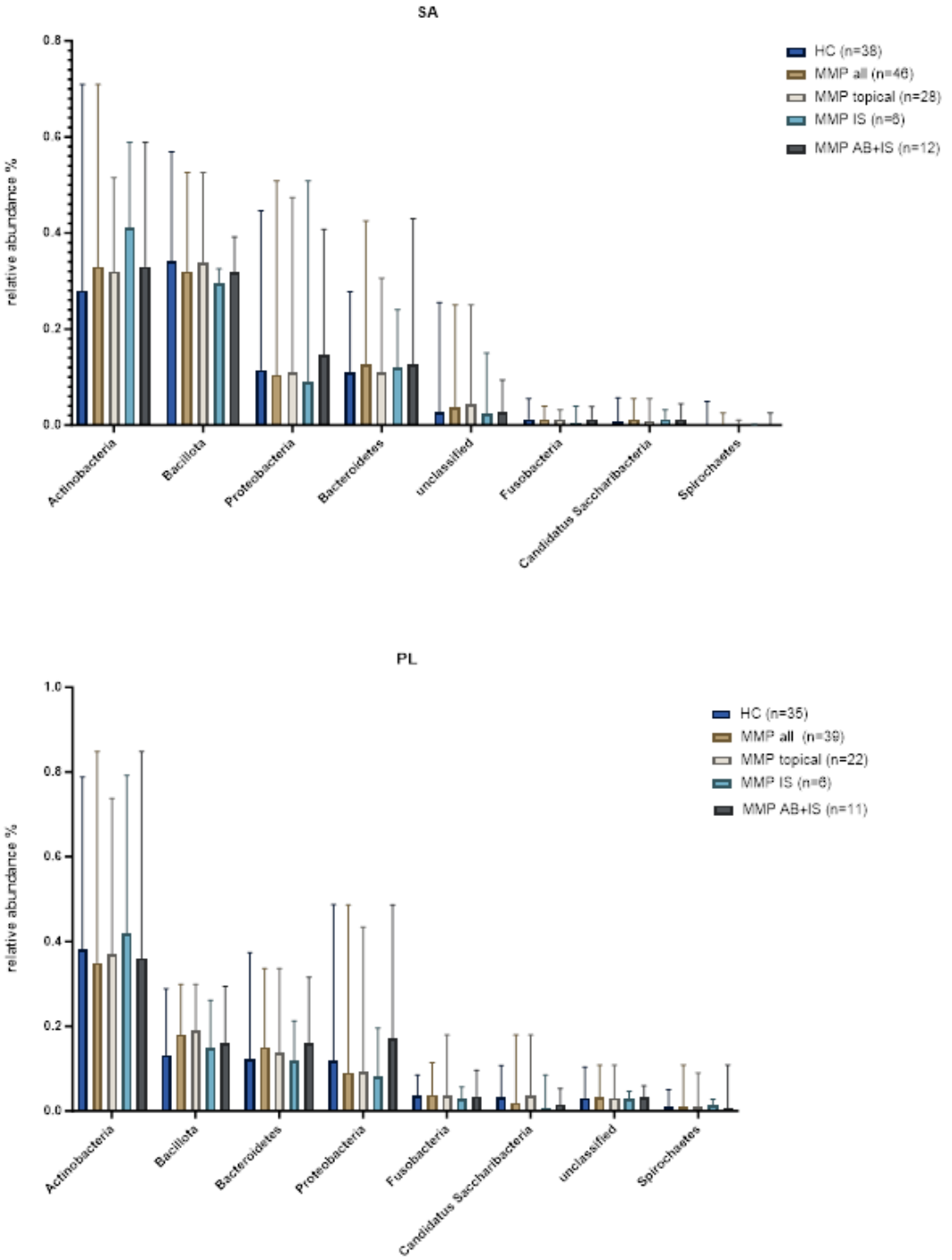


Figure 6.7. Relative abundance of microorganisms in treatment subgroups in MMP. *This graph is Illustrating the median relative abundance at the phylum level among various groups, including healthy controls, total MMP cases, subgroups within MMP undergoing topical treatment, those on immunosuppressants (IS), and the subset MMP on AB+IS.*

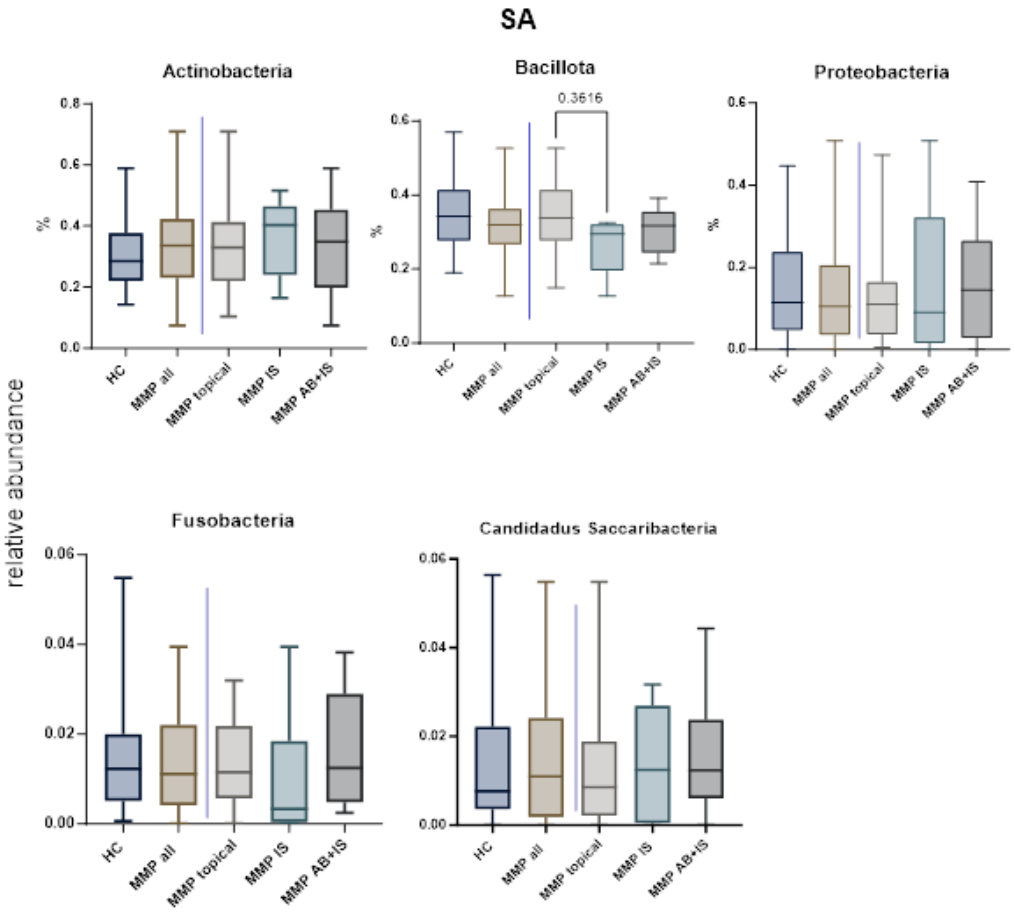


Figure 6.8.a Comparison between the median relative abundance of different phyla in saliva samples between treatment subgroups in MMP. *Statistical analysis using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test.*

These subgroups were compared. No significant differences in the relative abundance of the oral microbiome in saliva were detected between treatment groups. **Figure 6.8.a** However, patients on systemic immunosuppressant (IS) therapy had a lower abundance of Bacillota and Fusobacteria in saliva samples compared to those who used topical treatment or on low-dose systemic antibiotics (p=0.39 and p=0.97, respectively). Moreover, Actinobacteria were slightly overrepresented in IS group in both saliva and plaque samples.

No significant changes between groups were found in plaque samples. The representation of the Candidatus Saccharibacteria phylum in plaque samples was slightly lower in patients receiving immunosuppressive (IS) therapy (p=0.49). **Figure 6.8b**

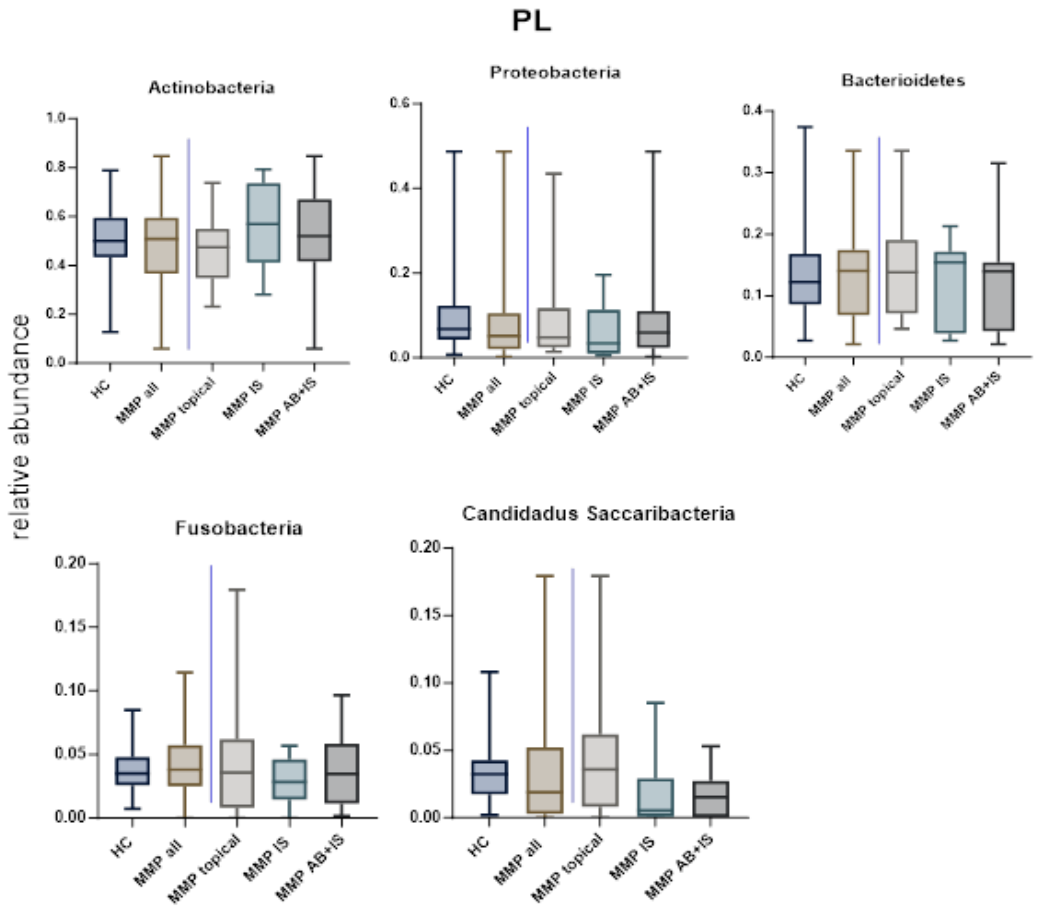


Figure 6.8.b Comparison between the median relative abundance of different phyla in plaque samples between treatment subgroups in MMP. *Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test.*

The OLP group also was subdivided into the OLP on topical, systemic immunosuppressants (IS) including hydroxychloroquine (HCQ) and the group on HCQ only. **Figure 6.9** There were no significant differences between treatment groups in saliva and plaque samples. A slight shift was observed in the group on immunosuppressants (IS) with a relative decrease in Candidatus Saccharibacteria in plaque samples (p=0.18). **Figure 6.10, 6.11.**

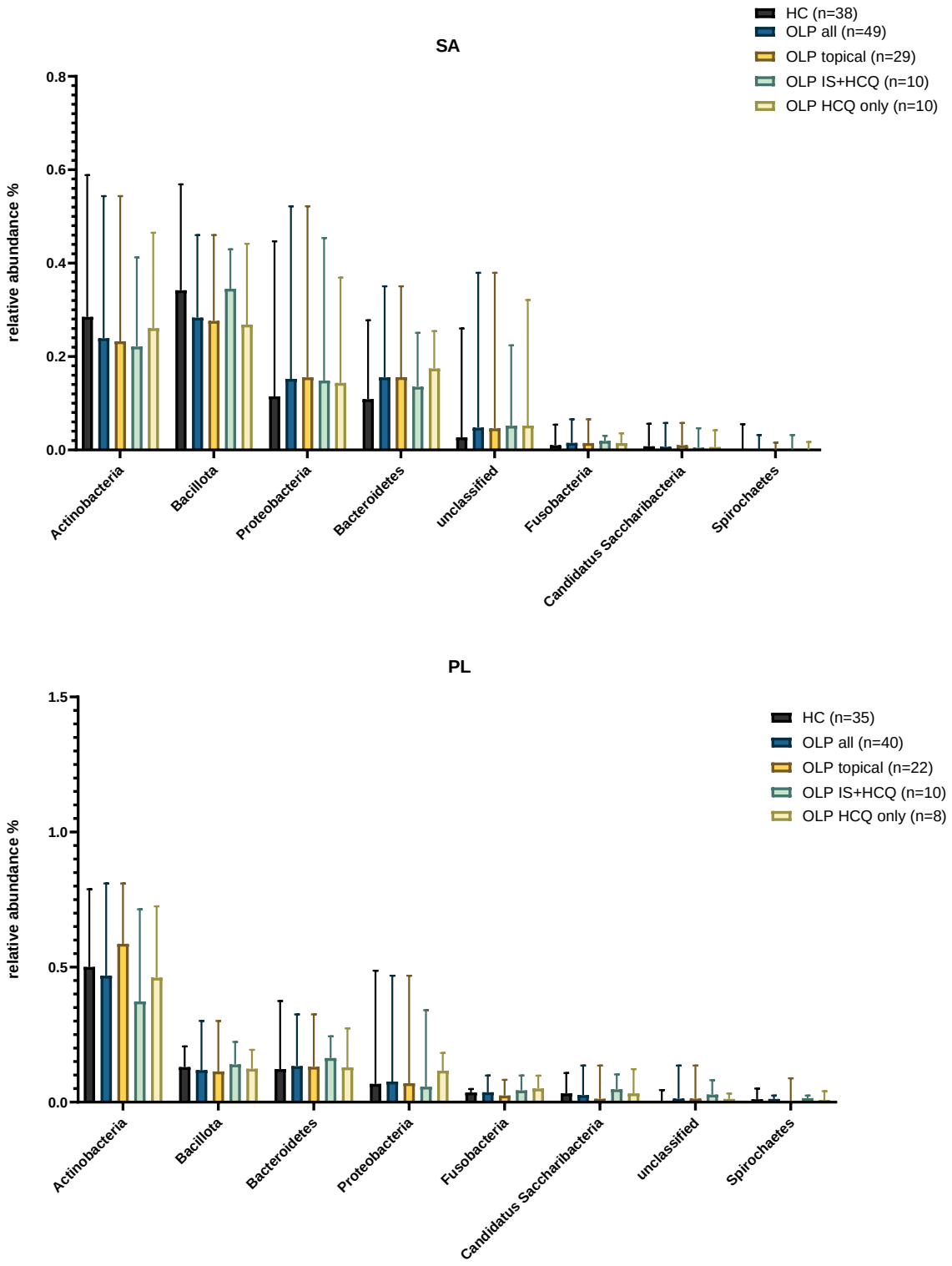


Figure 6.9. Relative abundance of microorganisms between treatment subgroups in OLP, *Illustrating the median relative abundance at the phylum level among various groups, including healthy controls, total OLP cases, OLP topical- topical corticosteroids, OLP IS+HCQ- on systemic immunosuppressants and hydroxychloroquine, OLP HCQ only- on hydroxychloroquine. Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test.*

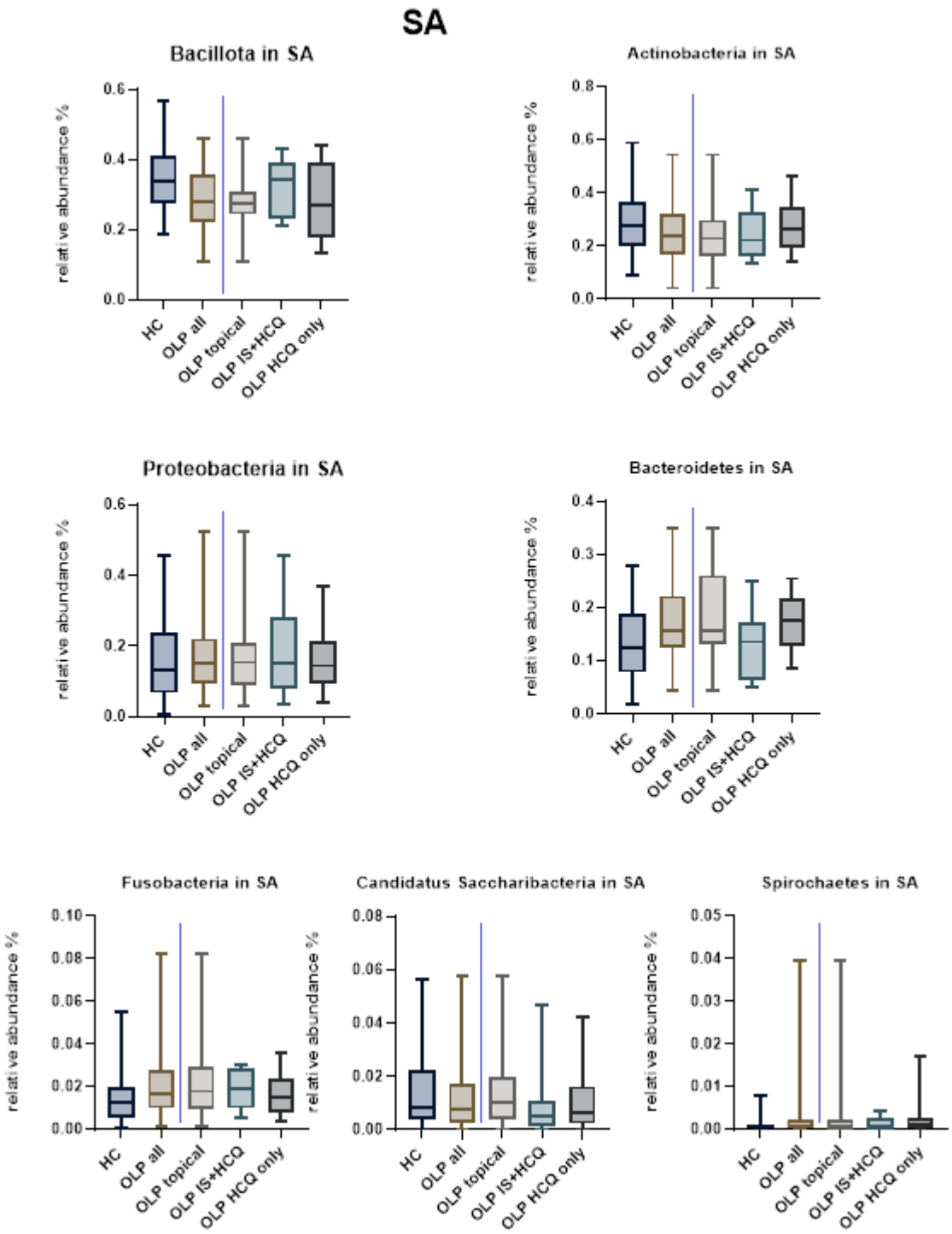


Figure 6.10. Comparison between the median relative abundance of different phyla in saliva samples between treatment subgroups in OLP*, including healthy controls, total OLP cases, OLP topical- topical corticosteroids, OLP IS+HCQ- on systemic immunosuppressants and hydroxychloroquine, OLP HCQ only- on hydroxychloroquine. Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test.*

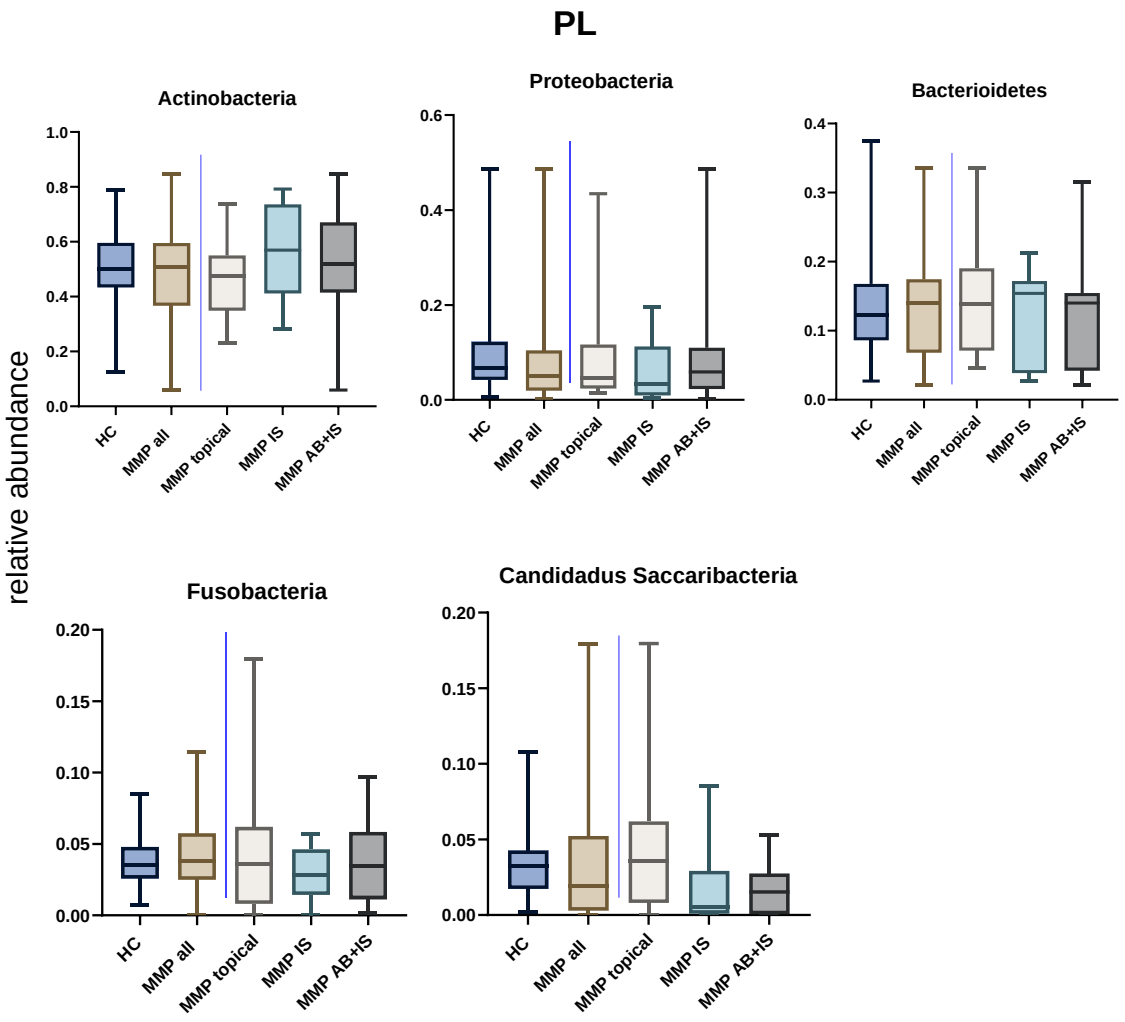


Figure 6.11. Comparison between the median relative abundance of different phyla in plaque samples between treatment subgroups in OLP*, including healthy controls, total OLP cases, OLP topical- topical corticosteroids, OLP IS+HCQ- on systemic immunosuppressants and hydroxychloroquine, OLP HCQ only- on hydroxychloroquine. Statistical analysis was done using the Kruskal-Wallis test followed by Dunn's multiple comparisons post hoc test.*

6.1.5 Changes of the oral microbiome at the species level.

Phylum identification provided a broad picture of the microbiome present in different sample sources. However, the data was then further analysed to identify microbial abundance at the species level, to investigate which were present and to start to identify interactions between different species to better understand their roles within the oral cavity associated with the diseases.

First, I found that saliva was comprised predominantly of gram-positive species, whereas plaque consists of gram-negative species. **Figure 6.12** At a species level comparing disease

groups, I found a significant difference in the microbiome composition between disease groups and healthy controls in saliva samples. There was a significant decrease in *Streptococcus salivarius* and *Streptococcus infantis* in OLP patients (q=0.006 and q=0.04, respectively). Additionally, a significantly reduced abundance of *Actinomyces marsseillensis, Hemophilla parainfluenza, Streptococcus mitis, Streptococcus australis, and Xylanimonas cellulosylitica* was found in the MMP group compared to HC. **Figure 6.13**

In plaque samples, *Campylobacter gracillis* was significantly increased in MMP (q=0.009) and *Capnocytophaga ochracea* species in OLP (q=0.04). *Fusobacterium nucleatum subp. animalis and Fusobacterium nucleatum subp. polymorphius* were higher in MMP and OLP respectively, however with no statistical significance. **Figure 6.14**

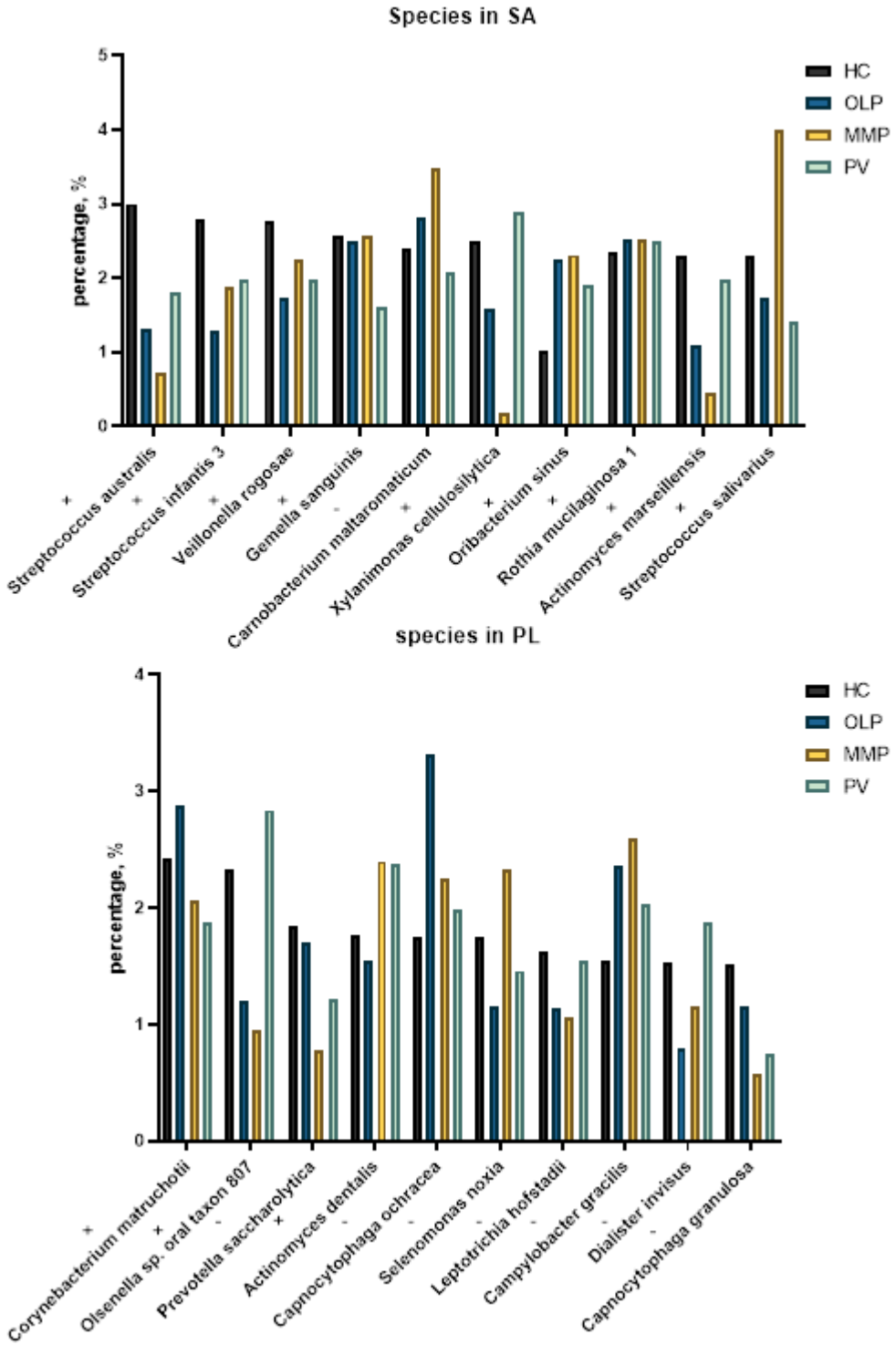


Figure 6.12 Average abundance of the most predominant species *in a) saliva (SA) b) plaque samples (PL)." +"- gram-positive and "–"gram-negative species.*

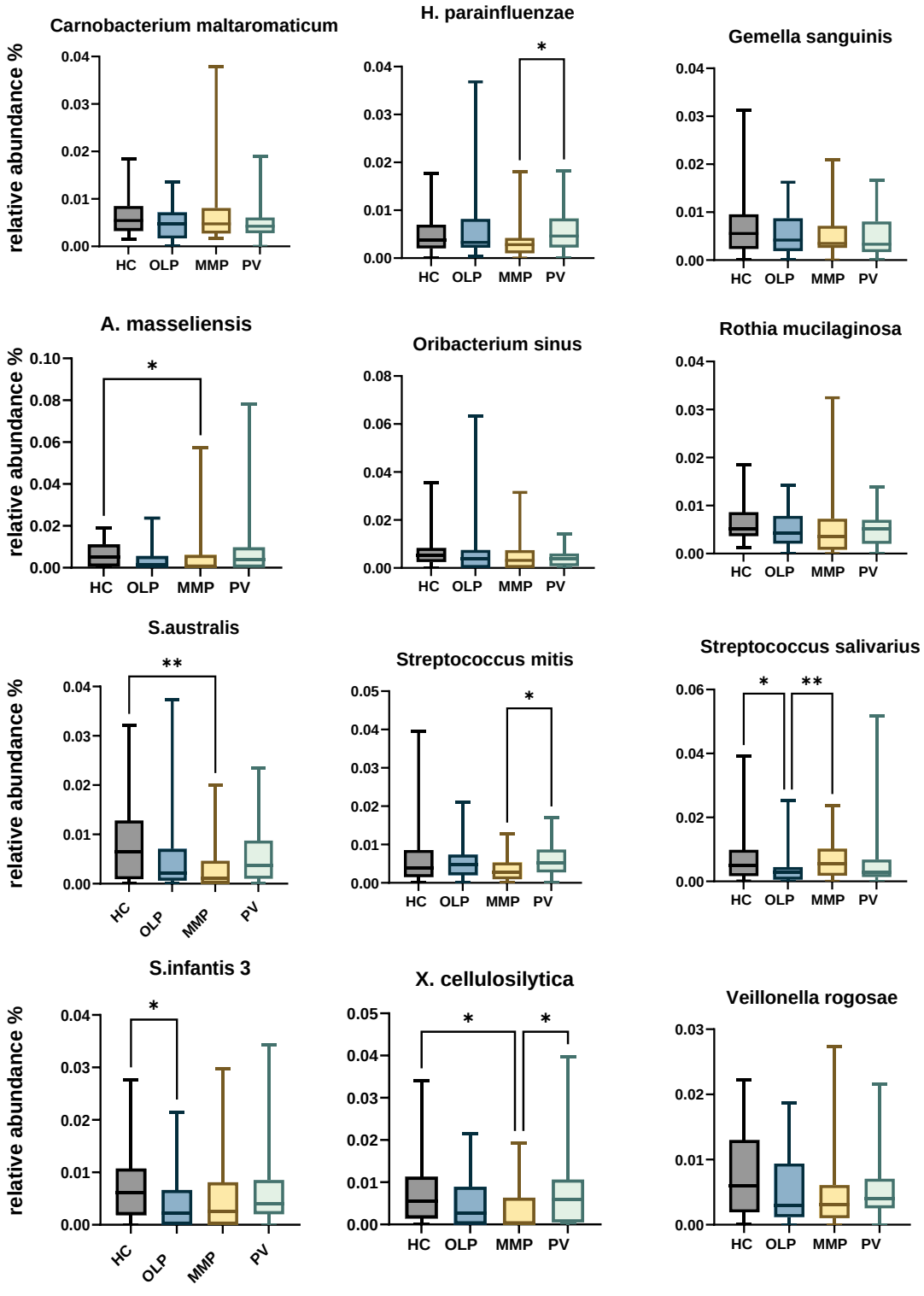


Figure 6.13 Comparison of relative abundance of different species between disease groups and HC in saliva samples. **p<0.05; ** p<0.01. Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test. P values generated were corrected for false discovery rate (FDR) set to 0.05 (Benjamini-Hochberg test) to generate q (FDR) values shown in* ***Appendix 19****.*

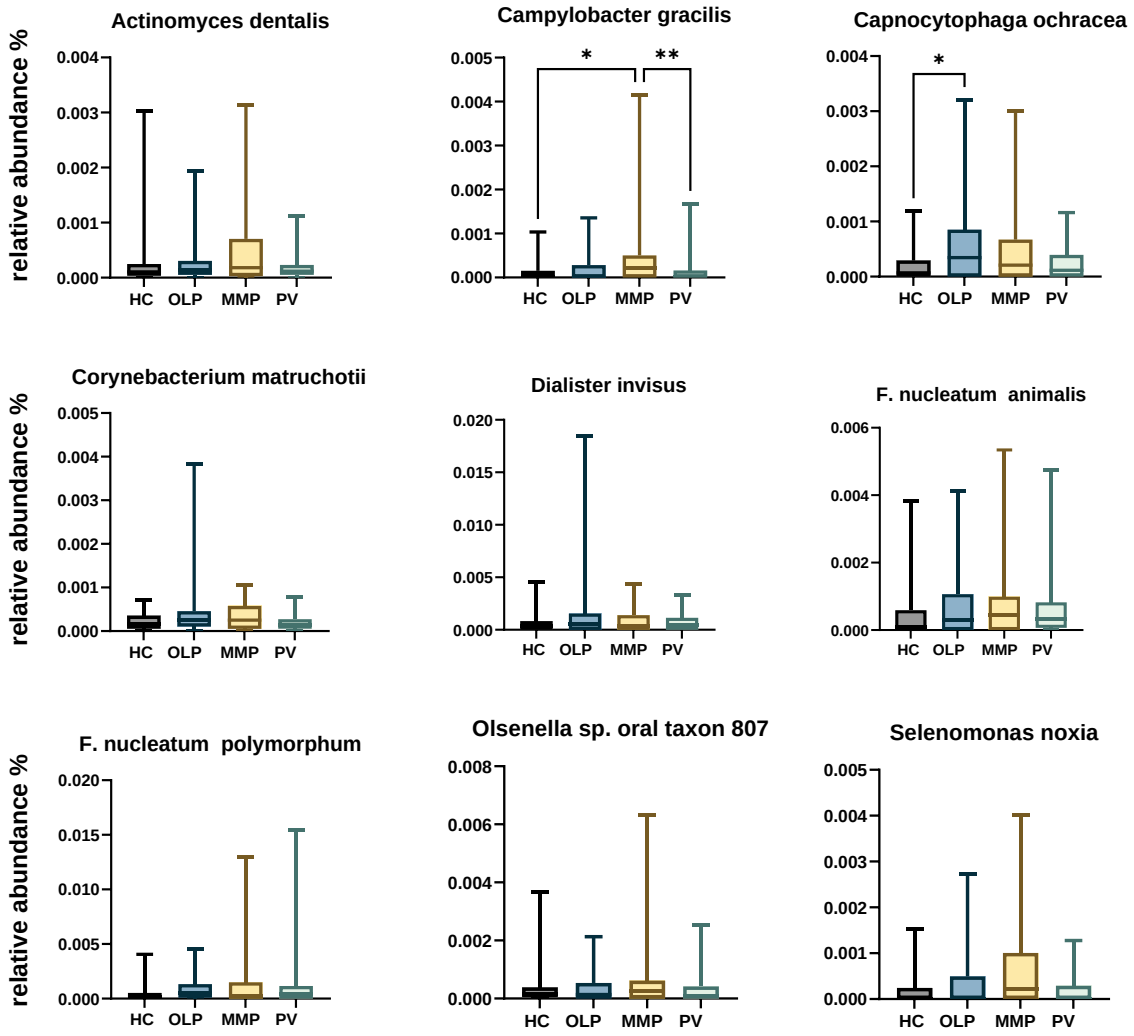


Figure 6.14 Comparison of relative abundance of different species between disease groups and HC in plaque samples. Statistical analysis done using Kruskal–Wallis followed by Dunn's multiple comparisons post hoc test **p<0.05; ** p<0.01. p values generated were corrected for false discovery rate (FDR) set to 0.05 (Benjamini-Hochberg test) to generate q (FDR) values shown in* ***Appendix 20****.*

6.1.6 Species associated with the clinical parameters in saliva samples.

To investigate the initial association between oral microorganisms and the clinical periodontal, immunological, and metabolomic variables, a Spearman rank correlation analysis was analysed. **Table 6.1** Simple linear regression was applied to understand whether microbial diversity can predict or explain variations in the disease severity in all three diseases. Both analyses provide a more comprehensive understanding of the relationship between ODSS and microbial diversity. While Spearman correlation analysis showed significant negative correlation between these two variables in SA samples of OLP group only (p=0.05), the linear regression was less significant (p=0.07). **Figure 6.15**

Further, a significant correlation was observed between certain species in each group of diseases in Spearman correlation rank analysis.

In the OLP group, *X. cellulosilytica, Actinomyces sp. ICM47, L. mirabilis, O. sinus, S. parasanguinis and S. salivarius, R. mucilaginosa 1* in saliva had a significant negative correlation with ODSS in OLP (p=0.04, p=0.012, p=0.004, p=0.014, p=0.034, p=0.002, p=0.07, respectively). In addition, from these species, *X. cellulosilytica and S. parasanguinis* inversely correlated with the PISA score (p=0.04 and p=0.0.5). Conversely, three periodontopathogens, *P. gingivalis, T. forsythia and T. denticola*, significantly correlated with PISA in these samples. No correlations of ODSS were found with periodontal pathogens.

The inflammatory markers associated with ODSS were positively correlated with the oral microbiome. IL-6 in saliva correlated negatively with *Actinomyces sp. ICM47* (p=0.07) and *O. sinus* (p=0.06). **Table 6.1**

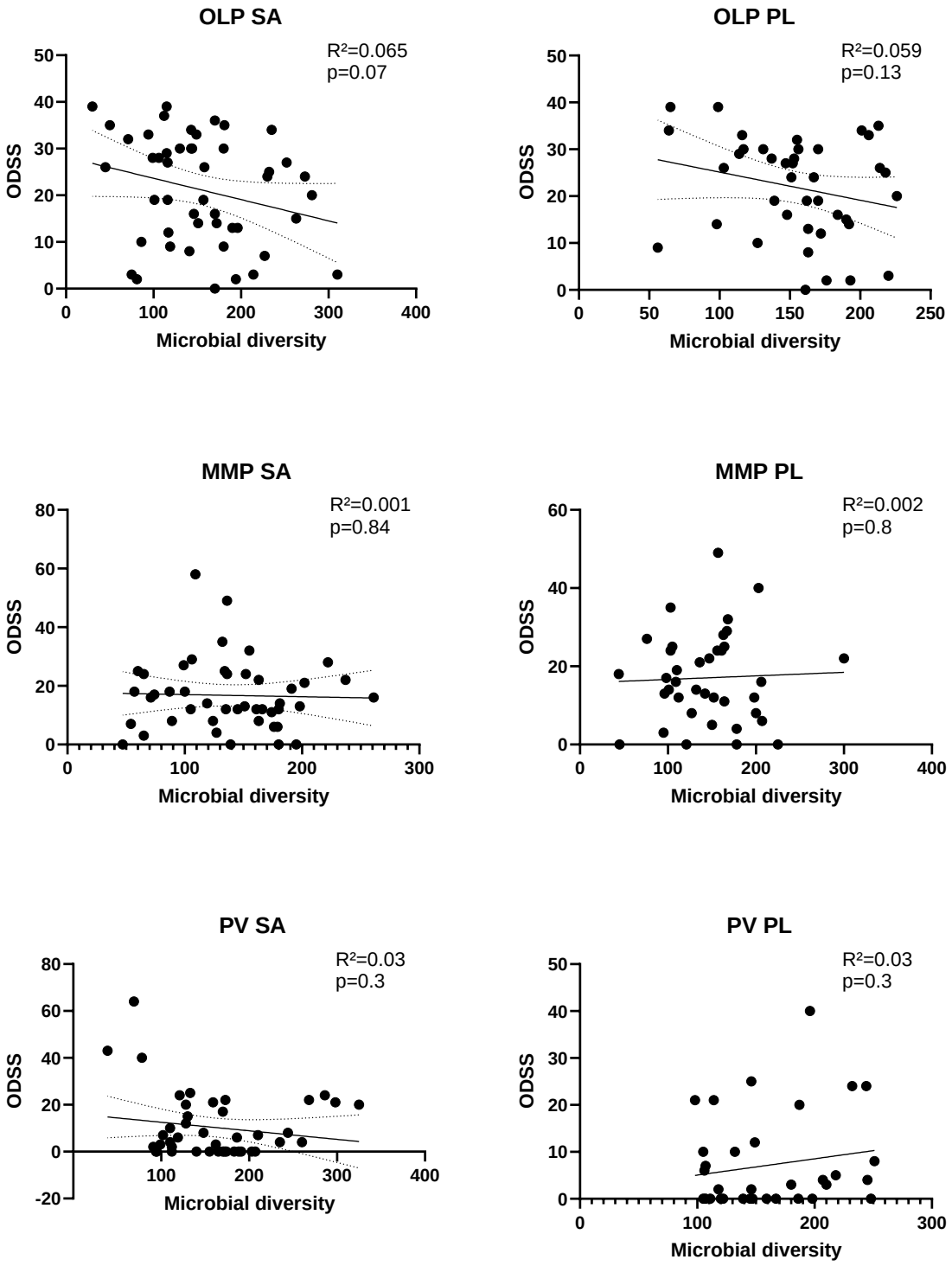


Figure 6.15. Simple linear regression analysis of association between ODSS and microbial diversity in saliva (SA) and plaque (PL) samples. *Coefficient of Determination and p values are indicated at the top right of each comparison.*

Table 6.1. Correlation between clinical, immunological and metabolomic variables in the salivary microbiome of OLP patients

p value	ODSS	PISA	Age	BMI	IL-6	IL-13	IL-8	Isovaleric acid	Cholesterol	*A. sp. ICM47*	*F. nucleatum polymorphum*	*L. mirabilis*	*O. sinus*	*P. gingivalis*	*R. mucilaginosa 1*	*S. parasanguinis*	*S. salivarius*	*T. forsythia*	*T. denticola*	*X. cellulosilytica*
ODSS		0.05	0.78	0.93	0.003	0.05	0.77	0.57	0.67	0.01	0.08	0.004	0.01	0.37	0.07	0.03	0.0025	0.61	0.86	0.04
PISA	0.05		0.10	0.06	0.78	0.22	0.05	0.05	0.75	0.34	0.002	0.67	0.17	0.07	0.0049	0.11	0.32	0.07	0.01	0.10
Age	0.78	0.10		0.13	0.03	0.06	0.34	0.15	0.09	0.32	0.88	0.11	0.31	0.33	0.71	0.07	0.66	0.53	0.44	0.78
BMI	0.93	0.06	0.13		0.07	0.74	0.31	0.27	0.28	0.75	0.36	0.55	0.94	0.40	1.00	0.13	0.03	0.43	0.87	0.93
Richness saliva	0.50	0.0016	0.91	0.50	0.18	0.52	0.05	0.01	0.73	0.03	>0.00001	0.52	0.05	0.96	0.92	0.89	0.32	0.48	0.0001	0.23
Shannon diversity saliva	0.05	0.27	0.77	0.64	0.11	0.61	0.36	0.01	0.94	0.01	0.0008	0.96	0.0001	0.60	0.08	0.55	0.25	0.44	0.01	0.0001
Richness PL	0.13	0.0012	0.48	0.72	0.14	0.27	0.82	0.37	0.79	0.15	0.07	0.57	0.86	0.52	0.48	0.29	0.71	1.00	0.86	0.53
Shannon diversity PL	0.06	0.02	0.36	0.96	0.02	0.10	0.42	0.41	0.61	0.22	0.03	0.20	0.78	0.26	0.63	0.13	0.88	0.94	0.39	0.20
IL-6 SA	0.003	0.78	0.03	0.07		0.004	0.11	0.01	0.52	0.07	0.87	0.52	0.06	0.96	0.33	0.64	0.43	0.29	0.42	0.15
IL-13 SA	0.05	0.22	0.06	0.74	0.004		0.07	0.97	0.14	0.31	0.58	0.59	0.43	1.00	0.52	0.64	0.81	0.74	0.91	0.38
IL-8 SA	0.77	0.05	0.34	0.31	0.11	0.07		0.02	0.80	0.99	0.30	0.56	0.88	0.11	0.32	0.56	0.83	0.33	0.04	0.19
Actinomyces sp. ICM47	0.01	0.34	0.32	0.75	0.07	0.31	0.99	0.17	0.78		0.73	0.82	0.0002	0.30	0.19	0.0020	0.0001	0.01	0.79	0.01
F. nucleatum polymorphum	0.08	0.0016	0.88	0.36	0.87	0.58	0.30	0.38	0.57	0.73		0.92	0.30	0.58	0.71	0.19	0.21	0.93	0.17	0.90
Lautropia mirabilis	0.004	0.67	0.11	0.55	0.52	0.59	0.56	0.20	0.14	0.82	0.92		0.86	0.51	0.58	0.34	0.91	0.79	0.83	0.90
Oribacterium sinus	0.01	0.17	0.31	0.94	0.06	0.43	0.88	0.82	0.20	0.0002	0.30	0.86		0.23	0.01	0.02	0.01	0.21	0.77	0.0001
Porphyromonas gingivalis	0.37	0.07	0.33	0.40	0.96	1.00	0.11	0.48	0.57	0.30	0.58	0.51	0.23		0.33	0.004	0.02	0.11	0.11	0.09
Rothia mucilaginosa 1	0.07	0.0049	0.71	1.00	0.33	0.52	0.32	0.29	0.86	0.19	0.71	0.58	0.01	0.33		0.34	0.02	0.92	0.80	>0.00001
Streptococcus parasanguinis	0.03	0.11	0.07	0.13	0.64	0.64	0.56	0.85	1.00	0.002	0.19	0.34	0.02	0.004	0.34		>0.00001	0.16	0.71	0.12
Streptococcus salivarius	0.002	0.32	0.66	0.03	0.43	0.81	0.83	0.50	0.68	0.0001	0.21	0.91	0.01	0.02	0.02	>0.00001		0.22	0.94	0.14
Tannerella forsythia	0.61	0.07	0.53	0.43	0.29	0.74	0.33	0.17	0.48	0.01	0.93	0.79	0.21	0.11	0.92	0.16	0.22		0.004	0.49
Treponema denticola	0.86	0.01	0.44	0.87	0.42	0.91	0.04	0.0026	0.55	0.79	0.17	0.83	0.77	0.11	0.80	0.71	0.94	0.0038		0.78
Xylanimonas cellulosilytica	0.04	0.10	0.78	0.93	0.15	0.38	0.19	0.70	0.94	0.01	0.90	0.90	0.0001	0.09	>0.00001	0.12	0.14	0.49	0.78	
Isovaleric acid SA	0.57	0.05	0.15	0.27	0.01	0.97	0.02		0.39	0.17	0.38	0.20	0.82	0.48	0.29	0.85	0.50	0.17	0.00	0.70
Cholesterol Pl	0.67	0.75	0.09	0.28	0.52	0.14	0.80	0.39		0.78	0.57	0.14	0.20	0.57	0.86	1.00	0.68	0.48	0.55	0.94

Spearman ,r	ODSS	PISA	Age	BMI	IL-6	IL-13	IL-8	Isovaleric acid	Cholesterol	*A. sp. ICM47*	*F. nucleatum subsp. polymorphum*	*L. mirabilis*	*O. sinus*	*P. gingivalis*	*R. mucilaginosa*	*S. parasanguinis*	*S. salivarius*	*T. forsythia*	*T. denticola*	*X. cellulosilytica*
ODSS	1.00	0.29	-0.04	0.02	0.43	0.30	0.05	0.09	-0.06	-0.36	0.26	-0.41	-0.35	0.13	-0.27	-0.31	-0.43	-0.07	0.03	-0.30
PISA	0.29	1.00	-0.25	0.47	0.04	0.20	0.32	0.31	-0.05	-0.14	0.45	-0.06	-0.21	0.26	-0.40	-0.24	-0.15	0.27	0.38	-0.24
Age	-0.04	-0.25	1.00	-0.37	-0.32	-0.29	0.16	0.23	0.25	0.15	0.02	-0.23	0.15	0.14	0.06	0.27	0.07	-0.09	0.11	-0.04
BMI	0.02	0.47	-0.37	1.00	0.45	-0.09	0.28	-0.31	-0.28	-0.08	0.23	-0.15	-0.02	-0.21	0.00	0.37	0.51	-0.20	0.04	-0.02
Richness saliva	-0.10	0.45	-0.02	0.17	-0.20	0.10	0.32	0.42	0.05	0.32	0.61	0.10	0.29	0.01	-0.02	-0.02	0.15	0.10	0.52	0.18
Shannon diversity saliva	-0.29	0.17	-0.04	0.12	-0.24	-0.08	0.15	0.39	0.01	0.38	0.47	-0.01	0.52	-0.08	0.25	0.09	0.17	0.11	0.39	0.52
Richness PL	-0.25	0.51	-0.12	0.10	-0.26	-0.20	-0.04	0.16	-0.05	0.24	0.30	0.09	-0.03	0.11	-0.12	-0.18	-0.06	0.00	0.03	0.11
Shannon diversity PL	-0.31	0.37	-0.15	0.02	-0.39	-0.29	-0.15	0.15	0.09	0.20	0.36	0.21	0.05	0.19	0.08	-0.25	-0.03	0.01	0.14	0.21
IL-6 SA	0.43	0.04	-0.32	0.45	1.00	0.43	0.27	-0.41	-0.10	-0.28	-0.03	-0.10	-0.29	-0.01	-0.15	-0.07	-0.12	-0.16	-0.12	-0.22
IL-13 SA	0.30	0.20	-0.29	-0.09	0.43	1.00	0.30	0.01	0.24	-0.16	0.09	-0.08	-0.12	0.00	-0.10	-0.07	-0.04	0.05	-0.02	-0.14
IL-8 SA	0.05	0.32	0.16	0.28	0.27	0.30	1.00	0.39	0.04	0.00	0.17	-0.10	-0.03	0.26	-0.16	0.10	0.04	0.16	0.33	-0.21
Actinomyces sp. ICM47	-0.36	-0.14	0.15	-0.08	-0.28	-0.16	0.00	0.22	-0.04	1.00	0.05	-0.03	0.52	-0.15	0.19	0.43	0.52	-0.39	-0.04	0.39
F. nucleatum polymorphum	0.26	0.45	0.02	0.23	-0.03	0.09	0.17	0.14	0.09	0.05	1.00	-0.01	0.15	0.08	-0.05	-0.19	-0.19	0.01	0.20	0.02
Lautropia mirabilis	-0.41	-0.06	-0.23	-0.15	-0.10	-0.08	-0.10	-0.21	0.22	-0.03	-0.01	1.00	-0.03	-0.10	-0.08	-0.14	0.02	0.04	-0.03	-0.02
Oribacterium sinus	-0.35	-0.21	0.15	-0.02	-0.29	-0.12	-0.03	-0.04	0.19	0.52	0.15	-0.03	1.00	-0.18	0.36	0.35	0.40	-0.18	0.04	0.53
Porphyromonas gingivalis	0.13	0.26	0.14	-0.21	-0.01	0.00	0.26	0.11	0.09	-0.15	0.08	-0.10	-0.18	1.00	-0.14	-0.41	-0.35	0.23	0.23	-0.25
Rothia mucilaginosa 1	-0.27	-0.40	0.06	0.00	-0.15	-0.10	-0.16	-0.17	0.03	0.19	-0.05	-0.08	0.36	-0.14	1.00	0.14	0.33	-0.02	-0.04	0.67
Streptococcus parasanguinis	-0.31	-0.24	0.27	0.37	-0.07	-0.07	0.10	-0.03	0.00	0.43	-0.19	-0.14	0.35	-0.41	0.14	1.00	0.65	-0.21	-0.06	0.23
Streptococcus salivarius	-0.43	-0.15	0.07	0.51	-0.12	-0.04	0.04	-0.11	-0.06	0.52	-0.19	0.02	0.40	-0.35	0.33	0.65	1.00	-0.18	0.01	0.22
Tannerella forsythia	-0.07	0.27	-0.09	-0.20	-0.16	0.05	0.16	0.22	-0.11	-0.39	0.01	0.04	-0.18	0.23	-0.02	-0.21	-0.18	1.00	0.41	-0.10
Treponema denticola	0.03	0.38	0.11	0.04	-0.12	-0.02	0.33	0.46	-0.09	-0.04	0.20	-0.03	0.04	0.23	-0.04	-0.06	0.01	0.41	1.00	0.04
Xylanimonas cellulosilytica	-0.30	-0.24	-0.04	-0.02	-0.22	-0.14	-0.21	0.06	-0.01	0.39	0.02	-0.02	0.53	-0.25	0.67	0.23	0.22	-0.10	0.04	1.00
Isovaleric acid SA	0.09	0.31	0.23	-0.31	-0.41	0.01	0.39	1.00	-0.14	0.22	0.14	-0.21	-0.04	0.11	-0.17	-0.03	-0.11	0.22	0.46	0.06
Cholesterol Pl	-0.06	-0.05	0.25	-0.28	-0.10	0.24	0.04	-0.14	1.00	-0.04	0.09	0.22	0.19	0.09	0.03	0.00	-0.06	-0.11	-0.09	-0.01

In the MMP group, a significant positive correlation of ODSS was observed with *Capnocytophaga sputigena* (p=0.03), L*. hofstadii* (p=0.033)*, Neisseria cinerea* (p=0.004), N*eisseria meningitidis (*p=0.01) and *Prevotella saccharolytica* (p=0.005). In contrast*, R. mucilaginosa* (p=0.004) *and Actinomyces meyeri (*p=0.03*)* negatively correlated with ODSS. No correlations were found with periodontal pathogens in the MMP group. PISA score was positively correlated with the red circle periodontal bacteria, *T. forsythia* and *T. denticola (*p=0.06 and p=0.034 respectively).

Inflammatory markers of MMP group correlated significantly with both Neisseria species associated with the ODSS. Particularly, IL-6 in serum, salivary IL-13 and MMP-3 were positively associated with *N. cinerea and N. meningitidis*. No associations were found between salivary metabolites and ODSS. However, isovaleric acid was positively associated with the *T. forsythia* and two ODSS-associated *Neisseria* species. **Table 6.2**

The MMP group was further stratified into subgroups based on the treatment, including topical, immunosuppressive (IS), and antibiotic (AB) therapies. The analysis revealed that among MMP patients undergoing immunosuppressive therapy, the previously observed correlations between the oral microbiome and ODSS, periodontal parameters, and metabolomics appeared to weaken. This suggests that the treatment regimen may have an impact on the microbiome and its associations with the disease. Immunosuppressive therapy is known to alter the immune response, and this may have downstream effects on the oral microbiome (Maier L et al., 2018) It is possible that the altered immune response and subsequent changes in the oral microbiome may contribute to the resolution of MMP symptoms. However, it is essential to have a cautious approach to interpretation of these findings. The subgroup analysis is constrained by the small sample size, which increases the risk of both type 1 and type 2 errors. Therefore, while these results provide valuable insights, they should be viewed with consideration of the limitations associated with subgroup analysis, and further research with larger sample sizes is needed to confirm findings.

Finally, in PV, a positive association with ODSS was observed with *E. corrodens* (p=0.01), *F. nucleatum* (p=0.03), *G. morbillorum* (p=0.0001), *G. elegans* (p=0.03), *H. sapiens* (p=0.02) and T*. vincentii* (p=0.02). Inverse correlation of ODSS was observed with *Actinomyces sp. ICM47* (p=0.003*), C. concisus (*p=0.01), *Isoptericola variabilis* (0.04)*, Lachnoanaerobaculum saburrum* (p=0.01*), S. parasanguinis(*p=0.02) *and S. salivarius* (p=0.001*).* From the cytokine perspective,

the ODSS associated cytokines IL-6, IL-1β and MMP-3 showed significant positive correlation with *F. nucleatum, G. morbillorum, H. sapiens and T. vincentii* ($p>0.05$*).* The salivary metabolites choline significantly correlated with *F. nucleatum, H. sapiens and T. vincentii.* **Table 6.3**

Table 6.2. Correlation between clinical, immunological and metabolomic variables in the salivary microbiome of MMP patients

P value	ODSS	PISA	Age	BMI	*A. meyeri*	*C. sputigena*	*L. hofstadii*	*N. cinerea*	*N. meningitidis*	*P. saccharolytica*	*R. mucilaginosa*	*T. forsythia*	*T. denticola*	IL-6 SA	IL-6 SE	IL-13 SA	IL-1b SA	IL17A SE	MMP-3 SA	MMP 3 SE	MMP 9 SA
ODSS		0.90	0.66	0.05	0.03	0.03	0.02	0.004	0.01	0.005	0.004	0.08	0.68	0.001	0.03	0.61	0.51	0.01	0.005	0.01	0.11
PISA	0.90		0.11	0.86	0.21	0.04	0.45	0.28	0.58	0.10	0.49	0.04	0.03	0.59	0.25	0.04	0.02	0.15	0.07	0.81	0.04
Age	0.66	0.11		0.60	0.74	0.48	0.02	0.55	0.82	0.36	0.17	0.65	0.96	0.44	0.84	0.05	0.31	0.93	0.21	0.69	0.91
BMI	0.05	0.86	0.60		0.67	0.30	0.20	0.32	0.32	0.78	0.72	0.47	0.87	0.50	0.32	0.21	0.08	0.04	0.04	0.23	0.43
Species richness SA	0.53	0.11	0.65	0.18	0.30	0.05	0.001	0.01	0.05	0.03	0.75	0.03	0.93	0.41	0.52	0.02	0.12	0.37	0.62	0.61	0.88
Shannon diversity SA	0.73	0.01	0.86	0.54	0.11	0.30	0.002	0.52	0.97	0.02	0.59	0.001	0.45	0.17	0.40	0.05	0.03	0.88	0.87	0.83	0.67
Species Richness PL	0.22	0.004	0.28	0.84	0.79	0.30	0.52	0.45	0.83	0.25	1.00	0.004	0.05	0.99	0.47	0.002	0.01	0.48	0.92	0.02	0.32
Shannon diversity PL	0.36	0.01	0.11	0.53	0.34	0.25	0.34	0.43	0.91	0.16	0.87	0.01	0.04	0.50	0.68	0.001	0.004	0.52	0.78	0.06	0.91
A. meyeri	0.03	0.21	0.74	0.67		0.28	0.69	0.42	0.19	0.32	0.01	0.04	0.30	0.47	0.06	0.18	0.04	0.07	0.78	0.71	0.47
C. sputigena	0.03	0.04	0.48	0.30	0.28		0.00	0.12	0.12	0.001	0.26	0.82	0.58	0.27	0.06	0.57	0.96	0.43	0.01	0.07	0.62
L. hofstadii	0.02	0.45	0.02	0.20	0.69	0.0001		0.06	0.20	0.0001	0.20	0.75	0.99	0.20	0.40	0.74	0.94	0.73	0.54	0.30	0.61
Neisseria cinerea	0.004	0.28	0.55	0.32	0.42	0.12	0.06		>0.00001	0.27	0.52	0.95	0.93	0.26	0.08	0.04	0.07	0.93	0.01	0.04	0.09
Neisseria meningitidis	0.01	0.58	0.82	0.32	0.19	0.12	0.20	>0.00001		0.68	0.60	0.49	0.27	0.11	0.01	0.04	0.09	0.59	0.01	0.06	0.13
Prevotella saccharolytica	0.005	0.10	0.36	0.78	0.32	0.001	0.0001	0.27	0.68		0.75	0.87	0.40	0.29	0.75	0.85	0.54	0.45	0.30	0.87	0.81
Rothia mucilaginosa	0.004	0.49	0.17	0.72	0.01	0.26	0.20	0.52	0.60	0.75		0.44	0.24	0.44	0.24	0.83	0.56	0.45	0.61	0.51	0.18
T. forsythia	0.08	0.04	0.65	0.47	0.04	0.82	0.75	0.95	0.49	0.87	0.44		0.02	0.02	0.18	0.20	0.05	0.82	0.06	0.06	0.44
T. denticola	0.68	0.03	0.96	0.87	0.30	0.58	0.99	0.93	0.27	0.40	0.24	0.02		0.41	0.02	0.36	0.19	0.12	0.81	0.15	0.50
IL-6 SA	0.001	0.59	0.44	0.50	0.47	0.27	0.20	0.26	0.11	0.29	0.44	0.02	0.41		0.01	0.01	0.01	0.57	0.00	0.32	0.53
IL6 SE	0.03	0.25	0.84	0.32	0.06	0.06	0.40	0.08	0.01	0.75	0.24	0.18	0.02	0.01		0.005	0.82	0.10	0.02	0.10	0.58
IL-13 SA	0.61	0.04	0.05	0.21	0.18	0.57	0.74	0.04	0.04	0.85	0.83	0.20	0.36	0.01	0.005		>0.00001	0.84	0.01	0.80	0.13
IL-1b SA	0.51	0.02	0.31	0.08	0.04	0.96	0.94	0.07	0.09	0.54	0.56	0.05	0.19	0.01	0.82	>0.00001		0.92	0.01	0.45	0.04
IL17A SE	0.005	0.07	0.21	0.04	0.78	0.01	0.54	0.01	0.01	0.30	0.61	0.06	0.81	>0.00001	0.02	0.01	0.01	0.71		0.02	0.10
MMP-3 SA	0.01	0.81	0.69	0.23	0.71	0.07	0.30	0.04	0.06	0.87	0.51	0.06	0.15	0.32	0.10	0.80	0.45	0.60	0.02		0.88
MMP 3 SE	0.11	0.04	0.91	0.43	0.47	0.62	0.61	0.09	0.13	0.81	0.18	0.44	0.50	0.53	0.58	0.13	0.04	0.88	0.10	0.88	
MMP 9 SA	0.85	0.01	0.63	0.23	0.14	0.76	0.50	0.10	0.07	0.96	0.71	0.001	0.09	0.49	0.78	0.03	0.0001	0.98	0.99	0.29	0.48
Butyric acid	0.85	0.01	0.63	0.23	0.14	0.76	0.50	0.10	0.07	0.96	0.71	0.001	0.09	0.49	0.78	0.03	0.0001	0.98	0.99	0.29	0.48
Isovaleric acid	0.83	0.001	0.57	0.03	0.03	0.36	0.47	0.06	0.05	0.21	0.70	0.001	0.19	0.70	0.82	0.001	>0.00001	0.90	0.22	0.52	0.40

Spearman, r	ODSS	PISA	Age	BMI	*A. meyeri*	*C. sputigena*	*L. hofstadii*	*N. cinerea*	*N. meningitidis*	*P. saccharolytica*	*Rothia mucilaginosa*	*T. forsythia*	*T. denticola*	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17A SE	MMP-3 SA	MMP 3 SE	MMP 9 SA
ODSS	1.00	0.02	0.07	-0.43	-0.32	0.33	0.34	0.43	0.41	0.42	-0.43	-0.26	-0.06	0.48	0.34	0.09	0.11	-0.38	0.44	0.42	0.34
PISA	0.02	1.00	-0.26	-0.04	0.20	0.32	0.12	0.18	0.09	0.26	0.11	0.32	0.34	0.09	-0.19	0.33	0.38	-0.24	0.30	-0.05	0.45
Age	0.07	-0.26	1.00	0.12	-0.05	0.11	0.36	-0.09	-0.03	0.14	-0.21	-0.07	0.01	-0.12	-0.03	-0.32	-0.17	-0.01	-0.20	-0.07	0.03
BMI	-0.43	-0.04	0.12	1.00	0.10	-0.24	-0.29	-0.23	-0.23	-0.06	-0.08	-0.17	0.04	-0.16	-0.24	-0.32	-0.44	0.50	-0.47	-0.32	-0.28
Species richness SA	0.10	0.25	0.07	-0.30	0.16	0.29	0.48	0.39	0.30	0.34	-0.05	0.33	0.01	-0.13	0.10	0.38	0.25	-0.14	0.08	0.09	-0.03
Shannon diversity SA	-0.05	0.43	0.03	-0.14	0.24	0.16	0.46	0.10	-0.01	0.36	0.08	0.47	0.12	-0.22	-0.13	0.32	0.34	0.02	0.03	0.04	0.09
Species Richness PL	-0.22	0.49	-0.19	0.06	0.05	0.18	0.12	0.13	0.04	0.20	0.00	0.48	0.34	0.00	0.13	0.53	0.45	-0.13	-0.02	-0.44	0.23
Shannon diversity PL	-0.16	0.47	-0.28	0.17	0.17	0.20	0.17	0.14	0.02	0.25	0.03	0.47	0.35	0.13	0.07	0.57	0.50	-0.12	0.05	-0.36	-0.03
A. meyeri	-0.32	0.20	-0.05	0.10	1.00	-0.17	-0.06	-0.13	-0.20	0.15	0.38	0.32	0.16	-0.12	-0.29	0.22	0.32	0.28	-0.05	-0.06	0.16
C. sputigena	0.33	0.32	0.11	-0.24	-0.17	1.00	0.56	0.24	0.24	0.47	-0.17	-0.04	0.09	0.18	0.29	0.09	0.01	-0.13	0.40	0.31	0.11
L. hofstadii	0.34	0.12	0.36	-0.29	-0.06	0.56	1.00	0.29	0.20	0.55	-0.20	0.05	0.00	-0.20	0.13	-0.05	-0.01	-0.06	0.10	0.18	0.11
Neisseria cinerea	0.43	0.18	-0.09	-0.23	-0.13	0.24	0.29	1.00	0.91	0.17	-0.10	0.01	-0.01	0.18	0.27	0.33	0.30	0.01	0.39	0.35	0.36
Neisseria meningitidis	0.41	0.09	-0.03	-0.23	-0.20	0.24	0.20	0.91	1.00	0.06	-0.08	-0.11	-0.17	0.26	0.39	0.34	0.27	0.09	0.39	0.32	0.33
Prevotella saccharolytica	0.42	0.26	0.14	-0.06	0.15	0.47	0.55	0.17	0.06	1.00	-0.05	-0.03	0.13	0.17	0.05	0.03	0.10	-0.12	0.17	0.03	0.05
Rothia mucilaginosa	-0.43	0.11	-0.21	-0.08	0.38	-0.17	-0.20	-0.10	-0.08	-0.05	1.00	0.12	-0.18	-0.12	-0.19	0.04	-0.10	0.12	-0.08	0.11	-0.29
T. forsythia	-0.26	0.32	-0.07	-0.17	0.32	-0.04	0.05	0.01	-0.11	-0.03	0.12	1.00	0.35	-0.36	-0.21	0.21	0.32	-0.04	-0.30	-0.32	0.17
T. denticola	-0.06	0.34	0.01	0.04	0.16	0.09	0.00	-0.01	-0.17	0.13	-0.18	0.35	1.00	-0.13	-0.35	-0.15	0.21	-0.25	-0.04	-0.25	0.15
IL-6 SA	0.48	0.09	-0.12	-0.16	-0.12	0.18	-0.20	0.18	0.26	0.17	-0.12	-0.36	-0.13	1.00	0.39	0.44	0.39	-0.09	0.73	0.18	0.14
IL6 SE	0.34	-0.19	-0.03	-0.24	-0.29	0.29	0.13	0.27	0.39	0.05	-0.19	-0.21	-0.35	0.39	1.00	0.45	0.04	0.27	0.38	0.29	-0.12
IL-13 SA	0.09	0.33	-0.32	-0.32	0.22	0.09	-0.05	0.33	0.34	0.03	0.04	0.21	-0.15	0.44	0.45	1.00	0.70	-0.03	0.44	0.05	0.32
IL-1b SA	0.11	0.38	-0.17	-0.44	0.32	0.01	-0.01	0.30	0.27	0.10	-0.10	0.32	0.21	0.39	0.04	0.70	1.00	-0.02	0.41	-0.14	0.43
IL-17A SE	-0.38	-0.24	-0.01	0.50	0.28	-0.13	-0.06	0.01	0.09	-0.12	0.12	-0.04	-0.25	-0.09	0.27	-0.03	-0.02	1.00	-0.06	-0.10	-0.04
MMP-3 SA	0.44	0.30	-0.20	-0.47	-0.05	0.40	0.10	0.39	0.39	0.17	-0.08	-0.30	-0.04	0.73	0.38	0.44	0.41	-0.06	1.00	0.41	0.36
MMP-3 SE	0.42	-0.05	-0.07	-0.32	-0.06	0.31	0.18	0.35	0.32	0.03	0.11	-0.32	-0.25	0.18	0.29	0.05	-0.14	-0.10	0.41	1.00	0.04
MMP-9 SA	0.34	0.45	0.03	-0.28	0.16	0.11	0.11	0.36	0.33	0.05	-0.29	0.17	0.15	0.14	-0.12	0.32	0.43	-0.04	0.36	0.04	1.00
Butyric acid	-0.03	0.39	0.08	-0.31	0.24	-0.05	0.11	0.26	0.29	0.01	-0.06	0.51	0.27	-0.12	-0.05	0.34	0.57	0.00	0.00	-0.20	0.16
Isovaleric acid	0.03	0.49	-0.09	-0.54	0.35	0.15	0.12	0.29	0.31	0.20	0.06	0.51	0.21	0.06	0.04	0.50	0.64	0.02	0.21	-0.12	0.18

Table 6.3. Correlation between clinical, immunological and metabolomic variables in the salivary microbiome of PV patients.

p value	ODSS	PISA	Age	BMI	*A. sp. ICM47*	*C. concisus*	*E. corrodens*	*F. nucleatum*	*G.morbillorum*	*G. elegans*	*H. sapiens*	*I. variabilis*	*L. saburreum*	*L. sp. ICM7*	*P. gingivalis*	*S. parasanguinis*	*S. salivarius*	*T. vincentii*	IL-6 SA	IL-1b SA	MMP-3 SA	Choline SA	Glucose PL
ODSS		0.02	0.55	0.49	0.00	0.04	0.01	0.03	0.00	0.03	0.02	0.04	0.01	0.02	0.33	0.02	0.001	0.02	>0.00001	0.03	>0.00001	0.04	0.01
PISA	0.02		0.01	0.22	0.04	0.02	0.28	0.01	0.01	0.01	0.01	0.05	0.002	0.85	0.01	0.17	0.12	0.56	0.26	0.08	0.06	0.10	0.90
Age	0.55	0.01		0.47	0.79	0.76	0.16	0.23	0.83	0.33	0.34	0.31	0.49	0.85	0.91	0.96	0.47	0.62	0.87	0.84	0.71	0.55	0.38
BMI	0.49	0.22	0.47		0.22	0.82	0.46	0.82	0.41	0.41	0.95	0.84	0.46	0.02	0.22	0.43	0.79	0.70	0.10	0.42	0.04	0.73	0.02
Richness saliva	0.39	0.47	0.49	0.96	0.01	0.001	0.11	0.20	0.05	0.03	0.002	0.05	0.29	0.0001	0.10	0.52	0.94	0.04	0.95	0.96	0.85	0.84	0.77
Shannon diversity saliva	0.41	0.78	0.93	0.91	0.02	>0.00001	0.03	0.50	0.04	0.02	>0.00001	0.08	0.12	0.0001	0.04	0.89	0.70	0.07	0.53	0.65	0.65	0.84	0.50
Richness PL	0.15	0.49	0.39	0.02	0.17	0.28	0.70	0.42	0.30	0.87	0.42	0.04	0.33	0.09	0.06	0.36	0.64	0.32	0.83	0.11	0.39	0.99	0.93
Shannon diversity PL	0.47	0.68	0.50	0.06	0.05	0.69	0.81	0.91	0.61	0.87	0.96	0.32	0.63	0.36	0.11	0.04	0.24	0.70	0.84	0.85	0.64	0.26	0.61
Actinomyces sp. ICM47	0.003	0.04	0.79	0.22		0.07	0.08	0.18	0.12	0.82	0.01	0.78	0.02	0.09	0.86	0.34	0.44	0.43	0.06	0.23	0.01	0.15	0.68
C. concisus	0.04	0.02	0.76	0.82	0.07		0.54	0.46	0.38	0.48	>0.00001	0.07	>0.00001	0.01	0.29	0.61	0.80	0.65	0.38	0.05	0.24	0.18	0.91
Eikenella corrodens	0.01	0.28	0.16	0.46	0.08	0.54		0.05	0.0002	0.003	0.63	0.18	0.08	0.36	0.13	0.01	0.00	0.04	0.07	0.72	0.22	0.52	0.01
F. nucleatum	0.03	0.01	0.23	0.82	0.18	0.46	0.05		0.05	0.83	0.02	0.59	0.12	0.19	0.22	0.62	0.11	0.03	0.01	0.003	0.002	0.003	0.74
Gemella morbillorum	0.0001	0.01	0.83	0.41	0.12	0.38	0.0002	0.05		0.001	0.14	>0.00001	0.04	0.56	0.21	0.002	0.004	0.002	0.04	0.03	0.02	0.10	0.07
Granulicatella elegans	0.03	0.01	0.33	0.41	0.82	0.48	0.003	0.83	0.00		0.61	0.03	0.05	0.05	0.37	0.03	0.06	0.22	0.22	0.66	0.38	0.50	0.28
Homo sapiens	0.02	0.01	0.34	0.95	0.01	>0.00001	0.63	0.02	0.14	0.61		0.02	0.0001	>0.00001	0.60	0.43	0.82	0.49	0.01	0.07	0.02	0.02	0.67
Isoptericola variabilis	0.04	0.05	0.31	0.84	0.78	0.07	0.18	0.59	0.00	0.03	0.02		0.002	0.88	0.08	0.03	0.29	0.15	0.26	0.33	0.29	0.22	0.01
L. saburreum	0.01	0.002	0.49	0.46	0.02	>0.00001	0.08	0.12	0.04	0.05	0.0001	0.002		0.14	0.82	0.03	0.05	0.97	0.05	0.24	0.16	0.25	0.95
L. sp. ICM7	0.02	0.85	0.85	0.02	0.09	0.01	0.36	0.19	0.56	0.05	>0.00001	0.88	0.14		0.56	0.23	0.98	0.64	0.06	0.05	0.07	0.18	0.19
P. gingivalis	0.33	0.01	0.91	0.22	0.86	0.29	0.13	0.22	0.21	0.37	0.60	0.08	0.82	0.56		0.86	0.81	0.52	0.59	0.56	0.74	0.76	0.21
S. parasanguinis	0.02	0.17	0.96	0.43	0.34	0.61	0.01	0.62	0.002	0.03	0.43	0.03	0.03	0.23	0.86		0.00	0.09	0.13	0.74	0.13	0.68	0.23
S. salivarius	0.001	0.12	0.47	0.79	0.44	0.80	0.002	0.11	0.004	0.06	0.82	0.29	0.05	0.98	0.81	>0.00001		0.10	0.05	0.24	0.10	0.97	0.23
T. vincentii	0.02	0.56	0.62	0.70	0.43	0.65	0.04	0.03	0.002	0.22	0.49	0.15	0.97	0.64	0.52	0.09	0.10		0.29	0.02	0.02	0.01	0.90
IL-6 SA	>0.00001	0.26	0.87	0.10	0.06	0.38	0.07	0.01	0.04	0.22	0.01	0.26	0.05	0.06	0.59	0.13	0.05	0.29		0.001	>0.00001	0.001	0.20
IL-1b SA	0.03	0.08	0.84	0.42	0.23	0.05	0.72	0.003	0.03	0.66	0.07	0.33	0.24	0.05	0.56	0.74	0.24	0.02	0.001		>0.00001	>0.00001	0.53
MMP-3 SA	>0.00001	0.06	0.71	0.04	0.01	0.24	0.22	0.002	0.02	0.38	0.02	0.29	0.16	0.07	0.74	0.13	0.10	0.02	>0.00001	>0.00001		0.001	0.34
Choline SA	0.04	0.10	0.55	0.73	0.15	0.18	0.52	0.003	0.10	0.50	0.02	0.22	0.25	0.18	0.76	0.68	0.97	0.01	0.001	>0.00001	0.001		0.92
Glucose PL	0.01	0.90	0.38	0.02	0.68	0.91	0.01	0.74	0.07	0.28	0.67	0.01	0.95	0.19	0.21	0.23	0.23	0.90	0.20	0.53	0.34	0.92	

Spearman, r	ODSS	PISA	Age	BMI	A. sp. ICM47	C. concisus	E. corrodens	F. nucleatum	G.morbillorum	G. elegans	H. sapiens	I. variabilis	L. saburreum	L. sp. ICM7	P. gingivalis	S. parasanguinis	S. salivarius	T. vincentii	IL-6 SA	IL-1b SA	MMP-3 SA	Choline SA	Glucose PL
ODSS	1.00	0.34	-0.09	0.15	-0.43	-0.30	0.40	0.31	0.56	0.32	0.34	-0.31	-0.36	-0.34	0.15	-0.34	-0.47	0.33	0.61	0.34	0.60	0.32	0.38
PISA	0.34	1.00	-0.37	-0.28	-0.31	-0.36	0.17	0.38	0.41	0.41	0.39	-0.30	-0.45	-0.03	0.41	-0.21	-0.24	0.09	0.18	0.29	0.30	0.27	0.02
Age	-0.09	-0.37	1.00	0.16	0.04	0.05	0.21	-0.18	-0.03	-0.15	-0.14	0.15	0.10	-0.03	-0.02	0.01	-0.11	-0.07	-0.03	-0.03	-0.06	-0.09	0.13
BMI	0.15	-0.28	0.16	1.00	0.28	-0.05	-0.17	-0.05	-0.19	-0.19	0.01	0.05	0.17	-0.50	0.27	0.18	0.06	-0.09	0.40	0.20	0.48	-0.09	0.48
Richness saliva	-0.13	0.11	-0.11	-0.01	0.36	0.49	0.24	0.19	0.29	0.33	-0.44	-0.29	0.16	0.53	0.25	-0.10	0.01	0.30	0.01	-0.01	0.03	0.03	0.04
Shannon diversity saliva	-0.12	0.04	-0.01	-0.03	0.33	0.56	0.32	0.10	0.30	0.36	-0.57	-0.26	0.23	0.53	0.31	-0.02	-0.06	0.27	-0.10	-0.07	-0.07	0.03	0.10
Richness PL	0.26	0.13	-0.15	0.62	0.24	-0.19	-0.07	0.15	0.18	0.03	0.14	-0.36	-0.17	-0.30	0.34	-0.16	-0.08	0.18	0.04	0.30	0.16	0.00	-0.02
Shannon diversity PL	0.13	0.07	-0.12	0.50	0.35	-0.07	0.04	0.02	0.09	-0.03	0.01	-0.18	-0.09	-0.17	0.28	-0.37	-0.21	0.07	-0.04	0.04	-0.09	-0.21	0.09
Actinomyces sp. ICM47	-0.43	-0.31	0.04	0.28	1.00	0.27	-0.26	-0.20	-0.23	0.03	-0.39	-0.04	0.35	0.25	-0.03	0.15	0.12	-0.12	-0.29	-0.19	-0.40	-0.22	0.06
C. concisus 2	-0.30	-0.36	0.05	-0.05	0.27	1.00	0.09	-0.11	-0.13	-0.11	-0.63	0.27	0.64	0.38	-0.16	0.08	0.04	0.07	-0.14	-0.31	-0.18	-0.21	-0.02
Eikenella corrodens	0.40	0.17	0.21	-0.17	-0.26	0.09	1.00	0.29	0.52	0.43	-0.07	-0.20	-0.26	0.14	0.23	-0.37	-0.45	0.30	0.28	0.06	0.19	0.10	0.36
F. nucleatum animalis	0.31	0.38	-0.18	-0.05	-0.20	-0.11	0.29	1.00	0.29	0.03	0.35	-0.08	-0.23	-0.20	0.18	-0.08	-0.24	0.31	0.42	0.45	0.45	0.44	-0.05
Gemella morbillorum	0.56	0.41	-0.03	-0.19	-0.23	-0.13	0.52	0.29	1.00	0.48	0.22	-0.56	-0.30	0.09	0.19	-0.45	-0.41	0.44	0.32	0.34	0.36	0.25	0.27
Granulicatella elegans	0.32	0.41	-0.15	-0.19	0.03	-0.11	0.43	0.03	0.48	1.00	-0.08	-0.31	-0.29	0.29	0.13	-0.31	-0.27	0.18	0.19	-0.07	0.14	0.11	0.16
Homo sapiens	0.34	0.39	-0.14	0.01	-0.39	-0.63	-0.07	0.35	0.22	-0.08	1.00	-0.35	-0.54	-0.57	0.08	-0.12	0.03	-0.10	0.39	0.28	0.35	0.36	0.06
Isoptericola variabilis	-0.31	-0.30	0.15	0.05	-0.04	0.27	-0.20	-0.08	-0.56	-0.31	-0.35	1.00	0.44	0.02	-0.26	0.32	0.16	-0.21	-0.18	-0.15	-0.16	-0.19	-0.40
L. saburreum	-0.36	-0.45	0.10	0.17	0.35	0.64	-0.26	-0.23	-0.30	-0.29	-0.54	0.44	1.00	0.22	-0.03	0.33	0.29	0.01	-0.31	-0.19	-0.22	-0.18	0.01
L. sp. ICM7	-0.34	-0.03	-0.03	-0.50	0.25	0.38	0.14	-0.20	0.09	0.29	-0.57	0.02	0.22	1.00	-0.09	-0.18	0.00	0.07	-0.29	-0.30	-0.28	-0.21	-0.20
P. gingivalis	0.15	0.41	-0.02	0.27	-0.03	-0.16	0.23	0.18	0.19	0.13	0.08	-0.26	-0.03	-0.09	1.00	0.03	-0.04	0.10	-0.09	0.09	-0.05	0.05	0.19
S. parasanguinis	-0.34	-0.21	0.01	0.18	0.15	0.08	-0.37	-0.08	-0.45	-0.31	-0.12	0.32	0.33	-0.18	0.03	1.00	0.66	-0.26	-0.24	-0.05	-0.24	0.06	-0.18
S. salivarius	-0.47	-0.24	-0.11	0.06	0.12	0.04	-0.45	-0.24	-0.41	-0.27	0.03	0.16	0.29	0.00	-0.04	0.66	1.00	-0.25	-0.30	-0.19	-0.25	0.01	-0.18
T. vincentii	0.33	0.09	-0.07	-0.09	-0.12	0.07	0.30	0.31	0.44	0.18	-0.10	-0.21	0.01	0.07	0.10	-0.26	-0.25	1.00	0.17	0.37	0.35	0.41	0.02
IL-6 SA	0.61	0.18	-0.03	0.40	-0.29	-0.14	0.28	0.42	0.32	0.19	0.39	-0.18	-0.31	-0.29	-0.09	-0.24	-0.30	0.17	1.00	0.50	0.80	0.51	0.20
IL-1b SA	0.34	0.29	-0.03	0.20	-0.19	-0.31	0.06	0.45	0.34	-0.07	0.28	-0.15	-0.19	-0.30	0.09	-0.05	-0.19	0.37	0.50	1.00	0.75	0.63	-0.10
MMP-3 SA	0.60	0.30	-0.06	0.48	-0.40	-0.18	0.19	0.45	0.36	0.14	0.35	-0.16	-0.22	-0.28	-0.05	-0.24	-0.25	0.35	0.80	0.75	1.00	0.50	0.15
Choline SA	0.32	0.27	-0.09	-0.09	-0.22	-0.21	0.10	0.44	0.25	0.11	0.36	-0.19	-0.18	-0.21	0.05	0.06	0.01	0.41	0.51	0.63	0.50	1.00	0.01
Glucose PL	0.38	0.02	0.13	0.48	0.06	-0.02	0.36	-0.05	0.27	0.16	0.06	-0.40	0.01	-0.20	0.19	-0.18	-0.18	0.02	0.20	-0.10	0.15	0.01	1.00

6.1.7 Species associated with the clinical parameters in plaque samples.

Spearman correlation coefficients between associated species and clinical, immunological and metabolomic profile showed fewer associations in plaque samples than in saliva. Nevertheless, ODSS in OLP negatively correlated with *T. denticola* (p=0.01). Salivary IL-6 levels correlated negatively with *A. dentalis* (p=0.04). No correlations were found between salivary metabolites and subgingival microbiome in OLP. **Table 6.4**

Table 6.4. Correlation between clinical, immunological and metabolomic variables in subgingival plaque samples of OLP patients. *The red colour represents the most significant p values.*

P value/Spearman r	ODSS	Spearman r	PISA	Spearman r	Species richness PL	Spearman r	*A. dentalis*	Spearman r	*T.denticola*	Spearman r
ODSS		1.00	0.28	0.18	0.13	-0.25	0.08	-0.28	0.01	-0.42
PISA	0.28	0.18		1.00	0.01	0.41	0.77	0.05	0.08	0.29
Species richness PL	0.13	-0.25	0.01	0.41		1.00	0.002	0.48	0.02	0.37
IL-6 SA	0.00	0.56	0.94	0.01	0.06	-0.31	0.04	-0.34	0.11	-0.27
IL-13 SA	0.01	0.43	0.31	0.18	0.10	-0.29	0.25	-0.20	0.19	-0.23
Ethanol	0.04	0.36	0.52	0.12	0.29	-0.19	0.69	0.07	0.24	-0.21
D. invisus	0.94	-0.01	0.06	0.30	0.01	0.41	0.002	0.47	0.21	0.21
O. sp. oral taxon 807	0.38	-0.15	0.06	0.31	0.002	0.48	>0.0001	0.56	0.09	0.28
Olsenella uli	0.34	0.16	0.02	0.38	0.03	0.35	0.11	0.26	0.61	0.08
S. massiliensis	0.87	0.03	0.01	0.39	>0.0001	0.58	0.37	0.15	0.29	0.17
T. forsythia	0.21	-0.20	0.003	0.46	0.004	0.45	0.19	0.21	0.001	0.51
T.denticola	0.01	-0.42	0.08	0.29	0.02	0.37	0.29	0.18		1.00

In MMP group *Olsenella oral taxon 807 and T. denticola* showed significant correlation with PISA (p=0.001 and p=0.01, respectively). Salivary IL-13 and IL-6 in serum showed positive association with *F. nucleatum.* Along with PISA score, isovaleric acid correlated positively with *D. invisus* (p=0.05*)*, *Olsenella oral taxon 807* (p=0.001), *O.uli (*p=0.005) *and T. forsythia* (p=0.02*)*. There were no significant correlations observed between ODSS and subgingival plaque microbiome in MMP. **Table 6.5**

Finally, In the PV group, ODSS significantly correlated only with *T. denticola* (p=0.33). **Table 6.6** MMP-3 positively correlated with ODSS and *F. nucleatum* (p=0.083).

Table 6.5. Correlation between clinical, immunological and metabolomic variables in subgingival plaque samples of MMP patients. The red colour represents the most significant p values.

P value/Spearman r	ODSS	Spearman r	PISA	Spearman r	Species richness PL	Spearman r	*C. gracilis*	Spearman r	*Dialister invisus*	Spearman r	*F. subsp. polymorphum*	Spearman r	*O. sp. oral taxon 807*	Spearman r	*O. uli*	Spearman r	*T. forsythia*	Spearman r	*T. denticola*	Spearman r
Age	0.54	-0.10	0.01	-0.44	0.06	-0.31	0.05	0.33	0.06	0.31	0.07	-0.30	0.42	-0.14	0.27	0.19	0.87	0.03	0.48	-0.12
ODSS		1.00	0.46	0.13	0.81	-0.04	0.40	-0.14	0.60	-0.09	1.00	0.00	0.13	-0.26	0.59	-0.09	0.22	-0.21	0.95	-0.01
PISA	0.46	0.13		1.00	0.03	0.35	0.09	-0.29	0.21	0.21	0.15	0.24	0.001	0.52	0.17	0.24	0.07	0.30	0.01	0.42
Species richness PL	0.81	-0.04	0.03	0.35		1.00	0.89	-0.02	0.54	-0.10	0.14	0.25	0.04	0.33	0.83	0.04	0.72	-0.06	0.49	-0.12
Isovaleric acid	0.47	0.12	0.002	0.50	0.03	0.36	0.20	-0.22	0.05	0.33	0.90	-0.02	0.001	0.51	0.005	0.46	0.02	0.39	0.09	0.29
Leucine	0.04	0.34	0.03	0.38	0.69	0.07	0.63	-0.08	0.46	0.13	0.55	-0.10	0.45	0.13	0.01	0.40	0.31	0.17	0.10	0.28
Valine	0.20	0.22	0.01	0.44	0.17	0.24	0.61	-0.09	0.20	0.22	0.98	-0.01	0.26	0.19	0.005	0.46	0.30	0.18	0.06	0.31
Isoleucine	0.26	0.19	0.01	0.43	0.18	0.23	0.60	-0.09	0.22	0.21	0.92	-0.02	0.35	0.16	0.01	0.43	0.42	0.14	0.11	0.27
Ethanol	0.98	0.00	0.05	0.34	0.47	0.12	0.13	-0.26	0.20	0.22	0.79	0.05	0.19	0.23	0.02	0.40	0.34	0.16	>0.0001	0.55
Choline	0.39	0.15	0.05	0.34	0.59	0.09	0.89	-0.02	0.52	0.11	0.99	0.00	0.54	0.11	0.38	0.15	0.43	0.13	0.55	0.10
IL-6 SA	0.001	0.54	0.49	0.12	0.17	-0.24	0.64	-0.08	0.63	-0.09	0.64	0.08	0.51	-0.12	0.46	-0.13	0.44	-0.14	0.39	0.15
IL6 SE	0.04	0.33	0.27	-0.19	0.97	-0.01	0.58	0.09	0.57	-0.10	0.03	0.36	0.82	-0.04	0.59	-0.09	0.51	-0.11	0.08	-0.29
IL-13 SA	0.19	0.23	0.02	0.41	0.38	0.15	0.80	-0.05	0.44	0.14	0.05	0.34	0.05	0.33	0.25	0.20	0.23	0.21	0.20	0.23
IL-17A	0.16	0.24	0.03	0.38	0.29	0.18	0.58	0.10	0.89	0.02	0.63	0.08	0.53	0.11	0.82	-0.04	0.46	0.13	0.33	0.17
MMP-3 SA	0.001	0.53	0.03	0.38	0.68	0.07	0.65	-0.08	0.54	-0.11	0.15	0.25	0.90	-0.02	0.96	-0.01	0.63	-0.09	0.48	0.13
MMP-3 SE	0.05	0.35	0.77	-0.05	0.35	-0.17	0.56	-0.11	0.15	-0.26	0.28	0.20	0.01	-0.43	0.09	-0.31	0.07	-0.32	0.64	-0.08
O.sp. oral taxon 807	*0.13*	*-0.26*	*0.001*	*0.52*	*0.04*	*0.33*	*0.64*	*0.08*	*0.02*	*0.38*	*0.57*	*0.10*		*1.00*	*0.09*	*0.28*	*0.02*	*0.39*	*0.12*	*0.26*
P. gingivalis	*0.12*	*-0.26*	*0.21*	*0.22*	*0.05*	*0.32*	*0.63*	*-0.08*	*0.06*	*0.31*	*0.78*	*0.05*	*0.003*	*0.48*	*0.15*	*0.24*	*0.09*	*0.29*	*0.91*	*-0.02*
S. massiliensis	*0.09*	*0.29*	*0.02*	*0.39*	*0.58*	*-0.09*	*0.14*	*-0.25*	*0.10*	*0.28*	*0.07*	*0.30*	*0.19*	*0.22*	*0.52*	*0.11*	*0.38*	*0.15*	*0.38*	*0.15*

Table 6.6 Correlation between clinical, immunological and metabolomic variables in subgingival plaque samples of PV patients. The red colour represents the most significant p value

P value /Spearman r	ODSS	Spearman r	PISA	Spearman r	*F. nucleatum animalis*	Spearman r	*T. denticola*	Spearman r
Age	0.22	-0.21	0.02	-0.39	0.04	-0.35	0.41	-0.14
ODSS		1.00	0.01	0.44	0.77	0.05	0.05	0.33
PISA	0.01	0.44		1.00	0.30	0.18	0.34	0.17
IL-6 SA	0.01	0.44	0.05	0.35	0.39	0.16	0.27	0.20
MMP-3 SA	0.002	0.52	0.003	0.51	0.08	0.31	0.27	0.20
Leucine	0.54	-0.11	0.61	0.09	0.01	0.45	0.63	-0.09
Valine	0.43	-0.14	0.96	0.01	0.01	0.42	0.72	-0.06
Isoleucine	0.39	-0.16	0.86	-0.03	0.03	0.37	0.86	-0.03
Propionic acid	0.04	-0.37	0.58	-0.10	0.74	-0.06	0.21	-0.22
Lysine	0.001	-0.55	0.82	0.04	0.97	-0.01	0.29	-0.19
Choline	0.05	0.34	0.18	0.24	0.11	0.28	0.18	0.24
Glucose	0.03	0.38	0.70	0.07	0.16	0.25	0.84	0.04
T. denticola	0.05	0.33	0.34	0.17	0.27	0.19		1.00

6.1.8 Effect of the diet on microbiome. (Preliminary results)

To find out whether diet affects the oral microbiome and severity of oral diseases, OLP and PV groups were divided into three groups: mixed (Western) diet (OLP n=35, PV n=29), vegetarian diet (OLP n=4, PV n=8) and pescatarian/flexitarian diet (OLP n=6, PV n=8), which is flexible eating that emphasises the addition of plant based foods and encourages meat to be consumed less frequently and in smaller portions (Derbyshire et.al., 2017). It was analysed particularly within OLP and PV groups, as it was possible to divide them into subgroups based on their diets. In contrast, in the MMP group, participants followed predominantly one type of diet. Preliminary results showed no significant changes were found, even though ODSS score in the vegetarian group among OLP patients was higher than the other two diet groups. In the PV group, the ODSS was higher in the pescatarian/flexitarian diet than in vegetarian and mixed diets. **Figure 6.16 a, 6.16 b**

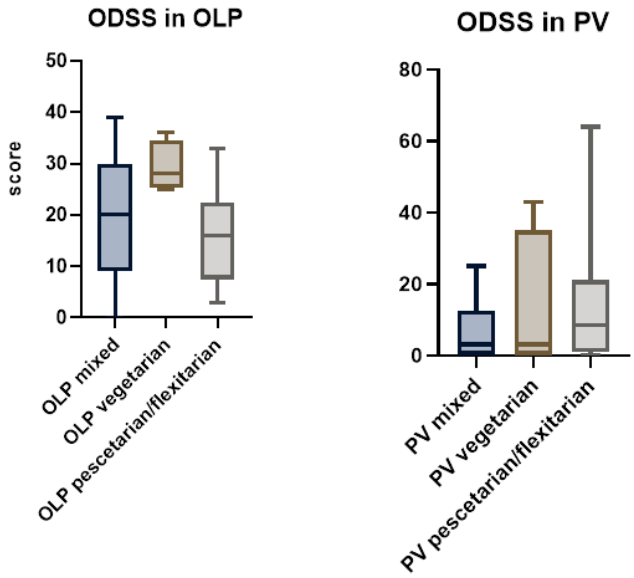


Figure 6.16 a. Effect of the diet on ODSS in OLP and PV: Mixed Western, Vegetarian, and Pescetarian/Flexitarian Diets.

However, a decrease of Actinobacteria and higher abundance of Bacteroidetes and Proteobacteria phyla were observed in a vegetarian group compared to the mixed and pescatarian diet.

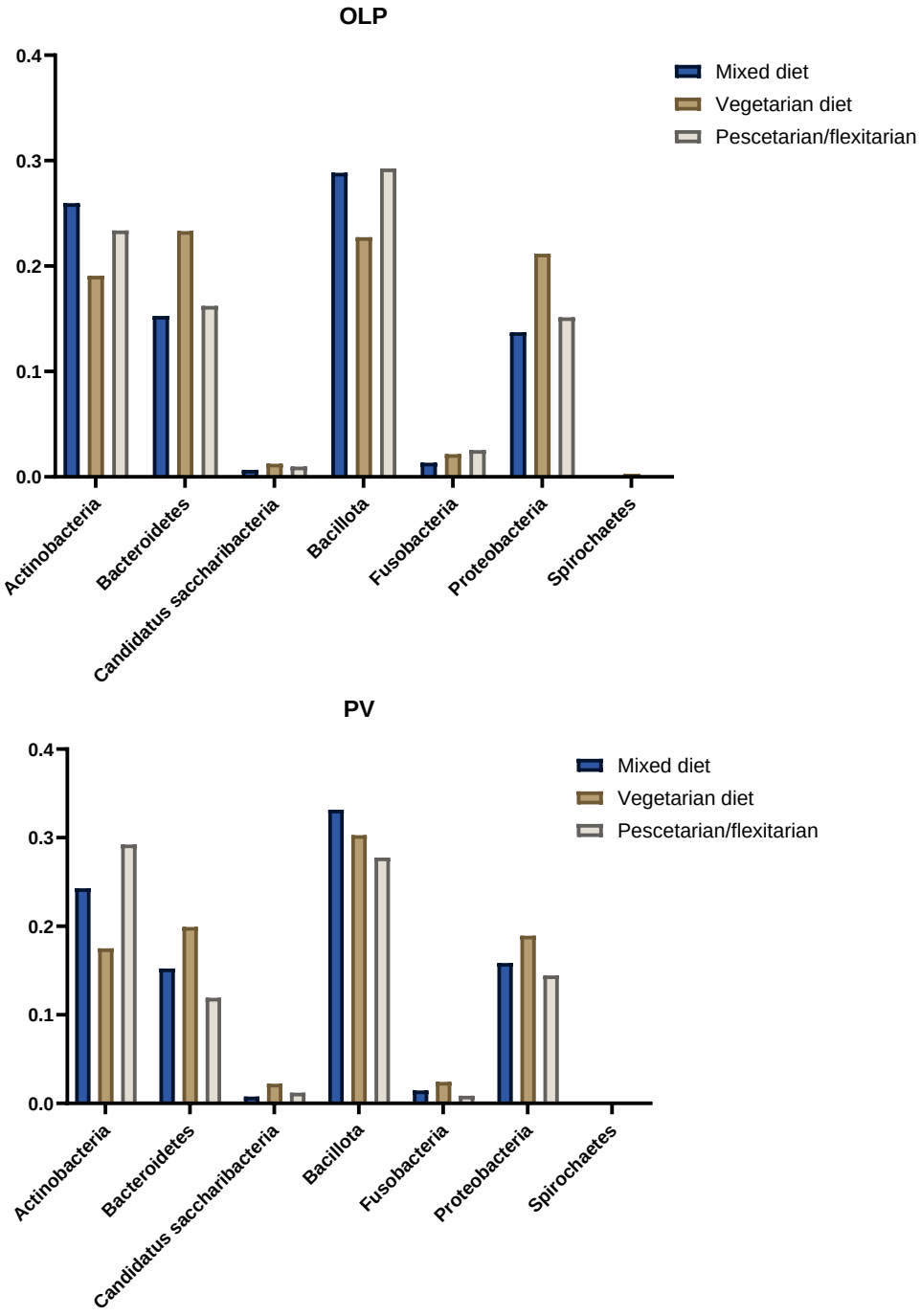


Figure 6.16 b. Effect of the different diet type on salivary microbiome in OLP and PV patients *on mixed western, vegetarian and pescetarian/flexitarian diet.*

6.2 Discussion

In this study, we aimed to determine the relationships between salivary and subgingival microbial profiles and MMP, PV and OLP disease groups.

Alpha diversity (representing microbial richness and evenness of the oral microbiome) did not differ significantly in saliva samples between the groups. However, the subgingival microbiome showed a significant decrease in diversity in all three groups of diseases compared to HC, with the lowest diversity being in the MMP. These results suggest that the oral microbiome in the subgingival region can be more directly affected by the mucosal diseases, leading to a reduction in microbial diversity. Moreover, the disease-associated changes in the oral environment, such as immune responses, could create conditions that are less suitable to a diverse microbiome. In this study I found that salivary IL-6 negatively correlated with microbial diversity in plaque samples of OLP patients only. Alternatively, routine in-clinic oral hygiene practices or ageing-associated teeth loss may contribute to the observed differences in microbial diversity. Further research in evaluating the impact of routine in-clinic oral hygiene practices on the oral microbiome could help to understand Investigating whether oral hygiene interventions contribute to changes in microbial diversity and how these practices differ among individuals with mucosal diseases.

Further, a Spearman correlation analysis showed that Shannon diversity in saliva and plaque was inversely correlated with disease severity in the OLP group. This finding is consistent with the previous microbiome studies in OLP (Hijazi et al., 2020). Low microbial diversity may be due to the overgrowth of potential pathogens, triggering or exacerbating disease states. Inflammation or immune imbalance, caused by OLP, MMP and PV, as mentioned earlier, may also create an unfavourable growth environment for microorganisms, thus reducing bacterial diversity. Other studies showed that a loss of microbial diversity in the gut has been associated with many human diseases, including inflammatory bowel disease, obesity, and type 2 diabetes (Gilbert et al., 2018). Moreover, Takeshita et al. reported that age-related tooth loss contributes to decreased alpha diversity of subgingival plaque in older age groups (Takeshita et al., 2016). A less diverse microbiome can result in a reduction in the production of essential metabolites and vitamins, which can further contribute to disease (Shoemark & Allen, 2015).

It is important in the analysis of data to use the most appropriate statistical test. While Spearman correlation provides insights into the direction and strength of the relationship between two variables, it doesn't offer a precise quantification of the extent to which one variable change when the other changes. Linear regression, on the other hand, enables an estimate the nature of this relationship more accurately. Therefore, simple linear regression was conducted to analyse the relationship between ODSS and microbial diversity in all three diseases. Interestingly, the closest association was observed in the OLP group, where more severe disease was associated with a less diverse oral microbiome, although the significance level was relatively modest (p=0.07). The observed association suggests a potential microbial dysbiosis in the oral microbiome of individuals with more severe OLP. This imbalance in microbial diversity may be indicative of specific microbial community shifts linked to the severity of the disease. This can be understood by that changes in the health of the oral tissues in OLP might create conditions that either support the growth or inhibit certain types of microorganisms. Strategies aimed at modulating the oral microbiome to restore diversity could be explored further as supplementary or preventive actions.

The oral microbiome composition at the phylum level showed 14 phyla in total from both saliva and plaque samples. Bacillota (37.7%), Actinobacteria (31.85%), Proteobacteria (12.97%), Bacteroidetes (12.32%), Fusobacteria (1.95%) were the five dominant phyla in saliva. In plaque samples Actinobacteria (54%), Bacillota (14.2%), Bacteroidetes (13.20%), Proteobacteria, and Fusobacteria (3.8%) were dominant. These findings slightly differ from the previous studies of OLP (K. Wang et al., 2016b) in saliva samples, with prevalence of Proteobacteria over Actinobacteria phylum. However, it is consistent with other studies using shotgun metagenomics analysis in Crohn's disease (Nomura et al., 2020; B. Sun et al., 2021). The discrepancy may be due to differences in sequencing methods, as previous studies were analysed using 16s rRNA gene sequencing, as well as bioinformatic analysis pipelines and the number of subjects included in different studies.

Further analysis revealed significant changes in the salivary microbiome at the phylum level of each disease group compared to HC. OLP group had a significantly decreased abundance of Gram-positive Bacillota and a significantly increased abundance of Gram-negative

Bacteroidetes, Fusobacteria and Spirochaetes in saliva samples. These results are consistent with a previous OLP studies (Y. Li et al., 2019).

In the PV group, a higher abundance of Bacteroidetes in saliva was observed compared to HC. Gram-negative bacteria are known to produce endotoxins that can cause inflammation and tissue damage (Marcano, Rojo, Cordoba-Diaz, & Garrosa, 2021), while Gram-positive bacteria are generally considered beneficial for oral health. An increased abundance of Gram-negative bacteria can also decrease the production of essential metabolites and vitamins, further contributing to the disease state (Kazor et al., 2003).

Oral microbiome compositions at the species level also showed changes in all three disease groups, where there was a significant decrease of gram-positive species *S. salivarius* and *S. Infantis* species in OLP group, *A. massiliensis, S. mitis, S. australis and X. cellulosilytica* in MMP saliva samples. Our findings are consistent with the results of He et al., where the authors found a decrease in *Streptococcus* genera in the buccal mucosa of OLP patients (He et al., 2017). Furthermore, the correlation analysis revealed an inverse relationship between disease severity and gram-positive species across all three groups of diseases. Specifically, in the case of OLP, species such as *Actinomyces sp. ICM47, O. sinus, S. parasanguinis, S. salivarius, and X. cellulosilytica* demonstrated a decrease in abundance as disease severity increased. In MMP, *A. meyeri and R. mucilaginosa* displayed a similar pattern, while in PV, it was observed that *Actinomyces sp. ICM47, S. salivarius, and S. parasanguinis* exhibited a reduction in abundance as disease severity increase. Clinically, these correlations suggest that the composition of the oral microbiome, particularly the presence of specific gram-positive species, may have implications for disease severity in OLP, MMP, and PV. Earlier, Filkins et al. showed that modest levels of *S. salivarius, S. parasanguinis, and S. milleri* group may increase microbial diversity and contribute to patient health (Filkins et al., 2012). *S. salivarius* is proposed as a probiotic due to its ability to suppress the proliferation of *S. pneumonia, S. mutans,* and certain Gram-negative species (Wescombe et al., 2009). Approaches to adjust the levels of certain beneficial bacteria may be considered to lessen the severity of the disease or as additional treatments.

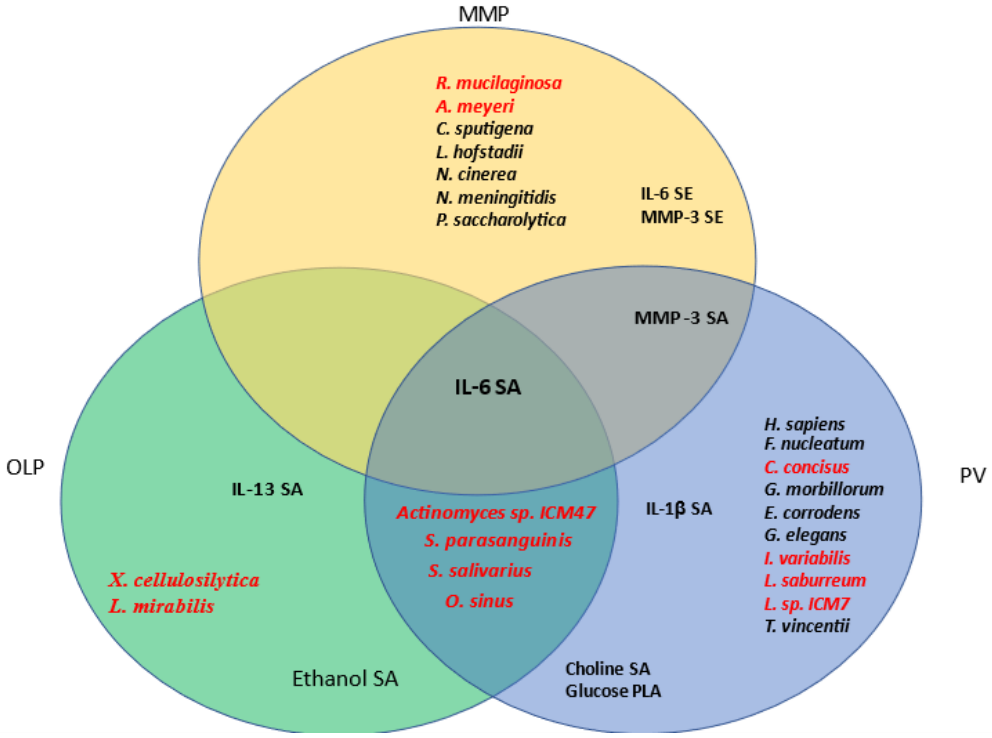


Figure 6.17 The bacterial species associated with oral disease severity scores, proinflammatory cytokines and metabolites*. The model displayed all species associated with ODSS in OLP, MMP and PV (P < 0.05). The species name in red were correlated negatively with ODSS, and those in black were correlated positively.*

Today, scientists state that the mediation of inflammatory disease is related not to a single bacteria but to how microorganisms interact in communities. In animal experiments, it has been observed that combinations of various bacteria increase pathogenicity compared to infections with a single type of bacterium, i.e the interaction between multiple bacterial species can lead to more severe disease outcomes. (Lamont et al., 2018). It was found that keystone pathogens can modulate the host immune response and promote the growth of other pathogenic microbes, leading to dysbiosis and disease. **Figure 6.18** For example, oral infection with *P. gingivalis* and *F. nucleatum* in a rodent model revealed more severe alveolar bone loss and inflammatory response when both bacteria were combined than with either species alone (H. Li et al., 2013). In MMP, the present study found a significant positive correlation between *C. sputigena* and the presense of *L. hofstadii*, *P. saccharolytica*. Moreover, in PV, there was a significant correlation between *E. corrodens* and the presence of *F. nucleatum*, *G. morbillorum*, and *T. vincentii* in saliva samples. Notably, all these microorganisms were positively correlated with the severity of the mucosal disease. Cooperative interactions among these microbes may contribute synergistically to the exacerbation of mucosal autoimmune diseases.

Treatment approaches focusing on adjusting the levels or interactions of certain groups of microbes may be considered to better handle or reduce the severity of mucosal autoimmune diseases. Addressing polymicrobial synergy could offer a new perspective in planning treatments.

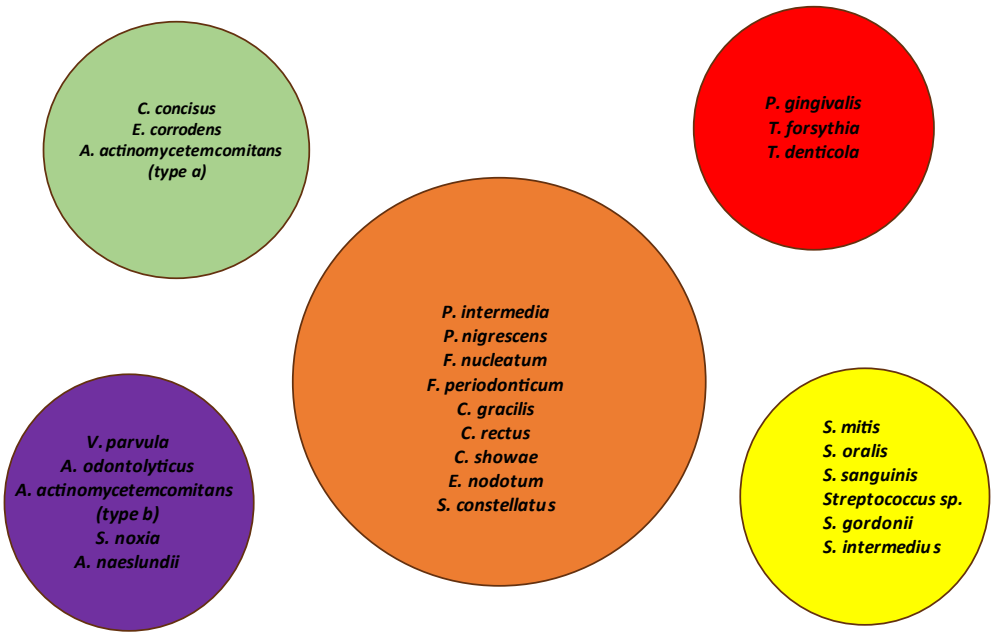


Figure 6.18 Socransky's microbial complexes in periodontitis. *Bacteria in the green, yellow and purple are considered early colonizers. Orange complex bacteria are associated with increased pocket depth and progressive attachment loss. They link early colonizers with more pathogenic bacteria that in the red complex. The red complex (keystone pathogens) bacteria are the final bacteria that colonize and lead to the destruction of the periodontium (Socransky & Haffajee, 2005).*

Early studies observed that patients with OLP, MMP and PV showed an increase in the incidence of PD compared to healthy individuals (Akman et al., 2008; Jascholt et al., 2017; Seckin Ertugrul et al., 2013a). In addition, these patients had worse periodontal parameters such as bleeding on probing, clinical attachment level of the periodontal ligament, probing depth, plaque index, and gingival recession. In this study, correlation analysis found periodontal pathogens, *T. denticola* correlated positively with ODSS in OLP and PV groups in subgingival plaque samples. Treponemes are members of the Spirochaetes phylum and are integral components of complex biofilms that form on tooth surfaces within the gingival crevice (Kolendrander et al., 2002). Remarkably, these treponemes play significant roles in the aetiology of various human diseases such as syphilis and yaws, caused by *Treponema pallidum*, as well as periodontal disease, necrotizing ulcerative gingivitis, endodontic infections, and dental abscesses, where species such as *Treponema denticola, Treponema*

lecithinolyticum, and Treponema socranskii are implicated (Sela et al., 2001; Foschi et al., 2006; Holt and Ebersole, 2006). The metabolic pathways of *T. denticola* remain relatively understudied. In *in vitro* studies, this bacteria primarily relies on serine, alanine, cysteine, and glycine, leading to the production of various fermentation byproducts, including acetate, lactate, succinate, formate, pyruvate, ethanol, carbon dioxide, hydrogen sulfide (H2S), and ammonia (Rother et al., 2001; Seshadri et al., 2004; Chu et al., 2008). These metabolites can accumulate in significant concentrations, potentially impacting the composition of bacterial species in polymicrobial biofilms and affecting host tissues (Kuramitsu et al., 2007). These compounds can penetrate periodontal tissues, disrupting host cell activity and immune responses. SCFA fermentation products are believed to contribute to disease progression (Niederman et al., 1997). Moreover, *T. denticola* is known to have extensive tissue penetration, and Treponemes were found within junctional epithelium cells (Saglie et al., 1982). When bacteria invade or internalise epithelial cells, they gain access to a nutrient-rich environment somewhat shielded from the host's immune system. *T. denticola* might use this intracellular space to persist and potentially replicate. When closely associated with epithelial cells, *T. denticola* employs strategies to evade this innate immune response (Brissette and Lukehart, 2007). TLRs on the surfaces of host cells play a crucial role in recognising pathogen-associated molecular patterns like LPS, flagellin, and peptidoglycan. Activation of TLRs leads to the induction of inflammation-related genes, which are essential for limiting infections. Dysregulation of this inflammatory response can result in overexpression and continued expression of inflammatory mediators, contributing to tissue destruction (Dauphinee and Karsan, 2006). These interactions and immune responses may contribute to the development of autoimmune conditions, potentially due to the phenomenon of molecular mimicry, where the host's immune system mistakenly targets its cells or tissues because of similarities to microbial components.

Compared with the healthy controls, non-parametric analysis of variance using Kruskal-Wallis test showed that species *C. ochracea* in OLP and *C. gracillis* in MMP groups were overrepresented. In both of these groups, the higher abundance of *F. nucleatum* was found. Similar results were observed in two studies of OLP with a rise in the *Capnocytophaga* genera in subgingival plaque of OLP compared to HC (Du et al., 2020; X. Wang et al., 2020). Okuda et.al described coaggregation and intracellular interaction through release of diffusible

molecules by *C. ochracea*, which play significant roles in forming a biofilm by *F. nucleatum* and *C. ochracea* (Okuda et al., 2012). Both species reside at the oral mucosa as commensals but may be opportunistic pathogens promoting PD and systemic diseases, such as periodontal disease, angina, lung infections, and gynaecological abscesses. (Hajishengallis, 2014). *F. nucleatum*, is a prevalent Gram-negative anaerobic bacterium, which can enter the bloodstream. Research has confirmed that *F. nucleatum* can travel from its initial colonization site in the oral cavity to other areas of the body. (Keku et al, 2013). As a result, it has the potential to contribute to a range of diseases, including cancer. *F. nucleatum* is implicated in various forms of PD, ranging from mild gingivitis to severe chronic periodontitis (Han et al., 2000). Its prevalence increases with disease severity, inflammation progression, or deeper tooth pocket development. One distinctive characteristic of *F. nucleatum* is its secretion of sulfur compounds, including H2S, which can lead to halitosis (Yoshimura et al., 2002). These compounds are produced through the breakdown of sulfur amino acids. *F. nucleatum* also modulates the immune system by inducing the expression of specific genes related to the immune response. These genes include cytokines, inflammation markers, protease inhibitors, and antimicrobial peptides (Stathopoulou et al., 2010). Additionally, *F. nucleatum* promotes the secretion of enzymes that degrade the ECM (Gursoy et al., 2008). The findings emphasise the need for further investigation into the systemic impact of *F. nucleatum* in individuals with mucosal autoimmune diseases. The presence of periodontal pathogens may contribute to the development of mucosal diseases though erosive gingival MMP and PV lesions may equally act as a potential reservoir for plaque accumulation and thus promote the growth of periodontal pathogens (Pollmann et al., 2018). Inflammatory processes are involved in the pathogenesis of all three diseases and PD (Kourosh & Yancey, 2011). Our study showed that in OLP, salivary IL-8 was associated with *T. denticola* in saliva. PISA score was earlier in this study associated with this cytokine. In MMP group, salivary IL-6, IL-1 β and MMP-9 were positively correlated with *T. forsythia*. In the PV group, all disease-associated bacteria, including *G. morbillorum, H. sapiens* and *F. nucleatum,* were positively associated with salivary IL-6, IL-1 β and MMP-3. Moreover, it was positively correlated with the severity of PV and was significantly correlated with PISA in all groups. The possible impact of periodontal bacteria on immune responses in conditions like OLP, MMP, and PV raises questions about its role in increased inflammatory responses in PD. This increase might contribute to the development of immunobullous diseases and OLP. To explore this theory, experimental

studies involving in vitro experiments, fluorescence in situ hybridization (FISH) studies, or animal models may be valuable. These studies will help understand exactly how oral bacteria affect the immune system and establish a clear connection between the rise of inflammatory mediators in PD and the onset of AIBD and OLP.

On the other hand, a release of cytokines in MMP, PV and OLP might affect the course of PD and exaggerate periodontal disease severity. The pathophysiologic mechanisms of OLP, MMP and PV are different. However, erosive lesions in these diseases may equally act as a potential reservoir for pathogen accumulation and thus promote PD.

Similarly, growing evidence supports the role of commensal bacteria in modulating the immune system, which could lead to autoimmune inflammation. Specifically, Ellebrecht et al. pointed out that regardless of the presence of circulating autoantibodies in patients with epidermolysis bullosa acquisita (a severe type of pemphigoid disease), what plays a significant role in the manifestation of the disease is the composition of the skin microbiome; it was found that cutaneous bacterial species richness and evenness is associated with protection from blister formation during EBA. (Ellebrecht et al., 2016).

Moreover, we should remember that MMP and PV patients are generally older and likely to suffer from PD. Age-related changes, such as xerostomia, change in the diet because of the loss of teeth or immunological changes, such as inflammageing and immunosenescence, would potentially have a systemic effect on health status in general (Nomura et al., 2020; Schwartz et al., 2021).

This study findings also showed that *T. denticola* was positively correlated with isovaleric acid, PISA score in the OLP group. Similarly, in the MMP group, the same metabolite was associated with T. *forsythia* and *N. meningitidis* and PISA. Proteolytic amino acid–degrading bacteria, including *Fusobacterium, Treponema, Prevotella* and *Porphyromonas* species, break down proteins and peptides, into amino acids and degrade them further to produce short-chain and branch chain fatty acids, and indole/skatole, which act as virulent and modifying factors in PD (N. Takahashi, 2015). As these two keystone periodontal bacteria are associated with PISA, the findings suggest that this fatty acid can contribute to initiating and promoting PD.

Monitoring the levels of these markers may help in the early detection and assessment of periodontal health.

Additionally, a positive correlation between ethanol and disease severity in the OLP group was noted. The literature suggests that in the oral cavity, ethanol derived from alcoholic drinks is converted by bacterial alcohol dehydrogenase into acetaldehyde, a carcinogen, which suggests that ethanol-derived acetaldehyde can cause oral cancer (Homann et al., 2001).

Finally, to investigate how treatment affects the microbiome, MMP and OLP patients were divided into three treatment subgroups: 1) topical treatment (topical corticosteroid creams), immunosuppressant (prednisolone, azathioprine, mycophenolate mofetil) and antibiotic group in MMP, and hydroxychloroquine (HCQ) in OLP. Immunosuppressants are commonly used to manage conditions like OLP, MMP, and PV (Knudson et al., 2010; J. F. Setterfield et al., 2000). The literature also indicates that immunosuppressive therapy may contribute to periodontal damage, a correlation between the dosage of prednisone and the occurrence of pathogenic bacterial species, including *F. nucleatum* and *P. gingivalis*, impacting periodontal health (Corrêa et al., 2017). However, other studies did not find changes from immunosuppressive treatment on PD parameters (Thorat et al., 2010). Similarly, Low et al. described gut microbiome on MMP patients and the effect of low-dose antibacterial and immunosuppressive medication on the microbiome, where no significant changes were found in patients on immunosuppressive and AB therapy (Low et al., 2022). In my study, Fusobacteria and Bacillota phyla showed lower abundance in saliva samples, and a trend to increase in Actinobacteria was noted in both saliva and plaque samples from MMP patients on IS therapy. In the OLP group, a higher abundance of Candidatus Saccharibacteria in plaque samples on IS+HCQ treatment was found. However, it's important to note that these findings did not reach statistical significance. This suggests that the oral microbiome may exhibit relative resilience to the effects of these treatments, aligning with prior literature. Interestingly, in MMP group, the results showed that the correlations between mucosal disease severity and microbiome completely disappeared in the subgroup of patients on immunosuppressive therapy and were partially affected on AB treatment (**Appendix 8**). Patients on topical treatment exhibited results similar to the overall MMP group, indicating

that the findings were not significantly influenced by the specific treatment type within this subgroup. However, it's essential to be cautious when interpreting these findings. In order to strengthen the validity of results and gain a more comprehensive understanding of how different treatments impact the oral microbiome, future research should prioritise adequately powered studies with larger sample size allowing for subset analysis. Additionally, the cross-sectional study nature of this study prevents any consideration of causal relationship between periodontitis-associated bacteria and MMP, PV, and OLP. Cross-sectional studies are susceptible to 'snapshot bias,' where the microbial composition observed at a single time point may not fully represent the long-term or dynamic characteristics of the microbiome. Longitudinal studies, as mentioned earlier in two previous chapters, would be better suited to explore temporal relationships and causality. Secondly, cross-sectional studies may not capture variations in disease severity or treatment effects accurately since they rely on a single assessment. The patients may be at different stages of their diseases, receiving various treatments, or experiencing fluctuations in their oral microbiome over time. Lastly, cross-sectional studies are also limited by potential selection bias and generalizability issues. The study sample may not fully represent the broader population, which can affect the external validity of the findings.

Likewise, the analysis of the microbiome composition across different diet groups in OLP and PV did not reveal significant differences, primarily due to the limited sample size within these groups. To address the potential for type I and type II statistical errors and enhance the robustness of future research findings, it is imperative that future studies prioritise larger sample sizes, providing greater statistical power to detect smaller effects or differences when they exist (Knight et al., 2018). Moreover, replication studies with an independent sample are a critical step in the scientific research process that enhances the reliability and validity of study results by confirming their consistency and reducing the risk of type 2 errors.

On account of the rarity of the AIBD and the fact that we are a tertiary referral centre for many of the patients in this study, it was not possible to recruit a sizeable number of treatment naive participants in a given time. Additionally, the recruitment period was significantly impacted by the Covid-19 pandemic. Moreover, excluding patients on medication would also limit the generalizability of the results, as many patients with oral

autoimmune diseases are on medication. This important confounding factor however can make comparison of data between studies and drawing generalizable conclusions, difficult.

However, our study stands has considerable strengths, including a significantly larger and more diverse population sample compared to previous microbiome studies described in literature. We used the latest shotgun metagenomics technique to investigate the microbiome in three distinct oral autoimmune diseases. This cutting-edge technique provides an unprecedented level of depth and precision in characterising the oral microbiome. By intricately associating the microbiome data with extensive clinical periodontal, immunological and metabolomic profiles, the study reveals complex relationships and potential mechanisms underlying these autoimmune conditions. This multi-omics approach elevates the understanding of the oral microbiome's role in health and disease, shedding light on its impact on the immune system and metabolic pathways in the context of these autoimmune conditions. Notably, the study delves into periodontal status and analyses specific periodontal bacteria, establishing a crucial link between oral health and autoimmune diseases, and further enhancing our understanding of the complex connections between the oral microbiome and broader health implications.

As a next step following the collection and analysis of the microbiome data in our current study, we are committed to enhancing our research by effectively integrating different datasets using a systems biology approach (Bernabe et al., 2018). While shotgun metagenomics has undeniably provided us with detailed insights into the microbial composition, our future studies will focus on exploring the functional potential, gene expression, and metabolic activities of these microbes (Karlsson et al., 2011). To facilitate this integration, we plan to employ a variety of robust methods. Co-occurrence networks will help us elucidate the complex relationships between different microbial species, shedding light on their interactions within the oral ecosystem. Functional pathway analysis will allow us to understand the specific biochemical pathways that these microorganisms are involved in, providing a deeper understanding of their roles in health and disease. Machine learning techniques will uncover patterns and correlations within the data, enabling us to make more precise predictions and interpretations. By embracing these advanced methodologies, we aim

to take our research to the next level, uncovering the complex connections between the oral microbiome and autoimmune diseases.

In conclusion, this study unveils notable alterations in the oral microbiome of patients with OLP, MMP, and PV, each displaying distinct microbial profiles. While these findings suggest dysbiosis in diseases groups, it is essential to note that the observed associations do not imply causality or assess susceptibility to PD. The complexity of microbial interactions in the oral cavity highlights the need for further research to elucidate the specific roles of these microorganisms in oral autoimmune diseases. The identification of unique microbial signatures in OLP, MMP, and PV patients may offer promising directions for future investigations to uncover potential disease-associated biomarkers and mechanisms underlying the progression of these conditions. Future research with a broader focus on in vitro, animal model and longitudinal investigations would provide a more comprehensive understanding of the relationships between oral microbiota, autoimmune diseases, and PD.

Chapter VII. Conclusions and future work

The aims of this study were:

1. To evaluate the role of periodontal status in these diseases and to investigate the relationship between periodontal status and the composition of the oral microbiome in large disease groups, while also assessing its correlation with disease severity.

2.To correlate cytokine responses: analyse and correlate cytokine responses in saliva and serum with the oral microbiome.

3. To explore the oral microbiome: examine the role of the oral microbiome in the inflammatory response observed in individuals with MMP and PV compared to both healthy individuals and those with OLP.

4.To investigate the oral metabolome: observe the oral metabolome and its association with the microbial composition of the oral microbiome, while also exploring its connection with disease severity in the respective groups.

To examine these objectives, clinical mucosal and periodontal assessments were performed, pro-inflammatory cytokines and matrixins were quantified in saliva and serum using Human Magnetic Luminex Screening Assay and ELISA methods, salivary and plasma metabolites and metabolic pathways were analysed by 1H-NMR Spectroscopy Analysis, and saliva and subgingival plaque microbiomes were identified using shotgun metagenomics analysis.

Summary of main findings.

1.Clinical findings.

The main findings of the study highlight differences in the demography, comorbidities, phenotype and severity of three oral autoimmune diseases, namely OLP, MMP and PV in consecutively recruited patients. The results show that all three diseases are more common in females, with OLP and MMP being more frequent in white European populations, while PV is more frequent in Asian subgroups. Interestingly, an increase in other autoimmune diseases, specifically thyroid disease, and type 2 diabetes mellitus (T2DM), was observed in MMP and OLP patients, with T2DM being linked to oral disease severity scores in PV patients. It is important to clarify that while this study identified the coexistence of T2DM with oral

diseases, the specific diabetes status prior to treatment was not known, and the potential for high-dose steroid therapy used to manage severe PV to induce steroid-induced diabetes mellitus is recognised (Hwang & Weiss, 2014).

Gingival inflammation was more prominent in MMP and OLP but not PV. In MMP, patients on antibiotic treatment had reduced gingival inflammation. Additionally, the correlation analysis indicated a significant positive association between ODSS and PISA score, and bleeding scores in both OLP and PV. These findings suggest that the oral microbiome may play an essential role in the pathogenesis of oral autoimmune diseases and that antibiotic treatment may have a beneficial effect on reducing inflammation.

2.Immune response.

The oral immune response was analysed in individuals with OLP, MMP and PV. In this investigation, two distinct groups of immune-mediated diseases exhibited an immunological response. While the specific causes of AIBD and OLP remain unknown, multiple cell types, including keratinocytes, CD4+ and CD8+ T-lymphocytes, macrophages, and mast cells, along with various cytokines and chemokines, contribute to the disease's activation.

The main findings indicate that the levels of several pro-inflammatory cytokines were elevated in the saliva and serum samples of individuals with OLP, MMP, and PV. In OLP patients, higher levels of salivary IL-6 and MMP-3 were observed compared to healthy controls, and IL-6 was elevated in serum. In addition, ODSS was associated with salivary levels of IL-6. In MMP patients, salivary IL-13, IL-6, MMP-3, and IL-17 were increased, and ODSS was positively correlated with salivary IL-6, MMP-3, and serum MMP-3 levels. In PV patients, salivary IL-6, IL-17, serum MMP-3, and IL-13 were elevated significantly, and ODSS was associated with salivary IL-6, IL-1β, and MMP-3 levels.

Our study showed no positive correlation between periodontal scores and inflammatory markers in OLP. In MMP, there was a significant positive correlation with salivary IL-1β, IL-13, MMP-3, MMP-9 and PISA score. These findings may indicate a potential association between elevated levels of these cytokines and proteases in the saliva of MMP patients and the development or progression of periodontal diseases, though further research is needed to confirm this relationship. In the PV group, only MMP-3 in saliva correlated with the

periodontal score. Interestingly, there was no statistically significant connection observed between the cytokine levels linked to ODSS and the scores related to periodontal disease in any of the studied groups. This suggests that while these cytokines may play a role in the severity of oral diseases, their influence on periodontal health in these specific conditions was not evident in this study. The different and unique profile of correlations between inflammatory response and periodontal scores in all these three diseases suggest that the pathogenesis of periodontal diseases with oral autoimmune diseases may not be straightforward and requires a more nuanced understanding of the underlying mechanisms.

To conclude, the study highlighted the significance of salivary IL-6 and MMP-3 as potential non-invasive biomarkers for disease severity in these oral autoimmune conditions. However, further research is needed to establish these salivary biomarkers' reliability and clinical utility. Future studies will benefit from expanding the patient cohort to enhance statistical power and validate these biomarkers in diverse populations.

3.The microbiome findings and its association with clinical and immunological parameters.

The first finding was a decrease in richness in the subgingival plaque microbiome in all three disease groups compared with healthy controls. However, the decrease was not statistically significant in the PV group possibly due to the sample size in PV group being smaller than in other groups. If the difference in diversity between groups is small, then a larger sample size may be required to achieve statistical significance. Additionally, OLP patients exhibited a significant decrease in the abundance of gram-positive Bacillota phylum and a notable increase in the abundance of Gram-negative Bacteroidetes, Fusobacteria, and Spirochaetes phyla in saliva samples. Finally, a Spearman correlation analysis in OLP demonstrated that gram-positive species were associated with lower disease levels and the Gram-negative species with higher disease severity. In this case, a decrease in Gram-positive bacteria, considered part of the healthy microbiota and an increase in Gram-negative bacteria (commonly associated with pathogenic species) could suggest a shift towards an imbalanced (dysbiotic) microbial composition.

In the MMP group, the Actinobacteria phylum was higher compared to HC. Furthermore, correlation analysis revealed significant associations between MMP disease severity and the abundance of *C. sputigena, L. hofstadii, N. cinerea, N. meningitidis*, and *P. saccharolytica*.

Intriguingly, these microorganisms were significantly associated with each other, which suggests a potential synergy among these microorganisms and their contribution to the disease state in MMP. All of these species are Gram-negative anaerobic commensal bacteria (except for *N. meningitidis*), which have been associated with various infections, including periodontal disease, respiratory tract infections, and abscesses (Ehrmann et al., 2016; Eribe & Olsen, 2008; Könönen et al., 2022*).*

In the PV group, the association between periodontal pathogenic bacteria and the disease appeared to be stronger than in OLP or MMP. The possible explanation for that could be in the clinical nature of these two latter diseases and their treatment strategies. OLP and MMP often involve the oral mucosa extensively, including the gingiva. The management of OLP and MMP may involve careful attention to oral hygiene practices, as in these conditions, the inflammation and changes in the oral mucosa can affect the periodontal tissues and lead to complications such as gingival inflammation, desquamative gingivitis, and periodontal attachment loss (Maderal et al., 2018). Since PV is more painful, patients avoid brushing and therefore there is more plaque accumulated. It is important to consider that the antibiotic treatment administered to MMP patients for an anti-inflammatory effect may have the beneficial effect of reducing the load of periodontal pathogens. PV typically has less prominent gingival involvement, resulting in a less specific periodontal treatment for PD. The primary focus of treatment for PV is typically systemic immunosuppressive therapy to control the autoimmune blistering process, associated with oral lesions. However, since PD worsens with age, PV patients potentially may develop slow-progressing periodontal changes associated with ageing. Moreover, because these patients do not receive aggressive periodontal treatment on account of painfulness of lesions, the subgingival plaque has time to develop into more of a pathogenic community commonly seen in periodontal disease, which may explain the observed associations with periodontal pathogens. It is important to note that these observations are based on the current understanding and available evidence, and further research is needed to establish a definitive causal relationship between periodontal bacteria and autoimmune conditions.

In the context of microbiome and inflammatory response, in OLP only salivary IL-8 demonstrated an association with *T. denticola* and PISA score in this study. Within MMP, salivary IL-6, IL-1β, and MMP-9 displayed positive correlations with *T. forsythia*. Additionally,

N. meningitidis was associated with increased levels of serum IL-6, IL-17, salivary IL-13, and MMP-3 in MMP. In PV group, all disease-associated bacteria, including *G. morbillorum, H. sapiens, and F. nucleatum*, exhibited positive associations with salivary IL-6, IL-1β, and MMP-3. Furthermore, this association was not only positively correlated with the severity of PV but also significantly linked to the PISA score across all groups. The potential impact of *F. nucleatum* on immune responses in conditions such as OLP, MMP, and PV could contribute to the development of immunobullous diseases and OLP.

4. Metabolome and metabolic activity in oral microbiome in AIBD and OLP.

Having characterised the oral microbiome in these patient groups, the oral (salivary) and systemic (plasma) metabolomes were characterised to identify any functional changes associated with disease.

First, this study identified salivary and plasma metabolites that may serve as indicators of metabolomic changes in patients with AIBD and OLP, emphasising the potential role of periodontal inflammation, and identifying specific biomarkers or metabolic changes that could provide insights into the underlying pathogenesis, disease severity, or potential interactions between oral inflammatory conditions and periodontal health.

The study's main findings were that salivary ethanol levels were positively correlated with oral disease severity in OLP. Most likely, it is associated with alcohol consumption, as indicated by data from dietary questionnaires. This association implies a detrimental impact of ethanol on OLP symptoms, potentially contributing to the development of more severe lesions in affected patients. Elevated cholesterol and glucose levels were also found in the plasma of patients with OLP, along with the identification of glycolysis and gluconeogenesis as significant metabolic pathways in OLP, which suggest disturbances in lipid and glucose/hexose metabolism. This may imply an altered metabolic state in OLP patients, confirmed with abnormally higher BMI in the OLP group, potentially influencing the inflammatory response and disease progression.

In MMP the data showed increased salivary arginine levels and significant associations between arginine biosynthesis and metabolism pathways with the disease. Increased arginine levels in MMP may indicate its involvement in the disease's inflammatory mechanisms. In addition, the significant association of arginine biosynthesis and metabolism pathways with

disease status suggests that there may be dysregulation, such as aberrant nitric oxide (NO) production. Excessive NO production can contribute to an inflammatory response by activating various pro-inflammatory signalling pathways, releasing pro-inflammatory cytokines and recruiting and activating immune cells.

Glucose levels in plasma were elevated across all three disease groups, and in PV, higher plasma glucose levels were associated with greater disease severity. The relationship of PV with T2DM was not directly evaluated in this study, but the elevated glucose levels observed in patients with these oral diseases, suggest an association with glucose metabolism disturbances. These imbalances in glucose metabolism may have significant implications, including cellular dysfunction, impaired tissue repair, and compromised barrier functions that are vital for maintaining the integrity and homeostasis of oral tissues (Drew et al., 2009). This dysregulation of glucose metabolism extends to its potential involvement in immunometabolism, where immune cells like T cells and B cells undergo metabolic shifts to meet the energy demands of specific immune processes. Finally, increased salivary choline levels were found and associated with disease severity in PV. These increased choline levels may suggest an alteration in acetylcholine signalling in the oral mucosa, which could affect immune cell activation, inflammation, and tissue homeostasis (Glunde et al., 2011).

Polyunsaturated fatty acids (PUFAs) in plasma were found to be increased in PV and associated with the disease severity. Dietary habits play a significant role in determining the fatty acid composition in plasma. PUFAs, particularly omega-3 fatty acids, are known to possess anti-inflammatory properties (Zhao et al., 2005). The increased levels of PUFAs may reflect an attempt by the body to counteract the inflammatory processes associated with PV, suggesting their potential role in restoring oral tissue integrity and function (Sheril et al., 2009). However, further research is necessary to fully understand the underlying mechanisms and implications of these findings.

Another salivary metabolite, a BCFA isovaleric acid, was associated with both the microbiome and the periodontal disease. It was found to be associated with the microbiome and the periodontal status in OLP and MMP. BCFAs have been shown to possess anti-inflammatory properties. They can inhibit the production of pro-inflammatory cytokines and chemokines, thereby dampening the inflammatory cascade. Isovaleric acid is a metabolite produced by predominantly Prevotella species and *P. gingivalis* in the oral microbiome by breaking specific

amino acids, such as the BCAAs (Zhang et al., 2018). It may serve as a normal by-product of microbial metabolism in moderate amounts without causing significant harm. In some cases, elevated levels of isovaleric acid may favour growth of certain bacteria, potentially leading to shifts in microbial community dynamics. This in turn, can influence oral health and contribute to development of oral diseases. However, it is essential to note that the presence of isovaleric acid alone may not directly cause oral health issues, but rather its association with other factors and microbial dysbiosis may contribute to such conditions. The association of isovaleric acid with the PISA score and increased microbial diversity, notably periodontal pathogens suggest that this metabolite may play a role in disrupting the microbial ecosystem. In doing so, the increase in this metabolite potentially drives dysbiosis community, promoting the growth of periodontal pathogens and worsening disease progression. Further investigation is needed to understand the underlying mechanisms involved fully and to explore potential therapeutic strategies targeting isovaleric acid and its related pathways.

Limitations and future work.

This study has several limitations, with one concerning the statistical power due to a relatively smaller sample size. The impact of the Covid-19 pandemic significantly reduced clinical activity, affecting patient recruitment. Despite the challenges of a limited participant pool, it is crucial in microbiome studies to establish strict criteria for recruitment to minimize the influence of local and systemic factors on the oral microbiota. In this study we maintained an equal distribution of age, sex, and BMI among study groups. For future research, considering patients with hidden conditions such as deficiencies in nutrients (iron, vitamin B12, folic acid), is important due to their known impact on oral epithelial cell turnover, potentially affecting the oral mucosal microbiota structure. Control measures for local environmental factors, such as sugar consumption and oral hygiene practices before sampling, should be implemented. Another confounding factor was the use of systemic immunosuppression in the treatment of mucosal diseases, influencing the oral microbiome, disease activity, and pathophysiology. Therefore, a future goal would be to minimize and control confounding factors. Addressing these limitations involves larger sample sizes to enhance statistical power and the use of regression models.

The cross-sectional design of this study limited the ability to establish causal relationships or assess the natural history of diseases. Integrating multi-omics data helps mitigate this limitation. However, studies on treatment-naive patients could offer insights into disease natural history. Longitudinal studies, including pre-treatment and after-treatment, would enhance understanding of treatment effects on the oral microbiome and its evolution during these diseases, potentially revealing associations with disease severity. Such studies have the potential to reveal associations between the oral microbiome and disease severity. To ensure the validity of the results, it is important to implement blinding and randomization techniques, which help distribute potential confounding factors evenly across the groups and reduce the risk of biased outcomes. Furthermore, comprehensive investigations into the effects of diet are necessary for a better understanding of disease development and progression.

Periodontal and mucosal disease scoring often involves subjective judgment, in clinical assessments or evaluations. Different observers may interpret symptoms or signs differently, leading to variability in scores. In this study disease severity was assessed objectively and by highly experienced clinicians who devised and validated the ODSS scoring methodology at Guy's Hospital. Similarly, the periodontal disease scores were undertaken by the lead periodontist or two of his trainees and thus scores were consistent between clinicians. Training sessions and calibration exercises were performed prior start of the study. However, implementing blinding techniques can prevent observers from knowing the disease status or treatment group of participants. This is relevant for interventional studies but probably not practical in a real-world observational study and cross-sectional study.

The main limitation of the immunologic study was that there were patients at different stages of their disease and each on different treatment regimens. This heterogeneity can result in variability in cytokine levels within each disease group and may have affected the strength of the association between cytokine response and the individual disease. Future studies could better consider stratifying the groups based on disease stage, treatment regimens with sequential samples analysed to understand the relationship between cytokines and disease severity. Moreover, Investigating the relationship between the host's genetics, specifically its immunogenetic aspects, by employing advanced technologies and methodologies in immunogenetics and whole genome sequencing, valuable insights can be obtained for more

precise diagnostics, personalized therapeutic strategies, and a deeper understanding of the complex interplay between genetics and autoimmune diseases.

To address limitations of the microbiome study for investigating the role of periodontal pathogens, further research should incorporate a stratified approach for each disease group based on periodontal disease severity. This adjustment would provide a more comprehensive understanding of the relationship between oral microbiome and disease severity within specific disease subgroups. Future research directions could explore the presence of specific microorganisms at other sites prone to involvement, such as the eye, to determine if there is an overlap with the oral microorganisms identified in this study, as well as the functionality of their genes. In addition, expanding the analyses used in this study to include other microorganism kingdoms, such as viruses, fungi, protozoa, and archaea, will provide a more comprehensive understanding of the oral microbiome ecosystem. Specifically, studying the mycobiome would be of interest, as Candida species infect a considerable proportion of OLP patients, and could help identify any differences in disease severity and periodontal involvement. Lastly, investigating combinations of bacteria associated with higher disease severity scores could uncover potential microbial signatures indicative of disease progression and severity.

Limitation of the metabolomic study was focussed on metabolite levels/profile without investigating gene expression or other molecular mechanisms involved in the metabolic pathways of these diseases. Future studies should aim to integrate metabolomic data with gene expression analysis to gain a more comprehensive understanding of the metabolic dysregulation of both host and microbiome in AIBD and OLP. Further studies should be conducted to validate the identified biomarkers' diagnostic and prognostic value, in larger cohorts of patients to ensure the accuracy and reproducibility of the biomarker measurements. Moreover, we started by using NMR technique to look broadly at the metabolites. Next, we plan to use a targeted method Mass Spectrometry (MS), which is more focused. Targeted MS allows us to see smaller details and find rare metabolites that might be important. The presence of confounding factors, such as diet, medication use, and comorbidities, can influence the composition of the metabolome. While the current study may not have integrated full dietary information due to time constraints, it is essential to recognize the potential value of future research for understanding the interplay between diet

and the microbiome, i.e. exploring correlations between dietary habits and specific microbial species or functional pathways within the microbiome, as well as stratifying participants based on their dietary habits and investigate how different dietary patterns are associated with distinct microbial profiles or functional characteristics. Moreover, integrating dietary information with metabolomic data would help to provide further insights into the relationship between diet, the microbiome and metabolite production. The intention is to integrate this dietary data into upcoming studies, particularly those that employ systems biology and modelling-based approaches. These approaches encompass systems modelling, machine learning algorithms, and longitudinal modelling. Their application will enable to explore temporal dynamics, predict how the microbiome responds to dietary patterns, identify causal relationships, and analyse longitudinal data. Ultimately, these advanced methodologies will provide a more profound understanding of how diet influences the microbiome and its interactions with health outcomes and enhance microbiome studies beyond a near-exclusive reliance on correlation analyses.

Based on the understanding that antigens from oral bacteria may cross-react with proteins targeted by antibodies in oral mucosa, the comprehensive screening of oral bacteria and characterization of their antigenic properties can be investigated. This investigation is essential for understanding the interaction between bacterial antigens, host immune cells, and antibodies, which can provide insights into the underlying mechanisms driving the inflammatory response and tissue damage in conditions such as MMP, PV, and OLP. Additionally, exploring the efficacy and safety of interventions like probiotics and antimicrobial agents in preclinical and clinical settings is important.

Overall, the identification of salivary IL-6, MMP-3, isovaleric acid, ethanol, arginine and choline, and species associated with them as potential biomarkers in AIBD and OLP opens up promising avenues for further research, clinical applications, and the development of personalized approaches for disease prediction, monitoring, and treatment.

In summary, this study demonstrates the presence of dysbiosis in all three disease groups, each with its distinct microbial, immunological and metabolomic profile. While the exact role of PD in oral mucocutaneous diseases remains unclear, this study highlights associations between periodontal pathogenic bacteria and OLP, MMP and PV. Furthermore, it demonstrates a positive correlation between the severity of oral disease and periodontal

scores in PV and OLP. Additionally, in PV, the study showed strong associations with periodontal pathogens. These findings suggest a potential link between periodontal health and development or progression of these diseases, indicating the importance of taking into consideration the periodontal status while managing and treating oral mucocutaneous diseases. Further research is needed to unravel the underlying mechanisms and establish the clinical significance of these associations. These results align with the widely accepted notion that saliva serves as a reservoir for the oral microbiota and can be a valuable non-invasive source for identifying disease-related biomarkers. By providing a comprehensive understanding of dysbiosis in the microbial community of OLP, MMP, and PV, this study highlights the potential microbial involvement in the progression of these diseases. It establishes a foundation for future investigations on specific bacteria implicated in these conditions.

Incorporating these suggestions and methodological improvements into future studies will help us to uncover the complex interactions between oral autoimmune diseases and microbiome which will help to understand pathogenesis of these diseases, enhancing the diagnosis and management of these conditions.

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Appendices

Appendix 1. IRAS Form

Faculty of Dentistry, Oral and Craniofacial Sciences
at Guy's, King's College and St Thomas' Hospitals

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NIHR IRAS ID: 207795

Title of Project: The Microbiome of Saliva

Names of Researchers: Professor Gordon Proctor, Professor Michael Escudier, Dr Mark Ide, Dr Jane Setterfield, Dr David Moyes,

Dear Dr

We are writing to inform you of a HRA approved research project (REC reference # 17/LO/0898) which is examining the microbiome of saliva and how it is altered in oral and systemic disease.

As part of the project, we will be accessing Electronic Patient Records of the participants with their consent. Your patient, xxxxxxxxxxx has consented to take part in the project.

Yours Sincerely,

Professor Gordon Proctor, on behalf of the research team.

Appendix 2. Inclusion and exclusion criteria.

(*exception: subgroup of 12 patients with MMP on systemic AB were later included)

Inclusion	Exclusion
>18 years old	No antibiotics prior 1-month prior sample collection*
	Pregnant women
	Presence of <1 tooth in the mouth

Appendix 3. Consent form

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: The Microbiome of saliva

Names of Researchers: Dr David Moyes, Professor Gordon Proctor, Professor Michael Escudier, Dr Mark Ide, Dr Jane Setterfield, Dr Sadia Niazi

Please initial box

1. I confirm that I have read and understood the information sheet dated _____ Version ___ for the above study. I have had the opportunity to consider the information and asked questions, which have been answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason without my medical care or legal rights being affected. ☐
3. I give permission for these individuals to analyse my samples ☐
4. I give permission for the research team to contact my dentist and GP ☐
5. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications ☐
6. I understand that the research team may use registered external organisations for the processing of my data and that in such cases my confidentiality and anonymity will be maintained. ☐
7. I agree that my samples and data can be used for ethically approved future research . ☐

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent	**Date**	**Signature**
_____	_____	_____
Name of Researcher	**Date**	**Signature**

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 4. Participant/patients information sheet

KING'S College LONDON
University of London

Guy's and St Thomas' NHS
NHS Foundation Trust

Participant Information Sheet for patient participants.

IRAS ID: 207795, Version 4, Date: 22/07/2019

Study title: The Microbiome of Saliva

We would like to invite you to participate in a research study. Before you decide, please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.
Thank you for reading this.

What is the purpose of the study?

The body contains microbes that are crucial for the maintenance of health and alterations in the types of microbe in the body are linked with disease. The objective of the study is to determine the microbes in saliva and to assess how it differs between people. A second objective is to determine the relationship between oral microbes and gut microbes in people.

Why have I been invited to take part?

We are recruiting patients at Guy's & St Thomas' Foundation Trust. You are suitable for this study if you are aged 18 years or over. You should not take part if you have been on a course of antibiotics up to 1 month prior to sample collection as antibiotic use can influence the composition bacteria in the body. You should not take part if you are pregnant.

Do I have to take part?

It is up to you to decide whether to take part or not. You are free to refuse. If you decide to take part you are still free to withdraw from the study at any time and without giving a reason. Samples and data already collected can be withdrawn up to 2 weeks after we have collected the sample, and the samples and data will be destroyed.

What will happen to me if I take part?

We will ask you some questions about your oral health and may make an assessment in order to see if you have gum disease or tooth decay. We may also ask you some questions about your diet. We would like you to give a sample of saliva by spitting into a plastic container for a period of 10 minutes (approximately 4 ml or 1 teaspoon). You may also be asked for samples of plaque from the surfaces of your teeth and these will be collected by a dental professional by lightly scraping the tooth surface near the gums. You may also be asked to give **small samples** (3ml) of blood which will be collected by a professional phlebotomist (blood collector). **If you are having endodontic treatment you may also be asked for collection of root canal samples, which will be collected by the dentist performing their root canal treatment. If you are attending an Oral Medicine clinic an oral swab may be taken.**

You may also be asked to provide a stool sample and we will give you a collection kit so that you can do this at home and post the sample to the laboratory.

Appendix 5. Dietary questionnaire sheet

Dietary Questionnaire								
Do you follow a special diet?						yes	no	
	Gluten free or wheat free diet							
	Diet for lactose intolerance							
	Low calorie/weight-controlled diet							
	Pescatarian diet (no meat, no poultry)							
	Vegetarian diet (no meat, no poultry and no fish)							
	Vegan plant-based diet							
	Other special diet							
Do you smoke?								
Dietary Questionnaire								
Daily Consumption								
Food components	Food photos		Never or less	1	2	3	4	More than 4
Bread								
Porridge								
Pasta								
Rice								
Other Grains								
Green leafy vegetables								

Potatoes							
Other vegetables							
Beans							
Other legumes							
Berries							
Citric fruits							
Other fruits							
Nuts							
Seeds							
Olive Oil							
Butter or margarine							
Mature cheese							

Young Cheese							
Animal derived milk or yogurt							
Plant derived milk or yogurt							
Pastries							
Sweets/ candy							
Red meat							
Red meat derived products							
Poultry							
Fish							
Fast or fried food							
Water							

Fresh-made pure fruit juices							
Fruit smoothie							
Low calorie or diet drinks							
Carbonized (fizzy) drinks							
Red wine							
White wine							
Beer							
Other alcoholic drinks							

Appendix 6. ODSS sheet

Guy's and St Thomas' NHS
NHS Foundation Trust

KCL Dental Institute
Department of Oral Medicine
DISEASE SEVERITY SCORE

KING'S College LONDON

LICHEN PLANUS

Name: ..... **Hospital Number** ..... **Date** .....

Predominant type (ring type): Reticular / Atrophic / Plaque / Desquamative gingivitis / Ulcerative

Site (possible score values)		Site score	Activity score (0 - 3), Double if site score = 2
Outer lips	(0-1)		
Inner lips	(0-1)		
R Buccal Mucosa	(0 or 1 [<50%] or 2 [>50%])		
L Buccal Mucosa	(0 or 1 [<50%] or 2 [>50%])		
Gingivae	(0-1 each segment)		
Lower R (distal)			
Lower central			
Lower L (distal)			
Upper R (distal)			
Upper central			
Upper L (distal)			
Dorsum tongue	(0 or 1 or 2)		
R ventral tongue	(0-1)		
L ventral tongue	(0-1)		
Floor of mouth	(0 or 1 or 2)		
Hard palate	(0 or 1 or 2)		
Soft palate	(0 or 1 or 2)		
Oropharynx	(0 or 1 or 2)		

Totals: SITE SCORE: ACTIVITY SCORE: PAIN SCORE (0-10):

VAS _____

TOTAL DISEASE SEVERITY SCORE:

Key

Activity Score

0 = no lesion at site

1 = mild erythema (e.g. on gingivae, papillae only or less than 3mm along margins)

2 = marked erythema (e.g. full thickness on gingivae, extensive with atropy or oedema on non-keratinised mucosa)

3 = Ulceration at this site

Site Score

0 if no lesion at site

1 if less than 50% of area affected

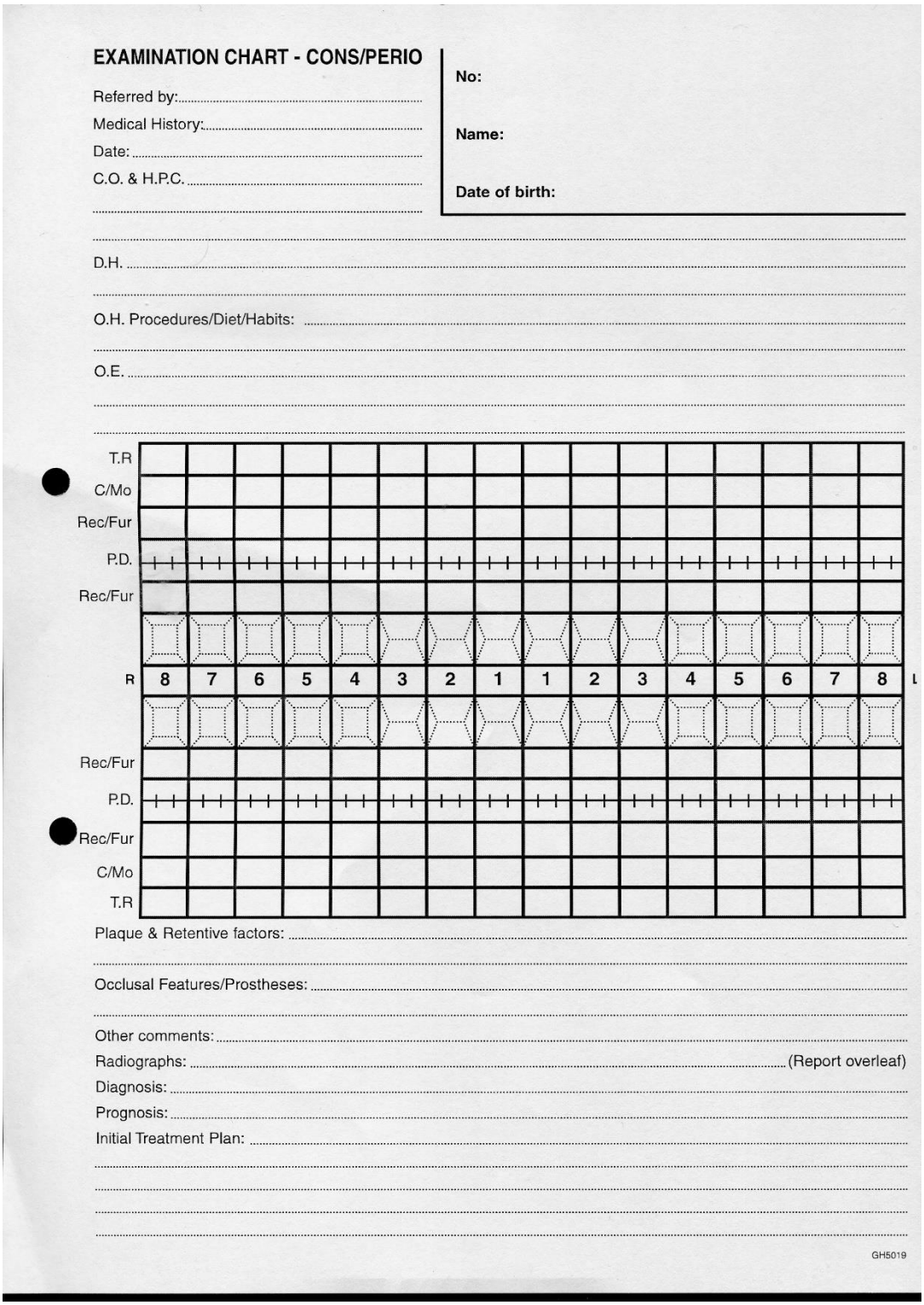
2 if greater than 50%. Not defined anatomically

Pain Score

Analogue scale from 0 (no discomfort) to 10 (unbearable pain)

Physician signature.....

Appendix 7. Periodontal examination chart.



EXAMINATION CHART - CONS/PERIO

Referred by:

Medical History:

Date:

C.O. & H.P.C.

No:

Name:

Date of birth:

D.H.

O.H. Procedures/Diet/Habits:

O.E.

	R	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8	L
T.R																		
C/Mo																		
Rec/Fur																		
P.D.																		
Rec/Fur																		
Rec/Fur																		
P.D.																		
Rec/Fur																		
C/Mo																		
T.R																		

Plaque & Retentive factors:

Occlusal Features/Prostheses:

Other comments:

Radiographs: (Report overleaf)

Diagnosis:

Prognosis:

Initial Treatment Plan:

GH5019

Appendix 8. Medical history sheet

IRAS Project ID: 207795

Guy's and St Thomas' NHS
NHS Foundation Trust

Medical History
Guy's and St Thomas' Dental Hospital

Patient Details (or label)

This form will be checked by the clinician.

Note: This medical history sheet should not replace a full history taking.

Hospital Number_____

Name _____

Date of Birth _____

	Yes	No
Are you in good health?		
Have you had any operations or serious illnesses?		
Are you attending a doctor, hospital clinic or specialist?		

Do you have, or have you had, any problems with your:

	Yes	No	Further Details
Chest e.g. asthma, bronchitis, shortness of breath or a persistent cough?			
Heart e.g. angina, 'murmur', rheumatic fever, a replacement valve or pacemaker?			
Blood Pressure? (high or low?)			
Circulation and Blood e.g. anaemia or prolonged bleeding?			
Stomach and intestine e.g. ulcers, gastric reflux, colitis, jaundice, or cirrhosis of liver?			
Kidneys e.g. chronic infections?			
Nervous system e.g. epilepsy, Parkinson's disease, multiple sclerosis or a stroke?			
Hormonal system e.g. diabetes or thyroid?			
Joints and bones e.g. arthritis, or replacement joints?			
Skin & Mucous Membranes e.g. eczema, psoriasis or ulcers?			
Allergies and Sensitivities e.g. allergy to penicillin or any other drugs or to rubber, foods or material?			
Are you taking any medications or drugs which are prescribed, bought over the counter or recreational? (e.g. steroids, warfarin ,chemotherapy or DXT)			If yes please list them below

Medication/Drugs (including dose and frequency)	Date started	Date stopped

Appendix 9. Clinical data

Dgs	Gender	ODSS	PISA	BS	LOAx	N of teeth	Age	SFR	Ethnicity	Treatment	Diet cluster	BMI	Diabetes
MMP	F	24	275.271	29.8851	1.67241	29	26	0.4	mixed	topical betnasol mouthwash (systemic antibiotic)	1	23.8	0
MMP	F	3					63	0.402	white	no treatment (no topical steroid)	1	34	0
MMP	F	12	0.36111	0	2.27083	32	48	0.8	asian	no treatment	1	22.3	0
MMP	F	58				no perio	86	0.3	white	MMF, Sulfupiridine, Pred	1	23.1	0

MMP	M	18	85.049	10.2151	1.99462	31	35	0.9	white	dapsone	1	24.8	0
MMP	M	18	0	0	1.88636	22	72	1.2	white	topical flexinase+niostatin, dermovate in orabase	1	27.2	0
MMP	F	6	200.809	14.3678	2.2069	29	55	1.1	white	topical steroid	2		0
MMP	M	4	121.775	13.0952	1.6131	28	59	1	white	no treatment	1	28.7	0
MMP	F	8	75.4158	4.7619	2.42857	28	55	0.45	white	Fluoxetine, Ramipril, Propranolol, Insulin and Atorvastatin.	3		1

MMP	F	7				11	64	0.23	white	topical mouthwash bethametazone, Metformin	1	29.3	1
MMP	M	25	102.257	15	3.31667	20	77	0.3	white	topical mouthwash	1		0
MMP	F	12	76.1266	8.04598	1.98276	29	61	0.6	white	topical MW, Clobetasol, citalopram, candesaran for magraine, frovapripran, domperidone,	2		0
MMP	F	12	108.081	16.092	1.61494	29	53	0.46	white	no treatment	1		0
MMP	M	22	311.641	62.5	1.55556	24	67	0.29	white	Topical prednisone MW, Tab candesartan, diazepam,	1	25.9	0

MMP	M	40	563.239	25	4.8125	32	60	0.63	asian	Aza, Sulfapyridine, Bethametazone MW	3	27.4	0
MMP	F	29	123.558	10.1449	5.65217	23	60	0.2	white	Flixonase and Nystatin mouthwash BD Dermovate and Orabase OD Fluconazole 50mg OD 10 days, adcal-3, citalopram, salbutamol, omeprazole, estrodiol, levothyroxine, dermovate topical	1	16.4	0
MMP	F	0	217.1	26.8116	2.47101	23	61	0.32	white	Flixotide spray; Flixonase Levothyroxine Beclomethasone inhaler – Fostiar Salbutamol inhaler, Montelucast since Sep2020 Flixonase mouthwash	1		0
MMP	M	25	293.972	31.5476	1.23214	28	57	0.5	white	Topical bethnasole MW, allopurinol	1	26.1	0

MMP	M	3	12.0643	1.66667	1.55556	30	64	0.45	white	Dapsone 50mg, Bethametazone MW, B12, Amlodipine 5mg	2		0
MMP	F	32	249.203	26.81	1.52174	24	76	0.3	white	Amitriptyline, Athorvostatin, amitriptilin, aspirin, ezetimibe, omeprazole, metformin, paracetamol, codein	1	27.5	1
MMP	F	13	136.943	17.7778	5.06667	15	86	0.2	white	MMF 1mg, Dermovate bethametazone, difflam MW, Alendronic acid, D3, levothyroxine,	1		0
MMP	F	12	381.457	24.1935	3.12903	31	44	0.5	white	1. Flixonase mouth rinse 2. Elocon Scalp twice per week	3		0
MMP	F	0	85.1155	11.73	1.24691	27	66	0.3	white	No treatment, peroxiteine, candevartan, rumivastatin	3		0

MMP	F	17	70.1678	7.97101	4.61594	33	67	0.2	asian	MMF, metformin, insulin, topical betamethasone	1	36	1
MMP	M	12	56.6788	3.96825	3.85714	21	86	0.32	white	Aza, Prednisolone, omperozole, Adcal, topical Dermovate	1	25.6	0
MMP	F	14	0	0	3.70513	26	90	0.2	white	topical flexinase mouthwash, atorvastatin, bisoprolol, losartan omeprazole, osteocopes, CalD3, nicorandir	1		0
MMP	F	19	110.341	7.24638	3.56522		79	0.35	white	Topical flexinase, dermovate, doxazosin, lansoprazole, semvastatin	1	28.1	0

MMP	F	16	24.7529	3.1746	3.09524	21	75	0.45	white	Topical, Dermovate, Bethnasole mouthwash, vitamin d,	1	21.7	0
MMP	M	8	136.097	14	2.02	25	73	0.3	white	Topical dermovate, vit C, cod liver oil, garlic tablets	3	24.6	0
MMP	F	13	85.7901	47.9167	3.3125	8	70	0.5	white	MMF, bisoprolol, omeprazole, Pred 5mg, Atorvastatin, Alendronic acid, Adcal	1		0
MMP	F	24	94.4481	14.6667	1.90667	25	81	0.7	white	Topical Bethametazone, Dermovate with orabaselevothyroxine, losartan, insulin	1	25.8	1
MMP	M	49	987.567	74.6914	4.70988	27	72	0.5	white	n/a	1	21.7	0

MMP	M	6	204.962	14.2857	6.95238	21	81	0.4	white	no treatment ramipril, metformin, atorvastatin	1		1
MMP	M	14	521.713	35.7143	3.83929	28	68	0.3	white	MMF+sulfapyridine, Nasonex, neostatin+flexinase, salbutamol, omeprosol	1	23.8	0
MMP	M	27	0	0	2.10417	24	90	0.3	white	pred 20mg, sulfapyridine 750 mg, topical	1		0
MMP	F	0	4.24305	0.98039	4.93137	17	86	0.35	white	Pred 4mg, Betnasol MW, Calcium, Vit D, Atorvastatin, Risedronate, Cetirizine, Folic Acid, Methotrexate, Furosemide, Metformin	1		1

MMP	F	5	65.0309	14.4444	3.52222	13	61	0.5	white	MMF, Dapsone, Flixonase, Nystatin, Vit B, metformin, levothyroxine, Lipitor, (Rituximab Aug 21) cyanocobolamin, rituximab	1		1
MMP	F	21	132.077	10.8974	3.62821	26	70	0.3	white	dapsone 50 mg od, Flexinase+niostatin, D+O fenotain, statins, levothyroxine	1		0
MMP	M	35	33.4581	3.7037	2.26543	26	39	0.5	white	Pred, Aza, dapsone, omeprazole, vit D, calcium	1	18.1	0
MMP	F	8	131.023	10.6667	2.51333	25	57	0.4	white	topical, bethametazone	3		1
MMP	F	11	793.218	65.4762	8.82143	14	63	0.5	white	doxycycline 100mg, betnesol MW, D+O calcium, levothyroxine, omeprazole	3		0

MMP	F	18			n/a		59	0.2	white	cardostatin, (rituximab jul 2021), MMP, dapsone, Pred	2		1
MMP	M	0	524.865	52.5	6.875	20	74	0.3	white	MMF 1g, dapsone 50mg, Pred 5 g, aspirin, omeprazole, nystatin	1	25.4	0
MMP	M	28	89.1584	15.942	4.73913	23	70	0.5	white	Topical treatment, code liver	1		0
MMP	F	16	654.306	27.1605	4.8642	27	63	0.4	white	topical (flexinase+niostatin), against blood pressure tab	1		0
MMP	F	12	138.036	9.67742	3.06452	31	66	0.3	asian	MMF 1 g, Dapsone 50mg, Pred 5 mg, alendronic acid, omeprazole	2		0

MMP	F	24	718.248	41.6667	5.90278	24	37	0.4	asian	pred 10 mg, Vit D3, Calcium, Vit B, C, Collagen, multivitamins	1		1
MMP	F	22	544.182	29.8246	6.89474	19	57	0.38	white	F+N, D+O topical	1		0

Dgs	Gender	ODSS	PISA	BS	LOAx	N of teeth	Age	SFR	Ethnicity	Treatment	Diet cluster	BMI	Diabetes
PV	M	20	165.307	47.5	2.1	20	72	0.9	asian	azathioprine 150 mg, Prednisone 20mg	2		0

PV	M	21	1052.21	52.6882	3.23656	31	46	1.2	asian	Azathioprine, Prednisone Flexinase, fluticazone, medication for oral Thrush	1		0
PV	F	64				28	56	1.4	asian	prednisolone, MMF, insulin, rampil, propranolol, antidepressants	3		1
PV	F	0	28.8958	2.38095	2.0119	28	59	0.41	white	Tab prednisone, topical F+N MW, Ad Cal, Vitamin D	1	31.8	0
PV	F	22	7.98205	0.61728	2.2037	27	68	0.5	asian	Tab prednisone, bethnasole MW, Dermovate in orabase, omeprazole, atorvastatin, Vit D, Risedronate	1	30.3	0
PV	F	17	25.7522	4.69	2.04688	32	38	0.8	mixed	Tab prednisolone	1		0
PV	F	24	256.349	23.6559	1.76344	31	49	0.7	asian	Aza, prednisone, allopurinol	1		0

PV	M	15	60.1745	5.55556	2.14815	27	60	0.9	white	Aza 200mg, Pred 15mg, Topical terbinafine, Nystatin, Fluticasone inhaler	1	31	0
PV	F	22	346.275	23.2143	2.66071	28	48	0.9	white	probiotics, Aza, prednisolone, MMF, Calcichew Alendronic acid, Topical flixonase orobase+clobetasone (rituximab in May 2019, probiotics	1	25.1	0
PV	M	8	162.327	25	1.64394	22	38	0.7	asian	Aza Pred, Lansoprazole calcichew, Vitamin D, bethametazone mouthwash	1		0
PV	F	12	394.434	30	1.73333	30	55	0.23	asian	prednisolone, bethametazone mouthwash, Metformin, Omeprazole, Atorvastatin, amlodipin,ramiprile, sertraline	3	29.3	1
PV	M	4	86.2939	8.33333	2.23214	28	53	0.3	black	Tab prednisolone, tenofovir, omeprozole	1		0

PV	F	0	78.1989	10	3.375	20	69	0.17	asian	prednisone and MMF	3	32.3	0
PV	M	20	9.42327	1.38889	1.77083	24	64	0.5	white	Aza 200mg+Pred 5 mg	1	25.2	0
PV	M	0	72.7462	9.25926	1.59259	27	48	0.9	white	prednisone 3g, MMFe 1g, aledronic acid, rituximab 1 year ago	1	20.9	0
PV	F	4	234.55	30.3571	1.66071	28	58	0.62	asian	MMF	3		0
PV	F	7		no		26	59	0.65	white	pred 5mg, MMF 1g BD, ad cal, omeprazole	3	24.3	0
PV	F	5	77.1319	8.92857	1.57738	28	33	0.9	asian	Pred 8mg, Topical Dermovate, sulfasalazine, omeprazole, alendronic acid, Vit C, Maexeni COCP, previous rituximab in march 2020	1	23.2	0

PV	M	8	218.442	16.6667	2.73333	30	45	0.63	white	Topical steroid cream, Aza finished in 24.11.20	1	29	0
PV	M	4	79.3936	6.25	2.31771	32	40	0.2	asian	Aza150 mg Betamethasone MW	3	29.3	0
PV	F	0	34.6795	3.57143	2	28	22	0.3	white	Azathioprine Prednisolone Adcal D3	1		0
PV	M	10	178.348	20.2381	4.92857	14	67	0.8	asian	Aza 50mg, pred 4mg, Flix MW, Difflam MW; Alendronic acid	3	21.6	0
PV	F	25	136.252	15.3333	1.85333	25	54	0.5	white	Azathioprine 75mg, Dermovate, Pred , Vid D, Calcium	1	27.1	0

PV	M	0	91.7232	12.96	1.3642	27	49	0.6	asian	Tab Prednisolone, Bethametazone MW	2	25.2	0
PV	F	0	236.06	19.44	2.03889	30	57	0.5	white	prednisolone, MMF, lansoprazole, vitamin D, C	1		0
PV	F	7	112.193	17.86	1.3631	27	47	0.2	other	MMF, Pred, Tenofovir, Femoston, Vit D, Calcium, Zink, got Rituximab in 2019	1	23.1	0
PV	F	6	22.2127	4.49	1.58974	26	72	0.4	asian	MMF, bethametazone mouthwash, amlodipin, calcichew, estriol cream, omeprazole, ramipril, travoprost, Adcal,	2	25.7	0
PV	F	0	0	0	1.41975	27	62	0.5	white	prednisolone 5mg, losartan, alendronic acid, adcal D3	1	26.8	0

PV	M	0	16.5137	2.46	1.41358	27	68	0.65	asian	Rituximab, MMF, Prednisolone 5 mg a, propranolol, ramipril , omeprazole, alendronic acid, atorvastatin	1	29	0
PV	M	0	72.5462	10.4167	1.84722	24	70	0.6	asian	Pred 3 mg, Aza 75 mg, Lansoprazole 15mg, Alendronic acid	2	22.6	0
PV	M	3	0	0	12.8333	3	68	0.4	white	Azathioprine 150 mg, Metformin	1		1
PV	F	10	306.883	43.3333	4.93333	15	60	0.3	white	MMF, hydrocortisone, adcal, hydroxicine, thyroxine, paroxetine, omeprozole, iron supplement, vit D, magnesium	1		0
PV	M	43			n/a		61	0.8	asian	antipsychotic and antidepressants	2		0

PV	M	2	43.9916	4.44	2.45556	30	53	0.4	white	prednisolone, Retuximab in Jan 2021), Alendronate, D3, omeprazole	1	20.9	0
PV	F	40	1639.78	82.2222	3.32222	30	31	0.3	asian	Azathioprine, topical Clobetasol gel, betnasol mouthwash	2		0
PV	F	3	209.719	24.359	2.33974	26	58	0.3	asian	MMF, Prednisone, calcium, atorvastatin, alendronic acid, topically dermovate	1		1
PV	F	0	65.4617	5	1.68889	30	61	0.3	asian	MMF, Pred, Alendronic acid, Adcal, Betnasol MW, nystatin	1		0
PV	F	0	28.2255	7.33333	1.36667	25	52	0.5	white	prednisolone, MMF 1g, Rituximab March , April 2021, adcal, lansoprasole, baclofen,	1		0

PV	F	0	310.006	25.5952	2.375	28	50	0.6	white	pred 5 mg, MMF 1.5 g, calcium, Vit D	3		0
PV	F	6	34.0028	3.1746	4.54762		78	0.3	white	Pred, MMF 5 g, omeprazole, prednisolone, statins	1	21.7	0
PV	F	21	16.0878	1.19048	2.44643	28	64	0.35	asian	MMF 500 BD, Rituximab in 2019, Pred 10 gm	1		0
PV	F	0	349.154	25	5.4	20	76	0.3	white	MMF, Pred, eyedrops for glaucoma, multivitamins, glucosamine	1		0
PV	F	0	28.0534	3.08642	2.89506	27	49	0.34	white	MMF, 2 g, Pred 5 mg, ramipril, sando k, omeprazole, alendronic acid, clacichew,	1		0

PV	F	0	32.8607	3.84615	2.16667	26	68	0.5	white	Aza, Pred, Bethametazone MW, Omeprazole, alendronic acid, Adcal	1		0
PV	M	0	29.052	2.38095	2.20238	28	55	0.3	white	recently stopped pred, edoxoban	2		0
PV	M	2	52.7443	2.89855	2.17391	23	58	0.3	asian	MMF 500 mg BD	1		0
PV	F	0	81.2366	8.04598	2.13218	29	41	0.4	asian	Aza 125 mg, Pred 5 mg, dermovate omeprazole, multivits, AdCal	2	18.7	0
PV	M	24	1512.2	61.2903	4.94624	31	51	0.6	asian	Aza, 5 mg Pred, bethnasole mouthwash, zero double emollient, dermovate with orabase	3		0

Dgs	Gender	ODSS	PISA	BS	LOAx	N of teeth	Age	SFR	Ethnicity	Treatment	Diet cluster	BMI	Diabetes
OLP	F	19	17.9205	1.38889	3.5		77	0.5	white	topical treatment F+N	3		0
OLP	M	30	202.957	22.619	1.95833	28	56	0.5	white	topical F+N, D+O	1	26.2	0
OLP	M	3	72.1428	6.98925	31	31	62	0.6	asian	Topical treatment Bethametazone mouthwash	1	24.6	0
OLP	F	7	816.592	52.4691	2.76543	27	58	0.3	asian	HCQ 200m, topical mouthwash	1	35.8	1

OLP	M	36	428.894	16.6667	3.25	24	62	0.35	asian	Topical bethametazone+nistatin MW, clopedagril, atorvastatin omeprazole, metphormin, thyroxine	2		1
OLP	M	9					32	0.3	asian	only topical Felxinase bethnasole mouthwash	3	24.5	0
OLP	F	9	0	0	1.73333	25	61	0.3	white	only topical bethnasole mouth wash	1		0
OLP	F	37	421.164	46.2963	2.22222	27	64	0.4	white	topical bethametazone	3		0
OLP	F	35	201.892	36.1111	2.31944	24	67	0.3	asian	HCQ, Flixanase, nystatin, vitamin D, statins Flix + nystatin MW	1		0
OLP	F	3	42.3584	2.5641	1.96154	26	41	0.5	asian	Progesterone pill	3		0

OLP	F	10	16.4289	2.22	5.48889	15	72	0.2	white	bethametazone mouthwash, sudocream, lotraderm, dermasol500, metformin, atorvastatin, aspirin, Vit D, clopidogrel	1		1
OLP	M	12	134.347	17.7778	1.76111	30	55	0.7	white	HCQ, dermovate in orabase MW, betamethasone MW, Vit D	1		0
OLP	F	26	208.82	16.6667	4.93651	21	57	0.4	asian	topical bethametazone multivitamins, cetirizine, simvastatin, metformin since 2011, levothyroxine	2	22.4	1
OLP	F	33	259.709	16.0714	3.84524	28	61	0.15	white	topical treatment with dermovate in orabase, bethametazone, on sulfasalazine, amlodipin, magnesium, nortriptyline	1	24.5	0

OLP	F	32	400.721	26.6667	2.76111	30	33	0.46	white	topical treatment Chlogecsidine MW, bethametazone, dermovate, Ventolin, bisoprolol	1	26.6	0
OLP	F	19	22.5728	3.7037	3.25	18	75	0.2	other (Hispanic)	Topical bethametazone mouthwash, Lipitor, vit d,	1		1
OLP	F	8	10.1515	0.69444	1.375	24	61	0.35	white	No topical treatment, betmiga, lansoprazole, sotoliprine for depression, cyfalopram, levothyroxine, estriol cream, dermovate	1	27.9	0

OLP	F	9	33.4264	3.08642	1.99383	26	55	1.3	white	Topical treatment, flixonase , omeprazoleBethametazone Dermovate ointment 0.05% mixed with orabase Mometasone 0.1% creamomeprosole, antihistamines,	1	33.6	0
OLP	F	20	726.635	56.1728	3.48765	27	56	0.21	n/a	Topical Betnasol mouth wash, Naproxen Amitriptyline Omeprazole Corsodyl mouth wash - helpful Atorvastatin	1	26.1	0
OLP	F	16	371.994	31.0345	2.1092	29	57	0.3	other (Hispanic)	Betamethasone MW Methotrexate 15mg oral weekly- folic acid the other 6 days Metformin Amlodipine,	3		1
OLP	F	2	16.7171	1.66667	1.36111	30	48	0.9	black	HCQ, pred 40mg, omeprazole; vid d, vit C; naproxen	1		0

OLP	F	26	389.951	38.0952	1.94048	28	49	0.4	white	HCQ; Cetirizine Flixotide inhaler Ventolin inhaler Flixonase + nystatin	1	36.4	0
OLP	M	25	419.391	28.85	4.4359	26	62	0.4	asian	Topical treatment Dermovate in orabase (clobetasone propionate 0.05%), Bethametasone MW, amlodipine, metformin, vit B12	2	26.3	1
OLP	F	0	85.5897	12.32	1.65217	22	72	0.4	white	MMF 1.5 g, soluble pred MW, +Niastatin	1	24.5	0
OLP	F	33	30.8539	7.41	1.62037	18	71	0.5	white	Nil, zinc	3	24	0
OLP	F	13	62.6886	10.9	2.30769	26	79	0.4	white	HCQ, Dermovate ointment Calcium D3	1		0
OLP	F	35	183.129	17.7778	2.05	30	45	0.35	white	flexinaze mouth wash	1		0

OLP	F	30	145.234	29.33	2.04667	25	55	0.6	n/a	F+N, D+O	2		1
OLP	F	29	478.5	76.1905	2.5478	14	65	0.55	white	Aza, Flixonse, Nystatin, Fluconasole ferros, levothyroxine,	1	33.5	0
OLP	F	27	154.9	17.8571	1.8988	28	70	0.6	white	multivitamins	1		0
OLP	F	19	251.671	28.6667	3.62667	25	65	0.5	white	topical treatment (davobet), metformin, octasa, simvastatin,	1		1
OLP	F	30	0	0	3.21429	28	75	0.35	asian	topical treatment, dexamethasone mouthwash, atorvastatin, vit D, calcium, magnesium	1	19.5	0

OLP	F	39	32.9604	5.76923	1.62179	26	68	0.6	white	Topical dermovate orabase; flexinase+nystatin	1		0
OLP	F	3	306.952	35.1852	2.22222	27	67	0.7	white	vemafaxine, atorvastatin	1	24	0
OLP	F	34	139.791	116.667	1.72222	6	58	0.4	other (Hispanic)	Topical treatment, D+O, F+N; inaclotide, lactulose, salbutamol, fentanyl, amitriptyline, fluticasone, lansoprasole, colecalciferol, pregabalin, citalopram, loratadine, ramipril, amlodipine	1	33.7	0

OLP	F	13	0	0	3.19444	12	66	0.3	white	Topical treatment, bethametazone, levothyroxine	1	28.2	0
OLP	F	27	220.909	11.3636	5.58333	22	65	0.3	white	HCQ, Ad Cal, topical treatment	1		1
OLP	F	28	44.301	8.92857	1.79167	28	55	0.7	white	MMF, Pred, Dermovate orabase, Flexinase+nystatin	1		0
OLP	F	24	10.9106	1.6129	1.18817	31	53	0.8	white	HCQ, floxitine, bethnasole mothwash	1		0
OLP	F	14	77.5033	9.44444	1.30556	30	60	0.5	white	Topical bethametazone, atorvastatin, ramipril,	1		0

OLP	F	15	89.0441	7.63889	3.71528	24	82	1.2	white	MMF 500 gm a day, lysonaprile 5 mg, Vit D	1		0
OLP	F	2	40.1977	5.88235	3.55882	17	70	0.3	white	HQC 200 mg, amlodipine 5mg,	1		0
OLP	M	14	34.9698	3.64583	2.09896	32	31	0.5	white	pred 30 bethametazone MW, Ayurvedic medication	1		0
OLP	F	30	145.403	18.3333	2.675	20	55	0.6	white	topical treatment, metformin, atorvastatin, glucoside	1		1
OLP	F	19	173.688	11.1111	4.15972	24	65	0.4	white	HCQ 200, glycoside, aspirin	1		1

OLP	F	28	107.525	11.7284	2.55556	27	65	0.4	white	Pred 1.25 mg, levothyroxine, alendronic acid, amitriptyline	1		0
OLP	M	34	92.6795	12.1212	3.10606	22	52	0.5	white	HCQ 200 mg, pred 20 mg, tacrolimus topically	1		0

OLP	F	16	74.453	14.2857	2.65079	21	57	0.4	white	HCQ, HLT, F+N mouthwash, antihistamine, multivitamins	3		0
OLP	M	39	48.6079	3.84615	3.24359	26	68	0.3	white	topical treatment (doxocicline+betamethazone+ nystatin), amitriptyline, atorvastatin, gliclozide, lansoprosole, novorapid penfill,	1		1

OLP	F	24	1594.65	72.4138	4.48276	29	38	0.45	asian	MMF, HCQ Pred mg, calcium, Ferrous furamate, Adcal, Vitamin D, Betnasol mouthwash, Peroxyn mouthwash	1		0
											1-mixed; 2-vegeterian; 3-Med/Pesc		

Appendix 10. Levels of cytokine and matrixins markers in saliva

	HC		OLP		MMP		PV	
	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)
GM-CSF	3.387±4.633	1.630 (0-23.22)	6.500±10.88	1.875 (0-46.11)	3.772±5.433	1.000 (0-22.08)	5.616±9.524	1.895 (0-53.05)
IL-6	9.871±36.23	1.460 (0-208.8)	40.33±77.12	5.730 (0-43.55)	27.84±45.31	12.33 (0-171.5)	45.95±111.7	5.230 (0-600.7)
TNF- α	6.623±9.986	1.600 (0.000-28.86)	8.192±8.735	6.840 (0.810-35.76)	13.36±13.14	13.80 (1.165-38.09)	14.36±16.73	7.235 (4.243-68.55)
IL-13	51.19±76.15	37.86 (0-365.0)	93.51± 81.55	88.16 (0-393.5)	185.5 ± 239.7	89.72 (0-994.4)	146.2 ± 139.6	90.09 (0-537.3)
IL-4	28.49±24.14	19.86 (6.40-79.79)	36.42±28.67	31.33 (3.96-152.2)	54.41±61.43	33.90 (6.44-250)	49.31±60.26	23.95 (10.34-264.3)
IL-1β	578.0±757.9	295.9 (12.15-3238)	1029±1168	698.9 (0-5663)	1527±2574	812.2 (25.92-13778)	1071±1086	688.0 (32.55-3802)
IL-8	938.2±829.1	644.0 (87.17-3520)	1418±1266	1210 (14.39-5123)	1227±989.0	942.3 (167.0-5123)	1075±757.3	853.2 (32.43-2572)
IL-17A	3.193±6.393	0.000 (0.000-30.62)	10.18±19.86	2.965 (0.000-106.3)	14.72±19.85	8.220 (0.000-94.90)	10.99±18.95	3.995 (0.000-96.53)
IL-23	674.3±1711	187.9 (0.000-6085)	280.3±297.8	153.5 (0.000-997.8)	289.9±244.8	160.6 (62.53-764.4)	1090±2926	136.2 28.26-11579)

IL-24	829.9±1301	429 (0.000-5323)	1653±3262	612.6 (0.00015185)	906.3±1281	368.7 (0.000-5446)	1261±2310	396.8 (0.000-12602)
MMP-2	677.4±831.0	356.7 (19.70-2430)	1071±1145	498.5 (0.000-3698)	2262±3772	686.5 (60.74-13990)	854.5±1008	531.3 (0.000-4332)
MMP-3	161.3±364.5	62.67 (1.050-2115)	1043±1756	356.9 (0.000-6904)	1670±4616	270.3 (16.42-25461)	1479±3931	205.1 (9.850-22278)
MMP-8	1529±993.2	1326 (64.10-3401)	1259±1417	837.5 (55.235512)	1252±1438	761.3 (0.000-5842)	2077±1830	1508 (51.24-6516)
MMP-9	1529±993.2	1326 (64.10-3401)	1259±1417	837.5 (55.23-5512)	1227±1428	744.5 (0.000-5842)	2077± 1830	1508 (51.24-6516)
MMP-13	104.3±203.5	45.33 (0.000-1057)	114.8±236.9	3.850 (0.000-1396)	139.3±301.5	28.13 (0.000-1752)	116.4±244.2	0.000 (0.000-1409)

P-value - difference between median of control and disease group using Kruskal-Wallis *test;* ****(p<0.0001); ***(p<0.001); **(p<0.01); *(p<0.05).

Appendix 11. Levels of cytokine and matrixins markers in serum

	HC		OLP		MMP		PV	
	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)	Mean (SD)	Median (min-max)
GM-CSF	0.9±1.52	0 (0-5.2)	9.7±34.9	0(0-187)	0.25±0.5225	0(0-2.050)	1.2±4.01	0(0-26)
IL-6	1.2±2.194	0.2(0-10.4)	1.83±1.81	1.21(0-5.93)	1.34±1.36	0.99(0-5.51)	1.76±2.67	1.02(0-17.23)
TNF- α	9.2±8.92	6.7(1.52-28.5)	4.9±5.01	2.68(0.6-17.76)	4.32±5.62	2.16(0.51-24.92)	4.46±3.34	3.7(0.5-14.02)
IL-13	94.58±148.8	51.1(0-568)	153.1±183	77.9(0-621)	147.4±135.9	104(0-514)	158.6±128	149(0-514)
IL-1β	1.66±3.41	0.12(0-18.87	4.3±9.51	0.24(0-50.2)	9.37±40.79	1.3(0-255.8)	9.78±39.95	1.43(0-255)
IL-8	14.25±17.10	9.07(1.85-95.25)	10.29±8.13	7.5(0-41.72)	12.2±13.01	7.2(4.7-48.5)	8.98±7.45	7.08(0-47.49)
IL-17A	0.52±1.05	0(0-4.62)	1.18±2.87	0(0-14.41)	4.19±18.86	0.17(0-126.8)	3.181±8.19	0.08(0-50.21
IL-23	91.19±97.07	54.32(0-256)	84.06±105.3	63.9(0-456)	116.3±86.95	113(0-257)	117±123	104.5(0-378)
IL-24	1254±2295	236(0-9930)	1230±2671	217(0-11927)	701.8±1261	179(0-5567)	2224±4485	252(0-27100)
MMP-2	22381±8966	25517 (4722-32279)	19145±5938	16759(11839-33652)	17634±4951)	17115(9451-30894)	20641±7777	17698(6278-35425)
MMP-3	8004±5524	6254(1314-2688)	11678±11804	8551(0-74352)	11502±12318	10106(2247-76472)	16829±19001	12144(0-112516)

MMP-8	564±1460	124.8(44.5-7283	526.8±1358	122.1(0-5789)	394±934.6	114.5(19.59-4354)	638±1520	124.9(0-7283
MMP-9	5585±2243	5859(1150-10386)	5201±2048	4869(1135-8660)	6854±3036	6942(1453-14398)	6360±2906	6033(4318-15199)
MMP-13	196.2±354.8	99.5(0-1576)	262±477.9	159(0-2800)	227.2±343.2	107.5(0-1642)	212±285	106(0-1642

Appendix 12. Correlation analysis in HC group.

P value/ Spearman R	PISA	Spearman r	Bleeding score	Spearman r	Microbial richness		Actinomyces dentalis	Spearman r	Campylobacter rectus	Spearman r	Fusobacterium nucleatum subsp. animalis	Spearman r	Olsenella sp. oral taxon 807	Spearman r	Porphyromonas gingivalis	Spearman r	Prevotella denticola	Spearman r	Prevotella oris	Spearman r	Tannerella forsythia	Spearman r	Treponema denticola	Spearman r	Eubacterium brachy	Spearman r
PISA		1.00	>0.0001	0.82	0.14	0.25	0.0003	0.56	0.0004	0.56	0.02	0.38	0.0004	0.56	0.02	0.39	0.05	0.33	0.02	0.39	0.02	0.39	0.03	0.36	0.02	0.39
Bleeding score	>0.0001	0.82		1.00	0.10	0.28	0.0004	0.57	0.0011	0.53	0.0024	0.50	0.0002	0.59	0.0007	0.54	0.01	0.43	0.01	0.45	0.0008	0.54	0.05	0.33	0.02	0.38
Microbial richness	0.14	0.25	0.10	0.28		1.00	0.0016	0.49	0.05	0.32	0.07	0.30	0.01	0.39	0.05	0.32	0.03	0.35	0.44	0.13	0.05	0.32	0.02	0.38	0.53	0.11
Butyric acid	0.03	0.37	0.0016	0.51	0.36	0.16	0.09	0.28	0.24	0.20	0.01	0.45	0.08	0.29	0.04	0.33	0.05	0.33	0.13	0.25	0.03	0.36	0.58	0.09	0.32	0.17
Isovaleric acid	0.06	0.31	0.01	0.45	0.25	0.19	0.06	0.32	0.04	0.33	0.0017	0.50	0.03	0.36	0.05	0.32	0.02	0.38	0.10	0.28	0.01	0.43	0.51	0.11	0.12	0.26
Tryptophan	0.07	0.30	0.18	0.23	0.78	-0.48	0.09	0.28	0.36	0.15	0.07	0.30	0.23	0.20	0.79	0.05	0.01	0.42	0.0045	0.46	0.58	0.09	0.79	-0.05	0.01	0.42
MMP 9 saliva	0.03	0.62	0.03	0.61	0.70	0.12	0.16	0.41	0.13	0.44	0.20	0.38	0.04	0.59	0.81	0.08	0.96	-0.02	0.87	0.05	0.25	0.34	0.24	0.35	0.51	0.20
Actinomyces dentalis	0.0003	0.56	0.0004	0.57	0.0016	0.5000		1.00	0.0002	0.56	0.0001	0.60	>0.0001	0.79	0.0007	0.53	0.0001	0.59	0.0006	0.53	0.0011	0.51	0.01	0.40	0.0012	0.51
Campylobacter rectus	0.0004	0.56	0.0011	0.53	0.05	0.32	0.0002	0.56		1.00	>0.0001	0.75	>0.0001	0.68	0.0005	0.54	0.01	0.42	0.0018	0.49	>0.0001	0.80	0.0001	0.60	0.0001	0.58
F. nucleatum subsp. animalis	0.02	0.38	0.0024	0.50	0.07	0.30	0.0001	0.60	>0.0001	0.75		1.00	>0.0001	0.67	0.0002	0.56	0.0001	0.58	>0.0001	0.62	>0.0001	0.70	0.0041	0.46	0.0002	0.57
Olsenella sp. oral taxon 807	0.0004	0.56	0.0002	0.59	0.01	0.40	>0.0001	0.79	>0.0001	0.68	>0.0001	0.67		1.00	0.01	0.39	>0.0001	0.62	0.0004	0.55	>0.0001	0.67	0.0049	0.45	>0.0001	0.61
Porphyromonas gingivalis	0.02	0.39	0.0007	0.54	0.05	0.32	0.0007	0.53	0.0005	0.54	0.0002	0.56	0.01	0.39		1.00	0.02	0.36	0.02	0.39	0.0001	0.59	0.01	0.40	0.02	0.38
Prevotella denticola	0.05	0.33	0.01	0.43	0.03	0.35	0.0001	0.59	0.01	0.42	0.0001	0.58	>0.0001	0.62	0.02	0.36		1.00	>0.0001	0.69	0.0042	0.45	0.08	0.29	0.0014	0.50
Prevotella oris	0.02	0.39	0.01	0.45	0.44	0.13	0.0006	0.53	0.0018	0.49	>0.0001	0.62	0.0004	0.55	0.02	0.39	>0.0001	0.69		1.00	0.01	0.41	0.18	0.22	>0.0001	0.78
Tannerella forsythia	0.02	0.39	0.0008	0.54	0.05	0.32	0.0011	0.51	>0.0001	0.80	>0.0001	0.70	>0.0001	0.67	0.0001	0.59	0.0042	0.45	0.01	0.41		1.00	0.0003	0.56	0.0032	0.47
Treponema denticola	0.03	0.36	0.05	0.33	0.02	0.38	0.01	0.40	0.0001	0.60	0.0041	0.46	0.0049	0.45	0.01	0.40	0.08	0.29	0.18	0.22	0.0003	0.56		1.00	0.11	0.27
Eubacterium brachy	0.02	0.39	0.02	0.38	0.53	0.11	0.0012	0.51	0.0001	0.58	0.0002	0.57	>0.0001	0.61	0.02	0.38	0.0014	0.50	>0.0001	0.78	0.0032	0.47	0.11	0.27		1.00

Appendix 13. Correlation analysis in MMP on a) topical treatment b) IS c) AB treatment.

a p value	ODSS	PISA	Age	BMI	A. meyeri	C. sputigena	L. hofstadii	N. cinerea	N. meningitidis	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	MMP 9 SA	Butyric acid SA	Isovaleric acid SA
ODSS		0.51	0.17	0.06	0.25	0.06	0.16	0.01	0.03	0.02	0.11	0.02	0.48	1.00	0.56	0.004	0.05	0.02	0.10	0.85	0.60
PISA	0.51		0.40	0.85	0.87	0.81	0.51	0.07	0.22	0.23	0.58	0.90	0.52	0.09	0.02	0.10	0.58	0.81	0.16	0.004	0.01
Age	0.17	0.40		0.99	0.26	0.15	0.02	0.77	0.43	0.34	0.30	0.64	0.64	0.25	0.86	0.61	0.79	0.83	0.06	0.41	0.27
BMI	0.06	0.85	0.99		0.27	0.26	0.57	0.37	0.37	0.90	0.86	0.69	0.52	0.53	0.32	0.01	0.49	0.34	0.24	0.43	0.08
Richness SA	0.20	0.11	0.51	0.16	0.86	0.10	0.01	0.01	0.05	0.06	0.87	0.15	0.90	0.14	0.88	0.06	0.73	0.05	0.60	0.23	0.18
Shannon diversity SA	0.96	0.02	0.51	0.56	0.59	0.56	0.01	0.51	0.95	0.01	0.63	0.20	0.23	0.11	0.09	0.55	0.71	0.49	0.32	0.05	0.03
Richness PL	0.43	0.07	0.31	0.97	0.67	0.37	0.74	0.70	0.92	0.42	0.20	0.43	0.83	0.14	0.02	0.63	0.56	0.01	0.40	0.53	0.45
Shannon diversity PL	0.09	0.02	0.01	0.36	0.29	0.15	0.78	0.92	0.38	0.37	0.69	0.27	0.64	0.17	0.09	0.65	0.62	0.17	0.45	0.33	0.14
A. meyeri	0.25	0.87	0.26	0.27		0.20	0.85	0.36	0.19	0.29	0.03	0.26	0.08	0.48	0.75	0.20	0.17	0.68	0.46	0.95	0.49
C. sputigena	0.06	0.81	0.15	0.26	0.20		0.001	0.01	0.01	0.04	0.60	0.81	0.08	0.70	0.67	0.73	0.04	0.01	0.25	0.49	0.71
L.hofstadii	0.16	0.51	0.02	0.57	0.85	0.001		0.03	0.16	0.01	0.58	0.15	0.95	0.93	0.72	0.84	0.78	0.21	0.01	0.96	0.75
Neisseria cinerea	0.01	0.07	0.77	0.37	0.36	0.01	0.03		0.00	0.13	0.43	0.41	0.20	0.35	0.45	0.39	0.03	0.09	0.09	0.24	0.17
Neisseria meningitidis	0.03	0.22	0.43	0.37	0.19	0.01	0.16	#######		0.44	0.51	0.16	0.04	0.26	0.53	0.64	0.05	0.14	0.17	0.16	0.11
P. saccharolytica	0.02	0.23	0.34	0.90	0.29	0.04	0.01	0.13	0.44		0.92	0.60	0.55	0.92	0.51	0.38	0.39	0.38	0.09	0.55	0.77
R. mucilaginosa	0.11	0.58	0.30	0.86	0.03	0.60	0.58	0.43	0.51	0.92		0.96	0.43	0.99	0.21	0.40	0.45	0.51	0.26	0.36	0.63
IL-6 SA	0.02	0.90	0.64	0.69	0.26	0.81	0.15	0.41	0.16	0.60	0.96		0.05	0.10	0.15	0.56	0.00	0.18	0.63	0.41	0.90
IL6 SE	0.48	0.52	0.64	0.52	0.08	0.08	0.95	0.20	0.04	0.55	0.43	0.05		0.09	0.79	0.42	0.01	0.12	0.68	0.24	0.82
IL-13 SA	1.00	0.09	0.25	0.53	0.48	0.70	0.93	0.35	0.26	0.92	0.99	0.10	0.09		0.001	0.34	0.14	0.81	0.82	0.09	0.00
IL-1b SA	0.56	0.02	0.86	0.32	0.75	0.67	0.72	0.45	0.53	0.51	0.21	0.15	0.79	0.001		0.26	0.41	0.21	0.32	0.02	0.00
IL-17 SE	0.004	0.10	0.61	0.01	0.20	0.73	0.84	0.39	0.64	0.38	0.40	0.56	0.42	0.34	0.26		0.49	0.45	0.63	0.11	0.24
MMP-3 SA	0.05	0.58	0.79	0.49	0.17	0.04	0.78	0.03	0.05	0.39	0.45	0.00	0.01	0.14	0.41	0.49		0.09	0.92	0.49	0.73
MMP 3 SE	0.02	0.81	0.83	0.34	0.68	0.01	0.21	0.09	0.14	0.38	0.51	0.18	0.12	0.81	0.21	0.45	0.09		0.68	0.23	0.75
MMP 9 SA	0.10	0.16	0.06	0.24	0.46	0.25	0.01	0.09	0.17	0.09	0.26	0.63	0.68	0.82	0.32	0.63	0.92	0.68		0.32	0.22
Butyric acid SA	0.85	0.004	0.41	0.43	0.95	0.49	0.96	0.24	0.16	0.55	0.36	0.41	0.24	0.09	0.02	0.11	0.49	0.23	0.32		0.00
Isovaleric acid SA	0.5961	0.011	0.266	0.082	0.4896	0.7149	0.7488	0.1746	0.1121	0.7676	0.6318	0.8973	0.8194	0.005	0.0041	0.2382	0.7253	0.7454	0.224	1E-07	

Spearman, r	ODSS	PISA	Age	BMI	A. meyeri	C. sputigena	L. hofstadii	N. cinerea	N. meningitidis	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	MMP 9 SA	Butyric acid SA	Isovaleric acid SA
ODSS	1.00	0.14	0.27	-0.55	-0.23	0.36	0.28	0.46	0.43	0.45	-0.31	0.44	0.15	0.00	0.12	-0.55	0.39	0.49	0.48	0.04	0.11
PISA	0.14	1.00	-0.18	-0.06	0.04	0.05	0.14	0.37	0.26	0.25	-0.12	0.03	-0.14	0.35	0.47	-0.35	0.12	-0.06	0.42	0.57	0.51
Age	0.27	-0.18	1.00	0.00	-0.22	0.29	0.44	0.06	0.16	0.19	-0.21	0.10	-0.10	-0.24	-0.04	-0.11	-0.06	-0.05	0.55	-0.18	-0.24
BMI	-0.55	-0.06	0.00	1.00	0.33	-0.34	-0.18	-0.27	-0.27	0.04	0.06	-0.13	-0.22	-0.22	-0.35	0.75	-0.23	-0.32	-0.54	-0.28	-0.58
Richness SA	0.26	0.33	-0.13	-0.42	0.03	0.33	0.51	0.50	0.38	0.37	-0.03	-0.29	0.03	0.31	0.03	-0.38	0.07	0.42	0.16	0.25	0.28
Shannon diversity SA	0.01	0.45	-0.13	-0.18	0.11	0.12	0.50	0.13	-0.01	0.48	0.10	-0.26	-0.25	0.33	0.35	-0.12	-0.08	0.15	0.30	0.40	0.45
Richness PL	-0.19	0.42	-0.25	-0.02	-0.10	-0.22	0.08	0.09	-0.02	0.20	-0.31	-0.19	0.05	0.36	0.54	-0.12	-0.15	-0.59	0.28	0.16	0.19
Shannon diversity PL	-0.40	0.53	-0.56	0.34	0.26	-0.35	0.07	-0.02	-0.22	0.22	0.10	-0.27	-0.11	0.34	0.41	-0.12	-0.12	-0.34	-0.25	0.24	0.36
A. meyeri	-0.23	0.04	-0.22	0.33	1.00	-0.25	0.04	-0.18	-0.26	0.21	0.42	-0.23	-0.35	0.15	0.07	0.27	-0.28	-0.09	0.23	-0.01	0.15
C. sputigena	0.36	0.05	0.29	-0.34	-0.25	1.00	0.61	0.47	0.48	0.39	-0.11	0.05	0.36	-0.08	-0.09	-0.07	0.42	0.54	0.34	-0.15	0.08
L.hofstadii	0.28	0.14	0.44	-0.18	0.04	0.61	1.00	0.41	0.28	0.48	-0.11	-0.29	-0.01	-0.02	0.08	-0.04	0.06	0.27	0.69	-0.01	0.07
Neisseria cinerea	0.46	0.37	0.06	-0.27	-0.18	0.47	0.41	1.00	0.88	0.30	-0.16	0.17	0.27	0.20	0.16	-0.18	0.44	0.37	0.49	0.25	0.29
Neisseria meningitidis	0.43	0.26	0.16	-0.27	-0.26	0.48	0.28	0.88	1.00	0.16	-0.13	0.29	0.42	0.24	0.14	-0.10	0.40	0.31	0.40	0.29	0.33
P. saccharolytica	0.45	0.25	0.19	0.04	0.21	0.39	0.48	0.30	0.16	1.00	0.02	0.11	-0.13	-0.02	0.14	-0.18	0.18	0.19	0.50	-0.13	0.06
R. mucilaginosa	-0.31	-0.12	-0.21	0.06	0.42	-0.11	-0.11	-0.16	-0.13	0.02	1.00	0.01	-0.17	0.00	-0.26	0.18	-0.16	0.14	-0.34	-0.20	-0.10
IL-6 SA	0.44	0.03	0.10	-0.13	-0.23	0.05	-0.29	0.17	0.29	0.11	0.01	1.00	0.40	0.35	0.30	-0.13	0.68	0.30	-0.15	-0.18	0.03
IL6 SE	0.15	-0.14	-0.10	-0.22	-0.35	0.36	-0.01	0.27	0.42	-0.13	-0.17	0.40	1.00	0.35	-0.06	0.18	0.51	0.35	-0.13	-0.25	-0.05
IL-13 SA	0.00	0.35	-0.24	-0.22	0.15	-0.08	-0.02	0.20	0.24	-0.02	0.00	0.35	0.35	1.00	0.65	-0.21	0.31	0.06	0.07	0.35	0.55
IL-1b SA	0.12	0.47	-0.04	-0.35	0.07	-0.09	0.08	0.16	0.14	0.14	-0.26	0.30	-0.06	0.65	1.00	-0.25	0.18	-0.30	0.30	0.48	0.56
IL-17 SE	-0.55	-0.35	-0.11	0.75	0.27	-0.07	-0.04	-0.18	-0.10	-0.18	0.18	-0.13	0.18	-0.21	-0.25	1.00	-0.15	-0.17	-0.15	-0.35	-0.26
MMP-3 SA	0.39	0.12	-0.06	-0.23	-0.28	0.42	0.06	0.44	0.40	0.18	-0.16	0.68	0.51	0.31	0.18	-0.15	1.00	0.38	-0.03	-0.15	0.08
MMP 3 SE	0.49	-0.06	-0.05	-0.32	-0.09	0.54	0.27	0.37	0.31	0.19	0.14	0.30	0.35	0.06	-0.30	-0.17	0.38	1.00	-0.13	-0.28	-0.08
MMP 9 SA	0.48	0.42	0.55	-0.54	0.23	0.34	0.69	0.49	0.40	0.50	-0.34	-0.15	-0.13	0.07	0.30	-0.15	-0.03	-0.13	1.00	0.30	0.36
Butyric acid	0.04	0.57	-0.18	-0.28	-0.01	-0.15	-0.01	0.25	0.29	-0.13	-0.20	-0.18	-0.25	0.35	0.48	-0.35	-0.15	-0.28	0.30	1.00	0.85
Isovaleric acid	0.11	0.51	-0.24	-0.58	0.15	0.08	0.07	0.29	0.33	0.06	-0.10	0.03	-0.05	0.55	0.56	-0.26	0.08	-0.08	0.36	0.852	1.00

b p value	ODSS	PISA	Age	A. meyeri	C. sputigena	L. hofstadii	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	Butyric acid SA	Isovaleric acid
ODSS		0.06	0.03	1.00	0.38	0.73	1.00	0.41	0.95	0.48	0.73	0.35	1.00	0.78	0.33	0.99	0.94
PISA	0.06		0.25	0.67	0.17	0.87	0.67	0.93	1.00	0.10	0.57	0.23	1.00	0.95	0.08	0.66	0.50
Age	0.03	0.25		0.83	0.80	0.30	0.83	0.39	0.83	0.83	0.50	0.43	0.63	0.83	0.50	0.45	0.62
BMI																	
Richness SA	0.27	0.80	0.07	0.33	1.00	0.07	0.33	0.65	0.45	0.92	0.40	0.95	0.62	0.68	0.75	0.06	0.10
Shannon diversity SA	0.99	0.66	0.45	0.33	0.62	0.07	0.33	0.70	0.35	0.80	0.17	0.52	0.73	0.78	0.75	0.00	0.02
Richness PL	0.20	0.02	0.45	0.67	0.07	0.67	0.67	0.93	0.95	0.14	0.57	0.08	0.73	0.68	0.08	0.50	0.42
Shannon diversity PL	0.27	0.24	0.53	1.00	0.05	0.47	1.00	0.27	0.52	0.80	1.00	0.08	0.62	0.23	0.08	0.56	0.80
A. meyeri	1.00	0.67	0.83		0.83	0.17	0.17	0.67		0.33			0.17		1.00	0.33	0.33
C. sputigena	0.38	0.17	0.80	0.83		0.37	0.83	0.88	0.30	0.33	0.65	0.10	0.93	0.10	0.17	0.62	0.83
Leptotrichia hofstadii	0.73	0.87	0.30	0.17	0.37		0.17	1.00	1.00	0.73	0.60	0.80	0.50	0.80	1.00	0.07	0.20
P. saccharolytica	1.00	0.67	0.83	0.17	0.83	0.17		0.67		0.33			0.17		1.00	0.33	0.33
Rothia mucilaginosa	0.41	0.93	0.39	0.67	0.88	1.00	0.67		0.83	0.24	0.22	0.50	0.53	0.73	0.75	0.70	0.93
IL-6 SA	0.95	1.00	0.83		0.30	1.00		0.83		0.68	0.27	0.68	1.00	0.08	1.00	0.35	0.13
IL6 SE	0.48	0.10	0.83	0.33	0.33	0.73	0.33	0.24	0.68		0.10	0.68	0.62	0.95	0.42	0.80	0.56
IL-13 SA	0.73	0.57	0.50		0.65	0.60		0.22	0.27	0.10		0.73	0.75	0.50	1.00	0.17	0.40
IL-1b SA	0.35	0.23	0.43		0.10	0.80		0.50	0.68	0.68	0.73		0.20	0.35	0.33	0.52	0.68
IL-17 SE	1.00	1.00	0.63	0.17	0.93	0.50	0.17	0.53	1.00	0.62	0.75	0.20		0.60	0.92	0.73	0.73
MMP-3 SA	0.78	0.95	0.83		0.10	0.80		0.73	0.08	0.95	0.50	0.35	0.60		1.00	0.78	0.45
MMP 3 SE	0.33	0.08	0.50	1.00	0.17	1.00	1.00	0.75	1.00	0.42	1.00	0.33	0.92	1.00		0.75	0.75
MMP 9 SA																	
Butyric acid	0.99	0.66	0.45	0.33	0.62	0.07	0.33	0.70	0.35	0.80	0.17	0.52	0.73	0.78	0.75		0.02
Isovaleric acid	0.94	0.50	0.62	0.33	0.83	0.20	0.33	0.93	0.13	0.56	0.40	0.68	0.73	0.45	0.75	0.02	

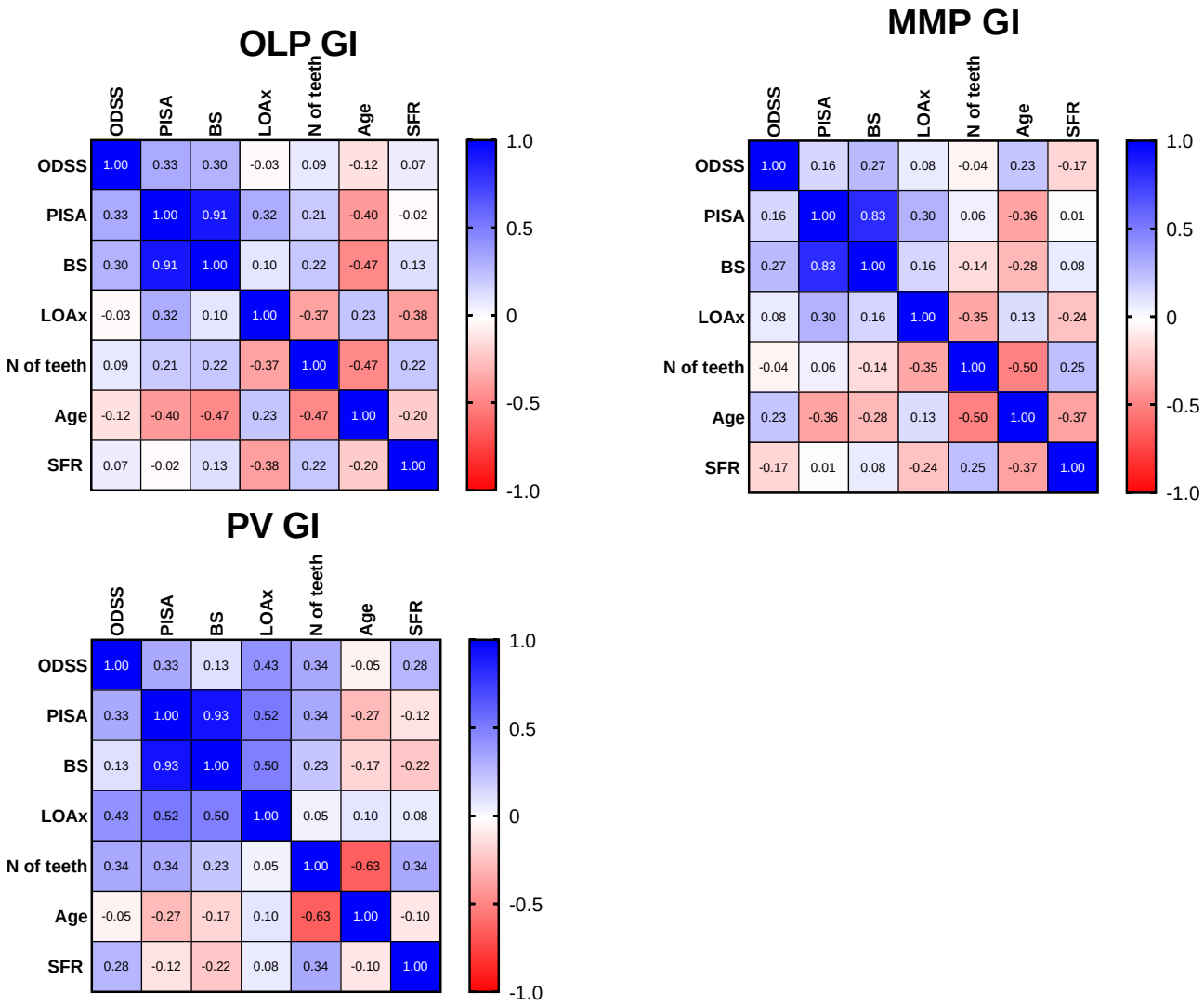
Spearman, r	ODSS	PISA	Age	A. meyeri	C. sputigena	L. hofstadii	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	Butyric acid SA	Isovaleric acid
ODSS	1.00	0.81	-0.89	0.00	0.43	-0.26	0.00	-0.44	0.10	0.38	0.21	0.60	-0.03	0.20	0.80	-0.03	0.06
PISA	0.81	1.00	-0.58	0.39	0.70	0.10	0.39	-0.06	0.00	0.77	0.36	0.70	-0.03	0.10	1.00	0.26	0.37
Age	-0.89	-0.58	1.00	0.42	-0.16	0.65	0.42	0.43	-0.15	-0.15	-0.34	-0.46	0.29	-0.15	-0.63	0.39	0.27
Richness SA	-0.55	-0.14	0.82	0.65	0.03	0.85	0.65	0.23	-0.50	0.09	-0.56	-0.10	0.27	-0.30	-0.40	0.83	0.77
Shannon diversity SA	-0.03	0.26	0.39	0.65	0.27	0.85	0.65	-0.20	-0.60	0.14	-0.72	0.40	0.21	-0.20	0.40	1.00	0.94
Richness PL	0.64	0.94	-0.39	0.39	0.82	0.27	0.39	-0.06	0.10	0.71	0.36	0.90	-0.21	0.30	1.00	0.37	0.43
Shannon diversity PL	0.55	0.60	-0.33	0.13	0.88	0.37	0.13	-0.55	0.40	0.14	0.05	0.90	-0.27	0.70	1.00	0.31	0.14
Actinomyces meyeri	0.00	0.39	0.42	1.00	0.42	0.77	1.00	0.40		0.65			0.70		0.26	0.65	0.65
C. sputigena	0.43	0.70	-0.16	0.42	1.00	0.47	0.42	-0.09	0.67	0.52	0.34	0.89	-0.10	0.89	0.95	0.27	0.15
Leptotrichia hofstadii	-0.26	0.10	0.65	0.77	0.47	1.00	0.77	0.02	0.00	0.17	-0.54	0.35	0.36	0.35	0.26	0.85	0.68
P. saccharolytica	0.00	0.39	0.42	1.00	0.42	0.77	1.00	0.40		0.65			0.70		0.26	0.65	0.65
Rothia mucilaginosa	-0.44	-0.06	0.43	0.40	-0.09	0.02	0.40	1.00	0.15	0.58	0.68	-0.41	0.31	-0.21	-0.40	-0.20	-0.06
IL-6 SA	0.10	0.00	-0.15		0.67	0.00		0.15	1.00	0.30	0.67	0.30	-0.11	0.90	0.50	-0.60	-0.80
IL6 SE	0.38	0.77	-0.15	0.65	0.52	0.17	0.65	0.58	0.30	1.00	0.87	0.30	0.27	0.10	0.60	0.14	0.31
IL-13 SA	0.21	0.36	-0.34		0.34	-0.54		0.68	0.67	0.87	1.00	0.21	-0.29	0.41	0.50	-0.72	-0.56
IL-1b SA	0.60	0.70	-0.46		0.89	0.35		-0.41	0.30	0.30	0.21	1.00	-0.78	0.60	1.00	0.40	0.30
IL-17 SE	-0.03	-0.03	0.29	0.70	-0.10	0.36	0.70	0.31	-0.11	0.27	-0.29	-0.78	1.00	-0.34	-0.20	0.21	0.21
MMP-3 SA	0.20	0.10	-0.15		0.89	0.35		-0.21	0.90	0.10	0.41	0.60	-0.34	1.00	0.50	-0.20	-0.50
MMP 3 SE	0.80	1.00	-0.63	0.26	0.95	0.26	0.26	-0.40	0.50	0.60	0.50	1.00	-0.20	0.50	1.00	0.40	0.40
Butyric acid	-0.03	0.26	0.39	0.65	0.27	0.85	0.65	-0.20	-0.60	0.14	-0.72	0.40	0.21	-0.20	0.40	1.00	0.94
Isovaleric acid	0.06	0.37	0.27	0.65	0.15	0.68	0.65	-0.06	-0.80	0.31	-0.56	0.30	0.21	-0.50	0.40	0.94	1.00

C p value

	ODSS	PISA	Age	BMI	A. meyeri	C. sputigena	L. hofstadii	N. cinerea	N. meningitidis	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	MMP 9 SA	Butyric acid SA	Isovaleric acid
ODSS		0.01	0.90	0.04	0.09	0.60	0.05	0.36	0.36	0.09	0.03	0.15	0.01	0.64	0.66	0.83	0.67	0.66	0.66	0.78	0.73
PISA	0.01		0.91	0.27	0.17	0.20	0.48	0.44	0.44	0.89	0.29	0.11	0.02	0.64	0.64	0.45	0.93	0.03	0.71	0.29	0.12
Age	0.90	0.91		0.94	0.38	0.88	0.60	0.55	0.55	1.00	0.28	0.97	0.69	0.78	0.92	0.51	0.95	0.98	0.58	0.31	0.71
BMI	0.04	0.27	0.94		0.27	1.00	0.15	0.33	0.33	0.53	0.03	0.83	0.06	0.50	0.77	0.40	0.55	0.92	0.67	0.43	0.43
Richness SA	0.56	0.29	0.39	0.11	0.73	0.06	0.15	0.91	0.91	0.36	0.63	0.43	0.33	0.10	0.47	0.80	0.33	0.33	0.88	0.04	0.02
Shannon diversity SA	0.22	0.11	0.56	0.72	0.31	0.35	0.64	0.91	0.91	0.65	0.52	0.05	0.60	0.50	0.95	0.65	0.51	0.70	0.62	0.13	0.10
Richness PL	0.08	0.11	0.88	1.00	0.89	0.24	0.98	1.00	1.00	0.89	0.12	0.30	0.73	0.54	0.71	0.48	0.43	0.44	0.98	0.95	0.74
Shannon diversity PL	0.19	0.18	0.82	0.83	0.94	0.08	0.53	1.00	1.00	1.00	0.18	0.33	0.74	0.27	0.85	0.65	0.68	0.71	0.52	0.63	0.48
Actinomyces meyeri	0.09	0.17	0.38	0.27		0.73	0.16	1.00	1.00	1.00	0.27	0.49	0.10	0.84	0.13	0.80	0.60	1.00	0.75	0.18	0.18
Capnocytophaga sputigena	0.60	0.20	0.88	1.00	0.73		0.04	0.82	0.82	0.02	0.51	0.91	0.83	1.00	0.65	0.23	0.94	0.08	0.13	0.75	0.41
Leptotrichia hofstadii	0.05	0.48	0.60	0.15	0.16	0.04		1.00	1.00	0.01	0.20	0.73	0.12	0.64	0.05	0.30	0.98	0.74	0.01	0.63	0.78
Neisseria cinerea	0.36	0.44	0.55	0.33	1.00	0.82	1.00		0.09	1.00	0.91		0.09	0.10	0.20	0.10	0.20	0.25	0.50	0.60	0.60
Neisseria meningitidis	0.36	0.44	0.55	0.33	1.00	0.82	1.00	0.09		1.00	0.91		0.09	0.10	0.20	0.10	0.20	0.25	0.50	0.60	0.60
Prevotella saccharolytica	0.09	0.89	1.00	0.53	1.00	0.02	0.01	1.00	1.00		0.37	0.76	0.59	0.60	0.40	0.20	0.89	0.50	0.25	0.80	1.00
Rothia mucilaginosa 1	0.03	0.29	0.28	0.03	0.27	0.51	0.20	0.91	0.91	0.37		0.23	0.23	0.67	0.95	0.44	0.89	0.74	0.96	0.64	0.82
IL-6 SA	0.15	0.11	0.97	0.83	0.49	0.91	0.73			0.76	0.23		0.56	0.65	0.78	0.65	0.34	0.78	0.96	0.31	0.23
IL6 SE	0.01	0.02	0.69	0.06	0.10	0.83	0.12	0.09	0.09	0.59	0.23	0.56		0.13	0.94	0.28	0.80	0.38	0.49	0.75	0.97
IL-13 SA	0.64	0.64	0.78	0.50	0.84	1.00	0.64	0.10	0.10	0.60	0.67	0.65	0.13		0.08	0.37	0.09	0.89	0.40	0.34	0.34
IL-1b SA	0.66	0.64	0.92	0.77	0.13	0.65	0.05	0.20	0.20	0.40	0.95	0.78	0.94	0.08		0.06	0.12	0.84	0.06	0.04	0.05
IL-17 SE	0.83	0.45	0.51	0.40	0.80	0.23	0.30	0.10	0.10	0.20	0.44	0.65	0.28	0.37	0.06		0.99	0.22	0.39	0.01	0.10
MMP-3 SA	0.67	0.93	0.95	0.55	0.60	0.94	0.98	0.20	0.20	0.89	0.89	0.34	0.80	0.09	0.12	0.99		0.50	0.20	0.91	0.78
MMP 3 SE	0.66	0.03	0.98	0.92	1.00	0.08	0.74	0.25	0.25	0.50	0.74	0.78	0.38	0.89	0.84	0.22	0.50		0.71	0.59	0.59
MMP 9 SA	0.66	0.71	0.58	0.67	0.75	0.13	0.01	0.50	0.50	0.25	0.96	0.96	0.49	0.40	0.06	0.39	0.20	0.71		0.66	0.75
Butyric acid	0.78	0.29	0.31	0.43	0.18	0.75	0.63	0.60	0.60	0.80	0.64	0.31	0.75	0.34	0.04	0.01	0.91	0.59	0.66		0.00
Isovaleric acid	0.73	0.12	0.71	0.43	0.18	0.41	0.78	0.60	0.60	1.00	0.82	0.23	0.97	0.34	0.05	0.10	0.78	0.59	0.75	0.00	

Spearman,r	ODSS	PISA	Age	BMI	A. meyeri	C. sputigena	L. hofstadii	N. cinerea	N. meningitidis	P. saccharolytica	R. mucilaginosa 1	IL-6 SA	IL6 SE	IL-13 SA	IL-1b SA	IL-17 SE	MMP-3 SA	MMP 3 SE	MMP 9 SA	Butyric acid SA	Isovaleric acid
ODSS	1.00	-0.82	0.05	-0.84	-0.54	0.17	0.61	0.40	0.40	0.54	-0.68	0.50	0.77	0.17	-0.16	0.08	0.15	0.19	-0.19	-0.10	-0.13
PISA	-0.82	1.00	-0.05	0.67	0.52	0.47	-0.27	-0.41	-0.41	0.14	0.40	-0.62	-0.77	-0.19	0.18	-0.31	-0.05	-0.89	0.18	0.40	0.57
Age	0.05	-0.05	1.00	-0.06	0.31	0.05	0.18	-0.30	-0.30	-0.01	-0.36	0.02	0.14	-0.10	0.04	0.23	0.03	-0.02	-0.24	0.36	0.14
BMI	-0.84	0.67	-0.06	1.00	0.60	0.03	-0.68	-0.66	-0.66	-0.36	0.87	0.15	-0.81	-0.41	-0.21	-0.57	-0.32	-0.20	-0.87	-0.46	-0.46
Richness SA	0.20	0.40	0.29	-0.72	0.12	0.58	0.47	0.10	0.10	0.32	-0.17	-0.28	0.32	0.56	0.26	0.09	0.35	-0.40	-0.07	0.67	0.75
Shannon diversity SA	-0.41	0.58	0.20	0.20	0.35	0.31	0.16	-0.10	-0.10	-0.16	0.22	-0.65	-0.18	0.24	0.03	0.16	0.24	-0.17	-0.21	0.52	0.56
Richness PL	-0.63	0.62	-0.07	0.32	0.07	0.44	0.02	0.00	0.00	-0.14	0.57	-0.43	-0.13	0.24	0.15	-0.29	0.33	-0.36	0.02	0.03	0.13
Shannon diversity PL	-0.49	0.54	0.09	0.50	0.05	0.62	0.24	-0.07	-0.07	-0.07	0.49	-0.40	0.13	0.41	0.08	-0.19	0.18	-0.18	-0.26	0.18	0.27
Actinomyces meyeri	-0.54	0.52	0.31	0.60	1.00	-0.10	-0.53	-0.15	-0.15	-0.22	0.38	-0.24	-0.54	0.07	0.53	0.24	0.20	0.02	0.25	0.49	0.49
Capnocytophaga sputigena	0.17	0.47	0.05	0.03	-0.10	1.00	0.63	-0.27	-0.27	0.72	-0.22	-0.05	0.07	0.00	-0.16	-0.41	0.03	-0.68	-0.60	0.12	0.29
Leptotrichia hofstadii	0.61	-0.27	0.18	-0.68	-0.53	0.63	1.00	0.00	0.00	0.69	-0.41	-0.13	0.49	-0.16	-0.66	-0.36	0.01	0.15	-0.84	-0.18	-0.10
Neisseria cinerea	0.40	-0.41	-0.30	-0.66	-0.15	-0.27	0.00	1.00	1.00	-0.15	-0.10		0.50	0.54	0.52	0.54	0.52	0.58	0.41	0.29	0.29
Neisseria meningitidis	0.40	-0.41	-0.30	-0.66	-0.15	-0.27	0.00	1.00	1.00	-0.15	-0.10		0.50	0.54	0.52	0.54	0.52	0.58	0.41	0.29	0.29
Prevotella saccharolytica	0.54	0.14	-0.01	-0.36	-0.22	0.72	0.69	-0.15	-0.15	1.00	-0.33	0.12	0.18	-0.36	-0.41	-0.54	-0.05	-0.41	-0.58	-0.17	0.06
Rothia mucilaginosa 1	-0.68	0.40	-0.36	0.87	0.38	-0.22	-0.41	-0.10	-0.10	-0.33	1.00	-0.42	-0.39	0.15	0.02	-0.27	-0.06	0.15	-0.02	-0.17	-0.09
IL-6 SA	0.50	-0.62	0.02	0.15	-0.24	-0.05	-0.13			0.12	-0.42	1.00	0.21	0.17	0.12	-0.18	0.37	-0.14	0.04	-0.38	-0.45
IL6 SE	0.77	-0.77	0.14	-0.81	-0.54	0.07	0.49	0.50	0.50	0.18	-0.39	0.21	1.00	0.52	-0.03	0.38	0.09	0.36	-0.29	0.12	0.02
IL-13 SA	0.17	-0.19	0.10	0.41	0.07	0.00	0.16	0.54	0.54	0.36	0.15	0.17	0.52	1.00	0.58	0.33	0.61	0.07	0.34	0.34	0.34
IL-1b SA	-0.16	0.18	0.04	-0.21	0.53	-0.16	-0.66	0.52	0.52	-0.41	0.02	0.12	-0.03	0.58	1.00	0.66	0.57	0.11	0.71	0.66	0.64
IL-17 SE	0.08	-0.31	0.23	-0.57	0.24	-0.41	-0.36	0.54	0.54	-0.54	-0.27	-0.18	0.38	0.33	0.66	1.00	0.01	0.55	0.35	0.80	0.59
MMP-3 SA	0.15	-0.05	0.03	-0.32	0.20	0.03	0.01	0.52	0.52	-0.05	-0.06	0.37	0.09	0.61	0.57	0.01	1.00	0.32	0.57	0.05	0.12
MMP 3 SE	0.19	-0.89	-0.02	-0.20	0.02	-0.68	0.15	0.58	0.58	-0.41	0.15	-0.14	0.36	0.07	0.11	0.55	0.32	1.00	-0.20	-0.25	-0.25
MMP 9 SA	-0.19	0.18	-0.24	-0.87	0.25	-0.60	-0.84	0.41	0.41	-0.58	-0.02	0.04	-0.29	0.34	0.71	0.35	0.57	-0.20	1.00	0.19	0.14
Butyric acid	-0.10	0.40	0.36	-0.46	0.49	0.12	-0.18	0.29	0.29	-0.17	-0.17	-0.38	0.12	0.34	0.66	0.80	0.05	-0.25	0.19	1.00	0.95
Isovaleric acid	-0.13	0.57	0.14	-0.46	0.49	0.29	-0.10	0.29	0.29	0.06	-0.09	-0.45	0.02	0.34	0.64	0.59	0.12	-0.25	0.14	0.95	1.00

Appendix 14. Correlation coefficient analysis in three disease groups with gingival involvement.



Appendix 15. Pre-library sample QC results.

Sample QC Methods

The quality and quantity of the samples are assessed spectrophotometrically with NanoDrop (Thermo Fisher, USA). The gDNA samples with A260/280:1.8-2.0 and A260/A230:2.0-2.2 is considered for the next step. For the RNA samples, A260/280:~2 and A260/230 2.0-2.2 is accepted as acceptable quality. The OD260 values of the samples are used for the calculation of concentration of samples. Since the concentration estimation based on the UV absorbance is not reliable, more precise concentration estimation of the samples are also done fluorometrically with Qubit (Thermo Fisher) or Victor Nivo Perkin Elmer Plate reader with appropriate kids for sample type.

Pre-library sample QC results.

#	SAMPLE ID	A260/A280	A260/A230	Spectrophotometric Con. (ng/ul)	Fluorometric Con. (ng/ul)
1	DM004-GW4	2,04	1,86	16,6	12,86
2	DM010-PS10	2,01	1,6	29,7	9,65
3	DM037-SO	2,03	0,91	4,5	2,66
4	DM043SL	1,87	2,35	216,5	3,25
5	DM047-MA	1,94	1,75	19,2	7,47

6	DM186	1,79	2,09	20,9	9,35
7	DM010	2,12	0,3	2,4	4,19
8	DM014	1,46	0,89	6,6	5,99
9	DM051	1,79	1,49	7,2	8,63
10	DM053	1,68	1,04	3,4	6,41
11	DM056	2,05	1,08	3,9	7,31
12	DM058	1,71	1,08	4,1	6,97
13	DM059	2,16	0,99	5,5	9,67
14	DM061	1,91	1,72	31,2	7,17
15	DM062	1,93	0,59	13,4	7,92
16	DM063	2,33	0,37	2,2	6,56
17	DM064	1,93	0,92	4,8	8,15
18	DM065	1,94	1,59	10,9	4,06
19	DM066	2,06	0,5	3	4,95
20	DM080	1,89	0,53	5	6,45
21	DM081	1,73	1,41	7,1	5,92
22	DM082	1,93	1,83	10,1	8,59
23	DM083	1,85	1,71	13,8	4,84
24	DM084	1,89	1,81	19,4	9,53
25	DM089	1,9	1,16	47	7,87
26	DM129	1,88	0,24	19,6	6,42

27	DM136	1,9	1,33	22,3	6,49
28	DM146	1,85	0,54	38,8	9,95
29	DM159	1,82	0,56	16,9	8,96
30	DM160	1,94	1,44	25	10,23
31	DM167	1,94	1,52	24,2	9,25
32	DM170	1,85	0,93	53	11,93
33	DM174	1,93	0,71	26,8	13,13
34	DM175	1,91	1,36	29,9	13,67
35	DM176	1,96	1,73	32,7	13,00
36	DM178	1,91	0,87	24,9	11,00
37	DM188	1,95	1,1	31	12,63
38	DM168	1,99	1,58	50,4	14,37
39	DM172	1,94	1,06	42,1	16,10
40	DM173	1,92	1,97	51,2	18,00
41	DM002	1,67	1,26	5,5	5,26
42	DM169	1,88	1,79	38,5	19,33
43	DM171	1,9	1,52	238,5	49,33
44	DM177	1,91	1,21	78,6	26,00
45	DM179	1,91	2,03	76,7	20,27
46	DM180	1,93	2,26	418,9	122,33
47	DM181	1,89	1,79	127,9	47,60
48	DM182	1,9	2,14	108	23,00

49	DM183	1,9	2,03	116,9	45,13
50	DM184	1,89	1,5	121,3	42,73
51	DM185	1,9	2,23	124,7	50,80
52	DM186	1,9	1,78	41,4	21,40
53	DM088-IM	1,88	2,34	282,7	147,60
54	DM089-AB	1,88	2,26	364,4	143,60
55	DM002-JO2	1,91	2,31	49,1	44,35
56	DM003-CB3	1,86	2,3	212,9	61,85
57	DM005-ND5	1,87	2,11	82	44,95
58	DM006-ML6	1,89	2,36	227,1	52,00
59	DM007-MR7	1,9	2,33	86,6	53,52
60	DM008-DR8	1,88	2,37	159,3	52,95
61	DM009-JB9	1,88	2,21	219	75,38
62	DM011-MS11	1,89	2,34	370,5	108,85
63	DM012-KH12	1,91	2,22	85,5	60,06
64	DM013-MD13	1,93	2,24	82,5	39,03
65	DM014-SF14	1,93	2,27	102,8	26,86
66	DM015-TM15	1,96	2,14	47,5	44,50
67	DM017-RE17	1,89	2,23	111,5	42,53
68	DM018-JL18	1,87	2,14	132,5	51,9
69	DM019-PO19	1,89	2,31	120,6	50,6
70	DM020-SP20	1,86	2,28	122,6	58,97

71	DM0021-TP21	1,88	2,24	95,7	59,79
72	DM0022-AS22	1,87	2,31	175,8	47,21
73	DM0023-PP23	1,83	1,91	23,5	19,65
74	DM0024-JLJ	1,88	2,31	140,2	60,98
75	DM0025-AJ	1,9	2,23	62,6	31,24
76	DM0026-GS26	1,89	2,39	368,5	96,90
77	DM0027-AR27	1,86	2,16	102,8	32,95
78	DM0028-CK28	1,87	2,3	177,3	62,90
79	DM0029-PM29	1,89	2,39	449,2	110,80
80	DM0030-ES30	1,85	2,38	65,1	35,10
81	DM031-SJD	1,84	2,37	283,2	73,05
82	DM032-JT	1,86	2,4	364,4	62,50
83	DM033-AM	1,85	2,36	259,3	74,40
84	DM034-PC	1,89	2,37	370,4	57,95
85	DM035-ME	1,89	2,37	296,8	131,40
86	DM036-TR	1,9	2,36	667,9	200,05
87	DM038-EY	1,87	2,33	112,7	79,97
88	DM039AM	1,88	2,33	150,5	107,70
89	DM40JC	1,85	2,19	49,4	25,02
90	DM41DW	1,88	2,23	86,6	20,52
91	DM042HP	1,89	2,38	474	71,32
92	DM044-BP	1,9	1,94	34	12,38

93	DM045-DC	1,88	2,34	231,8	86,65
94	DM046-VG	1,88	2,37	247,9	112,35
95	DM048-UK	1,87	2,32	365	65,37
96	DM049-RF	1,89	2,32	272,1	130,54
97	DM050-KB	1,88	2,31	224,7	87,15
98	DM051-DR	1,87	2,15	117,8	53,03
99	DM055	1,88	2,36	372,6	53,25
100	DM056-JW	1,87	2,29	203,6	37,30
101	DM057-AB	1,87	2,25	164,5	63,05
102	DM058-SD	1,86	2,27	143,3	64,30
103	DM059-AH	1,87	2,29	220,5	115,35
104	DM060-UA	1,88	1,64	146,2	35,60
105	DM061-LP	1,89	2,27	126,9	79,25
106	DM062-DP	1,99	2,13	17,8	11,03
107	DM063-MK	1,88	2,37	163,2	44,55
108	DM064-VP	1,89	2,38	286,2	227,20
109	DM065-MN	1,88	2,25	68,4	32,53
110	DM066-JW	1,88	2,29	147,2	76,30
111	DM67	1,89	2,38	53,1	26,47
112	DM068-RA	3,91	0,5	0,9	9,27
113	DM069-PC	1,88	2,33	226,7	77,50
114	DM070-J	1,89	2,1	137,5	27,95

115	DM071-AF	1,89	1,72	18,3	10,68
116	DM072-MK	1,88	2,37	208,4	80,80
117	DM073-LH	1,88	2,35	397,8	127,20
118	DM074-RM	1,88	2,34	197,3	83,55
119	DM075-KP	1,87	2,27	74,4	25,96
120	DM076-JW	1,88	2,29	222,4	115,05
121	DM077-SY	1,87	2,32	255	121,20
122	DM078-ZM	1,88	2,37	199,5	56,65
123	DM079-MF	1,87	2,2	169,7	49,00
124	DM080-JB	1,89	2,06	88,6	29,25
125	DM081-ET	1,89	2,33	203,1	99,20
126	DM082-JW	1,87	2,27	268,7	136,50
127	DM083-RA	1,83	0,93	23,1	9,10
128	DM084-MD	1,88	2,31	86,6	51,50
129	DM085-CM	1,87	2,31	177,8	50,95
130	DM087-LW	1,86	2,36	143,9	47,50
131	DM137-AC	1,86	2,03	829,7	60,53
132	DM016-KE16	1,94	1,78	16,8	12,33
133	DM090-EW	1,9	2,38	476,8	121,30
134	DM091-MCh	1,87	2,34	268,6	138,40
135	DM092-JW	1,88	2,38	362,7	87,60
136	DM093-SC	1,97	2,39	546,4	126,20

137	DM094-RK	1,72	1,04	152,4	88,25
138	DM095-MS	1,9	2,27	593,9	80,10
139	DM096-TP	1,88	2,34	101	44,65
140	DM097-JB	1,88	2,35	130,7	62,90
141	DM098-SB	1,89	2,35	307,1	81,90
142	DM099-SB	1,9	2,38	406,2	74,55
143	DM100-AR	1,88	2,35	214,5	82,55
144	DM101-SA	1,89	1,26	39,6	14,31
145	DM102-MF	1,89	2,36	189,3	60,25
146	DM103-VS	1,89	2,35	300,8	90,30
147	DM104-IS	1,89	1,78	127,9	50,42
148	DM105-JR	1,88	2,23	591,9	228,67
149	DM106-CR	1,89	1,17	242,4	266,00
150	DM108-JB	1,88	1,74	125,5	64,00
151	DM109-JB	1,9	2,09	495,4	286,67
152	DM110-DM	1,9	2,32	455,7	232,00
153	DM111-MG	1,87	1,35	145,2	75,67
154	DM112-EH	1,9	1,81	44,7	16,83
155	DM113-LC	1,88	2,15	438,1	292,00
156	DM114-	1,89	1,38	106,2	41,23
157	DM115	1,89	2,12	1087,6	233,33
158	DM116-KR	1,9	1,71	497,2	212,00

159	DM117-AW	1,88	0,87	187,8	94,50
160	DM118-NK	1,91	2,28	314,3	104,00
161	DM119-BW	1,91	2,32	595,1	258,67
162	DM120-JR	1,89	1,64	295,1	200,67
163	DM121-SR	1,9	2,34	480,8	199,33
164	DM122-JS	1,9	2,27	295,3	56,17
165	DM123-MD	1,91	2,31	514	200,00
166	DM124-WF	1,9	2,3	656,3	196,50
167	DM125-AMC	1,91	1,93	424,2	205,33
168	DM126-AOC	1,92	2,26	543,4	189,00
169	DM127-JR	1,89	2,2	258,1	130,67
170	DM128-KP	1,91	2,12	425,1	222,00
171	DM129-EG	1,89	1,37	129,3	44,93
172	DM130-JS	1,9	1,53	415,6	200,67
173	DM131-MK	1,9	2,35	359,1	70,83
174	DM132-DN	1,88	1,23	165,4	32,57
175	DM133-HJ	1,9	2,41	315,5	37,80
176	DM134-MF	1,92	2,25	718,5	73,80
177	DM135-PN	1,89	2,18	280,9	23,67
178	DM136-SC	1,88	2,24	358,7	118,50
179	DM138-AF	1,91	2,1	451,6	144,00
180	DM139-ZC	1,88	0,57	136,4	46,60

181	DM141-TH	1,89	1,97	420,9	139,00
182	DM142-RD	1,9	2,05	459,6	161,00
183	DM143-JED	1,9	2,2	413,3	127,00
184	DM144-RG	1,88	2,27	460,4	99,53
185	DM145-JS	1,87	2,17	171,6	41,33
186	DM146-MI	1,86	2,37	115,3	24,57
187	DM147-MP	1,88	2,21	191,7	43,80
188	DM148	1,88	2,23	166,5	68,60
189	DM149	1,88	2,36	180,8	77,33
190	DM150	1,88	2,14	275	30,65
191	DM151	1,89	2,1	371,7	39,23
192	DM188	1,89	2,29	300,5	188,33
193	DM153	1,88	1,42	128,8	63,87
194	DM154	1,9	2,17	482,3	167,67
195	DM155	1,88	2,28	175,1	83,67
196	DM156	1,89	2,19	330,1	122,33
197	DM157	1,89	2,08	345,6	118,67
198	DM158	1,88	1,34	143,6	78,20
199	DM159	1,89	2,3	516,1	162,00
200	DM160	1,9	2,3	475,5	118,00
201	DM161	1,88	2,16	229,2	130,00
202	DM162	1,89	2,15	290,8	146,00

203	DM163	1,87	1,32	236,6	104,50
204	DM164	1,89	2,08	473,7	95,27
205	DM165	1,9	1,91	468,8	128,00
206	DM166	1,89	2,27	334,8	165,50
207	DM167	1,88	2,12	204,1	122,33
208	DM168	1,88	2,12	186,2	97,93
209	DM169	1,89	2,28	160	67,73
210	DM170	1,87	0,75	83,1	42,40
211	DM171	1,88	2,13	219	97,00
212	DM172	1,88	2,21	222,6	135,00
213	DM173	1,87	2,14	158,7	59,60
214	DM174	1,91	2,33	659,1	164,00
215	DM175	1,86	2,21	194,3	86,90
216	DM176	1,89	2,25	373,6	129,00
217	DM177	1,88	2,31	293,5	134,67
218	DM178	1,89	2,38	466,8	156,67
219	DM179	1,86	1,85	214,5	108,67
220	DM180	1,88	2,28	286,5	81,90
221	DM181	1,87	1,78	156	69,27
222	DM182	1,89	2,33	414,2	115,00
223	DM183	1,88	2,19	373,5	142,33
224	DM184	1,91	2,32	576,3	143,67

225	DM185	1,88	2,29	310,5	100,33
226	DM187	1,87	2,4	892,6	304,00
227	DM036	1,89	2,02	20,6	26,50
228	DM037	1,8	1,43	13,9	19,87
229	DM038	1,89	1,9	15,6	19,63
230	DM039	1,73	1,33	6,5	12,75
231	DM040	1,92	1,93	28,5	25,60
232	DM041	1,79	1,51	10,2	14,17
233	DM042	1,91	2,06	32,9	26,40
234	DM043	1,86	1,84	13,3	13,30
235	DM044	1,92	1,56	13,1	11,03
236	DM046	1,76	1,55	14,4	17,33
237	DM047	1,85	1,98	31,7	28,00
238	DM049	1,85	1,73	34,1	21,00
239	DM055	1,81	1,76	18	20,17
240	DM057	1,84	1,75	59,4	29,53
241	DM060	1,9	2,03	83,1	43,67
242	DM068	1,9	1,76	15,5	12,07
243	DM069	1,94	1,77	33,7	14,53
244	DM070	1,91	0,55	15,6	13,55
245	DM073	1,94	1,58	13,7	13,33
246	DM074	1,89	1,83	22,9	21,13

247	DM085	1,89	2,25	60,8	33,40
248	DM092	1,88	1,77	104,7	41,20
249	DM093	1,88	1,88	47,9	32,33
250	DM094	1,89	1,4	41,4	33,4
251	DM098	1,9	0,69	27,1	19,2
252	DM099	1,89	1,1	32,6	27,47
253	DM100	1,89	0,75	62,5	67,40
254	DM101	1,88	2,06	80,8	14,17
255	DM102	1,86	1,68	94,2	34,27
256	DM104	1,88	1,13	154,4	47,93
257	DM105	1,91	1,38	465,7	200,00
258	DM106	1,89	1,4	219	88,20
259	DM107	1,85	1,21	71,3	30,07
260	DM108	1,88	1,61	119,6	53,87
261	DM109	1,88	1,29	45	21,90
262	DM110	1,86	1,09	110,3	45,40
263	DM111	1,87	1,14	45	18,67
264	DM113	1,89	2,01	216,6	77,00
265	DM115	1,89	2,22	178,4	64,80
266	DM116	1,9	2,04	99,3	36,67
267	DM117	1,91	1,7	61,2	19,23
268	DM118	1,86	1,86	62,9	28,53

269	DM119	1,87	2,11	203,4	77,27
270	DM120	1,88	2,14	166,2	32,60
271	DM121	1,87	1,03	53,3	18,37
272	DM122	1,79	1,29	46,4	14,17
273	DM123	1,87	2,27	730,8	164,00
274	DM125	1,88	1,01	126,3	49,80
275	DM127	1,89	1,64	92,1	35,93
276	DM128	1,9	1,78	253,3	100,53
277	DM130	1,9	1,35	258,3	121,33
278	DM132	1,9	2,06	21,2	25,73
279	DM133	1,87	2,26	251,4	78,00
280	DM135	1,88	2,21	258,5	44,35
281	DM137	1,87	1,52	54,6	27,30
282	DM138	1,86	1,21	79,1	15,30
283	DM139	1,86	0,73	73,2	15,43
284	DM143	1,89	1,68	36,6	12,80
285	DM145	1,88	0,77	18,6	22,00
286	DM147	1,91	1,69	55,5	24,53
287	DM149	1,89	1,91	232,5	27,00
288	DM150	1,89	1,21	67,1	21,80
289	DM151	1,88	1,95	120,9	21,60
290	DM152	1,87	1,77	89,2	14,47

291	DM154	1,85	2,05	170,9	79,93
292	DM156	1,86	1,98	77	38,07
293	DM157	1,87	1,3	69,3	19,53
294	DM158	1,88	1,34	36,7	18,30
295	DM163	1,91	1,82	54,4	19,17
296	DM164	1,93	2,07	51,8	23,07
297	DM165	1,89	1,75	95,4	34,80
298	DM166	1,9	2,17	273,1	101,80
299	DM075	1,82	1,37	8,6	6,83
300	DM076	1,92	1,02	6,8	6,02
301	DM078	1,8	1,14	6,1	5,58
302	DM087	1,98	1,98	15	5,99
303	DM088	1,83	1,63	12	6,94
304	DM090	1,89	0,72	13,7	8,11
305	DM112	1,92	0,19	8,3	5,85
306	DM124	1,82	1,45	13,9	6,90
307	DM140	2,01	0,21	10	5,15
308	DM144	1,98	0,19	4	6,64
309	DM148	1,87	0,48	10,5	8,82
310	DM162	2,01	0,24	7,1	5,78
311	DM114	1,91	1,13	13,9	21,47
312	DM142	1,99	0,07	13,2	13,53

313	DM091	2,13	0,92	5,1	4,81
314	DM008	1,86	0,88	12,2	2,89
315	DM023	0,97	0,96	1,5	1,17
316	DM052	1,33	0,54	4,2	2,55
317	DM072	1,48	0,66	3,2	2,06
318	DM086	1,64	0,56	1,2	1,20
319	DM097	1,93	0,23	4,3	2,72
320	DM141	2,57	0,22	3,1	2,25
321	DM153	1,61	0,52	6,5	1,85
322	DM155	1,5	0,26	5,9	2,75
323	DM161	2,95	0,38	2,3	1,96
324	DM029	1,86	1,91	25,9	14,27
325	DM077	1,81	1,7	15,3	11,13
326	DM095	1,94	1,09	12,7	11,40
327	DM103	1,93	0,61	11,2	10,90

Table 2. The samples failed at QC step

#	SAMPLE ID	Library Fail/Pass*
1	DM004	ABORTED
2	DM005	ABORTED
3	DM006	ABORTED
4	DM009	ABORTED
5	DM011	ABORTED

6	DM012	ABORTED
7	DM013	ABORTED
8	DM015	ABORTED
9	DM017	ABORTED
10	DM018	ABORTED
11	DM019	ABORTED
12	DM020	ABORTED
13	DM021	ABORTED
14	DM022	ABORTED
15	DM024	ABORTED
16	DM025	ABORTED
17	DM026	ABORTED
18	DM028	ABORTED
19	DM030	ABORTED
20	DM031	ABORTED
21	DM032	ABORTED
22	DM033	ABORTED
23	DM034	ABORTED
24	DM035	ABORTED
25	DM052-HW	ABORTED
26	DM053-AN	ABORTED

27	DM086-DE	ABORTED
28	DM107-DDP	ABORTED
29	DM140-UM	ABORTED
30	DM152	ABORTED
31	DM016	ABORTED
32	DM048	ABORTED
33	DM050	ABORTED
34	DM067	ABORTED
35	DM071	ABORTED
36	DM079	ABORTED
37	DM096	ABORTED
38	DM126	ABORTED

Appendix 16. Adjusted p-values for salivary metabolites with significant changes in relative abundance.

Metabolites	p-value	q- value	Discovery
Urea (HC vs. OLP)	0.0021	0.012	Yes
Citric acid (HC vs. MMP)	0.0021	0.013	Yes
Formate (HC vs. OLP)	0.002	0.01	Yes

*Adjusted p-values using Benjamini–Hochberg post hoc test with desired FDR (Q)=5%.

Appendix 17. Adjusted p-values for plasma metabolites with significant changes in relative abundance.

Plasma metabolites	p-value	q- value	Discovery
Apoliprotein (OLP vs. MMP)	0.004	0.02	Yes
Cholesterol (HC vs. OLP)	0.0017	0.011	Yes
Choline (HC vs. PV)	0.0003	0.002	Yes
Polyunsaturated Fatty acids (HC vs. OLP)	0.00031	0.0012	Yes
Polyunsaturated Fatty acids (HC vs. PV)	0.004	0.025	Yes
Glucose (HC vs. MMP)	0.008	0.016	Yes
Glucose (HC vs. OLP)	0.0031	0.01	Yes

*Adjusted p-values using Benjamini–Hochberg post hoc test with desired FDR (Q)=5%.

Appendix 18. Adjusted p-values for Phyla in saliva with significant changes in relative abundance.

Phylum in saliva	P-value	q- value	Discovery
Actinobacteria (MMP vs. PV)	0.0033	0.0198	Yes
Actinobacteria (OLP vs. MMP)	0.0077	0.0231	Yes
Bacillota (HC vs. OLP)	0.0178	0.047	Yes
Spirochaetes (HC vs. OLP)	0.0001	0.0002	Yes

*Adjusted p-values using Benjamini–Hochberg post hoc test with desired FDR (Q)=5%.

Appendix 19. Adjusted p-values for species in saliva with significant changes in relative abundance.

Species in saliva	P-value	q- value	Discovery
H. parainfluenzae (MMP vs.PV)	0.0150	0.0449	Yes
A. massiliensis (HC vs. MMP)	0.0061	0.0366	Yes
S. australis (HC vs. MMP)	0.0005	0.0032	Yes
S. mitis (MMP vs. PV)	0.0318	0.0955	Yes
S. salivarius (HCvs OLP)	0.0019	0.0058	Yes
S. salivarius (OLP vs MMP)	0.0016	0.0058	yes
S. infantis 3 (HC vs. OLP)	0.0067	0.0402	yes
X. cellulosylitica (HC vs. MMP)	0.0028	0.0109	Yes
X. cellulosylitica (MMP vs PV)	0.0036	0.0109	Yes

Appendix 20. Adjusted p-values for species in plaque samples with significant changes in relative abundance.

Species in plaque	P-value	q- value	Discovery
C. gracilis (MMP vs. PV)	0.0031	0.0093	Yes
C. gracilis (HC vs. OLP)	0.0006	0.0039	Yes
C. ochracea (HC vs. OLP)	0.0072	0.0434	Yes