Characterising the Oral Microbiome in Orofacial Granulomatosis

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Characterising the Oral Microbiome in Orofacial Granulomatosis

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To Dad

You always told me to keep my head high

Declaration

I declare that the contents of this thesis have not been used in any other submission for an academic award. All the sources of investigation have been acknowledged and the thesis does not exceed 50,000 words.

The studies presented in this thesis were conducted in the department of Medical and Molecular Genetics at King's College London, department of Oral Medicine and the department of Gastroenterology at Guy's & St Thomas' Hospitals.

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Abstract

Orofacial granulomatosis (OFG) is a rare disfiguring disease of unknown aetiology. It is characterised by deep seated granulomatous inflammation in the oral cavity which may also affect the lips and perioral region. The precise pathogenesis remains unknown but previous studies have demonstrated links with dietary sensitivity, allergy and Crohn's disease (CD). It has been suggested that an alteration in the oral microbiome could play a role in the disease. Clinically, OFG remains challenging to diagnose and treat, with a lack of objective biochemical markers for disease assessment and monitoring. The use of probiotics as treatment for OFG is an attractive option and has not been studied in great detail. Thus, the aims of this thesis were to:

1. Characterise the oral microbiome in OFG in an attempt to further illuminate the underlying pathogenesis of the disease.
2. Evaluate the salivary calprotectin assay for use as an oral diagnostic biomarker in OFG and CD.
3. Evaluate the use of a novel probiotic Lactobacillus brevis (CD2) in the treatment of active OFG.

Microbiome analysis was performed using 16S rRNA gene analysis and revealed no differences in diversity or richness in salivary bacterial communities. However, there was a relative abundance of Streptococcus salivarius in OFG and CD patients as compared with controls.

Salivary calprotectin analysis using Enzyme-Linked ImmunoSorbent Assay

(ELISA) was carried out over a two year period in 252 subjects. Salivary calprotectin levels were found to be significantly higher in OFG patients with concurrent CD, and patients with intra-oral involvement compared with controls. There was poor correlation with the Oral Disease Activity Score (ODAS) indicating salivary calprotectin levels to be a poor marker of OFG disease activity but could indicate the presence of concurrent gut CD.

The effect of CD2 Lactobacillus brevis lozenges were studied in 28 patients with OFG. These were well tolerated and were found to modestly reduce median ODAS but with a greater reduction in oral soreness.

The evidence presented in this thesis suggests that changes in the microbiome may be involved in the pathogenesis of OFG and CD. Salivary calprotectin appears to be of limited value as a marker of disease activity, however, there would be benefit from the identification and development of a biomarker of disease presence and activity. Streptococcus salivarius could be a potential biomarker for OFG and CD. Potential modulation of the microbiome with probiotic treatment appears to be well tolerated and beneficial.

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List of Abbreviations

ACE	Angiotensin converting enzyme
AMOVA	Analysis of molecular variance
Anti-TNFα	Anti-Tumour Necrosis Factor Alpha
APC	Antigen presenting cell
ASCA	Anti-*Sacchromyces cerevisiae* antibodies
ATG16L1	Autophagy-related 16-like 1
AUC	Area under the curve
B cell	B lymphocyte
bp	Base pair(s)
BPE	Basic periodontal examination
BSG	British Society of Gastroenterology
Casp8	Caspase-8
CDEIS	Crohn's Disease Endoscopic Index of Severity
Conc	Concentration
COVID-19	Coronavirus-19
CD	Crohn's disease
CG	Cheilitis granulomatosa
CRP	C-reactive protein
°C	Degrees Celsius
DAS	Derriford Appearance Scale
DNA	Deoxyribonucleic acid
EEN	Exclusive enteral nutrition
ELISA	Enzyme-Linked ImmunoSorbent Assay

FBC	Full blood count
FLG	Filaggrin
g	Gravitational force
g	Grams
GPA	Global physician assessment
GSTFT	Guy's & St Thomas' NHS Foundation Trusts
GWAS	Genome-wide association study
HAD	Hospital anxiety and depression scale
HC	Healthy controls
HDAC	Histone deacetylases
HOMD	Human Oral Microbiome database
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IgA	Immunoglobulin class A
IgE	Immunoglobulin class E
IGRA	Interferon-Gamma Release Assay
ILC	Innate lymphoid cell
IRGM	Immunity-related guanosine triphosphatase family M
kg	Kilograms
l	Litres
LDA	Linear Discriminant Analysis
LRRK2	Leucine-rich repeat kinase 2
Map	*Mycobacterium avium paratuberculosis*
MED	Minimum Entropy Decomposition
µg	Micrograms

µl	Microlitres
miRNA	MicroRNA
min	Minute(s)
ml	Millilitres
MRS	Melkersson-Rosenthal syndrome
n	Number
NAFLD	Non-alcoholic fatty liver disease
nm	Nanometre
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerization Domain
NS	Not significant
OAS	Oral allergy syndrome
ODAS	Oral disease activity score
OFG	Orofacial granulomatosis
OFG+CD	Orofacial granulomatosis with Crohn's disease
OTU	Operational taxonomic unit
p	*P*-value
PCoA	Principles coordinates analysis
PCR	Polymerase chain reaction
Q	Quartile
r	Correlation coefficient
RA	Rheumatoid arthritis
RAS	Recurrent aphthous stomatitis
RCT	Randomised controlled trial
RNA	Ribonucleic acid

rpm	Revolutions per minute
s	Seconds
SES-CD	Simple Endoscopic Scale for Crohn's Disease
SNP	Single nucleotide polypmorphism
SOP	Standard Operating Protocol
SS	Sjogren's syndrome
T cell	T lymphocyte
TB	Tuberculosis
TCR	T cell receptor
TE	Tris-EDTA
Th	Helper T cell
TNF	Tumour Necrosis Factor
UC	Ulcerative colitis
UK	United Kingdom
VAS	Visual analogue scale
Wnt	Wingless-related integration site
XBP1	X-box-binding protein-1

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Chapter 1 – Introduction

1.1 – Definition of Orofacial Granulomatosis

Orofacial granulomatosis (OFG) is a rare chronic disease characterised by lip swelling and oral inflammation. Histologically, there are deep-seated non-caseating granulomas and dilated lymphatics. The term 'Orofacial granulomatosis' was first introduced in an effort to provide a unifying entity by Wiesenfeld et al. describing “a constellation of signs resembling those of Crohn’s disease (CD) clinically and histologically in patients who do not appear to have abnormalities at any other site in the gastrointestinal tract.”[1] Thus, the term OFG, provides a unifying definition as historically these signs had been previously described with varying terminology.

Perhaps the earliest known description of OFG came from the Scottish Physician, Dr. Archibald Pitcairne (1652-1713), a founder member of the Royal College of Physicians in Edinburgh. In a letter from 1703, he comments on the health of Lady Anne Traquair, describing her to be suffering from “a little of a bloody flux” and ‘an old swelling on her lip”. To treat her condition, Pitcairne advised a prescription of *cavew* (acacia or uncaria tree resin extract), a widely astringent used to treat both oral and bowel disorders. Interestingly, this also appears to be the first description of OFG occurring concurrently with CD.[2] Indeed, it has been proposed that OFG be referred to as Pitcairne's disease.

In 1928, Melkersson reported a case of orofacial oedema and facial palsy.[3] Three years later, Rosenthal described a further feature of a fissured tongue leading to this

triad of signs being known as the Melkersson-Rosenthal syndrome (MRS).[1] Later in 1945, Miescher described a case series termed 'cheilitis granulomatosa' (CG).[4] This included features of lip swelling with non-caseating granulomas found on histopathological examination. Subsequently, Miescher's granulomatosa and CG have been used interchangeably.

It was in 1932 that Crohn et al. described a granulomatous disease of the bowel termed 'regional ileitis'.[5] This term was used as it was felt that the inflammatory changes were not exclusive to the terminal ileum and other parts of the gastrointestinal tract could be involved. As the oral cavity is considered to be continuous with the gastrointestinal tract and can be affected by granulomatous inflammation, the term 'oral CD' has also been used to describe patients with granulomatous inflammation of the oral cavity. In addition, a large proportion of patients with OFG also have microscopic gut granulomata with a small proportion also suffering from intestinal CD.[6] Thus given the first descriptions of CD and OFG, multiple links have been made between the two conditions which are therefore considered to be closely related. More recent research over the last two decades has suggested that although the conditions appear to be distinct, there are close links and overlaps.[7, 8]

As a result of these different terms there has been much confusion regarding a consistent definition for orofacial granulomatous disease. CG and OFG seem to be different terms for the same or overlapping disease and it appears that current consensus favours the use of 'OFG'.

OFG is a complex condition which has been historically difficult to define. OFG as a term appears to be too simplistic in defining the conditions as there is much heterogeneity in the disease. Developing a disease classification such as the Montreal classification for CD[9] may be a useful guide in how to describe and stratify the disease. This has the advantage of standardising future studies and treatment.

An important aspect in defining OFG includes the exclusion of other granulomatous diseases including sarcoidosis, tuberculosis and CD. Importantly, these differ from OFG in that other extra-oral systems are involved such as the respiratory and gastrointestinal systems. Recent studies have suggested that the clinical features of OFG and CD overlap.[6, 8, 10] However, on a genetic basis, the two conditions appear distinct.[11] The genetics of OFG and CD are discussed in greater detail in Chapter 1.4.5, whilst the relationship between OFG and CD is discussed in greater detail in Chapter 1.8.4. It has been proposed that patients can be broadly categorised into 3 groups:

- OFG only
- OFG with gut involvement
- OFG with CD (OFG+CD)

1.2 - Epidemiology of OFG

Being a rare disorder, there are no known specific epidemiological studies of OFG. A global prevalence of 0.8%[12] has been suggested but this seems very high.[13] The reported age of onset is highly variable[14] but appears to primarily affect children and young adults.[1, 6] Epidemiological studies would be difficult to perform given the

variations in classifying clinical features and nomenclature. The condition can present to different clinical specialties including Oral Medicine, Dentistry, Dermatology and Allergy resulting in it not always being recognised and potentially undiagnosed. It has been reported that Gastroenterologists can miss approximately half of oral lesions compatible with oral CD when compared with a dental examination.[15] Indeed, a recent Norwegian questionnaire study amongst dental clinicians revealed that 41.2% were aware of OFG and 24.6% had seen an OFG patient. It was suggested that OFG could be more common than previously thought and clinical awareness was promising, although the condition may still be underreported.[16] Studies have been published from around the world, however on this basis, the United Kingdom (UK) appears to have the largest number of cases. Scotland and particularly, Glasgow appears to have the highest prevalence, suggesting it may be most frequently seen in people of Celtic descent. Consequently, this has led to OFG being referred to as 'Celtic lip' in this region, however, this is not a widely accepted term. It has been reported that OFG appears to occur in greater frequency with concurrent CD in Northern Europe as compared to the South.[17] Data from Southeast England reporting the largest OFG series published to date indicates that males and females appear to be affected equally with the median age of disease onset being 23 years.[7] This correlates with most series where the median age being reported in the second and third decades.[18] Further studies are needed to provide accurate epidemiological data.

1.3 - Clinical Presentation of OFG

Being a complex disease, OFG can present in a variety of ways. Typically, it presents with clinical features of recurrent lip swelling and oral ulceration. Figures 1.1 - 1.6 demonstrate the clinical features of OFG.[6, 19] Persistent inflammation can result in disfiguring fibrotic disease such as permanent lip swelling (Figure 1.1) which is refractory to medical therapy and may require debulking surgery. Other features include gingival erythema[20] (Figure 1.2), mucosal tags (Figure 1.3) and 'cobblestoning' (Figure 1.4) caused by buccal oedema.[21] (Figure 1.5) 'Staghorning' is a unique sign where there is inflammation and swelling of the salivary gland duct orifices in the base of the mouth.[19] (Figure 1.6) Atypical features include associated perioral or facial inflammation[22] and rarely, fistulisation from the oral cavity.[23] Labial and angular fissures may be niduses for supra-added infection which can exacerbate the underlying inflammatory process.[24] Being facially-disfiguring, the disease carries a significant psychological burden causing distress for affected individuals.[8, 25] A small study has shown that most individuals with OFG report a good quality of life. However, it was found that up to 23% experience severe problems with depression and anxiety relating to their disease but independent to clinical severity.

Figure 1.1 – Lip swelling

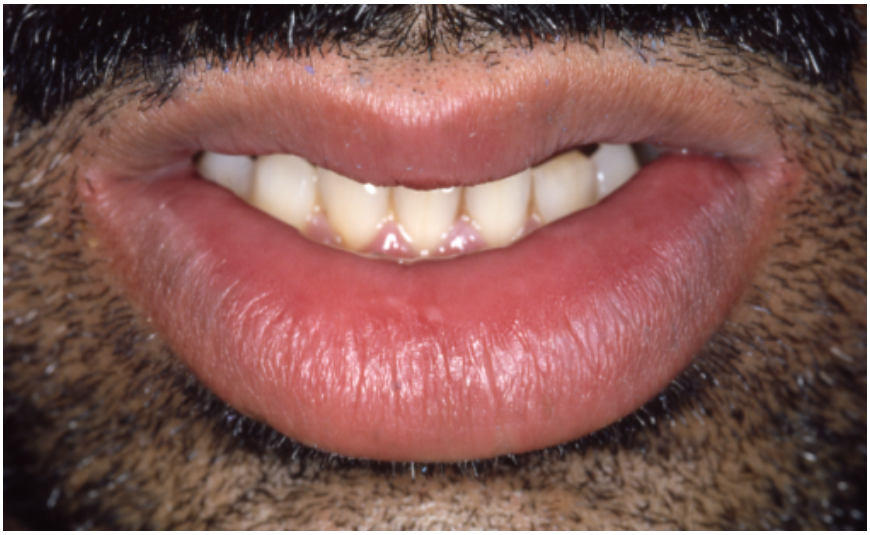


Figure 1.2 – Gingival involvement[19]

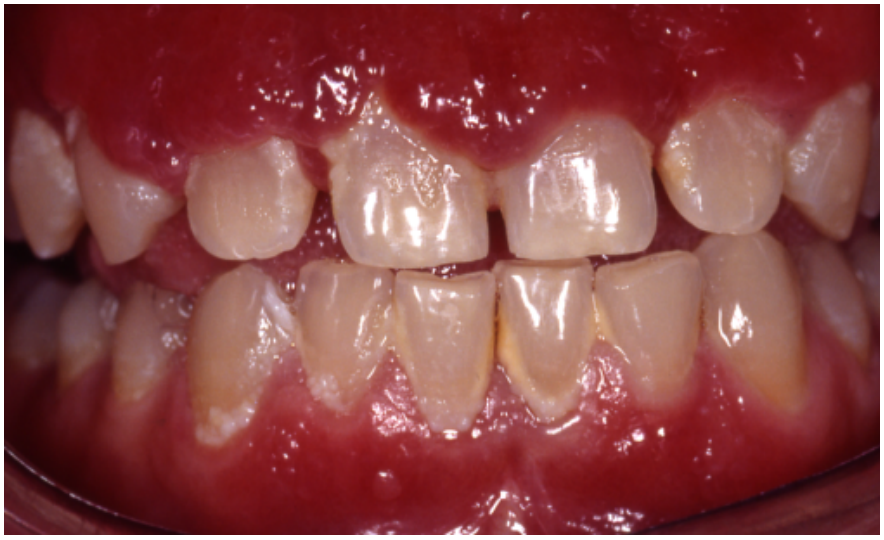


Figure 1.3 – Involvement of the buccal mucosa (tags)[19]

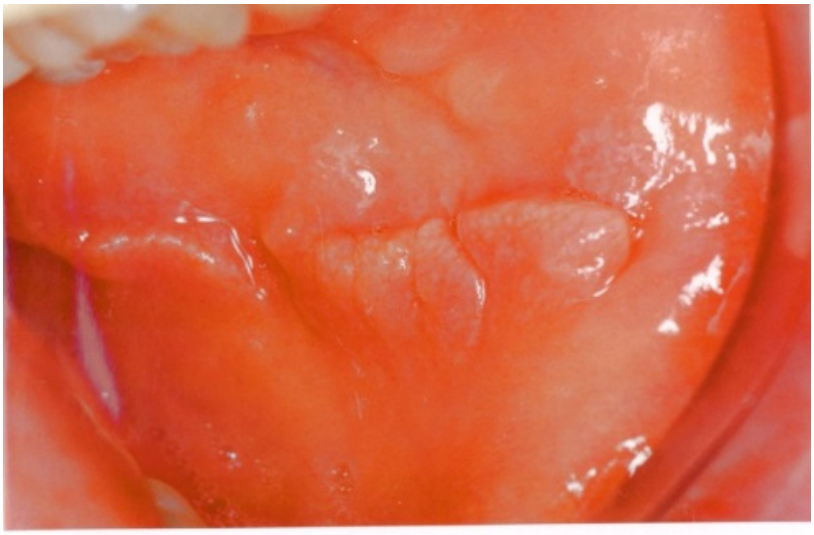


Figure 1.4 – Buccal 'cobblestoning'

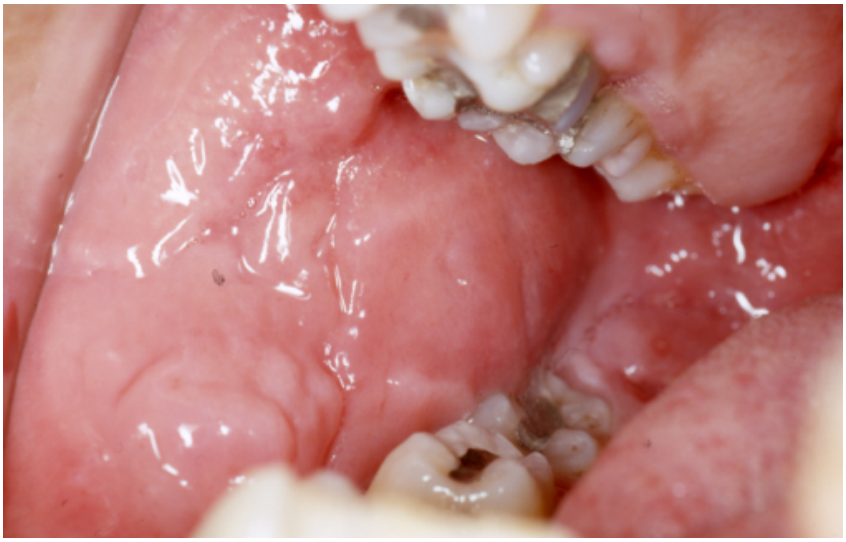


Figure 1.5 – Involvement of the buccal mucosa and sulcus showing oedema, thickening, fissuring, deep linear ulceration and mucosal tags[6]

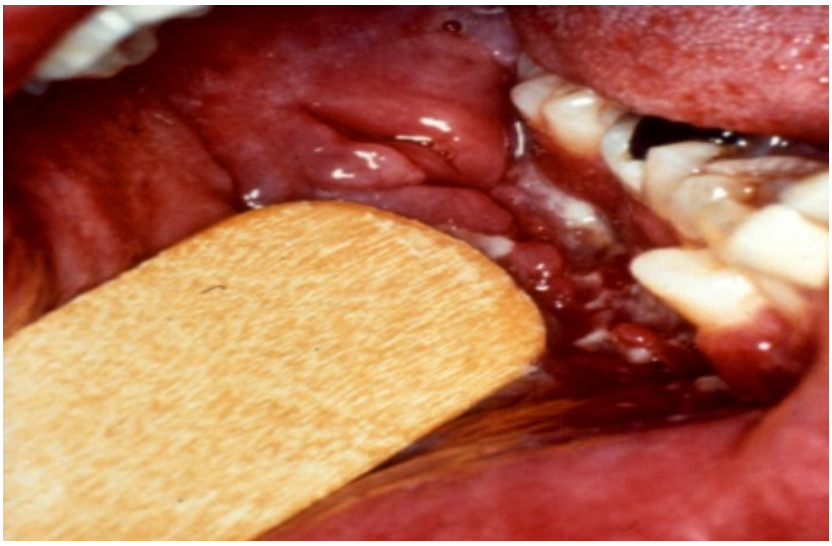
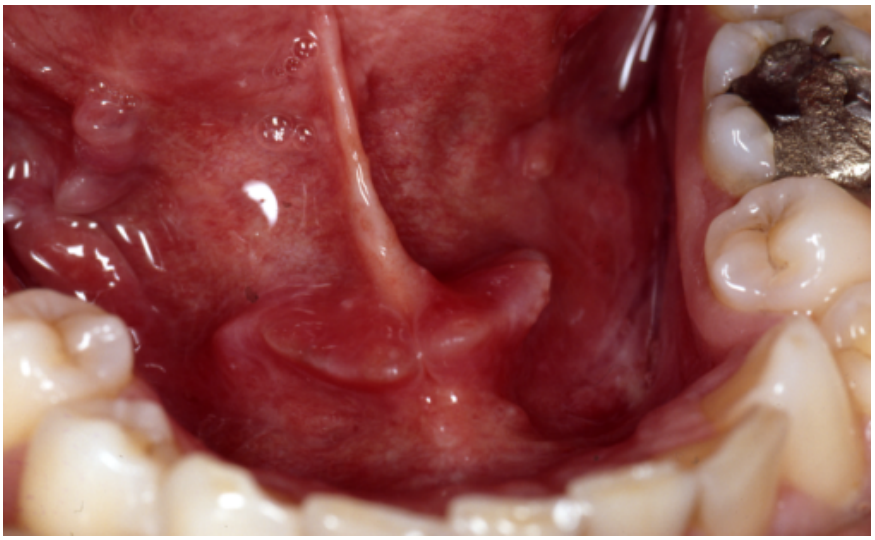


Figure 1.6 – 'Staghorning'[19]



It has been proposed that OFG be divided into two categories depending on the pattern of disease: anterior or posterior. The affected site is of clinical importance as it has been shown that patients with OFG and concurrent CD are significantly more likely to present with buccal sulcal involvement (posterior pattern), whereas patients in whom lip swelling (anterior pattern) is the sole or dominant feature are more likely to suffer from OFG only.[7, 26] Identifying patterns of OFG also allows for personal tailoring of treatment regimens as different regimens have different effectiveness in different patterns of disease. Overall, the risk of patients presenting with OFG and developing intestinal CD appears to be low with approximately 20% of patients developing CD within 25 years.[7]

1.4 - Aetiology of OFG

The precise cause of OFG is unknown. Given the complexity of the disease and its presentation, it is likely to have a multifactorial aetiology.

1.4.1 - Allergy

Much like with CD, OFG appears to be caused by exposure of the oral mucosa to an environmental trigger to which the subject is sensitive. The remarkably high incidence of allergy in OFG was first reported in 1986.[27] It was at this time that many studies demonstrated that certain foods and additives could trigger flare-ups of OFG. Table 1.1 lists commonly reported precipitants.[28-34] Hypersensitivity reactions to dental materials such as cobalt and amalgam have also been reported but these appear to be rare.[35-37] Reactions may mimic OFG, for example, a small series of patients treated

with silicone facial fillers has been recently described. These patients developed granulomatous inflammation and cheek oedema but these features were felt to be distinct from OFG.[38]

As discussed further in Chapter 1.7.1, subsequent studies involving the exclusion of specific dietary antigens such as cinnamon and benzoate showed significant success in treating most individuals with OFG.[19, 39] These findings add credence to an allergic component in the development of OFG. Interestingly, OFG has been found to share many clinical features with the oral allergy syndrome (OAS) with the key distinction being the absence of granulomatous inflammation in OAS.[40] In the largest study to date, allergy prevalence rates were determined in OFG patients and inflammatory bowel disease (IBD) patients. Individuals with OFG had allergy rates of 82% as compared to 22% in the general population.[40] The presence of concomitant CD, particularly perianal disease, was associated with an even higher allergy rate of 87%.[40] Additionally, 35% of allergic OFG patients had evidence of the OAS.[40] Interestingly, rates of allergy in ulcerative colitis (UC) were 18%[40] which were statistically comparable to 22% in the general population.[41]

OFG has conventionally thought to be a Th1-mediated disease[42] resulting in delayed hypersensitivity reactions. However, the smaller yet significant rate of atopy and recent finding of B cells which express IgE[43] also implicate a role for immediate hypersensitivity reactions involving Th2 cells.

Table 1.1 - Agents reported to precipitate OFG[28-34]

Wheat	Carvone
Dairy products	Carmosine
Chocolate	Sun yellow dye
Egg	Toothpaste
Peanut	Carrot
Cinnamaldehyde	Tomato
Benzoic acid	Pork
Alpha-lactolumin	Apple juice
Carbone piperitone	Orange juice
Cocoa	Monosodium glutamate

1.4.2 – Immunological

The immunopathology of OFG appears increasingly complex with early studies into the role of T cells proving contradictory. In 1997, a study investigating T cell receptor (TCR) V beta gene usage found that lesional T cells preferentially used the V beta 6 gene. The authors concluded that a local antigen was likely responsible for the V beta 6 T cell expansion and abnormal T cell response as this finding was not observed in normal oral mucosa.[44] However, a subsequent study found no differences in TCR V beta 6 usage between lesional and peripheral T cells. This suggested that there was a random influx of T cells at the affected sites with no specific antigen.[45] Later, a study of oral biopsies from 10 OFG patients found a predominantly Th1 environment which resembled that found in inflamed gut in patients with CD. The authors concluded that being granulomatous diseases, OFG and CD shared immunopathological features.[42] More recently, another study also found that there was no difference in TCR V beta gene repertoires at lesional and peripheral levels. This finding does not support the theory of a local antigen causing OFG.[46] Most recently, a study examining OFG biopsy infiltrates in OFG patients and those with concurrent CD, revealed that T cell profiles were significantly different between the two groups.[47] It was concluded that partly divergent immune mechanisms were responsible for the finding. Given the heterogeneity of OFG, it is likely that patients from these studies had differing disease phenotypes and thus different aetiological mechanisms leading to conflicting results. Immunopathologically, there do appear to be differences between OFG only and OFG with CD, suggesting overlapping yet fairly distinct diseases.

In 2010, a large study utilising immunohistochemistry analysis of oral biopsies from patients with active OFG identified a novel population of subepithelial dendritic B cell which expressed IgE.[43] It is not clear if these cells are present at disease onset or if there is an influx following local inflammation. This finding supports the role of an antigen in triggering an immediate hypersensitivity reaction and correlates with the high rates of allergy seen in OFG.[40]

Overall, there is strong evidence that the cell infiltrate in OFG has a prominent Th1 profile, however, there is significant clinical and immunological evidence of a Th2 and B cell response, likely to a dietary antigen. Thus the immunopathology of OFG would appear to include both cell-mediated and antibody-mediated immune responses.

1.4.3 – Infective

An infectious aetiology has been implicated in other granulomatous diseases such as sarcoidosis or tuberculosis, where the precise infectious mycobacterium is known. Much research has been carried out to discover a culpable organism in CD with studies indicating *Mycobacterium avium paratuberculosis* (*Map*) to be a potential causative agent.[48, 49] A zoonotic mechanism has been suggested based on observations that granulomatous intestinal lesions seen in cattle infected with *Map*, a condition known as Johne's disease, closely resemble those seen in CD.[50, 51] This theory is further supported by the correlation of rising incidence rates of Johne's disease and CD.[51] However, early studies were unable to successfully isolate *Map* in CD but technological advances have enabled numerous studies to confirm the presence of *Map* in CD.[50] The role of *Map* in the precise aetiopathogenesis of CD still remains to be elucidated.

Given the similarities and perceived relationship between OFG and CD, numerous studies have now been performed with the aim of detecting *Map* in OFG. The first such study was performed in 1993 which detected raised levels of antibodies against mycobacterium stress protein in OFG patients.[52] Conversely, two subsequent studies using PCR techniques were unsuccessful in detecting *Map* in larger series of OFG patients.[53, 54] As current results are contradictory, further studies are required to confirm or refute the presence of *Map* in OFG.

It has been suggested that *spirochete* infection may be the causative agent in the development of OFG.[55, 56] Specifically, the detection of high levels of antibodies against *Borrelia burgdorferi* has been reported[55], along with successful treatment by penicillin.[56] However, a subsequent study from 2000 examined biopsies from a case series of 12 CG patients.[57] This involved PCR techniques and serological measurement of antibodies against the *spirochete* and did not confirm the findings of the earlier studies. The authors thus concluded that the organism had no aetiological role in the development of OFG. Resultingly, at present there appears to be no compelling evidence for the role of a specific infective organism in OFG.

1.4.4 - Microbiological

It has previously been discovered that anti-*Sacchromyces cerevisiae* antibodies (ASCA) are more common in patients with CD as compared to UC and healthy controls.[58] It was proposed that ASCA could be used as a discriminating tool between CD and UC, however, subsequent findings did not support this theory.[59, 60] Based

on these findings, it was proposed that measurement of ASCA titres could be of clinical benefit in distinguishing between patients with OFG only and those with OFG and CD. Such a study was performed in 2004 looking at antibody titres for *Sacchromyces cerevisiae*, *Candida albicans* and *Streptococcus mutans*.[61] The patient groups studied were those with OFG only, OFG and CD, CD with no oral involvement and healthy controls. Serum IgA levels were raised in all groups but only salivary parotid IgA was raised in the OFG and OFG with CD groups. Interestingly, this suggests that increases in salivary IgA indicates oral cavity and specifically salivary gland involvement.[61] The observation of 'staghorning' in many OFG patients further supports this. Also, serum ASCA was raised in both groups with CD but not in OFG, thus suggesting clinical utility in measurement for predicting gut disease.[61] More recently, a study has identified 51 biomarkers, including antibodies against ASCA, which could predict the future development of CD.[62]

1.4.4.1 - The oral microbiome

The human microbiome refers to the microbial communities and their genetic information which reside in or on our bodies. More specifically, it has been defined as the collection of all genomes of microbes in an ecosystem.[63] The number of cells which constitute the microbiome are thought to far outweigh the number of human cells. The first descriptions of human microbiota came from Antonie van Leeuwenhoek and the use of early handcrafted microscopes. After observing scrapings from inside his mouth, he wrote in a letter to the Royal Society of London in 1683:[64]

"I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving."

He went on to describe these organisms in the mouth and faecal microbiota, determining differences between body sites and individuals, as well as in health and disease.[64] Thus ever since, there has been a wealth of interest into microbiota research particularly in identifying, cataloguing and discovering the role of these organisms with a surge in microbiome research over the last decade. This has primarily been driven by advances in microbiome analysis methods as described in Chapter 1.4.4.2, including genetic techniques enabling large-scale identification of microbial organisms which could not be previously identified using traditional in vitro cultivation methods.[65] International studies have now begun to explore microbial communities across different body sites and in various geographical populations. The hope is to better understand the potential causality of microbiota-related mechanisms of disease which could lead to the development of preventative and therapeutic strategies.[63]

The oral cavity is the gateway to the gastrointestinal tract and contains one of the largest ecology of microbial species in humans, second only to the colon in diversity. These organisms include viruses, protozoa, fungi, archaea and bacteria, which are responsible for two of the most commonly infectious diseases of humans: dental caries (tooth decay) and periodontal (gum) disease.[66]

A great challenge in microbiome research has been in trying to define what constitutes a normal or core human microbiome. Approximately 1000 bacteria have been identified in the oral cavity, although a typical cavity is thought to contain approximately 500

different species.[67] Less than one-third of these taxa have yet to be grown in vitro.[68] There are thought to be differences between the different oral cavity sites with samples from between the teeth, tongue, saliva and anterior/posterior mucosal cavities all showing different ecologies.[69] However, data from over 200 healthy individuals has shown a clear abundance and predominance for *Streptococcus* species from all oral mucosal sites.[70]

How the oral microbiome precisely develops is unknown, though it has been hypothesised that the origin of the foetal' microbiome comes from the mother. It has been logically suggested this occurs by maternal bacteria being transferred to the placenta via the bloodstream whilst mediated by pregnancy hormones. Maternal microbial cells are then presented to the foetal immune system with foetal antigen-presenting cells (APCs) interacting with the microbial antigens and returned to the foetal lymphoid system. Subsequently, foetal T cells develop prenatal tolerance and regard the maternal microbiome as non-threatening.[71] This would explain how the oral mucosa is highly immunotolerant and able to determine which microbial populations are friend and which are foe. 'Colonisation resistance' is the term which describes how oral microbiota are thought to inhibit pathogen colonisation due to factors such as signalling mechanism interference, fewer nutrients and binding site availability.[72] Additionally, some strains such as *Streptococcus salivarius*, directly inhibit the growth of Gram-negative species which have been associated with halitosis and periodontitis.[66]

Further acquisition of the oral microbiome is thought to be by vertical transmission from mother to newborn with the mode of delivery also determining exposure to either

skin (Caesarian section) or vaginal microorganisms.[73] Breastfeeding and horizontal transmission have also been shown to influence microbiome diversity.[71] Although the oral cavity is constantly exposed to various repeated environmental perturbations such as tooth brushing and eating, it's microbiome appears to exhibit a remarkable resilience.[74, 75] Longitudinal oral microbiome stability has been demonstrated in many studies including following antibiotic administration.[76, 77] Interestingly, a previous study showed that whilst the oral microbiome remained relatively robust in the face of antibiotic treatment, it produced a negative effect in the faecal microbiome.[77] In addition, mouthwash use and dental appliances treatment has also not been shown to greatly influence the oral microbiome.[78] Overall, despite acute changes, the oral microbiome appears to quickly recover to a homeostatic state in contrast with the gut microbiome.[75]

Host-microbial interactions are of great interest with the hope that altering the microbiome in targeted ways could lead to the prevention and treatment of diseases. Oral bacteria demonstrate specificity for colonisation sites and to each other which is mediated by adhesin-receptor binding.[79] Commensal colonisation also means there are less binding sites for pathogens.[66] Some bacteria may also have a role in maintaining health. For example, *Streptococcus salivarius* strain K12 produces a bacteriocin which inhibits the growth of bacteria associated with periodontitis and halitosis.[80, 81]

Studies have been carried out looking at the concept of a mobile oral microbiome, where oral cavity metabolism could affect another area in the body. Numerous studies have linked oral bacteria to other systemic diseases including bacterial endocarditis,

stroke, cardiovascular disease, pancreatic cancer and pneumonia.[68] There have been reports suggesting periodontitis to be a risk factor for neurological diseases[82] including Alzheimer's disease[83] and Parkinson's disease.[84, 85] However, it is important to note that such studies appear to be sporadic and speculative. A more compelling study has shown evidence of dysbiosis in both the oral and gut microbiomes of Rheumatoid Arthritis (RA) patients, with concordance between the two microbiomes.[86] Furthermore, microbiome differences were used to distinguish RA individuals from healthy controls and were used to stratify individuals according to therapeutic response.[86] The altered microbiome in RA was also shown correlate with the diagnosis and prognosis of RA.[86] More recently, research has been focused on investigating the oral-gut-liver axis exploring the interconnections among non-alcoholic fatty liver disease (NAFLD), periodontitis, and gut dysbiosis.[87]

One interesting mechanism which has been more widely explored involves the role of the microbiome with nitrate metabolism and cardiovascular health. Approximately 25% of ingested nitrate undergoes entero-salivary recirculation. Oral bacteria convert nitrate to nitrite which is then absorbed into the bloodstream via the gastric mucosa. Nitric oxide (NO) has an anti-hypertensive effect and plasma nitrite levels have been shown to decrease following the use of an antimicrobial mouthwash.[88, 89] Thus, the use of mouthwash and its effects on the oral microbiome may in turn lead to increases in blood pressure and affect cardiovascular health.[89, 90] Periodontal disease has also been linked with negative glycaemic control in diabetics.[91] Such potential changes in the oral microbiome could represent an opportunity to detect oral biomarkers in screening for systemic diseases.

1.4.4.2 - Oral microbiome analysis techniques

Previously, human microbiology research largely consisted of culture-based studies of microbes, however, these methodologies are costly, labour-intensive and not as comprehensive as newer molecular methods. Nevertheless, as per 2016, 31% of known oral taxa have not been cultured in vitro, bacterial culture still has a role in microbiology.[68]

Macro-level analysis of microbial communities has been possible using gradient gel electrophoresis with subsequent DNA sequencing. Homologous DNA sequences were digested using restriction fragment length polymorphism with the resulting fragment length used for genome mapping.[92] These methods were based on polymerase chain reaction (PCR) using specific primers to amplify regions of interest with subsequent analysis. One of the earlier sequencing methods involved DNA-DNA checkerboard hybridization[93] with subsequent analysis from DNA microarray.[68]

16S RNA sequencing has been the mainstay of sequence-based bacterial and microbiome analysis for decades as the region is highly conserved within species. This involves isolated DNA from a studied environment being amplified using PCR primers for 16S rRNA genes. Subsequent sequencing then helps identify the species with typically a >98.5% identity defining a species. Thus, a sequence with <98.5% similarity would be considered to identify a new species.[94, 95]

The significant increase in understanding of the microbiome has come about from the advent of next generation genetic sequencing techniques. These revolutionary methods

have increased accessibility allowing for whole community profiling and large scale high throughput sequencing projects to be completed in hours to days at a much lower cost. There are multiple sequencing platforms in use but they all share common features in methodology. Currently, the most widely used platform for bacterial community profiling is the Illumina MiSeq.[96]

1.4.4.3 - The oral microbiome in OFG

As previously discussed, there is no compelling evidence for an infective organism in the aetiology of OFG, however, it could be possible that the initial inappropriate immune response could be against an organism or community within the commensal oral microbiota.

To date, no studies appear to have been conducted specifically investigating the oral microbiome in OFG. In 2015, a study reported oral dysbiosis in a Chinese cohort with IBD however case and control numbers were both small being less than 10.[97] More recently, oral microbiome analysis in CD patients has shown a greater salivary microbial diversity compared with controls.[98] A recent review has published a summary of the recent studies reporting oral microbiota changes in IBD which is shown in Table 1.2.[99]

Table 1.2 – Oral Microbiota changes in IBD[99]

Disease	Subjects	Specimen	Oral Microbiota with Increased Abundance	Oral Microbiome with Decreased Abundance	Detection Method
IBD	HCs (*n* = 43) CD (*n* = 40) UC (*n* = 31)	Tongue and buccal mucosal brushings	*Bacteroidetes* *Spirochaetes* *Synergistetes*	*Fusobacteria* *Firmicutes*	HOMIM
Adult IBD with untreated chronic periodontitis	HCs (*n* = 15) CD (*n* = 15) UC (*n* = 15)	Gingivitis and periodontitis sites samples	*Prevotella Melaninogenica* *Staphylococcus aureus* *Streptococcus anginosus* and *Streptococcus mutans* in CD *Staphylococcus aureus* *Peptostreptococcus anaerobius* in UC		checkerboard DNA-DNA hybridization technique
Adult IBD	HC (*n* = 24) CD (*n* = 21) UC (*n* = 14)	Saliva	*Prevotella* *Veillonella* Bacteroidetes	*Streptococcus* *Haemophilus* Proteobacteria *Neisseria* in CD *Gemella* in CD	PCR
Pediatric CD	discovery cohort HC (*n* = 46) CD (*n* = 35) validation cohort HC (*n* = 31) CD (*n* = 44)	Subgingival plaque samples	*Capnocytophaga* *Rothia* TM7		PCR
Adult IBD	HC (*n* = 25) CD (*n* = 13) UC (*n* = 54)	Saliva	*Veillonellaceae* in CD Streptococcaceae and Enterobacteriaceae in UC	Neisseriaceae and Haemophilus in CD Lachnospiraceae and [Prevotella] in UC	PCR
Adult IBD	HC (*n* = 8) CD (*n* = 12) UC (*n* = 10)	Saliva	*Saccharibacteria* (TM7) *Absconditabacteria* (SR1) *Leptotrichia* *Prevotella* *Bulleidia* *Atopobium*	*Streptococcus* *Rothia*	PCR

1.4.5 - Genetic

There have been few studies exploring the genetic basis of OFG specifically. There seems to be a genetic predisposition as the condition has been observed to occur in families although it does not segregate in a Mendelian manner.[11] As discussed in Chapter 1.4.1, OFG demonstrates an increased prevalence of allergy. Atopy has been shown to have a strong genetic susceptibility with loss-of-function mutations in the filaggrin (FLG) gene being an important risk factor in the aetiology of atopic diseases.[100] These findings would support a possible genetic influence in the development of OFG. Additionally, the close clinical and histological associations between OFG and CD suggests a potential similar or shared genetic predisposition.

A previous study of 16 OFG patients showed an increased prevalence of Human Leukocyte antigen (HLA) genotypes A2/3, B7 and DR2/3/4 as compared with normal controls. It was therefore suggested that OFG had a defined HLA risk haplotype which could be a distinct factor in its development.[101] More recently, a larger genotyping study utilising Sanger sequencing of 201 OFG patients and 1023 healthy controls revealed a significant enrichment in NOD2 variants in OFG patients with concurrent CD.[11] The gene encoding NOD2 was the first susceptibility gene to be identified for CD[102, 103] and is involved in the recognition of bacterial molecules (peptidoglycans) stimulating an immune reaction. However, NOD2 was not seen to be associated with OFG only patients, which would support the observation that the conditions remain two separate entities on a genetic basis. In order to fully understand the genetic architecture of OFG, a genome-wide association study (GWAS) is needed.[11] These studies involve rapidly scanning markers, such as single nucleotide

polymorphisms (SNPs) across the complete genomes, of many people to find genetic variations associated with a particular disease.[104]

1.5 - Diagnosis of OFG

OFG is largely diagnosed on the basis of clinical features and can be complicated as clinicians may follow differing diagnostic criteria.[105, 106] As discussed in Chapter 1.2, there is heterogeneity in the classification of OFG. Additionally, multiple specialties have differing views as the condition overlaps Oral Medicine, Dentistry, Dermatology and Allergy. Thus there is a need for a unified standardised classification for OFG. A pragmatic diagnostic algorithm to aid diagnosis has recently been proposed.[107] Again, this is predominantly based on clinical features but also includes basic blood tests and less commonly radiological scans and biopsies. Additionally, the proposed algorithm does not adequately identify the OFG with CD or gut involvement cohort.

Histological examination with the presence of non-caseating epithelioid granulomas is definitive but is invasive and not always readily available. Biopsies have a high false negative rate as the granulomas are located within the lamina propria, frequently deep seated and so can be missed.[18] The granulomas are histologically indistinguishable from those found in CD and sarcoidosis. Other histological features are the presence of a marked chronic inflammatory infiltrate and lymphoedema of the corium and dilated lymphatics.[7] The lips and buccal mucosa are the most commonly affected sites.[7] Patients usually describe a history of flares of intermittent lip swelling with food triggers sometimes identified. Other reported symptoms include: oral discomfort/pain,

gum hypertrophy, ulceration and inflammation of the ductal orifices ('staghorning').[19]

Previous studies have reported a concurrent diagnosis of intestinal CD in 22% of OFG patients.[7] Thus, an existing diagnosis of CD or patients with significant systemic gastrointestinal symptoms should raise suspicion when diagnosing OFG. Patients with CD and OFG have been found to more commonly have the following features: ulcers, raised C-reactive protein (CRP) and an abnormal full blood count (FBC). Interestingly, the specific finding of buccal sulcal ulceration has been shown to be present at greater frequency in those OFG patients with concurrent CD.[7] These same patients also present less frequently with lip swelling.

Other granulomatous diseases such as sarcoidosis and tuberculosis are rare but should be considered if pulmonary or systemic involvement and may need to be excluded. If suspected, Chest X-ray, serum angiotensin-converting enzyme (ACE) and Tuberculosis (TB) Interferon-Gamma Release Assay (IGRA) should be performed. In patients with suspected concurrent CD, faecal calprotectin is a valuable non-invasive, cheap and readily available test to check for the presence of gut inflammation.[108] It is particularly useful as a screening test for concurrent CD in OFG patients and is frequently used to distinguish between functional and organic disease, such as IBD, in the community and outpatient setting.[109] Further definitive tests including ileo-colonoscopy and small bowel imaging such as small bowel video capsule endoscopy and MRI scan should be also considered.[109]

In the coronavirus 2019 (COVID-19) era, there have been reports of oral manifestations of COVID infection. A frequent symptom is dysgeusia which affects approximately 80% of infected individuals and the most common sites affected within the oral cavity in descending order are the tongue, labial mucosa and palate.[110, 111] There has been no obvious link reported in the literature with COVID-19 infection and the development or flaring of OFG.

1.6 – Oral and Salivary Biomarkers

Given the easy accessibility to the oral cavity, there has been much interest in potential oral biomarkers for disease screening and activity, as well as supplementing diagnostic and treatment methodologies. This reflects interest within the field of medicine as a whole regarding development of validated salivary biomarkers for conditions such as autoimmune and neoplastic disease.[112, 113] More recently, there has been a developing interest in drug monitoring, such as the measurement of salivary levels of steroids. Salivary biomarker research has been driven by the distinct advantages conferred by saliva, including non-invasive specimen collection and diverse opportunities for 'omics' based research.[114] Saliva has been shown to contain a large number of molecules, of which many directly correlate with their concentration in blood.[115] This is supported by the observation that saliva composition is regulated by the transcellular and paracellular passage of molecules from the blood to saliva.[115] However, the condition of the oral cavity and its effect on potential biomarkers is still being elucidated.

An important consideration regarding saliva collection is that the collection site of saliva has been shown to affect the biomarker concentration within the same individual.[116] Other factors which have been reported to affect salivation include periodontal disease and certain medications.[117] Important differences between stimulated and unstimulated saliva sample collection have also been reported. Stimulated saliva collected, such as collected after chewing, has been found to contain three times more bacterial species as compare with unstimulated saliva collection, such as using sterile paper points.[118] Thus, the need for consistency in study methodology such as sample collection and when comparing study results is crucial. Sample collection by passive drooling or expectoration has the advantage of being an easily reproducible consistent method and would contain a larger catchment of oral species for analysis.

Recently, novel biomarkers such as exosomes (extracellular vesicles) have been described.[119] Other examples of salivary biomarker research in relation to IBD include use of salivary microRNA for early detection of IBD and to distinguish between CD and UC,[120] Additionally, microbiome analysis studies have been used to differentiate between individuals with CD and healthy controls.[121, 122] As discussed earlier, raised levels of IgA have been detected specifically in individuals with OFG and OFG + CD but not in CD.[61] This concept of a ‘fluid biopsy’ may hold promise for use as a biomarker in CD pts to see if they have early OFG.

1.6.1 – Salivary calprotectin

With good representation of plasma proteins within saliva,[114] salivary proteomics has been an area of intense research within the field of salivary biomarkers, facilitated by the 'Human Salivary Proteome Initiative' launched in 2004 by the United States National Institute of Dental and Craniofacial Research. This initiative helped to catalogue the core salivary proteome, including calcium-binding proteins of the S100 family.[123] Calprotectin is a heterodimer of two S100 proteins (S100A8/S100A9) and is released by activated neutrophils, monocytes and macrophages, forming part of the innate immune response.[114, 124] The pro-inflammatory properties of calprotectin are thought to contribute to disease pathogenesis, such as RA, and have led to its use within disease monitoring, as for CD and faecal calprotectin testing.[124] Specifically, salivary calprotectin has been investigated as a disease marker for oral diseases including Sjogren's syndrome (SS)[125, 126] and periodontal disease.[114] Higher levels of salivary calprotectin have been noted in patients with SS compared with healthy controls and disease controls, leading to suggestions that salivary calprotectin could act as a biomarker for the diagnosis of SS and disease activity.[125, 126] Moreover, preliminary research suggests that salivary calprotectin could act as a biomarker for subgroups of SS patients at particularly high risk of lymphoma.[126] Salivary calprotectin has also been utilised to assess patients with oral candidiasis.[127] As mentioned earlier, salivary calprotectin concentration has specifically been shown to be affected by collection site.[116]

1.6.2 – Oral biomarkers in IBD

With regards to IBD, there has been a gaining interest for non-invasive biomarkers as a measure for disease activity. Until recently, there had been few studies involving patients exploring potential salivary markers. However, a recent systematic review of eleven studies with a total of 631 participants (255 with CD) from seven different countries concluded that there are several salivary biomarkers which can be used credibly for the early diagnosis and regular monitoring of IBD.[128] Due to their heterogeneity, the biomarkers were divided into four groups: oxidative status markers, inflammatory cytokines, MicroRNAs (miRNAs) and other biomarkers.[128] Furthermore, the most promising biomarkers with diagnostic potential appear to be oxidative stress mediators,[129-131] certain inflammatory cytokines,[121, 132, 133] exosomes, cortisol, amylase and mucin 5B[115] and selective miRNAs.[120] In 2021, data was published comparing concentrations of potential salivary parameters in 51 IBD patients (27 CD and 24 UC) with 51 healthy controls.[134] Salivary concentrations of S100A8/ calprotectin, myeloperoxidase and IgA were significantly decreased in IBD patients, with UC having decreased levels of TNF-R1 and catalase.[134] Of interesting potential clinical significance was the finding that myeloperoxidase and TNF-R1 concentrations showed high differential potential for CD and UC (AUC = 0.69 and 0.672 respectively).[134] In contrast, a small study of 23 newly-diagnosed IBD patients and 15 controls has shown that the median salivary calprotectin level was 4-fold higher in the IBD cohort and levels decreased following the initiation of treatment.[135] The study also found that salivary calprotectin levels were higher in unstimulated saliva samples compared with those collected by stimulation.[135] Interestingly, patients with CD have also been reported to present with hyposalivation.[136]

Faecal calprotectin remains a reliable validated biomarker but patients often report the collection of faeces to be unpleasant and burdensome.[108, 109] It has the advantages of being readily available, widely used and is regarded as a surrogate marker for endoscopic disease activity, frequently negating need for endoscopic assessment.[137] Nevertheless, endoscopic investigations remain the gold standard but are invasive and have a small risk of complications. Most recently, a study has been published comparing salivary and faecal calprotectin levels in 63 IBD patients and 11 controls. The calprotectin concentration in saliva was determined using a particle-enhanced turbidimetric immunoassay and revealed no significant correlation between salivary and faecal calprotectin levels.[138] Thus, the authors concluded that salivary calprotectin is unreliable for assessing IBD activity.

To date, there are no widely used specific biomarkers to aid in the diagnosis of oral inflammatory conditions such as OFG. Such a biomarker would be of objective value in screening for the disease and monitoring response to treatment over time. A validated oral biomarker in OFG could avoid the need for invasive biopsies in the future. Additionally, given that a small proportion of OFG patients also have intestinal CD, there is an unmet need for a non-invasive biomarker to identify those patients who would benefit from invasive gastrointestinal endoscopic investigations and those in whom it can be avoided. Moreover, in the COVID-19 era, there is an increasing need for remote testing which would make a salivary biomarker an even more attractive test. Finally, compared with blood sampling, the use of saliva testing involves less manipulation, avoids specialist phlebotomy technicians; samples are easier to ship and store, greater economic value and reduced healthcare costs.[139]

1.7 - Current Management and Interventions in OFG

There are many treatments for OFG although being a rare disease, few trials exist which have investigated treatment efficacy.[18, 140] Mild disease can often be managed conservatively with routine follow up with frequently no serious sequelae. The primary goal of treatment is to reduce orofacial swelling, painful ulceration and improve symptoms.[141] Reports of treatment response has variable with some in remission within 1 year and others still receiving treatment at 15 years.[142] There is no cure for OFG but by using combinations strategies, rates of improvement of up to 78.8% have been reported for tissue swelling and 70% for oral ulceration.[142] Pragmatically, OFG alone should be treated conservatively such as with dietary and topical therapies. However, with more extensive disease such as significant intra-oral involvement or concurrent CD, immunomodulatory and/or biologic treatment may be required earlier in the disease course. Effective management frequently requires a combination of dietary manipulation, pharmacotherapy and less commonly, surgical intervention.[143] Overall, there is no definitive uniform predictive model for how OFG may behave over time[140] although dividing disease locations into anterior and posterior patterns may be useful.[7]

An oral disease activity score (ODAS) has been developed which enables precise recording of disease severity, location within the oral cavity as well as any other pathological features. This was developed by the multidisciplinary OFG group at GSTFT and was first reported in 2006.[19] The scoring system was based on historical scoring systems used for Behcet's disease activity and is used at clinical examinations as an objective and clinically quantifiable measure of disease activity.[7] This provides

objective assessment and enables longitudinal monitoring treatment response at follow up and are valuable for clinical studies and research. Table 1.3 shows a chart of the ODAS used at GSTFT.[7] The chart combines site involvement, activity, and the type of lesions observed. Each involved site is scored individually for activity and lesion type, with the total activity score indicating global severity. A total activity score of 0 resembles normality or complete disease inactivity.

Analogous scoring indices exist for CD and are used in a similar fashion to document disease location, severity and monitor responses to treatment. The two most commonly used scores are the CD Endoscopic Index of Severity (CDEIS)[144] and Simple Endoscopic Scale for CD (SES-CD).[145] Both scoring indices have value despite limitations and are commonly used but remain unvalidated.[146]

Table 1.3 – Oral Disease Activity Score used in OFG[7]

Site <50% = 1, >50% = 2	Site score	Activity score (0-3) Double if site = 2	Types of lesion	Lesion legend	
Upper lip	0 1			1	Swelling
Lower lip	0 1			2	Tags
Angular chelitis	0 1			3	Ulceration
L Buccal Mucosa	0 1 2			4	Linear ulcers
R Buccal Mucosa	0 1 2			5	Erythema
L Buccal sulcus	0 1			6	Fissure
R Buccal sulcus	0 1			7	Nodules
Gingiva					
Lower R	0 1				
Lower central	0 1				
Lower L	0 1			Activity legend	
Upper R	0 1			0	Absent
Upper central	0 1			1	Mild
Upper L	0 1			2	Moderate
Dorsum tongue	0 1 2			3	Severe
R lateral tongue	0 1				
L lateral tongue	0 1				
Floor of mouth	0 1 2				
Hard palate	0 1 2				
Soft palate	0 1 2				
Oropharynx/fauces	0 1 2				
Total					
Other features				**Please indicate Y/N**	
Cobblestoning					
Fibrous banding/scarring					
Facial swelling					
Facial erythema					
Staghorning					

Basic measures such as promoting good oral hygiene and regular dental inspection should be encouraged, particularly as poor dentition and periodontitis can be exacerbated by potential immunomodulatory treatment.[6, 147] Additionally, OFG-associated gingivitis can be confused with periodontitis from poor oral hygiene. A multidisciplinary approach to management has been shown to be best incorporating specialists from Oral Medicine, Gastroenterology and Dietetics worked collaboratively.

1.7.1 - Dietary manipulation

As mentioned in Chapter 1.4.1, small studies involving dietary interventions have been carried out over the last decade with promising results.[19, 148, 149] Due to their ease of access and potential for disease response, they have become the first line treatment in OFG, being used in 86% of cases at GSTFT.[7, 39] Dietary intervention was developed following early observations that approximately at least 20% of patients with OFG reported sensitivities to certain foods, as shown in Table 1.1.[28, 29] It was observed that eliminating these foods reduced symptom recurrence. Subsequent studies involving patch testing in OFG patients demonstrated specific sensitivity to cinnamaldehyde (the main compound in cinnamon) and benzoate.[150, 151] Cinnamon originates from Sri Lanka, is harvested from cinnamon tree bark and is widely used as a spice in food flavouring. Benzoate is a widely used antimicrobial preserving agent and both substances are widely used as food additives.[39] Thus, the main dietary intervention is an exclusion diet of compounds containing cinnamon and benzoate.

Under the guidance of a dietician, response rates of 54-78% have been reported following an 8 week diet, with up to 23% of patients not requiring adjunctive

therapies.[39] Adherence to the diet can be challenging and relapse can occur once the diet is discontinued. Table 1.4 shows the most common sensitivities identified, predominantly through patch testing.[39, 151] Treatments are often used in combination and concurrently to provide maximal benefit. The goals of therapy are to induce and then maintain remission.

Phenolic acids are one of the main constituents excluded in the cinnamon- and benzoate-free diet and was trialled in small series of 10 patients. 7 of the 10 patients showed a response but the findings need to be studied further in a larger trial in the future.[149] A small series of 12 cases undergoing exclusive enteral nutrition demonstrated some response and could be a promising future intervention but compliance remains a challenge.[152] Chocolate and tomatoes have also been found to be particularly triggering in OFG, although the mechanism by which this occurs remains unclear.

Table 1.4 – Most common sensitivities in OFG[39, 151]

Compound	**Frequency of Sensitivity (%)**
Benzoic acid	36
Food additives	33
Perfumes and flavourings	28
Cinnamaldehyde	27
Cinnamon	17
Benzoates	17
Chocolate	11

1.7.2 – Pharmacological

There are multiple pharmacological treatments which work by reducing inflammation levels. These are conventionally used in a step-up fashion but greater disease burden may require a more top down approach. Disappointingly given the link with allergy, antihistamine therapy has not been shown to be beneficial in treating OFG.[153]

1.7.2.1 – Topical treatments

Application of topical corticosteroids has been efficacious for mild-moderate OFG. Benefit has been seen with betamethasone 500mg tablets dissolved in water and used as a mouthwash four times a day.[18] For more severe but localised lesions, clobetasol 0.05% ointment applied twice a day with orobase has been advantageous.[22] The topical NSAID, benzydamine, in the form of a mouthwash or spray may be a future treatment option and has shown potential benefit in other oral inflammatory conditions such as cancer-related mucositis.[154]

Calcineurin inhibitors including tacrolimus and pimecrolimus have demonstrated benefit, particularly in treating extra-oral OFG lesions.[23, 155] They have been shown to be safe with no significant side effects unlike observed with oral administration.

Topical antimicrobials and antifungals can also be used as adjunctive therapy, usually to treat superadded infections.[143] Angular stomatitis and lip fissures are common sites of infection in OFG. Successful responses have been reported

with azithromycin,[156] metronidazole,[1, 143] tetracyclines[157] and clofazimine.[158] Antifungal treatment may be required for oropharyngeal candidiasis which may occur in conjunction with active OFG or be a result of immunomodulatory therapy.

1.7.2.2 – Intra-lesional treatments

Intra-lesional steroid injections are usually reserved when treating persistent orofacial swelling predominantly affecting the lips.[159, 160] Triamcinolone has been successfully used in this fashion.[143] It may be indicated when there is no obvious wider involvement or when there has been an inadequate response to first line therapies including exclusion diets and topical therapies.[161] Significant results with a reduction in orofacial swelling has been seen within by 4 weeks and treatment can be repeated if needed.[161]

1.7.2.3 – Systemic treatments

Systemic treatment with oral corticosteroids can be effective and is usually reserved for more moderate-severe disease.[162, 163] They have the benefit of offering fast response and induction of remission rates but recurrence can occur upon stopping.[33] Typically, a course of prednisolone 40mg is given, with the dose reducing by 5mg per week over 8 weeks. Repeated or long term steroid use is associated with significant steroid side effects and an inadequate treatment response is an indication for step up immunomodulatory therapy.

There are many immunomodulatory therapies available as for the treatment of CD. These are indicated when first line therapies have been insufficient to control disease or there is more severe or systemic disease. OFG with concurrent CD is also a strong indication to commence systemic treatments.[109] Thiopurine drugs, azathioprine and mercaptopurine, remain a widely used and effective treatment as first line maintenance treatment in this scenario. However, importantly, azathioprine has been shown to be less effective for OFG only as compared with OFG and concurrent CD, further supporting the observation that they may be separate entities.[164] Although there are no published trials of methotrexate used in OFG, it is frequently used in CD and has been successfully used to treat an orofacial lesion related to CD.[165] Mycophenolate mofetil has also been beneficial to treat lip inflammation, both solely[166] and in combination with corticosteroids or tacrolimus.[142] Less commonly, thalidomide has been successfully used to treat OFG facial swelling and oral ulceration[162, 167, 168], however, its use remains limited by teratogenicity and side effects including peripheral neuropathy.

When there is an inadequate response to steroids and/or immunomodulator therapies, other potential differential conditions should be considered such as Behcet's disease, as such conditions may respond to specific correct treatment. If further management for OFG is required, then treatment is usually stepped up with the introduction of biologic drugs. First line treatment is with Anti-TNFα agents which are infliximab and adalimumab.[169-171] In a small series of 14 OFG patients, Infliximab has shown a 71% initial response which drops to 33% at 2 years.[172] Loss of response is not uncommon with biologics, usually due to antibody formation, however, sequential biologic use may be effective as in CD.[109] In the same series, 2 patients failing

infliximab subsequently responded to adalimumab.[172] Although biologics generally have a sound safety profile, any pre-existing sepsis should be treated prior to biologic initiation to prevent further complication such as peri-orbital cellulitis.[24] More recently, there have been successful case reports of ustekinumab being used to treat OFG and CD.[173, 174]

There are a number of other systemic therapies which have been less commonly used to manage OFG. Thalidomide has been successfully used to improve disease activity,[162] although its teratogenic potential considerably restricts its use. Historically, there have been case reports and small series reporting possible benefit with dapsone,[142, 169] mycophenolate mofetil,[166] pentoxifylline,[142] sulfasalazine.[175, 176] and clofazimine.[33, 158]

There is limited evidence that antimicrobials are of direct benefit in treating OFG and therefore they are less commonly used.[143] There are small studies possibly supporting the use of azithromycin,[156, 177] metronidazole[1] and tetracyclines.[157, 178]

1.7.3 – Probiotics

Probiotics have been broadly defined by consensus as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.”[179] Probiotics are substrates which are thought to act in via a variety of ways, including modulating immune function, producing organics acids and antimicrobial compounds, interacting with commensal microbiota, interfacing with the host, improving gut barrier integrity

and through the formation of enzymes.[180] The precise mechanisms remain unknown but it is thought that alteration in the gut flora and restoration of intestinal dysbiosis may be beneficial. The seven core genera of microbial organisms most frequently used in probiotic products are *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus, Escherichia,* and *Bacillus*.[181]

1.7.3.1 – The use of probiotics in inflammatory diseases

To date, there have been no compelling recommendations for probiotic use in adults.[180] Numerous clinical trials studying the potential benefits of probiotics have been carried out, however, there remains great contrast in results, with great heterogeneity in dosing regimens and clinical end-points and outcomes. There remains a great need for better designed, standardized and adequately powered randomised controlled trials (RCTs) with a better understanding of host characteristics and robust evaluation of safety data. Nevertheless, there have been numerous suggestions for the following conditions: necrotizing enterocolitis,[182] antibiotic-associated diarrhoea and *H. pylori* infection,[183-185] defecation frequency,[186, 187] infantile colic,[188] mild to moderate ulcerative colitis,[189] irritable bowel syndrome (IBS),[190] acute diarrhoea,[191] prevention of *Clostridium difficile*-associated diarrhoea[192, 193] and neonatal sepsis.[194]

More specifically, with regards to gut conditions, current British Society of Gastroenterology (BSG) guidance suggests that for IBS, probiotics, as a group, may be an effective treatment for global symptoms and abdominal pain, but it is not possible

to recommend a specific species or strain.[195] Additionally, a systematic review of RCTs of probiotics in CD has not shown any benefit.[196]

Interestingly, the use of oral probiotic supplements as an adjunctive therapy in RA have been evaluated in two separate unrelated double blind RCTs. Both studies found that RA patients who received *Lactobacillus casei* 01 over a two month period had statistically significant improvements in their disease activity as compared with controls.[197-199]

1.7.3.2 - The use of probiotics in oral diseases

Probiotics have been explored with promising results in some major oral diseases including periodontitis and dental caries.[200-207] Interestingly, it has been recently hypothesised that probiotics may be beneficial in treating oral aphthous lesions associated with CD by strengthening epithelial barrier function and antagonising pro-inflammatory cytokines,[208] however, this has not been studied or proven in a clinical setting.

As previously discussed in Chapter 1.4.4.1, the use of probiotics in oral diseases is an attractive therapeutic area as strains such as *Streptococcus salivarius* may be beneficial in treating conditions such as halitosis and periodontitis by inhibiting the growth of Gram-negative species associated with the conditions.[80, 81]

Promisingly, small studies using CD2 probiotic lozenges have been shown to be of benefit in treating numerous oral diseases including oral mucositis,[209, 210] Behcet's

disease[211] and recurrent aphthous stomatitis (RAS).[212] More recently, two RCTs using CD2 have been published showing benefit in treating caries in children.[213, 214] CD2 contains *Lactobacillus Brevis*, a species rich in arginine deaminase. This is thought to lead to a reduction in the inflammatory process by reducing the availability of arginine within the oral cavity, which prevents the growth of arginine–dependent inflammatory micro-organisms. The reduced availability of arginine to arginase leads to a minor production of polyamines. Consequently, there is reduced availability of arginine for NO-synthetase, thus reducing the production of pro-inflammatory NO.[215]

1.7.4 – Surgical

Surgery is solely reserved for medically-refractory disease or particularly in cases of burnt-out disease where the lip becomes firm and fibrosed necessitating debulking or corrective cheiloplasty.[140] It is often a last resort where medical management has inadequately controlled the disease[216] and is similar in concept to surgery for fibrostenotic disease in CD. However, unlike with CD, which carries a lifetime surgical interventional risk of around 75%,[109] surgical rates in OFG appear to remain relatively low.

1.7.5 – Psychological

The psychological impact of a facially disfiguring disease like OFG should not be underestimated. To date, there has been a lack of high quality research investigating the psychosocial needs of OFG patients. A preliminary qualitative assessment of the psychosocial impact in OFG has been carried out but not yet published. The study recruited 15 OFG patients (7 male, mean age 35 years) at GSTFT and data was captured from questionnaires. Patients reported a good quality of life based on the WHOQOL-BREF score, which incorporates physical, psychological, social and environmental domains.[217] Concerns relating to appearance and social anxiety was scored with the Derriford Appearance Scale (DAS)[218] (mean 41.9, range 24-66) and were within normal limits. Anxiety and depression was quantified using the Hospital Anxiety and Depression Scale (HADS)[219] (mean anxiety 7.61, range 3-13, mean depression 4.92, range 1-15) and were also within normal limits. However, maximum scores indicated 23% of patients experienced severe problems as a consequence of their disease, with severity not predicted by physical factors. Further research is needed to investigate the role of psychological support for patients.[162]

1.8 – Crohn's Disease

As there have been multiple links proposed between CD and OFG, it is important to discuss CD as this also informs and underpins aspects of OFG research.

CD is one of the two major forms of IBD, which has a worldwide prevalence of more than 6 million and rising.[220, 221] The highest incidence and prevalence is in Canada

and Northern Europe and it is more common in developed countries and particularly urban areas.[221] As with OFG, CD appears to affect both genders equally, with disease onset usually in the second to fourth decade of life and a smaller peak at 50 to 60 years of age.[221, 222]

1.8.1 – Aetiology and Pathophysiology of Crohn's Disease

The precise cause of CD remains unknown but is thought to be due to a complex interplay between genetic susceptibility and environmental factors resulting in an abnormal mucosal immune response possibly to an antigen present in the intestinal microflora.[222] Approximately 12% of patients have a family history of CD[223] and Ashkenazi Jews have a three-to-four fold higher risk of developing disease.[224] Twin studies have shown more monozygotic twin pairs with CD were concordant compared with dizygotic pairs, thus inferring heritability.[225] GWAS have identified 37 specific alleles for CD which has provided major insights into disease pathogenesis.[226, 227] Only 13.1% of disease heritability is accounted for by genetic variation indicating the significant involvement of other factors such as epigenetic and environmental factors.[228]

A number of environmental factors for the development of CD have been studied. The incidence of CD amongst Eastern countries adopting an increasingly Western lifestyle has increased sharply.[224] Cigarette smoking has been identified with a two-fold increase in developing CD[229] which interestingly, stands in contrast with UC, where smoking has been found to be a protective factor.[230] Childhood antibiotic exposure has also been shown to increase CD risk.[231] Reported dietary factors associated with

an increased CD risk include a reduction in dietary fibre and increased saturated fat intake.[232] Importantly, it has been shown that by modifying lifestyle factors, CD risk can be reduced.[233]

1.8.1.1 – The Microbiota in Crohn's Disease

The role of microbiota in CD has been of great interest. Dysbiosis is seen in CD patients with a decrease in Bacteroides and Firmicutes bacteria and an increase in Gammaproteobacteria and Actinobacteria.[234, 235] Mucosa-associated adherent-invasive *Escheria coli* is found to be in abundance in approximately one-third of CD patients.[236] These strains cross the mucosal barrier and invade epithelial cells. They survive and replicate within macrophages and provoke high concentrations of secretory TNFα.[236] In contrast, it has been found that there are reduced concentrations of the anti-inflammatory commensal bacterium, *Faecalibacterium prausnitzii* in CD.[237-240] Such findings have unfortunately not been successfully translated into clinical practice with strategies manipulating microbiota having failed.[222] Nevertheless, there are a number of clinical observations that have been made in CD which indicate a strong role for a microbial antigen in its pathogenesis. This is supported by the observation that by diverting the faecal stream away from diseased bowel (such as with the formation of a stoma), this has been shown to promote healing within the affected distal bowel.[109, 241] An interesting example of successful microbiota alteration with therapeutic benefit comes from faecal microbiota transplantation (FMT). RCTs have demonstrated that FMT is effective in treating primary and recurrent *Clostridioides difficile* infection above current standard of care using antimicrobial therapy.[242] Unfortunately, initial studies of FMT being used to

treat CD have been inconclusive,[243] however, better designed, larger future studies are required to properly determine if there is benefit. As discussed earlier in Chapter 1.4.3, it has been suggested that *Map* could be involved as an infective organism in CD but its precise role remains unknown and contentious.[50] CD patients have also been shown to harbour an expansion of caudovirales viruses and exhibit fungal dysbiosis.[222]

1.8.1.2 – Immunology in Crohn's Disease

There are thought to be significant alterations in the intestinal immune system in CD due to immune pathway dysregulation. Specifically, there appears to be a defect in the barrier function with disruption of the buffer zone of mucus and antimicrobial factors produced by the intestinal columnar luminal epithelium.[244] This is thought to promote bacterial translocation associated with IBD. Defects in autophagy-related genes[226] and intestinal tight junctions[245] also allow the dissemination of invasive bacterial species.

There are multiple pathway defects in the innate immune system which are also involved in CD. The host defence system to intracellular bacterial fragments is mobilised by NOD-like receptors which initiate immune protein gene transcription, producing protective cytokines.[222] Paneth cells secrete anti-microbial proteins, however, these are reduced due to genetic mutations in NOD2 and other genes including: ATG16L1, IRGM, Wnt, LRRK2, HDACs, Casp8 and XBP1.[246, 247] Innate lymphoid cells (ILCs) isolated from inflamed bowel in CD has shown increased gene expression of inflammatory cytokines including TNFα and interleukins.[222, 248]

Thus ILCs have been strongly implicated in CD pathogenesis due to their involvement in maintaining barrier integrity. These cytokines are also they key therapeutic targets for many biologic drugs used to treat IBD.

The intestinal inflammatory infiltrate in CD has been found to contain CD4-positive T-helper cells, specifically Th1 and Th17. The response of these effector T-cells to bacteria or fungi have been implicated in CD.[249] Treg cell dysfunction has also been reported in CD.[249] B-cells are thought to be involved but the precise nature of their role remains unclear. It has been suggested that they mount an immune response to luminal microbes as there are increased concentrations of antimicrobial antibodies including anti-*Saccaromyces cerevisiae* antibody, anti-I2 antibody, anti-outer membrane porin C antibody, antiflagellin antibody and antiglycan antibodies.[222, 250] Involvement of the B-cell system highlights another possible aetiopathogenic link between CD and OFG, as dendritic B-cells have been seen in OFG, as described in Chapter 1.4.2.[43] However, B-cells in CD are seen to produce IgA and IgG antibodies,[250] whereas those in OFG are class switched to IgE.[43]

1.8.2 - Clinical Presentation of Crohn's Disease

CD is a chronic progressive disease characterised by inflammation of the gastrointestinal tract which behaves in a relapsing and remitting fashion.[222] It can present insidiously and exhibits great heterogeneity. The most common symptoms are abdominal pain, weight loss and diarrhoea. Fatigue and anaemia are also common features and septic sequelae can occur resulting in fever. It can affect any part of the GI tract and most commonly involves the ileocaecal region. Historically, CD was

described as affecting any part of the gastrointestinal tract from mouth to anus. There are three main disease phenotypes: inflammatory, stricturing and penetrating disease. Approximately 30% of patients develop perianal disease which is frequently associated with worse outcomes.[251] Based on a genotype association study, it has been proposed that IBD be explained by three groups: colonic CD, ileal CD and UC.[252] The Montreal classification is used to describe in detail, the phenotype of CD based on age, disease location and behaviour.[253] It is beneficial from a longitudinal disease monitoring and treatment response perspective and provides a foundation for clinical trials. Table 1.5 shows the Montreal classification for CD.[253] The natural history is of cumulative bowel damage with inflammation evolving to fibrosis and carries a lifetime risk for surgery of approximately 50%.[254, 255] Extraintestinal features include skin, joint and eye involvement. Specific diseases associated with CD, but which run an independent course, include primary sclerosing cholangitis, erythema nodosum and axial arthropathies such as ankylosing spondylitis.

Table 1.5 – The Montreal Classification for Crohn's Disease[256]

Variable	
Age at diagnosis (years)	A1, <16
	A2, 17-39
	A3, >40
Location of disease	L1, ileal
	L2, colonic
	L3, ileocolonic
	L4, isolated upper disease*
Behaviour	B1, non-stricturing, non-penetrating
	B2, stricturing
	B3, penetrating
	p, perianal disease modifier†
*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present	
†p is added to B1–B3 when concomitant perianal disease is present	

CD is typically diagnosed based on clinical history and tests including biochemistry, radiology, endoscopy and histology. Initial blood tests may reveal raised inflammatory markers such as CRP and platelet count. Albumin levels, haemoglobin levels and haematinics may also be seen to fall. Stool biomarkers such as faecal calprotectin has been shown to have high sensitivity and specificity for gastrointestinal inflammation. It is widely used to distinguish between patients with active IBD and functional gut disorders where there is an absence of inflammation, potentially avoiding a need for extensive and invasive investigation in the latter group.[109, 257, 258] This is in contrast with the current use of salivary biomarkers and calprotectin as discussed in Chapter 1.6. Amongst known CD patients, elevated faecal calprotectin levels have shown strong correlation with current intestinal disease activity.[258] Moreover, elevated faecal calprotectin levels can act as a prognostic marker, with high baseline calprotectin levels significantly associated with disease progression.[258]

Endoscopic investigation, specifically, ileo-colonoscopy remains the gold standard investigation for diagnosing CD. Typical findings include serpiginous ulcers, discontinuous segmental inflammation (skip lesions) and nodular mucosal appearances (cobblestoning). Histological examination from biopsies show focal, patchy, chronic inflammation with the hallmark finding of CD being transmural lymphoid aggregates and fissuring ulcers.[109] Epithelioid granulomas are found in approximately half of cases and as discussed in Chapter 1.5, are indistinguishable from those found in OFG. On histological grounds, this finding supports another strong link between OFG and CD.

Cross-sectional imaging tests are carried out in CD to primarily investigate for the development of strictures and penetrating features of disease, such as fistulas. CT-enterography and MR-enterography are the most commonly used tests, although, contrast ultrasound has been shown to be an excellent modality for small bowel disease.[259] Akin to the use in OFG for investigating lip swelling, ultrasound carries no radiation and can be performed rapidly including at the bedside with the advantage of directly demonstrating inflammation to the subject being examined. CT and MR are more widely available and have the advantage of image records which aids multidisciplinary review and guides long term disease monitoring and treatment response.

1.8.3 – Current Management and Interventions in Crohn's Disease

There has been an increasing trend with a shift towards less reactive treatment and a more top down approach versus an historical step up approach to treatment in CD. As CD is so heterogeneous in presentation and disease course, a tailored approach with treat to target is most likely needed.[222, 260] Goals of therapy are to prevent cumulative bowel damage and to restore quality of life. Objective and aspirational endpoints include biochemical, radiological, endoscopic and histologic remission.

Initial treatment is usually with corticosteroids such as prednisolone or budesonide. These can achieve rapid remission and improve short-term outcomes but do not modify the disease course and are associated with significant side effects with prolonged exposure; Cushing's syndrome, diabetes, bone thinning and infections.[109] Steroid-sparing therapy is the mainstay of treatment and can achieve long term remission and

improve disease outcomes. Oral immunomodulators, including azathioprine, mercaptopurine, tioguanine and methotrexate are effective options.[261, 262] They take time to reach maximum efficacy, require regular blood monitoring but also have a favourable safety profile. Biologic agents include infliximab, adalimumab, ustekinumab and vedolizumab. These agents have the benefit of a rapid and high response rate with very good safety profiles. Although costs have reduced with the advent of biosimilars, access remains challenging in many healthcare systems. Antibiotic treatments are reserved for patients who develop septic complications are commonly used in perianal and penetrating disease phenotypes. Dietary modulation remains a under studied area in CD management, although exclusive enteral nutrition (EEN) has been shown to be effective in inducing remission, however, motivation and compliance remain challenging.[109] Surgical rates in CD remain high although improvements have likely been made with the introduction of biologic agents and EEN. Surgery is indicated for stricturing, penetrating and perianal disease, usually where response to Medical therapy has been insufficient or ineffective. Unfortunately, postoperative surgical recurrence rates remain high.

1.8.4 – OFG and Crohn's Disease

As discussed in Chapter 1.1, the precise aetiologies and relationship between OFG and CD remains elusive. One of the first descriptions of oral inflammation in CD was of a mucosal tag in the buccal mucosa of a patient with known CD.[263] Subsequent studies have reported prevalence of oral signs in CD from between 0.5 to 60%.[264-266] Typically, oral lesions seen in CD are non-specific and relate to disease activity and

vitamin deficiencies due to malabsorption from bowel inflammation.[109] Such deficiencies include iron, folic acid and vitamin B_{12}.

In a study involving ileo-colonoscopy in OFG patients with no gastrointestinal symptoms, 54% of patients were reported to have gut abnormalities with granulomas seen in biopsies in 68% of cases.[6] This raises the possibility that OFG may represent an alternative form of CD where there is a more severe oral and attenuated intestinal phenotype. As discussed in Chapter 1.5, concurrent intestinal CD has been found in 22% of OFG patients supporting a close link between the two conditions.[7] Additionally, OFG and concurrent CD patients have been found to be more likely to have buccal sulcal ulceration.[7] As mentioned in Chapter 1.3, 42.5% of patients with OFG and concurrent CD were found to develop OFG prior to their CD, with the median onset of OFG two years prior to CD development.[7] From personal observation in outpatient OFG clinics, patients with OFG and CD appear to be more likely to suffer from the presence of upper GI CD involvement and/or perianal disease. The latter has been referred has been referred to as 'top and tail CD.' These observations are further supported by the publication of a recent multicentre case series of OFG and CD patients.[8]

Overall, it appears that OFG and CD share common features, most noticeably granulomatous inflammation, but are distinct disorders on a genetic basis.[11] However, their close clinical presentation and histological similarities suggest they may represent parts of a complex spectrum of the same clinical disorder.[26]

1.9 – Summary

In summary, OFG is a chronic disfiguring condition of the lips and oral cavity characterised by granulomatous inflammation. It has close links with CD and allergy although the precise nature of these relationships remains unknown. The aetiologies of CD and OFG remains poorly understood and there has been a paucity of research into the oral microbiome for these two conditions.

Currently, the diagnosis is largely on clinical grounds and there is an unmet need for an simple objective marker of inflammation for OFG to objectively aid diagnosis, guide disease activity monitoring and gauge response to treatment.

Treatment for OFG has largely been with dietary intervention and immunomodulatory therapy. The potential benefit for a probiotic as treatment in OFG has never been explored.

The work presented in this thesis will contribute to the understanding of the oral microbiome in OFG and its aetiology. This could enable the use of salivary biomarkers and targeted treatments for patients including potential probiotic therapy.

1.10 – Aims and objectives

The aims of this thesis is to:

1. Characterise the oral microbiome in OFG using 16S rRNA gene community profiling in an attempt to illuminate the underlying aetiology of the disease.
    a. Explore if the oral microbiome varies between individuals with OFG, OFG + CD, CD and healthy controls. Potential factors which could affect the microbiome will also be investigated.
2. Evaluate the salivary calprotectin assay for use as an oral diagnostic biomarker in OFG and CD.
    a. Quantify salivary calprotectin levels in patients with OFG only, OFG+CD, CD only and healthy controls. Additionally, to determine whether salivary calprotectin could act as a biomarker for OFG by reflecting disease activity and help to distinguish between patients with OFG only and those with concurrent CD.
3. Evaluate the use of a novel probiotic Lactobacillus brevis (CD2) in the treatment of active OFG.
    a. Evaluate CD2 with regards to its tolerability and efficacy in reducing oral inflammation in patients with active OFG.

Chapter 2 – Methods

This chapter describes procedures which have been specifically optimised for the work presented in this thesis. The standard operating protocols are available in the laboratory.

2.1 – Subject Recruitment and Sample Collection

Whole saliva samples were collected from patients attending specialist outpatient clinics at Guy's & St Thomas' Hospitals (GSTFT), London, over a 2 year period. OFG patients were recruited from a specialist OFG clinic within the department of Oral Medicine at Guy's Hospital. Crohn's patients were recruited from specialist IBD clinics and the infusion suite at Guy's Hospital. Control subjects were predominantly recruited from staff volunteers from the Oral Medicine and Gastroenterology departments at GSTFT.

Eligible patients/subjects included were those aged above 16 years at the time of collection and each subject, including controls, provided informed verbal and written consent. The study was approved by the Local Research Ethics Committee (Approval No. 12/YH/0172; Yorkshire & The Humber REC).

The inclusion criteria for OFG and/or CD patients was a confirmed diagnosis and history of active or inactive disease. The diagnosis of OFG was based on clinical features including lip swelling and characteristically typical oral ulceration. Where available, histology results were also used to support the diagnosis. The diagnosis of CD was based on conventional clinical, biochemical, endoscopic, histological and

radiological criteria. Control subjects were excluded from participating if they reported a history of oral inflammatory disease or IBD. Patients/subjects being treated with antibiotics at the time of sampling were excluded from the study. Both subjects with disease and healthy controls had the same set of data variables recorded. Ethnicity was not recorded and smoking and alcohol use was not quantified but recorded if subjects currently used them or if had used in the past.

Patient/subject data variables were recorded. These were:

- Age at time of sample collection
- Sex
- ODAS[7]
- Any currently active concomitant medical conditions
- Basic Periodontal Examination (BPE) score[267]
- Global description of any dental disease
- Presence of gut CD (based on Montreal classification)[9]
- Any current drugs/medications (including immunomodulators/immunosuppressants) being used
- Use of mouthwash (yes current/ no)
- Smoking status (yes current/ never smoked)
- Use of alcohol (yes current/ never drank)

All patients underwent an oral examination detailing the sites of any involvement. The severity of OFG was recorded as part of a standardised ODAS, as described in Chapter 1.7 and shown in Table 1.3. All patients underwent a clinical assessment of their oral cavity including global physician assessment (GPA) and other oral signs were recorded,

particularly dental disease, active carious disease and other oral mucosal changes. The GPA was a record of the overall assessment of clinical severity of disease and was primarily listed as either mild/moderate/severe disease. Where possible, the examinations and disease score assessments were performed by the same clinician to limit inter-observer variation. For those with CD, details of disease phenotype (Montreal classification)[9], behaviour and surgical history was recorded.

Where possible and consented for, patients underwent a BPE to assess for gingival and periodontal disease. In the BPE, the mouth was divided into sextants and each sextant scored from 0-4 with 0 indicating no pocketing or bleeding in that sextant while 4 indicated advanced periodontitis. The scores for each sextant were summed to give a value between 0 and 24.[267] BPE scores were compared between groups. This variable was transformed by categorical grouping into three classes as shown in Table 2.1[267], and the differences in microbiome composition between BPE classes was assessed.

Table 2.1 – BPE Scoring and Disease Severity

Summed BPE Score	Global Periodontal Disease Severity
<2	Mild/ Low
2-10	Moderate/ Middle
>10	Severe/ High

Whole saliva was collected by asking patients/volunteers to allow their saliva to pool in the floor of the mouth before passively drooling or expectorating into a sterile universal container.[268] This was repeated until a minimum volume of at least 1ml had been obtained. As discussed in Chapter 1.6, to ensure accuracy the saliva samples were collected in a uniform standardised fashion from all individuals.

At least 2ml was collected per subject with 0.5ml required for DNA extraction. Sample collection was avoided if within 60 minutes of eating a major meal or consuming alcohol. Saliva samples were immediately placed on ice (snap freezing) and then transferred within 3 hours to a freezer for storage at -70°C. All samples were anonymised and coded.

2.2 - Characterising the Microbiome in OFG

The project outlined here consisted of 3 main steps:

1. DNA library preparation (extraction and PCR)
2. DNA sequencing
3. Statistical analysis

2.2.1 – DNA Extraction

Whole saliva samples were thawed in batches by removing from the freezer and allowed to warm to room temperature. A 500µL aliquot of each sample was pipetted and used for the DNA extraction. The remainder of each sample were reserved for calprotectin analysis which was performed separately at a later date and is described in Chapter 2.3. DNA was extracted by means of the Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich®, Poole, UK). This protocol was optimised for the extraction of gram positive bacteria (with the additional lysozyme step).

2.2.2.1 – Gram-Positive Bacterial Preparation

Samples were not prepared for gram-negative but only for gram-positive bacteria. Gram positive organisms were studied as these are largely commensal. For each sample, 0.5ml of thawed whole saliva was pipetted and centrifuged for 2 minutes at 16,000 x *g* to produce a pellet. The top liquid layer was removed and discarded leaving a pellet in the tube.

For cell lysis, 20µL of Proteinase K was added to the sample followed by 200µL of Lysis Solution C. The mixture was vortexed for 5 seconds and incubated at 55°C for 10 minutes.

Each pellet was resuspended in 200µL of Lysozyme solution (ThermoFisher Scientific®, Waltham, USA) and incubated for 30 minutes at 37°C. To obtain RNA-free genomic DNA, 20µL of RNase A Solution was added and incubated for 2 minutes at room temperature.

2.2.2.2 – DNA Isolation from Gram-Positive Bacteria

GenElute™ Nucleic Acid Binding Columns in 2mL collection tubes were prepared by washing with 500µL of Column Preparation Solution. This process maximised binding of DNA to the membranes resulting in higher yields. These were centrifuged at 12,000 x *g* for 1 minute and the eluate then discarded.

To prepare for binding, 200µL of 100% ethanol was added to the lysate and vortexed for 5-10 seconds. Using a wide bore pipette tip to reduce DNA shearing, the tube contents were transferred into the binding column. This was then centrifuged at 6,500 x *g* for 1 minute. The collection tube containing eluate was then discarded and the column placed in a new collection tube.

A first wash was performed by adding 500µL of Wash Solution 1 to the column and centrifuging for 1 minute at 6,500 x *g*. Again, the collection tube containing eluate was discarded and the column placed in a new collection tube.

A second wash was performed by adding 500µL of Wash Solution to the column and centrifuging for 3 minutes at 16,000 x *g*. Once the column was completely clear of ethanol, the collection tube containing eluate was discarded and the column placed in a new tube.

To elute the DNA, 200µL of Elution Solution was pipetted directly onto the centre of the column and the mixture incubated for 5 minutes at room temperature. This was then centrifuged for 1 minute at 6,500 x *g*. To improve the yield, a second elution was undertaken by adding an additional 200µL of Elution Solution and centrifuging again. The eluate containing pure genomic DNA was then placed in a freezer at -20°C allowing long term storage.

2.2.3 – 16S rRNA gene fusion PCR

PCR was performed to amplify the extracted DNA.[269, 270] This was performed using composite 16S fusion primers consisting of a broad range 16S template specific sequence along with unique 12-mer Golay 'barcode' sequences (on the forward primer only) and the Roche® GS-FLX-454 Titanium series adaptor sequences for the Lib-L kit emPCR method (for unidirectional sequencing of amplicons).[269] Table 2.2 shows the primer details.

Table 2.2 - Fusion primer details

Primer	Sequence 5'-3'
27FYM-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNAGAGTTTGATYMTGGCTCAG
519R-B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGWATTACCGCGGCKGCTG

The appropriate barcoded primers were used for each PCR reaction. 100 forward primers were used with 1 generic reverse primer. Table 2.3 lists the barcoded primers that were used. Table 2.4 shows one of the PCR reaction mixes that was set up on ice.

Table 2.3 - Fusion Primers

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-1	AACTCGTCGATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACTCGTCGATGAGAGTTTGATYMTGGCTCAG
27FYM-A-2	AACTGTGCGTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACTGTGCGTACAGAGTTTGATYMTGGCTCAG
27FYM-A-3	AAGAGATGTCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGATGTCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-4	AAGCTGCAGTCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGCTGCAGTCGAGAGTTTGATYMTGGCTCAG
27FYM-A-5	AATCAGTCTCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGAATCAGTCTCGTAGAGTTTGATYMTGGCTCAG
27FYM-A-6	AATCGTGACTCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAATCGTGACTCGAGAGTTTGATYMTGGCTCAG
27FYM-A-7	ACACACTATGGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACACTATGGCAGAGTTTGATYMTGGCTCAG
27FYM-A-8	ACACATGTCTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACATGTCTACAGAGTTTGATYMTGGCTCAG
27FYM-A-9	ACACGAGCCACA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGAGCCACAAGAGTTTGATYMTGGCTCAG
27FYM-A-10	ACACGGTGTCTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGGTGTCTAAGAGTTTGATYMTGGCTCAG
27FYM-A-11	ACACTAGATCCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTAGATCCGAGAGTTTGATYMTGGCTCAG
27FYM-A-12	ACACTGTTCATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTGTTCATGAGAGTTTGATYMTGGCTCAG
27FYM-A-13	ACAGACCACTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGACCACTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-14	ACAGAGTCGGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGAGTCGGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-15	ACAGCAGTGGTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGCAGTGGTCAGAGTTTGATYMTGGCTCAG
27FYM-A-16	ACAGCTAGCTTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGCTAGCTTGAGAGTTTGATYMTGGCTCAG
27FYM-A-17	ACAGTGCTTCAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTGCTTCATAGAGTTTGATYMTGGCTCAG
27FYM-A-18	ACAGTTGCGCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTTGCGCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-19	ACATCACTTAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATCACTTAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-20	ACATGATCGTTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATGATCGTTCAGAGTTTGATYMTGGCTCAG
27FYM-A-21	ACATGTCACGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATGTCACGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-22	ACATTCAGCGCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATTCAGCGCAAGAGTTTGATYMTGGCTCAG
27FYM-A-23	ACCACATACATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACCACATACATCAGAGTTTGATYMTGGCTCAG
27FYM-A-24	ACCAGACGATGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACCAGACGATGCAGAGTTTGATYMTGGCTCAG
27FYM-A-25	CAACACGCACGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACACGCACGAAGAGTTTGATYMTGGCTCAG
27FYM-A-26	CAACTATCAGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACTATCAGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-27	CAACTCATCGTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACTCATCGTAAGAGTTTGATYMTGGCTCAG
27FYM-A-28	CAAGATCGACTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAAGATCGACTCAGAGTTTGATYMTGGCTCAG
27FYM-A-29	CACACGTGAGCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACACGTGAGCAAGAGTTTGATYMTGGCTCAG
27FYM-A-30	CACAGCTCGAAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACAGCTCGAATAGAGTTTGATYMTGGCTCAG
27FYM-A-31	CACAGTGGACGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACAGTGGACGTAGAGTTTGATYMTGGCTCAG

27FYM-A-32	CACATCTAACAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACATCTAACACAGAGTTTGATYMTGGCTCAG
27FYM-A-33	CACATTGTGAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACATTGTGAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-34	CACGACAGGCTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGACAGGCTAAGAGTTTGATYMTGGCTCAG
27FYM-A-35	CACGGACTATAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGGACTATACAGAGTTTGATYMTGGCTCAG
27FYM-A-36	CACGTCGATGGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTCGATGGAAGAGTTTGATYMTGGCTCAG
27FYM-A-37	CACGTGACATGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTGACATGTAGAGTTTGATYMTGGCTCAG
27FYM-A-38	CACTACTGTTGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTACTGTTGAAGAGTTTGATYMTGGCTCAG
27FYM-A-39	CACTGGTATATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTGGTATATCAGAGTTTGATYMTGGCTCAG
27FYM-A-40	CACTGTAGGACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTGTAGGACGAGAGTTTGATYMTGGCTCAG
27FYM-A-41	CAGACATTGCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGACATTGCGTAGAGTTTGATYMTGGCTCAG
27FYM-A-42	CAGACTCGCAGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGACTCGCAGAAGAGTTTGATYMTGGCTCAG
27FYM-A-43	CAGAGGAGCTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAGGAGCTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-44	CAGATACACTTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATACACTTCAGAGTTTGATYMTGGCTCAG
27FYM-A-45	CAGATCGGATCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCGGATCGAGAGTTTGATYMTGGCTCAG
27FYM-A-46	CAGCACTAAGCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGCACTAAGCGAGAGTTTGATYMTGGCTCAG
27FYM-A-47	ACTCGATTCGAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGATTCGATAGAGTTTGATYMTGGCTCAG
27FYM-A-48	ACTCGCACAGGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGCACAGGAAGAGTTTGATYMTGGCTCAG
27FYM-A-49	ACTGACAGCCAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGACAGCCATAGAGTTTGATYMTGGCTCAG
27FYM-A-50	ACTGATCCTAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGATCCTAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-51	ACTGTACGCGTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACGCGTAAGAGTTTGATYMTGGCTCAG
27FYM-A-52	ACTGTCGAAGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTCGAAGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-53	ACTGTGACTTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTGACTTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-54	ACTTGTAGCAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTTGTAGCAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-55	AGAACACGTCTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGAACACGTCTCAGAGTTTGATYMTGGCTCAG
27FYM-A-56	CTAACGCAGTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAACGCAGTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-57	CTACACAAGCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACACAAGCACAGAGTTTGATYMTGGCTCAG
27FYM-A-58	CTACATCTAAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACATCTAAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-59	CTACGCGTCTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACGCGTCTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-60	CTACTACAGGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACTACAGGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-61	CTACTGATATCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACTGATATCGAGAGTTTGATYMTGGCTCAG
27FYM-A-62	CTAGAACGCACT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGAACGCACTAGAGTTTGATYMTGGCTCAG
27FYM-A-63	CTAGAGACTCTT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGAGACTCTTAGAGTTTGATYMTGGCTCAG
27FYM-A-64	CTAGCGAACATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGCGAACATCAGAGTTTGATYMTGGCTCAG
27FYM-A-65	CTAGTCAGCTGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGTCAGCTGAAGAGTTTGATYMTGGCTCAG

27FYM-A-66	CTATAGTCGTGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATAGTCGTGTAGAGTTTGATYMTGGCTCAG
27FYM-A-67	CTATCAGTGTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATCAGTGTACAGAGTTTGATYMTGGCTCAG
27FYM-A-68	CTATGCTTGATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATGCTTGATGAGAGTTTGATYMTGGCTCAG
27FYM-A-69	CTCAATGACTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCAATGACTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-70	CTCATGTACAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCATGTACAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-71	CTCCTACTGTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCCTACTGTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-72	CTCGAGAGTACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGAGAGTACGAGAGTTTGATYMTGGCTCAG
27FYM-A-73	CTCGATTAGATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGATTAGATCAGAGTTTGATYMTGGCTCAG
27FYM-A-74	CTCGCACATATA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCACATATAAGAGTTTGATYMTGGCTCAG
27FYM-A-75	CTCTCTACCTGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCTCTACCTGTAGAGTTTGATYMTGGCTCAG
27FYM-A-76	TAACAGTCGCTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAGTCGCTGAGAGTTTGATYMTGGCTCAG
27FYM-A-77	TAACTCTGATGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACTCTGATGCAGAGTTTGATYMTGGCTCAG
27FYM-A-78	TAAGCGCAGCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCGCAGCACAGAGTTTGATYMTGGCTCAG
27FYM-A-79	TACACACATGGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACACATGGCAGAGTTTGATYMTGGCTCAG
27FYM-A-80	TACACGATCTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACGATCTACAGAGTTTGATYMTGGCTCAG
27FYM-A-81	TACAGATGGCTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATGGCTCAGAGTTTGATYMTGGCTCAG
27FYM-A-82	TACAGTCTCATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGTCTCATGAGAGTTTGATYMTGGCTCAG
27FYM-A-83	TACATCACCACA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACATCACCACAAGAGTTTGATYMTGGCTCAG
27FYM-A-84	TACGATGACCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGATGACCACAGAGTTTGATYMTGGCTCAG
27FYM-A-85	TACGGTATGTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGGTATGTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-86	TACGTGTACGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGTGTACGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-87	TACTAATCTGCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTAATCTGCGAGAGTTTGATYMTGGCTCAG
27FYM-A-88	TACTACATGGTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTACATGGTCAGAGTTTGATYMTGGCTCAG
27FYM-A-89	TACTGCGACAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGCGACAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-90	TACTGGACGCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGGACGCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-91	TACTTCGCTCGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTTCGCTCGCAGAGTTTGATYMTGGCTCAG
27FYM-A-92	TAGACTGTACTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGACTGTACTCAGAGTTTGATYMTGGCTCAG
27FYM-A-93	TAGAGAGAGTGG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGAGAGTGGAGAGTTTGATYMTGGCTCAG
27FYM-A-94	TAGATCCTCGAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGATCCTCGATAGAGTTTGATYMTGGCTCAG
27FYM-A-95	TAGCACACCTAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCACACCTATAGAGTTTGATYMTGGCTCAG
27FYM-A-96	TAGCATCGTGGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCATCGTGGTAGAGTTTGATYMTGGCTCAG
27FYM-A-97	TCCTGAGATACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTGAGATACGAGAGTTTGATYMTGGCTCAG
27FYM-A-98	TCGAATCACAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGAATCACAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-99	TCGACTCCTCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACTCCTCGTAGAGTTTGATYMTGGCTCAG

27FYM-A-100	TCGAGACGCTTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGAGACGCTTAAGAGTTTGATYMTGGCTCAG
519-R-B	None	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGWATTACCGCGGCKGCTG

Table 2.4: PCR reaction mix

Component	**Volume; 1 reaction (µl)**	**Volume; 2 reactions (µl)**
Extensor Hi-Fidelity PCR mastermix	12.5	25
27-FYM (10 pmol/µl)	0.5	1
519-R (10 pmol/µl)	0.5	1
Water (PCR grade)	9.5	19
Total volume	23	46

The mixtures were vortexed and briefly spun down. Following this, a 23µl aliquot was placed into each 0.2ml PCR tube and 2µl of template added. The samples were placed on a thermal cycler and the following PCR programme '454 PCR` started: 5 mins at 95°C followed by 25 cycles of 95°C for 45s, 53°C for 45s, 72°C for 1min 30s and a final extension of 72°C for 15mins.

2.2.3.1 – PCR Efficiency and Purification

To confirm PCR efficiency and assess size and quality of the PCR amplicons, agarose gel electrophoresis was used. 1µl of PCR product was run with 5µl of loading dye at 1000 volts for 30 minutes. A 100 bp DNA ladder was also run along with the samples. PCR amplicons appeared as a single strong band (~640 bp) under UV light. It was ensured that the negative controls were free of any contamination.

PCR amplicons were purified using the QIAquick™ PCR purification kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions to remove unused primers and nucleotides (fragments smaller than 100 nucleotides in length). The solution was then eluted into 30µl of 0.1 X Tris-EDTA (TE) buffer resulting in 30µl of amplified and purified DNA.

The size and purity of the purified amplicons was checked using the Agilent® 2100 Bioanalyzer (Agilent®, Santa Clara, USA) along with the Agilent® DNA 1000 kit. A 1µl aliquot of the purified PCR product was run on a lane in the Agilent® chip following the manufacturers instructions for loading and running chips. The electropherograms were inspected to ensure the products were the correct size and free of any primer

dimers. None of the samples had any primer dimers and therefore repeat QIAquick™ PCR purification was not required.

2.2.3.2 – PCR Quantitation

The DNA standard provided with the PicoGreen® reagent (100 ng/µl) was thawed. Eight 1.5ml microcentrifuge tubes were labelled 1-8 with 594µl and 300µl of TE buffer solution added into tubes 1 and 2-8 respectively. 6µl of DNA standard was transferred to tube 1 and vortexed for 10 seconds. 300µl of DNA standard was then transferred to tubes 2 and vortexed for 10 seconds. A dilution series was then performed by transferring 300µl of DNA standard from one tube into the next for the subsequent tubes 2-8. Each tube was vortexed for 10 seconds prior to transfer. Tube 8 constituted the "no DNA control". 100 µl of each DNA standard dilution was transferred to the wells of column 12 of two 96- well black fluorometer plates (for duplicate measurements). The amounts of DNA per standard well are listed in Table 2.5.

Table 2.5 - DNA concentration of the 8-point standard curve for the fluorometric assay of amplicon libraries

Tube No	Well	DNA Concentration (ng/well)
1	A12	100.00
2	B12	50.00
3	C12	25.00
4	D12	12.50
5	E12	6.25
6	F12	3.13
7	G12	1.56
8	H12	0.00

99µl of 1x TE Buffer was transferred to the remaining 88 wells of each of the 96-well black fluorometer plates. Using a new tip for each sample, 1µl of each purified amplicon DNA sample was transferred to the appropriate wells of the fluorometer plates. The samples were then mixed by pipetting up and down using a new tip for each sample. The assay was carried out as described by the manufacturer of the Quant-iT™ PicoGreen® dsDNA Assay Kit, adding 100µl of a 1:200 dilution of PicoGreen® reagent to each well. Mixing was performed by pipetting using a new tip for each well. The R^2 value of the standard curve was then verified to be at least 0.98. The sample readings were verified to ensure they fell within the range of the standard curve.

Quantitation of the amplicons was performed by fluorometry using the Quant-iT™ dsDNA PicoGreen® assay kit (Invitrogen™, Carlsbad, USA) to determine ng/µl of each amplicon. This was then converted to molecules per µl using the following formula:

$$\text{Molecules/ µl} = \frac{\text{Sample conc.; ng/µl} \times (6.022\times10^{23})}{(656.6\times10^{9})\times (\text{amplicon length; bp})}$$

6.022×10^{23} = Avogadro's number (molecules/mole).

656.6 = the average molecular weight of nucleotide pairs in g/mole.

The PicoGreen® quantitation was performed twice on 2 separate occasions with the mean of the 2 values taken.

The amplicons were pooled for the final library in equimolar concentrations at a concentration of 1 x 10^9 molecules/µl diluting with 0.1 x TE buffer where necessary.

Finally, the library was stored at -20°C until proceeding to emPCR and unidirectional sequencing using the Lib-L kit emPCR kit.

2.2.4 – Sequencing data analysis

The mothur analysis suite version1.36.1[271] based on the Schloss standard operating protocol (SOP) (January 2016), was used for the pre-processing and analysis of sequences. The mothur-implemented AmpliconNoise algorithm was used to perform initial de-noising to reduce sequence errors in homo-polymer regions. Following de-noising, sequences with the following characteristics were removed from the dataset:

- Sequences with a length of less than 440 bases
- >2 mismatches in the primer
- >1 mismatch in barcode regions
- homopolymers of >8 bases

The remaining sequences were trimmed to remove the primers and barcodes and aligned to the SILVA 16S rRNA gene reference alignment.[272] An additional pre-cluster step was performed in mothur to merge sequences with 4 or fewer bases difference with that of a more abundant sequence. Sequence chimeras were identified and removed from the analysis using the UChime algorithm[273] as implemented by

mothur. Sequences were clustered into Operational Taxonomic Units (OTUs) at a sequence dissimilarity distance of 0.015 using an average neighbour algorithm. A distance of 0.015 was chosen rather than the more commonly used distance of 0.03 as many named oral bacterial species have a high sequence identity in their 16sRNA genes.[274] The sequences were then also classified using a mothur-implemented Naïve Bayesian classifier with the Human Oral Microbiome Database reference dataset v13.2[275] used as a taxonomic reference. Based on OTUs, the α-diversity of bacterial communities was analysed using numerous mothur-implemented approaches. Community diversity was calculated with Simpson's inverse diversity index and richness of the communities was assessed by the number of observed OTUs and the Chao1 richness index. Diversity estimates and richness were compared between groups using the Kruskal-Wallis test.

To compare the β-diversity of samples based on OTUs, the thetaYC metric (compares community structure by accounting for the relative abundance of taxa)[276] was used to generate distance matrices in mothur. Mothur-implemented Analysis of Molecular Variance (AMOVA)[277] was then performed to determine if any differences between the microbiomes of the experimental groups were statistically supported by differences in the distance matrix. LEfSe[278] which implements the Kruskal-Wallis rank-sum test, Wilcoxon rank-sum test and LDA (Linear Discriminant Analysis) was used to identify differentially abundant OTUs between the different subject groups.

2.2.5 – Minimum Entropy Decomposition (Oligotyping)

Minimum Entropy Decomposition (MED) was performed on the same samples used for the α- and β- diversity analyses, excluding the 19 samples falling below 3076 sequences. As described in Chapter 2.2.4, the mothur analysis suite was used to perform denoising, alignment, chimera removal and taxonomy assignation. Sequences identified as belonging to the genus *Streptococcus* were then extracted and formatted using the "mothur2oligo" tool (available from https://github.com/michberr/MicrobeMiseq/tree/master/mothur2oligo).

MED analysis[279] was performed using MED pipeline version 2.1 (available from https://meren.github.io/projects/med/). The MED algorithm is similar to the previously described oligotyping algorithm[280] and differentiates taxa on the basis of single-nucleotide differences in the positions of highest entropy. The parameters used were minimum substantive abundance of a MED node (-M)=7 and maximum variation allowed in each node (-V)=4 nt. The total number of *Streptococcus* sequences analysed was 564,342. Of these, 62,706 were removed as outliers due to the minimum substantive abundance parameter (-M, set to 60) and 8,262 were removed as outliers due to the maximum variation at each node parameter (-V, set to 5). Thus, after the refinement, 493,374 were analysed and classified into 370 MED nodes (oligotypes). Sequences representative of each oligotype were identified at species level by comparison to the Human Oral Microbiome database (HOMD) through the BLAST web tool accessible at http://www.homd.org.

2.3 - Investigating the use of Salivary Calprotectin in OFG and Crohn's Disease

Whole saliva samples were collected as outlined in Chapter 2.1. For the calprotectin project, it was deemed that additional ethical approval was not required as the biochemical test existed, was routinely available and was non-invasive. The samples were defrosted at room temperature and an 0.5ml aliquot initially used for microbiome analysis as outlined in Chapter 2.2.1. The remainder of the samples were left in the sample containers and stored at -20°C. These were then transferred in a dry ice container to the ViaPath laboratory at King's College Hospital, London.

2.3.1 – Salivary Calprotectin Quantification

The saliva samples were completely thawed to room temperature and then vortexed and centrifuged at 3000 rpm for 15 minutes.

Calprotectin quantification of the subject samples was performed using a specific enzyme-linked immunosorbent assay (ELISA) kit, CALPROLAB™ Calprotectin (CALPRO Labs®, Norway). Samples and standards were incubated in separate microtiter wells coated with calprotectin-binding monoclonal antibodies. After incubation and washing of the wells, bound calprotectin was allowed to react with enzyme-labelled, immunoaffinity-purified calprotectin-specific antibodies. Following this reaction, the amount of enzyme bound in the microtiter wells was proportional to the amount of calprotectin in the sample or standard. This was determined by incubation with a substrate for the enzyme giving a coloured product. The colour intensity was determined by absorbance using an ELISA plate reader set at 405nm, and was

proportional with the concentration of calprotectin in the standards and samples. The assay was calibrated using calprotectin purified from leukocyte extract.

2.3.2 - Statistical Analysis

Statistical analysis was performed using Minitab® version 18 (Minitab Inc., USA). The Ryan-Joiner test was used to assess consistency with a normal distribution. In view of the data distributions, non-parametric tests were used including Mann-Whitney test and Spearman's rank correlation with a p-value level of <0.05 being considered significant. For multiple comparisons, the Benjamini-Hochberg procedure was applied with a false discovery rate value of 0.1.[281]

2.4 - An Evaluation Study of *Lactobacillus Brevis* CD2 in OFG

This was a single centre prospective open label observational evaluation study of lactobacillus brevis CD2 lozenges in OFG.

2.4.1 – Subject Recruitment

Patients with active OFG were recruited from a specialist OFG clinic within the department of Oral Medicine at Guy's Hospital, London, over a 6 month period. Those with inactive or quiescent disease were not recruited and excluded from the study. Eligible patients recruited were those aged above 16 years and each patient provided informed verbal and written consent. As this was a clinical evaluation of an approved food supplement product and routine care of patients was not altered; it was deemed that additional ethical review was not required. It was envisaged that 20 patients would provide sufficient power to detect a difference in ODAS before and after treatment with CD2.

As described in Chapter 2.1, at baseline assessment and post-treatment, the following data was collected from patients:

- ODAS
- Severity of oral soreness

The ODAS was recorded as described in Chapter 2.1. The severity of oral soreness was recorded using a visual analogue scale (VAS). Figure 2.1 shows the specific VAS used in the study.

Figure 2.1 - CD2 Probiotic Evaluation Study: Oral Soreness Visual Analogue Scale

How sore has your mouth been in the last week?

Pre-CD2:

Not at all ---------- Worst soreness imaginable

Post-CD2:

Not at all ---------- Worst soreness imaginable

Do you think CD2 has improved your symptoms?

Not at all ---------- Yes, massive improvement

The presence of periodontal and dental carious disease were also recorded. Patients underwent a BPE with a standardised probe as described in Chapter 2.1. Additional information was recorded including concomitant medical conditions, previous drug treatment and the presence of concomitant intestinal CD. The proforma for data gathering can be seen in Appendix 1.

Patients with active OFG were given an 8 week course of standard dose of one CD2 lozenge four times a days. Instructions were provided to keep the lozenges refrigerated throughout the study.

Patients were asked to not start any new medications and no therapeutic interventions were allowed during the study period. Patients were followed up after 8 weeks of treatment for repeat assessment of response and as per routine clinical care. At each visit, the sites of involvement and severity of OFG were recorded using a GPA and the ODAS. To eliminate intra-observer bias, these scores were recorded by one senior clinician.

2.4.2 – Statistical Analysis

At the end of the study, the ODAS and GPA in OFG subjects were compared at 0 and 8 weeks of treatment. R© package version 3.1 (The R Foundation, USA) was used for the statistical analysis.

Chapter 3 – Characterising the oral microbiome in OFG and Crohn's Disease

3.1 – Introduction

As set out in Chapter 1.1, OFG is a rare disease characterised by chronic, granulomatous inflammation primarily affecting the oral cavity. It is characterised by lip swelling and oral ulceration. Histologically, it is similar to CD and clinically it has been found to be closely related to CD, with a proportion of patients having OFG+CD. The precise aetiology of OFG remains unknown and is likely to be multifactorial, but microbial interactions have been proposed to be involved. It is possible that an inappropriate immune response to a member of the oral microbiota could lead to inflammation in OFG.

The oral microbiome can be defined as the collection of all microbial genomes within the ecosystem of the oral cavity. As discussed in Chapter 1.4.4.2, a number of oral microbiome analysis techniques exist although 16S rRNA sequencing has been the mainstay of microbiome analysis. To date, there have been no studies looking specifically at the oral microbiome in OFG.

The aim of this study was to use 16S rRNA gene community profiling to determine and compare the salivary microbial composition of subjects with OFG and/or CD and healthy controls.

3.2 – Methods

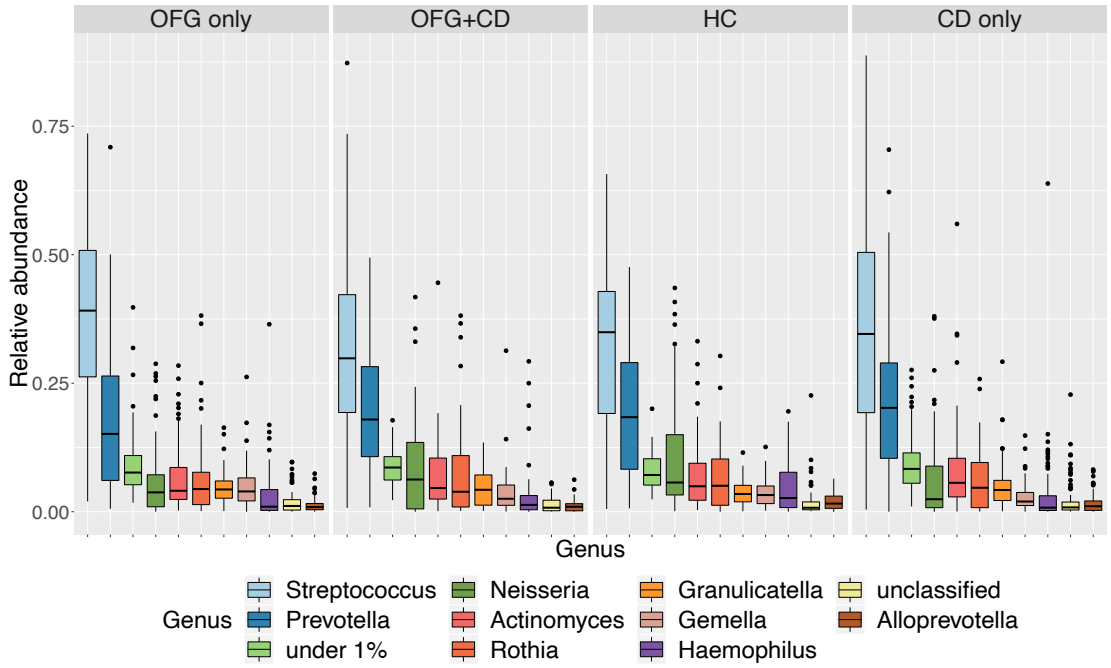
As described in Chapter 2.1, in total 261 subjects were recruited over a 2 year period. Unstimulated saliva samples and clinical information was collected from the subjects from a specialist outpatient clinic at GSTFT. Of these 261 subjects, 118 had OFG, 97 (62 female) had CD only and 46 (33 female) were healthy controls. Of the OFG patients, 78 (43 female) had OFG only and 40 (18 female) had both OFG+CD.

DNA was extracted from the samples and a DNA library prepared using PCR amplification. As described in Chapter 2.2.4, bacterial community profiles were obtained by sequencing the V1-V3 region of the 16S rRNA gene. The mothur analysis suite was used to data analyse the sequences. Oligotyping was performed by MED using the tools outlines in Chapter 2.2.5. Oligotypes were then compared to the HOMD.

3.3 – Results

In total, 1,630,578 sequences were obtained after denoising and quality filtering. Figure 3.1 is a box plot showing the relative abundance of the predominant bacterial genera found in the samples by subject group.[122] For most subjects, the genera, *Streptococcus* and *Prevotella*, dominated the communities. However, it was found that some subjects had very few or no streptococci.

Figure 3.1 - Box Plot of Bacterial Genera Abundance by Subject Group[122]



The predominant bacterial genera found are shown by subject group and distinguished by various colours. The group “under 1%” has combined all present genera at less than 1% relative abundance.

By sub-sampling to a level of 3076 sequences per sample, the data were normalised, which excluded 19 samples from the subsequent analyses. For the subsequent analyses, the subject group sizes were: OFG only (74), OFG+CD (38), CD only (85) and HC (45). There were no significant differences between the groups for the Chao1 or inverse Simpson's indices, nor for the average number of observed OTUs. Utilising the Kruskal-Wallis test, Table 3.1 shows salivary microbiota richness and diversity in subject groups.[122]

Table 3.1 – Richness and Diversity of the Salivary Microbiota in Subject Groups[122]

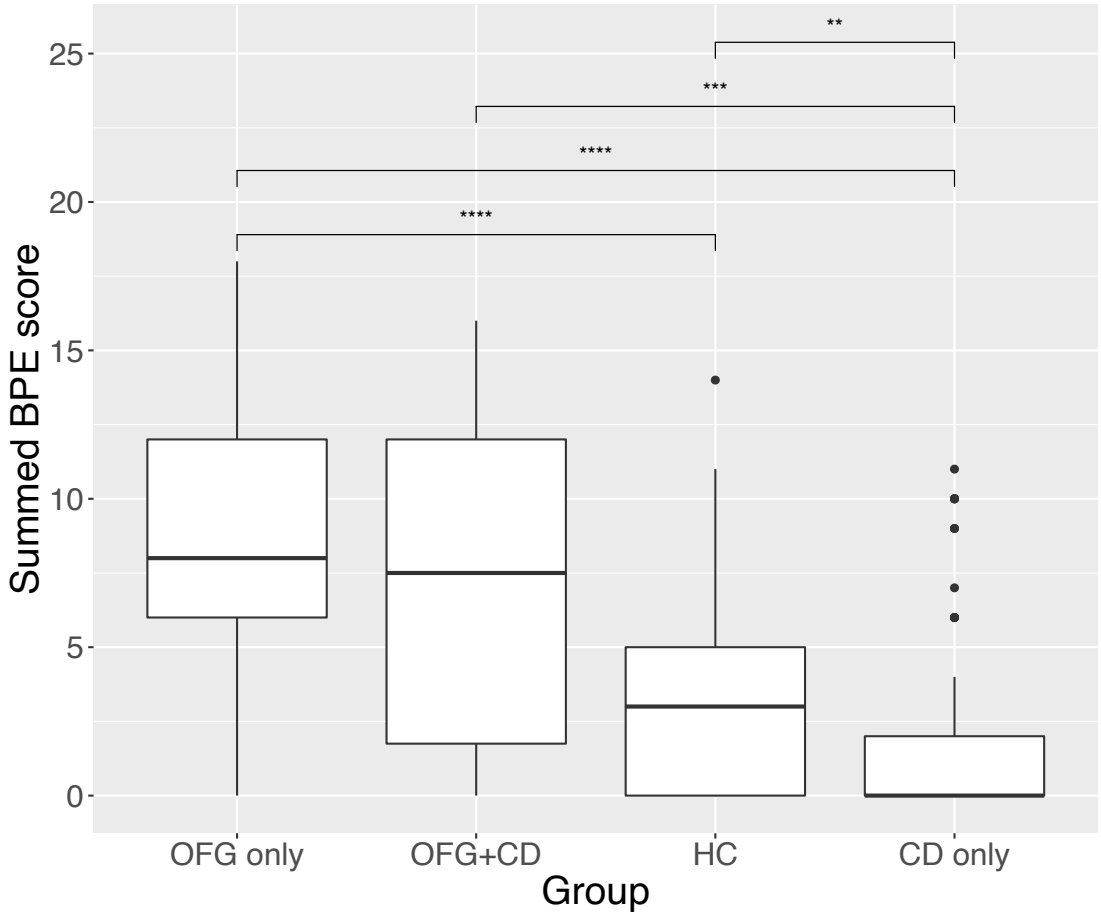
Subject group	n	observed OTUs (sd)	Chao1 (sd)	Inverse Simpson (sd)
OFG only	74	198.6	505.1	11.2
		54.2	152.5	6.2
OFG+CD	38	183.4	449.5	9.7
		57.2	125.6	4.8
HC	45	196.1	496.3	11.0
		53.5	161.1	4.6
CD only	85	203.5	493.4	9.2
		57.5	133.6	5.1

The composition of the microbial communities between the four subject groups was found to be significantly different (AMOVA, $p<0.001$). Pairwise comparisons of the individual subject groups were performed and CD appeared to be the primary driver of inter-group differences. For example, the CD only group was significantly different to both the HC group ($p<0.001$; significance threshold using Bonferroni correction: 0.008) and the OFG only group ($p<0.001$). The OFG+CD group was significantly different to HC ($p=0.006$) but the OFG only group was not significantly different to the HC group.

The mean age and gender distribution were not found to be significantly different between the four groups (age: Kruskal-Wallis test, gender distribution: Chi-Squared test). Immunomodulatory and anti-TNF therapy did not have a significant effect on the microbiome composition in subjects with OFG (OFG only and OFG+CD groups combined) (AMOVA).

207 of the 261 subjects had BPE scores recorded and were significantly different between phenotype groups ($p<0.001$, Kruskal Wallis). Figure 3.2 is a box plot of summed BPE scores as a proportion of the total microbiota.[122] Overall, there was a clear trend for OFG only subjects to have higher BPE scores than HC whereas CD only patients had lower BPE scores. Therefore, the effect of BPE score on microbiome composition was investigated. As described in Chapter 2.1 and shown in Table 2.1, BPE scores were assigned to three class variables: low, <= 2; middle, 2-10; high, >= 10. There was no significant difference in microbial composition between BPE class groups by AMOVA.

Figure 3.2 - Box Plot of Summed BPE Scores as a Proportion of Total Microbiota[122]



The summed BPE scores are shown as a box plot and as a proportion of the total microbiota. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual dots are outliers (**p<0.01; ***p<0.001; ****p<0.0001, Kruskal-Wallis test)

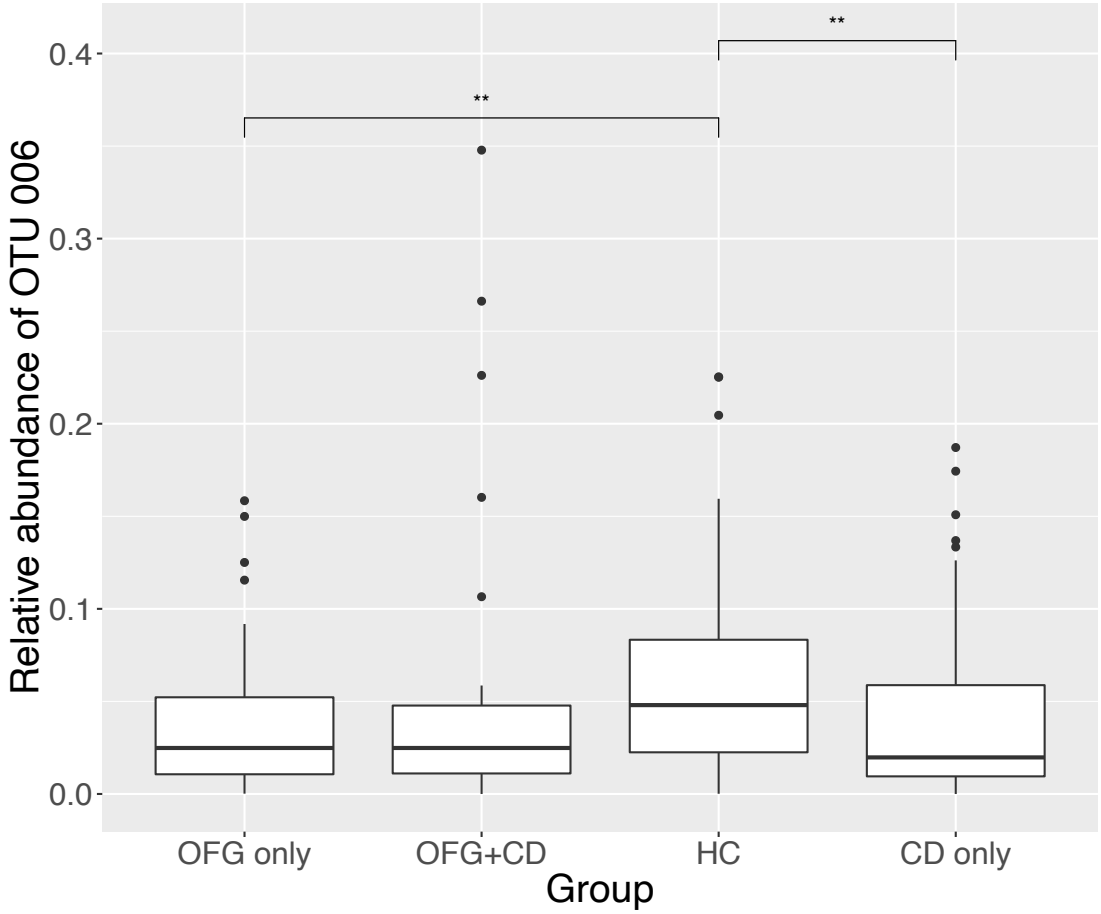
Table 3.2 shows the OTUs responsible for the differences in microbial composition seen between groups.[122] The default threshold of 2 was used for the logarithmic LDA score for discriminative features. 11 OTUs were found to be differentially represented between groups. 7 of these overrepresented OTUs were over-represented in the HC group, suggesting that the disease phenotypes were associated with loss of normal microbiota components. Most of the differentially represented OTUs were of relatively low abundance with only 3 of them (OTU 1, 2 and 6) present in the dataset at a relative abundance of greater than 0.01. OTU 6 was identified as *Haemophilus parainfluenzae* and its relative abundance was significantly reduced in OFG only and CD only compared to HC. Figure 3.3 shows a box plot of OTU 6 as a proportion of the total microbiota.[122] OTUs 1 and 2 were identified as unclassified members of the genus *Streptococcus* and were the most frequently detected OTUs in the study, making up 17.3 and 7.8 % respectively of the oral bacterial community across all subjects. Because *Streptococcus* species vary widely in the roles that they play in oral ecology and disease, Minimum Entropy Decomposition (MED) was used to lend more precision to identification at species-level.

Table 3.2 - OTUs overrepresented in subject groups (LeFSE)[122]

Group	OTU	Species overrepresented	Mean relative abundance*	log LDA
OFG only	152	*Catonella morbi*	0.0003	2.6
OFG+CD	065	*Lachnoanaerobaculum* unclassified	0.001	2.9
HC	002	*Streptococcus* unclassified (*mitis*-group)	0.78	4.5
	006	*Haemophilus parainfluenzae*	0.044	4.1
	032	*Prevotella sp.* HOT-299	0.003	3.3
	037	*Alloprevotella tannerae*	0.002	3.1
	067	*Bergeyella sp.* HOT-322	0.0009	2.8
	141	*Neisseria elongata*	0.0004	2.6
	162	*Prevotella sp.* HOT-305	0.003	2.7
CD only	001	*Streptococcus* unclassified (salivarius-group)	0.173	4.9
	047	*Fusobacterium nucleatum* subsp. *vincentii*	0.001	2.9

* - all samples

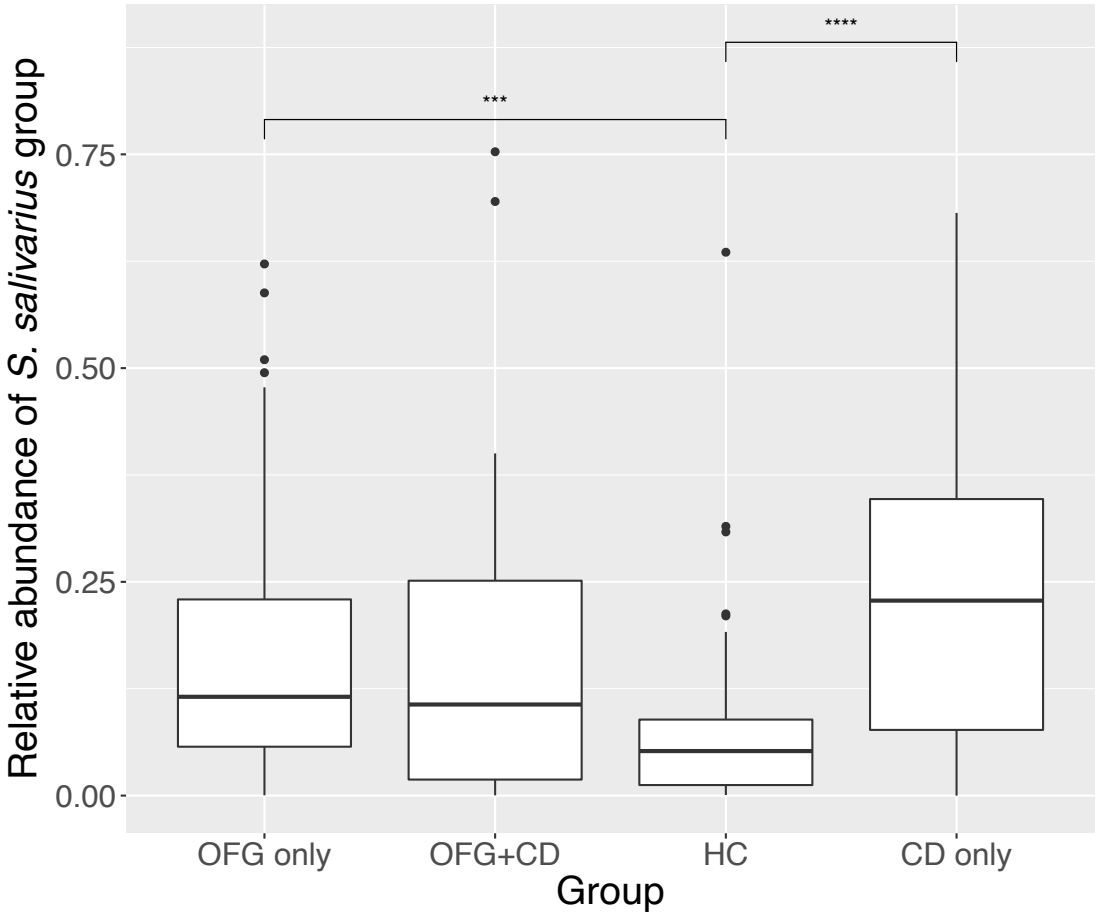
Figure 3.3 - Box Plot of OTU 6 as a Proportion of the Total Microbiota[122]



OTU 6 is shown as a box plot as a proportion of the total microbiota. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual dots are outliers (**p<0.01, Wilcoxon test)

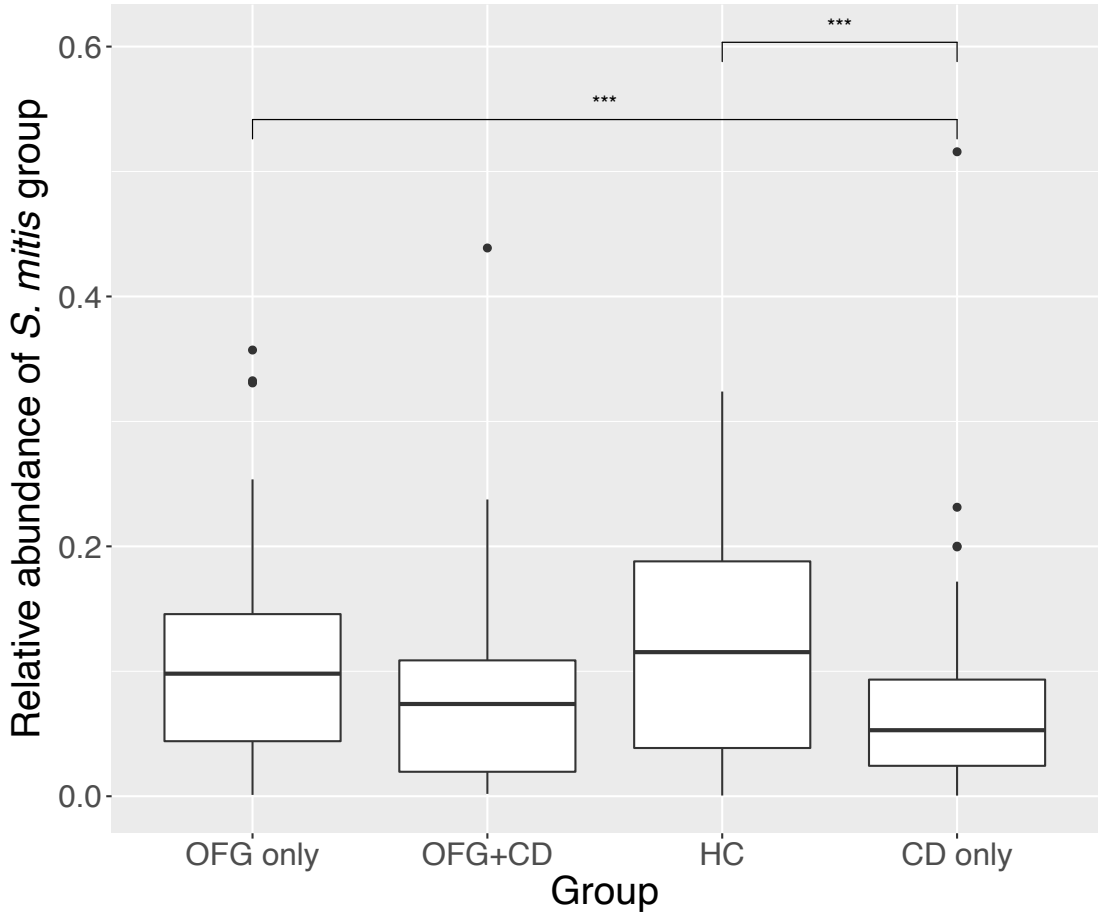
The *Streptococcus* sequences were binned into 370 oligotypes that were subsequently grouped by species after BLAST interrogation of the HOMD database. Where multiple species-level BLAST identifications were above 98.5% sequence identity, the identification was made to a group of species. The mean abundances of species and species groups making up more than 1% of the total microbiota were compared between groups, using Wilcoxon test with Bonferroni correction for multiple testing. Within the OFG only and CD only groups, there were significantly higher proportions of *S. salivarius* groups compared to HC. Figure 3.4 shows a box plot of the summed *S. salivarius* group oligotypes as a proportion of the total microbiota.[122] Significant differences among groups were also seen in the *S. mitis* group with HC and OFG only having significantly higher relative abundances than CD only. Additionally, the relative abundances of individual oligotypes whose mean relative abundance was over 0.5% were compared across the groups. Figure 3.5 shows a box plot of the summed *S. mitis* group oligotypes as a proportion of the total microbiota.[122] Significant differences between groups were also seen in 3 of the individual *S. salivarius* oligotypes. Figure 3.6 shows the proportions of individual *S. salivarius* oligotypes in subject groups.[122] Oligotype 2869 showed the largest differences with the OFG only, CD only and OFG+CD groups all having significantly higher relative abundance than the HC group.

Figure 3.4 - Box plot of the Summed *S. salivarius* Group Oligotypes as a Proportion of the Total Microbiota[122]



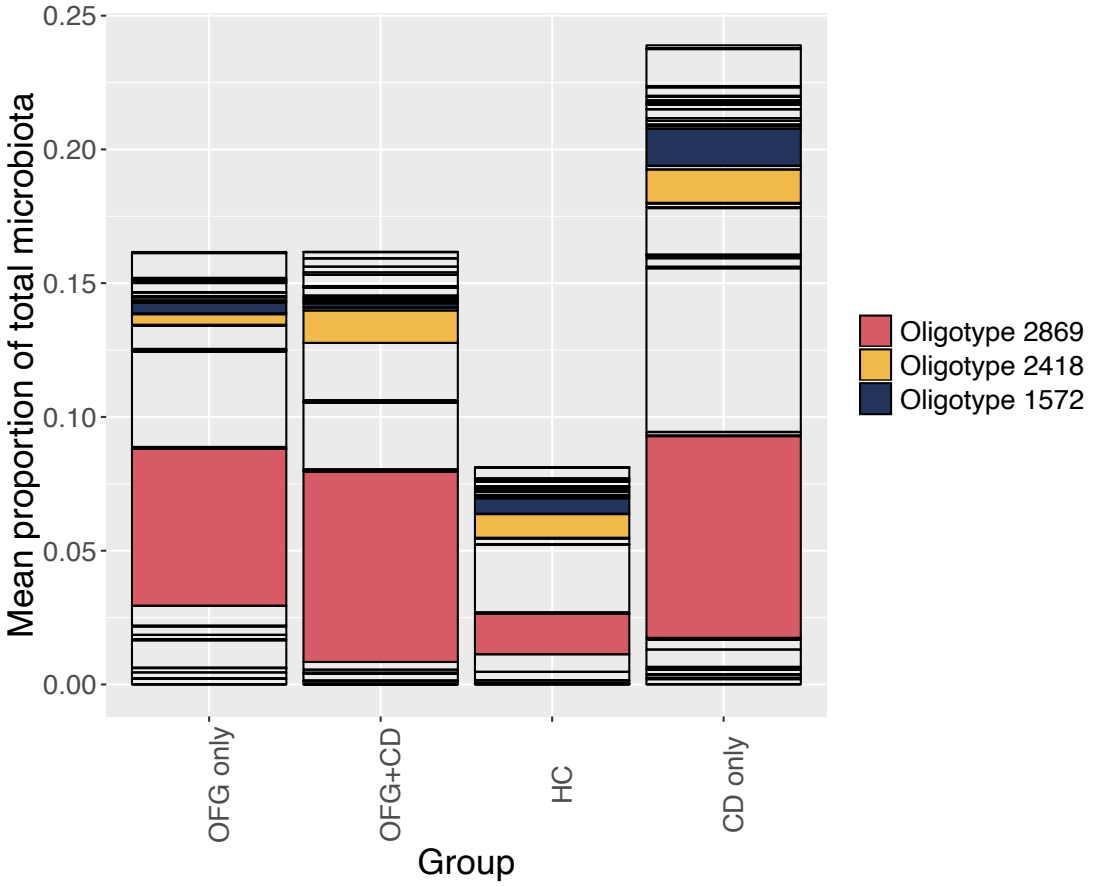
The group oligotypes of summed *S. salivarius* are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual dots are outliers (***$p<0.001$; ****$p<0.0001$, Wilcoxon test)

Figure 3.5 - Box plot of the Summed *S. mitis* Group Oligotypes as a Proportion of the Total Microbiota[122]



The group oligotypes of summed *S. mitis* are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual dots are outliers (***p<0.001, Wilcoxon test)

Figure 3.6 - Chart of the Proportions of individual *S. salivarius* oligotypes in Subject Groups[122]



Bar chart showing the mean proportions of the individual *S. salivarius* oligotypes in subject groups. Oligotypes highlighted in colour showed significant differences between groups (Kruskal-Wallis test)

3.4 – Discussion

The results of this study indicate that the oral microbiome in subjects with OFG is not markedly different to that of HC or subjects with CD, due to the findings that there were no differences in richness or diversity between the groups. Indeed, all subjects were found to have a typical oral microbiome compositional profile. This finding is in contrast to the results of numerous studies looking at the effect of CD on the composition of the intestinal microbiome, [282] where the faecal and mucosal microbiome is substantially altered. Richness is reduced [234, 283] and the phylum *Firmicutes* is relatively depleted, particularly anaerobes from the order *Clostridiales*, but raised proportions of *Proteobacteria*, mainly *Enterobacteriaceae*. This shift towards a less anaerobic bacterial community is thought to be the result of increased levels of reactive oxygen species produced as a part of the inflammatory response. [234] Specific genus-level microbial signatures of CD have been reported to be reduced levels of *Faecalibacterium*, an unknown *Peptostreptococcaceae*, *Anaerostipes*, *Methanobrevibacter*, an unknown *Christensenellaceae* and *Collinsella* and increased proportions of *Fusobacterium* and *Escherichia*.[284]

Patients with OFG were found to have higher BPE scores than HC although there was no corresponding difference in microbiome composition. The levels of BPE scores seen were quantitiaive and indicative of some degree of gingival inflammation. This may have been due to poorer oral hygiene in the patients due to the discomfort caused by the OFG lesions and regular cleaning. Importantly, BPE assessment was used as a marker to ensure any changes in microbiota were not related to periodontal disease.

However, it was therefore surprising to find that there was no difference in microbiome composition relating to BPE scores.

The lack of substantial alteration of the oral microbiome in OFG with or without gut CD most likely reflects the fact that the oral microbiome is extremely stable and not greatly affected by diet, [285] antibiotic administration [286] or inflammation. However, in the LEfSe analysis, there were some OTUs which showed differences in relative abundance between groups. There are a high number of comparisons performed in microbiome studies when OTU relative abundances are compared between patient groups which can lead to spurious associations being revealed by chance, even when significance thresholds, as in this study, are corrected for multiple comparisons. Therefore, such associations should be interpreted with caution. Of the 11 OTUs over-represented in particular subject groups, 7 were in the control group. This suggests that the major shift in OFG and CD was the relative loss of normal microbiota taxa. As discusssed earlier in Chapter 1.8.1, this pattern is also seen in IBD where the microbiome is found to have reduced concentrations of *F. Prausnitzii*,[237, 239] and particularly more so in CD subjects with ileal involvement.[238] Another important consideration in interpreting OTU association analyses is whether the size of the effect is biologically significant. Many of the OTUs found to be differentially abundant were present at extremely low levels and therefore only those present at a relative abundance of greater than 1% were considered further.

Levels of *H. parainfluenzae* were reduced in all patient groups compared to HC. *H. parainfluenzae* is a commonly occurring member of the normal microbiota and the

significance of this finding is unclear. In contrast, proportions of oligotypes belonging to the *S. salivarius* group, were found to be significantly raised in the OFG only and CD only groups. *S. salivarius* and related species are regarded as being asscoiated with health and are found primarily on the dorsum of the tongue and the pharyngeal mucosa.[287, 288] Indeed, strains of *S. salivarius* are used as probiotics with beneficial properties against oral conditions such as halitosis and pharyngitis [289] and have been shown to have anti-inflammatory properties *in vitro*, via down regulation of the NF-κB pathway. [290] However, it is not clear why the proportions of these species should be raised in OFG and CD but the diseases may change the oral mucosa in ways which promote the adherence and retention of these species. It was particularly interesting that one oligotype, 2869, was specifically elevated in subjects with OFG or CD. It seems that members of this oligotype which were found in multiple subjects, has a particular, and numerically strong, relationship with OFG. It is known that strains of a species can differ markedly in their biological and pathogenic properties. These findings are of particular interest as bacterial antigens, including streptococci are a known common target for IgE. Previous studies have identified infiltrates of dendritic B cells in the oral epithelium OFG patients which express surface IgE. [43] The lips, which are commonly involved in OFG, also have the highest number of B cells that are class switched to IgE.

Most recently, *S. salivarius* has been shown to contribute to another allergy-related condition, allergic rhinitis (commonly known as hay fever). [291] Using the same methodology of 16S rRNA sequencing, the microbiome of allergic rhinitis patients was found to be significantly more abundant in *S. salivarius* compared with healthy individuals. Furthermore, using ex vivo and in vivo models, it was demonstrated that *S. salivarius* appeared to contribute to the development of allergic rhinitis by promoting

inflammatory cytokine release and causing characteristic morphological changes in the nsaal epithelium. This is a surprising finding given that *S. salivarius* has commonly been thought to have anti-inflammatory effects and has been developed as a probiotic. [289] Neverthless, the findings of this recent study support the potential use of antibacterial therapies for allergic rhinitis [291] which may have more wide-reaching implications for other allergy-associated conditions including OFG.

In contrast to *S. salivarius*, *S. mitis*-group organisms were present at lower relative abundance in the subjects with CD, compared to OFG and controls. *S. mitis* is the commonest streptococcal species found in the human mouth.[288] The numbers of this group may have appeared to have been reduced because proportions of *S. salivarius* were raised, which would have affected their relative abundance by occupying binding sites.

Chapter 4 – Investigating the use of salivary calprotectin in OFG and Crohn's disease

4.1 – Introduction

As set out in Chapter 1.1, OFG is a chronic granulomatous inflammatory disorder of the oral cavity, with approximately 20% of cases associated with intestinal CD. Calprotectins are pro-inflammatory proteins released by activated leucocytes and faecal calprotectin assays are widely used to assist with diagnosing and monitoring intestinal Crohn's disease. [109] They have the advantages of being widely available, inexpensive, robust, quantitative and easy-to-use biomarkers. Perhaps their biggest advantage are that they can avoid non-invasive assessments such as colonoscopy and can be performed remotely. Therefore, this makes calprotectin assays an attractive option for testing salivary fluid in OFG. To date, there have been no studies exploring this unmet need.

The aim of this study was to to quantify salivary calprotectin levels amongst patients with OFG alone, OFG+CD, CD alone and HC, and to determine whether there is correlation between salivary calprotectin levels and OFG disease site, activity and severity.

4.2 – Methods

As described in Chapter 2.1, unstimulated whole saliva samples were collected from cases (adults with a diagnosis of OFG only, OFG+CD, or CD only) and controls over a 2 year period from a specialist outpatient clinic at GSTFT. A standardised ODAS was performed for each patient to record disease site and severity. Additional information was collected regarding presence of periodontal disease, smoking status and alcohol consumption.

Calprotectin quantification was performed using an ELISA kit, CALPROLAB™ Calprotectin (CALPRO Labs®, Norway). Statistical analysis was performed using Minitab® version 18 (Minitab Inc., USA).

4.3 – Results

Samples were collected from 252 subjects with an age range of 16-75 years: 79 had OFG only (mean age 41.7 years; 48 females), 42 had OFG+CD (mean age 44.8 years; 14 females), 97 had CD only (mean age 43.6 years; 63 females) and 34 were healthy controls (mean age 35.8 years; 22 females). Table 4.1 summarises the subject demographics and median ODAS and salivary calprotectin levels. Within all groups, there were 30 smokers, 136 who drank alcohol and 48 subjects taking immunomodulators and /or biologics.

Table 4.1 - Subject Demographics, Median ODAS and Salivary Calprotectin Levels

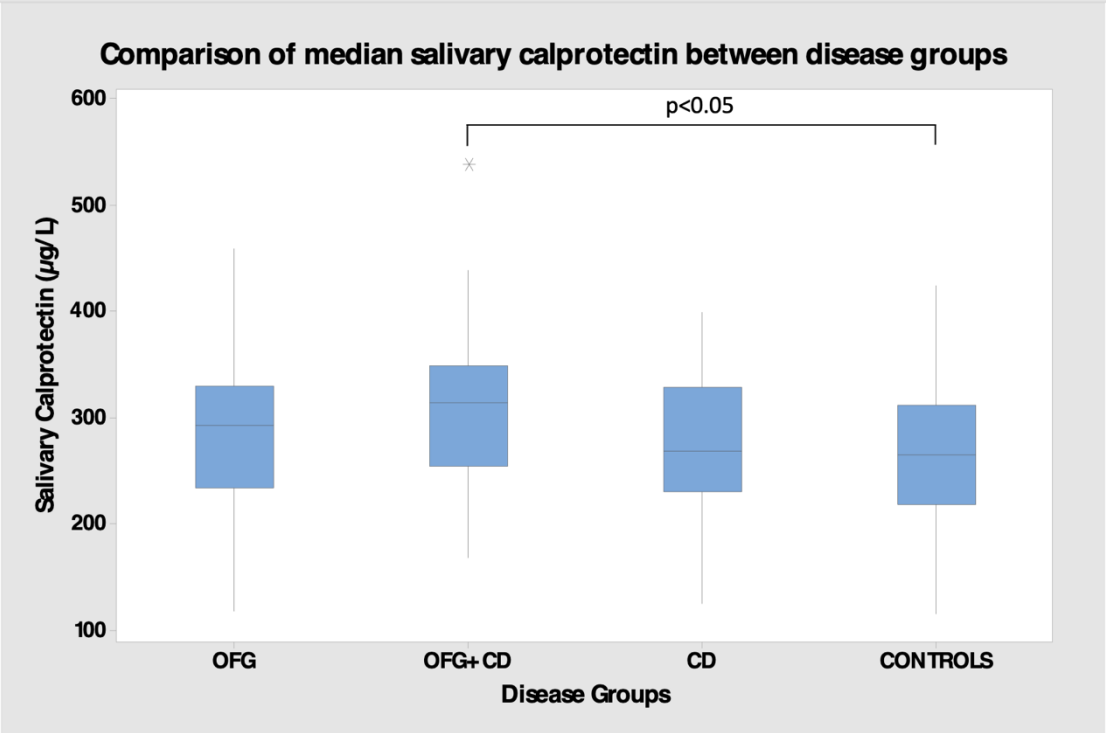
	OFG only	OFG + CD	CD only	HC
Mean Age (years)	41.7	44.8	43.6	35.8
Gender (% female)	48 (60.8%)	14 (33.3%)	63 (64.9%)	22 (64.7%)
Median ODAS (range)	4 (0-37)	4 (0-51)	N/A	N/A
Median Salivary Calprotectin (range) (μg/l)	293 (118–459)	315 (169-538)	268 (125-399)	264 (115-425)

Table 4.2 shows the range and median salivary calprotectin levels of all groups. Figure 4.1 shows a box plot with the variation of salivary calprotectin levels compared between subject groups. Calprotectin levels were significantly higher ($p=0.012$) amongst OFG+CD individuals (median 315 μg/l) compared with HC (median 265 μg/l). Table 4.3 shows the p-values between subject groups and HC.

Table 4.2 - Comparison of Salivary Calprotectin Levels between Disease Groups

Salivary Calprotectin (ug/L)						
Disease Group	n	Minimum	Q1	Median	Q3	Maximum
OFG	79	117.90	233.70	292.66	329.89	458.77
OFG+CD	42	168.7	254.8	314.5	349.4	538.2
CD	97	124.81	230.63	268.25	329.05	399.21
HC	34	115.0	218.6	264.6	312.2	424.6

Figure 4.1 – Box Plot of Salivary Calprotectin Levels between Disease Groups



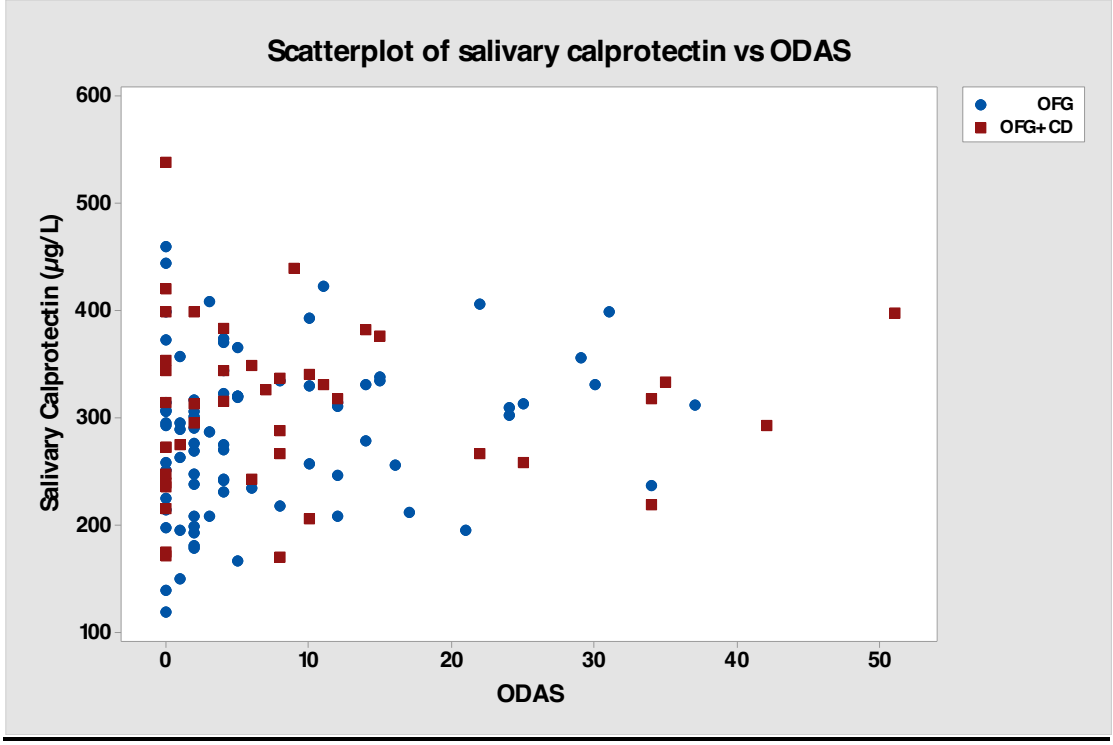
The salivary calprotectin levels for each disease group and controls are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual stars are outliers; statistically significant p-values are shown

Table 4.3 - Comparison of Salivary Calprotectin Levels with P-Values between Disease Groups

Disease Groups	p-value
OFG vs HC	P=0.151 (NS)
OFG + CD vs HC	P=0.012
CD vs HC	P=0.443 (NS)

Figure 4.2 shows a scatter plot of salivary calprotectin levels against ODAS in the OFG only and OFG+CD groups. There was no significant association between calprotectin levels and the ODAS in these groups however, as indicated in the graph there were few subjects with an ODAS greater than 15 in this study. Table 4.4 shows the p-values for the individual disease groups.

Figure 4.2 – Scatter Plot of Salivary Calprotectin Levels against ODAS



The scatter plot shows salivary calprotectin levels for OFG (blue dots) and OFG+CD (red dots) against ODAS.

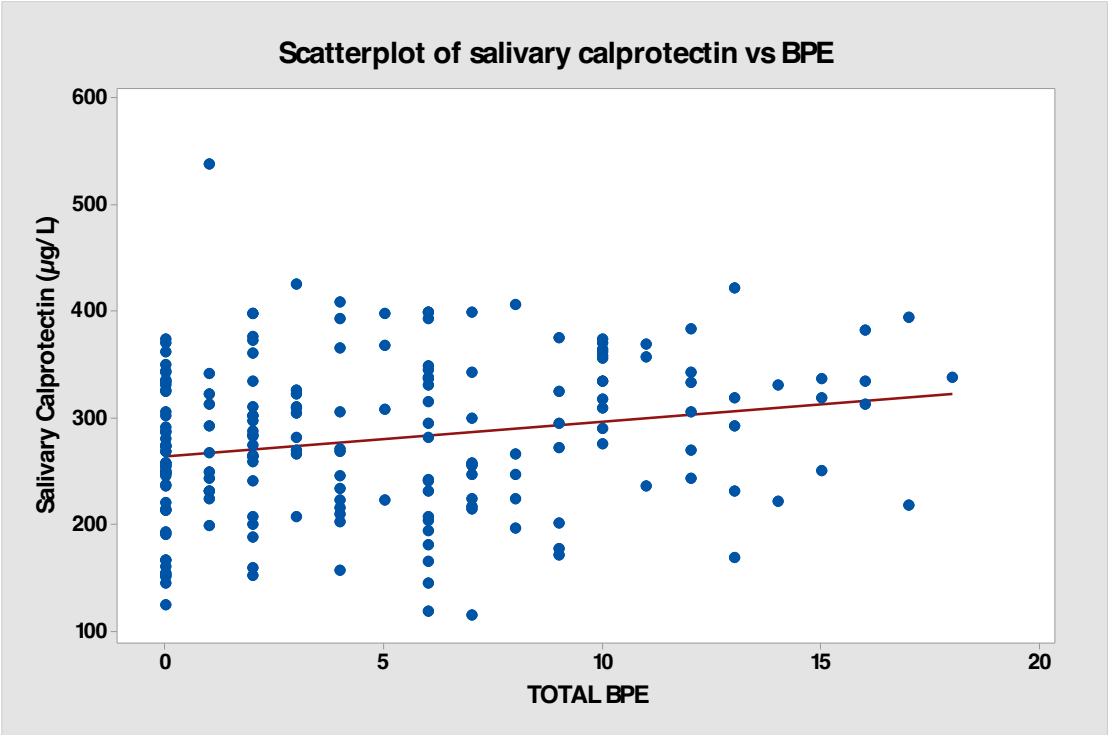
Table 4.4 - Comparison of Salivary Calprotectin Levels and ODAS with P-Values between Disease Groups

Disease Groups	R value	p-value
All OFG	0.164	P=0.072 (NS)
OFG only	0.211	P=0.062 (NS)
OFG + CD	0.091	P=0.565 (NS)

BPE data was collected where possible and there were corresponding BPE scores for 45 patients with OFG only, 20 patients with OFG+CD and all the CD only and HC. Figure 4.3 shows a scatterplot of salivary calprotectin levels versus BPE score for the subject groups and Figure 4.4 shows the same data by subject group. Figures 4.5 – 4.8 show the same data for each individual subject group.

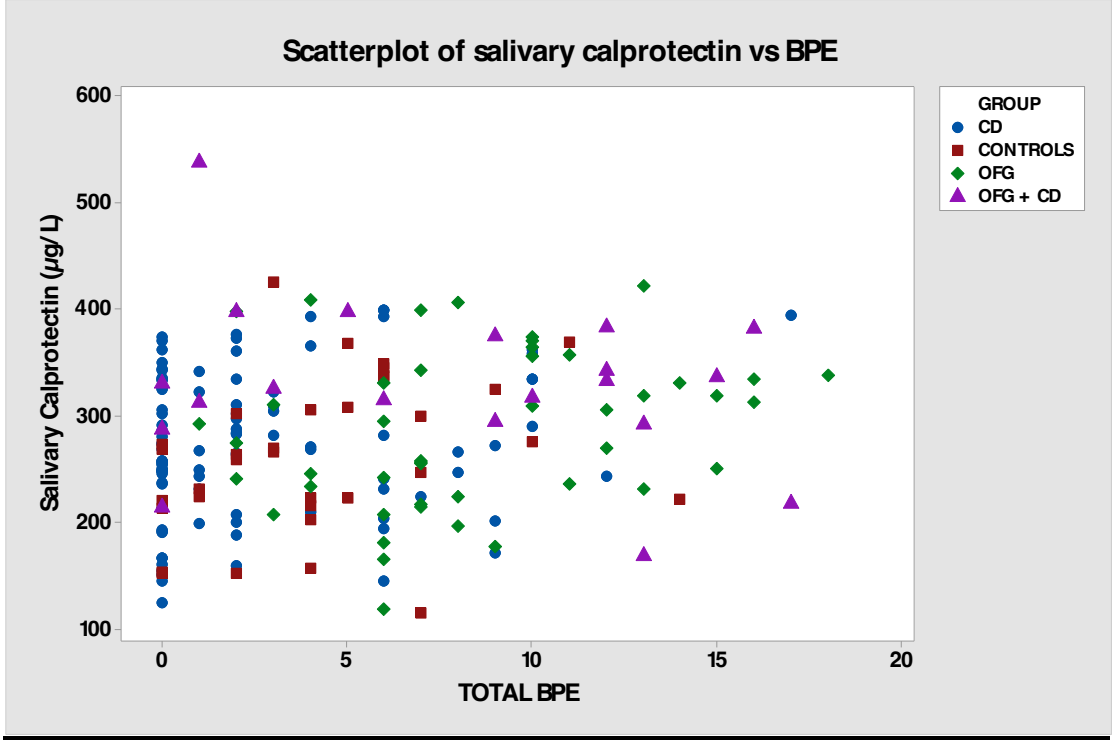
Overall, for all groups the Spearman rank correlation was r=0.219 (p=0.002, n=196) between the calprotectin and BPE score. There were no clear relations for the individual groups except the HC subjects which had a Spearman rank correlation of r=0.395 (p=0.021, n=34). The graph indicated that subjects with higher BPE scores tended to have higher salivary calprotectin levels.

Figure 4.3 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for All Subject Groups



The scatter plot (blue dots) shows salivary calprotectin levels for all subject groups against BPE scores. The line of best fit is shown in red.

Figure 4.4 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for Individual Subject Groups

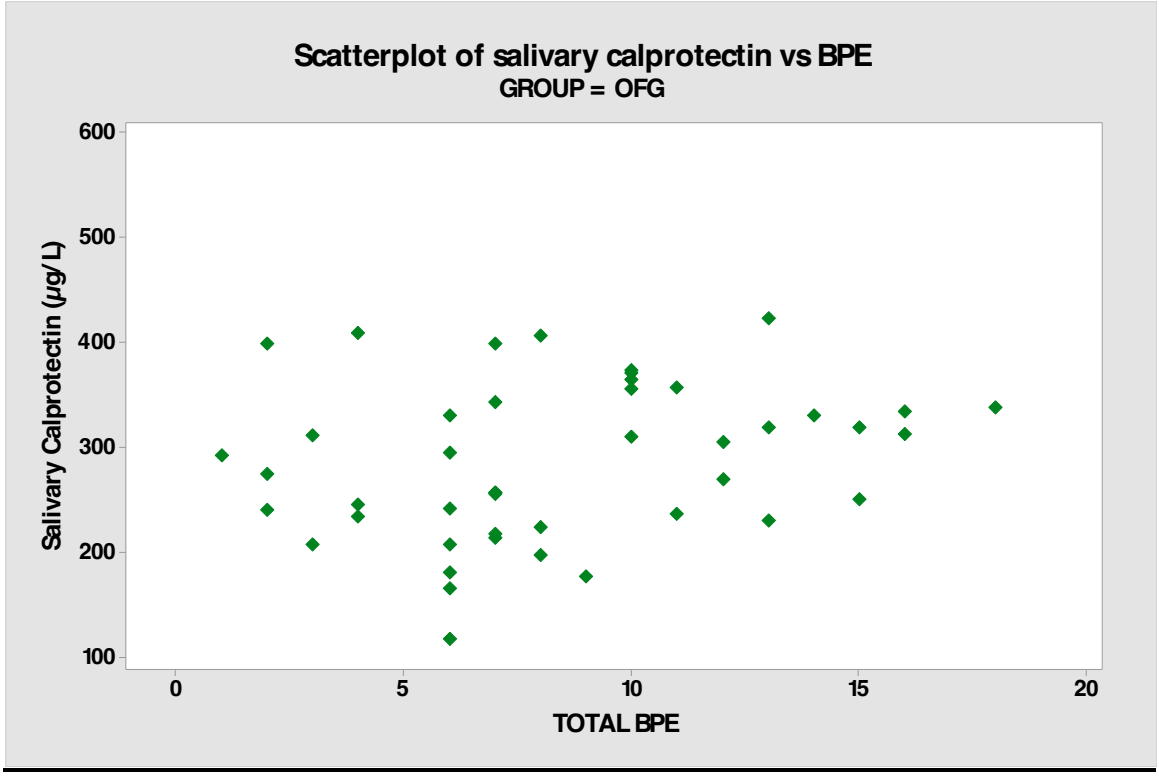


The scatter plot shows salivary calprotectin levels for all disease groups and controls (coloured symbols) against BPE scores.

Table 4.5 - Comparison of Salivary Calprotectin and BPE with R- and P-Values

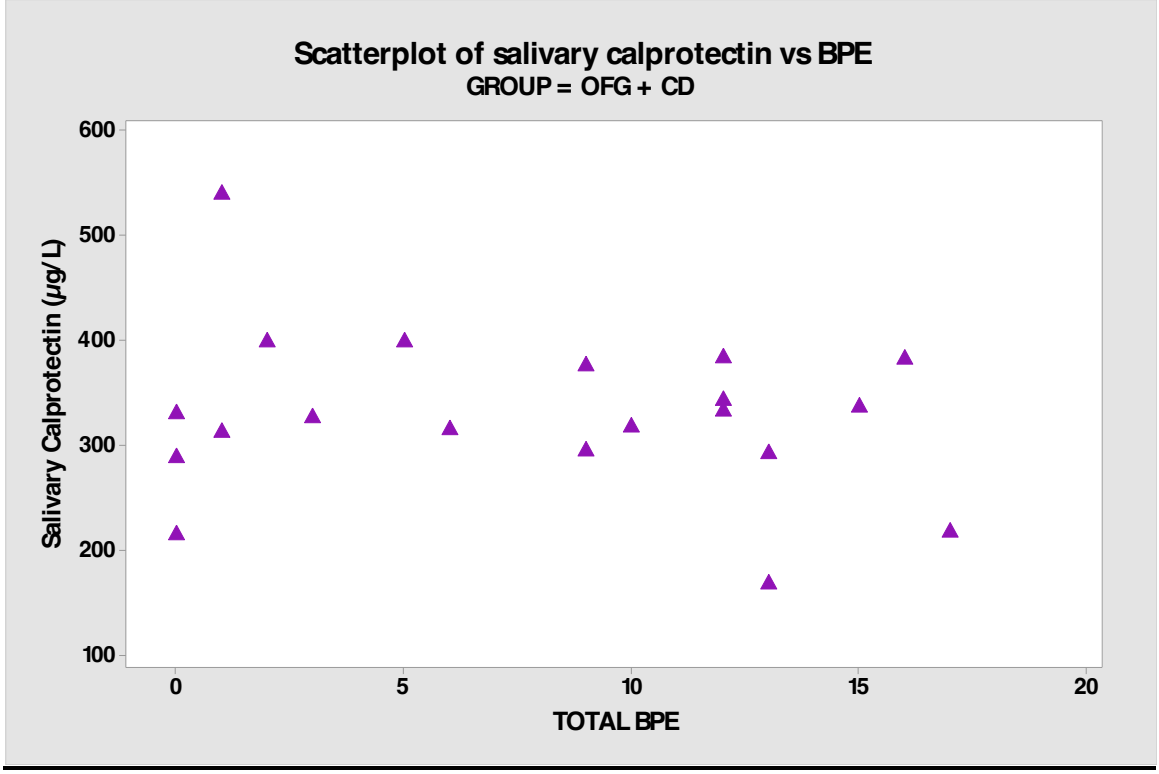
Disease Groups	R value	p-value
All Groups	0.219	P=0.002
OFG only	0.285	P=0.057 (NS)
OFG + CD	-0.032	P=0.894 (NS)
CD	0.156	P=0.127 (NS)
HC	0.395	P=0.021

Figure 4.5 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for OFG Only Group



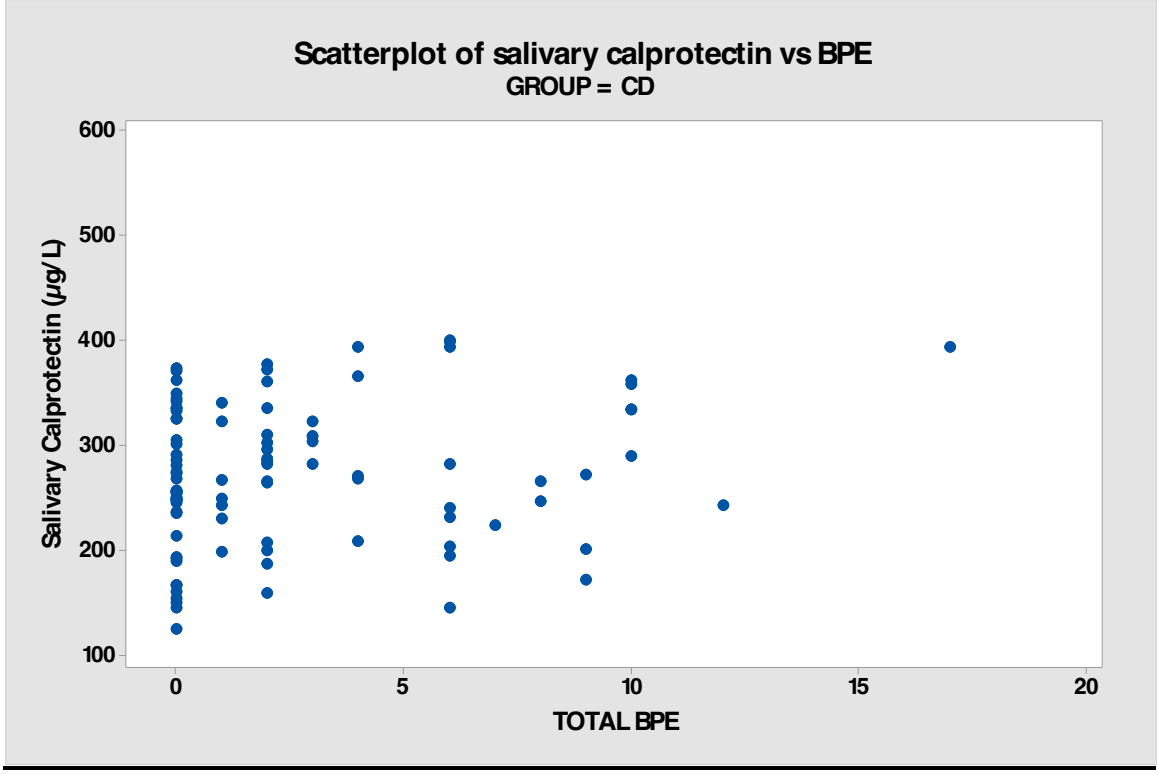
The scatter plot shows salivary calprotectin levels for OFG (green diamonds) against BPE scores.

Figure 4.6 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for OFG+CD Group



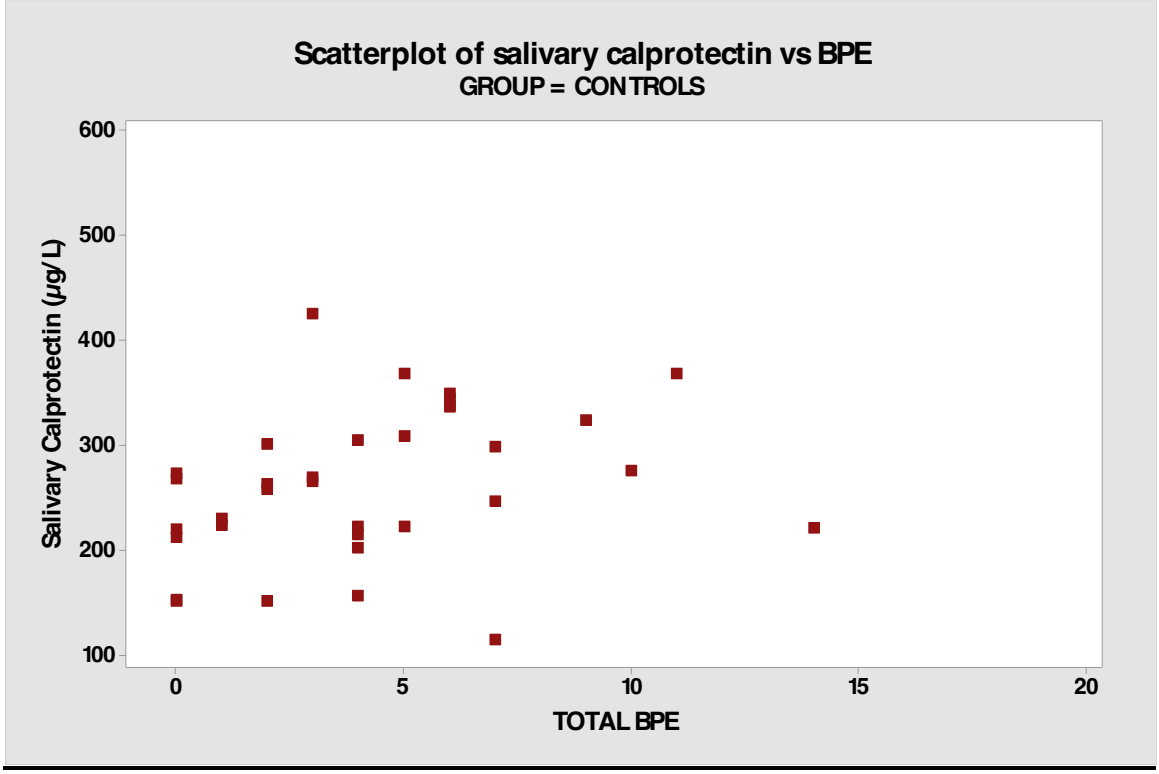
The scatter plot shows salivary calprotectin levels for OFG+CD (purple triangles) against BPE scores.

Figure 4.7 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for CD Group



The scatter plot shows salivary calprotectin levels for CD (blue dots) against BPE scores.

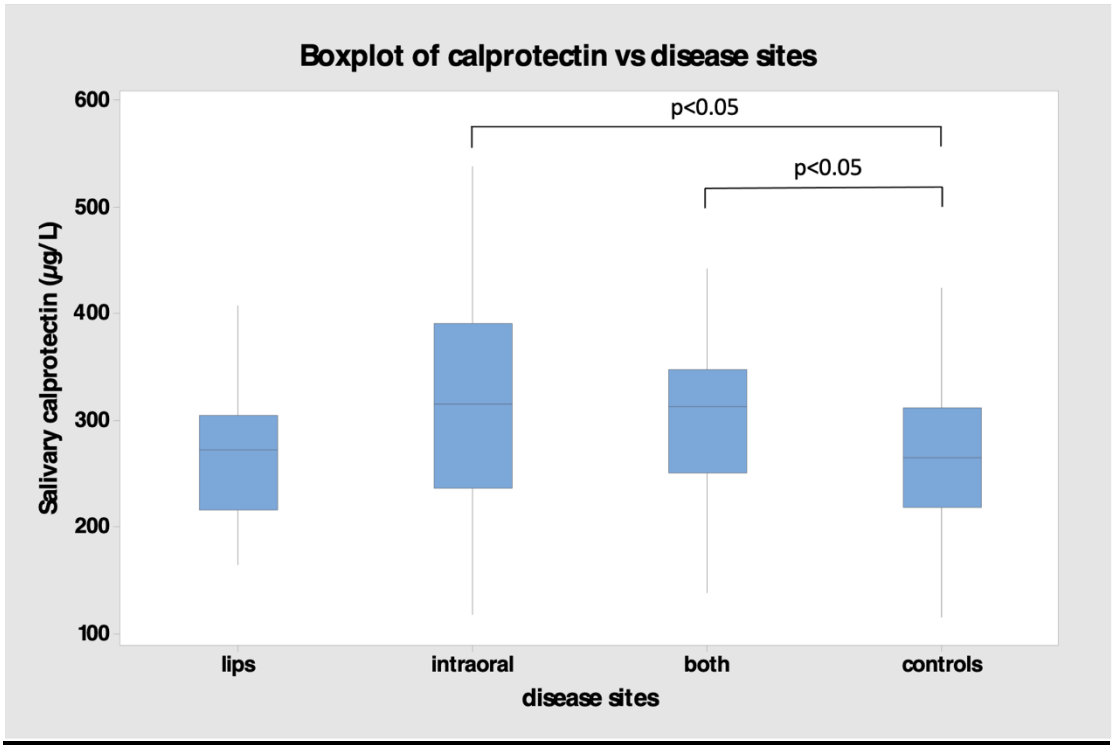
Figure 4.8 – Scatter Plot of Salivary Calprotectin Levels versus BPE Score for HC Group



The scatter plot shows salivary calprotectin levels for HC (red squares) against BPE scores.

Figure 4.9 shows a box plot of the variation in salivary calprotectin levels for the different disease sites. Patients with both lip and intra-oral disease involvement were found to have significantly higher ($p=0.015$) calprotectin levels (median 313 μg/l) compared with controls (median 265 μg/l). Calprotectin levels also appeared higher in patients with intra-oral involvement (median 315 μg/l) as compared with HC, however, the difference was not significant after allowing for multiple comparisons. There was no significant difference seen for patients with lip only (extra-oral) involvement compared with controls. Table 4.6 shows the p-values for the different disease sites.

Figure 4.9 – Box Plot of Salivary Calprotectin Levels between Disease Sites



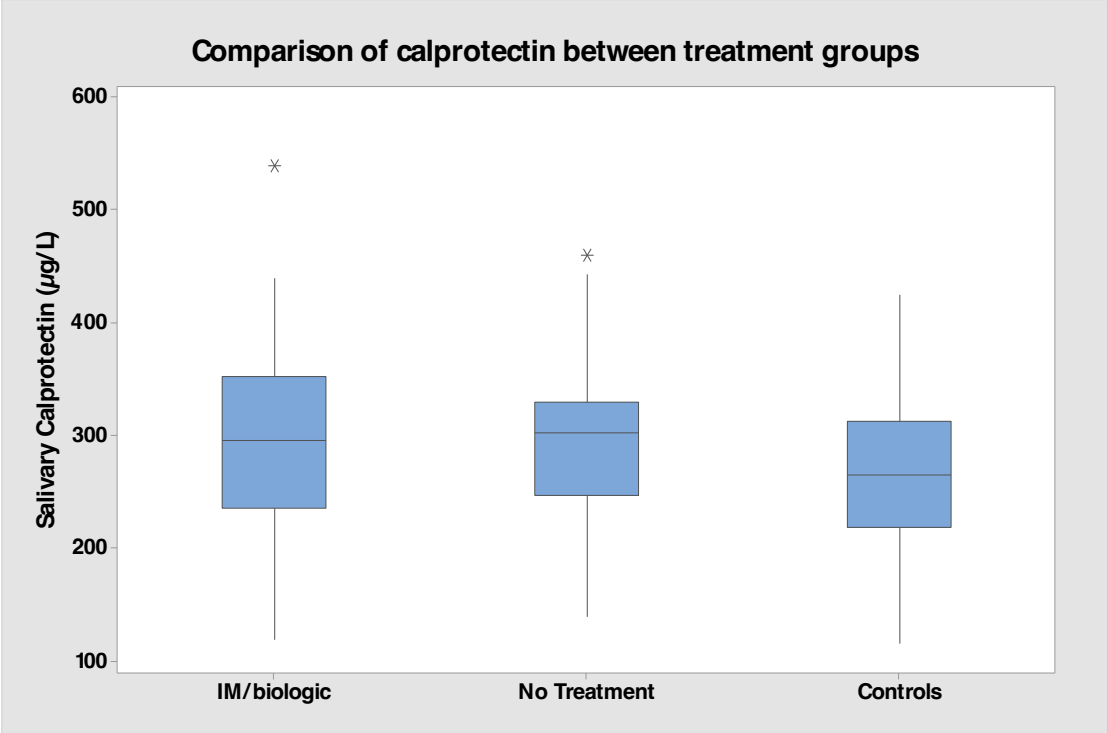
The salivary calprotectin levels for each oral site and controls are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; statistically significant p-values are shown

Table 4.6 – Table of P-Values for Salivary Calprotectin Levels between Disease Sites

Disease Site	p-value
Lips vs controls	P=0.873 (NS)
Intraoral vs controls	P=0.049
Both vs controls	P=0.015

Calprotectin levels were not found to be statistically different to the HC group when compared with patients receiving immunomodulator therapy (thiopurines, methotrexate, biologic drugs). Figure 4.10 shows a box plot of the calprotectin levels between treatment groups. Table 4.7 shows the p-values for the calprotectin levels between the treatment groups.

Figure 4.10 – Box Plot of Salivary Calprotectin Levels between Treatment Groups



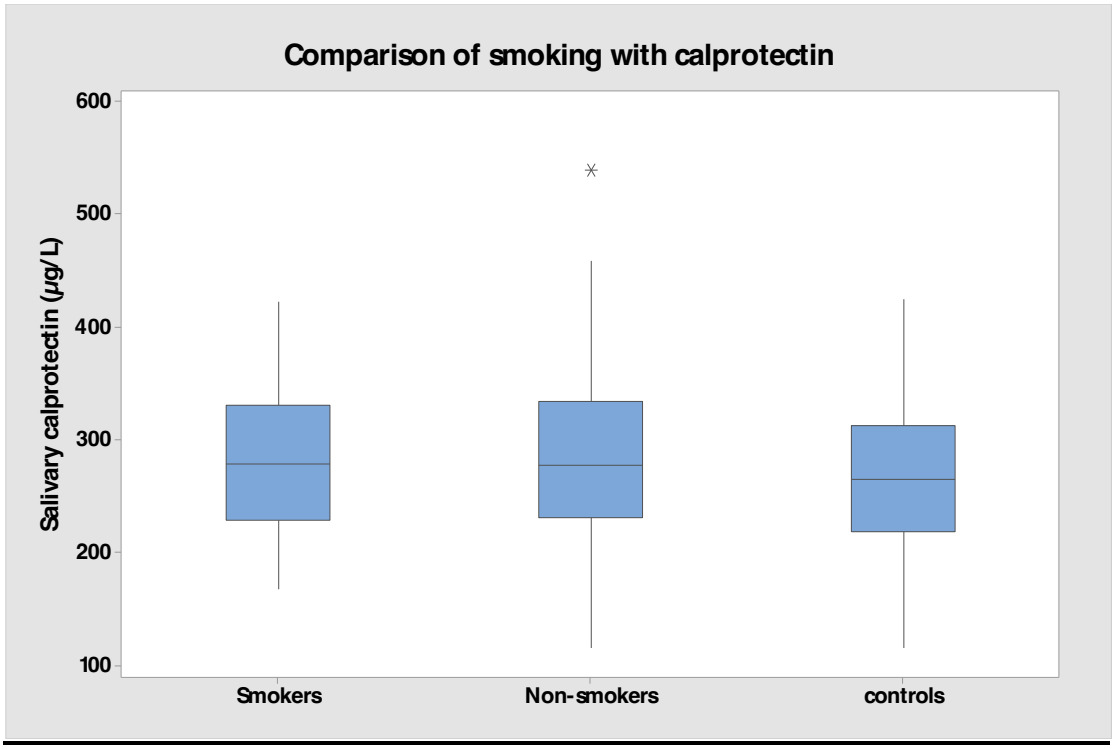
The salivary calprotectin levels for each treatment group and controls are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; individual stars are outliers

Table 4.7 – Table of P-Values for Salivary Calprotectin between Treatment Groups

Treatment Group	p-value
IM/Biologic vs controls	P=0.089 (NS)
No treatment vs controls	P=0.036

Calprotectin levels in smokers and non-smokers were not found to be statistically different to the HC group when compared. Figure 4.11 shows a box plot of the calprotectin levels based on smoking status. Table 4.8 shows the p-values for the calprotectin levels between the smoking status groups.

Figure 4.11 – Box Plot of Salivary Calprotectin Levels and Smoking Status



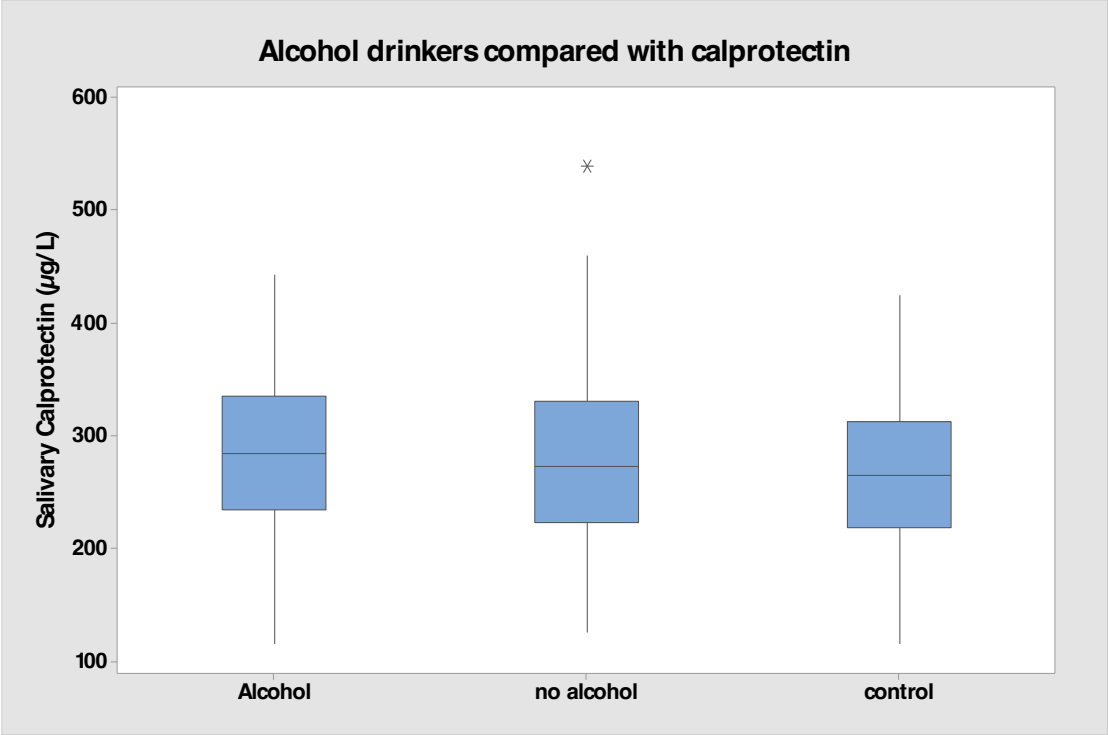
The salivary calprotectin levels for smokers, non-smokers and controls are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; the individual star is an outlier

Table 4.8 – Table of P-Values for Salivary Calprotectin with Smoking Status

Smoking Status	p-value
Smokers vs controls	P=0.400 (NS)
Non-smokers vs controls	P=0.166 (NS)

Calprotectin levels in subjects who did and did not use alcohol were not found to be statistically different to the HC group when compared. Figure 4.12 shows a box plot of the calprotectin levels based on alcohol use. Table 4.9 shows the p-values for the calprotectin levels between the alcohol use groups.

Figure 4.12 – Box Plot of Salivary Calprotectin Levels and Alcohol Use



The salivary calprotectin levels for alcohol drinkers, non-drinkers and controls are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles; the individual star is an outlier

Table 4.9 – Table of P-Values for Salivary Calprotectin with Alcohol Use

Alcohol Use	p-value
Alcohol use vs controls	P=0.114 (NS)
No alcohol use vs controls	P=0.338 (NS)

4.4 – Discussion

Historically, OFG has predominantly been a clinical diagnosis and this is the first study of a salivary biomarker in patients with OFG. Importantly and pragmatically, the study shows that salivary calprotectin samples can be easily collected from patients and tested from Oral Medicine and Gastroenterology Outpatient clinics. The demographics of the subjects were well matched although there was a younger mean age in the HC group as compared to the disease groups.

Salivary calprotectin levels were found to be significantly higher in specific patient cohorts with OFG such as those with concurrent CD as compared with HC. This suggests that testing salivary calprotectin levels in OFG patients could be of use in determining the presence of CD or could perhaps be at greater risk of developing CD over time. Further, this could be of clinical use as a predictor for CD in patients presenting with OFG who may have insidious bowel symptoms or do not have a formal diagnosis of CD. Calprotectin levels were also significantly higher in those with intra-oral OFG compared with controls. The presence of intra-oral OFG has already been shown to more closely linked to the presence of CD, particularly when there is buccal sulcal involvement and this also suggests the possibility that salivary calprotectin testing could be of value in OFG patients to try and determine if there is a greater risk of CD development.

A small effect of higher salivary calprotectin levels was also seen in OFG only and CD only patients , however, these did not reach statistical significance when compared with HC. Nevertheless, this suggests that CD patients may have a higher rate of periodontal

disease. A further greater powered study with larger numbers would be valuable to determine if there are statistically significantly greater salivary calprotectin levels in these disease groups. No relationship was seen in the salivary calprotectin levels of patients receiving immunomodulator therapy, tobacco or alcohol use.

Unfortunately, unlike faecal calprotectin measurement in CD, salivary calprotectin levels do not appear to be an adequate marker of OFG disease activity or severity. There was no clear relation between salivary calprotectin levels and oral inflammation activity, recorded as the ODAS, in OFG patients, both with and without CD.

Nevertheless, the idea of a salivary biomarker such as salivary calprotectin remains an attractive target for further research due to advantages such as simple, non-invasive point-of-care collection methods, simple laboratory extraction techniques and its quantitative nature. Given the current health climate with the COVID-19 pandemic, point-of-care tests will become increasingly valuable and may allow for remote monitoring of chronic inflammatory conditions such as OFG.

Chapter 5 – An Evaluation Study of *Lactobacillus Brevis* CD2 in OFG

5.1 – Introduction

As discussed in Chapter 1.1, OFG is a rare, chronic disfiguring condition affecting the oral mucosa and perioral region, and of unknown aetiology. Thousands of microbial species constitute the oral microflora and it has been suggested that microbial permutations may be involved in the inflammatory process of OFG.

Probiotics are preparations containing live micro-organisms which have been postulated to have effects on the microbiota. CD2 lozenges are a novel probiotic containing *Lactobacillus brevis* which have anti-inflammatory mechanisms, primarily via reduced arginine availability. Previous studies have shown that CD2 reduces oral inflammation in chemotherapy-induced oral mucositis,[209, 210] Behcet's disease[211] and aphthous ulceration.[212]

The aim was to evaluate the tolerability and efficacy of CD2 lozenges in reducing oral inflammation in patients with active OFG.

5.2 – Methods

This was a single-centre prospective open observational study which took place at Guy's Hospital, London, between February - August 2014. Patients with active OFG received an eight week course of CD2 lozenges taken four times per day. Patients were reviewed before and after treatment with the following primary parameters:

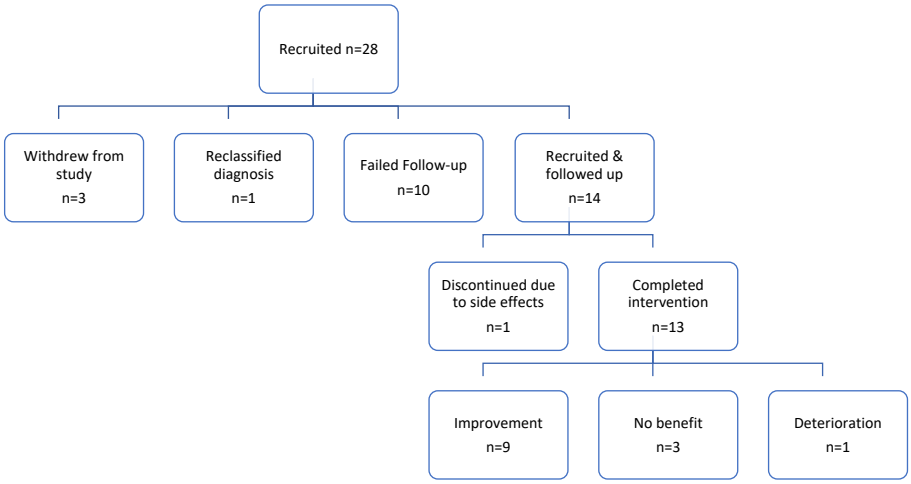
- ODAS
- VAS
- GPA

There was no control group. Results were analysed using a statistical package.

5.3 – Results

Over a 6 month period, 28 patients were recruited. From this group, 4 patients withdrew from the study (3 for non-compliance, 1 of whom had severe learning difficulties; 1 patient whose diagnosis was re-classified as RAS). 10 patients did not attend for follow-up leaving 14 patients for post-treatment analysis (7 males, 7 females). Figure 5.1 is a flowchart showing the subject numbers and overall outcomes as per GPA. The median age of the patients was 38.5 years with a range of 18-70 years. 5 of the 14 patients had a diagnosis of concurrent gastrointestinal CD.

Figure 5.1 – Flow Chart of Recruited Subjects and those Taking CD2

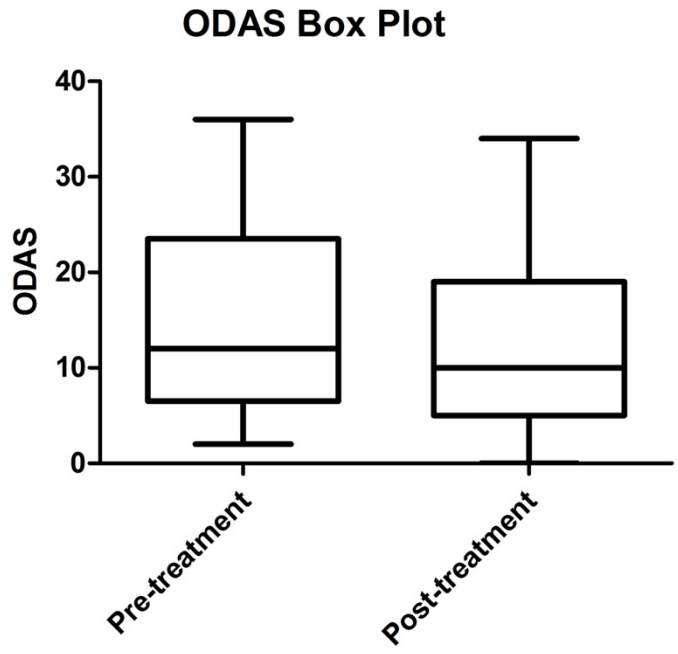


Flow chart showing the numbers of subjects taking CD2 and their overall outcomes following initial recruitment into the study.

The median ODAS prior to treatment was 14.5 with a range of 2-36. Prior to treatment, 8 patients were classified as having mild disease, 1 moderate and 5 severe. The median VAS prior to treatment was 40% with a range of 0-90%.

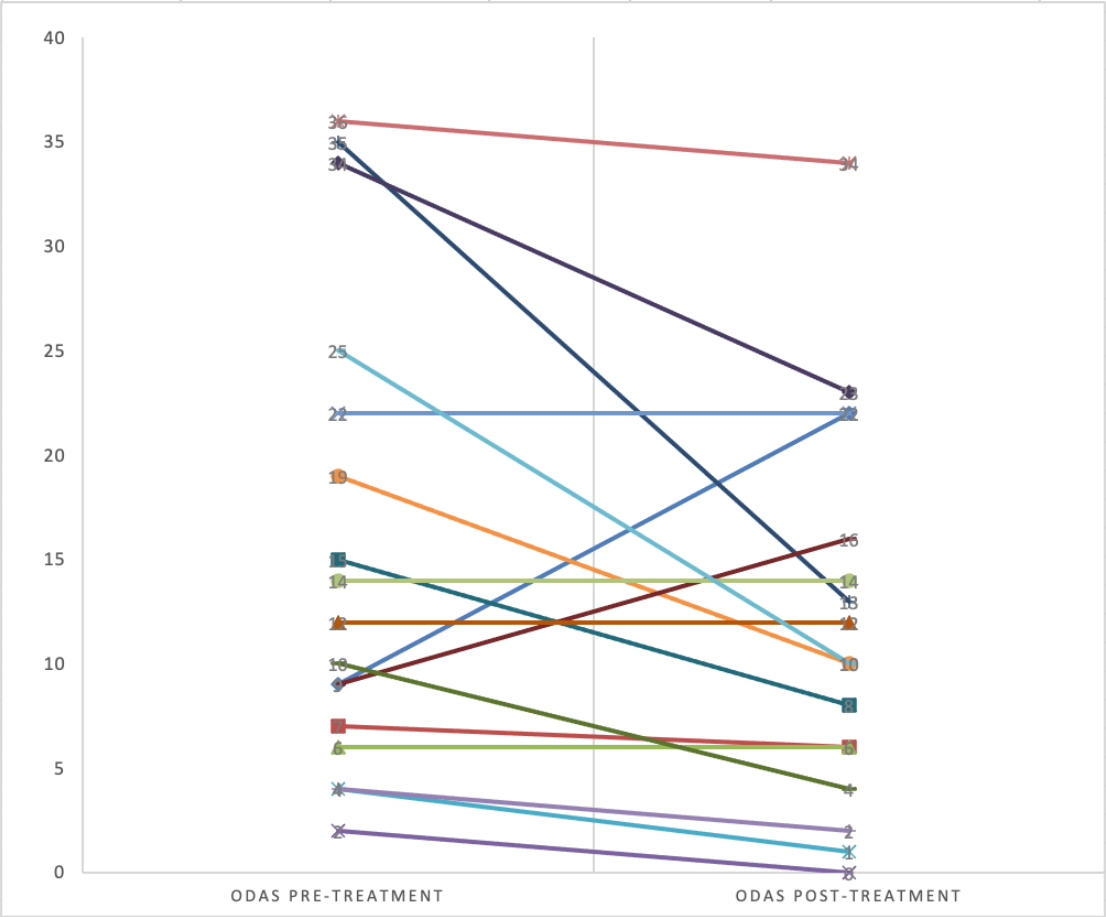
Following treatment, the median ODAS was 11.5 with a range of 1-34. At the end of treatment, 8/14 patients had mild disease, 5/14 moderate and 1/14 severe. Figure 5.2 shows a box plot of the pre- and post-treatment ODAS for each patient. Figure 5.3 shows a line graph of the ODAS values for each patient.

Figure 5.2 – Box Plot of ODAS Pre- and Post-Treatment



The ODAS for pre- and post-treatment groups shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles

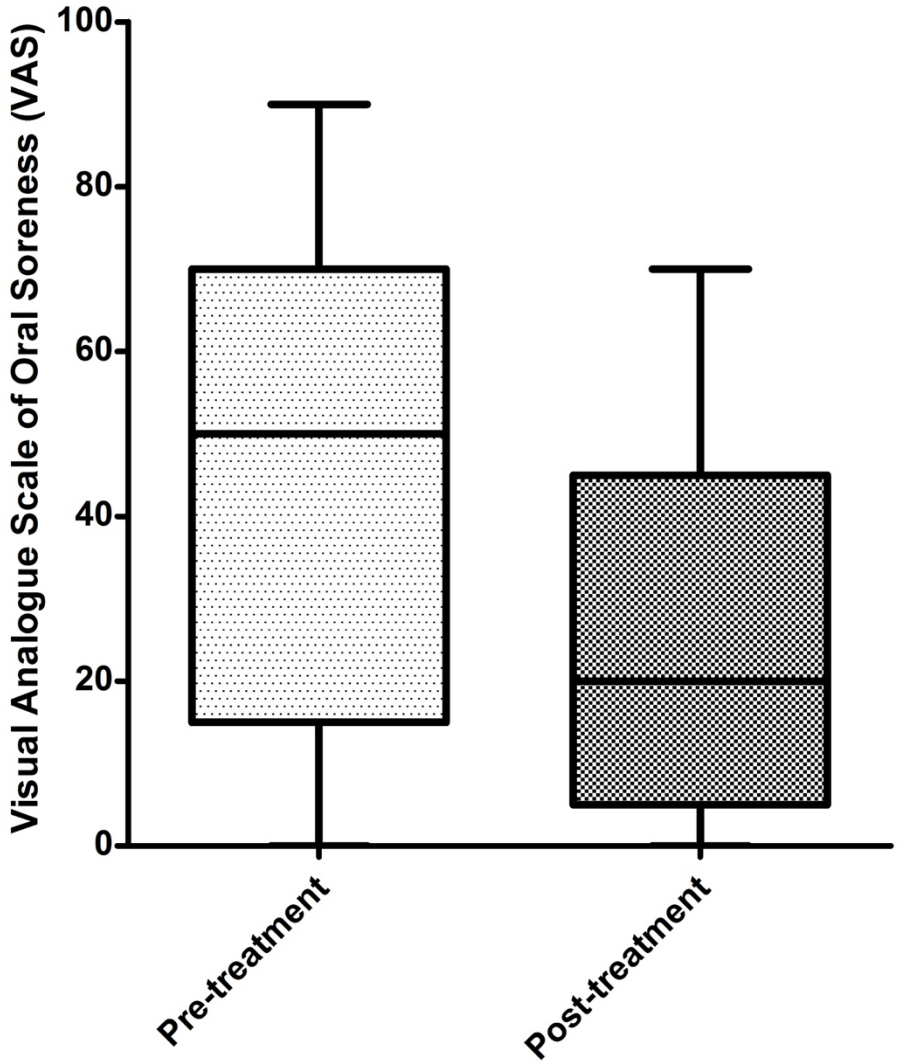
Figure 5.3 – Line Chart of ODAS Pre- and Post-Treatment



The ODAS shown for pre- and post-treatment groups as a line chart. The x-axis shows the pre- and post- treatment groups and the y-axis shows the ODAS. The different colour lines each represent individual patients and the connecting line shows the trend

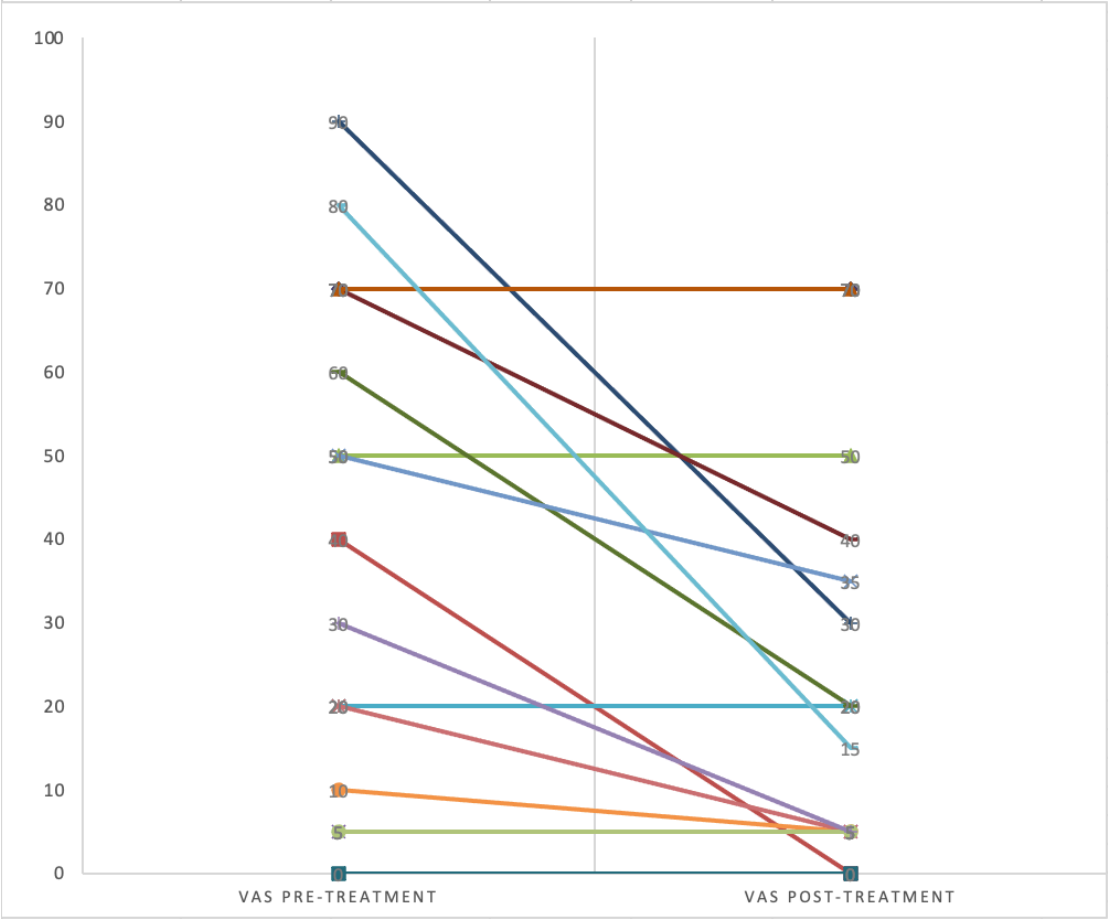
The median VAS post-treatment was 17.5% with a range of 0-70%. Figure 5.4 shows a box plot of the pre- and post-treatment VAS for each patient. Figure 5.5 shows a line graph of the VAS percentage scores for each patient.

Figure 5.4 – Box Plot of VAS of Oral Soreness Pre- and Post-Treatment



The VAS of oral soreness scores for pre- and post-treatment groups are shown as a box plot. The line inside the box is the median; upper and lower edges of the boxes are the first and third quartiles

Figure 5.5 – Line Chart of VAS of Oral Soreness Pre- and Post-Treatment



The VAS for oral soreness shown for pre- and post-treatment groups as a line chart. The x-axis shows the pre- and post- treatment groups and the y-axis shows the VAS. The different colour lines each represent individual patients and the connecting line shows the trend

The mean reduction in ODAS was 5. One patient's ODAS increased by 7 points and her disease progressed from mild to moderate but she reported a perceived reduction in oral soreness and felt CD2 was overall beneficial.

The mean improvement in oral soreness as measured by VAS was 31.1%. 5 of the 14 patients felt no improvement in oral soreness with treatment.

13/14 patients completed their treatment. 1 patient discontinued treatment after 1 week due to severe diarrhoea which she attributed to taking CD2. Her symptoms improved upon cessation. There were no serious adverse events in any of the patients.
1 patient complained that she disliked the taste of the lozenges. 2 patients found it inconvenient to have to keep the lozenges refrigerated although they were still able to complete the full recommended course.

5.4 – Discussion

This study represents the first evaluation study of a probiotic therapy in OFG. The primary aim was to evaluate the safety and tolerability of CD2 in patients with OFG. The secondary aim was to gain a basic assessment of the efficacy of CD2 in OFG. Although the study has significant limitations, these objectives were largely achieved.

Patients generally found CD2 quite tolerable and the only reported adverse reaction was an increased frequency of loose stool in 1 patient. These symptoms were relatively mild and rapidly resolved following cessation of CD2 with no obvious serious sequelae.

Most patients found the lozenges easy to take and the taste pleasant. However, pill burden and compliance could be a challenge as they frequently reported difficulty in remembering to take 4 lozenges per day and occasional skipped doses were not uncommon. Most patients reported no problems with refrigerating the lozenges and generally access was not an issue.

A number of limitations have to be acknowledged with this study. There was no blinding in recruitment and no study arm with placebo treatment. In addition, there were a number of confounding factors as many patients were already taking other immunomodulator treatment, although established medications were not altered. It had been intended for patients to be assessed at both 4 and 8 weeks as per the original

proforma but this proved to be logistically difficult and patients were reviewed at 8 weeks only.

An intention-to-treat-analysis was not performed due to the small number of patients and the limitations of not being a blinded randomized control trial. This would have included those patients who did not comply with treatment and were lost to follow-up. This primarily appears to be due to the disease course itself and logistics of follow-up rather than any effect of CD2 medication.

Due to the small numbers of patients involved, the performed study was not powerful enough to show a statistically significant result, although there appears to be a trend towards therapeutic benefit with CD2 as demonstrated by the reduction in ODAS, GPA and oral soreness.

Overall, CD2 appears to be safe, well-tolerated and of potential benefit in treating active OFG.

Chapter 6 – Conclusions

OFG is a rare, chronic inflammatory and disfiguring disease which predominantly affects young people. It is characterised by the presence of granulomatous inflammation in the oral cavity which may also affect the lips and perioral region. The precise aetiology and pathogenesis remains unknown but previous studies have demonstrated links with dietary sensitivity, allergy and a relationship with CD. The work presented in this thesis has atempted to clarify whether alterations in the microbiome could play a role in the disease. Also, due to a lack of objective biochemical markers for disease assessment and surveillance, OFG remains challenging to diagnose, thus salivary calprotectin was investigated as potential disease biomarker. Although, OFG responds well to dietary manipulation, the role of probiotic treatment to improve outcomes was investigated and is presented in this thesis.

6.1 – The oral microbiome in OFG and Crohn's Disease

To date, there have been no studies investigating the oral microbiome in OFG. The work presented in Chapter 3 demonstrates that the overall composition of the salivary microbiota in OFG, CD individuals and healthy subjects was grossly similar and resiliently stable. However, there were some significantly interesting differences in levels of two of the commonest groups of oral streptococcal commensals which warrant further investigation. In particular, *S. salivarius* was increased in both CD and OFG while *S. mitis* was decreased in CD only. As discussed in Chapter 3.4, strains of *S. salivarius* have been shown to have a benefical effect in certain oral diseases.[289] Additionally, based on the work in Chapter 5, this proposes a role for *S.salivarius* as

potential probiotic treatment in OFG. Recent studies have also shown *S.salivarius* to be safe as use as a probiotic in humans.[292, 293] The concept of microbial alteration in treating inflammatory dysbiosis and disease is further supported by recent clinical trial evidence supporting the use of FMT in treating psudomembranous colitis[294, 295] and UC.[296-299] Moreover, it has been shown that probiotic delivery via the oral route can be effective in treating IBD.[300]

It has previously been demonstrated that pathogenic gentic variants are known to confer a higher risk for CD and these are also enriched in OFG+CD.[11] Toolkits are being developed which combine genetic markers, such as the aforementioned, with family and lifestyle risk factors which can then be used to predict the development of CD intestinal inflammation.[301] Thus, the work presented in Chapter 3 provides an initial step in the investigation of salivary micobial biomarkers, such as *S. salivarius* for OFG as well as CD.

Additionally, given that the results presented in Chapter 4 show a poor correlation between a biochemical biomarker like calprotectin, the need for a microbial biomarker becomes ever more appealing.

6.2 – The use of salivary calprotectin in OFG and Crohn's disease

The need for remote testing and monitoring has become ever more relevant in the COVID-19 era due to the risk of close contact infection. This is particularly applicable

with rare diseases such as OFG, where remote testing could negate the need for costly, socially and environmentally unfriendly visits to specialist centres. Given the wide use of faecal calproectin in IBD, it was therefore attractive to consider salivary calprotectin as a potential biomarker for OFG disease activity.

The work presented in Chapter 4 is the first to study salivary calprotectin levels in OFG and the largest study to date to investigate levels in CD. It was found that salivary calprotectin levels were significantly higher in OFG+CD and patients with intra-oral involvement compared with HC. Unfortunately, salivary calprotectin levels do not appear to be an adequate marker of OFG disease activity due to their poor correlation with the ODAS. Whilst elevated salivary calprotectin levels have been found in OFG+CD and intra-oral involvement groups, inferences about OFG activity cannot be made from salivary calprotectin. Work from other groups have also reached similar conclusions.[134, 138]

6.3 - *Lactobacillus Brevis* CD2 probiotic treatment for OFG

The work presented in Chapter 5 is the first specific study of a probiotic as treatment in OFG. Given that increased concentrations of *S. salivarius* are found in OFG, as described in Chapter 3, and it has been efficaciously and safely used as a probiotic treatment; there is compelling evidence to support probiotic treatment in oral inflammatory conditions such as OFG.

The probiotic, CD2, has been successfully used in oral inflammatory conditions and in OFG, was found to be well tolerated and safe in a small cohort of patients. In this small pilot study, there was a modest improvement in ODAS and oral soreness supporting its use. This may be due to potential modulation of microbiome, such as tht adhernce of beneficial bacterial binding sites thus reducing binding opportunities for harmful microbiota.

Chapter 7 – Future Studies and Work

The studies in this thesis have contributed to the knowledge base of OFG, however, important questions remain to be answered. Over the last two decades, there has been a wealth of research and great strides have been made in investigating the human microbiome. Progressive microbiome analysis methods such as next generation sequencing has made this easier and will further enable characterisation in even greater detail. However, apart from cataloguing the microbiome, there is now a significant need for focused research to truly understand the significance of the microbiome, particularly with regards to its relationship with human cells and environmental factors, as well as its role in health and diseases, such as OFG.

Following the findings of the raised *S. Salivarius* concentrations in OFG, future work should be focused on confirming the association of specific *S. salivarius* strains with OFG by metagenomic analyses that enable strain differentiation [302], together with the isolation of representatives of this oligotype and investigation of its properties of relevance to OFG. By correlating the findings presented in Chapter 3 with host genetics, immune status, metabolomics along with other risk factors, further studies could help to develop prediction tools to identify those OFG patients who are at greatest risk of developing intestinal CD. This would enable earlier intervention and a possibility to improve disease outcomes. Additionally, a better understanding of the interactions between microbial shifts, inflammation and disease pathology would potentially lead to further targets for drug development and disease management strategies for this complex disease. Next generation sequencing techniques have advanced since this

study and the Illumina MiSeq platform is now most widely used. Currently, any future studies including replication studies would likely involve this platform. Additionally, the saliva samples studied were only prepared for gram positive bacteria. Future studies could also include preparations for gram negative bacteria, which have been shown to be increased in CD.

The results of the salivary calprotectin analysis highlight a number of areas for future research. Being a rare disease, OFG patient recruitment is difficult meaning high powered studies remain challenging. Further work should focus on classifying in detail, the different conditions within OFG. The development of a OFG classification model would be beneficial and would provide a firm foundation for more objective OFG research. As shown in Table 1.5, a robust example of this is the the Montreal classification for CD: age of onset, disease behaviour, disease location and the presence of any disease modifiers.[253, 256] Such a model is proposed in Table 7.1.

Table 7.1 – Proposed Novel Classification of OFG

Variable	
Age at diagnosis (years)	A1, <16
	A2, 17-39
	A3, >40
Location of disease	L1, Anterior (lips)
	L2, Posterior (sulcal)
	L3, Both anterior + posterior
Behaviour	B1, inflammatory
	B2, fibrosing
	B3, penetrating
	+CD, CD modifier
	+E, presence of allergy

It remains to be determined whether salivary calprotectin could have greater clinical utility if used in combination with other parameters, such as blood inflammatory markers and faecal calprotectin levels as part of an 'OFG/presence of CD' risk score. Further studies would be beneficial to determine if CD patients have higher rates of periodontal disease and whether this could be implicated or a factor in the pathogenesis of CD. Larger and longer term studies may reveal benefits of salivary calprotectin quantification, such as for OFG prognostication and determining treatment response. This phenomenon has been observed in CD, where higher baseline faecal calprotectin levels are associated with disease progression, irrespective of symptoms and can predict disease flares.

To further determine if CD2 is beneficial in treating OFG, a well-designed larger double-blind prospective RCT should be undertaken with the inclusion of comparator/HC groups to study the benefit and safety profile. Also as described earlier, more robust parameters of efficacy are needed to ensure an efficacious response is measured accurately. An RCT of *S. Salivarius* probiotic treatment, either solely or in combination with CD2, would be likely to show promise in improving OFG outcomes.

Finally, the role of the psychological impact in OFG is an underinvestigated area given the great impact the disease has patients' mental health. Further research into anxiety and depression scores with interventions such as counselling and support networks would be welcome.

Ultimately the biggest hindrance to OFG research is the rarirty of the disease compounded by its heterogeneous presentation which crosses multiple Medical and Dental specialties. Working with different centres to co-ordinate and unify efforts is most likely to lead to an improved understanding of the aetiopathogenesis of OFG and consequently, its management.

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Appendix

CRF Proforma : An evaluation of CD2 in orofacial granulomatosis

Recruiter Name:

Date Recruited:

Patient Name:

DOB:

Sex:

Hospital No:

Patient Study No:

ODAS & GPA (pre-treatment):

ODAS & GPA (4 weeks treatment):

ODAS & GPA (8 weeks treatment):

Dental Disease:

Medical History & Gut Crohn's:

Medications & Antibiotics:

Smoking & Alcohol:

Adverse Events: