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The Role of *Candida* in Oral Lichen Planus (OLP)

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BDSc (Hons)

A thesis submitted in total fulfilment of the requirements for the degree of

Doctor of Philosophy

December 2019

Melbourne Dental School

Faculty of Medicine, Dentistry and Health Science

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Declaration

This is to certify that:

1. This thesis comprises only my original work toward the degree of a PhD
2. Due acknowledgement has been made in the text to all other materials used
3. This thesis is less than 100,000 works in length, exclusive of tables, figures, bibliography and appendices as approved by the Research Higher Degrees Committee

Lara Marie DeAngelis

17th December 2019

Abstract

Purpose

Oral lichen planus (OLP) is a chronic condition characterised by T cell mediated destructions that is currently of unknown cause. OLP can be variably symptomatic with some patients experiencing no symptoms and others requiring extensive symptomatic management. *Candida spp.* can be found in association with OLP and due to this prophylactic treatment for *Candida spp.* is usually accounted for in the symptomatic management of OLP. This is despite current evidence not supporting concurrent use of antifungal therapy in the management of OLP with topical steroids.

A potential hypothesis for the cause of OLP is an interaction of host genetic susceptibility combined with an environmental trigger that initiates disease in the susceptible host. Another equally likely hypothesis is that OLP is a true autoimmune condition with autoimmunity directed against a currently unknown epithelial autoantigen. The oral cavity represents a unique microenvironment that plays host to many commensal and opportunistic microorganisms. The oral microbiota, specifically *Candida spp.*, could act as an aetiological trigger for the chronic T-cell mediated inflammation the defines OLP, specifically through activation of mucosal associated invariant (MAIT) cells. The role of *Candida* in the aetiopathogenesis and symptomatic management of OLP is currently unknown.

Hypothesis and Aim

The overall hypothesis was that *Candida* may play an aetiological role in the OLP disease process exerting an effect on T cells and cytokine expression and that adjunctive treatment is required in the symptomatic management of OLP. The overall aim of this study was to determine whether *Candida* plays an aetiological role in OLP as well as determine if specific treatment of *Candida* is required in symptomatic patients with OLP.

Materials and Methods

14 control and 7 OLP test patients, 3 assigned to the placebo and 4 assigned to the antifungal treatment group, completed the clinical study. Assessments of clinical appearance, symptoms, *Candida*, salivary acetaldehyde and medication use were made at 0, 6 and 12 weeks for OLP patients with assessments of *Candida* and salivary acetaldehyde made at baseline only for controls.

20 random OLP formalin fixed paraffin embedded (FFPE) samples were stained using a fluorescent multiplex immunohistochemistry (mIHC) protocol for the markers cluster of differentiation (CD)3, CD8, DAPI, interleukin 18 receptor 1 (IL18R1), CD161, MR-1 and T cell receptor (TCR) Vα7.2. The slides were scanned with the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA) to generate multispectral images (MSI). The MSI were then analysed with tissue segmentation and single antibody algorithms for both HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) to validate a method for quantitative analysis. Following validation of HALO™ (Indica Labs, USA) for quantitative analysis the above process was repeated on 89 FFPE biopsy tissue samples from 73 patients with OLP (28 asymptomatic, 30 symptomatic and 16 samples with concurrent *Candida* (9 symptomatic and 7 asymptomatic), for comparison with 15 patient samples of fibroepithelial polyp (FEP). All samples were tested for presence of *Candida* with periodic acid-Schiff (PAS) staining.

A BioPlex assay was performed to measure the cytokines interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin (IL) 17A, IL-18, IL-12p40, IL-12p70, IL-22 and IL-23. Supernatant for this experiment was collected at 8, 12 and 24 hours following prior incubation of peripheral blood mononuclear cells (PBMC) in PBMC media supplemented with either 10% v/v effluent derived from *C. albicans* biofilms or 10% v/v artificial salivary media (ASM). In addition, some wells were supplemented with either CD28 and/or phorbol 12-myristate 13-acetate (PMA)/Ionomycin. Flow cytometry was performed using TCRVα7.2, CD3, CD161, CD218a, CD4, CD8 and CD45 to define MAIT cells and T cell subsets. Prior to performing flow cytometry PBMC were incubated for 6 hours in PBMC media supplemented with either effluent derived from *C. albicans* biofilms or 10% v/v ASM with or without CD28.

Results

Results of this study showed no significant differences existed between the control group and the OLP test group at baseline with respect levels of salivary acetaldehyde, and *Candida* colony forming units (CFU). Downward trends were noted in both groups with respect to clinical appearance and subjective analysis of symptoms from baseline to 12 weeks. Trends noted from assessment of CFU and salivary acetylaldehyde levels between the test groups should be viewed with caution due low levels of detection at baseline and the wide spread of data.

Minor variability between the tissue segmentation algorithms with the trained algorithm for inForm 2.4.1 (PerkinElmer, USA) being the slightly less variable of the two. For quantitative cell analysis and identification of single antibody positive cells HALO™ (Indica Labs, USA) proved to be the least variable of the two trained algorithms.

The presence of MAIT cell phenotypes were confirmed within the subepithelial infiltrate of OLP. Reduced MAIT cell phenotype expression was noted in the presence of *Candida* and/or symptoms in OLP with decreased expression of CD161 noted in the presence of symptoms whilst decreased expression of TCRVα7.2 was noted in the presence of *Candida*.

Presence of PMA/Ionomycin and *Candida* effluent were factors that increased the expression of IFN-γ, TNF-α, IL-17A, IL-18, IL-22 and IL-23, cytokines that are associated with MAIT cell activation. Across all timepoints the presence of *Candida* effluent and CD28 resulted in upregulation of IL-18 and TNF-α. MAIT cells were not significantly affected by the presence of either effluent or CD28 suggesting that neither *Candida* effluent nor CD28 alone or the combination of the two were shown to induce MAIT cell proliferation.

Conclusion

Adjunctive treatment of symptomatic OLP with a topical antifungal did not significantly affect the presence of symptoms, erythema, CFU, *Candida spp.* or production of salivary acetaldehyde. HALO™ (Indica Labs, USA) was shown to be the more reliable program for mIHC quantitative cell analysis in FFPE OLP tissue. Analysis of mIHC in OLP FFPE tissue identified MAIT cells within the OLP inflammatory infiltrate with decreased expression of CD161 and TCRVα7.2 noted in the presence of symptoms and *Candida* respectively. Finally, *Candida* effluent was unable to induce proliferation of MAIT cells in PBMC. However, cytokines associated MAIT cell activation and OLP, specifically IFN-γ, TNF-α, IL-17A, IL-18, IL-22 and IL-23, were shown to be upregulated in the presence of *Candida* effluent derived from *C. albicans* biofilm.

Preface

Work towards thesis that has been submitted for other qualifications

This PhD is a combined degree with the DCD – Oral Medicine. Submission of the PhD and successful completion is a requirement for completing the DCD – Oral Medicine

Work towards thesis that was carried out prior to enrolment in degree

In 2015 the DCD – Oral Medicine degree was changed to a combined DCD and PhD degree. As the DCD has a research requirement the research project for the DCD, considered to be a masters level project, was converted to a PhD level project. Work carried out on the PhD prior to this conversion consisted of planning the Chapter 3 experiment, successful application for ethics and an ethics amendment as well as application for funding from the Melbourne Dental School

Publications

DeAngelis L. M., Cirillo N. and McCullough M. J. (2019). "The immunopathogenesis of oral lichen planus – Is there a role for mucosal associated invariant T cells?" Oral Pathology and Medicine **48 (7)**: 552-559

Abstracts

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Abbreviations

μ	Mean
4-NQO	4-nitroquinoline-1-oxide
ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
ASM	Artificial salivary media
AV	Aloe vera
BD	Betamethasone dipropionate with optimised vehicle
CD	Cluster of differentiation
CFU	Colony forming unit
CI	Confidence interval
COX	Cyclooxygenase
CW	Calcofluor white
CXCL	C-X-C motif ligand
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorting
FEP	Fibroepithelial polyp
FFPE	Formalin fixed paraffin embedded
FoxP3	Forxhead box protein 3
GVHD	Graft versus host disease
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – antigen D related
HPV	Human papilloma virus
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IFH	Inflammatory fibrous hyperplasia
IFN-γ	Interferon gamma
IHC	Immunohistochemistry

IL	Interleukin
IL18R1	Interleukin 18 receptor 1
INR	International normalised ratio
KOH	Potassium hydroxide
LC	Langerhans cell (define in the heading or text?)
LDR	Lichenoid drug reaction
LLA	Lower limit of agreement
LLLT	Low level laser therapy
MAIT	Mucosal associated invariant T cell
MHC	Major histocompatibility complex
mIHC	Multiplex immunohistochemistry
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MR-1	Major histocompatibility complex class 1 related protein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSI	Multispectral image
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRS	Numerical rating scale
OHIP-14	Oral health impact profile-14
OLL	Oral lichenoid lesion
OLP	Oral lichen planus
OLR	Oral lichenoid reaction
OR	Odds ratio
OSCC	Oral squamous cell carcinoma
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PROMs	Patient reported outcome measures
PT	Photodynamic therapy
RA	Rheumatoid arthritis
RANTES	Regulated on activation normal T-cell expressed and secreted
RNA	Ribonucleic Acid

RR	Relative risk
SD	Standard deviation
SDA	Sabouraud dextrose agar
SLE	Systemic lupus erythematosus
TBS + BSA	Tris-buffered saline with bovine serum albumin
TBST	Tris-buffered saline with Tween 20
TGF	Transforming growth factor
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TSA	Tyramide signal amplification
ULA	Upper limit of agreement
VEGF	Vascular endothelial growth factor

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1 Literature Review

1.1 Prevalence and Appearance of Oral Lichen Planus

OLP is a chronic mucocutaneous condition that has been estimated to affect the population at rates of 1-2% (Axell, 1976, Axell and Rundquist, 1987). OLP is a condition of variable appearance that can present as reticular, plaque like, atrophic/erythematous, papular, ulcerative and bullous forms (Axell and Rundquist, 1987). A 1986 study assessed 23,616 white Americans diagnosing 3,783 oral lesions over a period of 35 years with the overall prevalence of OLP was determined to be 1.1/1,000 population,1.2/1,000 for males and 1.1/1,000 for females (Bouquot and Gorlin, 1986). Of all the clinical presentations, reticular OLP is the form most commonly seen (Axell and Rundquist, 1987). A 2005 study determined the age adjusted incidence of OLP in a Japanese population by assessing 9,536 patients, 6,340 who attended annual examinations from 1995 to 1998 (Nagao et al., 2005). Over the study period 24 cases of OLP were diagnosed; the age-adjusted incidence rate per 100,000 per years observations for OLP was 59.7 in males (95% confidence interval (CI): 7.4-112.1) and 188.0 in females (95% CI: 96.0-280.1) (Nagao et al., 2005). Of the 24 OLP cases diagnosed, five were diagnosed in males with a mean age of diagnosis of 66.4 ± 6.3 years and 19 diagnosed in females with a mean age of diagnosis of 60.5 ± 8.3 years (Nagao et al., 2005). A more recent systematic review and meta-analysis assessed the global prevalence as well as the incidence estimates of OLP from 46 observational descriptive studies (Li et al., 2020). This study estimated the overall pooled prevalence of OLP was 0.89% in the general population and 0.98% in clinical patients (Li et al., 2020). The pooled prevalence in the general population was 1.55% (95% CI: 0.83%-2.89%) for females and 1.11% (95% CI: 0.57%-2.14%) for males whilst for clinical patients the pooled prevalence in the 1.69% (95% CI: 1.05%-2.70%) for females and 1.09% (95% CI: 0.67%-1.77%) for males (Li et al., 2020). Only a small number of the studies assessed provided age distribution (n=5) with the prevalence for patients < 40 0.62% (95% CI: 0.33%-1.13%) and 1.90% for those > 40 (95% CI: 1.16%-3.10%) with an overall higher prevalence of OLP noted in non-Asian countries (Li et al., 2020).

A 2008 review critiqued studies reporting on OLP prevalence with one study deemed usable from forty-five papers. The main issue cited was a lack of clear criteria with respect to distinguishing between OLP and lichenoid reactions (McCartan and Healy, 2008). OLP

can occur anywhere in the mouth, with the buccal mucosa, tongue, gingival tissues and alveolar ridge most commonly affected (Axell and Rundquist, 1987). A 2005 study of 674 Chinese patients with OLP showed 65.9% were women and 34.1% were men; reticular OLP was the most common subtype diagnosed (51.3%), followed by erosive (39.1%) and atrophic (9.6%) forms, with the majority of patients diagnosed with OLP being under 50 years of age (Xue et al., 2005). For all subtypes, the buccal mucosa was the site most frequently affected (Xue et al., 2005). A 2009 retrospective analysis of 808 northern Italian patients reviewed over a period of 6 months to seventeen years confirmed that 61% of those diagnosed were female with a mean age of diagnosis of 61 years; for males the mean age of diagnosis was 58 (Carbone et al., 2009b). The reticular and plaque like form of OLP was the predominate clinical presentation (60%) with less than half of the patients (40%) being symptomatic and only 12.3% exhibiting extra-oral manifestations (Carbone et al., 2009b). A study of 723 OLP patients attending a dermatology outpatient clinic showed the mean age at presentation was 47 years for females and 57 years for males with age of diagnosis ranging from 13 to 82 years; the majority of patients diagnosed (75%) were female with the erosive form of the OLP being the predominate subtype diagnosed at presentation and the buccal mucosa the site most frequently affected (Eisen, 2002). A retrospective analysis of 690 British patients with OLP also confirmed that reticular OLP was the most common variant in this cohort that was 68.7% Caucasian with 95% of the lesions noted being bilateral (Ingafou et al., 2006).

OLP is usually described as bilateral lesions with Wickham striae that may or may not present on an erythematous background (Sugerman et al., 2002, Roopashree et al., 2010). A 2010 retrospective analysis of 550 Spanish patients with OLP confirmed the majority of OLP cases (76.7%) diagnosed were in females with an overall mean age of diagnosis of 56.35 ± 13.67 years with atrophic-erosive OLP being the predominant subtype diagnosed (65.3%) (Bermejo-Fenoll et al., 2010). The majority of patients diagnosed with reticular-papular OLP were asymptomatic (94.9%) whilst the majority of patients diagnosed with atrophic-erosive OLP were symptomatic (Bermejo-Fenoll et al., 2010).

OLP on the gingival tissues typically presents as a desquamative gingivitis that extends beyond the borders of the marginal gingival tissues, a factor that distinguishes it from plaque induced gingivitis. Clinically, desquamative gingivitis caused by OLP appears indistinguishable from desquamative gingivitis caused by vesiculobullous disorders. In these cases immunofluorescence, direct and indirect, can provide additional information

to assist with diagnosis (Firth et al., 1990, Kulthanan et al., 2007). Vulvovaginal-gingival syndrome is another variant of lichen planus where desquamative lesions occur on the mucosal tissues of the vulva, vagina and gingivae (Eisen, 1994). On assessment of 22 patients with vulvovaginal-gingival syndrome most oral lesions presented as erosions with erythema on the gingival tissues (16/22) or reticulated gingival lesions (6/22) with the authors suggesting patients presenting with oral lesions should be screened for lesions involving other mucosal surfaces (Eisen, 1994). Cutaneous lichen may occur in the genital region as well as flexor aspects of the ankle and wrist, along with nail involvement, scarring of the scalp and lichen planus induced hair loss (Sugerman et al., 2000b, Sugerman and Savage, 2002, Roopashree et al., 2010).

1.2 Diagnostic Criteria and Lichenoid Lesions

Cytotoxic T cell infiltration in the region of the basal lamina results in apoptosis of basal keratinocytes and basement membrane destruction; the defining factors of the OLP disease process (Sugerman et al., 2002). The diagnostic criteria for OLP was defined by the World Health Organisation (WHO) in 1978 (WHO, 1978). This criteria specified clinical and histopathological guidelines to diagnose OLP with diagnosis requiring the presence of a reticular pattern, plaque, papules, annular or atrophic lesions with or without erosions that may contain bullae as well as ortho or parakeratinsation, Civatte bodies, liquefactive degeneration and a band like infiltrate confined to the superficial connective tissue consisting mainly of lymphocytes (WHO, 1978). Subsequently, a modified WHO diagnostic criteria was developed to help define the histopathological differences between OLP and oral lichenoid lesions (OLL) so as to aid in correct diagnosis and differentiation between the two conditions (van der Meij and van der Waal, 2003). This new criteria incorporated the original criteria for diagnosis but was stricter with regards to the need bilateral clinical presentation and both the clinical and histopathological criteria needing to be met to diagnose OLP with any variation resulting in a diagnosis of OLL (van der Meij and van der Waal, 2003). A recent study has further suggested modification to this diagnostic criteria including a checklist for clinical history and clinical presentation to be provided to the pathologist with the biopsy sample with the proposed checklist including information relating to history of systemic lupus erythematosus (SLE), graft versus host disease (GVDH), liver disease, use of tobacco or oral lesions that correlate with the commencement of medication (Cheng et al., 2016). This modified criterion would provide the pathologist with the clinical information required to exclude other conditions systemic conditions, such as SLE and GVHD that can mimic OLP, whilst also alerting the pathologist to habits that may explain pathological findings.

OLL are a distinct form of oral lesions that can resemble OLP both clinically and histopathologically. These lesions encompass lichenoid drug reactions (LDR) and OLL that form as a result of contact sensitivity, most commonly as a result of amalgam sensitivity (Koch and Bahmer, 1995, McCartan and McCreary, 1997, Thornhill et al., 2003, Issa et al., 2004, Ostman et al., 1996). When OLL occur due to dental materials, the oral lesions will form adjacent to the dental material. Allergens postulated to be involved in OLL as a result of contact sensitivity to amalgam include mercury, copper, metal zinc, palladium, silver, amalgam alloying and gold (Koch and Bahmer, 1995, Suter

and Warnakulasuriya, 2015). Patients presenting with OLL as a result of amalgam may represent true delayed hypersensitivity that forms as a result of release of metal haptans from the dental restoration (Laine et al., 1999).

For OLL resulting from contact with dental materials, patch testing can be considered for the assessment of contact sensitivity (Suter and Warnakulasuriya, 2015). However, a systematic review published in 2004 concluded that patch testing for OLL appeared to be of limited diagnostic value (Issa et al., 2004). In cases of a suspected OLL due to amalgam dental restorations, evidence exists that supports prophylactic restoration replacement as improvement may occur irrespective of a positive patch test result (Laine et al., 1992, Ostman et al., 1996, Thornhill et al., 2003, Wong and Freeman, 2003, Issa et al., 2004).

Microbes have also been implicated in the development of OLR with one study comparing OLR on the lower labial mucosa (n=25) with similar OLP lesions on the buccal mucosa (n=22) (Backman and Jontell, 2007). 22 LDR patients treated with chlorhexadine showed lesion resolution with treatment in 80% of cases indicating a microbial association with lesions potentially being triggered by plaque deposits on the anterior teeth (Backman and Jontell, 2007).

True LDR associated lesions will arise in direct temporal association following ingestion of a medication. Medications associated with the development of LDR include non-steroidal anti-inflammatory medications, penicillamine, anti-malarial medications and anti-hypertensive medications (Savage, 1958, Hay and Reade, 1978, Powell et al., 1983, Bagan et al., 2004). No specific diagnostic criteria currently exist for LDR. However, demonstrated resolution with withdrawal and recurrence with re-exposure to the causative medication can be considered diagnostic of an LDR. Recent systemic review on LDR concluded that no strong evidence currently exists to support causal relationships between the drug and lichenoid lesions in the oral cavity with the review showing standard protocol was not used in any of the cases to conclusively prove a causal relationship (Fortuna et al., 2017). Thus, in cases of lichenoid lesions it is paramount to ensure a correct diagnosis is made as OLP, OLL and LDR require different management protocols.

1.3 Cost of Managing Oral Lichen Planus

One significant consideration for OLP is the financial burden OLP places on both the public health system and the patient. The cost of OLP illness was estimated in a cross sectional study that assessed 100 OLP patients diagnosed with either mild (treated with medications) or severe (treated with both systemic and topical medications) disease (Ni Riordain et al., 2016). Costing was determined based on the number of visits to the Eastman Dental Hospital, medical and general dental practitioner, as well as cost of medications required per annum and number of biopsies performed (Ni Riordain et al., 2016) Overall, 2.64 appointments were attended by patients per year at an average yearly cost per patient per year of $US579.53 (£385.58) (Ni Riordain et al., 2016). To manage mild cases of OLP $US438.01 (£301.04) was required per patient per year whilst for severe OLP the cost per patient per year equate to $US964.67 (£663) (Ni Riordain et al., 2016). For an assumed prevalence of 1%, diagnosis and management of OLP would equate per year to 1.4-3.1 billion in the US alone.

1.4 Immunology Theories of Pathogenesis

Much is unknown about the pathogenesis and aetiology of OLP. The oral cavity has the capacity to generate an immune response that consists of both adaptive and innate components. The innate component lacks immunologic memory (Delves and Roitt, 2000) and consists of barrier defences and saliva. The adaptive immune remembers past microbial challenges and tailors the immune response to the pathogen. The main cells involved in the adaptive immune response are T lymphocytes, B lymphocytes and antigen presenting cells. The adaptive immune system also uses major histocompatibility complex (MHC) class I and II molecules, to generate the targeted immune response as well as distinguish self for non-self (Delves and Roitt, 2000).

OLP is characterised by a basal cell degeneration mediated by a chronic T cell inflammatory process. Histologically, OLP is characterised by vaculolar degeneration, parakeratosis or hyperkeratosis and saw tooth rete ridges (Krutchkoff et al., 1978, Sugerman et al., 1993, Sugerman et al., 2000a, Sugerman et al., 2002, Zhou et al., 2002).

The mechanism that results in the chronic disease state of OLP has not yet been elucidated. Below is an outline of those factors that may be involved in the aetiopathogenesis of OLP, specifically, matrixmetalloprotinases (MMP), T lymphocyte migration, mast cells, Langerhans cells (LC), macrophages, regulatory T cells, as well as chemokine and cytokine dysregulation.

1.4.1 Matrixmetalloprotinases

MMP are a family of proteinases implicated heavily in OLP pathogenesis as a potential cause basal lamina destruction (Rubaci et al., 2012). Specifically, MMP-2, MMP-7 (Rubaci et al., 2012), tissue inhibitors of metalloproteinases, MMP-10, MMP-3 and MMP-9 (Zhou et al., 2001, Roopashree et al., 2010). These proteins can cleave laminin and collagen type IV, proteins that are the major constituents of the basement membrane. An assessment of the *in vitro* rate of MMP-9 showed activation to be significantly higher in lesional T cells compared to peripheral blood cells (Zhou et al., 2001). These authors concluded that T cell derived MMP-9 may be involved in the pathogenesis of OLP and that specifically, basement membrane disruption may be the result

of MMP-9 facilitating both the migration of cytotoxic T cells and basal keratinocyte apoptosis (Zhou et al., 2001). The OLP disease state is ultimately a delicate balance between keratinocyte apoptosis, triggered by infiltrating cytotoxic T cells, and T cell

apoptosis triggered by resident keratinocytes (Roopashree et al., 2010, Sugerman et al., 2002).

1.4.2 T Lymphocyte Migration

T cells are the driving force of cell mediated immunity. The two main variants of T cells are helper T cells ($CD4^+$) that recognise MHC class II and aid in coordinating the adaptive immune response, and cytotoxic T cells ($CD8^+$) that recognise MHC class I and kill infected cells (Delves and Roitt, 2000, Vignali et al., 2008). Other T cell variants include regulatory T cells that have a suppressive role and are essential for limiting chronic inflammation and preventing autoimmunity, natural killer T cells, and memory T cells (Delves and Roitt, 2000). OLP is characterised by T cytotoxic cell infiltration within the superficial lamina propria with the cytotoxic T cell response directed at the basal keratinocytes (Sugerman et al., 2002, Roopashree et al., 2010).

Lesional and non-lesional T cell lines were cultured from one patient with cutaneous lichen planus (Sugerman et al., 2000a). Autologous keratinocytes were also cultured from the same patients from both lesional and non-lesional skin (Sugerman et al., 2000a). Results of this study showed lesional T cell clones demonstrated more cytotoxic activity against lesional keratinocytes than non-lesional T cell clones (Sugerman et al., 2000a). Interestingly, the majority of cytotoxic activity demonstrated from the lesional skin was $CD8^+$ mediated, in comparison to non-lesional skin which was $CD4^+$ mediated (Sugerman et al., 2000a). These results support the hypothesis of $CD8^+$ T cells being involved in the OLP disease process and that these cells may be reacting to an unknown antigen that is associated with MHC class I on keratinocytes (Sugerman et al., 2000a).

Significantly higher numbers of intraepithelial $CD8^+$ cells have also been noted in the basement membrane disruption zone compared to regions where the basement membrane was intact ($p < 0.05$) (Zhou et al., 2002). This chronic inflammatory process directed at the basal lamina zone is what defines OLP and many theories have been postulated as to how these $CD8^+$ cells enter the epithelium.

Two hypotheses have been proposed for T lymphocyte migration into the oral epithelium during OLP (Sugerman et al., 2002):

- Chance encounter hypothesis – $CD8^+$ cytotoxic T cells may be present in the oral epithelium during routine surveillance and encounter an unknown antigen to initiate the T cell response

- Directed migration hypothesis – cytokines are secreted directly by the oral keratinocytes and these secreted cytokines initiate and direct migration of T cells to the epithelium

Once T cells have migrated into the epithelium keratinocyte apoptosis will be initiated. One of the proposed mechanisms of keratinocyte apoptosis includes T cell release of TNF-α binding to the TNF-α R1 receptor on the keratinocyte surface. The T cell subsequently binds Fas on the keratinocyte surface to release granzyme B that enters the keratinocyte to activate the capsade cascade and induce keratinocyte apoptosis (Sugerman et al., 2002, Roopashree et al., 2010, Lavanya et al., 2011, Payeras et al., 2013).

1.4.3 Mast Cells

Mast cells have also been implicated in the pathogenesis of OLP with increased numbers of these cells detected in OLP (Zhao et al., 1997). It has been hypothesised that basement membrane disruption is facilitated by mast cells and this disruption allows the infiltration of cytotoxic T cells into the oral epithelium (Zhou et al., 2002). Zhao et al., 2001 showed that only 20% of mast cells were degranulated in controls compared to 60% in OLP. This same study demonstrated that OLP lesional T cells were able to produce and secrete regulated on activation normal T cell expressed and secreted (RANTES), a chemokine belonging to the CC chemokine family (Zhao et al., 2001). The effect of RANTES secretion was mast cell degranulation resulting in release of TNF- α (Zhao et al., 2001). Release of TNF- α upregulated the OLP lesional T cells causing T cell mediated keratinocyte apoptosis (Zhao et al., 2001). This cyclical mechanism of chronic inflammation process is what defines the OLP disease process.

Ramalingam et al., 2018 quantified mast cells in histopathological sections of OLP and oral lichenoid reaction (OLR) in an attempt to distinguish between the two lesions. OLR was defined as OLP like lesions that could encompass GVHD, OLL and LDRs (Ramalingam et al., 2018). This study included 21 cases of OLP, 21 cases of OLR and 10 normal mucosal control specimens (Ramalingam et al., 2018). Results showed significantly higher numbers of mast cells were present in OLP compared to OLR and normal mucosa ($p = 0.001$) (Ramalingam et al., 2018). Further, intergroup analysis demonstrated that both OLP and OLR had significantly higher numbers of mast cells when compared to control mucosa (Ramalingam et al., 2018). The authors proposed that mast cells count could aid in the differentiation between OLP and OLR (Ramalingam et al., 2018).

1.4.4 Langerhans Cells

LC have also been heavily implicated in the pathogenesis of OLP. LC are antigen presenting cells that reside in the epidermis. Within the oral mucosa LC are primarily found within the non-keratinised epithelium (Daniels, 1984). Higher numbers of LC have been shown in OLP compared to healthy control patients (Gustafson et al., 2007, Gueiros et al., 2012). Increased numbers of LC in OLP could be suggestive of a pathogenic role for the adaptive immunity in OLP (Gustafson et al., 2007, Gueiros et al., 2012). A study by Regezi et al., 1985 undertook immunohistochemistry (IHC) analyses utilizing S-100, a protein to identify LC, and a monoclonal antibody to human leukocyte antigen – antigen D related (HLA-DR), an antigen known to be involved with cell mediated immune reactions. This study concluded that LC expressed HLA-DR and S-100 more intensely in active OLP affected areas (Regezi et al., 1985).

A similar study assessing LC in OLP utilized IHC on 18 OLP and 10 healthy control patient biopsy material and stained for $CD4^+$, $CD8^+$ and LC/dendritic cells ($CD1a^+$) (Villarroel Dorrego et al., 2002). Immunostaining was also undertaken for integrin leukocyte function associated antigen (LFA-1), which is involved in leukocyte adhesion to the endothelium, vascular adhesion molecule one and ligand intercellular adhesion molecule one that both work to increase the interaction between cells, the endothelium and inflammatory sites (Villarroel Dorrego et al., 2002). Results of this study showed increased $CD4^+$, $CD8^+$ and $CD1a^+$ cells in OLP (Villarroel Dorrego et al., 2002). $CD4^+$ and $CD8^+$ cells were shown to express LFA-1 while both vascular adhesion molecule one and ligand intercellular adhesion molecule one were shown to be significantly higher, $p < 0.01$ and $p < 0.05$ respectively, in OLP patients compared to controls (Villarroel Dorrego et al., 2002). These authors suggested LC may be responding a currently unknown antigen that exists within the basal keratinocytes that triggers activation of LC and antigen presentation to $CD4^+$ cells that utilise adhesion molecules to promote $CD8^+$ induced epithelial destruction (Villarroel Dorrego et al., 2002).

1.4.5 Macrophages

Macrophages are involved in antigen processing and phagocytosis with abundance of these cells noted in OLP affected tissue (Regezi et al., 1985). Both macrophage distribution and presence has been assessed in tissue utilising 15 specimens from non-ulcerated OLP (Matthews et al., 1985). Macrophages were identified using lysozyme and α_1 antitrypsin with all specimens showing accumulation of cells positive for both

lysozyme and α_1 antitrypsin around the basement membrane (Matthews et al., 1985). Positivity counts were higher for lysozyme compared to α_1 antitrypsin with distribution occurring in similar tissue compartments (Matthews et al., 1985). 60% of positive cells were found to be present within the 125µm epithelial/sub-epithelial junctional zone, the area of epithelial cell destruction, supporting the hypothesis that macrophages may contributed to OLP pathogenesis (Matthews et al., 1985).

1.4.6 Regulatory T Cells

Regulatory T cells have also been hypothesised to play a role in OLP pathogenesis. Regulatory T cell development is dependent on expression of Forkhead box protein 3 (FoxP3) with FoxP3. In mice the $CD25^+CD4^+$ regulatory T cell population has been shown to predominately express FoxP3 (Hori et al., 2017). Using retroviral gene transfer of FoxP3 to naïve T cells the authors showed that FoxP3 converted the naive T cells in mice to a phenotype functionally similar naturally occurring $CD25^+CD4^+$ regulatory T cells (Hori et al., 2017). Results of this study suggested the FoxP3 gene may be an important regulator for the developmental differentiation of thymus and periphery regulatory T cells (Hori et al., 2017).

In OLP, the potential role of $FoxP3^+$ T cells in pathogenesis has been assessed. One study in 2010 utilised assessed the number of $FoxP3^+$ T regulatory cells in 23 OLP lesions, 7 reticular and 13 erythematous/erosive, and 12 controls using IHC. Results demonstrated fewer $FoxP3^+$ T regulatory cells in control tissue compared to OLP tissue where cells identified as $FoxP3^+$ T regulatory cells were found in abundance in the lamina propria (Tao et al., 2010). A negative correlation between the density of $FoxP3^+$ T cells and disease activity scores was also noted with significantly higher frequencies $FoxP3^+$ T regulatory cells detected in reticular OLP compared to the erythematous/erosive OLP (Tao et al., 2010). The data suggested $FoxP3^+$ T regulatory cells may be important contributors to OLP pathogenesis and that $FoxP3^+$ T regulatory numbers significantly correlate with both disease activity and OLP subtype (Tao et al., 2010).

A further study in 2012 assessed the presence of $FoxP3^+$ T regulatory cells utilising IHC in 10 cases of inflammatory fibrous hyperplasia (IFH) and 32 cases of OLP, 15 erosive and 17 reticular (Pereira et al., 2012). Reticular OLP exhibited a significantly higher epithelial thickness when compared to erosive OLP (Pereira et al., 2012). With regards to $FoxP3^+$ juxta-epithelial cells, the only significant difference that existed between the different lesions was between the IFH group and all cases of OLP ($p=0.019$) (Pereira et

al., 2012). The increased presence of $FoxP3^+$ T regulatory cells in OLP when compared to IFH could allude to a difference in aetiopathogenesis between the two conditions (Pereira et al., 2012).

A more recent 2016 study further investigated the role of impaired $CD4^+CD25^+$ regulatory T cells in OLP collecting venous blood from 40 OLP patients, 20 reticular and 20 erythematous/erosive OLP, and 22 healthy controls (Zhou et al., 2016). The authors further assessed the expression of FoxP3, transforming growth factor (TGF) β and IL-10, mediators involved in T regulatory immunosuppressive function as well as the frequency proinflammatory IL-17A and IFN-γ producing regulatory T cells (Zhou et al., 2016). In regulatory T cells FoxP3 protein levels were shown to be significantly elevated in both erythematous/erosive and reticular OLP compared to controls, with significantly elevated expression of FoxP3 messenger ribonucleic acid (mRNA) noted in both OLP cohorts when compared to controls (Zhou et al., 2016). Conversely, TGF-β mRNA expression was shown to be significantly lower in OLP compared to controls, with serum levels of TGF-β following the same trend. Interestingly, when compared to control percentages of $CD4^+FoxP3^+IL\text{-}17^+$ cells were shown to significantly higher the OLP cohort. The suppressive function of $CD4^+CD25^+$ was shown to be significantly impaired in the reticular OLP cohort with overall results supportive of the hypothesis that regulatory T cell functional impairments may be involved in the pathogenesis of OLP (Zhou et al., 2016).

In addition another recent 2016 study utilised IHC to assess T regulatory ($FoxP3^+$) and Th-17 ($IL\text{-}17A^+$) cells in 9 non-specific inflammation tissue and 10 OLP samples (Javvadi et al., 2016). OLP exhibited significantly higher numbers of $FoxP3^+$ cells whilst significantly higher frequencies of $IL\text{-}17A^+$ cells were detected in non-specific inflammatory tissue (p=0.021) (Javvadi et al., 2016). In OLP, $FoxP3^+$ cells were present at significantly higher frequency within the inflammatory infiltrate of the superficial connective tissue with the FoxP3 gene also being significantly up-regulated in the OLP; no such changes were noted with IL-17A expression (Javvadi et al., 2016). Based on these results the authors concluded $FoxP3^+$ cells potentially play a more prominent role OLP pathogenesis than $IL\text{-}17A^+$ cells (Javvadi et al., 2016).

A further 2016 study used IHC and double immunofluorescent staining, to identify $FoxP3^+$ cell subsets, examine functional phenotypes and determine if differing levels of disease resulted in differing numbers of $FoxP3^+$ cells (Schreurs et al., 2016). Atrophic

OLP exhibited the greatest frequency of $FoxP3^{+}CD4^{+}$ T cells with ulcerative OLP exhibiting the lowest frequency and a non-suppressive phenotype being the main phenotype observed in the $FoxP3^{+}CD4^{+}$ population; the suppressive phenotype was observed to be twice as high in reticular OLP when compared to atrophic OLP (Schreurs et al., 2016). Interestingly, the majority of $FoxP3^{+}CD4^{+}$ T cells were shown to express T-bet, a IFN-γ hallmark transcription factor that suggests these cells had an inherent capability to enhance inflammation, a factor that could explain the chronic nature of OLP (Schreurs et al., 2016).

Taken together these studies show $FoxP3^{+}$ is an important marker for the identification of regulatory T cells, cells that are important regulators of the immune response. Higher numbers of these cells found in OLP could be suggestive of a role in OLP pathogenesis. Interestingly, some studies have shown higher numbers of these cells in less active forms of OLP, reticular and atrophic (Tao et al., 2010, Schreurs et al., 2016) suggesting a decrease in regulatory T cells correlates with increased OLP disease activity. Furthermore, impaired suppressive function of regulatory T cells in OLP or a capacity for regulatory T cells to enhance inflammation (Schreurs et al., 2016, Zhou et al., 2016) may in part explain the ongoing T cell mediated inflammatory process that defines OLP.

1.4.7 Chemokine and Cytokine Dysregulation

Dysregulation within the cytokine and chemokine pathways has been postulated to play an important role in the immunopathogenesis of OLP. A study collected serum from 30 OLP and healthy control patients to evaluate the Th1/Th2 cytokine profile by measuring IL-2, IL-4, IL-5, IL-10, TNF-α and INF-γ (Pekiner et al., 2012). No statistically significant differences in the levels of IL-4, IL5, TNF-α and IFN-γ between OLP patients and controls were noted (Pekiner et al., 2012). However, IL-2 showed a significantly decreased tendency in OLP patients ($p < 0.05$) whilst IL-10 showed a significantly increased tendency in OLP patients ($p < 0.01$) compared to healthy controls (Pekiner et al., 2012). OLP lesions were classified as either reticular, erosive or bullous OLP and differences in the Th1/Th2 cytokine profiles similarly showed that serum levels of IL-2 were significantly decreased in reticular and erosive OLP patients compared to healthy controls ($p<0.05$), conversely a tendency for significantly increased IL-10 was demonstrated in reticular and erosive OLP compared to controls (Pekiner et al., 2012). The Th1 cells play an important role in cell-mediated immunity while Th2 cells are critical for humoral mediated immunity. The increase in IL-10 and

decrease in IL-2 in OLP is a suggestive of an imbalance towards a Th2 response (Pekiner et al., 2012). The authors suggested the results, specifically the shift towards Th2, was indicative of OLP being the result of a true delayed hypersensitivity (Pekiner et al., 2012).

Ichimura et al., 2006 used deoxyribonucleic acid (DNA) microarray analysis to determine chemokine expression and chemokine receptors in the epithelial cell layers. Biopsy specimens were obtained from the gingival tissue of 3 orthodontic patients undergoing third molar removal and the lesional lingual gingival tissue of 3 OLP patients without any systemic or periodontal disease (Ichimura et al., 2006). Levels of chemokine ligand 20 and its receptor chemokine receptor 6 were elevated in lesional OLP epithelium (Ichimura et al., 2006). LC expressing high levels of Langerin^{+} were detected in the epithelium of OLP suggesting the chemokine receptor 6 pathway is responsible for mediating infiltration of these cells (Ichimura et al., 2006). Within the OLP epithelial cell layers significantly higher expression of chemokine (C-X-C) motif ligand 9 (CXCL9), CXCL10, CXCL11 and chemokine ligand 5 was demonstrated in comparison to control epithelium (Ichimura et al., 2006). Three of these, CXCL9, CXCL10, and CXCL11, are specific for CXCR3, a chemokine receptor and chemokine ligand 5, the ligand of chemokine receptor 5 (Ichimura et al., 2006). Both chemokine receptor 5 and CXCR3 are selectively expressed on Th1 T cells suggesting infiltration of T cells in OLP is mediated through chemokine receptor 5 and CXCR3 (Ichimura et al., 2006).

In a recent study by Marshall et al., 2017b it was shown that constitutive expression of CD86 and CD40 is low in oral keratinocytes, specifically three strains of primary normal oral keratinocyte and the H357 squamous cell carcinoma cell line. Results of this study showed expression of these keratinocytes was enhanced by IFN-γ stimulation (Marshall et al., 2017b). Use of IHC to further evaluate the involvement of CD40 in OLP showing intense staining of CD40 in OLP tissues (Marshall et al., 2017b). Overall, this study shows both CD40 and CD86 potentially play an important pathophysiologic role in oral inflammatory diseases like OLP (Marshall et al., 2017b).

Moreover, production of keratinocyte CXCL 9/10/11 was assessed under basal and inflammatory conditions to determine the of pathological role of these chemokines in OLP by utilising semi-quantitative polymerase chain reaction (PCR), enzyme linked immunosorbent assay (ELISA), chemotaxis assays, and fluorescence-activated cell sorting (FACS) (Marshall et al., 2017a). CXCL 9/10/11 are chemokines induced by IFN-

γ that functions as a chemoattractant for activated T cells. This study demonstrated that levels of CXCL 9/10/11 were significantly higher in OLP compared to normal oral mucosa, demonstrating the ability of normal oral keratinocytes to produce chemotactic molecules and mediate T cell recruitment (Marshall et al., 2017a). This study further demonstrates a potential key role of for chemokine production in oral keratinocytes in OLP pathophysiology (Marshall et al., 2017a).

An assessment of CXCL9 and CXCL10 antimicrobial activity on oral microflora and the oral keratinocyte expression profiles was determined following exposure to both inflammatory and infectious stimuli (Marshall et al., 2016). *S. sanguinis*, a common oral microbe was used to stimulate the keratinocytes along with *E. coli* as the positive control (Marshall et al., 2016). It was shown that these oral epithelium derived chemokines, particularly CXCL9, demonstrated antimicrobial activity against *E. coli* and *S. sanguinis* (Marshall et al., 2016). Results also demonstrated that up-regulation of CXCL9/10 as a result of bacterial and inflammatory stimulation could be important in host-defence mechanisms as well as oral bacterial homeostasis and colonisation (Marshall et al., 2016). Thus, it could be hypothesised that dysregulation of the innate immunity is involved in the pathogenesis of OLP and that defence against the oral microflora could be critical to this dysregulation.

Expression of IL-22 and IL-23 was assessed in 80 cases of lichen planus, 38 cutaneous lichen planus and 42 OLP cases, along with 20 normal control samples, 10 samples from skin and 10 from oral mucosa (Chen et al., 2013). IL-23 is a cytokine first discovered in the year 2000 that shares the p40 subunit with IL-12 (Oppmann et al., 2000). Cellular expression of both cytokines was significantly higher both subepithelial and epithelial regions of lichen planus tissue compared to control (Chen et al., 2013). Expression of subepithelial IL-22 as well as epithelial and subepithelial IL-23 was significantly elevated in OLP compared to cutaneous lichen planus (Chen et al., 2013). Higher expression of these cytokines in OLP suggests Th-22 cells play an important role in oral mucosal defence against pathogenic microbes (Chen et al., 2013). A preliminary study comparing IL-22 expression in oral biopsies from OLP patients (n = 50) and normal oral mucosa (n = 19) by RT-qPCR and Western blot sh IL-22 mRNA was significantly increased in OLP when compared to controls (Shen et al., 2016). Assessment of miR-562 and miR-203, micro ribonucleic acids (miRNA) thought to be potential targets for IL-22, were also assessed (Shen et al., 2016). Results showed aberrant expression of miR-562 and miR-

203 was associated with high levels of IL-22 expression in HEK293 cells (Shen et al., 2016). The authors concluded there may be a potential role for IL-22 and the miRNA's associated with IL-22 in the pathogenesis of OLP (Shen et al., 2016).

The IL-17/IL-23 axis has also been studied in OLP with this axis thought to be involved in chronic inflammatory and immune disorders (Lu et al., 2014). Tissue samples collected from 14 reticular OLP, 13 erosive OLP and 10 normal mucosa controls and blood from 10/14 reticular OLP patients was used to assess the IL-17/IL-23 axis (Lu and Zeng, 2014). Results demonstrated an overexpression of IL-17 and IL-23 in OLP lesions compared to controls, suggesting a regulatory role for the IL-17/IL-23 axis in OLP (Lu et al., 2014). Monteiro et al., 2015 analysed the immunohistochemical expression of IL-17 and IL-23, to determine if differences in expression existed between reticular and erosive forms of OLP. 41 cases of OLP were analysed, 23 reticular and 18 erosive, along with 10 cases of IFH (Monteiro et al., 2015). No significant differences existed in the number of IL-17^{+} and IL-23^{+} lymphocytes detected in OLP and IFH samples (Monteiro et al., 2015). The number of IL-23^{+} lymphocytes was significantly higher erosive OLP compared to both the reticular OLP and IFH (Monteiro et al., 2015). Epithelial immunopositivity to IL-17 and IL-23 was also assessed with significantly higher levels noted in the OLP group for both IL-17 and IL-23 when compared to the IFH ($p = 0.12$ and $p = 0.011$ respectively) (Monteiro et al., 2015). The authors concluded that the above results supported a role for CD4^{+} Th17 cells and autoimmunity in the pathogenesis of OLP (Monteiro et al., 2015).

The expression of IL-17 and IL-23 in serum from patients with OLP alone, chronic periodontitis alone, patients with both OLP and chronic periodontitis and healthy controls has recently been reported with results showing that IL-17 levels were significantly elevated in the OLP patients with chronic periodontitis compared to both the OLP and healthy control group ($p < 0.05$ and $p < 0.01$ respectively) (Wang et al., 2013). Serum IL-23 levels were also significantly increased in the OLP patients with chronic periodontitis when compared to healthy controls ($p < 0.01$) (Wang et al., 2013). Elevated levels of IL-17 were noted in the female OLP patients with chronic periodontitis and erosive OLP (Wang et al., 2013). Interestingly, serum levels of IL-17 in the erosive OLP with chronic periodontitis group significantly correlated with plaque index and probing depths (Wang et al., 2013). These results suggest that IL-17 may play a potential role in the pathogenesis of OLP patients with chronic periodontitis, especially in females or patients with an erosive form of OLP (Wang et al., 2013).

The role of Th17 in two subtypes of OLP, reticular and erosive, and healthy controls was assessed by measuring IL-17 and IL-23 using ELISA with the role of microbial infection in the pathogenesis of OLP determined by utilisation of saliva to profile the microbial communities all cohorts (Wang et al., 2015). This study showed that the bacterial populations were significantly less rich in reticular ($p = 0.02$) and erosive ($p = 0.01$) OLP when compared to controls (Wang et al., 2015). No significant differences existed in salivary IL-17 concentrations between the reticular OLP group and healthy controls while IL-17 concentrations were significantly higher in the erosive OLP group when compared to controls and the reticular OLP group (Wang et al., 2015). Results of this study confirmed that microbial richness and diversity was negatively correlated with IL-17 concentration in saliva (Wang et al., 2015). These authors concluded that this mechanism should be further explored in future studies (Wang et al., 2015).

More recently the Th9 subset of $CD4^+$ cells (defined as $IL\text{-}9^+$ $IL\text{-}17^+$) has been assessed along with the Th17 subset of $CD4^+$ cells (defined as $IL\text{-}9^-$ $IL\text{-}17^+$) in OLP (Wang et al., 2017). Using flow cytometry peripheral blood from 41 OLP patients and 18 healthy controls was assessed for these subsets with significantly elevated levels of Th9 cells shown in both reticular and erosive OLP compared to controls (Wang et al., 2017). Th9 expression dominated in reticular OLP whilst Th17 expression was dominated in erosive OLP suggesting an immunopathogenic role for these subsets reticular and erosive OLP (Wang et al., 2017). Following on from this study the same author collected oral mucosa from 18 OLP, 9 reticular and 9 erosive, and 16 healthy controls to assess the role of Th9/IL-9 synergism with IL-17 and resultant production of MMP-9 (Wang et al., 2018). Quantitative PCR demonstrated that mRNA levels of IL-9 and MMP-9 were significantly elevated in of erosive OLP (Wang et al., 2018). Oral keratinocytes were co-cultured with $CD4^+$ T cells with significant elevations observed in Th9 and Th17 subsets noted and analysis of supernatant with ELISA demonstrating increased IL-9, IL-17 and MMP-9 (Wang et al., 2018). Stimulation of cultured $CD4^+$ T cells from OLP patients with IL-9 elevated the number of Th17 cells and stimulation of the Th17 cells with IL-9 resulted in elevated expression of MMP-9 (Wang et al., 2018). Increased levels of Th9/IL-9 and increased production of MMP-9 through Th17 synergism with IL-9 suggests a pathogenic role of the Th9 subset in OLP (Wang et al., 2018).

The role of IL-18 has also been assessed OLP. Zhang et al., 2012 measured both serum and salivary IL-18 in 103 Chinese patients, 50 with erosive and 53 with non-erosive OLP,

and 48 matched controls. In OLP, IL-18 levels were shown to be significantly higher in both serum and saliva when compared to controls and within the OLP cohort. Levels of IL-18 were shown to be significantly higher erosive forms of OLP compared to the non-erosive form (Zhang et al., 2012). The results suggest a potential role for IL-18 in OLP pathogenesis with IL-18 serum and salivary production potentially linked with an immune response to normal oral microbes.

Single nucleotide polymorphisms and haplotypes in the IL-18 gene in a cohort of 151 ethnic Chinese patients with OLP and 143 Chinese healthy controls showed significant differences in the *IL18*-607 genotype specifically, significantly higher frequencies of the CC genotype in OLP patients when compared to controls (Bai et al., 2007). The -137G/C polymorphism found in the IL-18 promoter region was also statistically associated with the erosive subtype of OLP (Bai et al., 2007). Analysis of haplotypes I-IV in the -607 and -137 polymorphisms showed significantly higher frequencies of haplotypes I and II in OLP patients when compared to controls whilst haplotype IV showed a significantly higher prevalence in control patients (Bai et al., 2007). Within this same study the authors also assessed serum levels of IL-18 in 30 OLP patients, with either erosive or non-erosive subtypes, and 30 controls. Both erosive and non-erosive OLP patients showed significantly elevated serum levels of IL-18 when compared to controls with the -137GG polymorphism noted in 21/30 OLP patients (Bai et al., 2007). Based on the findings it was concluded that the identified *IL18* polymorphisms may play a role in upregulation of IL-18 and the pathogenesis of OLP in this ethnic Chinese cohort (Bai et al., 2007).

1.5 Autoantigen and Other Theories of Pathogenesis

OLP shares many characteristics with autoimmune conditions including such as late onset, female predilection, chronicity, and T cell mediated reaction. Due to this, it has been theorised that the origin of OLP may be due to autoimmunity (Sugerman et al., 2002, Roopashree et al., 2010). However, OLP is not considered to be a true autoimmune condition as no auto-antigen responsible for initiation of the OLP disease process has been discovered. Whilst no auto-antigen has been discovered, many potential auto-antigens have been suggested as the autoimmune trigger. Potential auto-antigen candidates for OLP include heat shock protein (HSP), (Sugerman et al., 2002, Sugerman and Savage, 2002, Garcia-Garcia et al., 2013), hepatitis C (Carrozzo and Gandolfo, 2003, Carrozzo, 2008), human papillomavirus (HPV) (Syrjanen et al., 2011), Epstein Barr virus (Raybaud et al., 2018) with genetics, and stress comprising other theories of pathogenesis (Chaudhary, 2004, Ivanovski et al., 2005).

1.5.1 Heat Shock Protein

HSP are formed under normal conditions with the production of HSP increased during times of stress to prevent cellular mortality (Bayramgurler et al., 2004). HSP are also expressed conservatively in healthy cells and play an important role in normal biological processes, including the control of apoptosis, wound healing/repair and protection against ultraviolet damage (Bayramgurler et al., 2004). Assessment of the presence of HSP in cutaneous lichen planus and OLP has been undertaken using formalin-fixed paraffin embedded (FFPE) skin biopsy specimens from 39 cutaneous lichen planus, 20 psoriasis and 20 healthy control patients, assessing these for the presence of HSP 60 and 70 using IHC (Bayramgurler et al., 2004). Significantly altered levels of HSP were found in cutaneous lichen planus lesions compared to normal skin and psoriasis, supporting the theory that HSP may play a role in the pathogenesis of cutaneous lichen planus (Bayramgurler et al., 2004).

HSP 27 is a HSP present in the cytoplasm of many cells and is implicated in many biologic events, including the protection of cells from oxidative stress (Garcia-Garcia et al., 2013). In an evaluation of the presence of HSP 27 in different stages of OLP lesions, an analyses of 36 biopsy specimens from OLP patients and 10 healthy control used IHC, to assess HSP 27 staining intensity (Garcia-Garcia et al., 2013). Immunohistochemical findings showed increased expression of HSP 27 in the basal layer of OLP deemed active, moderately active atrophic, mild or inactive atrophic OLP while decreased expression

was observed in the superficial epithelium of all OLP groups when compared to controls (Garcia-Garcia et al., 2013). This study supports the theory that HSP 27 plays a role in the pathogenesis of OLP (Garcia-Garcia et al., 2013).

However, several studies do not support the role of HSP in the pathogenesis of OLP. In particular, an investigation of the expression of HSP 70 in OLP and oral non-dysplastic leukoplakia, showed no association between HSP 70 overexpression and the clinical presentation of OLP (Seoane et al., 2004). No statistically significant differences were noted in HSP 70 expression between OLP and normal mucosa specimens, although HSP 70 expression was significantly higher in the non-dysplastic leukoplakia specimens when compared to the OLP specimens (Seoane et al., 2004). These authors concluded that HSP 70 did not play an obvious role in the pathogenesis of OLP and the significantly higher expressions noted in non-dysplastic leukoplakia may be due to alterations of cellular activity or cellular proliferation (Seoane et al., 2004).

Further, an IHC analyses of HSP 27, 60, 70 and 90 expression, as well as the expression of the γδ T-cell receptor, a receptor thought to be involved in autologous HSP reactions, and CD3, in 22 OLP and 17 normal mucosa biopsy showed that normal mucosa expressed HSP 27, 60, 70 and 90 with the presence of few γδ T cells (Bramanti et al., 1995). In 6 of the 22 cases of OLP there was intense basal staining for HSP 27 with positive HSP 27 staining evident in all cases of OLP (Bramanti et al., 1995). HSP 70 staining was reduced in OLP compared to controls and confined only to the basal keratinocytes. Further, all cases of HSP 60 staining was confined to lower levels of keratinocytes (Bramanti et al., 1995). Whilst expression of HSP in OLP differed from controls, the differences demonstrated were not significant and the results of this study were ultimately inconclusive as to whether HSP is involved in the pathogenesis of OLP (Bramanti et al., 1995). Thus, although alteration in HSP's have been observed in some studies, the exact role in the immunopathogenesis of OLP is not overtly convincing.

1.5.2 Hepatitis C

The hepatitis C virus (HCV) is a single stranded, positive sense enveloped ribonucleic acid (RNA) virus that belongs to the Flavivirus family. HCV has been thought to play a potential role in the pathogenesis of OLP. The prevalence of hepatitis C in patients with OLP shows great regional variation with the greatest association occurring in Southern Europe and Japan (Carrozzo, 2008, Petti et al., 2011, Nagao and Sata, 2012). In a meta-analysis and case control study by Petti et al., 2011 it was determined that 2.1% of all

global OLP cases were associated with the HCV (95% CI 1.9-2.2%) (Petti et al., 2011). Due to this association some authors have advocated screening OLP patients for anti-HCV antibodies, HCV-RNA and alterations in liver function tests (LFT) in high risk areas such as Japan and the Mediterranean (Petti et al., 2011, Nagao and Sata, 2012, Halawani, 2014).

A 2010 systematic review and meta-analysis of HCV and lichen planus reviewed 39 articles including 33 assessing the seroprevalence of HCV in lichen planus patients and 6 articles assessing the prevalence of lichen planus in patients with HCV (Lodi et al., 2010). This study confirmed patients with lichen planus, both OLP and cutaneous, had a significantly higher risk of being seropositive for HCV compared to controls (odds ratio (OR): 4.9; 95% CI 3.6-6.6) (Lodi et al., 2010). Patients with HCV exhibited a similar high risk for having lichen planus (OR: 4.5; 95% CI 1.8-10.9) (Lodi et al., 2010). The association of lichen planus with HCV was apparent in Japan, United States of America and countries of the Mediterranean. However, the authors acknowledged the epidemiological data reviewed could be influenced by methodological biases specifically, small sample size, issues with disease classification, and recency of HCV infection (Lodi et al., 2010). While some studies have supported the association between OLP and HCV, it is important to note that HCV does not affect the majority of OLP patients (Petti et al., 2011).

Other studies have shown no clear association exists between OLP and chronic HCV. In an assessment of the HCV prevalence in 232 OLP patients using ELISA the prevalence of HCV was observed to be higher in controls, although this trend was not significant (Zhou et al., 2010). No correlation was noted between OLP and HCV with the authors suggesting HCV does not play a role in the aetiology of OLP in Chinese patients (Zhou et al., 2010). An earlier study investigating the possible epidemiological relationship between chronic HCV and OLP assessed three groups: group 1 consisting of 82 histologically confirmed OLP patients who were tested for HCV infection; group 2 with 165 patients with chronic HCV who were examined for OLP and group 3; the control group with 466 healthy individuals who were tested for OLP (Giuliani et al., 2007). An association was noted between HCV and OLP ($p < 0.05$), however the authors reported this association to be weak. Further, they suggested the reported association was likely the result of the frequency of each condition in the population (Giuliani et al., 2007). The authors also felt that no true pathogenetic association existed between the two conditions

and the weak association noted was determined by patient recruitment (Giuliani et al., 2007).

Many of the studies that assessed the association between HCV and OLP have had a retrospective case control design. This makes it impossible to definitively determine whether HCV exposure occurred before or after the development of OLP (Carrozzo, 2008). Thus, it is near impossible to determine if HCV plays a definitive role in the pathogenesis of OLP, or if patients with HCV and OLP simply share similar population characteristics (age, gender etc.). It is also important to note that medications used to treat HCV, specifically interferon α and ribavirin, have been implicated in the formation of LDRs (McCartan and McCreary, 1997, Serrano-Sanchez et al., 2010). Thus, for patients who have been treated for HCV there is no currently available method to ascertain definitively whether the presence of OLP like lesions are a true OLP or LDR.

1.5.3 Human Papilloma Virus

The role HPV plays in oropharyngeal and genital cancer is well known. HPV viral genes E6 and 7 have transforming properties (Yim and Park, 2005) with both HPV proteins acting as oncogenes. They cause the degradation and inactivation of the suppressor genes p53 and retinoblastoma enhancing cell cycle progression and increasing the likelihood of genetic mutations (Yim and Park, 2005). Studies have also evaluated the role that HPV may play in the pathogenesis of OLP, specifically, that viruses such as HPV have the capacity to alter host cell function leading to the expression of abnormal proteins and development of a disease state (Pol et al., 2015). In a case-controlled study of 30 normal mucosal biopsy specimens and 30 biopsy specimens with histopathologically diagnosed OLP, IHC was performed using the HPV-16 immunohistochemical marker on all specimens (Pol et al., 2015). 70% of the OLP specimens tested positive for HPV-16, all of the controls were negative for HPV-16 (Pol et al., 2015). This presence of HPV at rates of 70% in OLP is one of the highest reported with most other studies showing much, lower rates in the region of 9-30% (Jontell et al., 1990, Ostwald et al., 2003, Mattila et al., 2012). Given the high rate of the detection in OLP lesions, the authors concluded that HPV-16 potentially plays a role in OLP pathogenesis (Pol et al., 2015). They also suggested given the known oncogenic potential of HPV-16 that biopsy specimens should be screened for the presence of this virus (Pol et al., 2015).

An earlier study undertaken to identify the presence of HPV in erosive OLP used samples from 20 OLP patients assessed with two techniques, Southern blot analysis and a type

specific PCR, to determine the presence of HPV DNA (Jontell et al., 1990). HPV type-11 was present in 6/20 (30%) samples, while types 6, 16 and 18 were not identified in any samples (Jontell et al., 1990). With the PCR assay 65% of all samples were positive for HPV, specifically, HPV-11 was detected in 8/20 samples, HPV-6 in 5/20 samples and HPV-16 in 3/20 samples (Jontell et al., 1990). A similar study undertaken in 2003 showed an overall detection rate of HPV DNA in 10/65 OLP lesions (15.4%), 7.7% were positive for HPV-6/11, 6.2% for HPV-18 and 3.1% for HPV-16 (Ostwald et al., 2003). In this study HPV DNA presence was assessed in malignant (carcinoma), pre-neoplastic (leukoplakia and chelitis) and questionably pre-neoplastic (OLP) lesions (Ostwald et al., 2003). Presence of HPV DNA was noted at higher levels in the malignant and pre-neoplastic lesions including 51/118 carcinomas (43.2%), 16/72 leukoplakias (22.2%) and 3/12 cheilitic lesions (25%) (Ostwald et al., 2003). Presence of HPV -6 and 18 DNA was present in 41/118 carcinomas (34.7%), 12/72 leukoplakias (16.7%) and 2/12 cheilitic lesions (16.7%) (Ostwald et al., 2003). A successive increase in the detection rate of HPV-16 and 18 was noted from preneoplastic lesions (OLP) to malignant lesions (carcinoma) (Ostwald et al., 2003). These authors postulated that he presence of HPV 16 and 18 supports the involvement of HPV 16 and 18 in oral carcinogenesis (Ostwald et al., 2003), and the presence of HPV 16 and 18 may also provide some explanation for the malignant potential of OLP.

A systematic review undertaken in 2011 using a pooled estimate of 956 oral premalignant disease cases, including OLP and oral leukoplakia, and 675 controls showed association of HPV DNA, specifically HPV-16, with OLP (OR: 2.1 95% CI: 2.4-10.9) (Syrjanen et al., 2011). One explanation for this association was the presence of frequent ulceration in OLP resulting in the mucosa potentially being more susceptible to opportunistic infection (Syrjanen et al., 2011). The chronic use of steroids in OLP may also work to induce immune suppression and upregulate HPV replication (Syrjanen et al., 2011). Thus, presence of HPV in OLP may be entirely circumstantial due to conditions that facilitate opportunistic infection and replication.

1.5.4 Epstein-Barr Virus

The role the Epstein Barr virus (EBV) may play in the pathogenesis of OLP has also been investigated. One study compared serum from 22 patients with histologically diagnosed OLP with serum for 22 healthy controls and confirmed specific serum IgG antibodies towards the EBV early antigen were significantly higher in the OLP cohort

(Pedersen, 1996). It was interesting to note that in those patients that tested positive for the antibodies (20/22) there was a significant negative correlation between the duration of OLP symptoms and the optometric density values for the antibody (Pedersen, 1996). A more recent study assessed EBV in 99 OLP patients that were retrospectively selected based on clinical diagnosis, reticular (n = 66) and erosive/ulcerative (n = 33), and biopsy with 22 OLL patients with biopsies also retrospectively selected to act as controls (Raybaud et al., 2018). EBV was detected in biopsy samples using *in situ* hybridisation with IHC profiling performed on 21 OLP samples (Raybaud et al., 2018). Results confirmed the presence of EBV in 74% of all OLP samples with a significantly higher frequency of EBV detected in erosive OLP (83%) compared to reticular OLP (58%) (Raybaud et al., 2018). It should be noted that the EBV^+ cells in erosive OLP correlated with inflammatory parameters such as infiltration, disease activity and depth (Raybaud et al., 2018). For the EBV^+ cells detected in OLP the majority were shown to be $CD138^+$ plasma cells with a smaller number detected as $CD20^+$ B cells potentially alluding to a role for EBV in autoimmunity as these cells are involved in the immune regulation of autoimmune disease (Raybaud et al., 2018).

Conversely, some studies have found no relationship between EBV and OLP. One study assessed the presence of EBV with nested PCR in 24 OLP and 17 control patients using saliva, fresh tissue, plasma, and exfoliated cells (Vieira Rda and Ferreira, 2016). No significant differences were noted between OLP patients and controls for all samples with no clear relationship between EBV and OLP established (Vieira Rda and Ferreira, 2016). 68 biopsies comprising of 25 OLP, 26 genital lichen planus, 10 oral and 7 genital controls were assessed for EBV using *in-situ* hybridisation for EBER-1 and EBER2 as well as an RNA positive probe to ensure the presence of mRNA in the tissue samples (Danielsson et al., 2018). Whilst all samples were shown to have RNA present no samples were shown to be positive for EBER1 or EBER2 with results suggesting no aetiological relationship between EBV and lichen planus (Danielsson et al., 2018).

1.5.5 Genetics

The role of genes in the pathogenesis of OLP has been variably studied with one such study using microarray analysis to assess whether certain genes involved in cell proliferation, signal transduction, transcription, inflammation, angiogenesis and apoptosis may play a role in OLP (Tao et al., 2009). RNA samples were collected from mucosal specimens in 9 OLP and 9 control patients (Tao et al., 2009). Microarray

analysis was conducted using GeneChip and Real Time PCR for FoxP3, vascular endothelial growth factor (VEGF), angiopoietin 1, angiopoietin 2, MMP-1 and mammaglobin A (Tao et al., 2009). The expression profiles in OLP differed from the controls with upregulation of MMP-1, angiopoietin 1, angiopoietin 2 and downregulation of VEGF, FoxP3 and mammaglobin A noted in the OLP cases (Tao et al., 2009). Based on these results it can be theorised that changes in gene regulation may play a role in both the pathogenesis and chronicity of OLP.

Real time PCR was used to assess expression of genes involved inflammation and innate immunity in 23 healthy controls and 14 OLP/OLL patients with genes analysed including toll like receptor (TLR) genes specifically TLR1 and transmembrane factors that play a role in pattern recognition of microbial ligands (Adami et al., 2014). Inflammatory chemokine genes such as CXCL1 as well as IL-8 and CD14 were assessed (Adami et al., 2014). Overall increased expression in the epithelium of CD14, CXCL1, IL-8 and TLR1 genes was demonstrated in OLP patients (Adami et al., 2014). Overexpression of TLR1 and CXCL1 was also noted in OLP patients (Adami et al., 2014). OLP has been thought to be a condition that arises due to dysregulation of the adaptive immunity that results in abnormal T cell function. Results of this study showed increased expression of genes associated with the innate immunity, suggesting dysregulation of innate immunity may be involved in OLP pathogenesis (Adami et al., 2014).

Gene polymorphisms, specifically polymorphisms in genes implicated in the pathogenesis of infections, inflammation, or autoimmune conditions, may also play an important role in the aetiopathogenesis of OLP. A study assessing the expression of 14 functional gene polymorphisms in 32 OLP patients and 99 healthy controls found a polymorphism of the TNF receptor 2 with this receptor shown to be significantly higher in OLP patients compared to healthy controls (Fujita et al., 2009). This polymorphism has been implicated in autoimmune conditions, including SLE. It is possible this polymorphism may play an important role in the pathogenesis of OLP, specifically, with regards to susceptibility for this condition. IFN-γ is a potent cytokine involved with the activation of $CD8^{+}$ cells. Further, the presence of the INF-γ +874A/T polymorphism has been shown to be associated with OLP and could result in an increased susceptibility for this condition (Kimkong et al., 2012).

Interleukin 12 (IL-12) is a pro-inflammatory cytokine naturally produced by dendritic cells, that results in the clonal proliferation of T cells, specifically T-helper cells. Single

nucleotide polymorphisms of IL-12A have been shown to be associated with chronic inflammatory and autoimmune conditions. In a study of Chinese patients, 292 with OLP and 686 controls, the rs568408 variation in the IL-12A gene was shown to be significantly higher in OLP patients (Jiang et al., 2015). Expression of the rs568408 single nucleotide polymorphism variation in the IL-12A gene was also shown to be significantly higher in erosive cases of OLP compared to both healthy controls as well as non-erosive OLP (Jiang et al., 2015). Based on these results, the authors concluded that polymorphisms of the IL-12A gene could potentially be used as a biomarker for OLP, specifically, with regards to susceptibility for erosive subtypes (Jiang et al., 2015).

1.5.6 Stress, Anxiety and Depression

Psychosomatic factors, stress, anxiety and depression, may play a role in OLP pathogenesis. One study assessed differences in psychological factors between OLP and non-OLP patients, using the General Health Questionnaire and the Hospital Anxiety and Depression Scale (Chaudhary, 2004). The cohort consisted of 73 healthy patients with no mucosal disease (negative controls), 41 histopathologically proven OLP patients, and 36 burning mouth syndrome patients (positive controls) (Chaudhary, 2004). OLP patients showed significantly higher levels of stress ($p < 0.05$), anxiety ($p < 0.05$) and depression ($p < 0.05$) when compared to negative controls, while no significant differences existed between the OLP cohort and positive controls (Chaudhary, 2004). Based on these results the authors concluded that psychological stressors play an important role in the causation of OLP (Chaudhary, 2004).

Similarly, an evaluation using psychological personality profiles, the Minnesota Multiphasic Personality Inventory test, cortisol levels, and T cell markers CD3, CD4, CD8 and CD16 in 20 erosive OLP, 20 reticular OLP and 25 healthy patients demonstrated significantly higher levels of cortisol in the erosive OLP cohort compared with healthy controls (Ivanovski et al., 2005). OLP patients had significantly higher CD8 and CD4 counts compared to healthy controls (Ivanovski et al., 2005), a result that was not unexpected considering OLP is a T cell mediated condition. No statistically significant differences among the three groups were noted with respect to psychopathic deviant paranoia, psychasthenia, schizophrenia or hypomania (Ivanovski et al., 2005). Significant differences did exist between OLP patients and controls with regards to hypochondriasis, depression and hysteria (Ivanovski et al., 2005). This study concluded that in OLP

patients prolong emotive stress may result in psychosomatization and this might contribute to both the clinical expression and initiation of OLP (Ivanovski et al., 2005).

An earlier study analysed the importance of psychological factors in OLP patients with an attempt to identify characteristic personality traits of OLP patients assessing 100 OLP patients, divided into erosive and non-erosive, and 50 control subjects (Rojo-Moreno et al., 1998). Significantly higher anxiety levels were noted in OLP patients compared to controls (Rojo-Moreno et al., 1998). Despite these results the authors were unable to conclusively determine whether the psychological differences observed in OLP patients were a direct cause of OLP, or a consequence of OLP (Rojo-Moreno et al., 1998). It is important to note with these studies that it is unknown whether the depression/anxiety/stress follows or precedes the diagnosis of OLP. The diagnosis of OLP is not always straightforward and management is not always guaranteed to be successful. Patients with OLP may have higher levels of depression/anxiety/stress simply due the difficult nature of OLP and depression/anxiety/stress may not actually play a role in OLP pathogenesis.

Interestingly, a more recent study assessed salivary levels of dehydroepiandrosterone, a corticosteroid secreted by the adrenal gland that may be related to depression, in 31 OLP and 31 control patients, as well as symptoms of depression, anxiety and stress with no significant differences shown between the two cohorts (Girardi et al., 2011). Anxiety levels were higher in OLP patients with the difference between the OLP and control groups approaching significance ($p = 0.0611$) suggesting a possible association with anxiety in patients with OLP (Girardi et al., 2011). Overall, this study did not support depression/anxiety/stress or a neuroendocrine aetiology for OLP (Girardi et al., 2011). A recent systematic review assessed 14 papers, 13 of intermediate quality and one of high quality, with a significant association between OLP and psychological disorders noted in 10 studies (Cerqueira et al., 2018). Depression, anxiety and stress were the psychological disorders most studied with the authors concluding based on the above noted relationship that recognition of the emotional state of the patient during clinical practice is necessary for adequate monitoring and treatment (Cerqueira et al., 2018). Despite all these theorised mechanisms of pathogenesis, the exact cause and aetiopathogenesis of OLP remains unknown.

1.6 Mucosal Associated Invariant T cells

Mucosal associated invariant T (MAIT) cells T are a recently characterised subset of T cells thought play an important role in microbial immunity. MAIT cells can be isolated in peripheral blood and in peripheral blood are detected at rates of 1-10% (Tilloy et al., 1999, Gold et al., 2010, Cowley, 2014). What sets this subset of cells apart is an invariant T cell receptor (TCR) α chain, Vα7.2-Jα33 in humans and Vα19-Jα33 in mice, that is evolutionarily conserved (Tilloy et al., 1999, Gold et al., 2010). This contrasts with conventional T cells that typically express TCR repertoires that are both highly diverse and variable. TCRs contain heterodimers, two different protein molecules consisting of α, β, ɣ and δ protein chains. In humans, only a small number TCR contained the ɣδ heterodimer with the majority of TCR comprising of αβ heterodimer (Jarry et al., 1990, Kalyan and Kabelitz, 2013). In tissues, MAIT cells are mostly found in the gut mucosa and liver (Tilloy et al., 1999, Gold et al., 2010).

1.6.1 How Mucosal Associated Invariant T Cells Recognise Pathogens and Activation

What makes MAIT cells unique is restriction by a non-polymorphic class Ib MHC molecule, known as major histocompatibility class 1 related protein (MR-1) (Tilloy et al., 1999, Gold et al., 2010), with binding of MR-1 required for activation. The MR-1 protein is in all mammals, including humans and this protein is highly conserved (Tilloy et al., 1999, Gold et al., 2010). While highly conserved, MR-1 is also a ubiquitous protein expressed during a microbial challenge as demonstrated by *Mycobacterium tuberculosis* infection inducing MR-1 expression on lung epithelium (Gold et al., 2010). This reflects that MAIT cells are a subset of T-cells that exhibit conserved recognition properties and that micro-organisms with an inherent capacity to synthesise riboflavin have the ability to activate MAIT cells (Gold et al., 2010, Le Bourhis et al., 2010, Kjer-Nielsen et al., 2012, Gold et al., 2014). This is significant because in humans, riboflavin is not endogenously produced however, riboflavin is produced by microbes, including both *Candida* and bacteria (Tilloy et al., 1999, Cowley, 2014).

Le Bourhis et al., 2010 used an *in vitro* model to culture bone marrow-derived dendritic cells from MR-1-deficient and sufficient mice; subsequently infecting bone marrow-derived dendritic cells with *S. cerevisiae, C. albicans* and *C. glabrata* with all microbes shown to be capable of inducing a MAIT cell response in an MR-1 dependent manner (Le Bourhis et al., 2010). A further study obtained $CD8^+$ cells from the peripheral blood

of four donors and stimulated these cell via *ex vivo* exposure to *M. semgmatis, Salmonella typhimurium* and *C. albicans* infected A549 epithelial cells (Gold et al., 2014). MAIT cells were shown to be capable of detecting a diverse array of MR-1 restricted ligands and exhibited the ability to discriminate between the different ligands detected whilst also providing a basis for an adaptive immune response (Gold et al., 2014). This study also demonstrated that the MAIT cell TCR repertoire for *C. albicans* was less diverse than that demonstrated for the other two pathogens (Gold et al., 2014).

MAIT cells from healthy donor peripheral blood mononuclear cells (PBMC) were shown to display cytotoxic activity towards bacterially infected cells (Le Bourhis et al., 2013). Further, MAIT cells activated by epithelial cells (Hela cells) infected with *Shingella flexneri* has the ability to kill those epithelial cell expressing MR-1 (Le Bourhis et al., 2013). Interestingly, epithelial cells infected with *S. Typhimurium*, an invasive bacteria, were unable to induce a MAIT cell response (Le Bourhis et al., 2013). The authors speculated this could be the result of some *Salmonella* virulence factor that results in blocking of the mechanisms required for efficient loading into the MR-1 groove on MAIT cells (Le Bourhis et al., 2013)

The fact that MAIT cells can kill bacterially infected epithelial cells is significant as *Candida spp.* can superficially invade the epithelium and act as a potential trigger production of MR-1. A shift in the commensal oral flora can lead to overgrowth of *Candida* with this altered environment potentially being a trigger for MAIT cell activation and initiation of OLP. Persistence of this altered oral biofilm that includes *Candida* in the oral cavity could also account the longevity of the OLP disease process. Activation of MAIT cells via MR-1 bound riboflavin metabolites results in quick release of inflammatory cytokines such INF-ɣ, TNF and IL-17A that have the capacity to mediate an immune response (Gold and Lewinsohn, 2011, Cowley, 2014).

Recently, one study evaluated whether MAIT cells were able to discriminate between different human microbes (Tastan et al., 2018). 47 microbiota bacterial species, primarily commensals found on different mucosal tissues as well as some environmental strains, were assessed using an *in vitro* functional assay with T cells engineered for MAIT-TCRs (eMAIT-TCR) (Tastan et al., 2018). Bacteria assessed were graded into high and low stimulators of MAIT-TCRs with activation of MAIT cells correlating with either the level of riboflavin produced, measured with mass spectrometry, or macrophage infection with bacteria (Tastan et al., 2018). *Bacteroidetes* and *Proteobacteria* were able to induce

significantly higher levels of stimulation in MAIT-TCRs when compared to *Firmicutes* or *Actinobacteria* species (Tastan et al., 2018). Species that lack the riboflavin pathway were unable to stimulate the eMAIT-TCRs (Tastan et al., 2018). It was also shown that T-cells, specifically memory and effector T-cells, had the capacity to directly stimulate MAIT cells in an MR-1 dependent manner (Tastan et al., 2018). Findings of this study suggested that MAIT cells were able to discriminate between different bacteria by computation of the TCR signal and use this information to fine tune the functional response (Tastan et al., 2018).

MAIT cells exhibit high expression of receptors for IL-18, IL-12 and IL-23 (Dusseaux et al., 2011, Jeffery et al., 2016). Exposure to microbial by-products following infection can result in release of IL-12 and IL-18 with both these cytokines working together to coordinate a cell mediated immune response (Manigold et al., 2000). It has been shown that the $CD161^{++}CD8^{+}$ T cell population, including both the $TCRV\alpha^{+}$ (MAIT) and $TCRV\alpha^{-}$ subsets, were able to respond directly to stimulation with both IL-12 and IL-18 (Ussher et al., 2014). This was tested by stimulating PBMC with either IL-12 + IL-18 or *E. coli* with the addition or absence of an MR-1 blocking antibody (Ussher et al., 2014). The results showed the $CD161^{++}$ phenotype rather than MAIT cells specifically, was the phenotype that responded to stimulation with IL-12 + IL-18 with the $CD161^{++}$ phenotype expressing significantly higher levels of interleukin 18 receptor alpha (IL18Rα) when compared to the $CD161^{+}$ and $CD161^{-}$ subsets (Ussher et al., 2014). The results of this study show MAIT cells can be activated in an MR-1 independent manner, broadening the potential for their role in inflammatory and viral disease (Ussher et al., 2014).

1.6.2 Identification of Mucosal Associated Invariant T Cells and the Role of Cytokines

It has been shown by Martin et al., 2009 that MAIT cells can be detected by co-staining with antibodies directed against CD161 and TCRVα7.2 (Martin et al., 2009). Other markers able to be used for detection of MAIT cells in tissue sections include MR-1, IL18Rα, also known as interleukin 18 receptor 1 (IL18R1) or CD218a, and CD3. CD3 is T-cell co-receptor that works to activate cytotoxic T cells. IL18R is a receptor with that consists of both alpha and beta subunits that have a high affinity for IL-18. MAIT cell quantification in the literature has been undertaken with the above markers using peripheral blood for flow cytometry and IHC on fresh frozen or formalin fixed tissues samples (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016).

The development of the MR-1 tetramer is another way to identify MAIT cells (Corbett et al., 2014). The MR-1 tetramer is loaded with 5-amino-6-D-ribitylaminouracil (5-A-RU), a compound that is an early intermediate of bacterial riboflavin synthesis and able to form MAIT activating antigens in a way that does not require enzymatic catalysis (Corbett et al., 2014). Activating antigens formed include 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylidenaeamino)-6-D-ribitylaminouracil (5-OP-RU) (Corbett et al., 2014).

In adult human blood, the MAIT cell population consists of primarily $CD8^+$ T cells expressing the C-type lectin receptor CD161. A marker that can be used to identify T cells that produce IL-17, including the T helper 17 (Th17) cell subset (Martin et al., 2009, Dusseaux et al., 2011). It has been shown that unstimulated MAIT cells express high levels of RORC, a Th17 associated transcription factor (Dusseaux et al., 2011). Following stimulation MAIT cells have been shown to display a Th1/Th17 cytokine pattern (Dusseaux et al., 2011). The pathogenic role of the Th17 (inclusive of Th17 and Th17/Th1), Th2 and Th0 subsets of CD4 cells has been assessed in OLP (Piccinni et al., 2014). Cytokines associated with the Th17/Th1 subset included IL-17A, IL-17F, IL-23R, RORC and IFN-γ; IFN-γ, together with IL4 and IL-5 or IL-13 were associated with the Th0 subset; IL-4, IL5 and IL13 were associated with the Th2 subset (Piccinni et al., 2014). This study included tissue samples from 14 patients with either reticular or erosive OLP; normal mucosa was also taken from the same patients to serve as control tissue (Piccinni et al., 2014). Assessment of mRNA expression confirmed an increased expression of Th17 and Th0 mRNA in erosive OLP compared to control (Piccinni et al., 2014). In comparison increased expression of Th2 mRNA was observed in reticular OLP (Piccinni et al., 2014). The percentage of Th17 cells in reticular and erosive OLP was assessed by measuring CD161 surface expression after co-staining with CD3 and CD4 (Piccinni et al., 2014). Significantly higher expression of CD161 was demonstrated in erosive OLP (35%) compared to control (4%) ($p = 0.001$) (Piccinni et al., 2014). Results were supportive of a role for Th17, Th2 and Th0 in the pathogenesis of OLP, however, further research is required to clarify the exact pathogenic role of these subsets (Piccinni et al., 2014).

More recently it has been shown that the IL-17A G197A polymorphism was associated with an increased susceptibility of developing OLP (Gueiros et al., 2018). 83 patients with OLP and 99 healthy controls participated in the study and had oral mucosal cells

collected for IL-17A G197A phenotyping (Gueiros et al., 2018). Evaluation of serum IL-17A levels was observed in 42 OLP patients and compared to 23 healthy controls with results confirming significantly higher serum levels in OLP patients, both reticular and erosive (Gueiros et al., 2018). These results suggested that OLP could be strongly regulated by Th17 cells with IL-17A playing an important role in pathogenesis (Gueiros et al., 2018). MAIT cells are associated with Th17 (Dusseaux et al., 2011), It is possible that MAIT cells could be of the cell type that contributes to the Th17 cytokine pattern in OLP and contribute to IL-17A dysregulation in OLP.

In response to a microbial challenge MAIT cells release INF-γ, TNF, IL-17, and IL-22 (Le Bourhis et al., 2010, Dusseaux et al., 2011, Gibbs et al., 2017). Using the potent cell stimulator phorbol myristate acetate (PMA)/ionomycin, it was shown that MAIT cells were capable of producing the cytokines granzyme B, INF-γ and high levels of IL-17 (Dusseaux et al., 2011). INF-γ and TNF are important mediators required to activate the host immune response following viral or microbial infection. A recent study of MAIT cells in human oral mucosa showed the major mucosal MAIT cell subset displayed a tissue-resident and activated profile with a bias towards production of IL-17 and low perforin (Sobkowiak et al., 2019). This profile in buccal mucosa was determined by staining isolated mucosal cells for markers of activation, specifically HLA-DR and CD38 (Sobkowiak et al., 2019). Mucosal MAIT cells exhibited higher levels of HLA-DR compared to blood MAIT cells with slightly lower expression of CD38 noted in mucosal MAIT cells (Sobkowiak et al., 2019). Further investigation showed overall lower perforin levels in buccal MAIT cells compared to blood MAIT cells (Sobkowiak et al., 2019). MAIT cell functional profile was assessed in both blood and buccal mucosa by activation with PMA/ionomycin and using flow cytometry to stain for granzyme B, TNF, IFN-γ, IL-2 and IL-17 (Sobkowiak et al., 2019). Whilst levels of IL-2 and granzyme B were comparable, blood MAIT cells were shown to produce higher levels of TNF and IFN-γ, with buccal mucosal MAIT cells showing overall higher levels of IL-17 production (Sobkowiak et al., 2019).

A recent study of investigating female genital tract MAIT cells showed that stimulation with *E. coli* resulted in a bias towards expression of IL-22 and IL-17 (Gibbs et al., 2017). This is in contrast to MAIT cells derived from peripheral blood that were shown to primarily produce TNF, INF-γ and granzyme B when exposed to the same conditions (Gibbs et al., 2017). Preferential expression of IL-22, a cytokine involved in mucosal

immunity and barrier integrity, and IL-17 in mucosal MAIT cells could be suggestive of an important role for these cells in maintenance of mucosal barrier integrity and mucosal homeostasis (Gibbs et al., 2017). Diseases, such as OLP could disrupt production of IL-22 through T cell mediated epithelial destruction and affect mucosal barrier integrity. In OLP fungal colonisation has the potential to activate regional MAIT residing in the oral mucosa. Presence *C. albicans* and other common oral microbes may result in a bias towards expression of IL-22 and IL-17 similar to that shown in the female genital tract (Gibbs et al., 2017). Dysregulation of these cytokines have also been shown in OLP as described in section 1.4.7.

Umbilical cord blood, healthy donor blood and leukocyte cones, enriched blood that is the by-product of apheresis, were assessed by flow cytometry, *in vitro* stimulation as well as killing and proliferation assays using *E. coli* for antigenic stimulation (Kurioka et al., 2015). Results from this study showed the resting MAIT cells lacked inherent cytotoxic activity, with high levels of granzyme A and granzyme K and low levels of perforin and granzyme B noted in the resting state (Kurioka et al., 2015). On bacterial activation with *E. coli* the activated MAIT cells rapidly produced granzyme B and perforin, key proteins required for cytotoxic activity (Kurioka et al., 2015). This cytotoxic profile licenced the MAIT cells to kill the bacterially sensitised target cells with greater efficiency (Kurioka et al., 2015). Stimulation with *E. coli* showed MAIT cells markedly upregulated granzyme B and perforin by 18 hours with most cells expressing these molecules at 30 hours (Kurioka et al., 2015). Using a flow cytometry killing assay it was shown that the licenced MAIT cells, not *ex vivo* MAIT cells from the same donors, had the ability to kill *E.coli* exposed B cells lines in an MR-1 and degranulation dependent manner exhibiting the tight regulation of MAIT-cell cytotoxicity (Kurioka et al., 2015).

Based on the above a novel hypothesis for the pathogenesis of OLP is commensal oral microbes, such as *Candida,* may play a role in activating MAIT cells in an MR-1 dependent manner to release pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-22 and IL-17 whilst also mediating the destruction of *Candida* affected keratinocytes through release of perforin and granzyme B (DeAngelis et al., 2019).

1.6.3 Role of Mucosal Associated Invariant T Cells in Cancer, Autoimmune and Inflammatory Disease

The role MAIT cells play in different diseases is a currently expanding area of research. MAIT cells have been implicated in protective or pathogenic roles in multiple conditions

including cancer, autoimmune and inflammatory disease (Chiba et al., 2012, Gracey et al., 2016, Won et al., 2016, Tominaga et al., 2017, Raychaudhuri et al., 2019).

1.6.3.1 Mucosal Associated Invariant T Cells in Malignancy

The role of MAIT cells in mucosal associated cancers has been investigated. A recent study enrolled 20 healthy controls and cancer patients, 15 with gastric, 34 colon, 13 with lung, 13 with breast, 6 with hepatocellular and 18 with thyroid cancer (Won et al., 2016). Blood was collected from all patients and tissue from cancer affected and unaffected areas was collected from patients who underwent colorectomy (Won et al., 2016). Analysis with flow cytometry showed significantly lower percentages as well as absolute number of MAIT cells in mucosal associated cancers, specifically gastric, colon and lung cancers, when compared to healthy controls (Won et al., 2016). Circulating MAIT cell levels were shown to be significantly lower in mucosal associated cancer compared to non-mucosal associated cancers, but the capacity to produce anti-neoplastic and anti-microbial cytokines IL-17, IFN-γ and TNF-α was preserved in both groups (Won et al., 2016). MAIT cell numbers in mucosal associated cancers were correlated significantly with nodal staging as well as lymphocyte count, neutrophil count, haemoglobin and carcinoembryonic antigen levels counts (Won et al., 2016). On analysis of tissue specimens it was shown that MAIT cell levels were significantly higher in cancer affected tissue when compared to unaffected tissue and blood (Won et al., 2016). Higher levels of the chemokine receptor 6 and CXC receptor 6 were expressed on peripheral blood MAIT cells, with high expression of the corresponding chemokines chemokine ligand 20 and CXCL16 noted in cancer affected tissue, suggesting MAIT cells can migrate from blood to cancer affected colon tissue through chemokine signalling pathways (Won et al., 2016). Taken together these results confirm reduced circulating levels of MAIT cells with increased numbers in cancer affected tissue with an ability of these cells to migrate to cancer affected areas and percentage decrease correlating with increased N staging suggesting MAIT cells may play a role in oncoimmunity (Won et al., 2016).

1.6.3.2 Mucosal Associated Invariant T Cells in Multiple Sclerosis

MAIT cells have been implicated in the pathogenesis of multiple sclerosis (MS). One study collected blood from patients with 100 patients with MS, 15 with inflammatory bowel disease (IBD) and 63 healthy volunteers (controls) to determine the frequency, phenotype and activation potential of MAIT cells, whilst also assessing transmigration through an *in vitro* blood brain barrier (Salou et al., 2016). The MS cohort was divided

into relapsing/remitting MS (85), secondary progressive MS (7) and primary progressive MS (8) with frozen central nervous system samples from 13 patients, 42 lesions in total were also collected for immunofluorescent staining (Salou et al., 2016). MAIT cells in both relapsing/remitting MS and controls showed similar activation abilities, frequencies and phenotype, whilst progressive MS showed a decreased frequency when compared to the above groups that could potentially be explained by this cohort being older (Salou et al., 2016). Paired blood and frozen samples were assessed showing decreased MAIT cell frequency in the central nervous system with transmigration of MAIT cells through the *in vitro* blood brain barrier shown to be similar in MS and controls (Salou et al., 2016). Gene expression of MR-1, IL-12, IL-23 and IL-18 was analysed in frozen samples from 10 MS patients, samples consisted of paired active, chronic active and normal appearing (control) white matter with and without inflammatory infiltrate (Salou et al., 2016). MS lesions showed overexpression of IL-18, MR-1 and IL-23 to a lesser extent when compared to control samples (Salou et al., 2016). Whilst MAIT cell frequencies were low in the frozen samples results suggest a potential role for MAIT cells in MS.

A recent study collected peripheral blood from 30 patients with diagnosed MS and 30 healthy controls and compared MAIT cells between to two cohorts whilst also characterising type-17 differentiation (Willing et al., 2018). MAIT cells were assessed using flow cytometry and blood samples assessed for cytokines production (Willing et al., 2018). The MS cohort showed increased numbers of IL-17^{+} MAIT cells with IL-17 production correlating with expression of the surface marker CD127 (IL-7 receptor chain α); IL-7 treatment of PBMC in both the MS and healthy cohort also resulted in increased frequencies of IL-17^{+} MAIT cells (Willing et al., 2018). As IL-7R is involved in T cell homeostasis this enhancement of IL-7 signalling resulting in increased frequencies of type-17 differentiated MAIT cells could allude to a driver of functional alteration in MS (Willing et al., 2018).

1.6.3.3 Mucosal Associated Invariant T Cells in Skin Disease

A recent study by Li et al., 2016 quantitatively assessed the percentage of MAIT cells in normal skin, seborrheic keratosis, psoriasis, alopecia areata, and dermatitis herpetiformis tissue cryosections using immunofluorescent staining (Li et al., 2016). MAIT cells were identified using antibodies directed against CD3, IL18Rα and Vα7.2 with triple positive cells identified as MAIT cells (Dusseaux et al., 2011, Li et al., 2016). As per the original protocol the percentage of MAIT cells present was calculated as a proportion of all the

$CD3^+$ cells present (Dusseaux et al., 2011). The percentage of MAIT cells identified in dermatitis herpetiformis was significantly elevated when compared to the other groups analysed (Li et al., 2016). When identified MAIT cells were located with other T cells in the upper dermis (Li et al., 2016). The authors concluded that the elevated percentage of MAIT cells present in dermatitis herpetiformis possibly could elucidate to a role in the pathogenesis (Li et al., 2016).

1.6.3.4 Mucosal Associated Invariant T Cells in Inflammatory Joint Disease

An early study triggered collagen induced arthritis in $MR\text{-}1^{-/-}$ and $MR\text{-}1^{+/+}$ DBA/1J mice and assessed collagen II specific T cell responses, cytokine production and MAIT cell involvement in arthritis (Chiba et al., 2012). MAIT cells were shown to be stimulated in a TCR independent fashion by the cytokines IL-23 and IL-1β with IL-23 promoting production of IL-17 and IL-1β and inducing MAIT cell proliferation (Chiba et al., 2012). MAIT cells were also shown to enhance inflammation and exacerbate arthritis in the mouse models, suggesting a potential role for MAIT cells in human arthritis (Chiba et al., 2012). A recent study evaluated the role of MAIT cells in PBMC and synovial fluid mononuclear cells of 30 patients, 10 with psoriatic arthritis, 10 with rheumatoid arthritis (RA) and 10 with osteoarthritis (Raychaudhuri et al., 2019). Significantly higher frequencies of MAIT cells were noted in synovial fluid mononuclear cells of psoriatic arthritis and RA with compared to osteoarthritis, whilst the reverse was shown for PBMC MAIT cells with osteoarthritis showing significantly higher frequencies when compared to psoriatic arthritis and RA (Raychaudhuri et al., 2019). The majority of synovial fluid mononuclear cells from psoriatic arthritis patients were $CD8^+$ (> 80%) with small numbers of $CD4^+$ and natural killer cells, with both PBMC and synovial fluid mononuclear cells in psoriatic arthritis shown to produce significantly higher levels of IL-17A when compared to osteoarthritis and RA (Raychaudhuri et al., 2019). Significant upregulation of the IL-23 receptor was shown in both synovial fluid and peripheral blood MAIT cells of psoriatic arthritis and RA when compared to osteoarthritis, with the above results alluding to the fact that these MAIT cell may form part of the IL-23/IL-17 network and could play a pathogenic role in psoriatic arthritis (Raychaudhuri et al., 2019).

MAIT cells were investigated in untreated RA and spondylarthritis utilising peripheral blood from 12 controls, 10 RA and 10 spondylarthritis patients as well as synovial fluid from 8 RA and 8 spondylarthritis patients (Koppejan et al., 2019). Assessment of MAIT cell frequencies in peripheral blood and synovial fluid was undertaken using flow

cytometry; no significant differences in frequency were noted between the RA, spondylarthritis and control group (Koppejan et al., 2019). A significantly higher proportion of the peripheral blood MAIT cells were $CD4^+$ in RA compared to both spondylarthritis and control, with CD161 expression also shown to be significantly reduced in the RA cohort (Koppejan et al., 2019). To assess activation PBMCs were stimulated with *E. coli* for 24 hours prior to assessing MAIT cell activation using CD25 and CD69 expression with flow cytometry (Koppejan et al., 2019). Stimulation with *E. coli* resulted in significantly lower expression of CD25 and CD69 in RA compared to control and spondylarthritis (Koppejan et al., 2019). Based on the results it could be inferred that reduced MAIT cell function could contribute to early RA (Koppejan et al., 2019).

Another study assessed the role of MAIT cells in ankylosing spondylitis. Mononuclear cells were collected from peripheral blood and synovial fluid of patients with diagnosed ankylosing spondylitis, RA and healthy controls and analysed using flow cytometry (Gracey et al., 2016). When compared to controls, MAIT cell frequency was shown to be decreased in the ankylosing spondylitis cohort, however the frequency of the IL-17A$^+$ subset of MAIT cells was increased (Gracey et al., 2016). In synovial fluid MAIT cells in ankylosing spondylitis showed increased IL-17 and granzyme frequency when compared to RA, with increased IL-17 in ankylosing spondylitis MAIT cells shown to be dependent on priming with IL-7 (Gracey et al., 2016). More recently it was shown that increased IL-22 and IL-17A MAIT cells could be associated with ankylosing spondylitis (Toussirot et al., 2018). Peripheral blood was taken from 36 patients with ankylosing spondylitis and 55 healthy controls that were enrolled in the study and the PBMC's were analysed using flow cytometry (Toussirot et al., 2018). Whilst no differences in the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ cells existed between the two groups the proportion of MAIT cells and γδ cells were significantly lower in the ankylosing spondylitis group when compared to the controls (Toussirot et al., 2018). The IFNγ^+ $CD4^+$ and $CD8^+$ T cell subsets as well as the $CD4^+$ IL-17A$^+$/IFNγ^- subset were significantly lower in the ankylosing spondylitis group when compared to control (Toussirot et al., 2018). IL-22 and IL-17 producing MAIT cells were shown to be significantly higher in the ankylosing spondylitis group with the number of IFNγ^-/IL-17A$^+$ MAIT cells shown to be higher in females with ankylosing spondylitis only (Toussirot et al., 2018). Both of these studies show overall decreased MAIT cell numbers in ankylosing spondylitis with specific

increases in cytokine specific MAIT cell subsets alluding to a potential role for MAIT cells in ankylosing spondylitis (Gracey et al., 2016, Toussirot et al., 2018).

1.6.3.5 Mucosal Associated Invariant T Cells in Inflammatory Bowel Disease

The role of MAIT cells has been assessed in IBD, specifically Crohn's disease and ulcerative colitis. One study assessed 40 patients with severe IBD, 31 with Crohn's disease and 9 with ulcerative colitis, collecting blood and fixed frozen sections from test patients; blood was also sourced from healthy donors (Serriari et al., 2014). Using flow cytometry, it was shown that MAIT cells were significantly reduced in IBD patients when compared to healthy donors, with this decrease showing no correlation to disease activity (Serriari et al., 2014). Using confocal microscopy to assess 11 ileal biopsies from Crohn's disease patients it was shown that MAIT cell frequencies were significantly increased in areas of inflamed tissue when compared to healthy tissue (Serriari et al., 2014). Using the proliferation maker Ki67, MAIT cells expressing this were significantly higher in patients with Crohn's disease when compared to controls; ulcerative colitis and Crohn's disease patients secreted significantly higher levels of IL-17 compared the healthy donors (Serriari et al., 2014). IFN-γ was shown to be decreased in Crohn's disease whilst IL-22 levels were increased in ulcerative colitis (Serriari et al., 2014). From the results it was shown that MAIT cells in IBD showed an activated profile with recruitment of MAIT cells directed to the inflamed tissue (Serriari et al., 2014).

Hiejima et al., 2015 also attempted to quantify the percentage of MAIT cells using FFPE samples from patients with ulcerative colitis, Crohn's disease and normal control mucosae. Sections were stained with TCRVα7.2 first and then doubled stained with either MR-1, CD161, CD3, or IL18Rα (Hiejima et al., 2015). MAIT cells were identified as TCRVα7.2^{+}CD161^{+} cells and quantified by counting the number of MAIT cells in 30 crypts (Hiejima et al., 2015). This study confirmed the number of MAIT cells in patients with ulcerative colitis and Crohn's disease was lower than the number identified in control mucosae (Hiejima et al., 2015). Hiejima et al., 2015 suggested that based on these results further studies into the involvement of MAIT cells in IBD is required, as well as investigation into how manipulation of these cells could be used as for therapeutic management of IBD.

A more recent study also assessed the role of MAIT cells in IBD collecting blood and biopsy specimens from 40 IBD patients, 25 with ulcerative colitis and 15 with Crohn's disease, with 19 healthy patients used as controls (Tominaga et al., 2017). As with the

above studies flow cytometry showed significantly lower frequencies of MAIT cells in IBD patients when compared to controls (Tominaga et al., 2017). IHC performed on cryostat sections showed significantly higher frequencies of MAIT cells in the colons of IBD patients compared to healthy controls with increased frequencies of MAIT cells correlating with increased disease activity (Tominaga et al., 2017). No differences were noted in cytokine secretion between ulcerative colitis and Crohn's disease patients, however IL-17 and TNF-α production was significantly higher in IBD patients compared to controls (Tominaga et al., 2017). From the results of the above studies it can be inferred that MAIT cells are activated in IBD and congregate in areas of inflammation, with some studies showing increased frequencies of MAIT cells correlate with disease activity (Serriari et al., 2014, Hiejima et al., 2015, Tominaga et al., 2017).

1.6.3.6 Mucosal Associated Invariant T Cells in Systemic Lupus Erythematosus

Whilst MAIT cells have not been studied in OLP, the function of these cells has been studied in SLE, a condition that clinically and histologically mimics OLP in the oral cavity. Examination of the MAIT cell function and level in SLE, RA, ankylosing spondylitis and Bechet's disease showed circulating levels of MAIT cells were significantly reduced in both SLE and RA (Cho et al., 2014). In SLE the greatest deficiency of MAIT cell was noted in the double negative and $CD8^+$ cell subsets with IFN-γ production shown to be impaired in MAIT cells from SLE patients (Cho et al., 2014). In RA this was due to migration of the MAIT cells to the site of inflammation, in SLE MAIT deficiency correlated with disease activity (Cho et al., 2014). Programmed death protein 1 delivers inhibitory signals to balance activation, tolerance and immunopathology, in SLE the percentages of programmed death protein 1 expressing MAIT cells were significantly higher than in healthy controls (Cho et al., 2014). Chiba et al., 2017 used peripheral blood to also investigate the role of MAIT cells in SLE. This study also demonstrated a reduction in MAIT cells in SLE patients (Chiba et al., 2017). The reduction in MAIT cell numbers was due to enhanced MAIT cell death rather than a downregulation in surface markers (Chiba et al., 2017). This study also showed the activated status of MAIT cells, by expression of the activation maker CD69, in SLE reflected disease activity (Chiba et al., 2017). MAIT cells in SLE were also shown to be activated in an MR-1 independent manner by IL-15, IFN-γ, IL-12 as well as IL-18 by upregulation of CD69 expression (Chiba et al., 2017). These results suggest MAIT cells may play a role in the pathogenesis of SLE (Chiba et al., 2017).

1.6.3.7 *Mucosal Associated Invariant T Cells in Graft Versus Host Disease*

MAIT were recently investigated to determine if they played a regulatory role in GVHD (Varelias et al., 2018), another condition that can mimic OLP in the oral cavity. Mice, some that were MAIT cell MR-1$^{-/-}$ or IL-17A$^{-/-}$ deficient, underwent stem cell and bone marrow transplants and were assessed for GVHD (Varelias et al., 2018). Using the MR-1 tetramer loaded with activating or non-activating ligands it was showed recipient MAIT cells congregated in higher numbers in the liver, lung, small intestine and colon when compared to peripheral blood (Varelias et al., 2018). It was also demonstrated that colon-associated recipient MAIT cells were able to suppress expansion of pro-inflammatory donor Th1 and Th17 cells during GVHD (Varelias et al., 2018). Recipient MAIT cells in the colon were also found to be both activated and producing high levels of IL-17 at all times (Varelias et al., 2018). Mice deficient in IL-17A that were recipients of a bone marrow transplant developed GVHD at accelerated rates, suggesting a regulatory role for MAIT cells in GVHD that was related to generation of IL-17A (Varelias et al., 2018). Results of this study show that MAIT cells acted to both attenuate T cell responses in the colon whilst also controlling barrier functions and these cells play a pivotal role in bone marrow transplants patients (Varelias et al., 2018).

Another recent study assessed MAIT cell reconstitution following myeloablative and non-myeloablative allogenic haematopoietic stem cell transplants in 41 and 66 participants, respectively (Bhattacharyya et al., 2018). Blood was taken from all test patient and healthy controls with blood and stool samples also assessed for test patients at 0, 10, 20, 30, 60, 100 and 365 days (Bhattacharyya et al., 2018). Abundance of *Blautia spp*. in the stool of transplants recipients was assessed due to the association of reduced risk of developing acute GVHD; results showed higher abundance of both *Bifidobacterium longum* and *Blautia spp*. correlated with higher counts of MAIT cells in the blood (Bhattacharyya et al., 2018). Increased presence of blood MAIT cells early post-transplant was shown to reduce the risk of GVHD development (Bhattacharyya et al., 2018). A further study reported similar findings by demonstrating in the study cohort that post-transplant recovery of MAIT cells was closely related to the development of delayed onset acute GVHD (Kawaguchi et al., 2018). Acute GVHD developed at the median time of 30.5 days and patients with grade I-IV GVHD were shown to have significantly lower MAIT/T cell ratios at 60 days post-transplant and fewer MAIT cells than those without acute GVHD (Kawaguchi et al., 2018). Taken together these studies suggest post-

transplant recovery of MAIT cells could be used as a predictor for development of acute GVHD and guide GVHD prophylaxis (Bhattacharyya et al., 2018, Kawaguchi et al., 2018).

Altered number of MAIT cells in peripheral blood, evidence of congregation in disease affected tissue, altered cytokine production and activation independent of riboflavin producing microbes are the mechanism by which MAIT cells are implicated in neoplastic, inflammatory or autoimmune disease (Chiba et al., 2012, Gracey et al., 2016, Won et al., 2016, Tominaga et al., 2017, Raychaudhuri et al., 2019). Despite the above evidence the exact role of MAIT cells in these diseases is unclear and further research is required. OLP is a T cell mediated inflammatory disease (Sugerman et al., 2002, Roopashree et al., 2010) and it could be hypothesised the MAIT cells could play a regulatory or pathogenic role in OLP in ways similar to SLE (Cho et al., 2014, Chiba et al., 2017) or GVHD (Bhattacharyya et al., 2018, Kawaguchi et al., 2018, Varelias et al., 2018).

1.6.4 Mucosal Associated Invariant T Cells in the Oral Cavity

A recent study confirmed the presence of MAIT cells in buccal mucosa taking tissue and venous blood from 94 participants that were in two groups, healthy participants from the general population and patients undergoing orthognathic surgery (Sobkowiak et al., 2019). In situ staining with TCRVα7.2 and IL18Rα in normal buccal mucosa identified MAIT cells with these cells tending to cluster in the basement membrane zone residing in either the epithelium or connective tissue above and below the basement membrane respectively (Sobkowiak et al., 2019). Tissue from the buccal biopsies and peripheral blood were analysed by flow cytometry and it was shown that the percentage of MAIT cells in buccal mucosa ranged from 0.1-7% of total T cells, which was not significantly different from the matched blood samples (Sobkowiak et al., 2019). $CD8^+$ and $CD4^-CD8^-$ T cell populations were significantly higher in the buccal mucosa with the $CD4^-CD8^-$ and $CD4^+$ phenotype also shown to be significantly increased in the MAIT cell population (Sobkowiak et al., 2019). $CD4^+$ T cells and $CD8^+$ MAIT cells were significantly increased in blood when compared to buccal mucosa (Sobkowiak et al., 2019). For both blood and buccal mucosa $CD8^+$ and $CD4^-CD8^-$ were the dominant phenotypes (Sobkowiak et al., 2019). Thus, these results confirm MAIT cells reside in the oral mucosa and congregate to the basement membrane (Sobkowiak et al., 2019); this is significant as this is the zone of T-cell destruction in OLP and could allude to a pathogenic role for MAIT cells in OLP.

A further recent study confirmed significantly higher levels of expression of the MAIT cell markers TCR Vα7.2-Jα33, Vα7.2-Jα12 and Vα7.2-Jα20 in apical periodontitis when compared to control tissue with the Vα7.2-Jα33 being the most abundant marker (Davanian and Gaiser, 2019). Apical periodontitis tissue was also shown to express significantly higher levels of TNF-α, IFN-γ and IL-17A, cytokines associated with activated MAIT cells (Davanian and Gaiser, 2019). Interestingly, the MR-1 restricted MAIT cells identified in apical periodontitis tissues were primarily $CD4^+$ with the apical periodontitis microbiome showing high riboflavin-expression (Davanian and Gaiser, 2019). Whilst it was concluded that results were suggestive of MAIT cells playing a role in oral tissue barrier local defence it should be noted that use of 16S microbiota gene analysis this study did not allow for distinction between live and dead bacteria or exclusion of other microbes such as fungi (Davanian and Gaiser, 2019).

1.7 Scoring Systems and Quality of Life Assessment

OLP is a changeable condition characterised by periods of disease activity and quiescence. To date no universally accepted scoring system for both research and clinical practice currently exists, however, many have been proposed. Earlier scoring systems have been based upon a 3 or 5 point scale system to measure disease severity (Sloberg et al., 1983, Giustina et al., 1986, Eisen et al., 1990, Thongprasom et al., 1992). Other scoring systems use the level of site involvement to determine severity (Malhotra et al., 2008). Some scoring systems have been created for the sole purpose of objectively measuring changes in disease activity during intervention studies (Sloberg et al., 1983, Giustina et al., 1986, Eisen et al., 1990, Thongprasom et al., 1992, Harpenau et al., 1995). Silverman et al., 1991 was the first study that proposed using a visual analogue scale (VAS) for the subjective assessment of pain associated with OLP (Silverman et al., 1991).

The more detailed scoring systems measure OLP disease activity based on the presence or absence of reticular, atrophic/erythematous and erosive/ulcerative lesions (Chainani-Wu et al., 2001, Piboonniyom et al., 2005, Escudier et al., 2007, Chainani Wu et al., 2008). Use of these systems allows different sites in the oral cavity to be given a clinical score that in turn can be totalled to grade the entire oral cavity (Chainani-Wu et al., 2001, Piboonniyom et al., 2005, Escudier et al., 2007, Chainani Wu et al., 2008). Most of these systems weight the presence of erythema or ulcerations within the lesions higher than reticular or plaque-like lesions (Chainani-Wu et al., 2001, Piboonniyom et al., 2005, Escudier et al., 2007).

Use of a scoring system allows the clinicians or researcher to objectively measure changes in disease activity and attempts have been made to validate some of these systems (Chainani-Wu et al., 2008). Attempts have also been made to add subjective measures to evaluate pain and symptoms associated with OLP (Chainani-Wu et al., 2008, Park et al., 2012) as well as assess quality of life. One study undertook a comparative analyses of two different scoring systems, one scoring system by Escudier et al., 2007, the other by Malhotra et al., 2008 (Lopez-Jornet and Camacho-Alonso, 2010). The Spearmen correlation coefficient between the two systems was 0.54 with the differences between the two systems shown to be statistically significant ($p < 0.001$) (Lopez-Jornet and Camacho-Alonso, 2010).

A more recent study compared two existing OLP scoring systems (Gobbo et al., 2017). Photographs were evaluated and scored for 50 OLP cases using either the modified white/erosive/atrophic or reticular/erythematous/ulcerative scoring system (Gobbo et al., 2017). Pain was assessed for all cases using a VAS with the intra-oral sites examined before steroid therapy and three weeks following therapy (Gobbo et al., 2017). The Kendall-W coefficient was used to assess agreement between the three assessors with a coefficient of 0.89 and 0.83 at baseline and three weeks following therapy respectively for the reticular/erythematous/ulcerative scoring system (Gobbo et al., 2017). The white/erosive/atrophic scoring system showed correlation coefficients of 0.74 and 0.58 at baseline and three weeks following therapy respectively with the results confirming better reproducibility using the reticular/erythematous/ulcerative scoring system (Gobbo et al., 2017). The authors noted that a limitation of this study was both the number of assessors and patients (Gobbo et al., 2017).

For more in-depth analysis of quality of life the Oral Health Impact Profile 14 (OHIP-14) can be utilised. The OHIP-14 consists of 14 questions that aim to measure quality of life whilst also quantifying the patient's perception of the social impact of their oral condition (Slade, 1997). The OHIP-14 is a shorter version of the more extensive 49 question Oral Health Impact Profile 49 that has been shown to have good precision, reliability and validity when assessing quality of life for oral mucosal conditions (Slade, 1997).

More recently one study evaluated the use of patient reported outcome measures (PROMs), a standardised tool to for patients to evaluate aspects of their own health, in OLP (Wiriyakijja et al., 2018). The authors undertook a comprehensive review of the literature and aimed to assess the psychometric properties and interpretability of the range of PROMs used in OLP (Wiriyakijja et al., 2018). The authors identified a total of 41 PROMs from the 120 studies evaluated, 3 PROMs for oral symptoms, 30 for psychological status and 8 for quality of life (Wiriyakijja et al., 2018). From the results it was noted that a wide range of PROMs are used in clinical research with most of these not having any evidence of psychometric properties or interpretability for OLP patients (Wiriyakijja et al., 2018). It was noted that 6 PROMs, including the OHIP-14 and VAS, did demonstrate some psychometric properties in the OLP cohort, however there was no evidence for interpretability (Wiriyakijja et al., 2018). The authors advised that further studies are required to investigate PROMs in the OLP cohort (Wiriyakijja et al., 2018).

At this stage, no one OLP scoring system is universally accepted for use in either research or clinical practice. Further research is required to develop a universal, reproducible, validated disease scoring system that evaluates quality of life and can be applied to OLP in both research and clinical settings.

1.8 Treatment Modalities

Many therapeutic modalities currently exist for the symptomatic management of OLP. Topical steroids are the most currently accepted treatment modality with consideration for use of systemic steroids is in cases of refractory or widespread disease (Vincent et al., 1990, Lozada-Nur et al., 1994, Al-Hashimi et al., 2007). Other treatment modalities suggested for OLP include curcuminoids (Chainani-Wu et al., 2012), calcineurin inhibitors such as topical pimecrolimus (McCaughey et al., 2011) or topical tacrolimus (Siponen et al., 2017), cyclosporine (Voute et al., 1994), hydroxychloroquine (Eisen, 1993), levamisole (Won et al., 2009), low level laser therapy (LLLT) (Cafaro et al., 2010), photodynamic therapy (PT) (Mostafa et al., 2017), aloe vera (AV) mouthwash (Mansourian et al., 2011), topical chamomile (Lopez Jornet and Aznar-Cayuela, 2016) and topical tocopherol (Bacci et al., 2017).

1.8.1 Steroids

Steroids, or glucocorticoids, are the endogenous product of the adrenal glands. They have generalized effects on metabolism as well as anti-inflammatory and immunosuppressive effects. The use of topical corticosteroids in the treatment of OLP is considered the most currently accepted gold standard for treatment (Thongprasom et al., 2011). Cochrane review completed in 2011 concluded that, based on the then current available evidence, no one specific steroid therapy is more or less effective in reducing OLP associated pain (Thongprasom et al., 2011). It also concluded that the use of these medications has been implemented in clinical practice for many years, hence, to undertake a clinical trial at this stage to validate use would be considered unethical (Thongprasom et al., 2011, Lodi et al., 2012). A more recent Cochrane found three studies that compared topical steroids to placebo with only two of those studies proceeding to meta-analysis (Lodi et al., 2020). The authors concluded that for management of symptomatic OLP topical steroids may be more effective than placebo, however based on the studies assessed and the small number of participants it should be noted that the authors overall confidence for this finding was low (Lodi et al., 2020). Topical steroids are graded according to potency and can be listed as low potency, moderate potency, high potency or super-potent (Schoepe et al., 2006, Uva et al., 2012).

Negative side effects of steroids include increased osteoclast activation with decreased osteoblast function and in long term users this results in a significant risk for osteoporosis. There is also suppression of the response to injury that can result in poor wound healing,

hyperglycaemia, Cushing's syndrome, and inhibition of growth. Other issues with the use of steroids in the treatment of OLP includes the potential to pre-dispose the patient to a secondary candidal infection (Jainkittivong et al., 2007) that can occur at rate of up to 31% in patients treated with topical steroids (Vincent et al., 1990, Lodi et al., 2007). All the above can pose a significant concern as therapy for symptomatic OLP can be protracted.

1.8.1.1 Clobetasol Propionate

Clobetasol propionate is a super-potent topical steroid that has been used in the management of oral mucosal conditions such as OLP, mucous membrane pemphigoid, pemphigus vulgaris and erythema multiforme (Lozada-Nur et al., 1994). Clobetasol has been shown to be an effective treatment for the management of symptomatic OLP with the ability to significantly improve symptoms and lesion extension within 3 weeks (Lodi et al., 2007). An assessment of the efficacy of both 0.05% clobetasol propionate ointment and 0.05% fluocinonide ointment supported in a 4% hydroxyethel cellulose gel has been compared with placebo in the management of atrophic/erosive OLP (Carbone et al., 1999). All groups received antimycotic treatment consisting of a 0.12% chlorhexidine mouthwash and miconazole gel with 60 patients enrolled in the study, divided into the three treatment groups described and instructed to use the medications for 6 months with reviews every 2 months and 6 months post-treatment (Carbone et al., 1999). A total of 50 completed the study; some improvement was noted in 20% of placebo group patients compared to some improvement noted in 90% of patients treated with fluocinonide and all CP patients treated with clobetasol (Carbone et al., 1999). 25% of lesions resolved with use of fluocinonide compared to 75% with clobetasol and 0% with placebo; overall clobetasol achieved better resolution than fluocinonide (Carbone et al., 1999). 6 months following treatment 65% of patients treated with clobetasol were stable compared to 55% treated with fluocinonide; no patients experienced candidosis whilst on treatment (Carbone et al., 1999). The authors concluded that use of clobetasol, a very potent topical corticosteroid, may be useful in controlling OLP with use of miconazole gel and chlorhexidine mouthwash a safe prophylactic treatment for candidosis (Carbone et al., 1999).

Clobetasol propionate 0.05% in Orabase administered in trays with nystatin 100,000 IU/cc efficacy was assessed in erosive gingival lesions, 11 with OLP and 22 with mucous membrane pemphigoid (Gonzalez-Moles et al., 2003). Participants were assessed for

improvement and side-effects at 10 follow-up visits every 2 weeks for the first 12 weeks then at weeks 16, 20, 24 and 48 (Gonzalez-Moles et al., 2003). No adverse effects were noted during treatment with all patients experiencing a complete response to treatment, defined as resolution of pain and ulceration (Gonzalez-Moles et al., 2003). 93.9% of patients experienced a complete response with regards to recovery of daily activity whilst 21.2% experienced a complete response with regards to recovery of atrophy (Gonzalez-Moles et al., 2003). Whilst the authors concluded the above regime was effective in managing severely erosive gingival lesions it should be noted that this study lacks both a placebo control for comparison and blinding.

A more recent study assessed two different concentrations of clobetasol propionate supported in a cellulose bioadhesive gel, 0.025% and 0.05%, in a randomised controlled trial with each group also receiving a 0.12% chlorhexadine mouthwash and miconazole gel for anti-mycotic prophylaxis (Carbone et al., 2009a). 30 patients were enrolled in the trial, 15 in each arm; patients were instructed to use the medication for 2 months and were followed up 2 months post-treatment (Carbone et al., 2009a). Both groups showed significant improvements from baseline after 2 months of treatment with the respective medications with no statistically significant differences noted between the two preparations or any significant increases in blood cortisol levels (Carbone et al., 2009a). None of the participants developed oropharyngeal candidosis with the authors concluding use of a higher concentration of clobetasol did not provide further significant therapeutic benefit (Carbone et al., 2009a).

1.8.1.2 Betamethasone Dipropionate

Betamethasone dipropionate is another high potency topical steroid that has also been used for the management of oral ulcerative conditions including recurrent aphthous stomatitis and OLP (Uva et al., 2012, Schoepe et al., 2006). A comparison of the efficacy of intralesional compounded betamethasone (5 mg betamethasone dipropionate and 2 mg betamethasone disodium phosphate per mL) to intralesional compounded triamcinolone acetonide (control) has been undertaken in a randomised control trial with 61 participants with erosive OLP (Liu et al., 2013). Patients received intralesional injections once/week for 2 weeks, if the lesions had healed prior to the second injection no further treatment was provided, if the erosion had not healed following treatment or recurred within 3 months following treatment systemic management was instituted (Liu et al., 2013). No significant differences were noted between the two groups with regards to pain reduction

however, the betamethasone group experienced significant reductions in healing percentage of erosion and erosion area when compared to control (Liu et al., 2013). The betamethasone group also had a significantly lower proportion of lesion recurrence at 3 months compared to the control group with the authors concluding that intralesional betamethasone could be used in the management of erosive OLP (Liu et al., 2013).

1.8.1.3 Dexamethasone

Dexamethasone is a steroid medication used for the treatment of including rheumatic disease, skin diseases, and allergies. Topical dexamethasone use has been studied in the management of OLP. Preparations studied for OLP management include a 0.5 mg tablet dissolved in 20 mL of water or 0.5 mg/2mL compounded mouthwash used three times/day (Hambly et al., 2016). Both of these treatment modalities were reviewed in 9 patients over 7 weeks in a single blind cross-over trial (Hambly et al., 2016). This study concluded overall that topical dexamethasone was useful in the treatment of OLP, however, the compounded mouthwash was superior to patient made mouthwash due to its convenience and patient perception of faster onset of action (Hambly et al., 2016).

1.8.2 Curcuminoids

Curcuminoids are components of turmeric that have been shown to possess anti-inflammatory properties. The efficacy of curcuminoids 2000 mg/day as an adjunct to corticosteroids in the management of OLP was assessed in a phase II placebo controlled trial (Chainani-Wu et al., 2007). The aim was to enrol 100 consecutive patients with OLP, however, interim analysis during the trial showed no difference between the placebo and curcuminoid group resulting in early termination (Chainani-Wu et al., 2007). 33 OLP patients were enrolled, with 28 completing the study, all received 60 mg/day prednisone for the first week and were either given curcuminoid 2000 mg/day or placebo for the entire duration of the study (Chainani-Wu et al., 2007). Whilst curcuminoids were well tolerated no conclusions could be drawn from the results due to early termination, however, initial analysis indicated that a less 2% chance existed that the curcuminoids would show a significantly better outcome than placebo (Chainani-Wu et al., 2007). In a more recent randomized placebo-controlled trial to study the efficacy of curcuminoids in the management of 20 OLP patients a percentage change from baseline in clinical signs and symptoms was shown to be significant in the curcuminoid group (Chainani-Wu et al., 2012). Whilst the study had limited power due to the small sample size the authors

concluded that curcuminoids 6000 mg/day in three divided doses may prove efficacious in the management of OLP (Chainani-Wu et al., 2012).

A systematic review has been undertaken recently to evaluate the current evidence on safety of curcuminoid use in OLP, efficacy of curcuminoids in treating OLP and efficacy of curcuminoids compared to use of corticosteroids with 58 articles sourced in the literature and 9 articles selected for analysis (Lv et al., 2018). A total of 259 OLP patients were included in the review with 7 studies noting significant differences in lesion appearance following management with curcuminoids (Lv et al., 2018). Of the three controlled clinical trials that compared curcuminoids to corticosteroids all of these studies showed no statistically significant differences in clinical appearance or severity of pain (Lv et al., 2018). With all of the studies reviewed few or no adverse effected were reported with only one study reporting diarrhoea (Lv et al., 2018). Whilst larger randomised controlled trials are required to further investigate use of curcuminoids in OLP the current evidence shows curcuminoids can be used safely as an adjunct to corticosteroid treatment in the management of OLP (Lv et al., 2018).

1.8.3 Calcineurin Inhibitors

Calcineurin inhibitors work to block calcineurin, a calcium and calmodulin dependent serine/threonine protein phosphate that is required for T cell activation. Topical calcineurin inhibitors commonly used in oral medicine include pimecrolimus and tacrolimus (McCaughey et al., 2011). Significant side effects associated with calcineurin inhibitors include an increased risk of skin cancer development with long term topical use on the skin.

A recent study assessed the safety and efficacy of both tacrolimus 0.1% ointment and pimecrolimus 1% cream applied twice/day in the management of refractory erosive OLP with 30 patients, 15 in each arm, completing the 8-week study with a follow-up period of 6 months post-treatment (Arduino et al., 2014). The daily cost of treatment was comparable for both medications with some patients in both groups experiencing comparable minor side effects such as burning sensation, xerostomia or gastroesophageal reflux (Arduino et al., 2014). Both medications resulted in significant improvement in clinical signs and symptoms with no significant differences noted between the two treatments, however, the authors noted pimecrolimus appeared more effective (Arduino et al., 2014). Both pimecrolimus 1% and tacrolimus 0.1% twice/daily appeared to be both

a safe and efficacious treatment option for the management of recalcitrant OLP (Arduino et al., 2014).

1.8.3.1 Pimecrolimus

The efficacy of 1% pimecrolimus cream in the management of erosive OLP has been assessed in a placebo-controlled, double-masked study with 20 participants prescribed either placebo gel or 1% pimecrolimus cream and instructed to apply the cream to the affected area twice daily; patients were assessed bi-weekly for a duration of 4 weeks (Swift et al., 2005). 18 patients completed the study with the 1% pimecrolimus group showing significant decreases in erythema at the study midpoint ($\alpha = 0.005$) and endpoint ($\alpha = 0.075$) with a trend for decreased ulceration at the study midpoint that was not maintained (Swift et al., 2005). The test group showed a significant decrease in pain scores measured with VAS compared to virtually unchanged scores recorded by the control group (Swift et al., 2005). A significant increase in areas of reticulation was noted in the control group at both midpoint and endpoint (Swift et al., 2005). Whilst small and short term this study confirmed that 1% pimecrolimus cream was effective in erosive OLP and significantly reduced both erythema and pain (Swift et al., 2005).

A double-blind randomised controlled trial of 1% pimecrolimus cream to determine efficacy in the management of erosive OLP was undertaken with 12 patients with erosive OLP completing the trial, applying either pimecrolimus 1% or placebo to ulcerated lesions twice/day for 4 weeks (Passeron et al., 2007). Patients in the pimecrolimus groups showed significant improvement with scores decreasing from 6.83 at day 0 to 3.33 at day 28 ($p = 0.04$) compared to no significant change in the control group at days 0 and 28 with scores of 4.67 and 3.33 respectively (Passeron et al., 2007). Treatment was well tolerated in both the pimecrolimus and control groups with only minor side effects, such as transient burning, reported (Passeron et al., 2007). Blood levels of pimecrolimus were not detected in controls and found above threshold in the test group with mean levels of 2.32ng/ mL on day 14 and 2.84ng/ mL on day 28 (Passeron et al., 2007). All patients who showed improvement whilst on pimecrolimus subsequently relapsed 1-month post-treatment with the authors acknowledging that long term use of pimecrolimus is required maintain symptom reduction (Passeron et al., 2007). A 6-week trial compared topical pimecrolimus 1% cream twice/day to a vehicle cream in the management of erosive OLP (McCaughey et al., 2011). Overall 1% pimecrolimus cream was shown to be superior to the vehicle in reducing overall severity, pain, erythema and erosion size (McCaughey et

al., 2011). The efficacy of topical calcineurin inhibitors has also been compared with topical steroid therapy. These two studies concluded that there was no evidence to suggest a difference between treatment with steroids or calcineurin inhibitors with regard to pain reduction in OLP (Thongprasom et al., 2011, Lodi et al., 2012).

1.8.3.2 Tacrolimus

An open label phase II non-comparative study was conducted to determine the efficacy of 0.1% tacrolimus in the management of recalcitrant or medication dependent OLP (Kaliakatsou et al., 2002). Tacrolimus 0.1% ointment was prepared in a paraffin base and all patients asked to apply the ointment to the affected areas twice a day for 8 weeks with follow-up during the 8 week study period and 22 weeks post-application (Kaliakatsou et al., 2002). 17 patients completed the study with no serious side effects reported; levels of tacrolimus in the therapeutic range were reported in 8/17 patients (Kaliakatsou et al., 2002). All patients exhibited a significant reduction in OLP scores after the full 8 weeks of treatment ($p < 0.002$) with a mean surface areas reduction of 73.3% that was shown to be significant ($p < 0.001$) (Kaliakatsou et al., 2002). The greatest reduction in lesion area occurred between weeks 1-5; after this time the response appeared to plateau (Kaliakatsou et al., 2002). Significant reductions in lesion surface area and patient reported pain were noted in the first week of therapy ($p<0.001$ and $p<0.001$ respectively) along with significant reductions in the McGill Pain Questionnaire and OHIP-14($p < 0.001$ and $p < 0.001$ respectively) (Kaliakatsou et al., 2002). Relapse occurred in 76.5% of patients with the mean time of relapse being 4 weeks post-treatment cessation (Kaliakatsou et al., 2002). 0.1% topical tacrolimus was shown to be effective in the management of OLP, however, it should be noted this study lacked a control group for comparison.

A comparison of 0.1% topical tacrolimus ointment with 0.1% triamcinolone acetonide in the management of symptomatic OLP enrolled 40 patients, 20 in each arm, with both groups applying the respective topical medications 4 times/day for 6 weeks (Laeijendecker et al., 2006). In the tacrolimus group 6 of the 20 experienced complete healing, 12 showed some improvement, with 2 showing no improvement; whilst in the triamcinolone group 2 of the 20 experienced complete healing, 7 showed some improvement and 11 showed no improvement (Laeijendecker et al., 2006). The tacrolimus group experienced a better initial response to treatment than the TA group ($p = 0.007$) with both groups experiencing similar levels of side effects (Laeijendecker et al., 2006). Whilst use of topical tacrolimus resulted in a better clinical response, both

topical tacrolimus and TA experienced similar levels of relapse post treatment (72% and 78% respectively) (Laeijendecker et al., 2006).

The effectiveness of topical tacrolimus, topical triamcinolone acetonide and placebo was assessed in a pilot randomised controlled trial in symptomatic OLP (Siponen et al., 2017). Patients were advised to use the medications three times/day and were assessed at 3 and 6 weeks with follow-up at 9 weeks if required as well as a 6 month post first phase treatment review (Siponen et al., 2017). A less than 20% reduction in OLP clinical score resulted in the patient being switched to topical tacrolimus for 6 weeks (Siponen et al., 2017). Both tacrolimus and triamcinolone were significantly more effective reducing clinical signs of OLP than placebo at week 3 with no significant differences in efficacy noted between either treatment (Siponen et al., 2017). Whilst both TA and tacrolimus proved more effective than placebo (Siponen et al., 2017), one limitation of this study was the small initial sample size and drop-out experienced during follow-up.

1.8.4 Cyclosporine

Cyclosporine is a fungal peptide with potent immunosuppressive activity but no effect on the acute inflammatory reaction. The efficacy of cyclosporin A 0.025% adhesive paste applied topically 4 times/day was assessed in 9 patients with symptomatic recalcitrant OLP with at least 4 months follow up and all patients had previously failed topical or systemic corticosteroid therapy (Voute et al., 1994). None of the patients had complete remission and 5 showed either no response or an increase in adverse symptoms leading the authors to conclude that topical cyclosporin A was not an effective treatment for management of recalcitrant OLP (Voute et al., 1994). A similar experiment with 14 patients with recalcitrant OLR found that 7 of the 14 demonstrated a partial reduction in signs and symptoms with the authors concluding that bioadhesive topical cyclosporine may be a useful adjunct in oral lichenoid reactions (Epstein and Truelove, 1996).

Another study compared cyclosporin A, 500 mg as a 5-minute swish and spit mouthwash three times/day, and 1 mg/mL triamcinolone acetonide paste 3 times/day, with patients advised not to eat or drink for 30 minutes after application and assessments at baseline, 2 days, 1, 2, 4 and 6 weeks during active treatment, as well as 6 and 12 months post-treatment (Sieg et al., 1995). After 6 weeks of treatment slight but transient improvement was noted in both groups with not additional benefit gained with an additional 6 weeks of cyclosporin A treatment following use of triamcinolone (Sieg et al., 1995). Neither

treatment was able to exhibit long-term improvement in disease activity with ultimately no significant differences existed between the two treatment regimens (Sieg et al., 1995).

A further randomised, double blind controlled trial to compare the effectiveness of topical 0.025% clobetasol propionate and topical 1.5% cyclosporin, both supported in a bioadhesive gel, in the management of erosive and atrophic OLP with 40 patients enrolled and instructed to apply the medication twice/day (Conrotto et al., 2006). Chlohexadine 0.12% mouthwash used three times/day and miconazole gel applied once/day for antimycotic prophylaxis (Conrotto et al., 2006). Patients continued use of the prescribed medication for 2 months and examined prior to starting treatment, every week during treatment and 2 months post-treatment (Conrotto et al., 2006). No significant differences existed between the two groups with regards to improvement of symptoms, however, significantly more patients treated with clobetasol showed clinical improvement compared to those treated with cyclosporin (95% vs. 65% $p = 0.004$) (Conrotto et al., 2006). Cyclosporin was more effective in inducing stability than clobetasol with 77% of patients being stable 2 months post-therapy compared to 33% ($p = 0.04$); patients treated with clobetasol also experienced significantly more side effects than those treated with cyclosporin ($p = 0.04$) (Conrotto et al., 2006). The daily cost of cyclosporin was estimated as 1.82€ compared to 0.35€ per day for clobetasol; whilst clobetasol induced greater clinical improvement the long term results were less stable than cyclosporin (Conrotto et al., 2006).

1.8.5 Hydroxychloroquine

Hydroxychloroquine is an antimalarial medication that has been used to reduce inflammation in patients with rheumatoid disorders and lupus. Hydroxychloroquine increases lysosomal pH in antigen presenting cells to block the TLRs on plasmacytoid dendritic cells. Hydroxychloroquine has been shown to improve healing and symptoms of both OLP and cutaneous lichen planus (Eisen, 1993, Atzmony et al., 2016). In a recent systematic review and meta-analysis of treatment for cutaneous lichen planus, hydroxychloroquine was shown to be effective in achieving an overall response (Atzmony et al., 2016). In an open trial 10 OLP received hydroxychloroquine 200-400 mg/day as monotherapy for 6 months with no adverse effects reported (Eisen, 1993). Patients were reviewed every 4-8 weeks during treatment with 9/10 experiencing an excellent response with pain relief and reduction in erythema usually observed 1-2 months following therapy (Eisen, 1993). Resolution of erosions typically occurred

between 3-6 months with 3/6 patients with erosions at baseline experiencing complete healing by 6 months (Eisen, 1993).

1.8.6 Levamisole

Levamisole is an immunomodulatory agent with the capacity to restore the normal phagocytic function of macrophages and neutrophils whilst also modulating T cell mediated immunity. The efficacy of levamisole 50 mg 3 times/day for 3 consecutive days, then no therapy for 4 consecutive days was evaluated in 11 patients with OLP (Won et al., 2009). Treatment was continued until lesion clearance was demonstrated with patients demonstrating clinical improvement after 2 weeks of treatment with lesion clearance demonstrated at an average of 6.2 weeks (Won et al., 2009). No significant side effects, except mild itching in one patient were noted (Won et al., 2009). The authors concluded that levamisole monotherapy could be a useful and safe treatment for the management of chronic OLP (Won et al., 2009).

1.8.7 Low Level Laser

LLLT uses 40 mW with an output of 7mW to facilitate laser biostimulation so as to stimulate the tissues regenerative capabilities. Using a prospective cohort of 13 cases of OLP unresponsive to standard therapy, a study has shown the outcome of these patients treated with a 904nm pulsed laser, based on reduction of lesion size, VAS of pain and stability of symptoms (Cafaro et al., 2010). Overall patients showed a significant reduction in symptoms, including reduction of lesion size and reported pain, with no LLLT reported complications (Cafaro et al., 2010). This study concluded that LLLT could be used as a possible treatment for patients with OLP unresponsive to standard therapy (Cafaro et al., 2010). A comparative pilot study assessed the efficacies of LLLT and topical corticosteroids, 0.5 mg in 5 mL 4 times/day, in the management of erosive OLP, with 30 patients enrolled and 24 completing the study (Jajarm et al., 2011). Results of the study showed significant symptom improvement from baseline in both groups. Both groups showed reductions in pain score, appearance and clinical severity with no significant differences noted between the two groups when comparing for relapse and clinical response (Jajarm et al., 2011). Thus, it would appear that LLLT is as effective as topical corticosteroids in the management of OLP.

1.8.8 Photodynamic Therapy

Photodynamic therapy (PT) is a minimally invasive treatment that utilises light, oxygen and a photosensitising agent to initiate cellular destruction via a free radical oxidative process. Use of PT to manage erosive lesions in OLP was assessed in a comparative study of toluidine mediated PT and topical corticosteroids, dexamethasone 0.5 mg in 5 mL, in the management of 25 patients with erosive OLP with evaluation weekly during the first month and further reviewed 2, 3 and 4 weeks post-treatment (Jajarm et al., 2015). Both the corticosteroid and PT groups showed significant improvement in OLP score with no significant changes being noted between the two groups (Jajarm et al., 2015). With regards to improvement in pain and efficacy indices, the control group showed significantly more improvement than the PT group (Jajarm et al., 2015). Whilst PT was effective in reducing signs and symptoms of erosive OLP, greater reductions in signs and symptoms was achieved with use of topical corticosteroids (Jajarm et al., 2015).

A more recent similar study assessing the efficacy of PT in the management of erosive OLP comparing PT with conventional treatment using topical corticosteroid (Kenakort-A-orabase) enrolled 20 patients with a total of 37 lesions randomly assigned to two groups; group A was advised to apply a thin layer of the topical corticosteroids three times/day for 2 months, whilst group B received PT with methylene blue as a photosensitiser once/week for 2 months (Mostafa et al., 2017). Both groups showed significant decreases in lesions size at 2 months with the PT groups showing a greater decrease compared to the control group ($p = 0.0001$ and $p = 0.022$ respectively) (Mostafa et al., 2017). The PT group also showed greater decreases in VAS scores than controls with the authors concluding PT was a better treatment than topical corticosteroids for the management of erosive OLP (Mostafa et al., 2017).

Another study comparing PT with methylene blue efficacy with dexamethasone solution (0.5 mg in 5 mL) 4 times/day in the management of OLP enrolled 30 patients that were equally divided into two group groups and evaluated at 15, 30, 60 and 90 days (Bakhtiari et al., 2017). Subjective change was assessed using a VAS with objective changes in lesion appearance measured using a scoring system (Bakhtiari et al., 2017). No significant differences existed between the two groups with the results of this study confirming PT was as effective as topical dexamethasone in the management of OLP (Bakhtiari et al., 2017).

1.8.9 Aloe Vera

Natural alternatives such as AV mouthwash have also been assessed in the management of OLP. A randomised, placebo-controlled double-blinded trial of AV gel recruiting 54 patients who were assessed at enrolment, 2, 4, 6- and 8-weeks post therapy found that 22 of the 27 (81%) patients treated with AV showed a good response (≥ 50% reduction in clinical score) to treatment over 8 weeks of treatment, with 2 (7%) experiencing complete remission (Choonhakarn et al., 2008). For those treated with placebo, no patients experienced full remission and only 1/27 (4%) experienced a good response (Choonhakarn et al., 2008). No serious side effects were experienced in either group with AV gel being significantly more effective in OLP management than placebo ($p = 0.005$) (Choonhakarn et al., 2008).

A separate and more recent study divided 46 erosive or atrophic OLP patients randomly into two treatment groups and prescribed patients either 0.1% triamcinolone acetonide paste or AV mouthwash 4 times/day for 2 months (Mansourian et al., 2011). Both treatment groups showed significant reductions in OLP signs, specifically lesion reduction and symptoms, immediately after treatment as well as 2 months post-treatment (Mansourian et al., 2011). Thus, AV mouthwash was determined to be an effective substitute for triamcinolone in the management of erosive or atrophic OLP (Mansourian et al., 2011). Further, another randomised blinded controlled trial compared the efficacy of AV and triamcinolone acetonide in 40 patients with OLP with patients randomly assigned to two groups and advised to apply the topical medications 3 times/day for 8 weeks (Reddy et al., 2012). Both the AV and triamcinolone groups showed reductions in the OLP clinical score, with the AV group showing a more marked reduction that was significantly higher at 2, 3, 6 and 8 weeks compared to the triamcinolone group (Reddy et al., 2012). Change in burning sensation was similar across both groups with the authors concluding that whilst further research is required, AV gel was effective in reducing both signs and symptoms of OLP and could be considered as an alternative treatment option to topical steroids (Reddy et al., 2012).

A 2017 systematic review and meta-analysis for the efficacy of AV in the management of OLP searched for clinical trials comparing either corticosteroids or placebo with 5 trials and two case reports undergoing qualitative analysis and three undergoing meta-analysis (Ali and Wahbi, 2017). This review concluded that the studies assessed confirmed effectiveness of AV in the management of OLP and use of AV was not inferior

to either placebo or topical steroid (triamcinolone acetonide) (Ali and Wahbi, 2017). Whilst AV showed promising results the authors advised further randomised controlled trials are required (Ali and Wahbi, 2017).

1.8.10 Topical Chamomile

A recent randomised double-blinded study divided 60 patients with OLP randomly into two treatment groups; 30 treated with 2% *Chamaemelum nobile* and 30 treated with a placebo gel (Lopez Jornet and Aznar-Cayuela, 2016). Both treatment groups used the respective medications three times/day for 1 month and those treated with chamomile showed significant reductions in symptoms including reduction in pain ($p < 0.001$) and burning sensations ($p < 0.001$) (Lopez Jornet and Aznar-Cayuela, 2016). 92% of patients treated with chamomile showed a partial or total response to treatment compared to a partial response only in 17% of the placebo group patients (Lopez Jornet and Aznar-Cayuela, 2016). No adverse effects were experienced in either treatment group and the authors concluded that topical chamomile could be an effective alternative treatment modality in the management OLP (Lopez Jornet and Aznar-Cayuela, 2016).

1.8.11 Tocopherol

Tocopherol is a liposoluble antioxidant that belongs to the vitamin E family and is a compound that occurs naturally in many foods. An exploratory study of the use of topical tocopherol as a possible management strategy for reticular OLP was undertaken in a double-blind, placebo, crossover study design with a total of 33 patients completing the study (Bacci et al., 2017). The patients were split into two groups and instructed to use their respective treatments three times/day for one month with a two week wash out period prior to switching to the alternative treatment and repeating the same process for another month (Bacci et al., 2017). While no significant differences were noted between the two treatments with respect to symptoms, topical tocopherol was shown to be effective in reducing OLP lesion size ($p<0.05$) (Bacci et al., 2017).

Ultimately many topical and systemic treatment modalities have been trialled with the intent of managing the symptoms of OLP. There is a lack of high-quality evidence to support the use of any one treatment modality over another. To date use of topical corticosteroids remains the most clinically acceptable gold standard of treatment (Thongprasom et al., 2011). Further high-quality randomised placebo-controlled trials are required to definitively determine the best management protocol for OLP.

1.9 The Role of Candida in Oral Lichen Planus

Patients presenting with intra-oral lesions, including potentially malignant lesions, such as OLP and oral leukoplakia, as well as malignant lesions such as oral squamous cell carcinoma (OSCC) are frequently identified with *Candida spp.* (Hatchuel et al., 1990, Jainkittivong et al., 2007, Zeng et al., 2009, Masaki et al., 2011). Higher frequency and colonisation rates of *Candida spp.* have been identified in patients with oral cancer (Alnuaimi et al., 2015). With specific reference to OLP, The presences of intra-oral yeast has been variably reported in the literature with rates of varying from 20-40% for controls and 40-80% for OLP (Jainkittivong et al., 2007, Masaki et al., 2011). Studies have shown that patients treated with topical steroids to manage OLP are more likely to harbour *Candida spp.*, in particular *C. albicans* (Pereira Tdos et al., 2014). However, in those patients who have been identified as having oral candidosis it has been suggested by some that correct identification of the *Candida* species is required to allow for appropriate antimycotic therapy (Kragelund et al., 2013).

1.9.1 Detection of Oral Yeast

Isolation of *Candida spp.* from the oral cavity is not indicative of infection and diagnosis of oral candidosis must be evaluated with clinical signs and symptoms (Farah et al., 2010). Common tests for the quick and easy identification of hyphae include a smear of the site that can be fixed for staining with the periodic acid Schiff (PAS) stain (Williams and Lewis, 2000, Coronado-Castellote and Jimenez-Soriano, 2013). Use of potassium hydroxide (KOH), a stain that degrades skin and cellular debris for rapid identification of dermatophytes, can also be used alone or in conjunction with calcofluor white (CW), a stain that assists with identification of hyphae by causing the hyphae elements to fluoresce under ultraviolet light (Haldane and Robart, 1990). CW has several advantages for use including ease of use, rapid diagnosis of *Candida* hyphae and requiring only approximately 30 seconds for slide preparation (Bhavasar et al., 2010). The main disadvantage of using CW is that a fluorescent microscope is required in conjunction with a standard microscope, a significantly more expensive purchase for an arguably minor increase in diagnostic yield. Photo documentation of the fluorescence on the slide is also required (Bhavasar et al., 2010). Smear techniques are commonly used in clinical practice as they are quick, easy and can be carried out chair side.

The oral rinse technique involves the patient holding or rinsing for one minute with 10 mL of sterile phosphate buffered saline and can be used to culture *Candida* on an agar

medium, such as Sabouraud dextrose agar (SDA), to count the number of colony forming units (CFU) (Borromeo et al., 1992, Williams and Lewis, 2000). A more recent advance on this technique is use of a concentrated oral rinse technique combined with PCR to provide a rapid technique that allows for both identification and speciation of *Candida spp.* (White et al., 2004, Liguori et al., 2007). It is important to note that at this stage no one single test used to identify *Candida* in the oral cavity has the ability to determine when the transition between commensal and pathogenic states will occur (Manfredi et al., 2013).

1.9.2 Oral Candidosis

Candida spp. is associated with many other conditions in the oral cavity, including pseudomembranous candidosis (McCullough and Savage, 2005, Farah et al., 2010), erythematous stomatitis, (McCullough and Savage, 2005, Terai and Shimahara, 2005, Farah et al., 2010), angular chelitis (Öhman et al., 1986, Warnakulasuriya et al., 1991), median rhomboid glossitis (Goregen et al., 2011) and chronic hyperplastic candidosis (Cawson and Lehner, 1968, Lamey et al., 1991, Sitheeque and Samaranayake, 2003). *Candida* is a commensal oral organism that is present in the mouths of approximately 30-50% of healthy individuals (Arendorf and Walker, 1980, Mun et al., 2015). The most important *Candida* species in relation to the oral cavity is *C. albicans* with other *Candida spp.* isolated in the oral cavity including *C. tropicalis, C. glabrata, C. parapsilosis, C. stellatoidea, C. krusei* and *C. kyfer* (McCullough et al., 1996).

Candida spp. are opportunistic commensal organisms of the oral cavity and the development of oral candidosis is usually the result of an imbalance between fungal virulence and host defence. The balance between health and disease states in oral candidosis may be in part due to the presence of medical conditions (MacPhail et al., 2002, Greenspan et al., 2004, Javed et al., 2014), tobacco smoking or use of medications that can disrupt to the oral flora (Terai and Shimahara, 2005). Oral candidosis is strongly associated with endocrine disorders and immunosuppression. Infections resulting from *Candida spp.* are especially prevalent in patients who are human immunodeficiency virus (HIV) positive with a low CD4 count and high viral load (MacPhail et al., 2002, Greenspan et al., 2004, Naidu et al., 2013, Javed et al., 2014).

1.9.3 Fungal Virulence Factors

Fungal virulence factors of *Candida spp.* include the ability to invade epithelial cells, through endocytosis or active penetration, resulting in transmigration and breaching of

epithelial barriers (Dalle et al., 2010). *Candida spp.* have the ability to bind to epithelial cells and secrete hydrolases. Specifically, *Candida spp.* can secrete aspartic proteases, esterase, β-haemolysis, phospholipase class B and lipases to facilitate epithelial cells invasion (Dalle et al., 2010, Pakshir et al., 2013). Different species of *Candida* show different profiles with relation to phospholipase, β-haemolytic and esterase activity. *C. parapsilosis* shows less phospholipase activity when compared to strains of *C. albicans* (Pakshir et al., 2013).

The ability for *Candida spp.* to form biofilms of varying densities also determines its virulence and pathogenicity. Those species able to form biofilms with higher cell densities having a greater inherent capacity to form active infection (Silva et al., 2009, Silva et al., 2010). Studies have shown a relationship between *Candida* genotypes and virulence properties related specifically to OLP (Zeng et al., 2008). *C. albicans* genotypes A and C are found primarily in erosive forms of OLP, while genotypes A and D have been found primarily in non-erosive forms of OLP (Zeng et al., 2008). Phospholipase activity in genotypes A and C have been shown to be significantly higher than phospholipase activity in genotypes B and D with the type A genotype showing the strongest ability to adhere to the epithelium (Zeng et al., 2008). Based on this study there is some evidence to support the theory that *Candida spp.* presence may play a role in OLP pathogenesis, especially with regards to erosive forms of OLP.

1.9.4 Oral Yeast Carriage in OLP

The normal, healthy oral yeast carriage has been shown to be 48.3% in 203 patients attending for general and specialist dental treatment who had no clinical sign or symptoms of oral candidosis, with no statistically significant associations noted in carriage when comparing age, gender or presence of a removable oral prosthesis (Mun et al., 2015). Smoking status and the presence of active caries was shown to be positively correlated to oral yeast carriage with current smokers being seven times more likely to harbour *Candida* compared to non-smokers (Mun et al., 2015).

A study in 2009 reviewed patients for *C. albicans* from a cohort of 128 healthy controls and 300 patients with OLP, including patients with both the erosive and non-erosive OLP (Zeng et al., 2009). It was shown the erosive form of OLP harboured more *C. albicans* compared to control or non-erosive forms of OLP; this relationship was considered significant ($p = 0.001$). Control patients showed a higher *C. albicans* carriage (20.3%) compared to the patients with non-erosive OLP (15.0%) (Zeng et al., 2009). This study

also reported that the virulence factors of *C. albicans*, such as phospholipase activity and adhesion, were shown to be significantly more marked in OLP patients compared to control (Zeng et al., 2009). The authors concluded is possible that some isolates of *C. albicans* that possess specific virulence factors could act as co-factors that contribute to the development of OLP, especially the erosive form of OLP (Zeng et al., 2009). A more recent study assessed the prevalence and phenotypic variation of *Candida* species in 64 patients with non-erosive OLP, 16 patients with erosive OLP, and 80 healthy controls (Arora et al., 2016). 50% of the erosive cases, 28% of the non-erosive cases and none of the controls showed evidence of *Candida* (Arora et al., 2016). *C. albicans* was the predominant species found in non-erosive OLP, whilst non-*C. albicans* species, including *C. glabrata* and *C. krusei*, were more prominent in erosive OLP (Arora et al., 2016). The non-*C. albicans* isolates showed a higher resistance to commonly used antifungals when compared to the *C. albicans* isolates, highlighting the need for antifungal susceptibility testing prior to provision of medication (Arora et al., 2016).

In contrast, it has also been argued that no significant relationship exists between OLP and *Candida. spp* and that *Candida spp.* have no effect on the development or symptomatology of OLP (Holmstrup and Dabelsteen, 1974, Mehdipour et al., 2010, Artico et al., 2014). In a study of 21 erosive OLP patients and 21 controls, no significant differences existed between the two groups with regards to the presence of *Candida spp.* and *Candida* was not confirmed to be an aetiological factor for erosive OLP in this study (Mehdipour et al., 2010). The frequency of *Candida spp.*, xerostomia and salivary flow rate in 38 patients with OLP, 28 non-OLP patients with mucosal lesions and 32 control patients with no mucosal pathology were studied and it was found that the frequencies of hyposalivation and xerostomia were similar across all groups, however, control patients showed significantly higher frequencies of *Candida spp.* compared to OLP and non-OLP patients (Artico et al., 2014). *C. albicans* was the predominate species in all groups and salivary flow rate was not considered to be a significant factor for *Candida* colonisation (Artico et al., 2014). The significance of simply finding *Candida* in association with OLP or any other oral lesion is currently unknown. An early 1974 review of 43 biopsies of typical OLP reported that only one sample, 2% of all samples, was shown to be invaded by *Candida* (Holmstrup and Dabelsteen, 1974). OLP showed less susceptibility to *Candida* invasion than oral leukoplakia, where approximately 1/3 of leukoplakia lesions are invaded by *Candida* (Holmstrup and Dabelsteen, 1974).

The types and strains of *Candida* that inhabit oral leukoplakia and OLP have been purported to be different from those isolated from the normal oral mucosa of healthy patients with the oral cavity comprising different aetiological niches for yeasts (Krogh et al., 1987b). In OLP, *C. albicans* is typically the dominating species isolated (Krogh et al., 1987b). However, non-*C. albicans* species have been shown in higher frequency in OLP patients compared to controls, especially in OLP patients with diabetes (Masaki et al., 2011). In a study of 37 OLP patients that assessed different *Candida spp.* diagnostic techniques and how these may affect treatment decisions, it was shown that 16 of the 37 patients were diagnosed with oral candidosis based on clinical signs and symptoms as well as the presence of hyphae on cytosmear (Kragelund et al., 2013). More non-*albicans* *Candida* species were detected by cytobrush sampling compared to the oral rinse/culture technique; it was determined that of the 22 OLP patients who received treatment, 27% should have been offered alternative therapy based on the diagnostic results (Kragelund et al., 2013).

It is possible that patients with OLP may have impaired T cell immune responses that affect the patients ability to react to microbial antigens (Simark-Mattsson and Eklund, 2013). One study assessed mononuclear cells from 8 patients with OLP and 8 healthy controls by either stimulating with purified protein derivative, *C. albicans* and phytohemagglutinin or leaving the cells unstimulated and the measuring production of inflammatory cytokines (Simark-Mattsson and Eklund, 2013). Patients with OLP showed significantly reduced cellular proliferative responses to purified protein derivative and *C. albicans* compared to controls as well as reduced production of TNF-α and INF-γ when stimulated with purified protein derivative (Simark-Mattsson and Eklund, 2013). Ultimately, patients with OLP showed reduced immunological responses to microbial antigens when compared to age, sex and gender matched controls (Simark-Mattsson and Eklund, 2013), a factor that may encourage microbial infection.

It is not known whether the presence of *Candida* in OLP and other oral lesions is coincidental or if changes to the local environment induced by these lesions create ecological niches that facilitates overgrowth and colonisation of *Candida spp.* (McCullough et al., 2002, Hebbar et al., 2013).

1.10 The Malignant Potential of Oral Lichen Planus

The malignant potential of OLP has been a subject of much contention. Specifically, many questions to whether OLP is truly an oral potentially malignant condition with an inherent ability to undergo a mutagenic change.

1.10.1 Lichenoid Dysplasia and Oral Lichenoid Lesions

Questions have been raised as to the histopathological diagnostic criteria used to diagnose OLP. In 1985, strict histopathological criteria were employed to differentiate OLP from lichenoid dysplasia, a new clinical entity described for those lesions that have the presence of epithelial dysplasia with coincident lichenoid features (Krutchkoff and Eisenberg, 1985). These authors concluded that reports of malignant transformation in OLP should be viewed with scepticism unless accurate documentation with regards to histopathological diagnosis had been provided (Krutchkoff and Eisenberg, 1985). Subsequently, a new histopathological diagnostic criterion to diagnose OLP was developed to help define the histopathological differences between OLP and OLL so as to aid in correct diagnosis and differentiation between the two conditions (van der Meij and van der Waal, 2003). While one study showed an increased malignant transformation rate of OLL, this increase was not significant with results suggesting only a trend in the OLL cohort (van der Meij et al., 2003). A further study of 192 patients with either OLP or OLL reported no increase in the development of carcinomas in patients with OLP, whilst patients with OLL showed a 142 fold increase (van der Meij et al., 2007). The difference between the malignant transformation rates of OLL and OLP was also shown to be significant (van der Meij et al., 2007).

The reported rates of malignant transformation for OLP vary from less than 1% per annum (Eisen, 2002, van der Meij et al., 2007, Carbone et al., 2009b, Bombeccari et al., 2011), greater than 1 % per annum (Silverman Jr et al., 1985, Holmstrup et al., 1988, Silverman Jr et al., 1991), to as high as 3.5% (Fitzpatrick et al., 2014).

A recent meta-analysis of 20,095 patient with both OLL (419) and OLP (19,676) lesions was undertaken using data pooled from 57 studies and the overall pooled proportion for the OLP malignant transformation from the 57 studies was 1.1% (95% CI: 0.9%, 1.4%) (Aghbari et al., 2017). Pooling data from 14 studies that used the WHO 2003 diagnostic criteria resulted in a slightly lower pooled proportion of 0.9% (95% CI: 0.5%, 1.3%) (Aghbari et al., 2017). The risk of malignant transformation was higher amongst patients

diagnosed with OLL with a pooled proportion of 2.5% (95% CI: 1%, 4%) (Aghbari et al., 2017). These authors also found that the most common sites for malignant transformation were the tongue (1.05%), followed by the buccal mucosa (0.7%), gingiva/lips (0.6%) and floor of mouth (0.5%). Another recent systematic review and meta-analysis of 82 studies and 26,742 patients defined a combined malignant transformation rate for OLP of 1.1% (95% CI: 0.8-1.5) with a slightly higher rate for OLL of 1.9% (95% CI: 0.2-5.0) and lichenoid reactions 1.7% (95% CI: 0.0-5.5) (Gonzalez-Moles et al., 2019). This study concluded that while consistent results were reported the malignant transformation rates were underestimated and underestimation was the result of the restrictive diagnostic criteria employed in the studies assessed, specifically some papers excluding cases of OLP with histologically diagnosed dysplasia, inadequate patient follow-up and/or the low quality of the studies reviewed (Gonzalez-Moles et al., 2019).

1.10.2 Oral Lichen Planus Subtype

While the rate of malignant transformation of OLP is low compared to other potentially malignant lesions, there is a need for ongoing mucosal surveillance. This is especially important for patients with plaque-like and the ulcerative/atrophic/erosive forms of OLP that are associated with the highest risk of malignant transformation (Holmstrup et al., 1988, Eisen, 2002, Bombeccari et al., 2011). The recent meta analyses demonstrated that the rate of malignant transformation was 1.7% for erosive OLP, 1.3% for atrophic OLP, while only 0.1% for reticular OLP (Aghbari et al., 2017). In line with other oral cancer research, smokers and alcoholics show significantly higher rates of OLP malignant transformation, (OR: 2.95% CI (1.3, 3.2) $p = 0.004$) and (OR: 3.5 95% CI (1.5, 8.0) $p = 0.003$) respectively, compared to non-smokers and non-alcoholics (Aghbari et al., 2017). HCV positive patients also showed an increased rate of OLP malignant transformation (OR: = 5.95% CI (1.6, 16.1) $p = 0.007$), compared to HCV negative patients (Aghbari et al., 2017). Another study assessed the risk of malignant transformation in a cohort of Italian patients ($n = 402$) diagnosed with OLP of which 19.3% were HCV positive (Gandolfo et al., 2004). During the follow-up period that averaged 4.9 years two men and 7 women were diagnosed with OSCC and it was shown that irrespective of clinical subtype or therapy, patients with OLP were at an increased risk of developing OSCC (Gandolfo et al., 2004). The relative risk for development of OSCC for those with HCV compared to those without was 3.16 (0.8-12.5) (Gandolfo et al., 2004). Whilst the results were suggestive that presence of HCV infection did increase the risk of developing OSCC

the authors did note that it was difficult to interpret the results based on other cofounders associated with HCV infection such as cirrhosis (Gandolfo et al., 2004).

A more recent 2019 systemic review and meta-analysis also confirmed the above findings with risk factors for malignant transformation including lesions located on the tongue (relative risk (RR): 4.1, 95% CI 2.4-7.0, $p = 0.004$), use of alcohol (RR: 2.3, 1.1-4.6, $p = 0.02$) and use of tobacco (RR: 2.0, 1.3-3.1, $p = 0.002$) with the association with hepatitis C approaching significance (RR: 4.5, 95% CI 1.0-20.2, $p = 0.053$) (Gonzalez-Moles et al., 2019). One finding that should be highlighted in this study was that lesions defined as erosive and/or atrophic were exclusively associated with an increased malignant transformation risk (RR: 4.1, 95% CI: 2.4-7.0 $p < 0.001$) (Gonzalez-Moles et al., 2019). Inclusion or exclusion of dysplasia in the diagnostic criteria is contentious, some studies included in this analysis did not have epithelial dysplasia as an exclusion criterion, and it should be noted that the malignant transformation rate was significantly higher in this subset ($p < 0.001$) (Gonzalez-Moles et al., 2019).

Presence and severity of dysplasia in OLP and other oral potentially premalignant lesions on histopathological examination has also been associated with an increased risk of malignant transformation (Bornstein et al., 2006, Warnakulasuriya et al., 2011). Thus, biopsy is an essential part of the diagnosis and management of OLP, particularly in those patients with ulcerative/erosive OLP and other risk factors for oral malignancy, including smoking and regular consumption of alcohol.

1.10.3 Gene Expression and Polymorphisms

It is important to consider that fact that OLP patients may have a genetic predisposition that could increase the risk of a malignant change. Specifically, altered gene expression for FoxP3, VEGF, angiopoietin 1, angiopoietin 2, MMP1 and SCGB2A as well as gene polymorphisms in INF-γ +874A/T and IL-12A that change the inflammatory profile in OLP leading to ongoing inflammation, a known risk factor for carcinogenesis (Fujita et al., 2009, Tao et al., 2009, Kimkong et al., 2012, Jiang et al., 2015). MYC is a family of proto-oncogenes that play an important role in cell proliferation, apoptosis, differentiation and metabolism. c-MYC is a gene from the MYC family shown to be involved in OSCC (Segura et al., 2013). A study assessing from 10 OLP patients that progressed to OSCC, including samples taken before and after OSCC formation, and 12 OLP controls assessed c-MYC expression using microarray analysis, IHC and fluorescent *in situ* hybridisation (Segura et al., 2013). c-MYC overexpression was noted in the OSCC and pre-OSCC

samples, 91% and 47% respectively with no overexpression noted in the control groups (Segura et al., 2013). Overexpression of c-MYC was noted in 73% of samples in the OSCC group, 87% samples in the pre-OSCC group, and 44% of control patients with the differences between the groups being statistically significant ($p = 0.019$) (Segura et al., 2013). OLP patients that progressed to OSCC showed significant MYC gains and c-MYC expression compared to controls, suggesting MYC status may be predictive of OLP patients with a higher risk of malignant progression (Segura et al., 2013).

1.11 *Candida's* role in Carcinogenesis

There has been increasing interest in the role *Candida spp.* may play in oral carcinogenesis. In 1966 the potential link between chronic hyperplastic candidosis and oral carcinogenesis was first reported (Cawson, 1966). This particular form of oral candidosis is thought to be associated with an increased risk of malignant transformation (Darling et al., 2012) and is differentiated from true oral leukoplakia by the fact that treatment with antifungals usually results in some form, either partial or complete, of lesion resolution (Cawson and Lehner, 1968). Studies have also shown a correlation for increasing colonization of *Candida* species with increasing degree of dysplasia (McCullough et al., 2002, Hebbar et al., 2013) as well as differences in *Candida* strain colonisation between oral cancer patients and non-oral cancer cohort (Alnuaimi et al., 2015). Oral cancer patients have been shown to harbour both significantly higher frequencies and colonisation rates of oral yeast compared to patients without oral cancer with significantly higher frequencies of *C. albicans* genotype A noted in the oral cancer cohort (Alnuaimi et al., 2015).

However, whether or not *Candida* plays a role in enhancing the malignant potential of oral lesions is yet to be conclusively answered as it is possible this correlation is coincidental and simply reflects a change in the local environment (McCullough et al., 2002, Hebbar et al., 2013). Whilst *Candida spp.* has many different inherent pathogenic capabilities that have been hypothesised to contribute to an enhanced mutagenic potential of a lesion, a definitive cause and effect relationship is yet to be established.

1.11.1 Nitrosamines

Candida has the ability to produce endogenous nitrosamines from precursor nitrites and amines (Krogh et al., 1987a). In a rat model nitrosamine compounds, such as nitrosomethylbenzylamine, were shown to induce tumours in rat oesophagus (Lijinsky et al., 1982). Krogh et al. 1987b showed certain *Candida* species and strains had the ability to produce endogenous N-nitrosobenzylmethylamine, specifically the *C. albicans* biotypes 051, 147, 151, 153, 157 and 353 were the highest producers of this endogenous nitrosamine (Krogh et al., 1987b). It is interesting to note that these strains with the highest nitrosation potential were isolated from oral lesions associated with more advanced precancerous changes, thus supporting a potential causative role of oral yeast in oral carcinogenesis (Krogh et al., 1987a).

1.11.2 Promotion of Carcinogenesis

Using a rat model with the water-soluble carcinogen 4-nitroquinoline-1-oxide (4-NQO), a compound used in research to mimic tobacco, a study in 1992 showed *C. albicans* strain 151 exhibited a similar ability to promote neoplastic change when combined with 4-NQO as the known promoter phorbol-12,13-didecanoate (O'Grady and Reade, 1992). Results indicated that this strain of *C. albicans* may participate in neoplastic transformation (O'Grady and Reade, 1992). A similar experiment using a mouse model undertaken in 2009 showed *C. albicans* infected mice were able to initiate hyperplasia with fewer inflammatory infiltrates when exposed to 4-NQO compared to control mice challenged with 4-NQO alone (Dwivedi et al., 2009). The test group infected with *C. albicans* showed histologically detectable dysplasia after prolonged 4-NQO exposure, thus supporting the hypothesis that *C. albicans* may play a role in promotion of oral dysplasia (Dwivedi et al., 2009).

1.11.3 Acetaldehyde

The role of oral yeast with the ability to produce pro-carcinogens has also been explored. This includes the ability of certain oral micro-organisms, including yeasts and *Streptococci*, to metabolise ethanol to produce acetaldehyde (Vakevainen et al., 2002, Kurkivuori et al., 2007). Acetaldehyde is the genotoxic metabolic by-product of ethanol metabolism by the enzyme alcohol dehydrogenase. Chronic alcohol exposure in a rat model has been shown to result in significant changes in the rat mucosa (Maier et al., 1994). The rats fed ethanol showed significant enlargement of the basal cell nuclei in high risk mucosal sites including the floor mouth, base of tongue and edge of the tongue ($p < 0.01$) (Maier et al., 1994). The ethanol fed rats also showed a significantly higher percentage of cells in the S-phase of the cells cycle and a significantly reduced mean epithelial thickness of the floor of mouth mucosa (Maier et al., 1994). These results indicate that the oral mucosa of rats is susceptible to chemical carcinogens (Maier et al., 1994).

Potential mechanisms of acetaldehyde carcinogenesis include the ability of acetaldehyde to produce free radicals that can induce double stranded DNA breaks (Rajasinghe et al., 1990). Another possible mechanism of carcinogenesis is the potential ability of acetaldehyde to switch off the protein O^6-methylguanine-DNA methyltransferase, a protein involved in DNA repair (Wilson et al., 1994). Extrapolating on this theory that switching off O^6-methylguanine-DNA methyltransferase results in decreased DNA repair

and a higher chance of point mutations that in turn leads to oncogenesis. Acetaldehyde has also been shown to have the ability to form DNA adducts that again may play a role in carcinogenicity (Theruvathu et al., 2005).

Clinically, this is relevant it has been demonstrated that salivary acetaldehyde is produced almost immediately after sipping from an alcoholic beverage and that this exposure continues for at least 10 minutes (Linderborg et al., 2011). The study concluded that repeated small sips of strong alcoholic beverages with the production of high levels of acetaldehyde may increase the risk of carcinogenesis in the upper digestive track (Linderborg et al., 2011). Ethanol is present in mouthwashes and can be found at levels anywhere between 5-27% volume (Lachenmeier et al., 2009). Simply rinsing with these alcohol containing mouthwashes has been shown to result in the production of significant levels of salivary acetaldehyde (9-85 μM) (Lachenmeier et al., 2009). This is also significant as patients may use alcohol containing mouthwashes on a regular basis. Taking these risks into consideration it would not be advisable to recommend to patients the long term use of an alcohol-containing mouthwashes (McCullough and Farah, 2008).

Smoking, together with drinking, increases the exposure of the upper digestive tract to acetaldehyde in saliva via a synergistic relationship between alcohol and tobacco (Salaspuro and Salaspuro, 2004). Marttila et al., 2013 used gas chromatography to show that cultures of oral microbes from OSCC, oral lichenoid disease and control patients were all shown to be capable of producing acetaldehyde at carcinogenic levels measuring > 100 μM, when incubated in the presence of 22 mM ethanol. However, the mean acetaldehyde production was greater by microbes cultured from smokers compared to non-smokers with the difference being significant ($p = 0.033$) (Marttila et al., 2013b). This suggests that smoking potentially has the ability to increase the production of acetaldehyde in the oral cavity (Marttila et al., 2013b).

Using gas chromatography, Nieminen et al., 2009 tested the ability of different non-*C. albicans* species to produce acetaldehyde *in vitro* during ethanol and glucose incubation. Levels of acetaldehyde > 200 μM were considered high, 40-200 μM moderate and < 40 μM considered low (Nieminen et al., 2009). Results showed all *Candida spp.* isolated and tested produced moderate or high amounts of acetaldehyde when challenged with 11 μM of ethanol (Nieminen et al., 2009). Gainza-Cirauqui et al., 2013 isolated *C. albicans* from 28 patients with potentially malignant oral disorders, including OLL, OLP, oral leukoplakia, and 6 healthy controls. Using gas chromatography the level of acetaldehyde

was measured when *C. albicans* was challenged with glucose, ethanol-glucose and red wine (Gainza-Cirauqui et al., 2013). It was concluded that *C. albicans* was able to produce mutagenic amounts, > 100 μM, of acetaldehyde in all solutions containing ethanol, however the controls were the highest producers of acetaldehyde (Gainza-Cirauqui et al., 2013). In all groups, isolates from patients who were both smokers and drinkers produced the highest amounts of acetaldehyde suggesting that these behaviours may favour adaptational changes that can upregulate the metabolism of acetaldehyde by *Candida* (Gainza-Cirauqui et al., 2013).

A recent study grew fungal biofilms from oral cancer and non-oral cancer patients to assess differences in biofilm mass, biofilm activity, phospholipase, proteinase, esterase as well as acetaldehyde production (Alnuaimi et al., 2016). Results showed the *Candida* isolated from the oral cancer cohort was significantly more virulent with significantly higher phospholipase and proteinase activity as well as significantly higher biofilm mass and biofilm metabolic activity when compared to *Candida* isolated from the non-cancer cohort (Alnuaimi et al., 2016). A significantly higher prevalence of high ethanol-derived acetaldehyde producing *Candida* was also noted in the oral cancer cohort (Alnuaimi et al., 2016). Univariant analyses showed all these factors to be significant risk factors for oral cancer, while multivariant analyses showed phospholipase activity and high biofilm activity to be factors that have an influence on oral cancer (Alnuaimi et al., 2016). Taken together these results support a positive association between oral cancer promotion and the ability of *Candida* to form biofilms, metabolise alcohol to acetylaldehyde and produce hydrolytic enzymes (Alnuaimi et al., 2016)

1.11.4 Inflammation

Oral carcinogenesis has been associated with the upregulation of certain inflammatory and pro-inflammatory cytokines that can result in uncontrolled cell division. *Candida spp.* has the ability to release proteases that damage epithelial cells and transmigrate across cell barriers to invade epithelial cells (Dalle et al., 2010), all factors that result in inflammation that may lead to DNA alterations and tumour formation (Yoshida et al., 2013). One possible mechanism for how inflammation induces carcinogenesis is the upregulation of pro-inflammatory cytokines and growth factors, including TNF-α, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important factor that regulates many genes responsible for cell growth, anti-apoptosis and cell cycle (Liu et al., 2012, Piva et al., 2013), FoxP3, CD8 (Piva et al., 2013), TGF-β (Piva et al.,

2013, Lu et al., 2004) and interleukins, specifically IL-1α, IL-8 and IL-17 (He et al., 2012, Chen et al., 1998).

To assess the role inflammation plays in oral carcinogenesis the expression of cellular markers, cytokines and transcription factors were evaluated in cases of oral epithelial dysplasia and OSCC (Piva et al., 2013). It was concluded that the overexpression of the inflammatory mediators TNF-α and NK-κB favoured transformation and invasion and that controlling these inflammatory mediators may help prevent malignant transformation (Piva et al., 2013). Using a murine model with 4-NQO to induce tongue tumours it was shown that early induction of NF-κB and cyclooxygenase 2 activation was involved in early carcinogenesis (Liu et al., 2012). Further, the effect that TGF-β1 had on OSCC has been reviewed and the overexpression of TGF- β1 in early stages of OSCC was postulated to generate a microenvironment that favours tumour promotion (Lu et al., 2004).

A mouse model assessed the ability of *C. albicans* to affect metastatic progression in a cytokine dependent manner, specifically by measuring the inflammatory mediators TNF-α and IL-18 in both test and control group mice (Rodriguez-Cuesta et al., 2010). The test group was infected with *C. albicans* and both test and control groups were challenged with b16M cells to induce melanoma liver metastasis (Rodriguez-Cuesta et al., 2010). Results showed the *C. albicans* test group had enhanced small diameter liver metastasis compared to the controls and a statistically significant increase in metastatic volume to the control mice (Rodriguez-Cuesta et al., 2010). Use of the antifungal ketoconazole lowered the severity of the metastatic process in the *C. albicans* infected mice to levels indistinguishable from controls (Rodriguez-Cuesta et al., 2010). The controls received no benefit from this treatment, suggesting that the pro-inflammatory microenvironment created in response to *C. albicans* supports melanoma metastasis in the liver in test mice (Rodriguez-Cuesta et al., 2010)

1.12 Use of Topical Antifungal Therapy

The role *Candida spp.* plays in exacerbating the symptoms of OLP is currently unknown. Due to this, the question arises as to whether concurrent antifungal therapy is required when managing symptomatic OLP patients with corticosteroids so as to prevent secondary candidosis or decrease the risk of the development of cancer.

1.12.1 Antifungals

Antifungals are split into two broad classes, one being azole antifungals that work by inhibiting fungal p450 enzymes limiting the formation of ergosterol, a component essential for fungal cell membrane formation (Ellepola and Samaranayake, 2000). The other class are polyene antifungals that by binding to ergosterol in the fungal cell membrane creating a membrane perforation (Ellepola and Samaranayake, 2000). Antifungals can be used topically and systemically to manage oral candidosis with systemic agents usually being reserved for recalcitrant cases of oral candidosis and patients with immunodeficiency (McCullough and Savage, 2005). Common topical antifungals used to manage oral candidosis include amphotericin B lozenges, miconazole gel, nystatin suspension and nystatin cream (McCullough and Savage, 2005, Farah et al., 2010).

All antifungals have the capacity to cause side effects, including nausea, diarrhoea and hepatotoxicity. Another issue with anti-fungal therapy includes the risk of interactions with other medications the patient may be taking. One significant interaction is the interaction of Warfarin with azoles, including topical miconazole. A recent assessment of the interaction of topical miconazole and topical nystatin with warfarin in a cohort of individuals has been reported with 17 patients who were using both warfarin and topical miconazole and a further 30 patients using both topical nystatin and warfarin (Hellfritzsch et al., 2017). In the patients using warfarin and topical miconazole, 65% experienced an increase in their international normalised ratio (INR) with the INR increasing from 2.5 (95% CI: 2.1-2.8) to 3.8 (95% CI: 2.8-4.8) with a mean INR increase of 1.4 (95% CI 0.3-2.4) (Hellfritzsch et al., 2017). No such change was noted in any patients who were taking warfarin with concurrent topical nystatin (Hellfritzsch et al., 2017). A more recent case series documented 4 cases of significant INR elevation in patients treated for oral candidosis with miconazole gel with three of the 4 cases resulting in death that could be attributable to this interaction (Pemberton, 2018).

In a retrospective study of 43 patients on anti-coagulation management with warfarin prior to the use of topical antifungal therapy with miconazole oral gel, miconazole vaginal suppositories or nystatin solution results showed 44% reported bleeding complications, with 23.3% of patients requiring a transfusion of fresh frozen plasma to stop bleeding (Kovac et al., 2012). Of the 32 patients taking miconazole oral gel, 15 were affected with the mean INR increasing significantly from 2.4 (range 1.9-3.2) before treatment to 8.8 (range 4.9-16.9) after treatment (Kovac et al., 2012). Of the 8 patients taking nystatin solution, 4 were affected, with the INR increasing significantly from 2.5 (range 1.9-3.5) before treatment to 10.6 (range 4.5-19.3) after treatment (Kovac et al., 2012). Miconazole oral gel and topical nystatin both exerted a strong effect on warfarin, resulting in an increase in the INR and a need to reduce the weekly dose of warfarin to manage complications (Kovac et al., 2012).

The above studies highlight the fact that even topically applied antifungals have the ability to significantly affect warfarin metabolism and the need to consider warfarin dose adjustments or alternative medications when the use of topical antifungals is required (Kovac et al., 2012, Hellfritzsch et al., 2017).

1.12.2 Chlorhexadine

Chlorhexadine could be used as an alternative to antimycotic therapy. It has been demonstrated that chlorhexadine gluconate at concentrations of 0.005% and 0.0025% caused statistically significant reductions in the cell surface hydrophobicity of *C. albicans* (Anil et al., 2001). Chlorhexadine gluconate has also been shown to supress germ tube formation of *C. albicans* isolates when the isolates were exposed for 30 minutes to subtherapeutic concentrations of chlorhexadine gluconate at 0.00125%, 0.0025% and 0.005% (Ellepola et al., 2012). Compared to unexposed controls chlorhexadine gluconate was shown to suppress germ tube formation in *C. albicans* isolates by 13.72% (0.00125%), 46.16% (0.0025%) and 72.46% (0.005%) showing that chlorhexadine gluconate has the ability, even at subtherapeutic concentrations, to suppress *C. albicans* pathogenicity (Ellepola et al., 2012).

The efficacy of chlorhexadine gluconate rinse in the prevention of oral candidosis was assessed in a pilot study that included 38 children with HIV with patients assessed at monthly intervals (Barasch et al., 2004). Patients with no clinical lesions but positive *Candida* culture (n = 9) were placed on 0.12% chlorhexadine gluconate mouthwash once/day for 90 days whilst those with oral candidosis were placed on the same

mouthwash twice/day for 90 days (n = 9) (Barasch et al., 2004). All patients were assessed monthly with *Candida* testing completed at 90 days as well as before and after use of chlorhexadine mouthwash (Barasch et al., 2004). At baseline 50% of all patients had positive oral cultures for *C. albicans* with 12/18 patients included in the final CFU analysis; 6 were excluded due to poor adherence to the treatment protocol (Barasch et al., 2004). Baseline CFU reduced significantly from 6.18 ± 2.19 pre-treatment to 2.72 ± 3.15 post-treatment ($p = 0.009$) with results suggesting use of 0.12% chlorhexadine gluconate rinse could be effective in both prevention and treatment of oral candidosis in children with HIV (Barasch et al., 2004).

Chlorhexadine rinse has also been assessed as an intervention to prolong the relapse time for oral candidosis in 75 patients with HIV or acquired immunodeficiency virus (Nittayananta et al., 2008). All patients were prescribed clotrimazole and examined fortnightly until all oral candidal lesions had completely resolved (Nittayananta et al., 2008). Following this patients were randomly assigned to the 0.12% chlorhexadine (n = 37) or 0.9% saline (n =38) group and advised to rinse 3 times/day (Nittayananta et al., 2008). All subjected were reassessed fortnightly until they experienced a new episode of oral candidosis (Nittayananta et al., 2008). No significant differences were noted between the two groups, however those with lower lymphocyte counts were shown to have significantly shorter oral candidosis free periods ($p = 0.034$) (Nittayananta et al., 2008). The authors ultimately concluded that the lack of significance could be due to the small sample size and ultimately further research was required to assess the efficacy of chlorhexadine in the maintenance of oral candidosis free periods in this cohort (Nittayananta et al., 2008).

1.12.3 Secondary Candidosis

It has been shown that use of topical corticosteroids in the management of erosive OLP will increase *Candida spp.*, specifically *C. albicans*, in the oral cavity (Pereira Tdos et al., 2014). A randomised controlled trial of the efficacy of topically applied clobetasol propionate in the absence of presence of topical miconazole was assessed in a double blinded clinical trial showing that both the test and control groups exhibited significant improvements following treatment (Lodi et al., 2007). Secondary candidosis occurred at a rate of 30% in the control group however, use of topical miconazole treatment did not improve efficacy, or outcomes such as lesions extension or pain (Lodi et al., 2007).

A recent, multicentre, retrospective study reviewed 315 patients seen in four oral medicine practices who were treated with topical steroids with and without concurrent antifungal therapy for at least two weeks (Marable et al., 2016). The overall incidence of fungal infection in those treated with steroids alone was 13.6% with no statistically significant differences existing between those treated with concurrent antifungal therapy and those treated with steroids alone (Marable et al., 2016). Patients exhibiting a non-reticular OLP were more likely to develop a secondary fungal infection compared to patients with a reticular OLP pattern (20% vs. 9.3% $p = 0.008$) (Marable et al., 2016). While no statistically significant differences existed between the two treatment groups, those treated with antifungal therapy reported a greater overall subjective improvement (Marable et al., 2016).

It is difficult to assess the significance of the results of this multicentre, retrospective analysis, in part due to the lack of control with regards to corticosteroid therapy received by the patients over the treatment period, as well as the type of antifungal therapy received (Marable et al., 2016). Topical corticosteroids were used in 96.2% of patients, while systemic corticosteroids were used in 3.8% of cases, with the type of topical steroids used including clobetasol, dexamethasone rinse, flucinonide gel or a betamethasone-clotrimazole combination (Marable et al., 2016). Each of these topical steroids varies in potency from super-potent to high/moderate potency (Schoepe et al., 2006, Uva et al., 2012). These variations of steroid potency and application have a large confounding effect on the validity of the results from this study. Patients using super potent topical steroids, such as clobetasol, and systemic steroids are more likely to experience the effects of secondary candidosis compared to use of moderate potency topical steroids (Lodi et al., 2007).

A more recent randomised, placebo-controlled pilot study assessed the efficacy of probiotics on recurrent candidosis in OLP patients (Keller and Kragelund, 2018). Patients were randomly assigned to the probiotic or placebo group and advised to dissolve the lozenges in the mouth three times/day for 16 weeks; patients requiring symptomatic management of OLP or treatment for diagnosed candidosis were managed accordingly (Keller and Kragelund, 2018). Whilst no significant differences with respect to reducing recurrent oral candidosis was noted between the two groups, results of this study are difficult to interpret due to low numbers of participants (Keller and Kragelund, 2018).

1.12.4 Current Recommendations

Due to the current lack of evidence it cannot be determined if adjunctive antifungal therapy is truly effective in the management of OLP (Thongprasom et al., 2011, Lodi et al., 2012). Whilst the scientific evidence is lacking, based on the potential risk of secondary candidosis associated with corticosteroid therapy (Vincent et al., 1990, Jainkittivong et al., 2007), adjunctive antifungal therapy is often prescribed by clinicians simply in an attempt to reduce this potential risk (Carbone et al., 1999). However, prescribing any medication is not without risks. One significant risk with prescribing antifungal therapy long term is the potential for the development of antifungal resistance (Nguyen et al., 1996), an important consideration in patient with OLP who may require lifelong management after diagnosis. Ultimately, further research is required in this area to determine definitively whether *Candida spp.* plays a role in the symptomatology of OLP and whether adjunctive antifungal therapy is truly required in management of symptomatic OLP.

1.13 Hypothesis

1. That adjunctive antifungal therapy is required for the symptomatic management of oral lichen planus and inflammation will be reduced in the oral lichen planus group treated with adjunctive anti-fungal therapy.

2. That *Candida* levels will be lower in the oral lichen planus patients treated with adjunctive antifungal therapy. However, intra-oral acetaldehyde production and species will be similar across both test groups.

3. That HALO™ (Indica Labs, USA) image analysis software will be more sensitive for single antibody and tissue segmentations analysis of multiplex immunohistochemistry than inForm 2.4.1 (PerkinElmer, USA) image analysis software.

4. That oral lichen planus affected biopsy tissue will have higher numbers of mucosal associated invariant T cells than control tissue. Further, that oral lichen planus biopsy tissue that has concurrent presence of *Candida* will have higher numbers of MAIT cells than oral lichen planus biopsy tissue without *Candida*

5. That the number of mucosal associated invariant cells and the production of inflammatory cytokines will increase in peripheral blood mononuclear cells after exposure to *C. albicans* biofilm effluent

1.14 Aims

1. To assess antifungal therapy effectiveness as an adjunctive treatment in the management of symptomatic patients with oral lichen planus and observable variation in symptoms and clinical appearance associated with using topical steroids and adjunctive topical antifungals

2. To assess oral yeast presence, amount, species and acetaldehyde production associated with treatment of symptomatic patients with oral lichen planus

3. To determine variation between HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) quantitative analyses

4. To determine the number of MAIT cells between oral lichen planus effected biopsy tissue and mucosal control biopsy tissue, as well as determine if oral lichen planus affected biopsy tissue with *Candida* has higher numbers of MAIT cells observable than OLP affected biopsy tissue without *Candida*

5. To determine the production of inflammatory cytokines and percentage of mucosal associated invariant T cells after peripheral blood mononuclear cells are exposed to *C. albicans* biofilm effluent

2 Materials and Methods

2.1 Clinical Pilot Study

A pilot study was conducted to determine whether concurrent antifungal use is required when managing symptomatic OLP patients with topical corticosteroids. This study also aimed to assess whether patients with OLP were more at risk of harbouring *Candida spp.* than the general population; whether there was a variation in *Candida* species and whether salivary acetaldehyde production varied between OLP patients and controls.

2.1.1 Sample size

We aimed to enroll 96 participants, 64 control patients and 32 test patients, 16 in each arm. The purpose of including controls was to help achieve the aims, specifically to compare *Candida* levels between OLP and the normal population as well as differences in levels of salivary acetaldehyde between OLP and control patients. Studies have shown the presence of oral yeast be highly variable with rate of colonization ranging from 40-80% for OLP and 20-40% for controls (Jainkittivong et al., 2007, Lodi et al., 2007, Masaki et al., 2011). Taking the average, we can expect presence of yeast to be approximately 60% for cases with OLP and 30% for controls.

Sample size calculations were based on matched pairs and the methods described in James J. Schlesselman, (1982) Case-Control Studies: Design, Conduct, Analysis (Schlesselman, 1982). The number of intended cases and controls to be recruited for this research project was more than adequate to obtain results that would have been statistically significant to a power of 0.8 (32 cases, 64 control).

2.1.2 Patients

The aim was to enrol 32 test subjects and 64 healthy control subjects matched by age, denture status and sex to the test subjects from the Oral Medicine clinic at the Royal Dental Hospital of Melbourne. Due to difficulties with recruitment, only 7 test subject and 14 controls were enrolled in the study.

2.1.2.1 Exclusion Criteria

All potential participants, those with OLP and those without, were assessed for eligibility prior to enrolling in the study. Any non-English speaking patient and patients unable to give self-consent were not considered eligible to enrol. All patients who had been treated within the last 2 weeks with any medication to manage their oral condition, including

either topical or systemic antifungals and/or corticosteroids, were also deemed not eligible to enrol. Any patient with HIV, those wearing full or partial dentures, or those with a recent history of oral candidosis with/without treatment in the last 2 weeks were also not eligible to enrol.

Only test subjects (new diagnosis or existing patients under follow-up) above 18 years of age with symptomatic OLP requiring treatment were included in the study. OLP patients were diagnosed by both clinical and histopathological assessment, according to the World Health Organisation (WHO) criteria (WHO, 1978) with both clinical appearance and histopathology needing to be supportive of a diagnosis of OLP. In potential participants with OLP any patient with a history of allergy to either of the proposed test medications or placebo, or those taking a medication that might interact with the test medications were excluded from participating in the study.

2.1.3 Randomisation

Prior to the clinical study a computer randomisation procedure was undertaken to equally separate patients into the two test groups. When giving consent patients were given a unique identifying number. Using excel a second column of numbers was randomly generated to correspond to the identifying number. An even number placed the patients in group A whilst an odd number placed them in group B.

The test group used a combination of miconazole nitrate gel (20 mg/g) (Daktarin®, Jannsen-Cilag Pty Ltd, Australia) and betamethasone dipropionate with optimised vehicle (BD) cream (0.5 mg/g) (Diprosone OV®, Merck, Sharp & Dohme Pty Ltd, Australia). The placebo group used BD cream and a placebo compounded by the distributing pharmacist.

Randomisation was undertaken in lots of 10 with the results sealed in an envelope and given to the pharmacist. The pharmacist would randomly choose an envelope and use the provided randomisation to allocate the patients to the appropriate test group. Randomising in lots of 10 ensured equal numbers per group for each 10 patients enrolled.

2.1.4 Management

All patients were prescribed BD cream. Placebo or miconazole nitrate (20 mg/g) (Jannsen-Cilag Pty Ltd, Australia) were placed in unmarked tubes and given to patients to use in conjunction with BD cream as per the randomisation protocol described in 2.1.3. All patients were instructed to use both creams as per the current management protocol

in use by the Oral Medicine Department at the Royal Dental Hospital of Melbourne. As per this protocol, patients were instructed to mix equal half pea sized amounts of BD cream and test medication together and apply this mixture to the affected areas three times daily after meals. Participants were also instructed not to eat, drink or rinse for 30 minutes after application. Both groups were instructed to continue this regime for 12 weeks. Participants were told to bring their medications to review appointments so they could be weighed at each appointment.

2.1.5 Test Patient and Control Assessment

Patients in both test groups were reviewed at 6 weeks and 12 weeks. At the 6-week review any patient who presented with symptomatic secondary candidosis or a history of worsening symptoms were to be assessed, managed accordingly and removed from the active study. Those patients who did not present with the above history were advised continue their respective regimes as described for a further 6 weeks. Of the 7 patients enrolled none presented with secondary candidosis or worsening symptoms and all 7 completed the study. All tests described below were undertaken at 0, 6 and 12 weeks for the OLP test patients. Microbial testing and measurement of salivary acetaldehyde only was undertaken for controls. One clinician (LD) trained in oral medicine performed all the clinical assessment and lab analysis for this study.

2.1.5.1 Medical and Habit Questionnaire

At the enrolment appointment all patients completed a questionnaire detailing their current medical history, use of current medications, drinking, smoking and mouthwash use habits. The medical and medication history was used as a basis to confirm eligibility to enrol.

2.1.6 Measuring Changes in Inflammation

Prior to commencement of treatment a full mucosal examination was undertaken along with pre-treatment digital photographs. Measurements of reticular/hyperkeratotic lesions, erythema/erosive lesions and ulceration was undertaken as per the criteria originally described by Piboonniyom et al., 2005 (Piboonniyom et al., 2005). An example of this scoring system is shown in Table 2.1

Site	Reticular / hyperkeratotic area (R)		Erythematous / erosive area (E)				Ulcerative area (U)			
Dorsal Tongue	0	1	0	1	2	3	0	1	2	3

Table 2.1: *Example of OLP scoring system*

The algorithm used to calculate a score for each site was $\sum R + \sum E (E \times 1.5) + \sum U (U \times 1.5)$ (Piboonniyom et al., 2005). Sites measured included the dorsal tongue, ventral tongue, upper/lower labial mucosa, left buccal mucosa, right buccal mucosa, floor of mouth, hard palate, soft palate/tonsillar pillars, maxillary gingivae and mandibular gingivae.

Based on this system a severity score was generated for each affected region in the mouth and a totalled to give an oral severity score. Using this system, a test site was chosen for each patient. This test site was determined as the highest scoring site affected with OLP at the time of initial examination. Should multiple sites have an equal score the most symptomatic of the highest scoring sites was chosen. The site chosen encompassed the entire surface of the mucosa. For the purpose of microbial testing in controls the test site chosen was matched to the test patient site.

2.1.7 Subjective Assessment

Test patients were questioned as to the ease of use of the respective medications and pain associated with OLP at its worst. Patients were asked to assign a number to these questions using a 10-point numerical rating scale (NRS). Test patient experience was also assessed using the OHIP-14 (Slade, 1997) at 0, 6 and 12 weeks. The OHIP-14 is a series of 14 questions that measures quality of life and quantifies the patient's perception of the social impact of their oral condition (Slade, 1997). There are 5 possible answers to each question, "never", "hardly ever", "occasionally", "fairly often" and "very often", ranked from 0 to 4 with maximum total score of 56. For the OHIP-14 the combined total score was assessed in each OLP test group at 0, 6 and 12 weeks.

2.1.8 Microbial Testing

A test site was chosen at baseline for test patients as the site to assess for *Candida* infiltration; this site was the same site used for testing in the age and sex matched controls. Two cytological examinations with KOH and CW (Sigma-Aldrich, USA) were used to assess for fungal forms and hyphae as well as an oral rinse technique to assess for colony forming units (CFU) in the oral cavity.

2.1.8.1 Cytological Testing

A sterile tongue depressor was used to scrape the mucosa of the test site two times. The material collected was placed on two slides and treated immediately. One was treated with 10% potassium hydroxide (KOH) alone and the other with 10% KOH followed by

calcofluor white (CW) (Sigma-Aldrich, USA) (Haldane and Robart, 1990). To assess for the presence of fungal organisms the slides with KOH were examined by light microscopy and the KOH plus CW slides (Sigma-Aldrich, USA) were examined using fluorescence microscopy.

2.1.8.2 Oral Rinse Technique

A modified oral rinse technique was used to culture *Candida spp*. Each participant was asked to rinse for 30 seconds with 10 mL of sterile distilled water that was collected in a sterile container (Mun et al., 2015). Once collected the mixture was homogenised by vortexing for 30 seconds and 1 mL of the mouthwash was pipetted and spread with a sterile spreader onto culture plates containing ChromAgar (Department of Microbiology and Immunology, The University of Melbourne, Australia). A second mL was similarly placed on Sabouraud dextrose agar (SDA) with Chloramphenicol and Gentamicin (Department of Microbiology and Immunology, The University of Melbourne, Australia). Additionally, to help determine the degree of colonisation, two tenfold serial dilutions were also plated on separate culture plates containing SDA with Chloramphenicol and Gentamicin (Department of Microbiology and Immunology, The University of Melbourne, Australia). The media plates were incubated at 37°C for 48 hours and assessed for growth of *Candida* with preliminary phenotyping from ChromAgar.

2.1.9 Measurement of Salivary Acetaldehyde

Saliva samples were collected from all test subjects and control subjects to assess levels of acetaldehyde. Patients were initially asked to rinse with 10 mL distilled water for 30 seconds and expectorate into a sterile container. This baseline sample was also used to determine the presence and degree of colonisation as described in 2.1.8.2. Patients were then asked to rinse with 27% alcohol mouthwash for 30 seconds; expel the mouthwash and wait 60 seconds. After 60 seconds patients were asked to rinse with another 10 mL distilled water for 30 seconds and expectorate into a sterile container. The post alcohol distilled water mouthwash expectorated samples were frozen at -20ºC.

Post mouthwash expectorated samples were batch analysed using gas headspace chromatography (University of Melbourne Chemistry Department) (Nieminen et al., 2009, Gainza-Cirauqui et al., 2013, Marttila et al., 2013a). Mutagenic production of salivary acetaldehyde was taken as > 100 µM (Theruvathu et al., 2005, Marttila et al., 2013a, Gainza-Cirauqui et al., 2013). Acetaldehyde production post-mouthwash use was measured at 0, 6 and 12 weeks in test subjects, and at enrolment for controls.

2.1.10 Bias

To avoid potential sources of bias during the selection process a rigorous inclusion/exclusion criterion was introduced to ensure patients originate from the same general population to avoid confounding results. A standard form was used to collect information about the patient's medical history, habits and to score the severity of the patient's condition. A form to standardise the treating clinician/researcher's interactions with the patient was used to help reduce interviewer bias. Use of standard measurements to assess erythema (Piboonniyom et al., 2005), symptoms (Slade, 1997) and a clinical lab-based design was also employed to eliminate recall bias by using objective rather than subjective measurements of data.

2.2 Multiplex Immunohistochemical Study

An immunohistochemical study utilising a multiplex immunofluorescent technique, was employed to quantify mucosal associated invariant T (MAIT) cells in formalin fixed tissues samples. The aim of this study was to determine whether there was a variation in the number of MAIT cells present between OLP tissues with/without symptoms, OLP tissues with/without *Candida* and control tissue.

2.2.1 Sample Collection

This retrospective analysis included 90 formalin fixed paraffin embedded tissue blocks sorted into 5 test groups. The test groups included 30 samples of asymptomatic OLP, 30 symptomatic OLP samples, 15 OLP samples with *Candida*, both asymptomatic and symptomatic, and 15 fibroepithelial polyp (FEP) samples that served as mucosal controls. The tissue samples were sourced from the Melbourne University Histopathology Service at the Royal Dental Hospital of Melbourne. All samples were confirmed to be from oral mucosal tissue and chosen based on a histopathological diagnosis of OLP, presence of *Candida* for the OLP with *Candida* cohort and presence/absence of symptoms at the time of biopsy determined by the clinical description provided by the clinician on the biopsy report. Asymptomatic/minor activity OLP was defined as a report of no symptoms or very limited occasional discomfort. Symptomatic/high activity OLP was defined as OLP with reported ulceration and/or presence of ongoing symptoms on the biopsy report. Tissue from three patients with a diagnosis of oral Crohn's disease/orofacial granulomatosis served as positive controls as MAIT cells have been shown to be present in intestinal inflammatory bowel disease (Hiejima et al., 2015).

2.2.2 Sample Preparation

Formalin fixed paraffin embedded (FFPE) biopsy samples were collected from the 5th floor histopathology laboratory at the Royal Dental Hospital of Melbourne. Sections (4 μM) were cut and mounted onto Superfrost^{TM} Plus (Thermo Fischer Scientific, USA) slides. Cutting of sections was performed with technical assistance from laboratory staff in 5th floor histopathology laboratory at the Royal Dental Hospital of Melbourne.

2.2.3 Periodic Acid Schiff Staining

One set of samples was stained with periodic acid-Schiff (PAS) stain. The sections were bought to water and treated with diastase if required for 10 minutes. The sections were then washed well in running tap water and periodic acid was applied to all the sections for 5 minutes. After 5 minutes the sections were rinsed under running tap water and then

further rinsed with distilled water. Schiff's reagent was applied for 10 minutes and the slides were rinsed under tap water for 5-10 minutes. Mayer's Haematoxylin was applied to the sections for 30 seconds and differentiated with blue as needed. Finally, the sections were washed under running water. Using light microscopy glycogen, mucin and *Candida* were identified by magenta staining while the cell nuclei stained blue with the PAS stain. PAS staining was performed as above with assistance from laboratory staff in the 5th floor histopathology laboratory at the Royal Dental Hospital of Melbourne to confirm allocation to the correct group.

2.2.4 Single Antibody Validation

Antibodies against T cell receptor (TCR) Vα7.2 (Miltenyi Biotec, Germany), CD3 (Abcam, UK), interleukin 18 receptor 1 (IL18R1) (Abcam, UK), CD161 (Abcam, UK), CD8 (Abcam, UK) and major histocompatibility complex class 1 related protein (MR-1) (Biorbyt, UK) were validated with positive controls using 3,3'-Diaminobenzidine (DAB) (DAKO, Germany) at the manufacturers recommended dilution. All antibodies except anti-TCRVα7.2 (Miltenyi Biotec, Germany) were validated on human tonsil, provided by the Centre for Advance Histology and Microscopy, whilst TCRVα7.2 was validated on Crohn's disease (Hiejima et al., 2015).

Briefly, the slides underwent an automated dewax procedure (Jung AutoStainer XL, Leica Biosystems, Germany) where the slides were rinsed in xylene for two rounds of 4 minutes and once again in xylene for 3 minutes. The slides were then rinsed in 100% ethanol for three rounds of 1 minute; further rinsed in 70% ethanol for 1 minute and finally rinsed in water for 1 minute. The slides were then washed in phosphate buffered saline for 5 minutes prior to antigen retrieval. Antigen retrieval was achieved by placing the slides in pH 6 sodium citrate buffer and heating in a pressure cooker at 125°C for 3 minutes. Following heat antigen retrieval, the slides were washed for 2 x 3 minutes in PBS then held for 5 minutes in 3% H_2O_2. The slides were further washed in PBS for 3 x 2 minutes and hydrophobic pen applied around the tissue sections. The slides were blocked for 5 minutes using tris-buffered saline with bovine serum albumin (TBS + BSA) buffer in a humid chamber for 5 minutes. Following blocking the primary antibodies were applied using the manufacturers recommended dilution.

For CD3 (Abcam, UK) and CD8 (Abcam, UK), the optimal dilution for multiplex staining had been determined by the Centre for Advanced Histology and Microscopy and this dilution of 1:500 was used for validation. All other antibodies were validated using

the manufacturers recommended dilution. Primary antibodies were incubated in a humid chamber for 1 hour prior to washing with tris-buffered saline with Tween 20 (TBST) for 3 x 2 minutes. Slides were incubated with secondary antibodies for 30 minutes and washed in TBST for 3 x 2 minutes. DAB (DAKO, Germany) was applied to the slides and incubated at 5-10 minutes prior to rinsing with distilled water and counterstaining with haemoxylin on the autostainer (Jung AutoStainer XL, Leica Biosystems, Germany).

2.2.5 Single Antibody Titration

To define optimal staining conditions, serial dilutions of all antibody reagents were evaluated, except for anti-CD3 (Abcam, UK), anti-CD8 (Abcam, UK). Optimum staining conditions for the anti-CD3 and anti-CD8 antibodies, together with the optimum concentration for the nuclear counterstain stain 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA), had been previously defined at the Centre for Advanced Histology and Microscopy at the Victorian Comprehensive Cancer Centre. Positive, unstained and isotype control samples were evaluated alongside dilutions except for CD3 (Abcam, UK) and CD8 (Abcam, UK). For testing of CD3 (Abcam, UK) and CD8 (Abcam, UK) positive and unstained controls were evaluated at the validated concentrations. Both CD3 (Abcam, UK) and CD8 (Abcam, UK) were antibodies derived from supernatant and as such protein concentration could not be determined with certainty. For CD3 (Abcam, UK) a range of concentration was supplied and so an isotype control could be run to validate concentration. Abcam could not provide a protein concentration or concentration range for CD8 (Abcam, UK), thus no isotype control could be run. Serial dilutions were conducted using DAB as per the protocol described in 2.2.4 and using the tyramide signal amplification (TSA)® fluorescein detection kit (PerkinElmer, USA) to validate the concentrations in fluorescence.

The final optimum antibody dilutions for fluorescence staining chosen are shown Table 2.2 Representative images of positive staining for each antibody are shown Figure 2.1. Nuclear counterstain DAPI (Sigma-Aldrich, USA) was used at the optimised concentration of 1:1000. All antibodies were optimised first as monoplex slides prior to being optimised on Crohn’s disease/orofacial granulomatosis samples as multiplex slides to determine the antibody order of application (Parra et al., 2017).

	CD3	CD8	IL18R1	CD161	MR-1	TCRVα7.2
Primary Antibody	Rabbit anti-CD3 (Abcam, UK)	Mouse anti-CD8 (Abcam, UK)	Rabbit anti-IL18R1 (Abcam, UK)	Rabbit anti-CD161 (Abcam, UK)	Rabbit anti-MR-1 (Biorbyt, UK)	Recombinant anti-TCRVα7.2 (Miltenyi Biotec, Germany)
Isotype	Rabbit IgG	Mouse IgG2b	Rabbit IgG	Rabbit IgG	Rabbit IgG	Recombinant surface isotype
Dilution	1:500	1:500	1:1500	1:400	1:2000	1:500
Staining Pattern	Membrane	Membrane	Membrane	Membrane	Membrane	Membrane

Table 2.2: *Antibodies used for mIHC, optimised dilutions and staining pattern of antibodies*

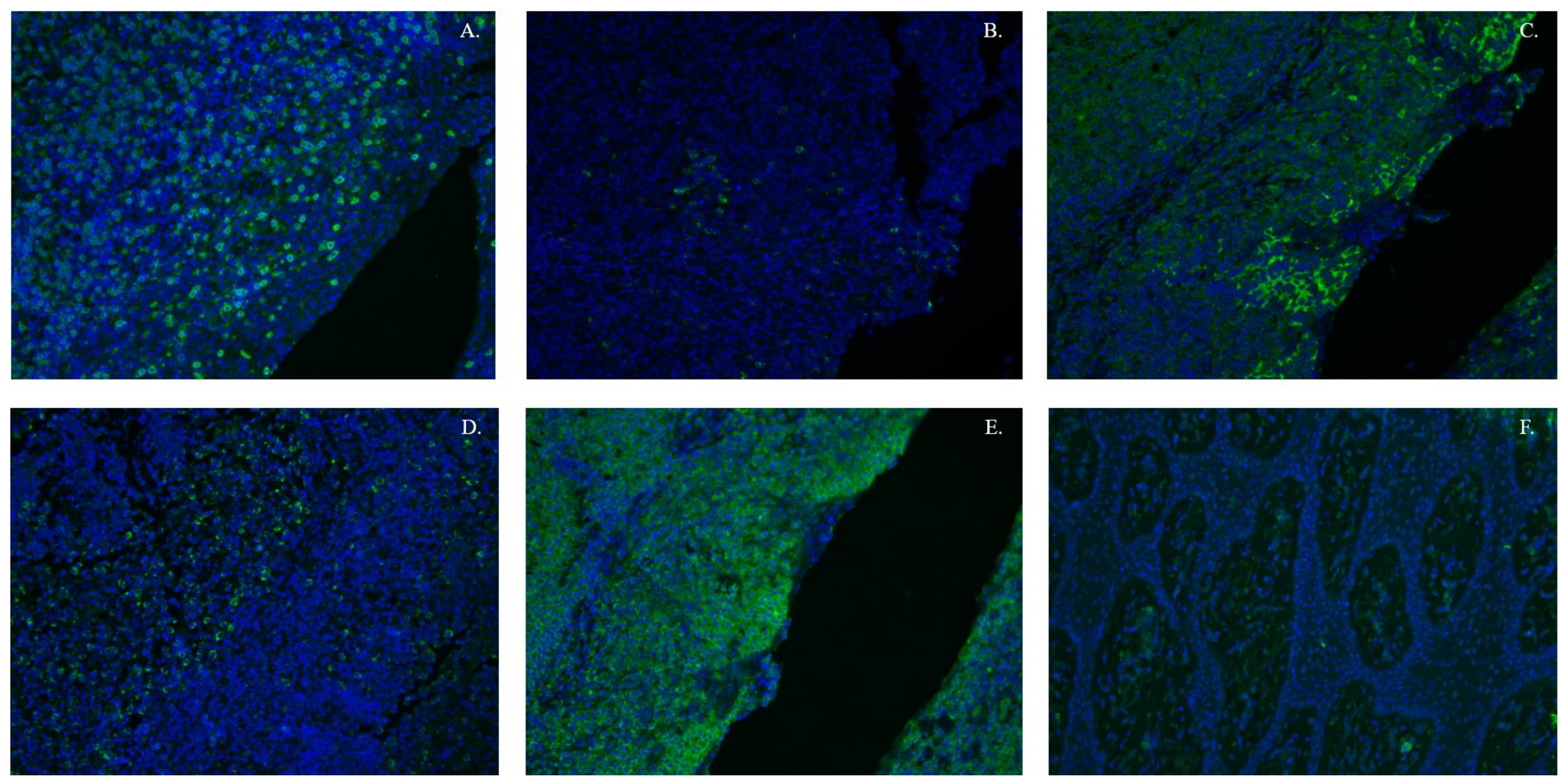

Figure 2.1: *Representative examples of positive antibody staining. A. CD3 (tonsil), B. CD8 (tonsil), C. IL18R1 (tonsil), D. CD161 (tonsil), E. MR-1 (tonsil), F. TCRVα7.2 (oral Crohn's disease) (Magnification 200 X)*

2.2.6 Validation of Antibody Order and Fluorochromes

The six primary antibodies were trialled in various orders with different fluorophores. This was to ensure minimal interference between the different antibody targets whilst maximising detection. DAPI (Sigma-Aldrich, USA) was applied as the nuclear stain following application of all primary antibodies.

TCRVα7.2 (Miltenyi Biotec, Germany) showed the weakest staining and was applied first and with Opal 520 (PerkinElmer, USA) to allow for maximum detection. CD3 (Abcam, UK) showed the strongest stain and hence was applied last with the Opal 690 (PerkinElmer, USA) that had the weakest signal. To prevent signal overlap CD8 (Abcam, UK) was placed at the signal furthest away from CD3 (Abcam, UK) with the Opal 540 (PerkinElmer, USA) fluorophore. The other antibodies and fluorophores were tested in varying combinations. Various orders of application were also trialled to determine the ideal order for optimal detection. The final order of application and fluorophores chosen as they provided the strongest signals for detection are given in Table 2.3.

It was noted during optimisation of multiplex staining that background staining existed, especially with the first three antibodies anti-TCRVα7.2, anti-CD161 and anti-IL18R1. In order to reduce background in fluorescent staining and primary antibody attenuation the concentration of NaCl (Ruck et al., 1990, Bruno et al., 1992) was titrated in the TBS + BSA blocking buffer using serial dilutions. 0.25 M NaCl in a 1:1 ratio with TBS + BSA was chosen as the blocking buffer as it showed maximum attenuation with the least amount of background.

Order	Antibody	Fluorophore
1	TCRVα7.2 (Miltenyi Biotec, Germany)	Opal 520 (PerkinElmer, USA)
2	CD161 (Abcam, UK)	Opal 620 (PerkinElmer, USA)
3	IL18R1 (Abcam, UK)	Opal 570 (PerkinElmer, USA)
4	MR-1 (Abcam, UK)	Opal 650 (PerkinElmer, USA)
5	CD8 (Abcam, UK)	Opal 540 (PerkinElmer, USA)
6	CD3 (Abcam, UK)	Opal 690 (PerkinElmer, USA)

Table 2.3: *Antibody order and fluorophore used*

2.2.7 Multiplex Immunohistochemistry

Once the multiplex staining order and optimums fluorophores for each antibody were determined the panel was applied to all slides. The sections underwent a dewax procedure (Jung AutoStainer XL, Germany) as described in 2.2.4. Briefly, the slides were rinsed in xylene for two rounds of 4 minutes and once again in xylene for 3 minutes. The slides were then rinsed in pure ethanol for three rounds of 1 minute before being rinsed in 70% ethanol for 1 minute and finally rinsed in water for 1 minute. The slides were washed in PBS for 5 minutes prior to antigen retrieval. Antigen retrieval was performed as described in 2.2.4. Briefly the slides were placed in pH6 sodium citrate buffer and heated in a pressure cooker. The slides were subsequently washed for 3 x 2 minutes in PBS. Endogenous peroxidase activity was quenched by rinsing the slides for 10 minutes in 3% hydrogen peroxide. The slides were further washed for 3 x 2 minutes in PBS and hydrophobic pen applied around the tissue. The slides underwent protein blocking for 10 minutes with TBS + BSA and 0.25 M NaCl in a 1:1 ratio. The addition of 0.25 M NaCl for protein blocking was undertaken to limit background staining through non-specific ionic interactions and attenuation of primary antibody staining (Ruck et al., 1990). The slides were washed for 3 x 2 minutes with TBST prior to undergoing further blocking with avidin (DAKO, Germany) for 20 minutes. The slides were washed for 3 x 2 minutes in TBST and blocked with biotin (DAKO, Germany) for a further 20 minutes.

The first primary antibody was then applied and incubated in a humid chamber at room temperature for 1 hour. The slides were then washed in TBST and the secondary antibody was applied and incubated at room temperature for 30 minutes. After 30 minutes the slides were washed with TBST for 3 x 2 minutes. After the washes the Opal fluorophore (PerkinElmer, USA) diluted in amplification buffer was applied to the slides for 6 minutes. The slides were then washed for 3 x 2 minutes in TBST. After the washes the slides were placed in sodium citrate pH6 buffer and heated in the microwave for 2 minutes and 30 seconds. After cooling the slides were washed for 3 x 2 minutes in TBST and the cycle repeated as above with the next primary antibody.

The primary order included, TCRVα7.2 (Miltenyi Biotec, Germany) 1:500 dilution Opal 520 (PerkinElmer, USA), CD161 (Abcam, UK) 1:400 dilution Opal 620 (PerkinElmer, USA), IL18R1 (Abcam, UK) 1:1500 dilution Opal 570 (PerkinElmer, USA), MR-1 (Biorbyt, UK) 1:2000 dilution Opal 650 (PerkinElmer, USA), CD8 (Abcam, UK) 1:500 dilution Opal 540 (PerkinElmer, USA) and CD3 (Abcam, UK) 1:500 dilution Opal 690 (PerkinElmer, USA). The secondary antibodies used included strep-avidin horse radish peroxidase (HRP) 1:100

dilution for TCRVα7.2 (Miltenyi Biotec, Germany), anti-mouse HRP (PerkinElmer, USA) 1:1000 dilution for CD8 (Abcam, UK) and anti-rabbit HRP (PerkinElmer, USA) 1:1000 dilution for all other antibodies.

Once all 6 primary and secondary antibodies had been applied and the final cycle completed, DAPI 1:1000 was applied to all the slides and incubated in a humid chamber at room temperature for 2 minutes. After 2 minutes the slides were all washed for 3 x 2 minutes in PBS and mounted with coverslips using Citifluor hard set (Electron Microscopy Sciences, USA). This research project was undertaken at the Centre for Advance Histology and Microscopy at the Victorian Comprehensive Cancer Centre.

2.2.8 Slide Scanning

Full tissue multi-channel fluorescent scans were taken with the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA) at 10X magnification from 420 nm to 720 nm (excitation spectra) to generate one lower power single stack multispectral image (MSI) per slide (Stack et al., 2014). The optimum exposure time for each antibody was defined using one of the fibroepithelial polyp control tissue slides. Five random fields were chosen and imaged at 200X magnification using the stamp application in Phenochart (PerkinElmer, USA). These fields had a final resolution of 0.5 µm/pixel with an image size of 1338 µm x 1000 µm. These fields were scanned at high resolution with the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA) at 20 nm wavelength intervals spanning from 420 nm to 720 nm. These captures were combined to generate 5 high powered single stack MSI per slide. Images of monoplex stained sections on tonsil for DAPI, CD161, CD3, CD8, IL18R1 and MR-1 and Crohn's disease for TCRVα7.2 were scanned at the appropriate wavelength for the individual fluorophores described in 2.2.6. These monoplex slides were imaged at 20X magnification for use as spectral controls for digital analysis.

2.2.9 Image Analysis

Single cell analysis was undertaken on each MSI using the tissue imaging software inForm Cell Analysis 2.4.1 (PerkinElmer, USA). On one slide the tissue was highly folded leading to high signal and overexposure during imaging; hence no further analysis could be undertaken for that sample. The final multiplex workflow as described in Figure 2.2.

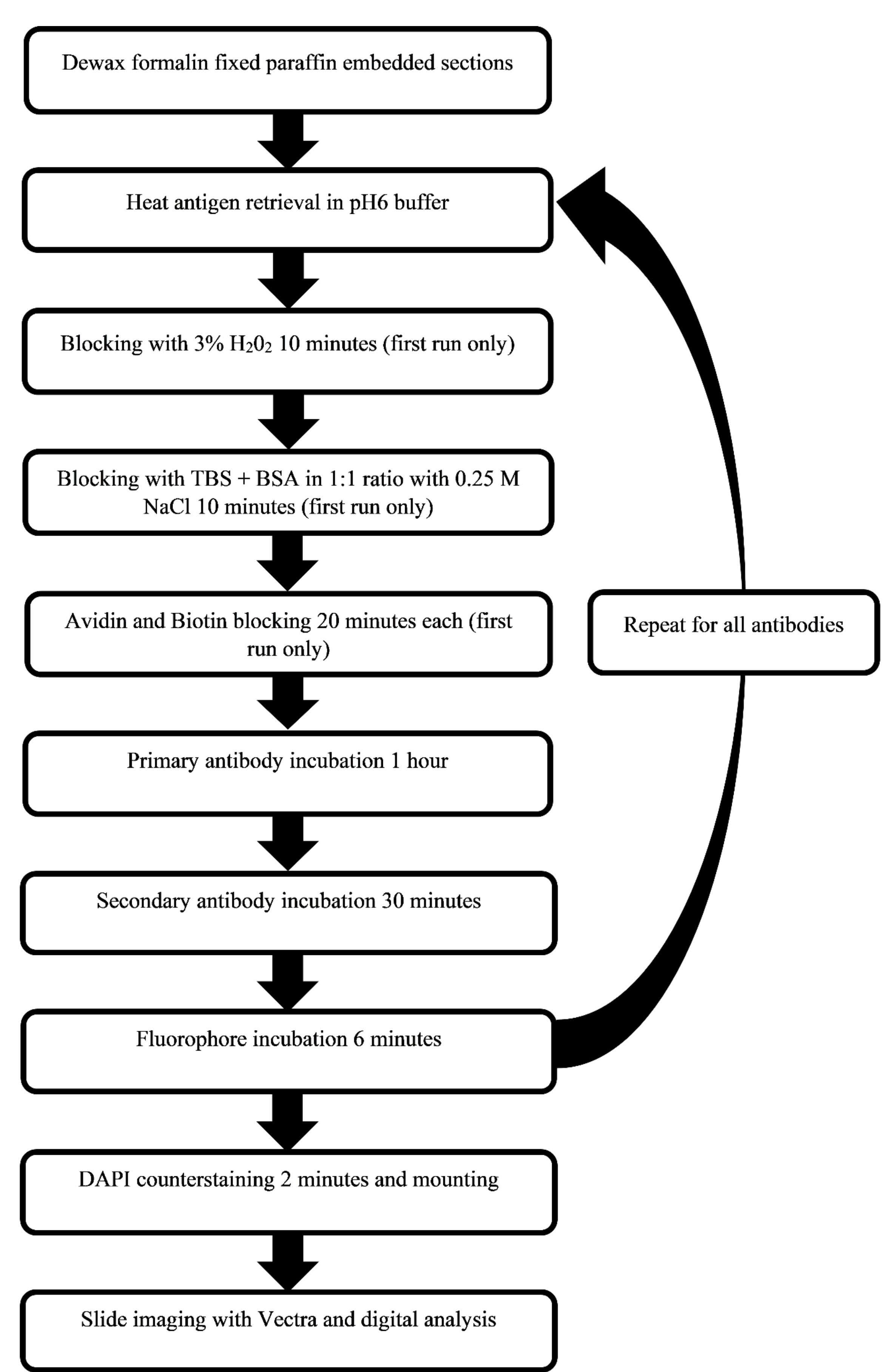

Figure 2.2: *Flow chart of sample preparation for mIHC*

2.2.9.1 inForm 2.4.1 Single Antibody Analysis for 20 Oral Lichen Planus Samples

Single antibody controls were loaded into inForm 2.4.1 (PerkinElmer, USA) to generate a spectral library. This spectral library facilitated association of each fluorochrome component with a component within the MSI (Parra et al., 2017). An algorithm was trained for single antibody analysis. Briefly, tissue was segmented into three defined regions “tissue”; areas containing unfolded tissue with cells; “tissue folds”, areas where the tissue was folded over itself; and “not-tissue”, areas containing no tissue or cells. The areas defined as “not-tissue” and “tissue folds” were excluded from further analysis. The algorithm was trained by selecting areas representative of the three tissue categories. Single cell identification was determined by using the cell segmentation tool. The phenotyping tool was used to identify positive cells for each marker. 20-40 positive cells per marker were manually selected to train the phenotyping algorithm. Cells could only be identified as positive if they were positive for nuclear staining, thus all cells identified were positive for DAPI. Once the algorithm was deemed trained it was applied to the MSI from 20 random OLP slides using batch analysis for single antibody identification. Using inForm 2.4.1 (PerkinElmer, USA) and the spectral library component images were generated for analysis with HALO™ (Indica Labs, USA) software.

2.2.9.2 HALO™ Single Antibody Analysis for 20 Oral Lichen Planus Samples

Component images generated by inForm 2.4.1 (PerkinElmer, USA) as outlined in 2.2.9.1 were loaded into HALO™ (Indica Labs, USA). The five MSI fields per slide were merged using the merge function to generate one overlay of the 5 fields. The spectral library information was transmitted with the component images allowing for identification of antibody markers. Trainable tissue segmentation was undertaken using the classifier tool in HALO™ (Indica Labs, USA). Briefly, tissue was segmented into three defined regions “tissue”; areas containing unfolded tissue with cells; “tissue folds”, areas where the tissue was folded over itself; and “not-tissue”, areas containing no tissue or cells. The areas defined as “not-tissue” and “tissue folds” were excluded from further analysis. Algorithm training was undertaken by manually selecting examples of the three above defined categories. After tissue classification the algorithm was trained to identify single cells using DAPI to segment the nuclei. The analysis tool was used to train the algorithm to identify antibody positive cells based on threshold values. Currently, no validated thresholds e been published to determine antibody positivity or negativity. Antibody thresholds were therefore set using positive examples of staining for each antibody. The thresholds for detection for each antibody are as shown in Table 2.4. All single antibody positive staining identified were set to co-localise for DAPI to ensure only cells were

identified, thus excluding artefactual staining. Once the algorithm was deemed trained it was applied to the same 20 OLP images from 2.2.9.1 using batch analysis.

Dye	Fluorophore	Marker	Threshold intensity
1	DAPI (Sigma-Aldrich, USA)	DAPI	0.14
2	Opal 690 (PerkinElmer, USA)	CD3	0.03
3	Opal 540 (PerkinElmer, USA)	CD8	0.23
4	Opal 620 (PerkinElmer, USA)	CD161	0.33
5	Opal 570 (PerkinElmer, USA)	IL18R1	0.46
6	Opal 650 (PerkinElmer, USA)	MR-1	0.33
7	Opal 570 (PerkinElmer, USA)	TCRVα7.2	0.34

Table 2.4: *Threshold intensities used for HALO™ analysis*

2.2.9.3 HALO™ Phenotyping Analysis for 89 Oral Lichen Planus Samples

Component images were generated for all 89 samples using inForm 2.4.1 (PerkinElmer, USA) as described in 2.2.9.1. Tissue segmentation and trainable single antibody phenotyping was undertaken for each antibody with the settings for each antibody as described in 2.2.9.2. Once the algorithm was deemed trained it was applied to all the merged images from the 89 samples using batch analysis. Following batch analysis HALO™ (Indica Labs, USA) was used for phenotype quantification of cells positive for 2-5 antibodies plus co-localization with DAPI to exclude artefacts. All phenotypes included CD3. The percentage of MAIT cell phenotypes was determined by dividing the number of phenotype positive cells by the total number of CD3 positive cells in the sample (Dusseaux et al., 2011).

2.3 Cell Culture Study

The cell culture study was conducted using commercially available human mononuclear cells derived from peripheral blood, PBMC (Sigma-Aldrich, USA and Lonza, Switzerland). Cells were sourced from two different companies, 10 vials of 1 x10^7 PBMC (Sigma-Aldrich, USA) all from the same donor and lot and 3 vials of 5 x 10^7 PBMC (Lonza, Switzerland) all from the same donor and lot. The PBMC sourced from Sigma-Aldrich (Sigma-Aldrich, USA) were used for the optimisation experiments and the PBMC from Lonza (Lonza, Switzerland) were used for the final experiments.

2.3.1 Optimisation of Test Media

PBMC culture medium contained Roswell Park Memorial Institute (RPMI) 1640 media supplemented with L-glutamine (Sigma-Aldrich, USA), 10% heat inactivated foetal bovine serum (Sigma-Aldrich, USA) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, USA). The medium was vacuum filtered with a 0.22 µm filter prior to use. PBMC's were cultured in PBMC media supplemented with either effluent derived from *C. albicans* biofilms supported in 25% artificial salivary media (ASM) and 25% non-effluent ASM (Arzmi et al., 2018). Both *Candida* effluent and ASM were 0.22 µm filtered prior to use. Determination of the concentration of effluent in PBMC culture media was assessed using the assay described below.

2.3.1.1 Quantification of Viable Cells Using MTS Assay

The CellTitre 96 ® AQueous One Solution Cell Proliferation Assay (Promega, USA) contains a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). This assay was used to measure proliferation and viability and was prepared as per the manufacturer's instructions.

To prepare the cell suspension PBMC's (Sigma-Aldrich, USA) were resuspended in PBMC media to a concentration of 1 x 10^6 vital cells mL^{-1}. 50 µL containing 5 x 10^4 vital cells mL^{-1} were seeded into three different 96 well plates (Corning, USA) with 50 µL of pre-dispensed 2X concentration test media in triplicate wells. The final concentration of test media included 10%, 20%, 50% and 0% (100% PBMC media control) ASM or *C. albicans* effluent. Media only controls were also run in triplicate on the same 96 well plate to serve as absorbance controls as the media contained constituents that could cause background absorbance.

Plates were incubated in a humidified incubator at 37ºC and 5% CO_2 for 2, 6 and 24 hours prior to the addition of 20 µL of CellTitre 96 ® AQueous One Solution (Promega, USA) per 100

µL of medium. As per the manufacturer's recommendation for PBMC, the plates were incubated for a further 4 hours at 37ºC and 5% CO_2. The absorbance was then recorded at 490 nm using a 96-well plate reader (Victor3, 1420 multilabel counter, PerkinElmer, USA). To determine the absorbance reading, measurements from the control wells were subtracted from the corresponding test wells. The number of live cells in culture is directly proportion to the quantity of formazan product produced by the MTS assay as measured by absorbance at 490 nm.

2.3.1.2 Preparation of Cell Media

The results of the optimisation experiment in 2.3.1 indicated that 10% *C. albicans* effluent and 10% ASM (v/v) was optimal. This concentration was therefore selected as the concentration for all subsequent cell culture experiments as it exhibited the greatest preservation of PBMC vitality.

Media was prepared at 2X concentration to allow for dilution to a final concentration of 10% effluent derived from *C. albicans* biofilm (v/v) or 10% ASM (v/v) with the addition of cell suspension. This was because PBMC were resuspended in 100% PBMC media rather than test media prior to culture to allow for maximum preservation of cell vitality. Prior to use in experiments the prepared media was mixed thoroughly and warmed in a water bath at 37ºC.

2.3.2 Preparation of Cell Suspension and Collection of Supernatant

PBMC (Lonza, Switzerland) were suspended in PBMC media to a concentration of 8 x 10^5 vital cells mL^{-1}. 500 µL of cell suspension, equivalent to 4 x 10^5 vital cells mL^{-1} were seeded into 24-well plates (Corning, USA) containing 500 µL of 2X concentration test media per well. The cells were seeded in three replicate plates and incubated in the final concentrations of test media as shown in Table 2.5.

Addition of CD28 1.25 µg/mL to some test media was to provide a co-stimulatory signal for T-cell activation (Dias et al., 2017b, Dias et al., 2017a, Gibbs et al., 2017). Phorbol 12-myristate 13-acetate (PMA)/Ionomycin 1/500 was added in the final 6 hours of incubation to wells that were used as positive control for cytokine production in the experiment described in 2.3.3.

Test group	Media final concentrations
1	10% (v/v) *C. albicans* effluent with PBMC media
2	10% (v/v) *C. albicans* effluent with PBMC media and CD28 1.25 µg/mL (BD Biosciences, USA)
3	10% (v/v) *C. albicans* effluent with PBMC media, CD28 1.25 µg/mL (BD Biosciences, USA) and 1/500 PMA/Ionomycin (Invitrogen, USA)
4	10% (v/v) *C. albicans* effluent with PBMC media and 1/500 PMA/Ionomycin (Invitrogen, USA)
5	10% (v/v) ASM with PBMC media
6	10% (v/v) ASM with PBMC media and CD28 1.25 µg/mL (BD Biosciences, USA)
7	10% (v/v) ASM with PBMC media, CD28 1.25 µg/mL (BD Biosciences, USA) and 1/500 PMA/Ionomycin (Invitrogen, USA)
8	10% (v/v) ASM with PBMC media and 1/500 PMA/Ionomycin (Invitrogen, USA)

Table 2.5: *Final concentrations of cell culture test media used for cell culture and collection of supernatants*

The plates were incubated at 37ºC and 5% CO_2 for 8, 12 and 24 hours, with PMA/ionomycin (Thermo Fischer Scientific, USA) added in the final 6 hours of incubation. Following incubation, cell suspensions per well were collected in a 1.5 mL sterile Eppendorf tube (Eppendorf, Germany) and centrifuged at 800g at 20ºC for 5 minutes. The supernatant was collected in a sterile Eppendorf tube (Eppendorf, Germany) and stored at -80ºC.

2.3.3 BioPlex Assay

IL-17A, IL-22, IL-23, TNF-α, INF-γ, IL-12 and IL-18 levels were measured using two custom BioPlex kits (Bio-Rad Laboratories, USA). One kit measured IL-17A, IL-12p40, IL-12p70, TNF-α, INF-γ and IL-18 while the other measured IL-22 and IL-23. Both IL-12p40 and IL12p70 were both measured as IL-12p70 is the active subunit of IL-12 whilst IL-12p40 is also subunit of IL-23. PMA/Ionomycin was used a positive control for cytokine production in control wells as described in Table 2.5.

Stored supernatant collected from the experiment described in 2.3.2 were defrosted from -80ºC and kept on ice. The assays from the custom BioPlex kits (Bio-Rad Laboratories, USA) were performed as per the manufacturer's instructions. Briefly, 50 µL of beads were added to the wells of the 96 well plate. 2 x 100 µL wash buffer was used to wash the beads prior to adding 50 µL of standards, samples and controls to the wells. The 96 well plate was then incubated at 850rpm on an orbital shaker at room temperature for 30 minutes. The samples were then washed with 3 x 100 µL of wash buffer prior to the addition of 25 µL of detection antibody. The plate was again incubated at 850rpm on a shaker at room temperature for 30 minutes followed by 3 x 100 µL washes with wash buffer. 50 µL streptavidin PE was added to all wells and the plate incubated at 850rpm on the orbital shaker at room temperature for 10 minutes. Three further washes were performed with 100 µL wash buffer. The samples were then resuspended in 125 µL of assay buffer and placed on the orbital shaker at 850rpm for 30 seconds. A protocol was made for the assay and analytes information entered into the protocol as well as the 8 standards made up of 4-fold serial dilutions to create the standard curve for later analysis. A map of the plate with the standard curves, blanks and samples were added to the protocol. The 96 well plate was analysed using the BioPlex suspension array system to determine the concentration of each cytokine on the BioPlex 200 system (Bio-Rad Laboratories, USA).

Fold changes were determined by dividing concentration of cytokines expressed by cells incubated in *Candida* effluent by concentration of cytokines expressed by cells incubated in ASM (Arzmi et al., 2018).

2.3.4 Flow Cytometric Analysis

PBMC were resuspended in PBMC media to a concentration of 2 x 10^6 vital cells mL^{-1}. 1 mL of cell suspension was seeded into a 12 well plate (Corning, USA) containing 1 mL of 2X concentration test media. The final concentration of cells was 2 x 10^6 vital cells per well. The cells were seeded in triplicate in the 12 well plate (Corning, USA) and incubated in the final concentrations of test media shown in Table 2.6. The plates were incubated at 37ºC and 5% CO_2 for 6 hours prior to preparation for flow cytometry.

Test group	Media final concentrations
1	10% *C. albicans* effluent (v/v) with PBMC media
2	10% *C. albicans* effluent (v/v) with PBMC media and CD28 1.25 µg/mL (BD Biosciences, USA)
3	10% (v/v) ASM with PBMC media
4	10% (v/v) ASM with PBMC media and CD28 1.25 ug/mL (BD Biosciences, USA)

Table 2.6: *Final concentrations of test media used for cell culture prior to flow cytometry*

After incubation the cell suspensions were collected in a sterile 2 mL Eppendorf tube (Eppendorf, Germany) and centrifuged at 800g at 20ºC for 5 minutes. The supernatant was removed and discarded. The cell pellets were then resuspended in fluorescent activated cell sorting (FACS) wash buffer. FACS was buffer was prepared by combining 2% w/v bovine serum albumin (Bovogen Biologicals Pty Ltd., Australia), 2 mM Ethylenediaminetetraacetic acid and 0.02% v/v sodium azide in PBS and vacuum filtering with a 0.22 µm filter (Corning, USA). The resuspended cells were diluted to 1 x 10^6 cells per 100 µL FACS wash.

Cells were prepared for flow cytometry by incubating the PBMC with 2 µL of Fc block (BD Biosciences, USA) for 10 minutes on ice. The titred amount of antibody was then added as per Table 2.7 and mixed by pipetting 3 times. The samples were incubated at 2-8ºC in the refrigerator for 30 minutes wrapped in foil. After 30 minutes 1.8 mL of FACS wash was added to each tube and mixed by drawing in and out of the pipette 3 times. The cells were then centrifuged at 800g at 20ºC for 5 minutes. The supernatant was aspirated and discarded leaving the cell pellet. The samples were then vortexed to loosen the cell pellet.

Antibody	Fluorophore	Volume
TCRVα7.2 (Bio-Legend, USA)	BV421	5 µL
CD161 (BD Biosciences, USA)	APC	20 µL
CD218a (BD Biosciences, USA)	PE	5 µL
CD4 (BD Biosciences, USA)	APC-Cy7	2 µL
CD45 (BD Biosciences, USA)	FITC	4 µL
CD8 (BD Biosciences, USA)	BV500	5 µL
CD3 (BD Biosciences, USA)	PE-Cy7	4 µL

Table 2.7: *Volumes of antibody used for cell staining prior to flow cytometric analysis*

1.8 mL of FACS wash buffer was added to each tube and mixed by pipetting 3 times and the samples centrifuged again at 800g at 20ºC for 5 minutes. The supernatant was aspirated and discarded leaving the cell pellet. The samples were again vortexed to loosen the cell pellet. For live/dead cell discrimination 5 µL of 7-aminoactinomycin D (7-AAD) (BD Biosciences, USA) was added to all samples and the 7-AAD control sample prior to incubation for 10 minutes on ice according to the manufacturer's instructions. Finally, 200 µL of FACS wash buffer was added to each tube prior to analysis.

Anti-Mouse Ig, κ/Negative Control beads (BD Biosciences, USA) were used to run single colour control samples for compensation. PBMC's were subjected to negative control conditions, incubated for 6 hours in 10% v/v ASM, was used for the 7-AAD control and unstained PBMC control. Prior to analysis, all samples were transferred to Falcon® 5 mL round bottomed tubes (Corning, USA).

Cells were analysed using the Melbourne Dental School LSR Fortessa X-20 SORP (BD Biosciences, USA). Initial analysis to define the population during acquisition included forward and side scatter gates, set to define cell populations and to exclude aggregates. Side scatter vs. 7-AAD 2D plots were used to exclude dead cells. TCRVα7.2 vs. CD3 plots were used to define the TCRVα7.2 positive subset of T cells. To define the MAIT cell population, TCRVα7.2 vs. CD161 and TCRVα7.2 vs. CD218a plots were used. Gating was then performed using CD4 and CD8 to determine positive and negative populations. The CD3 population was also defined via CD3 vs. CD45 plots. Gating was then performed in CD4 vs. CD8 plots to determine positive and negative populations. Final analysis of flow cytometry data was performed using FlowJo analysis software (Becton Dickinson, USA).

2.4 Ethics Approval

All experiments were performed in accordance with strict ethical guidelines set by the NHMRC. The pilot study and immunohistochemistry study were approved by the University of Melbourne Human Ethics Sub-Committee, project numbers 1442765.3 and 1749368.1 respectively. The cell culture study was approved by the University of Melbourne Human Ethics Advisory Group, project number 1750145.1.

3 Clinical Pilot Study

3.1 Abstract

Oral lichen planus (OLP) is an often painful, chronic, immune mediated, mucosal condition that affects the oral cavity. Due to the chronicity, symptoms are frequently present long term and require appropriate ongoing therapeutic management. *Candida spp.* form part of the normal oral flora and have the potential to cause opportunistic infection if conditions in the oral cavity facilitate overgrowth. Long term use of a topical corticosteroid can place OLP patients at an increased risk of opportunistic fungal infection. However, the use of adjunctive topical antifungals in the symptomatic management of OLP has not yet been validated. The role that *Candida spp.* play in the OLP disease process, how its presence affects the management and malignant potential of OLP, is also currently unclear.

The aim of this study was to assess the efficacy of adjunctive antifungal therapy in the treatment of symptomatic OLP as well as assess the variation in symptoms and clinical appearance following treatment with either adjunctive placebo or antifungal. This study also aimed to assess the changes in oral yeast presence, species and changes in salivary acetaldehyde production following treatment. 14 control and 7 OLP test patients, 3 assigned to the placebo group and 4 assigned to the antifungal group, completed this study. Assessments of clinical appearance, symptoms, *Candida*, salivary acetaldehyde and medication use were made at 0, 6 and 12 weeks for test patients with assessments of *Candida* and salivary acetaldehyde made at baseline only for controls.

Results of this study showed that no significant differences existed between the control group and the OLP test group at baseline with respect levels of salivary acetaldehyde, and *Candida* colony forming units (CFU). Due to the limited number of test patients enrolled, meaningful statistical analyses were unable to be performed to compare between the OLP test groups. However, downward trends were noted in both groups with respect to clinical appearance and subjective analysis of symptoms from baseline to 12 weeks. Trends noted from assessment of CFU and salivary acetaldehyde levels between the test groups should be viewed with caution due low levels of detection at baseline and the wide spread of data. Ultimately, further research is required to assess whether adjunctive treatment with an antifungal is required in the symptomatic management of OLP with steroids.

3.2 Introduction

The role *Candida spp.* plays OLP symptomatology and malignant potential is unclear. *Candida* is a yeast often identified in the oral cavity that forms part of the commensal oral flora with a capacity to cause opportunistic infections under favourable conditions. *Candida spp.* are frequently identified in oral potentially premalignant lesions such as OLP as well as oral malignant lesions (Hatchuel et al., 1990, Jainkittivong et al., 2007, Zeng et al., 2009, Masaki et al., 2011). In those patients identified as having oral candidosis, correct identification of *Candida spp.* is paramount for appropriate anti-mycotic therapy (Kragelund et al., 2013). With respect to OLP it has been shown that those treated with topical steroids are more likely to harbour *Candida spp.*, with *C. albicans* being the predominant species (Pereira Tdos et al., 2014).

Some studies have shown higher rates of *Candida* carriage in OLP compared to controls with erosive OLP patients showing a higher rate of carriage than both controls and non-erosive OLP (Zeng et al., 2009). *C. albicans* virulence factors such as phospholipase activity and adhesion, have been shown to be significantly more marked in OLP patients compared to the controls ($p = 0.001$) suggesting *C. albicans* may play a role in the development of OLP, specifically erosive OLP (Zeng et al., 2009). With respect to OLP clinical variants, one study showed *C. albicans* was the predominant species found in non-erosive OLP, whilst in erosive OLP non-*C. albicans* species dominated (Arora et al., 2016). Regarding treatment the non-*C. albicans* isolates showed greater resistance to commonly used antifungals highlighting the need for susceptibility testing prior to the provision of treatment (Arora et al., 2016).

Other studies have shown no significant relationship between *Candida* and erosive OLP (Mehdipour et al., 2010). Artico et al., 2014 assessed the frequency of *Candida spp.,* salivary flow rate and xerostomia in patients with OLP, non-OLP mucosal lesions and healthy controls. Whilst the frequencies of hyposalivation and xerostomia were similar across all groups, the control group showed higher frequencies of *Candida spp.* when compared to both the OLP and non-OLP cohorts with *C. albicans* being the predominant species isolated (Artico et al., 2014). The presence of *Candida* in OLP and other oral mucosal disease may be entirely coincidental and reflective of changes in the local environment, such as hyperkeratosis and roughness, creating an ideal environment for *Candida* favours colonization and overgrowth (McCullough et al., 2002, Hebbar et al., 2013).

Oral micro-organisms, including oral yeasts, have been shown to possess the ability to metabolise ethanol to produce the carcinogenic compound acetaldehyde (Vakevainen et al.,

2002, Kurkivuori et al., 2007). Potential mechanisms of acetaldehyde carcinogenesis include inductions of double stranded DNA breaks (Rajasinghe et al., 1990), switching off O^6-methylguanine-DNA methyltransferase, a protein involved in DNA repair (Wilson et al., 1994) and formation of DNA adducts (Theruvathu et al., 2005).

Oral microflora can metabolise ethanol resulting in the production of salivary acetaldehyde. Salivary acetaldehyde has been shown to be produced from ethanol in alcoholic beverages almost immediately with exposure to acetaldehyde persisting for at least 10 minutes (Linderborg et al., 2011). Ethanol at concentrations of 5-27% volume can be found in mouthwashes (Lachenmeier et al., 2009). It has been shown rinsing with mouthwash can result in the production of significant levels of salivary acetaldehyde (Lachenmeier et al., 2009), a factor that could be a significant contributor to the malignant potential of OLP.

For the symptomatic management of OLP, use of topical corticosteroids is considered first line therapy. Use of topical corticosteroids in the management of OLP has been shown to increase *Candida spp.*in the oral cavity, specifically increase *C. albicans* (Pereira Tdos et al., 2014). Due to the current lack of controlled studies the evidence is lacking to support the use of adjunctive antifungal therapy in the management of OLP (Thongprasom et al., 2011, Lodi et al., 2012). However, adjunctive antifungal therapy may be prescribed by clinicians for prophylaxis (Carbone et al., 1999) due to the risk of secondary candidosis that may be associated with corticosteroid therapy (Vincent et al., 1990, Jainkittivong et al., 2007). However, use of adjunctive antifungal therapy is not without risks such as the development of antimicrobial resistance (Nguyen et al., 1996) or clinically significant interactions with other prescribed medications like warfarin (Hellfritzsch et al., 2017). Both are important considerations as treatment for patients with symptomatic OLP can be lifelong in some instances.

The aim of this study was to assess and compare the efficacy of adjunctive topical antifungal therapy with adjunctive topical placebo in the treatment of symptomatic OLP with topical corticosteroids. This study also aimed as to assess variation in symptoms and clinical appearance following treatment with either adjunctive topical antifungal or topical placebo. Finally, this study further aimed to assess the changes in oral yeast presence, species and changes in salivary acetaldehyde production following treatment.

3.3 Materials and Methods

3.3.1 Patients

This study aimed to enrol 32 test subjects and 64 health control subjects matched by age, denture status and sex to the test subjects from the Oral Medicine clinic at the Royal Dental Hospital of Melbourne as described in 2.1.1 and 2.1.2. Briefly, sample size calculations were based on matched pairs and the methods described in James J. Schlesselman, (1982) Case-Control Studies: Design, Conduct, Analysis (Schlesselman, 1982) taking into account the expected presence of oral yeast in OLP and controls as determined by previous studies (Jainkittivong et al., 2007, Lodi et al., 2007, Masaki et al., 2011). Due to difficulties with recruitment, only 7 test subjects and 14 controls were enrolled in the study.

3.3.1.1 Exclusion Criteria

All potential participants, those with OLP and those without, were assessed for eligibility prior to enrolling in the study as described in 2.1.2.1. Briefly, non-English speaking patients, patients unable to give self-consent, patients with HIV, dentures, and patients treated within the last 2 weeks with any medication to manage their oral condition, were deemed not eligible to enrol. All OLP test subjects were above 18 years of age with symptomatic OLP requiring treatment. Further, both the clinical and histopathological assessment were required to be supportive of a diagnosis of OLP. Those with a history of allergy to the medications, or those taking a medication that might interact with these medications, were excluded from participating in the study.

3.3.2 Randomisation

Randomisation was performed to separate consecutive OLP patients into the two test groups. Randomisation was undertaken as described in 2.1.3. Briefly, all participants were assigned a number on enrolment. Using excel a second column of numbers was randomly generated to correspond to the identifying number. If the number was even patients were assigned to the test group (miconazole nitrate gel (20 mg/g) (Daktarin®, Jannsen-Cilag Pty Ltd, Australia) plus betamethasone dipropionate (BD) cream (0.5 mg/g) (Diprosone OV®, Merck, Sharp & Dohme Pty Ltd, Australia). If the number was odd, they were assigned to the placebo group (placebo plus BD cream). The pharmacist distributing the test medications was the only person who knew which group the patients were assigned, ensuring the researchers were blinded during the project.

3.3.3 Treatment

All OLP patients were managed as described in 2.1.4. Briefly, all patients were prescribed BD cream (0.5 mg/g) (Diprosone OV®, Merck, Sharp & Dohme Pty Ltd, Australia). Placebo or miconazole nitrate gel (20 mg/g) (Daktarin®, Jannsen-Cilag Pty Ltd, Australia) in unmarked tubes was given to the patients to use in conjunction with BD cream. Patients were instructed to mix equal half pea sized amounts of both creams together and apply this mixture to the affected areas three times daily after meals with no eating, drinking or rinsing for 30 minutes after application.

3.3.4 Clinical Assessment

As detailed in 2.1.5 patients in both test groups were reviewed at 6 weeks and 12 weeks. All tests described below were undertaken at 0, 6 and 12 weeks for the OLP test patients. Microbial testing and measurement of salivary acetaldehyde at baseline only was undertaken for controls.

As described in 2.1.5.1, all patients, test and control, completed a questionnaire detailing their current medical history, use of current medications, drinking, smoking and mouthwash use habits at the enrolment appointment. The medical and medication history was used as a basis to confirm eligibility to enrol.

3.3.4.1 Clinical Appearance

Prior to commencement of treatment a full mucosal examination was undertaken along with pre-treatment digital photographs as previously described in 2.1.6. Briefly, measurements of reticular/hyperkeratotic lesions, erythema/erosive lesions and ulceration was undertaken as per the criteria originally described by Piboonniyom et al., 2005 (Piboonniyom et al., 2005). Each affected site and the whole mouth were scored. A test site, determined as the highest scoring site affected with OLP at the time of examination, was chosen for each test patient. The chosen site was matched in the controls for the purpose of microbial testing.

3.3.4.2 Subjective Assessment

Test patients were questioned as to the ease of use of the respective medications as well as the pain associated with OLP as described in previously in 2.1.7. Patient responses were measured using a numerical rating scale (NRS) and the OHIP-14 (Slade, 1997).

3.3.4.3 Microbial Testing

Microbial testing was performed as previously described in 2.1.8. Briefly, cytological examinations with potassium hydroxide (KOH) and KOH plus calcofluor white (CW) (Sigma-

Aldrich, USA) were used to assess for fungal forms and hyphae, as well as an oral rinse technique to assess for CFU.

3.3.4.3.1 Cytological Testing

Cytological testing was performed as previously described in 2.1.8.1. Briefly, a sterile tongue depressor was used to scrap the test site twice with the material collected placed onto two slides. One was treated with 10% potassium hydroxide (KOH) alone; the other with 10% KOH followed by calcofluor white (CW) (Sigma-Aldrich, USA) (Haldane and Robart, 1990) prior to assessment with either light or fluorescence microscopy.

3.3.4.3.2 Oral Rinse Technique

A modified oral rinse technique was used to culture *Candida spp* and performed as previously described in 2.1.8.2. Briefly, each participant rinsed for 30 seconds with 10 mL of sterile distilled water that was collected in a sterile container (Mun et al., 2015). The mixture was homogenised by vortexing the mixture for 30 seconds and 1 mL pipetted and spread onto a ChromAgar plate (Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia). A further 1 mL was pipetted onto Sabouraud dextrose agar (SDA) with Chloramphenicol and Gentamicin plate (Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia) plates. Two tenfold serial dilutions on SDA with Chloramphenicol and Gentamicin were performed prior to incubating the plates for 48 hours at 37°C.

3.3.4.4 Measurement of Salivary Acetaldehyde

Acetaldehyde production post-mouthwash use was measured at 0, 6 and 12 weeks in test subjects, and at enrolment for controls. Assessment of salivary acetylaldehyde was performed as previously described in 2.1.9. Briefly, patients were asked rinse with 10 mL of distilled water for 30 seconds and expectorate into a sterile container. Patients then rinsed with a 27% alcohol mouthwash for 30 seconds prior to expectorating. After 60 seconds they were asked to rinse again with 10 mL distilled water for 30 seconds and expectorate into a sterile container. The post mouthwash samples were frozen at -20ºC prior to batch analysis with gas headspace chromatography, performed by the University of Melbourne Chemistry Department (Nieminen et al., 2009, Gainza-Cirauqui et al., 2013, Marttila et al., 2013a). Mutagenic production of salivary acetaldehyde was taken as > 100 µM (Theruvathu et al., 2005, Gainza-Cirauqui et al., 2013, Marttila et al., 2013a).

3.3.5 Statistical Analysis

To enable comparison between the test and control groups at baseline two sample T-tests were used to compare CFU and salivary acetaldehyde production. Paired T-tests were used to assess the amounts of medication used by the OLP test group at 6 and 12 weeks. A p-value of less than or equal to 0.05 was taken as significant. For the OLP test groups multiple tests were performed including measures of inflammation, cytology, quantification of salivary acetaldehyde, assessment of CFU and *Candida* species, and subjective measures of symptoms. Due to the limited number of patients enrolled meaningful statistical analysis was unable to be performed and descriptive statistics has been used to present the study results.

The above T-tests were chosen based on the assumption that the data was normally distributed. All data was analysed using Minitab® 18.

3.4 Results

3.4.1 Patient Characteristics

14 controls and 7 OLP test patients completed baselines assessments for this study. The mean age at enrolment for both the control group and OLP cohort was 61 years with 29% of enrolled patients being male and 71% being female with both. All current smokers in the control group had been smoking for greater than 30 years with smoking frequency ranging from 1 cigarette per month to 15 cigarettes per day. In the OLP cohort, the only current smoker reported a smoking history of 50 years with a frequency of 5 cigarettes per day. Past smokers in the control group reported smoking histories ranging from 10-25 years, with a past frequency of smoking ranging from 5-20 cigarettes per day. Only one past smoker existed in the OLP test group with a smoking history of 35 years and past frequency of smoking that was unable to be determined.

In the control group those who currently consumed alcohol had a frequency of consumption ranging from twice per month to 3 times per day, with duration of drinking ranging for 5 to 50 years. In the OLP test group those who currently consumed alcohol had a frequency of consumption ranging from once per month to 6 times per day, with a duration of drinking ranging from a few years to 50 years. The types of alcohol commonly consumed in both groups included beer, wine, whisky and spirits. Current users of mouthwash in the control group did so at frequencies ranging from once per week to twice per day with duration of use ranging from 5 months to 30 years. Current users of mouthwash in the OLP test group did so at frequencies ranging from irregular use to 3 times per day, with duration of use ranging from 2 weeks to a few years. In both groups, those using mouthwashes had difficulty recalling if they were using an alcohol containing or alcohol-free mouthwash. The patient demographics are summarised in Table 3.1.

	Control	OLP
Age (years, μ ± SD)	61 ± 10.8	61 ± 11.1
Male (%)	4 (29%)	2 (29%)
Female (%)	10 (71%)	5 (71%)
Current Smoker (%)	3 (21%)	1 (14%)
Past Smoker (%	4 (29%)	1 (14%)
Non-smoker (%)	7 (50%)	5 (71%)
Current Alcohol Consumption (%)	6 (43%)	4 (57%)
No Alcohol Consumption (%)	8 (57%)	3 (43%)
Regular Mouthwash Use (%)	5 (35%)	3 (43%)
No Regular Mouthwash Use (%)	9 (64%)	4 (57%)

Table 3.1: *Distribution of patient demographics between controls and test patient groups*

Legend: μ represents the mean and SD represents the standard deviation

3.4.2 Oral Lichen Planus and Control Assessment of *Candida* at Baseline

14 controls and 7 OLP test patients completed baseline assessments for this study. Mean ± SD CFU for controls was 774 ± 1248 whilst mean ± SD CFU for all test subjects at baseline was 960 ± 2238. Two sample T-test analysis comparing CFU between controls and test subjects at baseline showed no significant differences existed between the two groups (p = 0.843). Cytological assessment using KOH as well as KOH plus CW was negative for all patients in both groups. The species of *Candida* was detected using ChromAgar (Department of Microbiology and Immunology, The University of Melbourne, Australia). Of the 14 control patients 7 (50%) cultured *Candida* with one patient culturing two different species. *C. albicans* (50%) was the most prevalent species cultured, followed by other *Candida* species (37.5%) and *C. Krusei* (12.5%). Three of the 7 OLP patients (42.9%) cultured *Candida* with *C. albicans* (100%) being the only species cultured.

3.4.3 Oral Lichen Planus and Control Assessment of Salivary Acetaldehyde at Baseline

14 controls and 7 OLP test patients completed baseline assessments for this study. One control sample was unable to be retrieved from storage and results could not be assessed for this sample. The mean ± SD salivary acetaldehyde readings for controls was 16.5 ± 9.2 µM, whilst mean ± SD salivary acetaldehyde readings for all OLP test subjects at baseline was 6.5 ± 15.4 µM. Two sample T-test analysis comparing levels of salivary acetaldehyde showed no significant differences existed between the two groups at baseline (p = 0.152).

3.4.4 Test Patient Assessments

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group, with one test patient missing the 6-week review.

3.4.4.1 Assessment of Medication Use

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group, with one test patient from the antifungal group missing the 6-week review meaning assessment of medication use could not be performed for this patient at this timepoint. The mean ± SD of BD and test medication used is shown in Table 3.2.

Slight percentage increases in the amount of BD and test medication used for the placebo group were recorded from 6 to 12 weeks, 17% and 12% respectively. Percentage change in the amount of BD and test medication used in the antifungal group were recorded from 6 to 12

weeks, 12% increase and 1% decrease respectively. There were no statistically significant differences in the amount of medications used either between groups or over time.

	Placebo **μ ± SD** **(g)**	**Antifungal** **μ ± SD** **(g)**
0 - 6 Week BD	18.5 ± 15.9	15.0 ± 5.2
6 - 12 Week BD	21.6 ± 12.8	16.8 ± 11.2
0 – 6 Week Test Medication	16.1 ± 16.4	18.2 ± 2.0
6 - 12 Week Test Medication	18.0 ± 13.0	17.8 ± 12.9

Table 3.2: *Change in CFU for the two OLP test groups at baseline, 6 and 12 weeks*

Legend: μ represents the mean and SD represents the standard deviation

3.4.4.2 Assessment Changes in Symptoms

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group, with one test patient in the antifungal group missing the 6-week review. Hence, assessment of symptoms could not be performed for this patient at this timepoint. The mean ± SD of OHIP-14 and VAS scores is shown in Table 3.3.

Assessment with both the combined OHIP-14 scores and NRS showed a downward trend in mean ± SD scores in both OLP groups from baseline to 12 weeks suggesting symptoms improved over time with treatment. Patients in the placebo groups showed an 88% reduction in OHIP-14 score with the antifungal group showing a similar 81% reduction in score. Patients in the placebo group showed a 75% reduction in NRS at 12 weeks following treatment while patients in the antifungal group showed a 73% reduction in NRS at 12 weeks following treatment. Interval plots demonstrating the change in OHIP-14 and NRS scores for both groups over time are shown below (Fig. 3.1)

	Placebo **μ ± SD**	**Antifungal** **μ ± SD**
Baseline OHIP	14.3 ± 7.4	6.5 ± 7.1
6 Week OHIP	5.7 ± 3.1	2.3 ± 2.5
12 Week OHIP	1.7 ± 1.2	1.3 ± 1.5
Baseline NRS	4.0 ± 2.7	2.8 ± 2.1
6 Week NRS	2.0 ± 1.0	1.3 ± 1.5
12 Week NRS	1.0 ± 1.7	0.8 ± 1.0

Table 3.3: *Change in OHIP-14 and NRS score for the two OLP test groups at baseline, 6 and 12 weeks*

Legend: μ represents the mean and SD represents the standard deviation

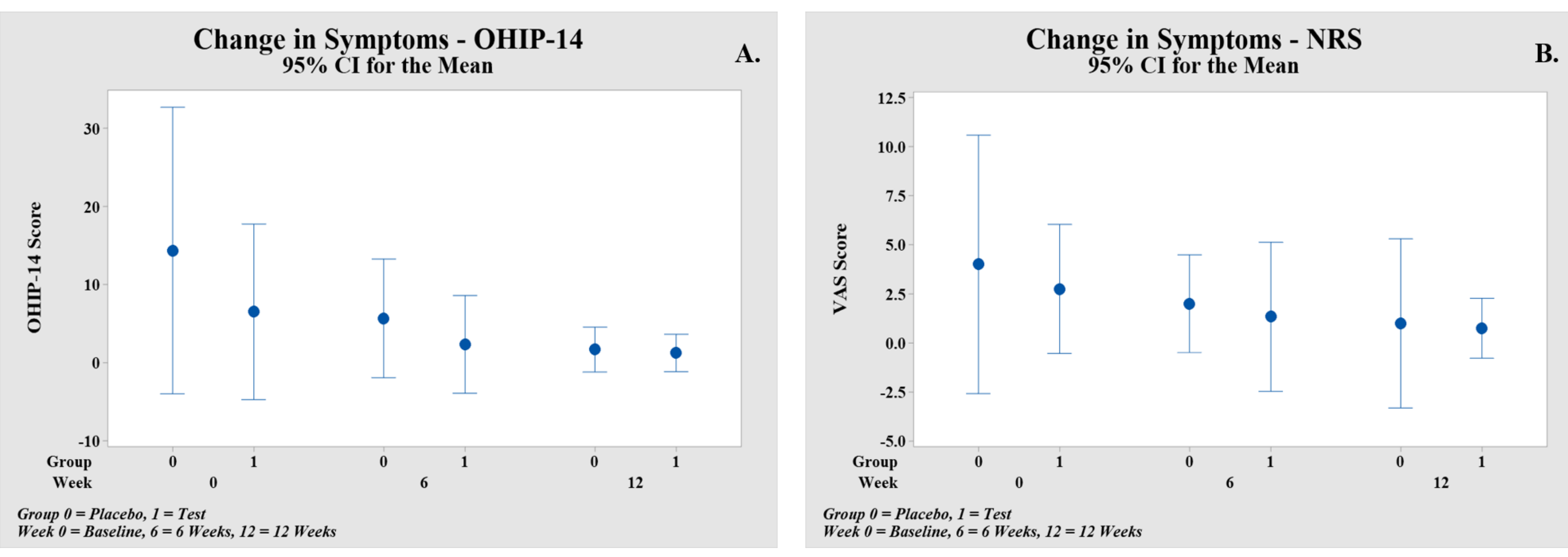

Figure 3.1: *Interval plots showing the change in symptoms assessed by OHIP-14 (A.) and NRS score (B.) for the two OLP test groups at baseline, 6 and 12 weeks*

3.4.4.3 Clinical Appearance

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group, with one test patient in the antifungal group missing the 6-week review meaning assessment of inflammation could not be performed for this patient at this timepoint. Assessment of inflammation was performed using a standardised OLP scoring system (Piboonniyom et al., 2005). For all patients except one, the test site chosen was either the left or right buccal mucosa; for one patient assigned to the antifungal group the test site chosen was the mandibular gingivae. The mean ± SD of whole mouth and test site scores is shown in Table 3.4.

With respect to the whole mouth score, both OLP groups showed a general downwards trend for mean ± SD score reduction from baseline. The placebo group showed an overall reduction in whole mouth score of 54% at 12 weeks following treatment, and the antifungal group showing a 29% reduction in whole mouth score at 12 weeks following treatment. A similar downward trend was demonstrated in the test site scores over time for both groups. The placebo group showed an overall reduction in test site score of 57% at 12 weeks following treatment, and the antifungal group showing a 43% reduction in test site score at 12 weeks following treatment. Interval plots showing the change in clinical appearance over time for the whole mouth and test site for both groups are shown below (Fig. 3.2). Representative test site images for one OLP patient at 0 and 12 weeks are also shown below (Fig. 3.3).

	Placebo μ ± SD	Antifungal μ ± SD
Baseline Whole Mouth Score	14.8 ± 8.8	8.8 ± 3.1
6 Week Whole Mouth Score	7.5 ± 5.2	7.8 ± 3.0
12 Week Whole Mouth Score	6.8 ± 4.1	6.3 ± 1.8
Baseline Test Site Score	7.0 ± 1.5	4.4 ± 1.4
6 Week Test Site Score	3.5 ± 0.9	3.5 ± 0.9
12 Week Test Site Score	3.0 ± 0.9	2.5 ± 0.0

Table 3.4: *Change in whole mouth and test site score for the two OLP test groups at baseline, 6 and 12 weeks*

Legend: μ represents the mean and SD represents the standard deviation

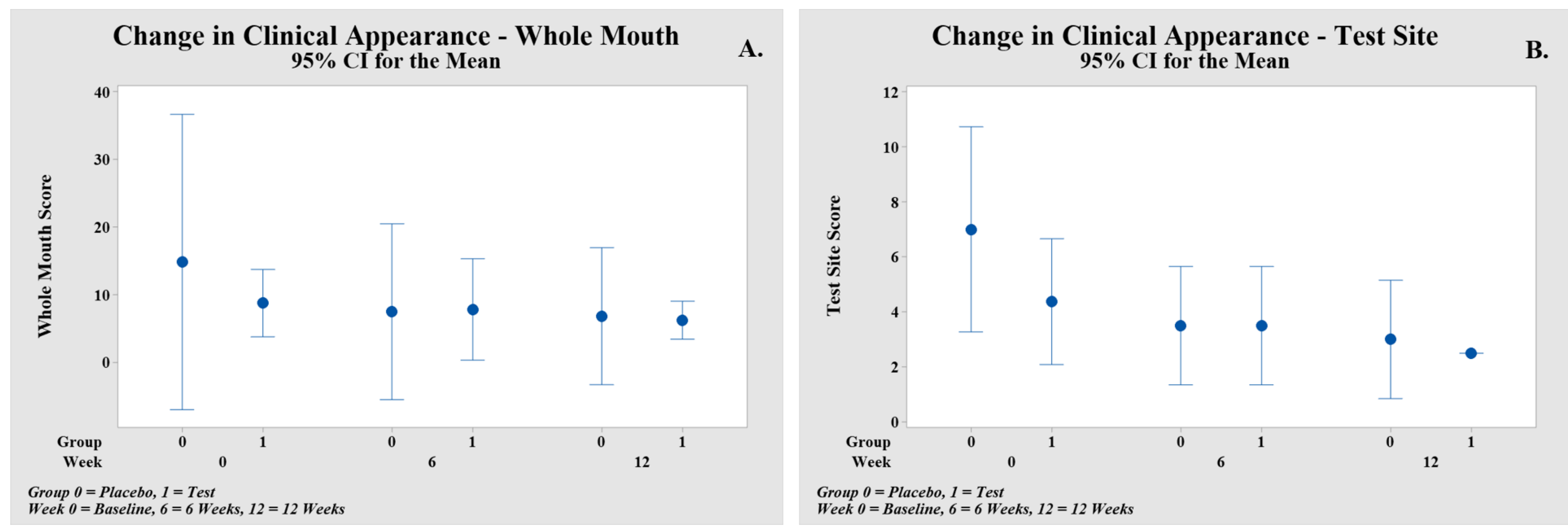

Figure 3.2: *Interval plots demonstrating the change in whole mouth (A.) and test site score (B.) over time for the two OLP test groups at baseline, 6 and 12 weeks*

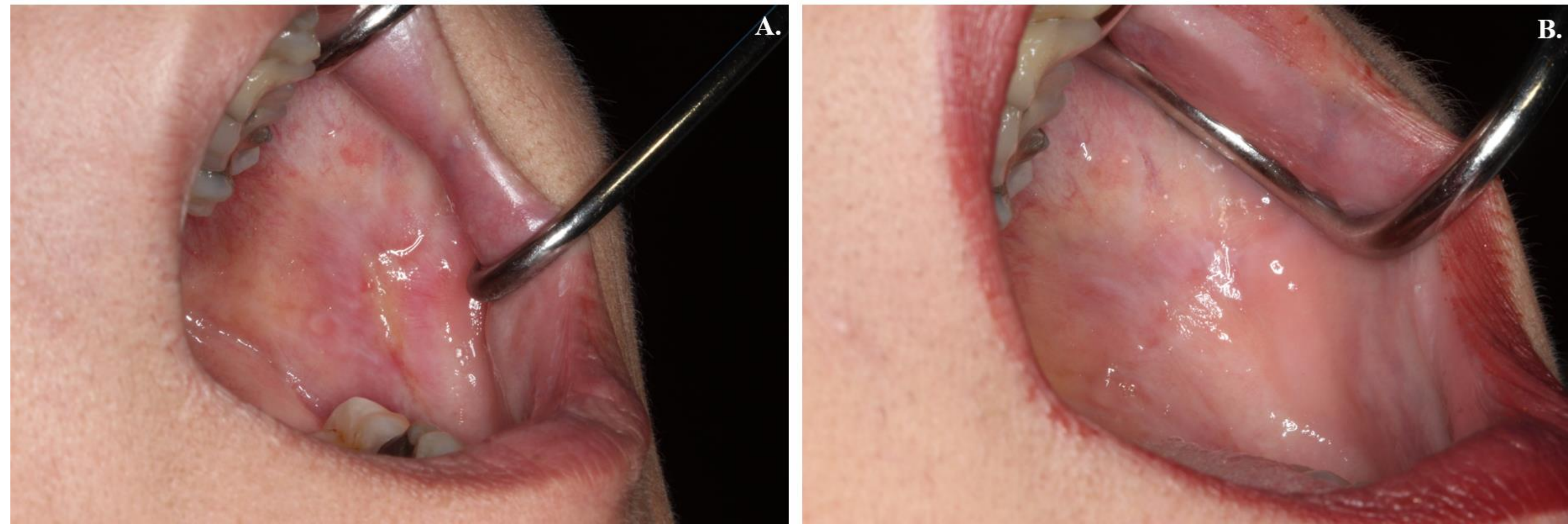

Figure 3.3: *Representative images of OLP test patient assigned to the antifungal group, test site left buccal mucosa. A. baseline photograph taken at enrolment B. photograph taken a 12 weeks post treatment after study completion*

3.4.4.4 Assessment of Candida

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group. One test patient in the antifungal group missed the 6-week review meaning assessment *Candida* could not be performed for this patient at this timepoint. No hyphae were detected in either group using either KOH or KOH plus CW for cytological assessment. The mean ± SD of the CFU readings are shown in Table 3.5.

In each OLP test group only two patient's cultured *Candida* with *C. albicans* and *C. krusei* being the only strains cultured in either group. Table 3.6 demonstrates the strains of *Candida* cultured in both test groups at baseline, 6 and 12 weeks.

With respect to CFU assessment the placebo group showed a downward trend in CFU mean ± SD from baseline to 6 weeks and an upward trend from 6 to 12 weeks following treatment (Fig. 3.1). The large standard deviations in these results show a large variability exists within the data that is reflective of the small sample size. Percentage change in CFU from baseline in the placebo group was a 66% reduction at 6 weeks and a 17% reduction from baseline at 12 weeks with a 143% increase in CFU from 6 to 12 weeks following treatment. Conversely the antifungal group showed a consistent downward trend over the study period. While percentage change in CFU from baseline to 12 weeks in the antifungal group following treatment was 100% it should be noted that only low numbers of *Candida* were cultured from the antifungal group at baseline. Interval plots demonstrating the change in CFU over time for both groups are shown below (Fig. 3.4).

	Placebo **μ ± SD**	**Antifungal** **μ ± SD**
Baseline CFU	2000.0 ± 3464.0	180.0 ± 347.0
6 Week CFU	687.0 ± 1138.0	6.7 ± 5.8
12 Week CFU	1667.0 ± 2887.0	0.0 ± 0.0

Table 3.5: *Change in CFU for the two OLP test groups at baseline, 6 and 12 weeks*

Legend: μ represents the mean and SD represents the standard deviation

	Baseline	6 Weeks	12 Weeks
Placebo			
Patient 1	*C. albicans*	*C. albicans*	*C. albicans*
Patient 2	N/A	N/A	N/A
Patient 3	N/A	*C. Krusei*	N/A
Antifungal			
Patient 1	N/A	N/A	N/A
Patient 2	*C. albicans*	*C. Krusei*	N/A
Patient 3	*C. albicans*	*C. albicans*	N/A
Patient 4	N/A	#	N/A

Table 3.6: *Distribution of Candida species for two OLP test groups at baseline, 6 and 12 weeks*

Legend: # denotes missing results as patient did not attend for review

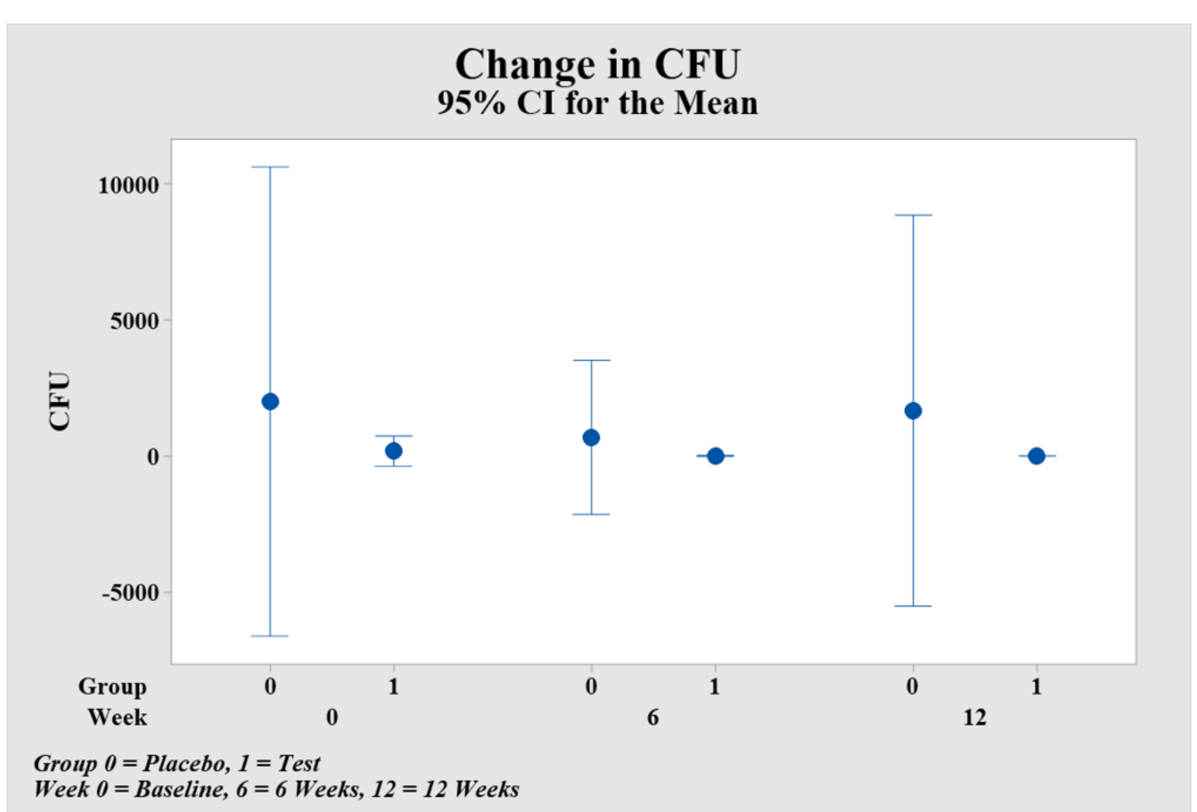

Figure 3.4: *Interval plots demonstrating the change in CFU for the two OLP test groups at baseline, 6 and 12 weeks*

3.4.4.5 Assessment of Salivary Acetaldehyde

7 OLP test patients completed this study, 3 in the placebo group and 4 in the antifungal group, with one test patient in the antifungal group missing the 6-week review meaning assessment salivary acetylaldehyde could not be performed for this patient at this timepoint. Another sample from a patient in the antifungal group for the 6-week timepoint was unable to be retrieved from storage and could also not be analysed. The mean ± SD of salivary acetaldehyde measurements post alcohol containing mouthwash use is shown in Table 3.7.

The placebo group showed a downward trend in salivary acetaldehyde production at 6 weeks following treatment with an upward trend at 12 weeks following treatment. The antifungal group showed an upward trend in mean ± SD at both 6- and 12-weeks following treatment. Percentage change from baseline in the placebo group was an 88% reduction at 6 weeks and a 54% reduction in salivary acetaldehyde production at 12 weeks following treatment. A 287% increase in salivary acetaldehyde production occurred from 6 to 12 weeks following treatment in the placebo group. Percentage change from baseline in the antifungal group was a 30% increase at 6 weeks and a 236% increase at 12 weeks in salivary acetaldehyde production following treatment. A 159% increase in salivary acetaldehyde occurred from 6 to 12 weeks following treatment in the antifungal group. In both treatment groups only low levels of salivary acetaldehyde were detected with high variability in the data. Interval plots demonstrating the change in salivary acetaldehyde over time for both groups are shown below (Fig. 3.5).

	Placebo μ ± SD	Antifungal μ ± SD
Baseline Salivary Acetaldehyde (μM)	13.7 ± 23.8	1.1 ± 1.9
6 Week Salivary Acetaldehyde (μM)	1.6 ± 2.6	1.4 ± 2.0
12 Week Salivary Acetaldehyde (μM)	6.3 ± 5.7	3.6 ± 6.4

Table 3.7: *Change in salivary acetaldehyde for the two OLP test groups at baseline, 6 and 12 weeks*

Legend: μ represents the mean and SD represents the standard deviation

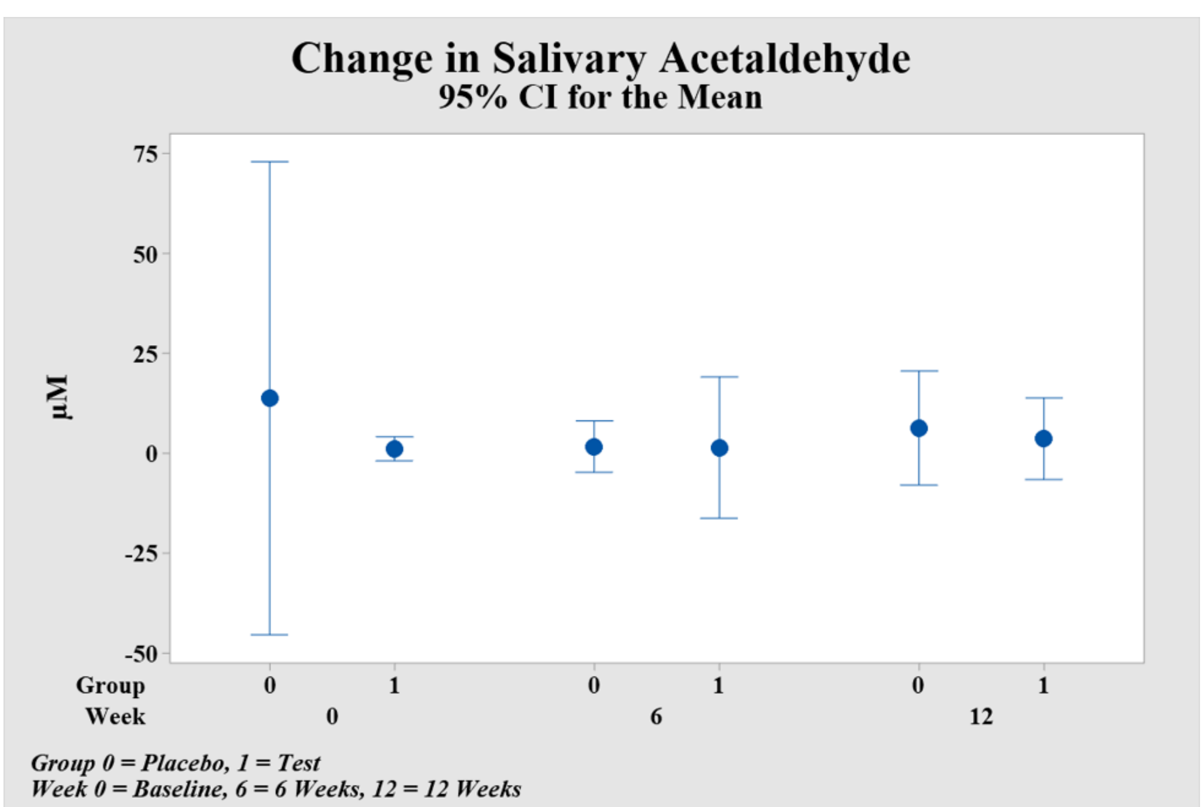

Figure 3.5: *Interval plots demonstrating the change in salivary acetaldehyde over time for the two OLP test groups at baseline, 6 and 12 weeks*

3.5 Discussion

This study aimed to assess the efficacy of adjunctive antifungal therapy in the treatment of symptomatic OLP assessing the variation in symptoms and clinical appearance following treatment with either adjunctive topical placebo or antifungal and quantifying oral yeast presence, species and changes in salivary acetaldehyde production. Due to the limited number of OLP test patients enrolled in this study, 7 in total, 3 assigned to the placebo group and 4 to the antifungal group, the sample sizes of the groups were too small to allow for meaningful statistical analysis to be performed for this cohort.

The control group in this study was age, sex and denture status matched to the OLP test cohort with two control patients enrolled for every OLP test subject. Both the OLP and test cohorts showed similar characteristics with respect to smoking, drinking and mouthwash use habits (Table 3.1). In some studies, higher frequencies of *Candida* colonisation have been reported in patients with OSCC and OLP when compared to controls (Zeng et al., 2009, Alnuaimi et al., 2015). In this study, baseline comparisons between the total OLP cohort and control group showed no significant differences existed between the two groups CFU. Also, with respect to the OLP and in keeping with the literature (Krogh et al., 1987b), *C. albicans* was the dominant species cultured at baseline. More variability exists in the control cohort with respect to non-*C. albicans* species. However, even in the control group *C. albicans* was still the dominant species cultured.

Salivary acetaldehyde production showed no significant differences between the control group and the total OLP cohort at baseline. None of the patients in the control group or OLP cohort at baseline produced levels of acetaldehyde greater than 100µM, a level quoted in the literature with the potential to induce mutagenic changes (Theruvathu et al., 2005, Gainza-Cirauqui et al., 2013, Marttila et al., 2013a). In assessing the whole OLP test cohort no significant differences were noted in the amount of BD or test cream used over the course of the study period.

Downward trends were noted in both OLP test groups with respect to clinical appearance and subjective analysis of symptoms from baseline to 12 weeks. However, these trends should be interpreted with caution as both test groups received BD cream in conjunction with the respective test medication, either topical placebo or topical antifungal. Due to low number of enrolled patients it is not known whether reduction in clinical appearance and symptoms is due to BD alone or BD used in conjunction with the test medication.

Studies do exists showing use of topical corticosteroids in OLP will result in clinical and symptomatic improvement (Liu et al., 2013).

Cytological testing with KOH as well as with KOH plus CW was negative for all control patients and all OLP test patients at all time points. With respect to the two OLP test groups only two patients' in each group cultured *Candida* with *C. albicans* and *C. krusei* being the only strains cultured in either group. Whilst the antifungal test group showed a consistent downward trend in CFU from baseline (100% reduction at 12 weeks), the placebo group showed a downward trend from baseline to 6 weeks (66% reduction) with an upward trend at 6 to 12 weeks (143% increase) following treatment. Whilst percentage change in CFU from baseline to 12 weeks shows complete reduction of CFU, it should be noted that only low numbers of *Candida* were cultured from the antifungal group at baseline. Based on this, and the low number of patients, in this treatment group it is impossible to infer whether this 100% reduction in CFU is due to the concurrent use of a topical antifungal or a random occurrence. Studies have shown that use of topical corticosteroids in OLP increases *Candida spp.* colonisation and may result in secondary candidosis (Vincent et al., 1990, Jainkittivong et al., 2007, Pereira Tdos et al., 2014). The large standard deviations noted, especially in the placebo group, are indicative of the large variability that exists within the data that again is reflective of the small sample size. This makes it difficult to infer if the upward trend in CFU noted in the placebo group from 6 to 12 weeks is due to consistent use of BD cream or natural variation.

No patients in the placebo or antifungal group produced salivary acetaldehyde at mutagenic levels (Gainza-Cirauqui et al., 2013, Marttila et al., 2013a). In both treatment groups levels of only low levels of salivary acetaldehyde were detected with the high variability in the data attributed to the low number of patients enrolled. Due to this it cannot be inferred whether the downward and upward trends noted are due to the test medication used (placebo or antifungal) or the result of natural variation.

Based on the results it cannot be inferred whether adjunctive treatment with an antifungal had any significant effect the presence of symptoms, erythema, *Candida, Candida* species or production of salivary acetaldehyde. In line with the previous literature the results of this study cannot support the use of adjunctive antifungal therapy in the management of symptomatic OLP (Thongprasom et al., 2011, Lodi et al., 2012).

Ultimately, further research is required to fully assess whether adjunctive treatment with an antifungal is required in the symptomatic management of OLP with topical steroids. For future studies, a multicentre study design should be considered as enrolling patients from multiple centres will optimise the study cohort.

3.6 Conclusion

Results of this study showed no significant differences existed between the control group and entire OLP cohort at baseline with respect to CFU and salivary acetaldehyde levels. Meaningful statistical analyses could not be performed to assesses between the OLP test groups due to the limited number of enrolled patients. Whilst downward trends were noted in both OLP test groups with respect to clinical appearance and subjective analysis of symptoms, due to the small sample size these trends should be interpreted with caution. Due to the large variability within the data and small sample size it is unknown whether the upward trend noted from assessment of CFU in the placebo group from 6 to 12 weeks and the downward trend noted in the antifungal group over 12 weeks is due to the adjunctive treatment used or natural variation. Similarly, the upward trends in salivary acetaldehyde levels over 12 weeks in the placebo group; the downward trend noted at 6 weeks with an upward trend from 6 to 12 weeks in the antifungal group should also be viewed with caution. This is in part due low levels of detection of salivary acetaldehyde at baseline and the wide spread of data.

Ultimately, due to low levels of enrolment and small test group sample size it cannot be inferred whether adjunctive treatment with a topical antifungal had any significant effect the presence of symptoms, erythema, *Candida spp.* or production of salivary acetaldehyde. Hence, further research is required to assess whether adjunctive treatment with an antifungal is required in the symptomatic management of OLP with steroids.

4 Multiplex Immunohistochemical Study – Validation of a Method for Quantitative Cell Analysis

4.1 Abstract

Mucosal associated invariant T (MAIT) cells are a recently characterised subset of T cells that are thought to play a role in immunity and immune-mediated diseases, including oral lichen planus (OLP). The aim of this study was to determine variation between HALO ™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) quantitative analysis using a panel that can be used for identification MAIT cells. Twenty oral lichen planus (OLP) biopsy samples chosen at random were stained using a fluorescent multiplex immunohistochemistry (mIHC) protocol using the markers CD3, CD8, DAPI, interleukin 18 receptor 1 (IL18R1), CD161, major histocompatibility complex class 1 related protein (MR-1) and T cell receptor (TCR) Vα7.2. The slides were scanned with the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA) to generate multispectral images (MSI). The MSI were subsequently analysed using trained algorithms for both HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) to classify as well as segment tissue and quantitatively analyse single antibody positive cells.

Results of this study showed only minor variability between the tissue segmentation algorithms with the trained algorithm for inForm 2.4.1 (PerkinElmer, USA) being the slightly less variable of the two. For quantitative cell analysis and identification of single antibody positive cells HALO™ (Indica Labs, USA) demonstrated the least amount of variability of the two trained algorithms. Hence, HALO™ (Indica Labs, USA) analysis showed the least variability of the two methods of analysis and was subsequently chosen as the validated method for quantitative cell analysis.

4.2 Introduction

OLP is a mucocutaneous condition characterised by chronic subepithelial cytotoxic (CD8) T cell accumulation with destruction of the basal lamina zone (Sugerman et al., 2002, Roopashree et al., 2010). MAIT cells are a recently characterized subpopulation of T cells that possess the highly conserved invariant T-cell receptor α chain Vα7.2-Jα33 (TCRVa7.2) (Tilloy et al., 1999, Gold et al., 2010), a contrast to other T cells that typically possess inherently diverse TCR repertoires. Whilst MAIT cells have been studied in multiple diseases, to date, the presence and role of MAIT cells in OLP has not been explored. MAIT cells can be activated by MR-1 (Tilloy et al., 1999, Gold et al., 2010) by riboflavin by-products produced by microbes; including certain oral bacteria and *Candida* (Tilloy et al., 1999, Cowley, 2014).

The role *Candida* plays in the aetiopathogenesis of OLP is currently unknown. However, some studies support increased *Candida* colonisation in OLP (Jainkittivong et al., 2007, Masaki et al., 2011). Given that MAIT cells can be activated in response to a microbial infection increased colonisation of *Candida* in OLP could trigger MAIT cell activation through MR-1, resulting in the observed chronic T cell mediations inflammation. MAIT cells can be identified using markers such as TCRVα7.2, a marker for MAIT cell; CD161, a marker of Th17 cells; IL18Rα (IL18R1 or CD218a), the interleukin 18 receptor alpha subunit; and CD3, a T cell marker (Martin et al., 2009, Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016). These cells can be further classified with IHC as $CD4^+$ or $CD8^+$ T cells.

Fluorescent mIHC is an immunohistochemical process where primary specific antibodies, conjugated to fluorophores that are excited by different wavelengths of light, facilitate the detection of multiple makers on one tissue section (Stack et al., 2014). A profound benefit of fluorescent mIHC is the ability not only to identify multiple cell phenotypes, but also the ability to gain insight as to the architecture of a lesion or disease. Fluorescent mIHC can be either direct or indirect. For direct fluorescent IHC the fluorophore is conjugated to the primary antibody of interest, for indirect the fluorophore is conjugated to a species specific secondary antibody that binds to the primary antibody (Stack et al., 2014).

This technique can be applied to both fresh frozen and formalin fixed paraffin embedded (FFPE) samples. The use of a TSA system is advantageous due to specific covalent binding (Bobrow et al., 1989, Bobrow et al., 1991) allowing for the covalently bonded

fluorescent signal remaining through multiple rounds of IHC. This also circumvents a problem when antibodies are raised in the same species as primary/secondary antibodies are removed with each round of IHC whilst the fluorophore remains bound to the tissue. The Opal mIHC method uses a TSA system to detect multiple targets (Stack et al., 2014, Parra et al., 2017), with up to 6 antibodies and one nuclear stain occurring on one tissue section. Prior to performing mIHC each individual marker must be validated with positive and negative controls and optimised in singleplex (Stack et al., 2014)

Imaging is the cornerstone of mIHC analysis and can be accomplished with confocal laser scanning microscopy. This can be undertaken where each fluorophore is scanned individually, or on a multichannel fluorescent microscope that has been equipped with a multispectral detector (Stack et al., 2014). That latter requires representative single staining controls to develop the spectral library so the MSI can be linearly unmixed for later analysis (Dickinson et al., 2001, Mansfield, 2014). One of the main advantages of the MSI technology its ability to overcome the difficulties encountered with imaging and quantifying multiple markers, such as spectral and spatial overlap (Mansfield, 2014) with the ability to attenuate for autofluorescence if required. MSI technology has been shown to improve both the reliability and accuracy of quantitative analysis, as well provide greater information with relation to protein expression when compared to conventional analysis with red-green-blue images (Liu et al., 2016).

To analyse these images digital pathology software is required to facilitate single cell analysis and target quantification. Image analysis software usually falls into three main categories; area based, cell based, and object based measurements (Aeffner et al., 2016). Software packages, such as HALO™ (Indica Labs, USA) and inForm (PerkinElmer, USA) support mIHC analysis. Conventionally analysis of IHC has been though visual assessment by a trained professional, usually a pathologist. However, with mIHC, visual assessment of multiple markers on one tissue section can prove difficult even when aided by MSI (Mansfield, 2014), highlighting the need for analytical software.

Digital pathology has revolutionised both research and diagnostic pathology allowing for analysis of images without the use of a microscopy platform and use of software that can batch process multiple slides in short amounts of time with trained algorithms. The purpose of the present study was to validate a fluorescent mIHC panel of makers that could be used for the identification of MAIT cells in oral mucosa, to determine if there is a difference between HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA)

for quantitative cell analysis and validate a method of quantitative cell analysis by comparing tissue classification and quantitative single antibody algorithms.

4.3 Materials and Methods

4.3.1 Patients

Twenty FFPE tissue blocks were sourced from OLP patients at random for validation of the quantitative method to be used for mIHC from a larger sample of 74 OLP patients. FFPE samples were selected based on the biopsy report confirming a histopathological diagnosis consistent with OLP. Expression of single antibody analysis for six antibodies and one nuclear marker was undertaken with mIHC and scored using two different quantitative cell analysis software packages, HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA). This project was approved by the University of Melbourne Human Ethics Sub-Committee, project number 1749368.1.

4.3.2 Tissue Samples

FFPE tissue blocks were obtained and 4µm thick sections were cut from each of the 20 tissue blocks and mounted onto Superfrost™ Plus (Thermo Fischer Scientific, USA) slides for subsequent mIHC staining.

4.3.3 Multiplex Immunohistochemistry

Fluorescent mIHC staining was performed as outlined in 2.2.7 to determine the expression of the MAIT cell biomarkers in the 20 OLP tissue sections. Briefly, the order of antibody application and primary antibody dilutions were TCRVα7.2 (Miltenyi Biotec, Germany) 1:500 dilution Opal 520 (PerkinElmer, USA), CD161 (Abcam, UK) 1:400 dilution Opal 620 (PerkinElmer, USA), IL18R1 (Abcam, UK) 1:1500 dilution Opal 570 (PerkinElmer, USA), MR-1 (Biorbyt, UK) 1:2000 dilution Opal 650 (PerkinElmer, USA), CD8 (Abcam, UK) 1:500 dilution Opal 540 (PerkinElmer, USA), CD3 (Abcam, UK) 1:500 dilution Opal 690 (PerkinElmer, USA) and DAPI 1:1000.

Positive control slides known to express the molecular target were used for each antibody and served as spectral controls for digital analysis. Human tonsil was used as the positive control for CD3 (Abcam, UK), IL18R1 (Abcam, UK), CD161 (Abcam, UK), CD8 (Abcam, UK), MR-1 (Biorbyt, UK) and DAPI (Sigma-Aldrich, USA). Slides from a patient demonstrating oral Crohn's disease served as control tissue for TCRVα7.2 (Miltenyi Biotec, Germany). CD3, IL18R1 (IL18Rα), CD161, MR-1 and TCRVα7.2 were chosen for this mIHC panel as these markers have been used with fluorescent IHC in previous MAIT cell studies (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016, Gibbs et al., 2017). CD8 was chosen as this has been previously shown to be the

predominant cell of the OLP chronic inflammatory infiltrate (Sugerman et al., 2000a), with DAPI used for nuclear counterstaining.

4.3.4 Quantitative Analysis

Slide scanning was performed as described in 2.2.8. Briefly, all slides were initially scanned at 100X (resolution of 1µm/pixel) magnification using the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA). The MSI were then loaded into Phenochart (PerkinElmer, USA) and 5 random fields were chosen for high resolution imaging at 200X (0.5µm/pixel) magnification to generate 5 1338µm x 1000µm images for quantitative single antibody analysis.

The same researcher who performed the mIHC (LD), also trained algorithms for both analytical software packages. Algorithms for both inForm 2.4.1 (PerkinElmer, USA) and HALO™ (Indica Labs, USA) were trained for tissue segmentation and single antibody identification as described in 2.2.9.1 and 2.2.9.2.

4.3.5 Statistical Analysis

Bland-Altman plots were used to assess the agreement between the digital methods of tissue segmentation and single cell analysis for the 6 antibodies plus nuclear stain. These plots are used to assess for differences between two techniques by plotting against the averages of the two techniques (Bland and Altman, 1999). Upper (ULA) and lower (LLA) limits of agreement are determined as the mean of the difference plus or minus 1.96 times the standard deviation (Bland and Altman, 1999). However, prior to this assessment the normality of the distribution of the data was assessed using probability plots and representative images of these plots shown in Figure 4.1. The probability plots showed that log transformation of the data was required to undertake this analysis. The data was transformed using a natural log + 0.01 (1%) transformation for better fit with a normal distribution and to account for zero values within the dataset.

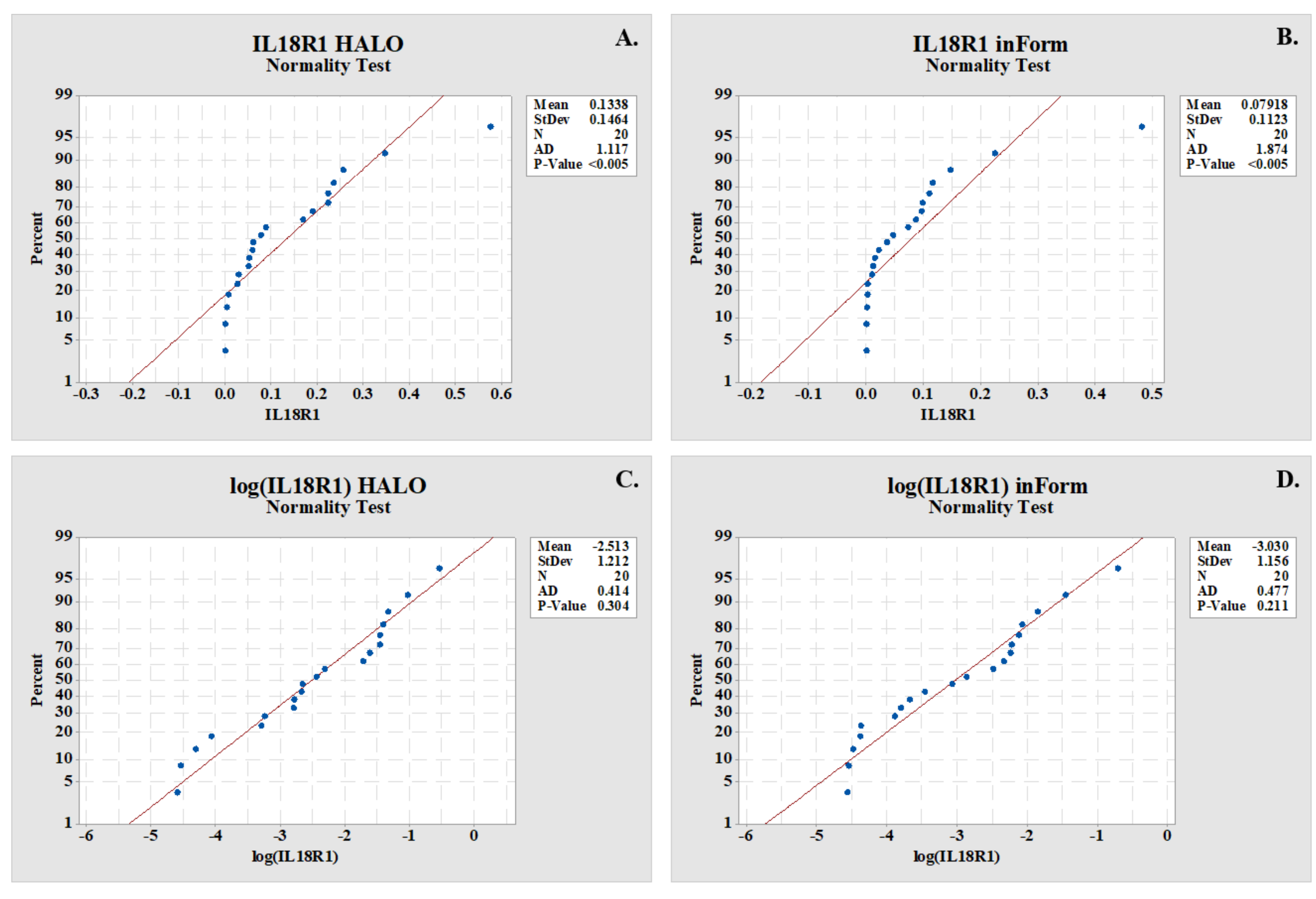

Figure 4.1: *Representative examples of normality testing for IL18R1. A. IL18R1 HALO™ untransformed, B. IL18R1 inForm 2.4.1 untransformed, C. IL18R1 HALO™ log transformed D. IL18R1 inForm 2.4.1 log transformed*

4.4 Results

4.4.1 Patient Characteristics

The study population consisted of 20 patients with histopathologically diagnosed OLP, 16 (80%) female and 4 (20%) male with a mean age of 64.2 years at the time of OLP diagnosis. Seven (35%) were greater than 70 years of age and 13 (65%) were less than 70 years of age at the time of diagnosis.

4.4.2 Comparison for Tissue Segmentation Algorithm between HALO™ and inForm 2.4.1

For the tissue segmentation algorithm tissue was defined into three categories: “tissue”; areas containing unfolded tissue with cells; “tissue folds”, areas where the tissue was folded over itself; and “not-tissue”, areas containing no tissue or cells. Comparison of “tissue” segmentation showed one comparison beyond the LLA (Fig. 4.2 A.); comparison of “not tissue” segmentation showed only one comparison was beyond the ULA (Fig. 4.2 B.); whilst comparison of “tissue folds” segmentation showed no comparisons beyond the ULA or LLA (Fig. 4.2 C.). The Bland-Altman plots generated from the log transformed data are shown in Figure 4.2 and distribution of the raw data is shown in Figure 4.3.

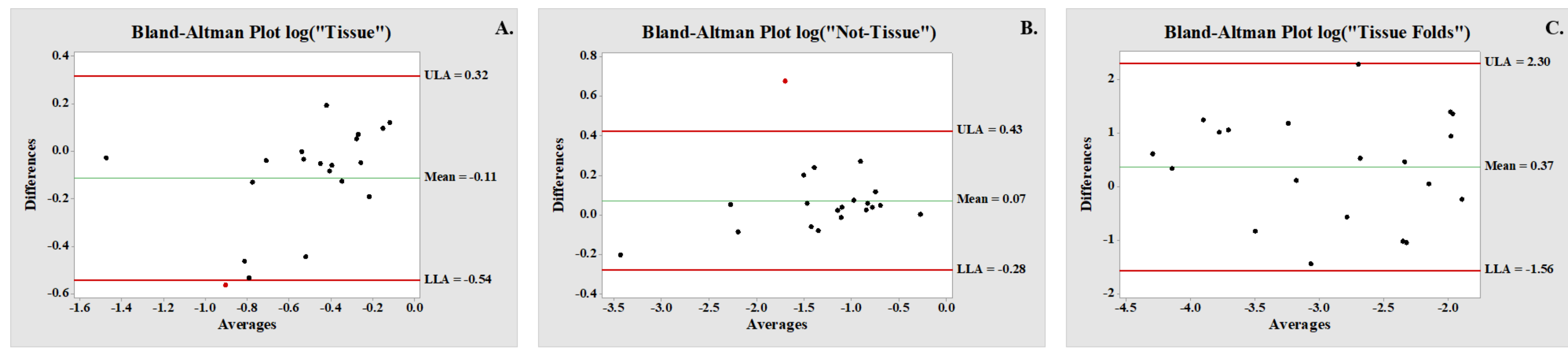

Figure 4.2: *Bland-Altman plots generated for tissue segmentation comparison performed on log transformed data. A. "Tissue", B. "Not-Tissue", C. "Tissue Folds".*

Legend: Red dots represent comparisons beyond the limits of agreement

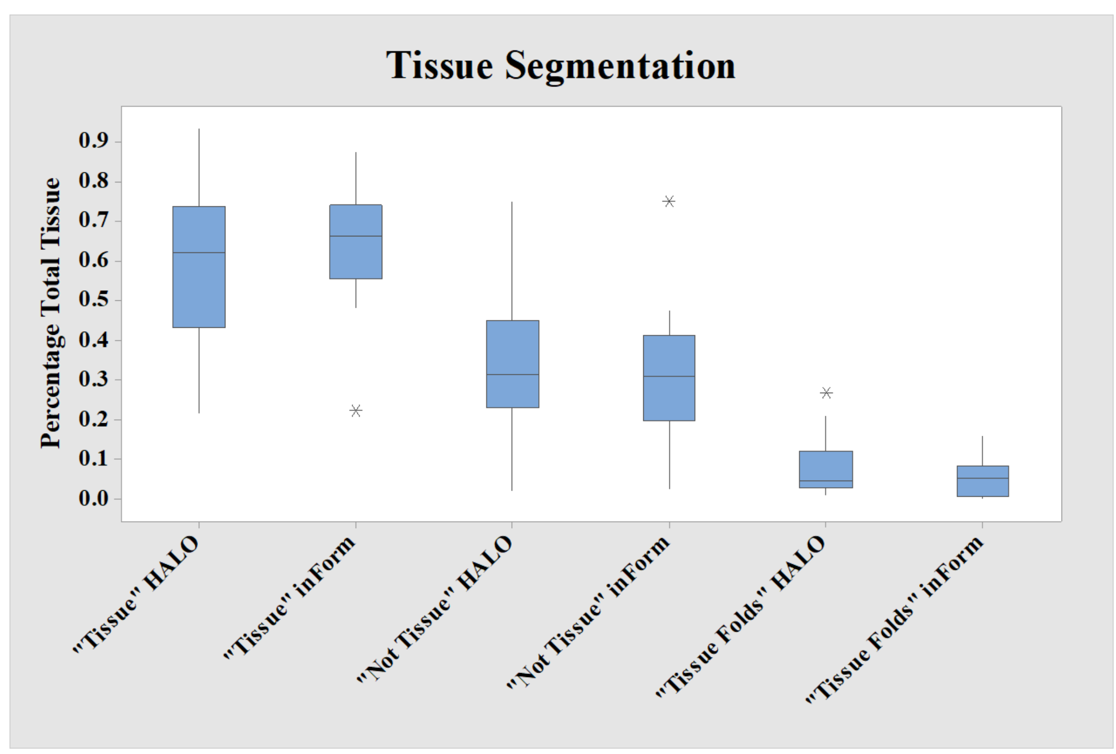

Figure 4.3: *Boxplots demonstrating raw data for HALO™ and inForm 2.4.1 tissue segmentation*

An analysis of the variability observed for these two methods of analysis was undertaken on the log transformed data. To analyse the amount of variability present across all 20 samples using each of the two methods, the standard deviation present across the 5 images of each sample was divided by that samples mean value and expressed as a percentage. Percentage variability was low, less than 11% for all variables (Table 4.1). inForm 2.4.1 (PerkinElmer, USA) showed less variability than HALO™ (Indica Labs, USA) for "tissue" and "tissue fold" segmentation, 10.3% and 6% respectively, whilst HALO™ (Indica Labs, USA) showed 3.2% less variability than inForm 2.4.1 (PerkinElmer, USA) for "not tissue" segmentation.

Tissue Segmentation	Percent Variability (%)	Difference (%)
“Tissue” HALO	33.8	**-10.3**
“Tissue” inForm	**23.5**	
“Not Tissue” HALO	52.0	**3.2**
“Not Tissue” inForm	**55.2**	
“Tissue Folds” HALO	99.1	**-6.0**
“Tissue Folds” inForm	**93.1**	

Table 4.1: *Percentage variability between tissue segmentation algorithms for HALO™ and inForm 2.4.1performed on log transformed data*

4.4.3 Comparison for Single Antibody Analysis between HALO™ and inForm 2.4.1

Bland-Altman plots of the log transformed data for each single antibody was compared between HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA), and this showed that only CD161 had no comparisons beyond the limits of agreement, with all other markers showing one or more comparisons that were beyond the limits of agreement (Figure 4.5 A.). CD3 showed one comparison beyond the ULA (Figure 4.5 B.), CD8 showed two comparisons beyond the LLA (Figure 4.5 C.), IL18R1 showed one comparison above and one below the limits of agreement (Figure 4.5 D .), MR-1 showed one comparison above the ULA (Figure 4.5 E.), TCRVα7.2 showed one comparison above and one below the limits of agreement (Figure 4.5 F.) and DAPI showed two comparisons above the ULA (Figure 4.5 G.).

The Bland-Altman plots generated from the log transformed data are shown Figure 4.4 and distribution of the raw data is shown in Figure 4.5.

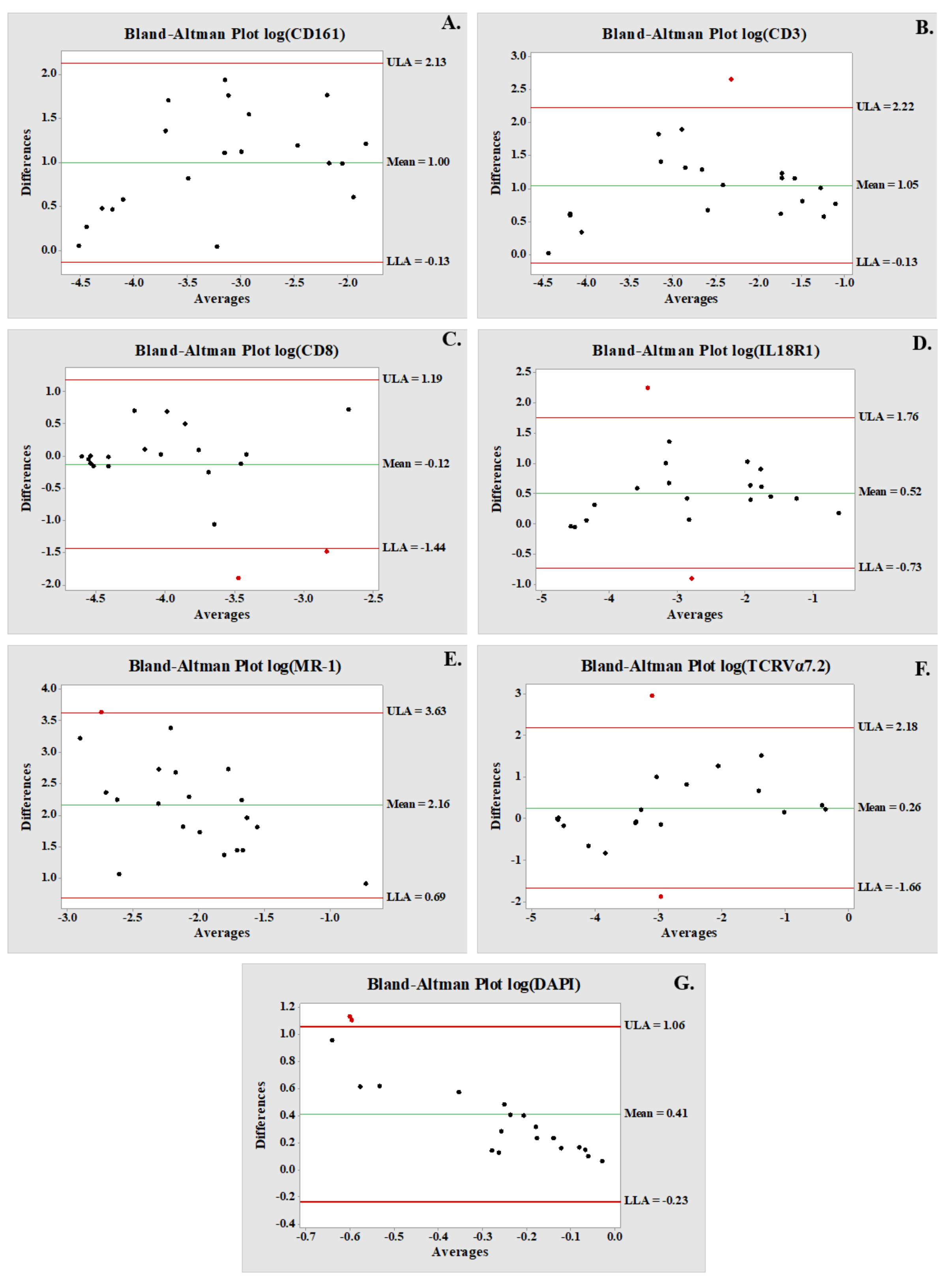

Figure 4.4: *Bland-Altman plots generated for single antibody analysis performed on log transformed data. A. CD161, B. CD3, C. CD8, D. IL18R1, E. MR-1, F. TCRVα7.2 and G. DAPI.*

Legend: Red dots represent comparisons beyond the limits of agreement

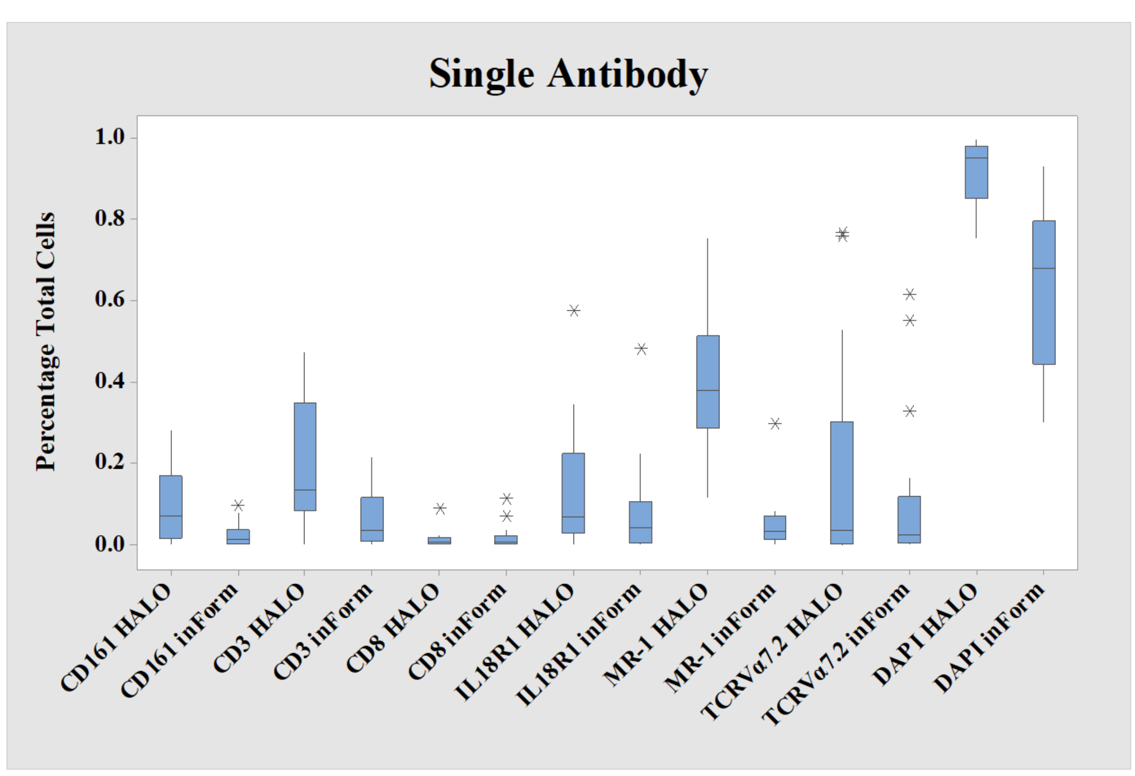

Figure 4.5: *Boxplots demonstrating raw data for HALO™ and inForm 2.4.1 single antibody analysis*

An analysis of the variability observed for these two methods of analysis was undertaken on the log transformed data. To analyse the amount of variability present across all 20 samples for each antibody the standard deviation present across the 5 images of each sample was divided by that samples mean value and expressed as a percentage. This analysis (Table 4.2) revealed that all the antibodies tested, except CD8, exhibited less variability with the HALO™ (Indica Labs, USA) analysis compared with inForm 2.4.1 (PerkinElmer, USA) analysis. Specifically, CD161 showed 27.7% less, CD3 29% less, IL18R1 32.4% less, MR-1 87.1% less, TCRVα7.2 22.7% less and DAPI 22.7% less variability with HALO™ (Indica Labs, USA) analysis when compared inForm 2.4.1 (PerkinElmer, USA) analysis. CD8 showed a slight 2.4% increase in variability with HALO™ (Indica Labs, USA) analysis when compared to inForm 2.4.1 (PerkinElmer, USA) analysis (Table 4.2).

Antibody / methods	**Percent Variability (%)**	**Difference (%)**
CD161 HALO	92.6	**27.7**
CD161 inForm	**120.3**	
CD3 HALO	78.6	**29.0**
CD3 inForm	**107.6**	
CD8 HALO	158.1	**-2.4**
CD8 inForm	**155.7**	
IL18R1 HALO	109.4	**32.4**
IL18R1 inForm	**141.9**	
MR-1 HALO	40.7	**87.1**
MR-1 inForm	**127.8**	
TCRVα7.2 HALO	143.7	**22.7**
TCRVα7.2 inForm	**166.4**	
DAPI HALO	8.5	**22.7**
DAPI inForm	**31.1**	

Table 4.2: *Percentage variability between single antibody analysis for HALO™ and inForm 2.4.1 performed on log transformed data*

Representative images of antibody phenotyping following tissue segmentation to exclude "non tissue" areas and "tissue folds" from further analysis and following nuclear and cell segmentation to identify individual cells with HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) are shown in Figure 4.5. Subjectively, HALO™ (Indica Labs, USA) showed a greater ability to segment single cells using DAPI for nuclear segmentation than inForm 2.4.1 (PerkinElmer, USA), especially in the region of the epithelial tissue as shown in Figure 4.6.

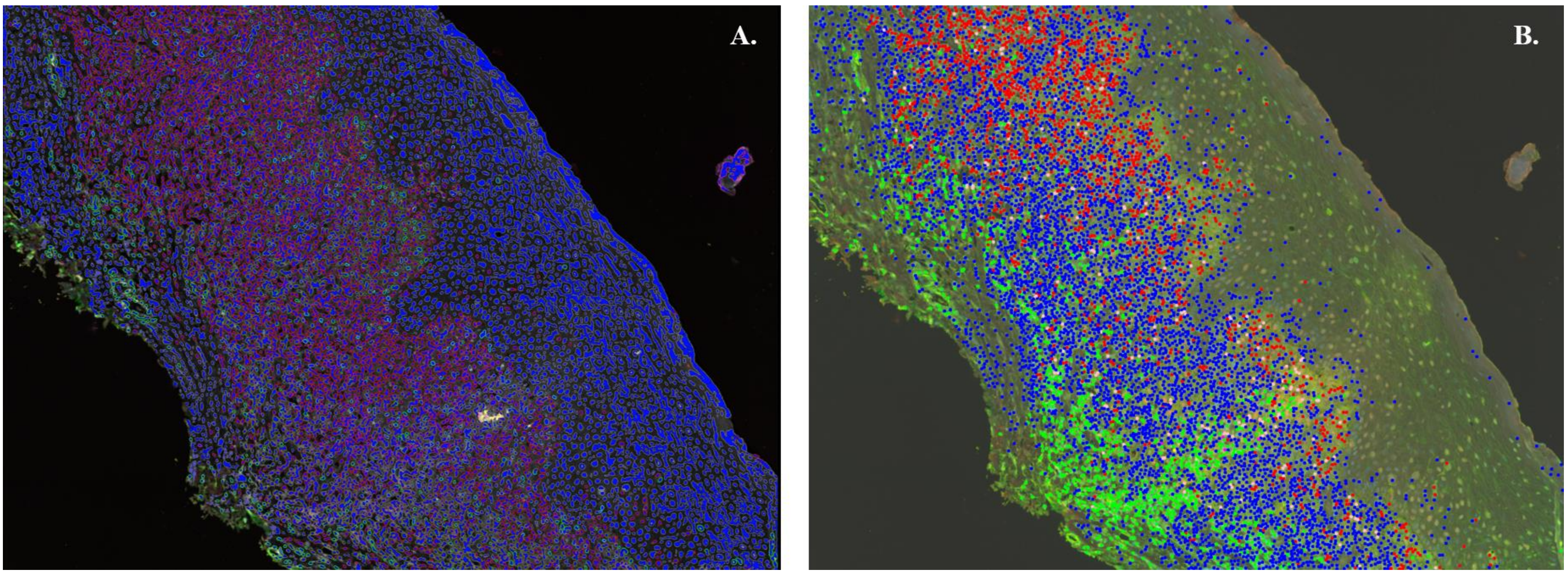

Figure 4.6: *Representative images of phenotyping performed on areas classified as tissue imaged at 200X magnification. A. Phenotyping performed with HALO™ B. Phenotyping performed with inForm 2.4.1*

Legend HALO™: Blue nucleus = DAPI positive, red membrane = CD3 positive, orange membrane = CD8 positive, cyan membrane = CD161 positive, yellow membrane = IL18R1 positive, magenta membrane = MR-1 positive and green membrane = TCRVα7.2 positive

Legend Inform: Blue dot = DAPI positive, red dot = CD3 positive, orange dot = CD8 positive, pink dot = CD161 positive, yellow dot = IL18R1 positive, magenta dot = MR-1 positive and green dot = TCRVα7.2 positive

4.5 Discussion

This study has validated a method for quantitative cell analysis in a fluorescent mIHC study using 6 antibody markers and one nuclear stain. This combination of markers is a panel that can be used to identify MAIT cells using colocalization to for the phenotyping of cells positive for multiple makers. These markers have been previously used with fluorescent IHC to identify MAIT cells in skin, vaginal and gastrointestinal mucosa (Hiejima et al., 2015, Li et al., 2016, Gibbs et al., 2017). A recent study confirmed the presence of MAIT cells in this tissue using in situ staining with TCRVα7.2 and IL18Rα in normal buccal mucosa (Sobkowiak et al., 2019) with MAIT cells shown to reside in the epithelium just above the basement membrane (Sobkowiak et al., 2019). To our knowledge this is the first time these markers have been used in a fluorescent mIHC panel in OLP. This panel was applied to 20 OLP samples and analysed using inForm 2.4.1 (PerkinElmer, USA) and HALO™ (Indica Labs, USA). Comparisons between the tissue segmentation algorithms were only slightly less variable using inForm 2.4.1 (PerkinElmer, USA). For single cell analysis and identification of single antibody positive cells HALO™ (Indica Labs, USA) consistently proved to be the least variable method of analysis.

Fluorescent mIHC using the Opal method has been shown to be a highly reproducible method that facilitates staining of multiples markers on one slides negating the need for multiple singly stained tissue sections (Stack et al., 2014, Parra et al., 2017, Mezheyeuski et al., 2018). Quantitative cell analysis of Opal mIHC stained tissues is reliant on the MSI and spectral library being able to spectrally separate between the markers, using a tissue classifier to separate out areas of interest for measurement and use of cell segmentation software to identify single cells for quantitative measurement (Mansfield, 2014).

Analysis is also dependent on the individual analysing the images and their skills in defining the parameters of positivity and negativity; something that contributes to the subjectivity and inherent biases of this process (Aeffner et al., 2017). Whilst lacking the inherent capacity of a pathologist to fully interpret the staining, digital analysis using trained algorithms can be advantageous as they are both reproducible and objective (Aeffner et al., 2017, Acs et al., 2019). The above qualities, plus easy to use interfaces, are invaluable to research with researchers trained to use this technology independently. Input from a pathologist during digital workflow analysis can assist in guiding both technicians and scientists (Aeffner et al., 2016).

Variabilities during tissue staining and processing may also prove problematic during later stages of digital analysis, as the algorithm, unlike a trained pathologist, does not have the capacity to make judgement calls or account for these processing variabilities. Factors that negatively impact the quality of a fluorescent image may include concentrations of antibodies and fluorophores, resulting in either weak signals or increased background, autofluorescence, or bleaching due to improper storage or prolonged exposure to light (Lichtman and Conchello, 2005). It is also important for the operator to ensure consistency during cutting, processing and staining of sections to limit variability. If using a manual rather than automated workflow, smaller numbers of slides should be processed in batches to also decrease variability in staining (Parra et al., 2017).

The Opal mIHC method uses a TSA system to detect multiple targets (Stack et al., 2014, Parra et al., 2017), however, one study reported fluorophore crosstalk occurred between markers where the emission spectra overlapped (Mezheyeuski et al., 2018). This is a potential problem that can complicate analysis and lead to inaccurate interpretations of data or even an inability to further analyse the data (Mezheyeuski et al., 2018) whilst also leading to an increase in false positives and reducing the specificity of this technique. To ensure the integrity of mIHC proper antibody and fluorophore titration prior to conducting the final experiment is therefore required (Parra et al., 2017, Mezheyeuski et al., 2018).

Whilst the tissue segmentation algorithms appeared comparable, variabilities in the algorithm determination of positivity is a potential cause of the variability in analysis. HALO™ (Indica Labs, USA) used threshold values to determine antibody positivity having an objective cut off point for positive and negative cells. Conversely, inForm 2.4.1 (PerkinElmer, USA) marked each cell with a percentage of confidence for antibody positivity, hence some cells identified as positive for a given marker could have a confidence of $< 50\%$. Subjectively, HALO™ (Indica Labs, USA) appeared more likely to segment single cells compared to inForm 2.4.1 (PerkinElmer, USA) in tissue classified regions and HALO™ (Indica Labs, USA) appeared more likely to determine antibody positive cells than inForm 2.4.1 (PerkinElmer, USA).

A recent study assessing the inter-platform and inter-operator reproducibility of Ki67 analysis for HALO™, QuantCenter and QuPath was undertaken initially with one operator and subsequently with multiple operators (Acs et al., 2019). This study showed that both inter-platform and inter-operator reproducibility was very high (Acs et al., 2019), nevertheless it is important to note this study assessed reproducibility for only one

maker, Ki67, instead of the 7 assessed in the present study. It is reasonable to expect that an increase in complexity of analysis will occur with an increase in the number of markers measured, and potentially a diminishment of reproducibility.

To our knowledge, this is the first study to compare inForm 2.4.1 (PerkinElmer, USA) and HALO™ (Indica Labs, USA) for digital analysis of tissue samples from OLP. Although considerable variability was expected, the method that most consistently diminished this variability, HALO™ (Indica Labs, USA), was chosen for all subsequently analyses.

4.6 Conclusion

The results of this present study indicate that HALO™ (Indica Labs, USA) shows less variability than inForm 2.4.1 (PerkinElmer, USA) for single antibody analysis, whilst variability for tissue segmentation was only slightly less variable with inForm 2.4.1 (PerkinElmer, USA). The findings from this study indicated that overall HALO™ (Indica Labs, USA) was the more reliable program for mIHC quantitative cell analysis, hence this was the program chosen for analysis in subsequent experiments

5 Multiplex Immunohistochemical Study – Mucosal Associated Invariant T Cell Phenotypes in OLP

5.1 Abstract

Mucosal associated invariant T (MAIT) cells possess the T cell receptor (TCR) Vα7.2 and are restricted by the highly conserved molecule, major histocompatibility complex class 1 related protein (MR-1). MAIT cells are activated by riboflavin derived MR-1 bound intermediates that are not produced endogenously in humans. Riboflavin can be produced by microbes including *Candida* and bacteria. OLP, a T cell mediated inflammatory condition of unknown cause, has been associated with concurrent candidal infection. The interaction between MAIT cells and oral *Candida* and the role this may play in the aetiopathogenesis of OLP is currently unknown.

The aim of this study was to determine if MAIT cells are present in OLP and whether the number of MAIT cells changed due to the presence of *Candida* or patient symptoms. This study also aimed to quantify single antibody and T cell phenotypes.

Eighty-nine formalin fixed paraffin embedded (FFPE) biopsy tissue samples from patients were assessed, including samples from 74 patients with OLP (28 asymptomatic, 30 symptomatic) and 16 samples with concurrent *Candida* (9 symptomatic and 7 asymptomatic), for comparison with 15 patient samples of oral fibroepithelial polyp (FEP). All samples were tested for presence of *Candida* with periodic acid-Schiff (PAS) staining. Cells positive for MAIT cell phenotypes were identified using a multiplex immunohistochemistry (mIHC) staining technique for the cellular markers CD3, interleukin 18 receptor 1 (IL18R1), TCRVα7.2, CD161, CD8 and MR-1.

Results confirmed the presence of MAIT cell phenotypes within the subepithelial infiltrate of OLP. The presence of *Candida* and/or symptoms in tissue reduced MAIT cell phenotype expression with decreased expression of CD161 generally noted in the presence of symptoms and decreased expression of TCRVα7.2 generally noted in the presence of *Candida*. Expression of MR-1 was not significantly different between biopsy tissue from OLP patients or FEP tissue, and expression of MR-1 was not significantly affected by the presence of either *Candida* or symptoms.

From the results it can be inferred that the presence of either *Candida* or symptoms in patients with OLP either limits MAIT cell presence or that patients with poorer MAIT cell expression are more likely to have an inherent susceptibility for candidal infection and be symptomatic.

5.2 Introduction

OLP is a chronic mucocutaneous condition of unknown cause that affects 1-2% of the population (Axell, 1976, Axell and Rundquist, 1987) presenting more frequently in women around the time of middle age. The OLP inflammatory disease mechanism is characterised by a persistent cytotoxic T cell inflammatory process directed against the basal lamina of the epithelium (Sugerman et al., 2002, Roopashree et al., 2010). What triggers and maintains this inflammatory process is currently unknown, however, heat shock protein (HSP) (Sugerman et al., 2002, Sugerman and Savage, 2002, Garcia-Garcia et al., 2013), hepatitis C virus (HCV) (Carrozzo and Gandolfo, 2003, Carrozzo, 2008), human papilloma virus (HPV) (Syrjanen et al., 2011), genetics and stress (Chaudhary, 2004, Ivanovski et al., 2005) have all been postulated as the causative immune triggers.

MAIT cells are a unique population of T cells present in peripheral blood at rates of 1-10% (Tilloy et al., 1999, Gold et al., 2010, Cowley, 2014). MAIT cells can also be found in both the gut mucosa and liver (Tilloy et al., 1999, Gold et al., 2010). What makes these cells a potential aetipathogenic factor in OLP is the highly conserved invariant TCR α chain (TCRVa7.2) that can be activated by riboflavin intermediates bound by MR-1 (Tilloy et al., 1999, Gold et al., 2010, Cowley, 2014) .

Oral microbes, including *Candida,* may produce the riboflavin by-products required for MAIT cell activation. The ongoing presence of commensal oral microbes, such as *Candida,* may also account for the ongoing chronic inflammation in OLP. MAIT cells have been shown to be activated by epithelial cells infected with *Shingella flexneri*, an invasive bacteria with the MAIT cells killing infected cells expressing MR-1 (Le Bourhis et al., 2013). This may be significant in the oral cavity as *Candida spp.* can superficially invade the epithelium, potentially triggering MR-1 production, activation of MAIT cells and subsequent death for the infected keratinocyte.

An expanding area of research is the role MAIT cells may play in disease. Quantitative assessment of MAIT cells in normal skin, seborrheic keratosis, psoriasis, alopecia areata and dermatitis herpetiformis showed that MAIT cells identified in dermatitis herpetiformis were significantly elevated compared to the other groups analysed (Li et al., 2016). The authors concluded that the elevated levels of MAIT cells could suggest a potential aetiopathogenic role for MAIT cells in this condition (Li et al., 2016). Further, quantification of MAIT cells in ulcerative colitis, Crohn's disease and normal control mucosae showed significantly lower numbers of MAIT in these conditions than controls

(Hiejima et al., 2015). In peripheral blood higher numbers of activated caspase expressing MAIT cells were noted in ulcerative colitis and Crohn's disease compared to health controls and that reduced MAIT cell numbers in these conditions was suggestive of increased apoptosis (Hiejima et al., 2015). Increased expression of IL-22, an interleukin that limits mucosal inflammation, was noted in the peripheral blood of ulcerative colitis patients (Hiejima et al., 2015). Reduced number of MAIT in inflammatory bowel disease (IBD) suggests MAIT cells play a protective role against mucosal inflammation (Hiejima et al., 2015). The authors concluded that the results inferred MAIT cells have pathological role in IBD and suggested a possible role for therapeutic manipulation of MAIT cells in IBD (Hiejima et al., 2015).

Systematic lupus erythematosus (SLE) is a condition that, when present in the oral cavity, clinically and histologically mimics OLP. An assessment of both the function and number of MAIT cells in multiple diseases, one of them being SLE, confirmed MAIT cells were significantly reduced in SLE (Cho et al., 2014). Furthermore, in the SLE cohort MAIT cell deficiency was correlated with disease activity (Cho et al., 2014). More recently a further investigation of the role of MAIT cells in SLE also confirmed a reduction of MAIT cells in SLE patients (Chiba et al., 2017). Utilising peripheral blood this study confirmed reduction in MAIT cell numbers was due to enhancement of MAIT cell death rather than surface marker downregulation (Chiba et al., 2017). Interestingly, the activated status of MAIT cells in SLE reflected disease activity (Chiba et al., 2017). Based on these results the authors suggested a potential role for MAIT cells in SLE pathogenesis (Chiba et al., 2017). As SLE mimics OLP in the oral cavity, it is possible to extrapolate from these studies that MAIT cells may play a pathogenic role in OLP, with *Candida* potentially acting as a trigger.

At present, the role that MAIT cells may play in the aetiopathogenesis of OLP has not been investigated. The aim of the present study was to determine if MAIT cells are present in the oral mucosa in patients with OLP, and whether the number of MAIT cells was affected by the presence of *Candida* or symptoms. This study further aimed to quantify single antibody and T cell phenotypes associated with MAIT cell markers.

5.3 Materials and Methods

5.3.1 Patients

Patients and samples for this study were identified and selected as described in 2.2.1. Briefly, 90 FFPE tissue blocks were selected after reviewing biopsy reports and sorted into 5 test groups. The test groups included 30 samples of asymptomatic OLP, 30 symptomatic OLP samples, 15 OLP samples with *Candida*, both asymptomatic and symptomatic, and 15 FEP samples that served as mucosal controls. The tissue samples were sourced from the Melbourne University Histopathology Service at the Royal Dental Hospital of Melbourne. An additional 3 blocks with a diagnosis of oral CD/orofacial granulomatosis were also selected to serve as positive control tissue for IHC analysis. This project was approved by the University of Melbourne Human Ethics Sub-Committee, project number 1749368.1.

5.3.2 Tissue Samples

4µm thick sections were cut from each of the 90 tissue blocks and mounted onto Superfrost™ Plus (Thermo Fischer Scientific, USA) slides for subsequent PAS and mIHC staining.

5.3.3 Periodic Acid-Schiff Staining

PAS staining was performed as detailed in 2.2.3. Briefly the sections were bought to water, washed and periodic acid was applied to all the sections for 5 minutes. After 5 minutes the sections were rinsed, and Schiff's reagent was applied for 10 minutes followed by further washing. Mayer's Haematoxylin was applied to the sections for 30 seconds and differentiated with blue prior to the final wash. Using light microscopy glycogen, mucin and *Candida* were identified by magenta staining, while the cell nuclei stained blue with the PAS stain. PAS staining was performed with assistance from laboratory staff in the 5th floor histopathology laboratory at the Royal Dental Hospital of Melbourne to confirm allocation to the correct group. One sample in the OLP asymptomatic group was identified as having *Candida* with PAS staining and was reassigned to the OLP *Candida* asymptomatic group. A representative image of PAS staining with hyphae in the superficial epithelium is shown in Figure 5.1

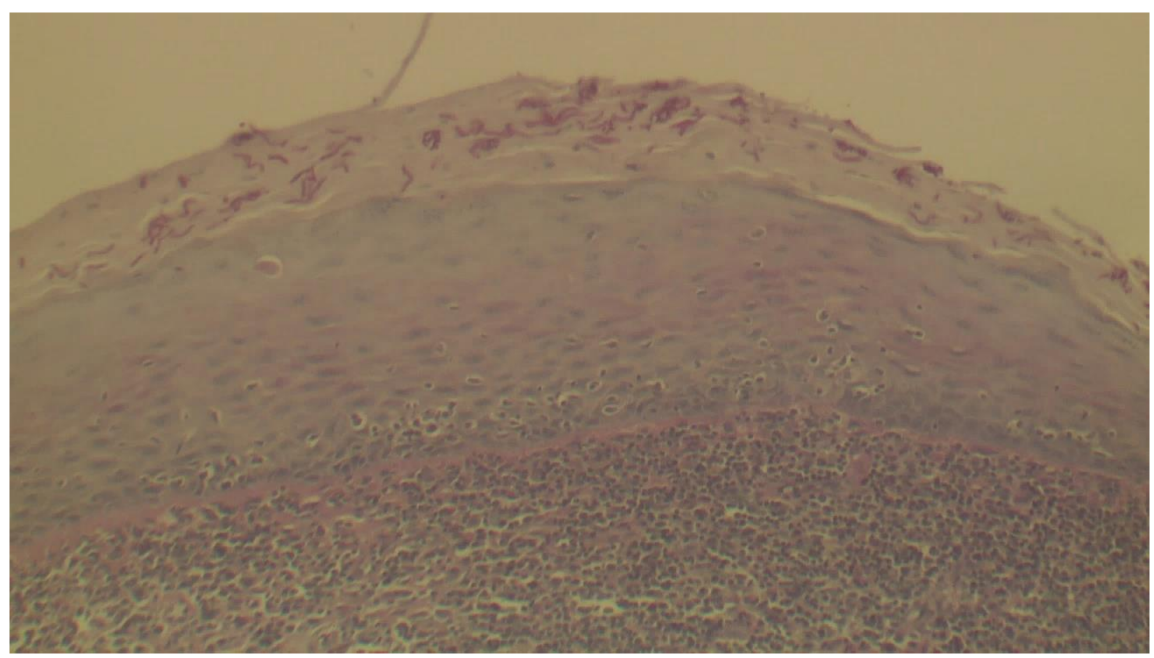

Figure 5.1: *OLP sample with candidal hyphae infiltrating the superficial epithelium 200X magnification*

5.3.4 Multiplex Immunohistochemistry

Fluorescent mIHC staining was performed as outlined in 2.2.7 following optimisation as outlined in 2.2.4 and 2.2.5. Briefly, the order of antibody application and primary antibody dilutions were TCRVα7.2 (Miltenyi Biotec, Germany) 1:500 dilution Opal 520 (PerkinElmer, USA), CD161 (Abcam, UK) 1:400 dilution Opal 620 (PerkinElmer, USA), IL18R1 (Abcam, UK) 1:1500 dilution Opal 570 (PerkinElmer, USA), MR-1 (Biorbyt, UK) 1:2000 dilution Opal 650 (PerkinElmer, USA), CD8 (Abcam, UK) 1:500 dilution Opal 540 (PerkinElmer, USA), CD3 (Abcam, UK) 1:500 dilution Opal 690 (PerkinElmer, USA) and DAPI 1:1000.

Staining was performed in batches over 3 days with positive and negative control slides used for each antibody with the positive control slides also serving as spectral controls for digital analysis. Human tonsil was used as the positive control for CD3 (Abcam, UK), IL18R1 (Abcam, UK), CD161 (Abcam, UK), CD8 (Abcam, UK), MR-1 (Biorbyt, UK) and DAPI (Sigma-Aldrich, USA) with oral Crohn's disease serving as the control tissue for TCRVα7.2 (Miltenyi Biotec, Germany).

As described in 4.3.3 CD3, IL18R1 (IL18Rα), CD161, MR-1 and TCRVα7.2 were chosen for this mIHC panel as these markers have been used in previous studies to identify MAIT cells in tissue and determine MR-1 expression (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016, Gibbs et al., 2017). Phenotypes used for MAIT cell identification in tissue have included triple positive CD3 + IL18R1 + TCRVα7.2 and DAPI + CD161+ TCRVα7.2 phenotypes (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016) or double positive CD3 + TCRVα7.2 and TCRVα7.2 + IL18Rα phenotypes (Gibbs et al., 2017). CD8 was also chosen for this panel for quantification of cytotoxic T cell phenotypes as this is the predominant cell of the OLP chronic inflammatory infiltrate (Sugerman et al., 2000a). DAPI was required for nuclear counterstaining and identification of cells. Following mIHC all samples were visually assessed and deemed suitable to proceed to slide scanning and quantitative analysis.

5.3.5 Quantitative Analysis and Phenotyping

Slide scanning was performed on all 90 slides as described in 2.2.8. Briefly the entire tissue section on the slide was outlined and scanned at 100X (resolution of 1µm/pixel) magnification using the Vectra® Automated Multispectral Imaging System (PerkinElmer, USA). The MSI were loaded onto Phenochart (PerkinElmer, USA) and 5 random fields chosen using the stamp function and imaged at 200X (0.5µm/pixel)

magnification. This generated 5 1338µm x 1000µm images for quantitative single antibody analysis. One OLP asymptomatic sample was over exposed and could not be further analysed. The final workflow for quantitative analysis shown in Figure 5.2.

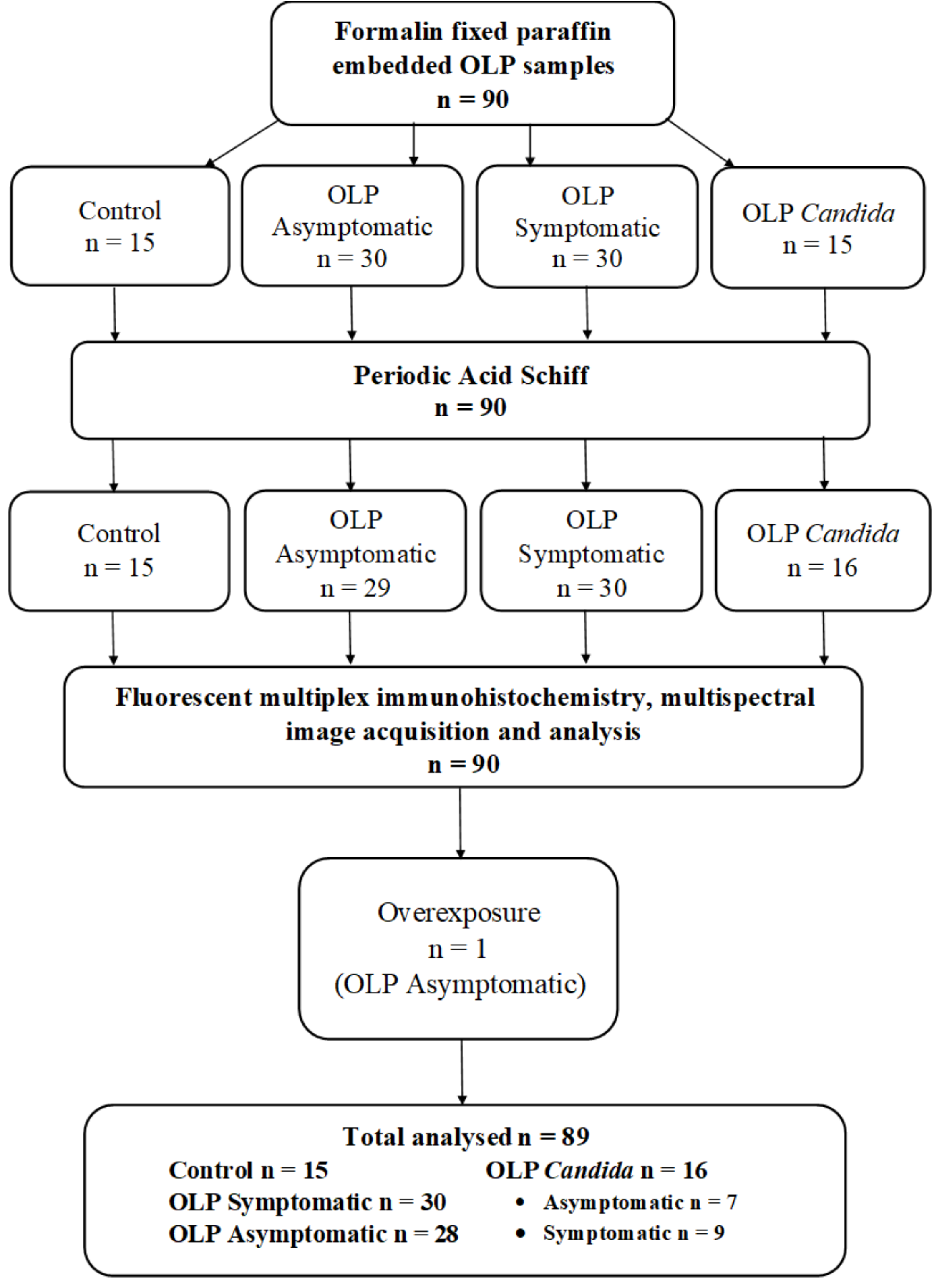

Figure 5.2: *mIHC workflow with a total of 89 samples quantitatively analysed*

Phenotyping of cells was undertaken after HALO™ (Indica Labs, USA) was trained to recognise cells fluorescing at the correct wavelength for each antibody and was performed as outlined in 2.2.9. Briefly, component images were generated for the 5 MSI for each sample (total 445 images) using inForm 2.4.1 (PerkinElmer, USA) as described in 2.2.9.1. These images were loaded into HALO™ (Indica Labs, USA) and the 5 MSI per sample merged to form 89 merged images. Tissue segmentation and trainable phenotyping was undertaken for each antibody using representative examples to train the algorithms as described in 2.2.9.2 prior to the 89 merged images undergoing batch analysis. As described in 2.2.9.3 the co-localisation setting was used to phenotype cells positive for more than one marker.

All phenotypes analysed were restricted by DAPI to ensure only nucleated cells were included in the final analysis. Double positive staining with DAPI plus CD3, CD8, CD161, MR-1, IL18R1 and TCRVα7.2 was used to quantify all 6 markers to determine variations in number associated with the different treatment groups and, as in other studies, to assess the expression of MR-1 in tissue (Hiejima et al., 2015). Triple positive T cell phenotypes for DAPI + CD3 + CD8, DAPI + CD3 + CD161, DAPI + CD3 + IL18R1 and DAPI + CD3 + TCRVα7.2 were also enumerated.

These phenotypes were analysed to firstly identify cytotoxic T cells (CD3 + CD8) as these are the predominant cell in OLP (Sugerman et al., 2000a). The other three phenotypes (CD3 + CD161, CD3 + IL18R1 and CD3 + TCRVα7.2) were chosen as MAIT cells are T cells (CD3) that express high level of the above markers (Gibbs et al., 2017). Thus, using combinations of the above markers, T cells positive for MAIT cell markers were identified. Finally, cell's whose phenotypes expressed either 4 or 5 of the markers were identified as MAIT with previous studies using some of these combinations to quantify MAIT cells in tissue (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016). The maximal number of markers used to identify MAIT cells in these studies were 3, utilising a mIHC technique allowed for identification of 4 makers plus DAPI. The cells expressing the following combinations of markers were enumerated: DAPI + CD3 + CD161 + TCRVα7.2; DAPI + CD3 + IL18R1 + TCRVα7.2; DAPI + CD3 + CD161 + IL18R1; and DAPI + CD3 + CD161 + IL18R1 + TCRVα7.2. The percentage of these cell phenotypes in each sample was determined by dividing the total number of CD3 positive cells in the sample (Dusseaux et al., 2011).

5.3.6 Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post hoc testing was used to assess the means of all groups. General linear models were fitted to further analyse the OLP treatment groups to determine the effect the presence of symptoms, *Candida,* and symptoms plus *Candida* had on phenotype expression. For ANOVA the equality of the variances was assessed using the multiple comparisons test and Levene's test using Minitab® 18, with the results indicating transformation of the data to better fit the model was required. To determine normality and skewness of the data an assessment of the residuals and residual vs. fits was for untransformed and log transformed data was undertaken using for both ANOVA and the general linear models. Representative images of these plots for both the transformed and untransformed data for DAPI + CD161 analysis with ANOVA and a general linear model is shown in Figures 5.4 and 5.5.

Thus, a natural log transformation was required to undertake statistical analysis. Some data had 0 (zero) values which was accounted for by adding 1% to the natural log transformation. No log transformation was required for assessment of age as the data was normally distributed with one-way ANOVA used to assess between the different groups. Finally, chi-squared analyses were undertaken to assess for associations related to gender. Significance was defined as a p value less than or equal to 0.05.

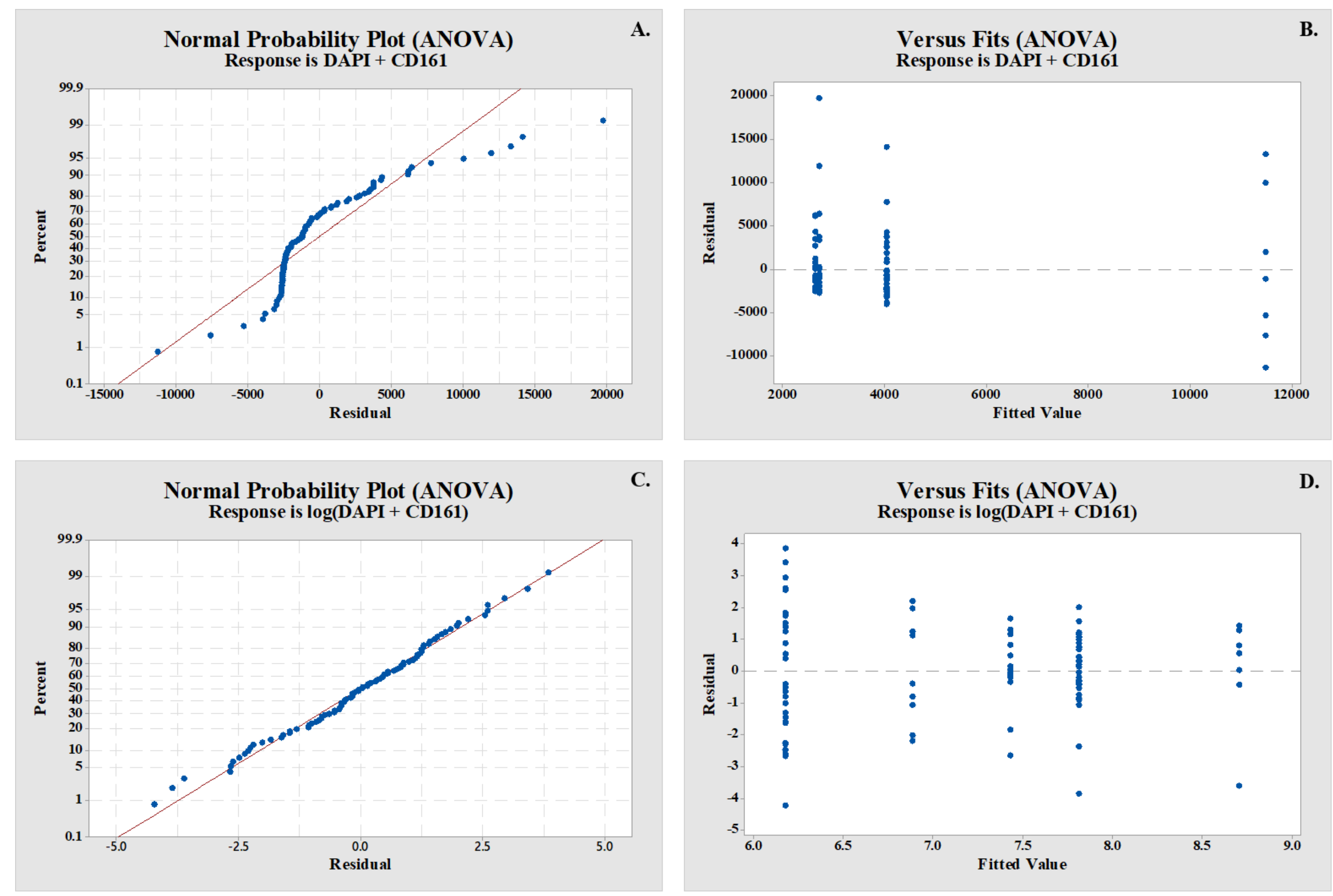

Figure 5.3: *Representative examples of normality of the residuals and residuals vs. fit plots for DAPI + CD161 with one-way ANOVA analysis. A. normality plot untransformed data B. residuals vs. fit untransformed data, C. normality plot log transformed data, D. residuals vs. fit log transformed data*

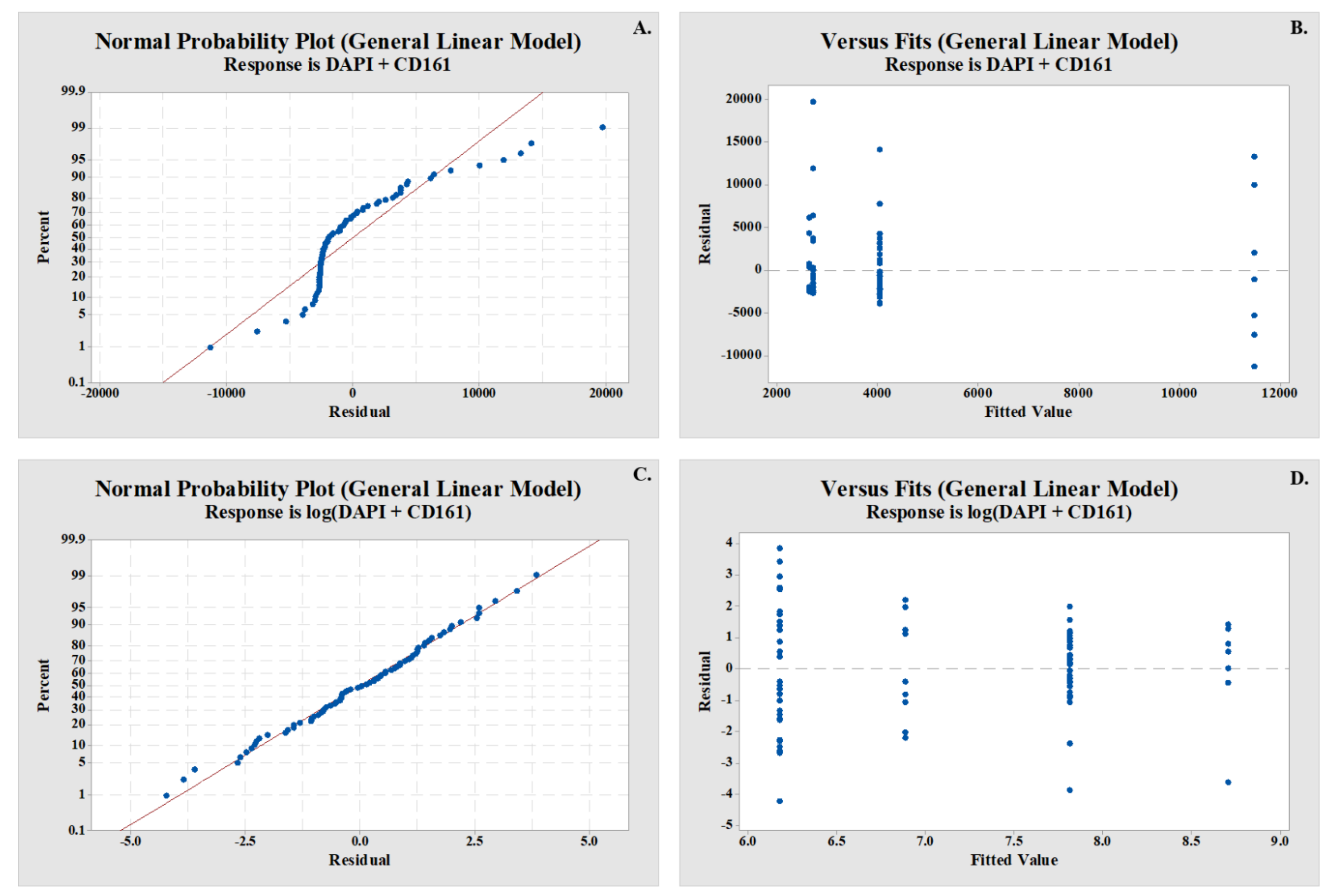

Figure 5.4: *Representative examples of normality of the residuals and residuals vs. fit plots for DAPI + CD161 with general linear model analysis. A. normality plot untransformed data B. residuals vs. fit untransformed data, C. normality plot log transformed data, D. residuals vs. fit log transformed data*

5.4 Results

5.4.1 Patient Characteristics

The study population consisted of 89 FFPE tissue samples sourced from 88 patients, 65 (74%) female and 23 (26%) male with a mean age of 61.1 years at the time of diagnosis. 22 (25%) were greater than 70 years of age and 66 (75%) were less than 70 years of age at the time of diagnosis. The age and sex distributions for the 5 treatment groups is summarised in Table 5.1. No significant differences existed between the age ($p = 0.308$) and sex ($p = 0.565$) distributions between the 5 different treatment groups. This table also defined the percentage of the sample the classifier defined as tissue with one-way ANOVA of the log transformed data showing significantly more tissue was analysed in the control group compared to the OLP asymptomatic group ($p = 0.005$).

	Sex (M: F)	Age μ ± SD	% Tissue Analysed μ ± SD
Control	1:6.5	55.3 ± 11.9	78.9 ± 9.8*
OLP Asymptomatic	1:1.8	59.6 ± 15.6	54.9 ± 21.0*
OLP Symptomatic	1:4.0	64.4 ± 12.0	60.2 ± 21.7
OLP *Candida* Asymptomatic	1:2.3	59.1 ± 14.7	76.5 ± 17.6
OLP *Candida* Symptomatic	1:3.5	63.1± 14.7	68.9 ± 16.9

Table 5.1: *Distribution of sex, age and percentage of tissue analysed for the all groups using raw data*

*Legend: * represents significance (< or = 0.05), μ represents the mean and SD represents the standard deviation*

5.4.2 Single Phenotypes

Double positive staining was determined by co-localisation of DAPI with CD3, CD8, CD161, MR-1, IL18R1 and TCRVα7.2 Representative images demonstrating positive staining for single phenotypes are shown below (Figs. 5.5, 5.6, 5.7, 5.8, 5.9 and 5.10)

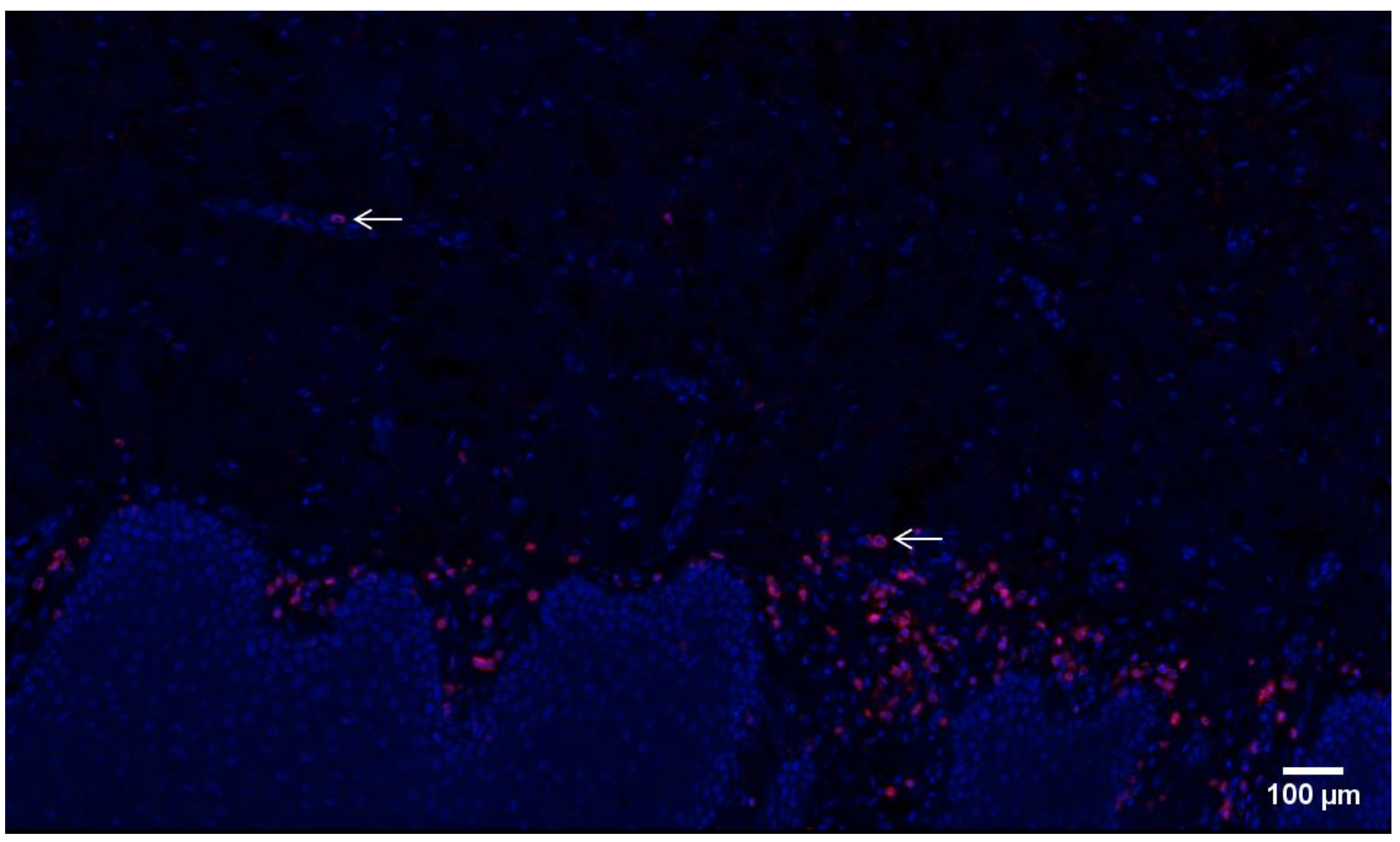

Figure 5.5: *Representative field of view image of DAPI + CD3 phenotype positive cells (red) from control sample*

Legend: White arrow identifies phenotype positive cell

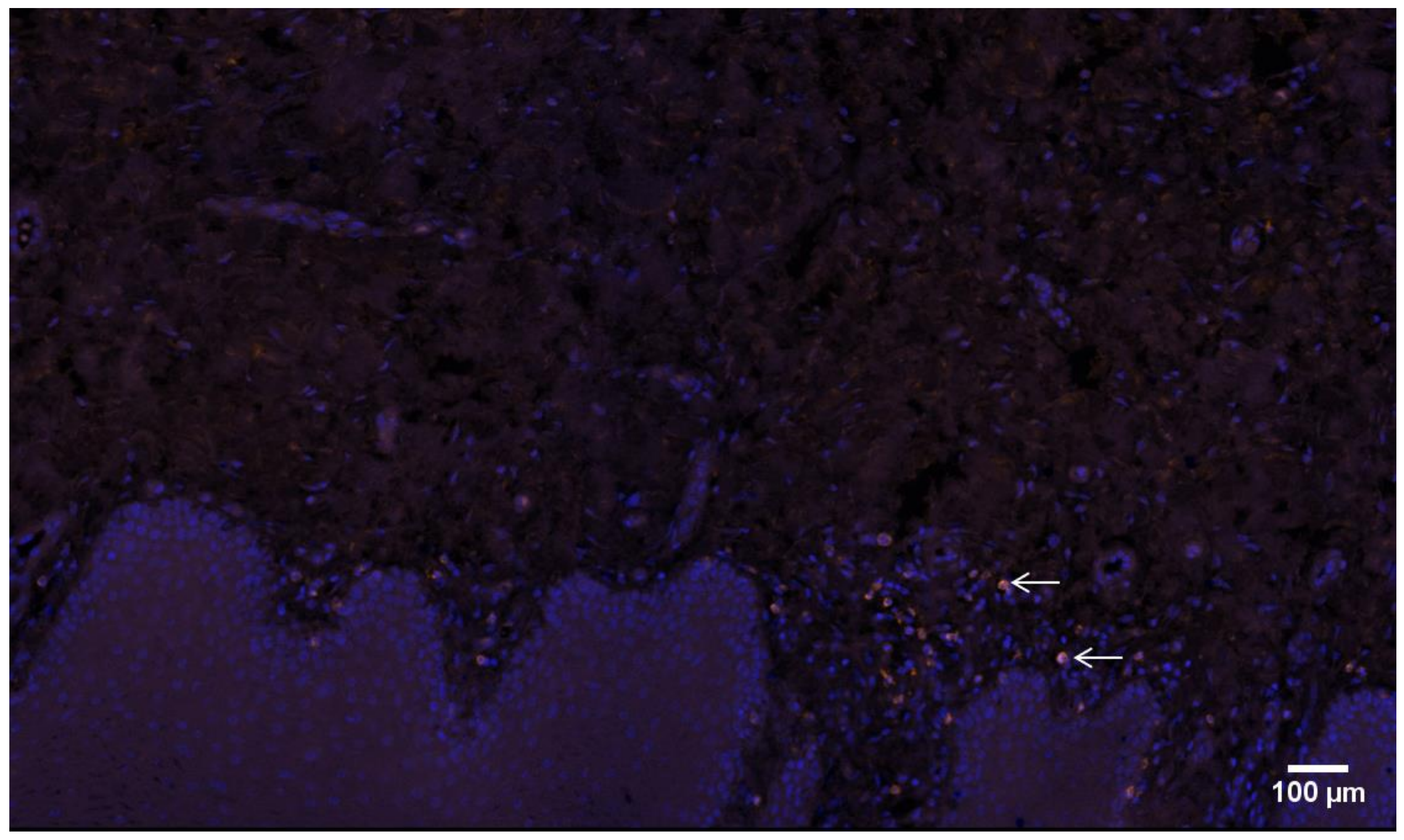

Figure 5.6: *Representative field of view image of DAPI + CD8 phenotype positive cells (orange) from control sample*

Legend: White arrow identifies phenotype positive cell

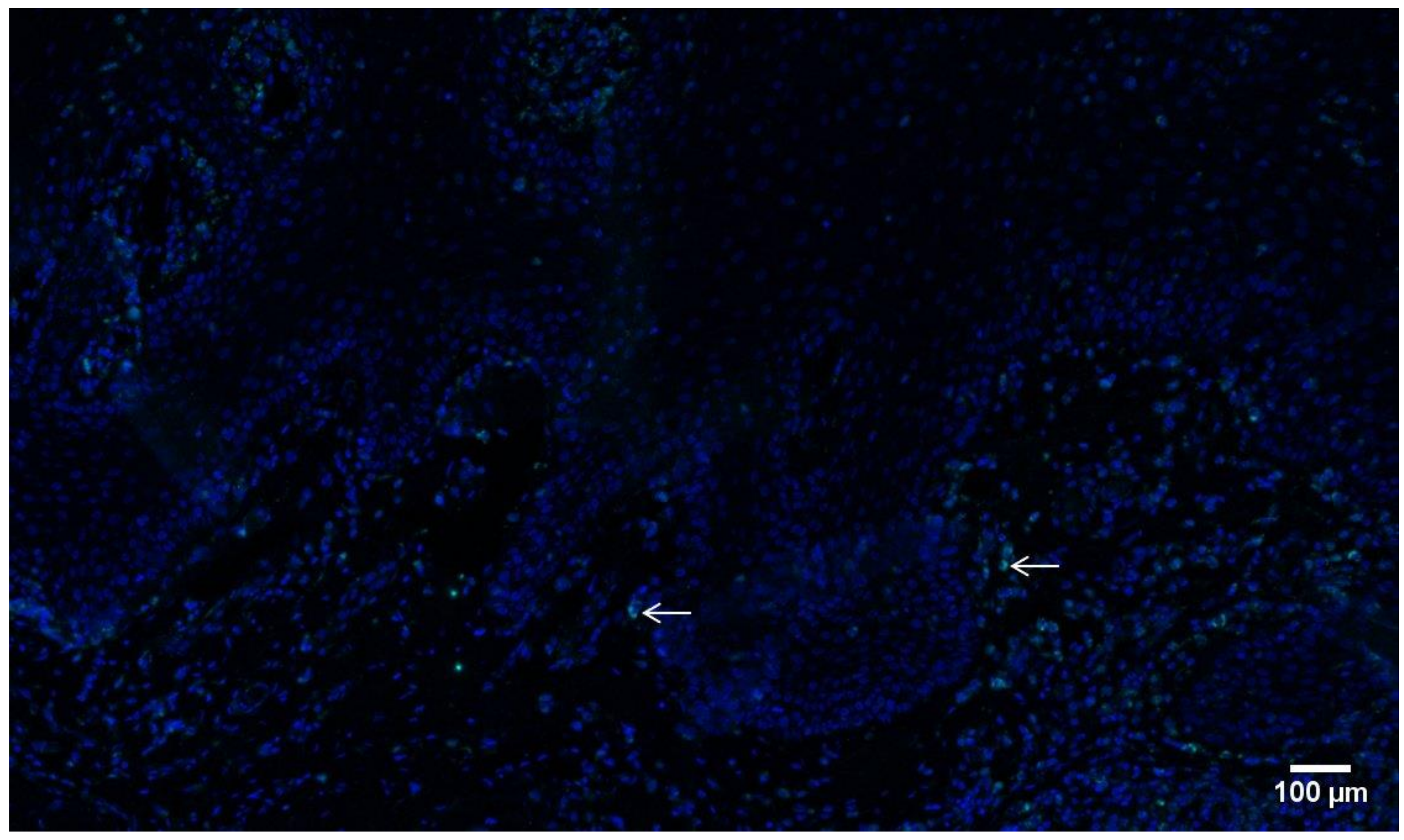

Figure 5.7: *Representative field of view image of DAPI + CD161 phenotype positive cells (cyan) for OLP Candida asymptomatic sample*

Legend: White arrow identifies phenotype positive cell

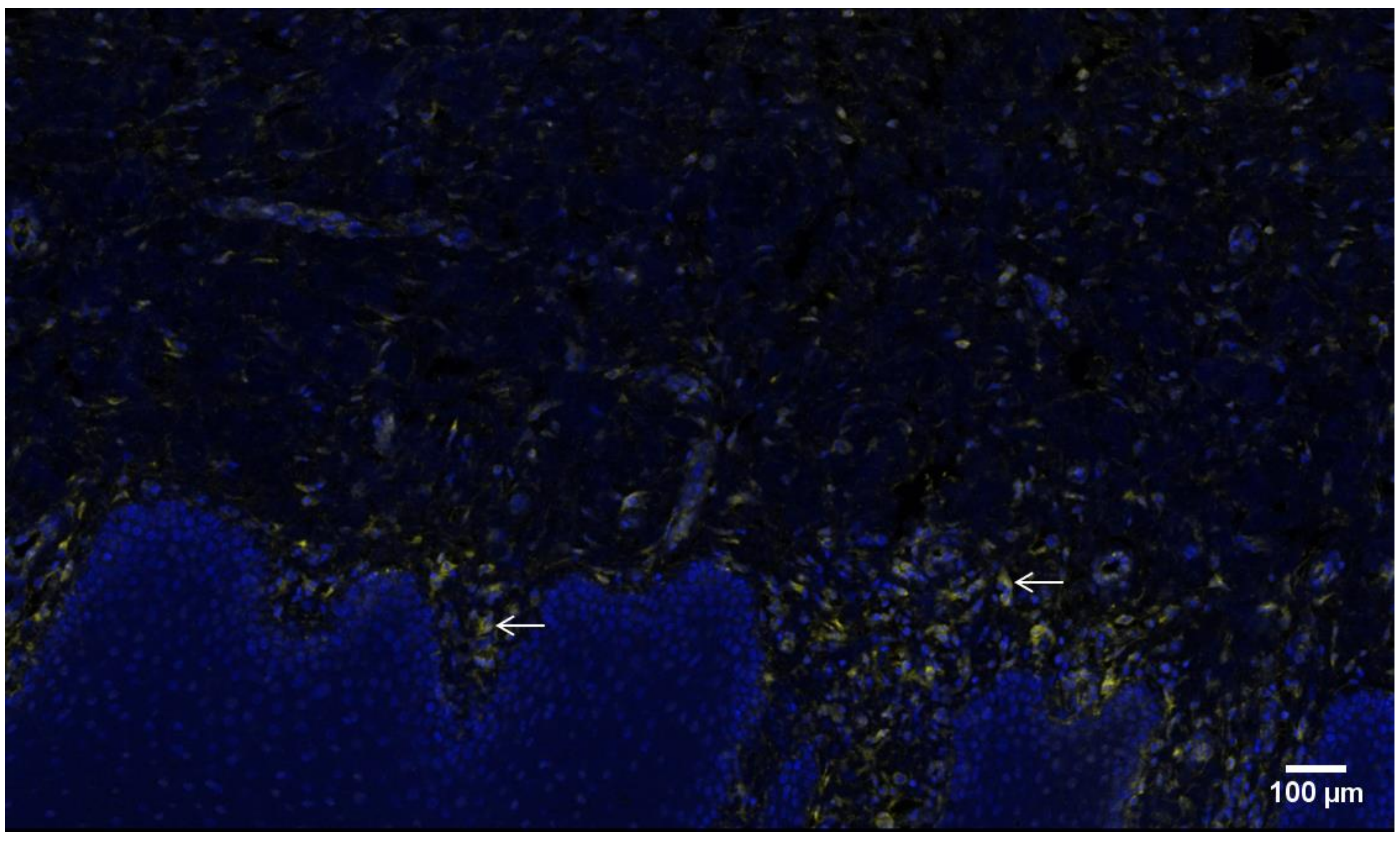

Figure 5.8: *Representative field of view image of DAPI + IL18R1 phenotype positive cells (yellow) from control sample*

Legend: White arrow identifies phenotype positive cell

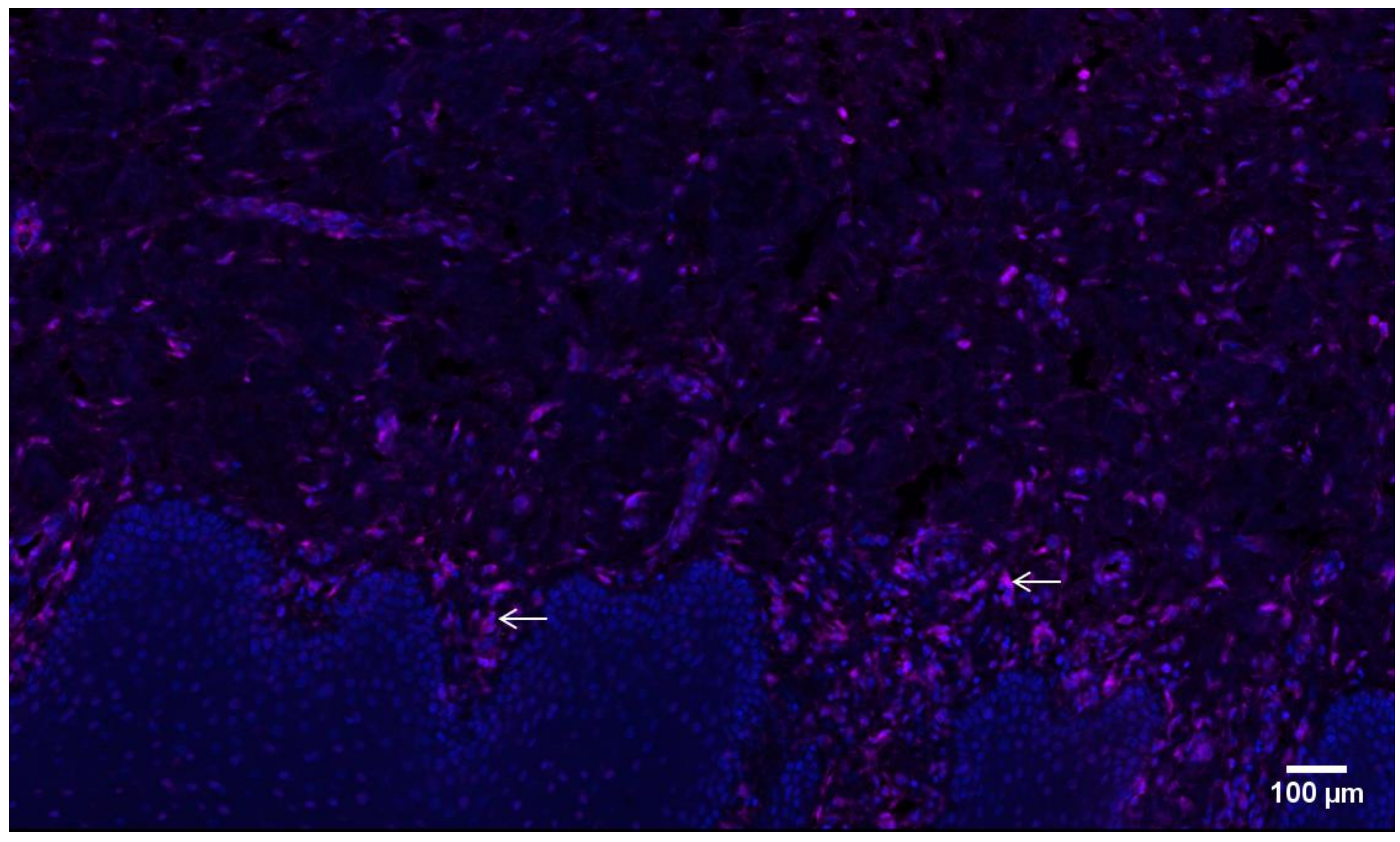

Figure 5.9: *Representative field of view image of DAPI + MR-1 phenotype positive cells (magenta) from control sample*

Legend: White arrow identifies phenotype positive cell

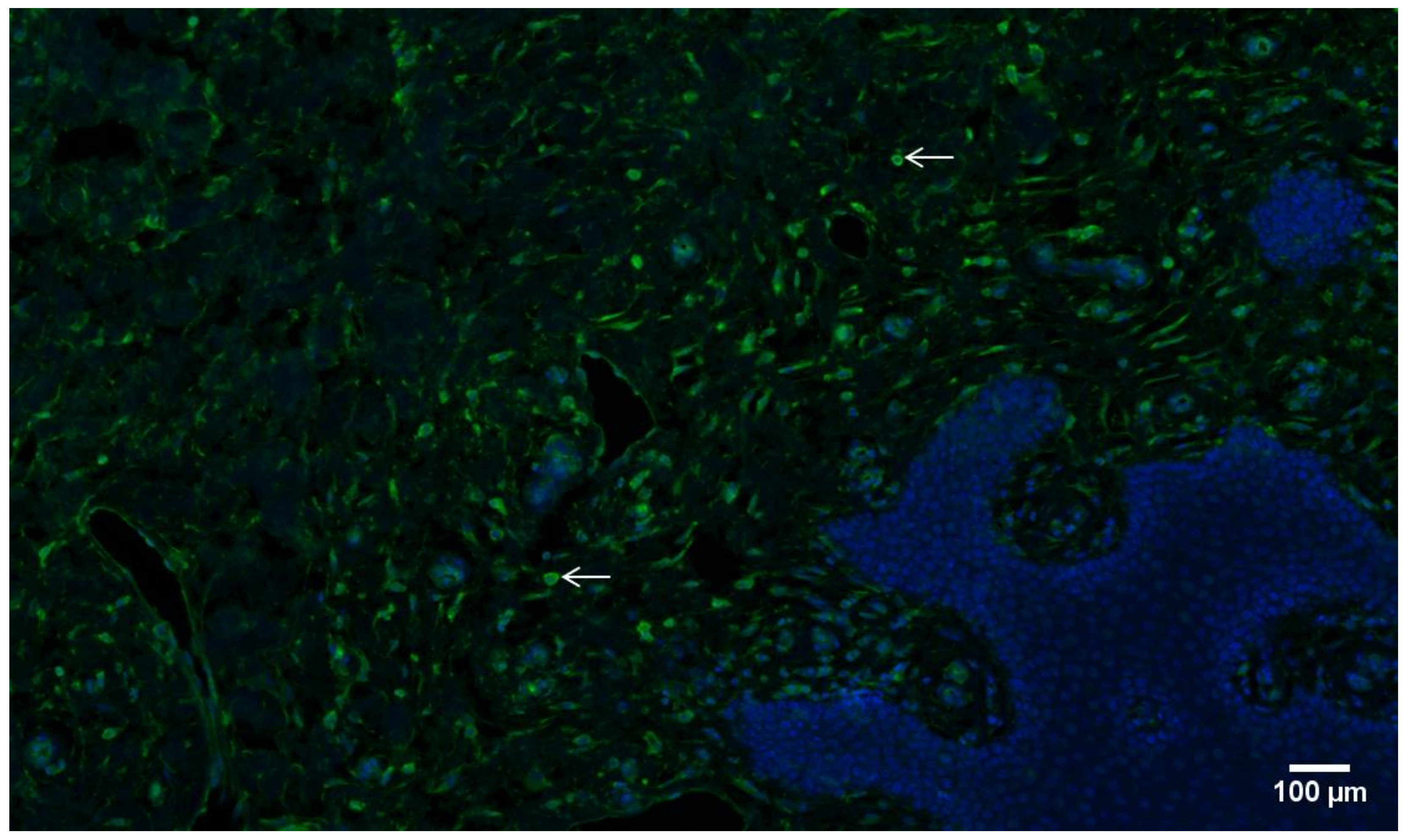

Figure 5.10: *Representative field of view of DAPI + TCRVα7.2 phenotype positive cells (green) from control sample*

Legend: White arrow identifies phenotype positive cell

The distribution of the means and standard deviations of the raw data for each phenotype with respect to the each of the clinical groups shows wide variation (Table 5.2). An analysis of the distribution of each of these single antibodies was undertaken on the transformed data.

	DAPI + CD3 μ ± SD	DAPI + CD8 μ ± SD	DAPI + CD161 μ ± SD	DAPI + IL18R1 μ ± SD	DAPI + MR-1 μ ± SD	DAPI + TCRVα7.2 μ ± SD
Control	1,645 ± 2,263	3,095 ± 10,020	2,629 ± 2,413	4,073 ± 3,534	9,489 ± 5,593	2,958 ± 5,223
OLP Asymptomatic	6,845 ± 5,509	410 ± 878	4,033 ± 3,936	4,683 ± 4,928	14,586 ± 7,809	3,270 ± 5,648
OLP Symptomatic	3,674 ± 3,446	379 ± 835	2,712 ± 5,020	4,630 ± 6,827	11,032 ± 6,761	7,266 ± 7,556
OLP *Candida* Asymptomatic	2,913 ± 3,936	461 ± 601	11,480 ± 9,085	10,755 ± 11,114	11,411 ± 7,829	184 ± 415
OLP *Candida* Symptomatic	6,731 ± 605	853 ± 960	2,635 ± 3,229	2,949 ± 6,110	8,033 ± 5,914	3,595 ± 6,903

Table 5.2: *DAPI plus single marker phenotype positive cells for all groups using the raw data*

Legend: μ represents the mean and SD represents the standard deviation

5.4.2.1 DAPI + CD3

Significant differences existed among the 5 groups with regards to the number of DAPI + CD3 positive cells ($p = 0.017$). Post hoc testing revealed the OLP asymptomatic group had a significantly higher number of DAPI + CD3 positive cells compared to the OLP *Candida* asymptomatic group ($p = 0.024$) (Fig. 5.11). The number of DAPI + CD3 positive cells was significantly associated with the presence of *Candida* and symptoms together (Degree of freedom (DF) = 1, F-value = 6.52, $p = 0.013$). Biopsy samples from asymptomatic OLP patients that did not have any observable *Candida* on their PAS stained tissue, had statistically significantly higher levels of DAPI + CD3 cells (2.51 units higher: 95% CI; 0.18, 4.83, $p = 0.030$) than the asymptomatic OLP patients with observable *Candida*.

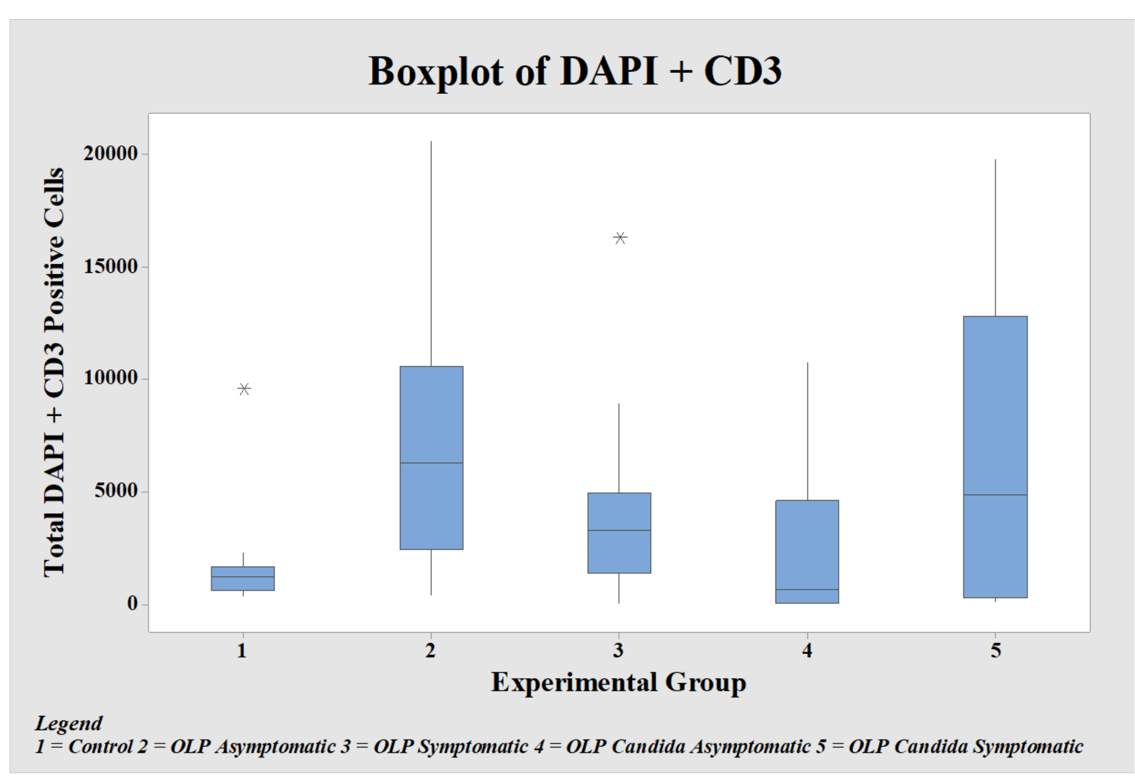

Figure 5.11: *Boxplot of total DAPI + CD3 positive cells for all groups using raw data*

5.4.2.2 DAPI + CD8

No significant differences existed between the 5 groups with regards to the number of DAPI + CD8 positive cells ($p = 0.056$) (Fig. 5.12). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the number of DAPI + CD8 positive cells.

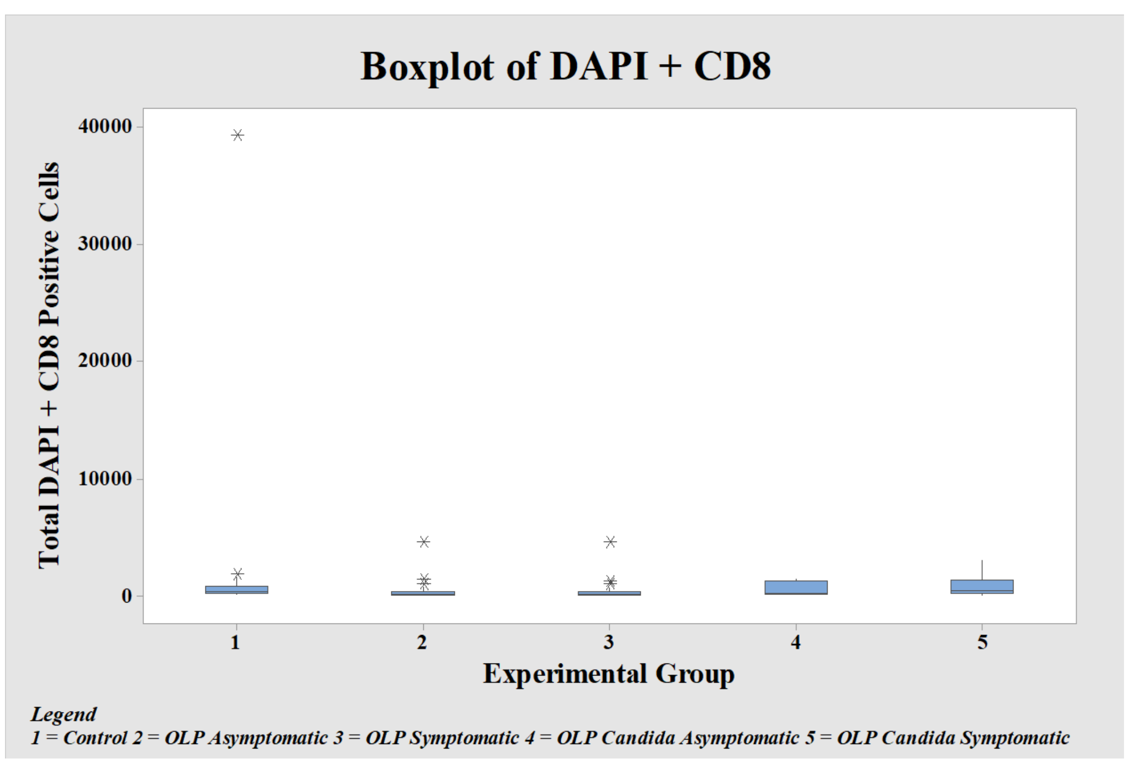

Figure 5.12: *Boxplot of total DAPI + CD8 positive cells for all groups using raw data*

5.4.2.3 DAPI + CD161

Significant differences existed between the 5 groups with regards to the number of DAPI + CD161 positive cells ($p = 0.001$). Both the OLP asymptomatic and OLP *Candida* asymptomatic groups had a significantly higher number of DAPI + CD161 positive cells compared to the OLP symptomatic group ($p = 0.003$ and $p = 0.004$ respectively) (Fig. 5.13). The effect of *Candida* and symptoms together was not significant ($p = 0.853$). The individual explanatory variables from the main effects model was significant for symptoms (degree of freedom (DF) = 1, F – value = 12.38, $p = 0.001$). The effect of symptoms was quantified by the regression coefficient and the estimate indicates an increase of 0.86 (95% CI 0.37, 1.35) in DAPI + CD161 cells in the absence of symptoms.

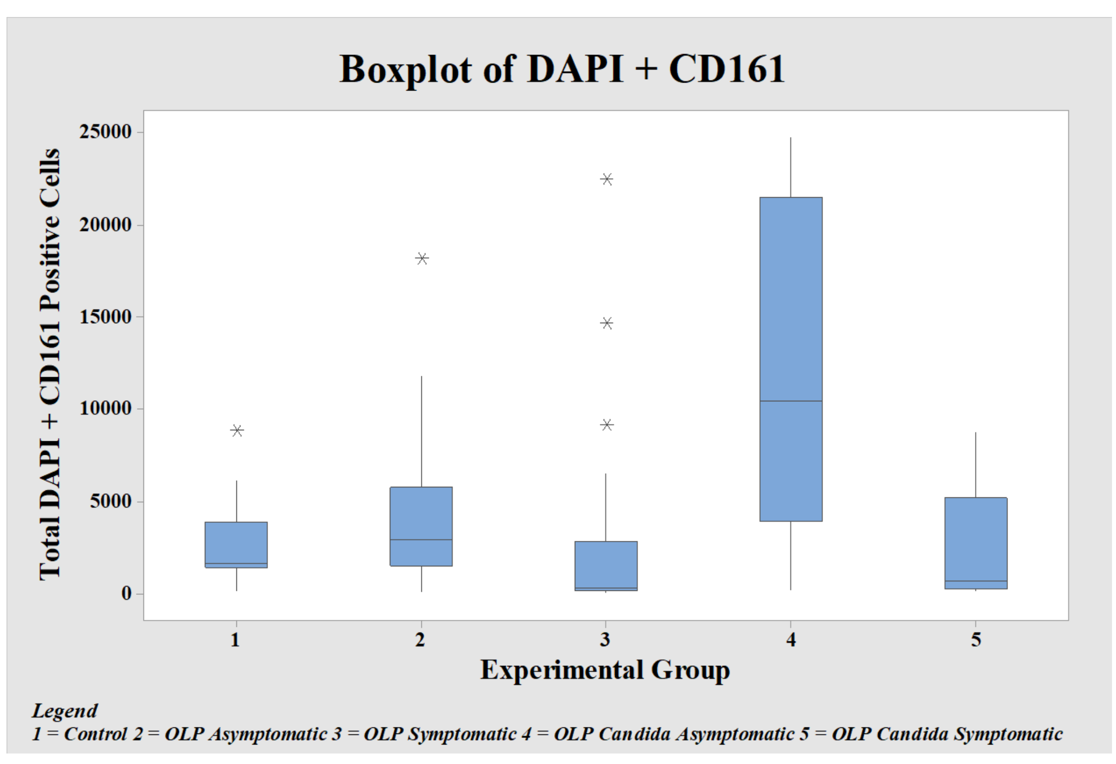

Figure 5.13: *Boxplot total DAPI + CD161 positive cells for all groups using raw data*

5.4.2.4 DAPI + IL18R1

No significant differences existed between the 5 groups with regards to the number of DAPI + IL18R1 positive cells ($p = 0.466$) (Fig. 5.14). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on DAPI + IL18R1 expression, however *Candida* presence approached significance in the main effects model ($p = 0.052$).

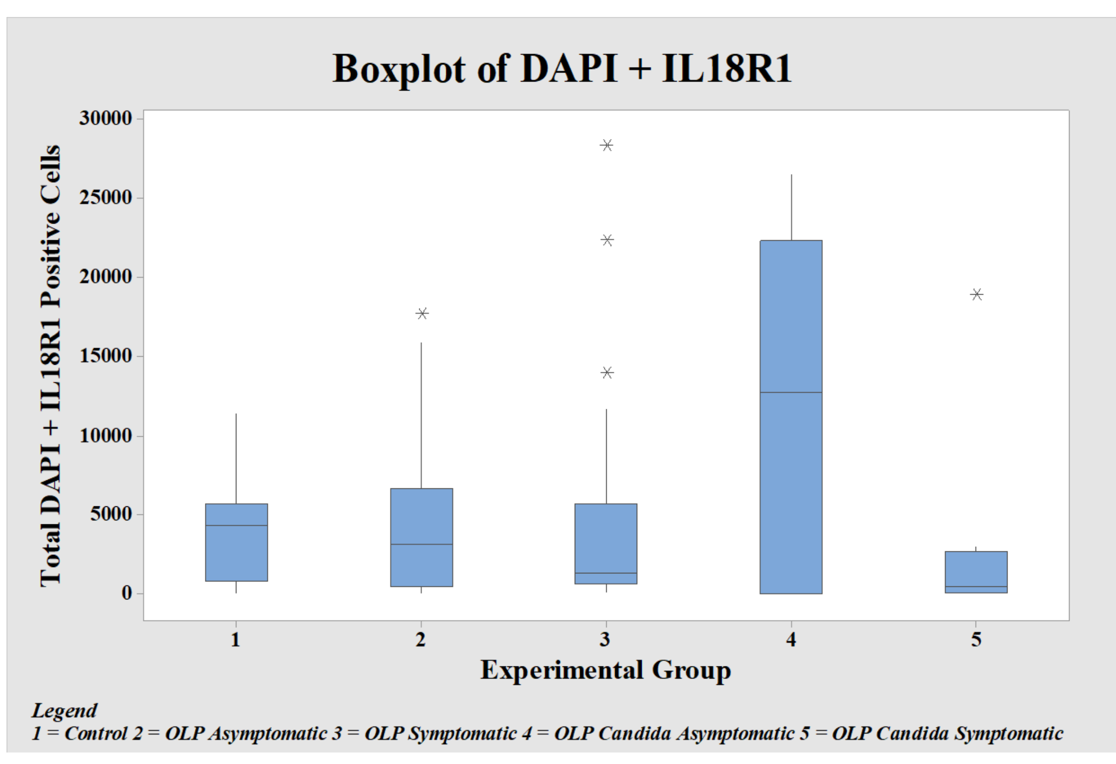

Figure 5.14: *Boxplot of total DAPI + IL18R1 positive cells for all groups using raw data*

5.4.2.5 DAPI + MR-1

No significant differences existed between the 5 groups with regards to the number of DAPI + MR-1 positive cells ($p = 0.211$) (Fig. 5.15). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the number of DAPI + MR1 positive cells.

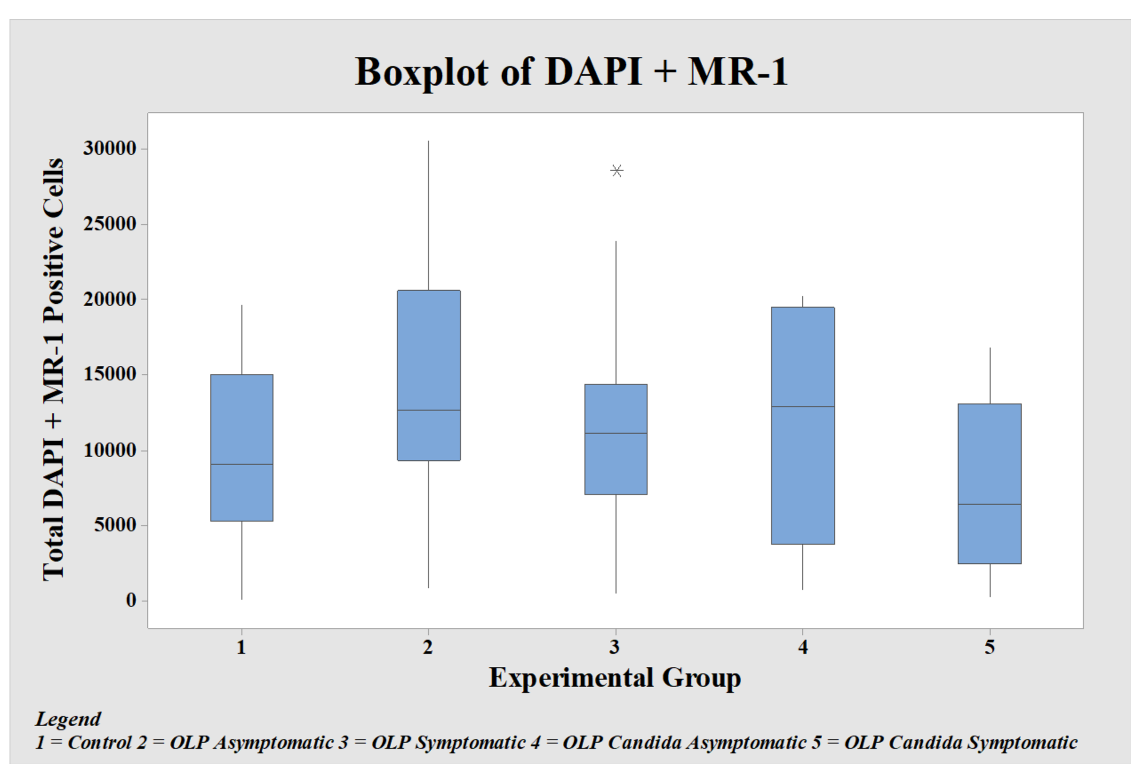

Figure 5.15: *Boxplot of total DAPI + MR-1 positive cells for all groups using raw data*

5.4.2.6 DAPI + TCRVα7.2

Significant differences existed between the 5 groups with regards to the number of DAPI + TCRVα7.2 positive cells ($p = 0.002$). Both the OLP *Candida* asymptomatic and OLP *Candida* symptomatic groups had a significantly lower number of DAPI + TCRVα7.2 positive cells compared to the OLP symptomatic group ($p = 0.006$ and $p = 0.017$ respectively) (Fig. 5.16). The effect of *Candida* and symptoms together was not significant ($p = 0.705$). A description of the individual explanatory variables from the main effects model revealed presence of *Candida* had a significant effect on the number of DAPI + TCRVα7.2 positive cells (DF = 1, F – value = 13.50, $p = 0.000$). The effect of *Candida* was quantified by the regression coefficient and the estimate indicates a decrease of 1.84 (95% CI -2.82, 0.84) DAPI + TCRVα7.2 positive cells in the presence of *Candida*.

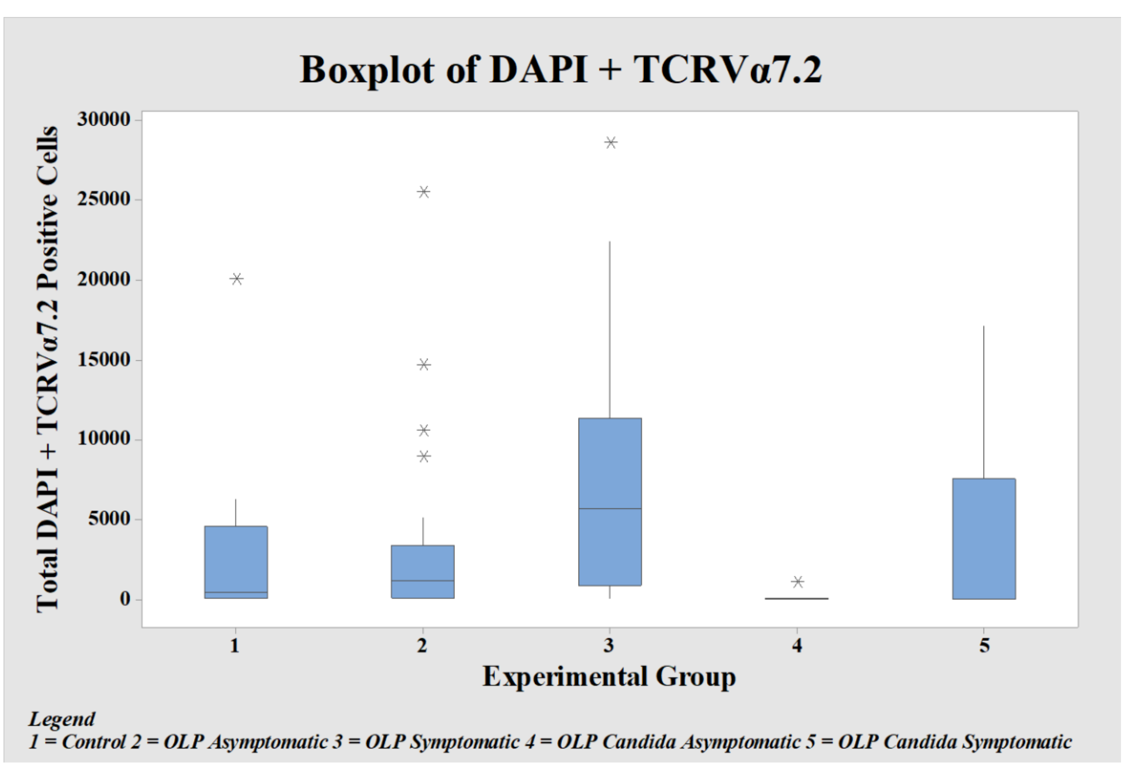

Figure 5.16: *Boxplot of total DAPI + TCRVα7.2 positive cells for all groups using raw data*

5.4.3 T Cell Phenotypes

Triple positive T-cells phenotype were determined by staining with DAPI + CD3 and the markers CD8, CD161, IL18R1 or TCRVα7.2. T cell phenotypes when present tended to congregate in the subepithelial infiltrate where the majority of CD3 cells were present (Fig. 5.17). The distribution of the means and standard deviations of the raw data, defined as percentage of total CD3 positive cells, for each phenotype with respect to the different groups, revealed considerable diversity (Table 5.3).

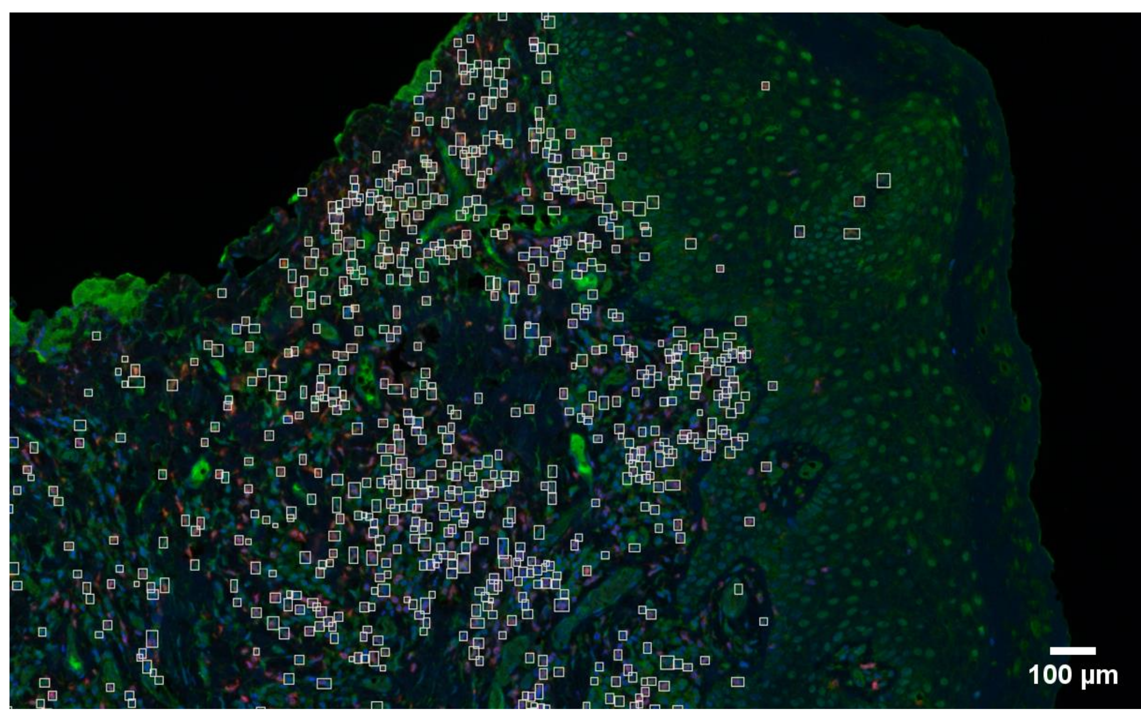

Figure 5.17: *Representative field of view image of T cell phenotyping. OLP asymptomatic sample after DAPI + CD3 + TCRVa7.2 phenotype analysis*

Legend: White boxes identify the DAPI + CD3 + Va7.2 positive cells

	DAPI + CD3 + CD8 μ ± SD (%)	**DAPI + CD3 + CD161 μ ± SD (%)**	**DAPI + CD3 + IL18R1 μ ± SD (%)**	**DAPI + CD3 + Va7.2 μ ± SD (%)**
Control	10.0 ± 21.9	17.6 ± 14.7	24.9 ± 19.8	11.8 ± 20.3
OLP Asymptomatic	1.4 ± 3.5	14.3 ± 14.8	20.7 ± 19.1	17.6 ± 26.0
OLP Symptomatic	1.1 ± 2.8	5.7 ± 11.9	17.1 ± 24.5	30.2 ± 33.6
OLP *Candida* Asymptomatic	2.9 ± 6.6	10.3 ± 15.1	21.5 ± 25.9	0.3 ± 0.6
OLP *Candida* Symptomatic	3.4 ± 5 .9	7.7 ± 8.6	12.7 ± 28.4	13.9 ± 27.9

Table 5.3: *Triple positive T cell phenotypes as a percentage of the total CD3 population for all groups using the raw data*

Legend: μ represents the mean and SD represents the standard deviation

5.4.3.1 DAPI + CD3 + CD8

Significant differences existed between the 5 groups with regards to DAPI + CD3 + CD8 positive cells as a percentage of the total number of CD3 cells ($p = 0.000$). Both the OLP asymptomatic and OLP symptomatic groups showed a significantly lower percentage of DAPI + CD3 + CD8 cells compare to the control group ($p < 0.001$ for both groups) (Fig. 5.18). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the percentage of DAPI + CD3 + CD8 positive cells. It was noted that staining for CD8 was generally weak in the OLP samples (Fig. 5.19) compared to controls leading to difficulty with detecting CD8 positive cells and colocalization with CD3.

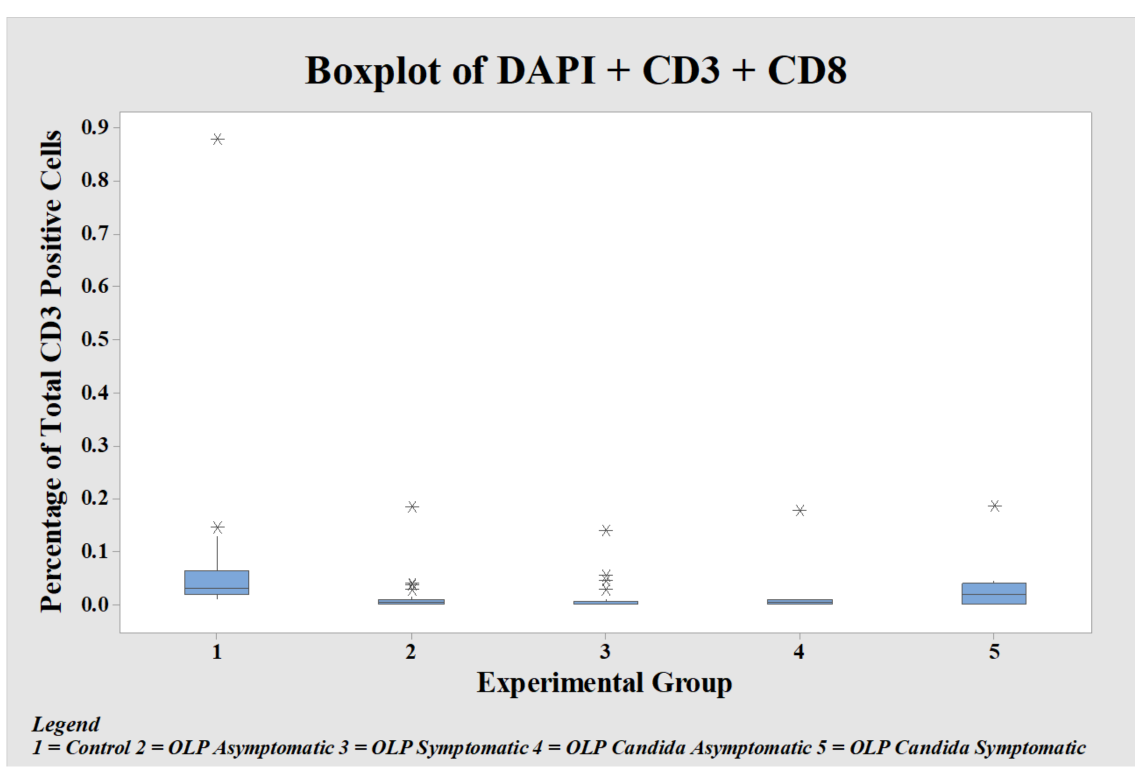

Figure 5.18: *Boxplots of DAPI + CD3 + CD8 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

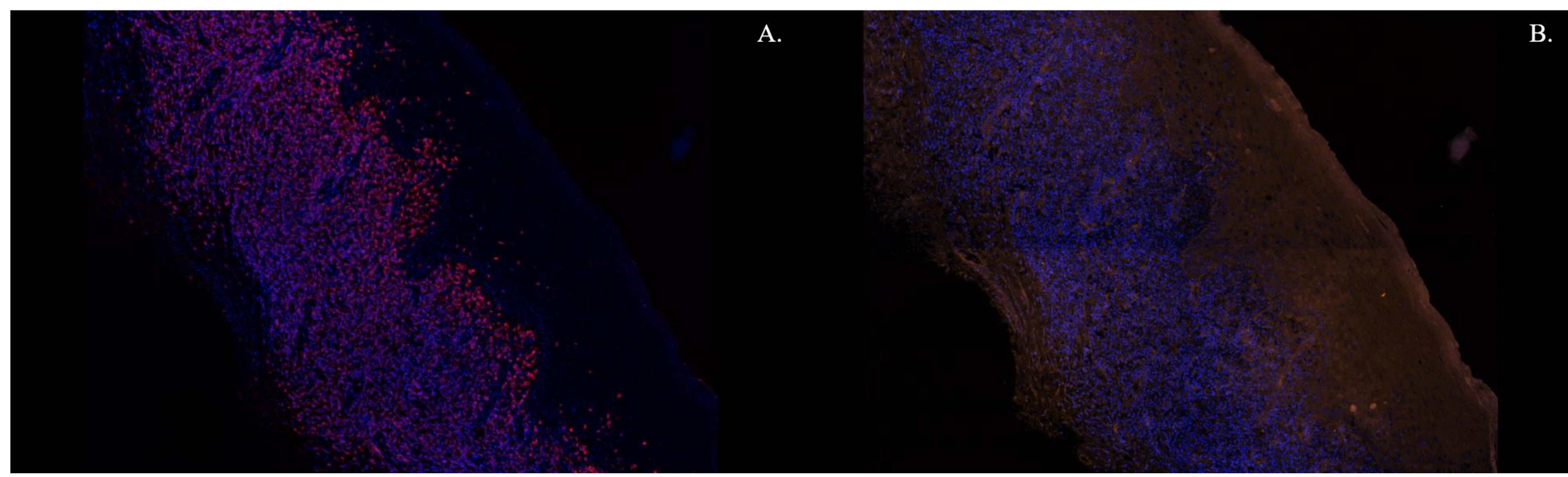

Figure 5.19: *Representative images at 200X magnification of CD3 and CD8 positive staining in OLP Candida symptomatic sample. A. representative image of positive CD3 staining cells (red) B. representative image of positive CD8 staining (orange)*

5.4.3.2 DAPI + CD3 + CD161

Significant differences existed between the 5 groups with regards to DAPI + CD3 + CD161 positive cells as a percentage of the total number of CD3 cells ($p = 0.000$). The OLP symptomatic group showed a significantly lower percentage of DAPI + CD3 + CD161 cells compared to both the control and OLP asymptomatic groups ($p < 0.001$ for both groups) (Fig. 5.20). The effect of *Candida* and symptoms together was not significant ($p = 0.061$). The individual explanatory variables from the main effects model was significant for symptoms (DF = 1, F – value = 7.61, $p = 0.021$). The effect of symptoms was quantified by the regression coefficient and the estimate indicates a decrease of 0.39 (95% CI-0.74, 0.06) in DAPI + CD3 + CD161 phenotype expression in the presence of symptoms.

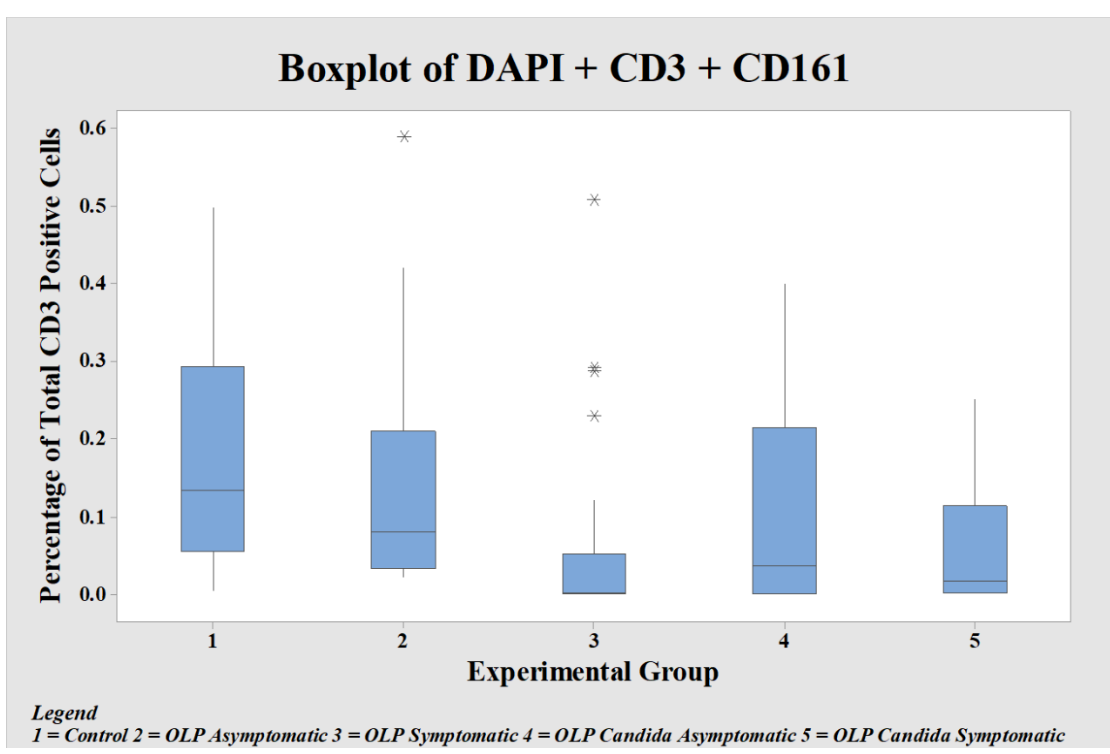

Figure 5.20: *Boxplots of total DAPI + CD3 + CD161 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.3.3 DAPI + CD3 + IL18R1

No significant differences existed between the 5 groups with regards to DAPI + CD3 + IL18R1 positive cells as a percentage of the total number of CD3 cells ($p = 0.080$) (Fig. 5.21). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the percentage of DAPI + CD3 + IL18R1 positive cells.

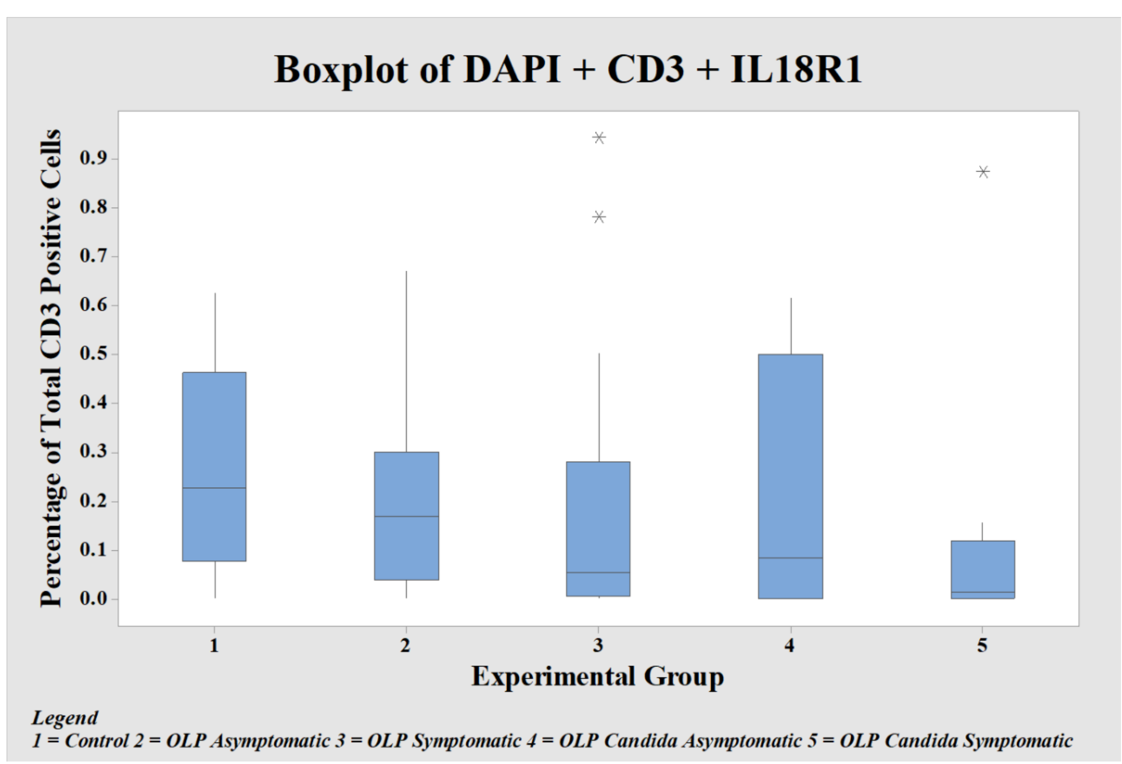

Figure 5.21: *Boxplots of total DAPI + CD3 + IL18R1 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.3.4 DAPI + CD3 + TCRVα7.2

Significant differences existed between the 5 groups with regards to DAPI + CD3 + TCRVα7.2 positive cells as a percentage of total CD3 cells ($p = 0.007$). The OLP *Candida* asymptomatic group showed a significantly lower percentage of DAPI + CD3 + TCRVα7.2 cells compared the OLP symptomatic group ($p = 0.006$) (Fig. 5.22). The effect of *Candida* and symptoms together was not significant ($p = 0.758$). The individual explanatory variables from the main effects model was significant for *Candida* (DF = 1, F – value = 11.26, $p = 0.001$). The effect of *Candida* was quantified by the regression coefficient and the estimate indicates a decrease of 0.76 (95% CI -1.22, 0.31) in DAPI + CD3 + TCRVα7.2 phenotype expression in the presence of *Candida*.

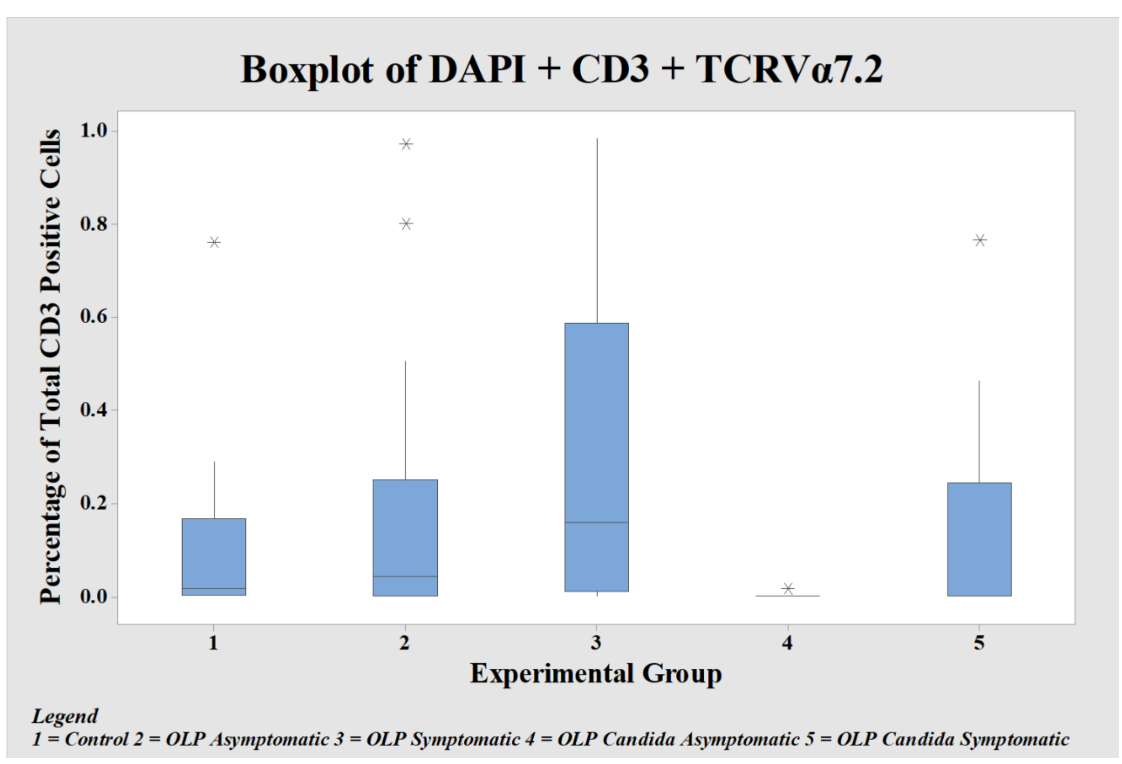

Figure 5.22: *Boxplots of total DAPI + CD3 + TCRVα7.2 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.4 Mucosal Associated Invariant T Cell Phenotypes

MAIT cell phenotypes when present tended to congregate in the subepithelial infiltrate where the majority of CD3 cells were present (Fig. 5.23). The distribution of the means and standard deviations of the raw data, defined as percentage of CD3 positive cells, for each phenotype with respect to the different groups is shown in Table 5.4.

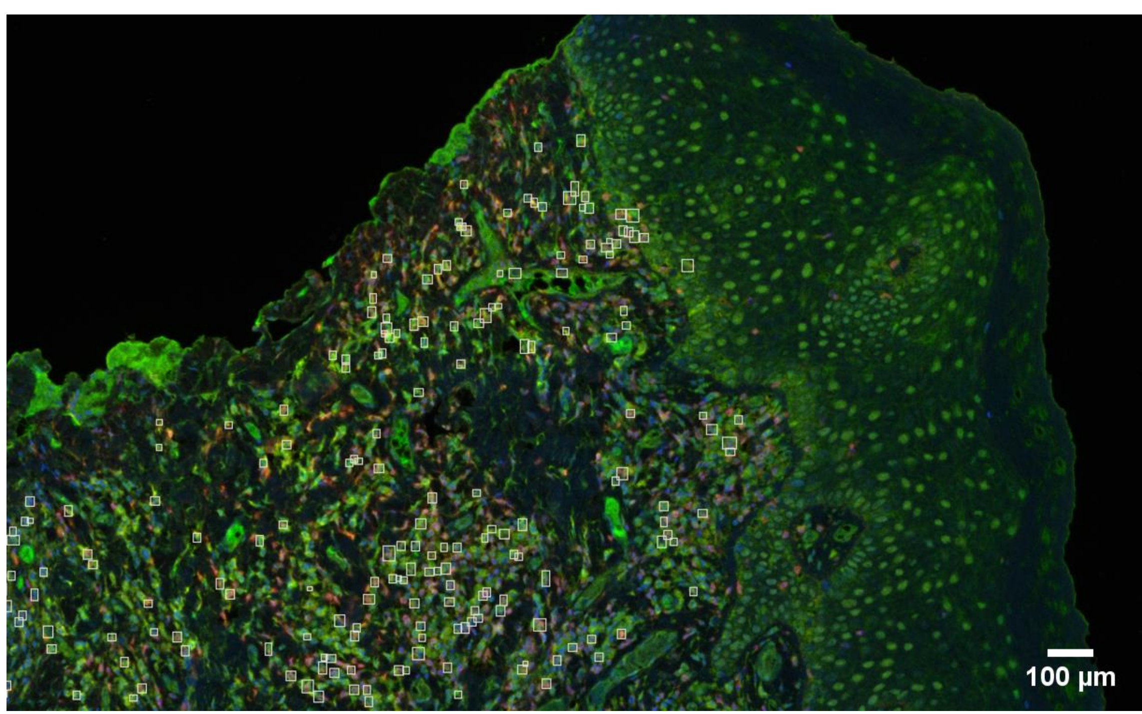

Figure 5.23: *Representative field of view image of MAIT cell phenotyping. OLP asymptomatic sample after DAPI + CD3 + IL18R1 + TCRVa7.2 phenotype analysis*

Legend: White boxes identify the DAPI + CD3 + IL18R1 + TCRVa7.2 positive cells

	DAPI + CD3 + CD161 + Va7.2 μ ± SD (%)	DAPI + CD3 + IL18R1 + Va7.2 μ ± SD (%)	DAPI + CD3 + CD161 + IL18R1 μ ± SD (%)	DAPI + CD3 + CD161 + IL18R1 + Va7.2 μ ± SD (%)
Control	2.8 ± 3.6	5.1 ± 10.8	13.7 ± 14.4	1.9 ± 2.8
OLP Asymptomatic	2.7 ± 4.9	4.1 ± 7.4	9.5 ± 11.9	2.0 ± 4.4
OLP Symptomatic	1.6 ± 3.6	7.0 ± 17.2	5.1 ± 10.6	1.5 ± 3.4
OLP *Candida* Asymptomatic	0.2 ± 0.4	0.1 ± 0.3	8.3 ± 13.6	0.1 ± 0.2
OLP *Candida* Symptomatic	3.4 ± 7.0	8.0 ± 23.9	1.5 ± 3.5	1.1 ± 3.3

Table 5 4: *MAIT phenotypes as a percentage of the total CD3 population for all groups using the raw data*

Legend: μ represents the mean and SD represents the standard deviation

5.4.4.1 DAPI + CD3 + CD161 + TCRVα7.2

No significant differences existed between the 5 groups with regards to DAPI + CD3 + CD161 + TCRVα7.2 positive cells as a percentage of the total number of CD3 cells ($p = 0.129$) (Fig. 5.24). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the percentage of DAPI + CD3 + CD161 + TCRVα7.2 positive cells.

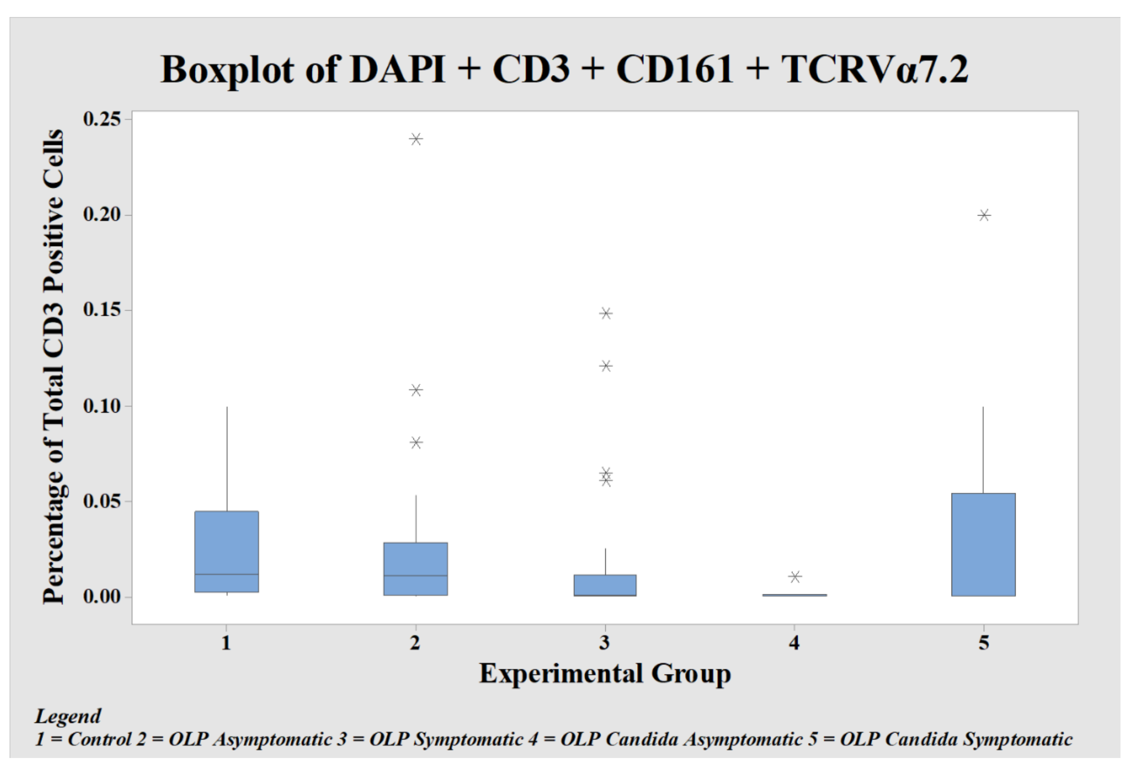

Figure 5.24: *Boxplots of total DAPI + CD3 + CD161 + TCRVα7.2 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.4.2 DAPI + CD3 + IL18R1 + TCRVα7.2

No significant differences existed between the 5 groups with regards to DAPI + CD3 + IL18R1 + TCRVα7.2 positive cells as a percentage of the total number of CD3 cells ($p = 0.245$) (Fig. 5.25). The effect of *Candida* and symptoms together was not significant ($p = 0.746$). The individual explanatory variables from the main effects model was significant for *Candida* (DF = 1, F – value = 4.70, $p = 0.034$). The effect of *Candida* was quantified by the regression coefficient and the estimate indicates a decrease of 0.36 (95% CI -0.69, 0.030) in DAPI + CD3 + IL18R1 + TCRVα7.2 phenotype expression in the presence of *Candida*.

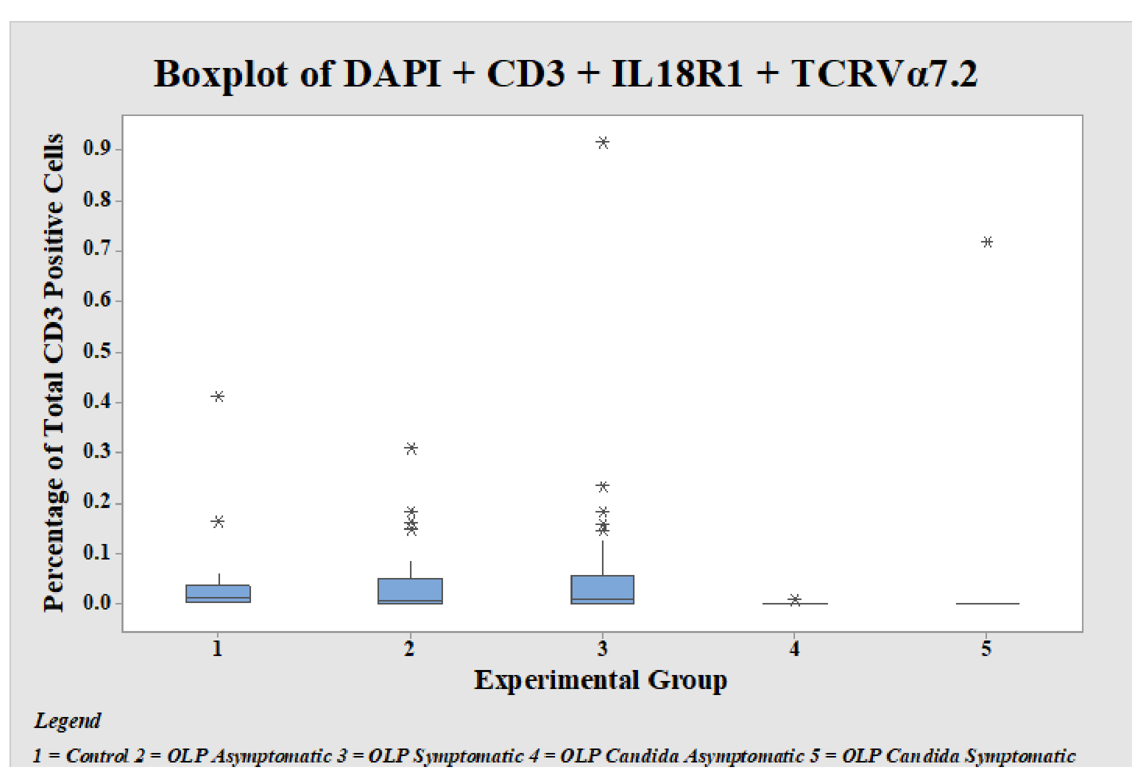

Figure 5.25: *Boxplots of total DAPI + CD3 + IL18R1 + TCRVα7.2 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.4.3 DAPI + CD3 + CD161 + IL18R1

Significant differences existed between the 5 groups with regards to DAPI + CD3 + CD161 + IL18R1 positive cells as a percentage of the total number of CD3 cells (p = 0.002). The OLP symptomatic and OLP *Candida* symptomatic groups showed a significantly lower percentage of DAPI + CD3 + CD161 + IL18R1 positive cells compared to the control group (p = 0.010 and p = 0.015 respectively). The OLP symptomatic group also showed a significantly lower percentage of DAPI + CD3 + CD161 + IL18R1 positive cells compared to the OLP asymptomatic group (p = 0.046) (Fig. 5.26). The effect of *Candida* and symptoms together was not significant (p = 0.948). The individual explanatory variables from the main effects model was significant for symptoms (DF = 1, F – value = 14.55, p = 0.012). The effect of symptoms was quantified by the regression coefficient. The estimate indicates a decrease of 0.44 (95% CI -0.78, 0.100) in DAPI + CD3 + CD161 + IL18R1 phenotype expression in the presence of symptoms.

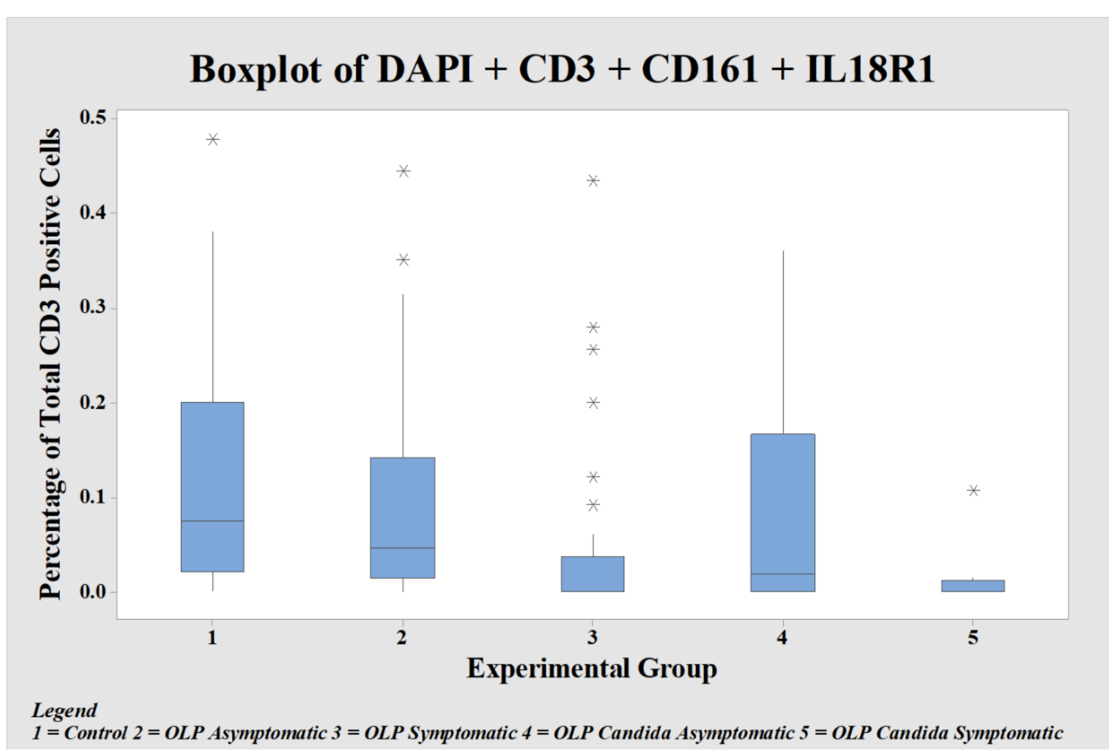

Figure 5.26: *Boxplots of total DAPI + CD3 + CD161 + IL18R1 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.4.4 DAPI + CD3 + CD161 + IL18R1 + TCRVα7.2

No significant differences existed between the 5 groups with regards to DAPI + CD3 + CD161 + IL18R1 + TCRVα7.2 positive cells as a percentage of the total number of CD3 cells ($p = 0.310$) (Fig. 5.27). The presence of *Candida,* symptoms, or *Candida* and symptoms together had no effect on the percentage of DAPI + CD3 + CD161 + IL18R1 + TCRVα7.2 positive cells.

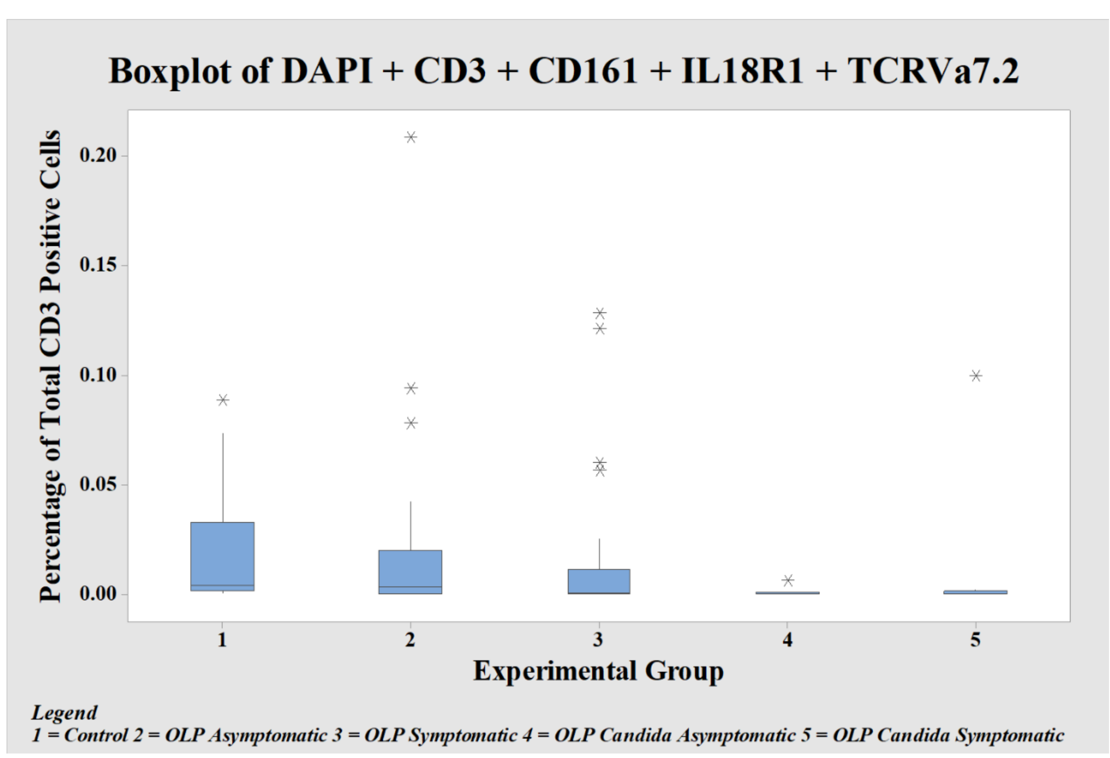

Figure 5.27: *Boxplots of total DAPI + CD3 + CD161 + IL18R1 + TCRVα7.2 positive cells as a percentage of total CD3 positive cells for all groups using raw data*

5.4.5 Overall Phenotype Expression

The presence of *Candida* had a suppressive effect on CD3 single phenotype cells that was significant in asymptomatic OLP whilst CD161 cells were significantly downregulated in symptomatic OLP when compared to asymptomatic OLP with and without concurrent *Candida*. The presence of symptoms in OLP also resulted in significant decreases in the number of T cell phenotypes expressing CD161 compared to both control and asymptomatic OLP. T cell phenotypes expressing TCRVα7.2 were significantly decreased in the presence of *Candida* in asymptomatic OLP. The MAIT cell phenotype expressing both CD161 and IL18R1 was significantly decreased in the presence of symptoms or symptoms with *Candida* compared to control. Cytotoxic T cells were shown to be significantly lower in both the OLP asymptomatic and symptomatic groups when compared to the control group.

Single cell and T cell phenotypes associated with CD161 as well as the MAIT cell phenotype expressing CD161 and IL18R1 were shown to be decreased in the presence of symptoms. Conversely, single cell and T cell phenotypes associated with TCRVα7.2 as well as the MAIT cell phenotype expressing IL18R1 and TCRVα7.2 were shown to be decreased in the presence of *Candida*.

5.5 Discussion

OLP is a T cell mediated, chronic, inflammatory condition of unknown cause and the role that the oral microbiota may play in coordinating at T cell response against the oral keratinocytes has not been fully elucidated. Studies have shown bacterial inflammatory stimulated up-regulation of CXCL9/10 and over-representation of these markers in OLP, suggesting a role for innate immunity in OLP pathogenesis with defence against oral microflora being critical to this dysregulation (Marshall et al., 2016, Marshall et al., 2017a). The role of *Candida* in OLP pathogenesis is currently unclear. The present study assessed the presence of single cell, T cell and MAIT cell phenotypes and the effect that the presence of *Candida* and symptoms had on the expression of these phenotypes. The results demonstrate that the presence of either *Candida* or symptoms in patients with OLP decreased MAIT cell numbers. Alternatively, the results are suggestive of MAIT cell expression in OLP being more likely associated with symptoms and an inherent susceptibility for candidal infection. To date, this is the first study to characterise the presence of MAIT cells in OLP, and the first to undertake this using mIHC.

Previous studies have relied on double or triple staining protocols with CD3, CD161, IL18R1 and Va7.2 to identify MAIT cells in histological tissue specimens (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016, Gibbs et al., 2017). In the present study CD3 plus TCRVa7.2, CD161 and IL18R1 were used to identify phenotype positive cells. Further, MR-1 was used to quantify MR-1 expression and CD8 for the identification of cytotoxic T cell phenotypes by co-localisation with CD3 and CD8. CD161, IL18R1 and TCRVa7.2 were all co-localised with CD3 to identify T cells expressing MAIT cell markers with the CD3 + TCRVa7.2 phenotype similar to that previously used to identify MAIT cells in tissue (Gibbs et al., 2017). To increase the likelihood of identifying MAIT cells, phenotyping was subsequently performed using DAPI, CD3 and a combination of at least two of the following markers CD161, IL18R1 or TCRVa7.2 (Dusseaux et al., 2011, Li et al., 2016).

We have also demonstrated that, when present, MAIT phenotype positive cells tended congregate within the subepithelial lymphocytic infiltrate where the majority of CD3 cells were located. A recent study confirmed the presence of MAIT cells in this tissue using in situ staining with TCRVα7.2 and IL18Rα in normal buccal mucosa (Sobkowiak et al., 2019). This study showed the MAIT cells identified in buccal mucosa were found in close proximity to the basement membrane and were situated either just above the basement

membrane in the epithelium or beneath the basement membrane in the connective tissue (Sobkowiak et al., 2019). The tendency of MAIT cells to congregate in the connective tissue in OLP rather than both above and below the basement membrane could be explained by the intense subepithelial inflammatory infiltrate present in OLP.

The presence of *Candida* has been variably reported in the literature with rates of 40-80% in OLP and 20-40% in controls (Jainkittivong et al., 2007, Masaki et al., 2011). Of the *Candida* species present in the oral cavity, *C. albicans* is the most important (McCullough et al., 1996). It has been shown that *C. albicans, C. glabrata* and *Saccharomyces cerevisiae* can induce a MAIT cell response in a manner that is MR-1 dependent (Le Bourhis et al., 2010). In the present study no significant differences were observed between any of the OLP test groups and control group with regards to expression of MR-1. The lack of variation with MR-1 expression suggests that expression of MR-1 was not a critical factor in determining variations in expression of MAIT cell phenotypes in the different groups. Furthermore, the general linear model demonstrated that expression of MR-1 in tissue was not significantly affected by the presence of either *Candida* or symptoms.

Suppression of the CD3 single phenotype cells was significant in asymptomatic OLP in the presence of *Candida*. Significant decreases in the T cell phenotype expressing CD161 and the MAIT cell phenotype expressing CD161 and IL18R1 were also demonstrated in cases of symptomatic OLP compared to control. For the MAIT cell phenotype the significant decreases were demonstrated in symptomatic OLP with and without concurrent *Candida*. In OLP the MAIT cell phenotype expressing both IL18R1 and TCRVα7.2 as well as the TCRVα7.2 single cell and T cell phenotypes were shown to be decreased in the presence of *Candida*. Conversely, the MAIT cell phenotype expressing both CD161 and IL18R1 as well as the CD161 single cell and T cell associated phenotypes were shown to be decreased in the presence of symptoms. Dysregulation of MAIT cells has been implicated in multiple conditions including dermatological conditions, IBD and SLE (Cho et al., 2014, Hiejima et al., 2015, Li et al., 2016, Chiba et al., 2017). Histologically and clinically SLE mimics OLP with previous studies having shown decreased numbers of MAIT cells that correlate with SLE disease activity (Cho et al., 2014, Chiba et al., 2017). With regards to OLP in the present study, presence of symptoms or *Candida* in OLP had a suppressive effect on CD161 and TCRVα7.2

phenotype expression which in turn would result in decreased numbers of MAIT cells expressing these markers.

The presence of cytotoxic T cell phenotypes (DAPI + CD3 + CD8) was found to be significantly lower in both the OLP asymptomatic and symptomatic groups when compared to the control group which was unexpected as this is thought to be the predominant phenotype expressed the OLP inflammatory infiltrate (Sugerman et al., 2000a). However, this may be related, at least in part, due to weak staining for CD8 throughout the present study. This is unlikely related to the length of time the specimen had been stored, as the same sections were used for each antibody, thus eliminating antibody specific variability due to samples source. Although factors such as delay in fixation, fixative used, dehydration, drying, storage humidity and temperature can all play an important role in the success of antigen retrieval (Engel and Moore, 2011, Xie et al., 2011), this variability should have occurred with all antibodies, not just CD8.

The preservation of T-lymphocyte surface membrane antigens in paraffin embedded tissues have been previously shown to vary related to delays in fixation time, exposure to temperatures above 4ºC and pH (Pollard et al., 1987). In an assessment of CD3, CD4 and CD8 stability following fixation, CD3 was previously shown to be the most stable with CD8 being the most affected by sub-optimal processing and CD4 being intermediately affected (Pollard et al., 1987). Thus, a lack of robustness in the CD8 surface membrane with regards to variations in tissue fixation could also account for decreased CD8 expression in the OLP cohort. Due to the above issues with staining no further assessment of CD8 was undertaken beyond identifying the DAPI + CD3 + CD8 phenotype. Perhaps the observed variability in the present study's OLP cohort for CD8 is an accurate depiction of reality, while previously reported high levels may have been skewed as these studies did not necessarily include patients with and without symptoms, or the presence of *Candida*. It is possible that the above could be the result of $CD4^+$ rather than $CD8^+$ cells being present in the inflammatory infiltrate. However, this cannot be inferred with the current panel and future studies should include CD3, CD8 and CD4.

The MAIT cell phenotypes DAPI + CD3 + IL18R1 + TCRVα7.2 and DAPI + CD3 + CD161 + IL18R1 and the T cell phenotype DAPI + CD3 + TCRVα7.2 are all phenotypes that could be used to identify MAIT cells. The results of the present study show that MAIT cells may play a role in the aetiopathogenesis of OLP with decreased MAIT cell numbers associated with the presence of *Candida* or symptoms.

5.6 Conclusion

MAIT cells were shown to be present within the OLP and when present congregated within the OLP inflammatory infiltrate. Expression of MR-1 had no effect on determining variations in expression of MAIT cell phenotypes within OLP or control groups and MR-1 expression was not significantly affected by the presence of either *Candida* or symptoms. Significant differences existed between the different treatment groups and the expression of MAIT cell phenotypes in tissue. The presence of *Candida* or symptoms in tissue was generally associated with a decrease in expression of phenotype positive cells with decreased expression of CD161 noted in the presence of symptoms and decreased expression of TCRVα7.2 noted in the presence of *Candida*. *Candida* and symptoms together were significantly associated with DAPI + CD3 expression. Taken together these results demonstrate that patients with poorer MAIT cell expression are more likely to be symptomatic with an inherent susceptibility for candidal infection.

6 The Effect of Effluent on Peripheral Blood Mononuclear Cells

6.1 Abstract

Mucosal associated invariant (MAIT) cells can be activated by microbial derived riboflavin intermediates, from either bacteria or *Candida*, bound by MR-1. However, it has also been shown that the MAIT T cell receptor (TCR) repertoire for *C. albicans* is less diverse than the repertoire for other bacteria. MAIT cells exhibit high expression of receptors for IL-18, IL-12 and IL-23. In response to a microbial challenge, MAIT cells release the cytokines INF-γ, TNF, IL-17 and IL-22. Overexpression of IL-23, IL-22, IL-17 and IL-18 has been shown in OLP.

The aim of this study was to determine if the percentage of MAIT cells and the production of inflammatory cytokines changes when peripheral blood mononuclear cells (PBMC) are exposed to effluent derived from *C. albicans* biofilms.

Optimisation of the experimental conditions was conducted to determine the optimal ratio for culture. PBMC media supplemented with either 10% v/v effluent derived from *C. albicans* biofilms, or 10% v/v artificial salivary media (ASM) as the control, was chosen for all experiments. A BioPlex assay was performed to measure the cytokines IFN-γ, TNF-α, IL-17A, IL-18, IL-12p40, IL-12p70, IL-22 and IL-23. Supernatant used for this experiment was collected at 8, 12 and 24 hours after incubating PBMC in PBMC media supplemented with either 10% v/v effluent derived from *C. albicans* biofilms, or 10% v/v ASM. In addition to the test or control media, some positive control wells were supplemented with either CD28 and/or PMA/Ionomycin. Flow cytometry was performed using TCRVα7.2, CD3, CD161, CD218a, CD4, CD8 and CD45 to define MAIT cell populations and T-cell subsets within this population. Prior to performing flow cytometry, PBMC were incubated for 6 hours in PBMC media supplemented with either 10% v/v effluent derived from *C. albicans* biofilms, or 10% v/v artificial salivary media (ASM), with or without CD28.

Presence of PMA/Ionomycin and *Candida* effluent were significant factors that increased the concentration of IFN-γ, TNF-α, IL-18, IL-22, IL-23 and IL-17A. For IL-18 and TNF-α incubation in the presence of *Candida* effluent and CD28 together resulted in upregulation of cytokine expression. MAIT cells identified using flow cytometry were

not significantly affected by the presence of effluent derived from *C. albicans* biofilms or CD28.

Taken together these results indicate effluent derived from *C. albicans* biofilms does not induce MAIT cell proliferation but does have the ability to induce production of MAIT cell associated cytokines.

6.2 Introduction

MAIT cells can be activated by microbial riboflavin derived intermediates produced by bacteria and *Candida* and bound by MR-1 (Tilloy et al., 1999, Cowley, 2014). Le Bourhis et al., 2010 infected MR-1 sufficient and deficient bone marrow-derived dendritic cells *Saccharomyces cerevisiae* , *C. albicans* and *C. glabrata* showing that these fungi were able induce an MR-1 dependent MAIT cell response (Le Bourhis et al., 2010). Further to this an *in vitro* model showed that MAIT cells were able to detect and discriminate between a diverse array of MR-1 restricted ligands whilst also providing a basis for an adaptive immune response (Gold et al., 2014). However, it has also been shown that the MAIT TCR repertoire for *C. albicans* is less diverse than the repertoires for *Mycobacterium smegmatis* and *Salmonella typhimurium* (Gold et al., 2014).

In response to a microbial challenge, MAIT cells release the cytokines INF-γ, IL-17, TNF and IL-22 (Le Bourhis et al., 2010, Dusseaux et al., 2011, Gibbs et al., 2017), however they do so in a tissue-specific manner. For example, female genital tract derived MAIT cells have been shown to exhibit a bias towards expression of IL-22 and IL-17 when stimulated with *E. coli* with preferential expression of these cytokines potentially playing an important role in maintenance of mucosal barrier integrity and mucosal homeostasis (Gibbs et al., 2017).

Overexpression of IL-22 and IL23 has been shown in both OLP and cutaneous lichen planus (Chen et al., 2013). Higher expression of subepithelial IL-22 as well as higher expression of epithelial and subepithelial IL-23 could be suggestive of a Th-22 response against pathogenic microbes (Chen et al., 2013). Shen et al., 2016 undertook a preliminary study to compare IL-22 expression in oral biopsies in OLP and normal mucosa. An assessment of micro ribonucleic acid (miRNA) 562 and miRNA-203 was also undertaken as these miRNA's were identified through bioinformatic software as being potential target miRNA's of IL-22 (Shen et al., 2016). High levels of IL-22 expression were noted along with aberrant expression of miR-562 and miR-203, with the authors concluding there may be a potential role for IL-22 and the associated miRNAs of IL-22 in the pathogenesis of OLP (Shen et al., 2016).

MAIT cells exhibit high expression of receptors for IL-18, IL-12 and IL-23 (Dusseaux et al., 2011, Jeffery et al., 2016). Following exposure to microbial by-products, IL-12 and IL-18 can work together to initiate a cell mediated immune response (Manigold et al., 2000). With respect to OLP, the rs568408 single nucleotide polymorphism variation in

the IL-12A gene has been shown to occur significantly more often in OLP patients, especially those with the erosive form of OLP, when compared to both healthy controls and non-erosive forms of OLP (Jiang et al., 2015). IL-18 has also been explored in OLP with IL-18 expression shown to be significantly higher in OLP compared to controls, with erosive forms of OLP exhibiting significantly higher expression of IL-18 compared to non-erosive OLP (Zhang et al., 2012). Over-production of IL-18 may play a pathogenic role in OLP expression of IL-18 potentially linked to a MAIT cell response to oral microbes.

The IL-17/IL-23 axis has been studied in OLP and is thought to be involved in both chronic inflammatory and immune mediated disorders (Lu et al., 2014). Lu et al., 2014 assessed the IL-17/IL-23 axis in healthy controls and OLP, both erosive and reticular forms. Overexpression of IL-17 and IL-23 was noted in OLP lesions when compared to controls suggesting a potential regulatory role for the IL-17/IL-23 axis in OLP (Lu et al., 2014). Wang et al., 2015 also assessed the role the Th17 subset of cells in OLP, both reticular and erosive forms, and healthy controls using IL-17 and IL-23. The role of microbial infection in OLP has also been assessed by using saliva to profile microbial communities in both the control and test patients (Wang et al., 2015). Both reticular and erosive OLP patients exhibited microbial populations that were significantly less rich and diverse when compared to controls (Wang et al., 2015). Furthermore, in the same patients the concentration of IL-17 was significantly higher in the erosive OLP when compared to both the healthy controls and the reticular OLP (Wang et al., 2015). Overall, salivary microbial richness and diversity was significantly diminished in the OLP populations, which could be suggestive of a role for the microbiota in the pathogenesis of OLP (Wang et al., 2015). Overexpression of salivary IL-17 in OLP may also be suggestive of an inflammatory response to microbial pathogens in OLP (Wang et al., 2015), alluding to a role in pathogenesis.

Using IHC, Monteiro et al., 2015 assessed expression of IL-17 and IL-23 in both reticular and erosive forms of OLP and inflammatory fibrous hyperplasia (IFH) (Monteiro et al., 2015). Results showed no significant differences in the number if IL-17^{+} and IL-23^{+} lymphocytes between OLP and IFH samples; however, a significantly higher number of IL-23^{+} lymphocytes were noted in cases of erosive OLP compared both IFH and reticular OLP (Monteiro et al., 2015). The authors concluded that the above results supported a

role for $CD4^+$ Th17 cells and autoimmunity in the pathogenesis of OLP (Monteiro et al., 2015).

The aim of the present study was to determine if the production of inflammatory cytokines and the percentage of MAIT cells change when PBMC are exposed to effluent derived from *C. albicans* biofilms.

6.3 Materials and Methods

As described in 2.3 a cell culture study was conducted using commercially available human mononuclear cells derived from PBMC. Briefly, cells were sourced from two different companies, 10 vials of 1 x10^7 PBMC (Sigma-Aldrich, USA) all from the same donor and lot and 3 vials of 5 x 10^7 PBMC (Lonza, Switzerland) all from the same donor and lot. The PBMC sourced from Sigma-Aldrich (Sigma-Aldrich, USA) were used for the optimisation experiments and the PBMC from Lonza (Lonza, Switzerland) were used for the final experiments.

6.3.1 Optimisation of Test Media

Optimisation of the culture medium for the PBMC was performed as described in 2.3.1. Briefly, PBMC media was made from RPMI 1640 media containing L-glutamine (Sigma-Aldrich, USA) and was supplemented with 10% heat inactivated foetal bovine serum (Sigma-Aldrich, USA) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, USA). All complete media were vacuum filtered with a 0.22 µm filter before use. PBMC were cultured in PBMC media supplemented with either effluent derived from *C. albicans* biofilm supported in 25% ASM or 25% non-effluent ASM (Arzmi et al., 2018); both effluents were 0.22 µm vacuum filtered prior to use. Determination of the optimal concentration of effluent in PBMC media was assessed using the assay described below.

6.3.1.1 Quantification of Viable Cells Using MTS Assay

The CellTitre 96 ® AQueous One Solution Cell Proliferation Assay (Promega, USA) was used to measure proliferation and viability. This assay was performed as per the manufacturer's instructions and as described in 2.3.1.2. Briefly, PBMC's (Sigma-Aldrich, USA) were resuspended PBMC media to a concentration of 1 x 10^6 vital cells mL^{-1}. 50 µL, equivalent to 5 x 10^4 vital cells, were seeded into 3 different 96 well plates (Corning, USA) with 50 µL of pre-dispensed 2X concentration test media in triplicate wells. Final concentrations of media tested were 10%, 20%, 50% and 0% (100% PBMC media control) ASM or *C. albicans* effluent in PBMC media. Media only controls were also run in triplicate on the same 96 well plate to serve as absorbance controls.

The plates were incubated in a humidified incubator at 37ºC and 5% CO_2 for 2, 6 and 24 hours prior to addition of 20 µL of CellTitre 96 ® AQueous One Solution (Promega, USA). As per the manufacturer's recommendation, plates were incubated for 4 hours at 37ºC and 5% CO_2 prior to measuring absorbance at 490 nm using a 96-well plate reader

(Victor3, 1420 multilabel counter, PerkinElmer, USA). Final absorbance was calculated by subtracting the absorbance readings of the media only controls from the absorbance of the media/cell combinations. The number of live cells in culture is directly proportion to the quantity of formazan product produced by the MTS assay as measured by absorbance at 490 nm.

6.3.1.2 Preparation of Cell Media

Based on the results of the above optimisation experiment, 10% *C. albicans* effluent or ASM (v/v) in PBMC media was selected as the cell media for all cell culture experiments as it exhibited the greatest preservation of PBMC vitality.

Media was prepared at 2X concentration to allow for dilution to a final concentration of 10% *C. albicans* effluent (v/v) or 10% ASM (v/v) with the addition of cell suspension. Prior to use in experiments the prepared media were mixed thoroughly and warmed in a water bath at 37ºC.

6.3.2 Preparation of Cell Suspension and Collection of Supernatant

Collection of supernatants was performed as described in 2.3.2. Briefly, PBMC (Lonza, Switzerland) were suspended in PBMC media to a concentration of 8×10^5 vital cells mL^{-1}. 500 µL of cell suspension, equivalent to 4×10^5 vital cells, were seeded into 24-well plates (Corning, USA) pre-prepared with 500 µL of 2X test media. The cells were seeded in triplicate and incubated in ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v *Candida* effluent), CEPMA (10% v/v *Candida* effluent with PMA/Ionomycin), CE28 (10% v/v *Candida* effluent with CD28), CE28PMA (10% v/v *Candida* effluent with CD28 and PMA/Ionomycin).

Addition of 1.25µg/mL CD28 to some test media, as demonstrated in other MAIT cell culture studies (Dias et al., 2017a, Dias et al., 2017b, Gibbs et al., 2017), was carried out as CD28 is a marker expressed on T- cells that provides a co-stimulatory signal for T-cell activation. PMA/Ionomycin was used as a positive control for cytokine expression, as reported in other studies (Dias et al., 2017b, Gibbs et al., 2017). The plates were incubated at 37ºC and 5% CO_2 for 8, 12 and 24 hours with PMA/Ionomycin (Thermo Fischer Scientific, USA) added in the last 6 hours. The timepoints of 8, 12 and 24 hours were chosen based on the optimisation experiments in 6.3.1. Twenty-four hours was chosen as the last timepoint to show late changes in cytokine production. 8 hours was chosen as the

first timepoint rather than 6 or 2 hours to allow for incubation with the test media prior to addition of PMA/Ionomycin in some wells with 12 hours as the middle timepoint. The cell suspension was collected in a 1.5 mL sterile Eppendorf tube (Eppendorf, Germany) and centrifuged at 800g at 20ºC for 5 minutes to pellet the cells. The supernatant was then collected and stored in a sterile Eppendorf tube (Eppendorf, Germany) at -80ºC.

6.3.3 BioPlex Assay

A custom BioPlex assay was performed as described in 2.3.3 according to the manufacturer's instructions. Briefly, IL-17A (IL-17), IL-22, IL-23, TNF-α, IFN-γ, IL-12p40, IL-12p70 and IL-18 concentrations was measured using two custom BioPlex kits (Bio-Rad Laboratories, USA). The above cytokines were chosen because IL-17, IL-22, TNF-α and IFN-γ are produced by activated MAIT cells with IL-18 and IL-12 shown to be involved in MAIT cell activation (Manigold et al., 2000, Martin et al., 2009, Dusseaux et al., 2011). IL-22, IL-23, IL-17 and IL-18 have also been shown to be upregulated in OLP with IL-12A gene polymorphisms noted in OLP (Chen et al., 2013, Wang et al., 2013, Lu and Zeng, 2014, Piccinni et al., 2014, Jiang et al., 2015, Wang et al., 2015, Shen et al., 2016). Both subunits of IL-12 were measured as IL-12p40 is a subunit of IL-23, whilst IL-12p70 is the active subunit of IL-12.

Stored supernatant collected as described in 2.3.3 was defrosted from -80ºC and kept on ice. Fifty microlitre of beads were added to the wells of the 96 well plate prior to washing and addition of standards, samples and controls. Following 30-minute incubation and washing, the detection antibody was added. Following another 30-minute incubation, streptavidin PE was added to the wells and incubated for 10 minutes followed by washing. The samples were then resuspended in 125 µL wash buffer and after 30 seconds data was acquired on a BioPlex System (Bio-Rad Laboratories, USA). Fold changes were calculated by dividing the concentration of cytokine produced by cells in effluent by the concentration of cytokine produced by cells incubated in ASM (Arzmi et al., 2018).

6.3.4 Flow Cytometric Analysis

Flow cytometry was performed as described in 2.3.4. Briefly, PBMC were suspended in PBMC media to a concentration of 2 x 10^6 vital cells mL^{-1} and 1 mL of cell suspension seeded per well into a 12 well plate (Corning, USA) pre-prepared with 1 mL of test media at 2X concentration. The cells were seeded in triplicate with final the concentrations of control and test media including ASM (10% v/v ASM), ASM28 (10% v/v ASM with

CD28), CE (10% v/v *Candida* effluent) and CE28 (10% v/v *Candida* effluent with CD28), prior to incubation at 37ºC with 5% CO_2 for 6 hours. Six hours was chosen based on the optimisation experiments in 6.3.1, as this timepoint represented the longest incubation period still preserving the viability of cultured cells

Following incubation, the cell suspension was collected in a sterile 2 mL Eppendorf tube (Eppendorf, Germany) and centrifuged at 800g at 20ºC for 5 minutes prior to removing the supernatant. The cells were then resuspended in fluorescent activated cell sorting (FACS) wash buffer and diluted to 1 x 10^6 cells per 100 µL FACS wash. 2 µL of Fc block (BD Biosciences, USA) was added to the cells for 10 minutes on ice prior to addition of titred antibodies as described in Table 6.1. The samples were incubated at 2-8ºC for 30 minutes and wrapped in foil prior to the addition of 1.8 mL of FACS was buffer. The cells were centrifuged at 800g at 20ºC for 5 minutes and the supernatant discarded to leave 100 µL of cell pellet. Cell wash and centrifugation steps were performed twice. 5 µL of 7-aminoactinomycin D (7-AAD) (BD Biosciences, USA) was added to all samples and the 7-AAD control and prior to incubating for 10 minutes on ice according to the manufacturer's instructions. 200 µL of FACS wash was then added to each tube prior to analysis.

Anti-Mouse Ig κ/Negative Control beads (BD Biosciences, USA) were used to run single colour fluorescence compensation controls. PBMC subjected to negative control conditions, specifically, incubation for 6 hours in PBMC media with 10% v/v ASM, were used for the 7-AAD control and unstained PBMC control. Acquisition was performed using the LSR Fortessa X-20 (BD Biosciences, USA).

Antibody	Fluorophore	Volume
TCRVα7.2 (Bio-Legend, USA)	BV421	5 µL
CD161 (BD Biosciences, USA)	APC	20 µL
CD218a (IL18Rα) (BD Biosciences, USA)	PE	5 µL
CD4 (BD Biosciences, USA)	APC-Cy7	2 µL
CD45 (BD Biosciences, USA)	FITC	4 µL
CD8 (BD Biosciences, USA)	BV500	5 µL
CD3 (BD Biosciences, USA)	PE-Cy7	4 µL

Table 6.1: *Volumes of antibody used for cell staining prior to flow cytometric analysis*

6.3.5 Statistical Analysis

To test for differences between the effluent test groups in the MTS and BioPlex assays and between the control and test media at the relevant timepoints, ANOVA one-way with Tukey's post hoc testing was used. This same test was used to assess differences between control and test media over time. Fold changes were calculated and reported in chapter 6 for cytokines where greater than 50% of the concentrations were within the range of the standard curve (IL-18 only) with all other fold changes reported in Appendix II. Changes of 0.5-fold decrease or 2-fold increase were used as cut-off standards to evaluate cytokine production. For the Bioplex assay and flow cytometry experiment independent T-tests were used to assess the means of the test media, control media and between the matched test and control media groups. Independent T-tests were also used to assess for differences between the CD161 and CD218a MAIT and non-MAIT cell populations.

To determine whether the data was normally distributed or needed to be transformed, an assessment of the normality of the residuals and residual vs. fits was undertaken. Due to multiple outliers in IFN-γ a natural log transformation plus 1 was undertaken to permit approximation of a normal distribution for analysis of this cytokine. No other transformations were required with all the raw data reported in Appendix II. For all analyses, significance was defined as a p-value less than or equal to 0.05. Analyses were performed using with Minitab® 18 and Microsoft® Excel® for Office 365 and GraphPad Prism 8.3.0.

6.4 Results

6.4.1 Quantification of Viable Cells

PBMC showed significant changes in growth over 2, 6 and 24 hours (Tables 6.2 and 6.3). Over time PBMC in the 10%, 20% and 50% ASM groups showed significantly higher cell growth at 2 and 6 hours when compared to 24 hours (Fig. 6.1 A.). This trend was repeated in the 10%, 20% and 50% effluent groups (Fig. 6.1 B.). No such significant increase in cell growth was observed in the 0% media group.

Significantly higher viability in the 0% media as well as the 10% and 20% ASM groups was observed when compared to the 50% ASM group (Fig 6.2 A.) Significantly higher viability was noted in the 0% media when compared to the 10%, 20% and 50% effluent groups at 24 hours (Fig. 6.2 B.). At 24 hours significantly higher viability was observed in the 0% media when compared to the 20% and 50% ASM groups (Fig. 6.2 B.). All other comparisons between groups were not significant. At 2 hours, significantly lower viability was noted in the 0% media and 50% effluent groups was observed when compared to 10% effluent (Fig. 6.2 A.). Significantly lower viability was also observed in the 0% media as well as the 20% and 50% ASM groups when compared to the 10% ASM group (Fig. 6.2 A.). At 6 hours, 10% effluent showed significantly higher viability than both the 20% and 50% effluent groups at 6 hours. Significantly lower viability was noted in the 20% effluent when compared to the 50% effluent group at 6 hours (Fig. 6.2 A.). Taken together, the data show that the general trend was significantly decreased viability by 24 hours

Independent T-test analyses showed significantly higher viability at 6 hours in the 10% and 20% effluent groups when compared to the 10% and 20% ASM groups respectively (Fig. 6.4 B.) No other significant differences existed between the effluent and the matched ASM groups a 2, 6 and 24 hours (Fig. 6.3 A. and B.)

	2 hours (Absorbance 490 nm) μ ± SD	**6 hours (Absorbance 490 nm) μ ± SD**	**24 hours (Absorbance 490 nm) μ ± SD**	**Change in viable cells over time ANOVA p-value**
0% Media	0.16 ± 0.01	0.20 ± 0.02	0.15 ± 0.03	0.085
10% ASM	0.26 ± 0.03	0.21 ± 0.00	0.10 ± 0.01	0.000*
20% ASM	0.19 ± 0.01	0.20 ± 0.00	0.01 ± 0.01	0.005*
50% ASM	0.12 ± 0.03	0.12 ± 0.01	0.00 ± 0.00	0.000*
Change between groups ANOVA p-values	0.001*	0.001*	0.008*	

Table 6.2: *PBMC viability as measured by absorbance at 490 nm over time at differing concentrations of ASM (0%, 10%, 20% and 50%) determined with one-way ANOVA*

*Legend: * significance as determined by one-way ANOVA with p - value less than or equal to 0.05*

	2 hours (Absorbance 490 nm) μ ± SD	6 hours (Absorbance 490 nm) μ ± SD	24 hours (Absorbance 490 nm) μ ± SD	Change in viable cells over time ANOVA p-value
0% Media	0.16 ± 0.01	0.20 ± 0.02	0.15 ± 0.03	0.085
10% Effluent	0.30 ± 0.06	0.24 ± 0.01	0.06 ± 0.05	0.002*
20% Effluent	0.19 ± 0.05	0.19 ± 0.03	0.02 ± 0.03	0.003*
50% Effluent	0.17 ± 0.03	0.11 ± 0.01	0.01 ± 0.01	0.000*
Change between groups ANOVA p-values	0.022*	0.000*	0.004*	

Table 6.3: *PBMC viability as measured by absorbance at 490 nm over time at differing concentrations of Candida effluent (0%, 10%, 20% and 50%) determined with one-way ANOVA*

*Legend: * significance as determined by one-way ANOVA with p - value less than or equal to 0.05*

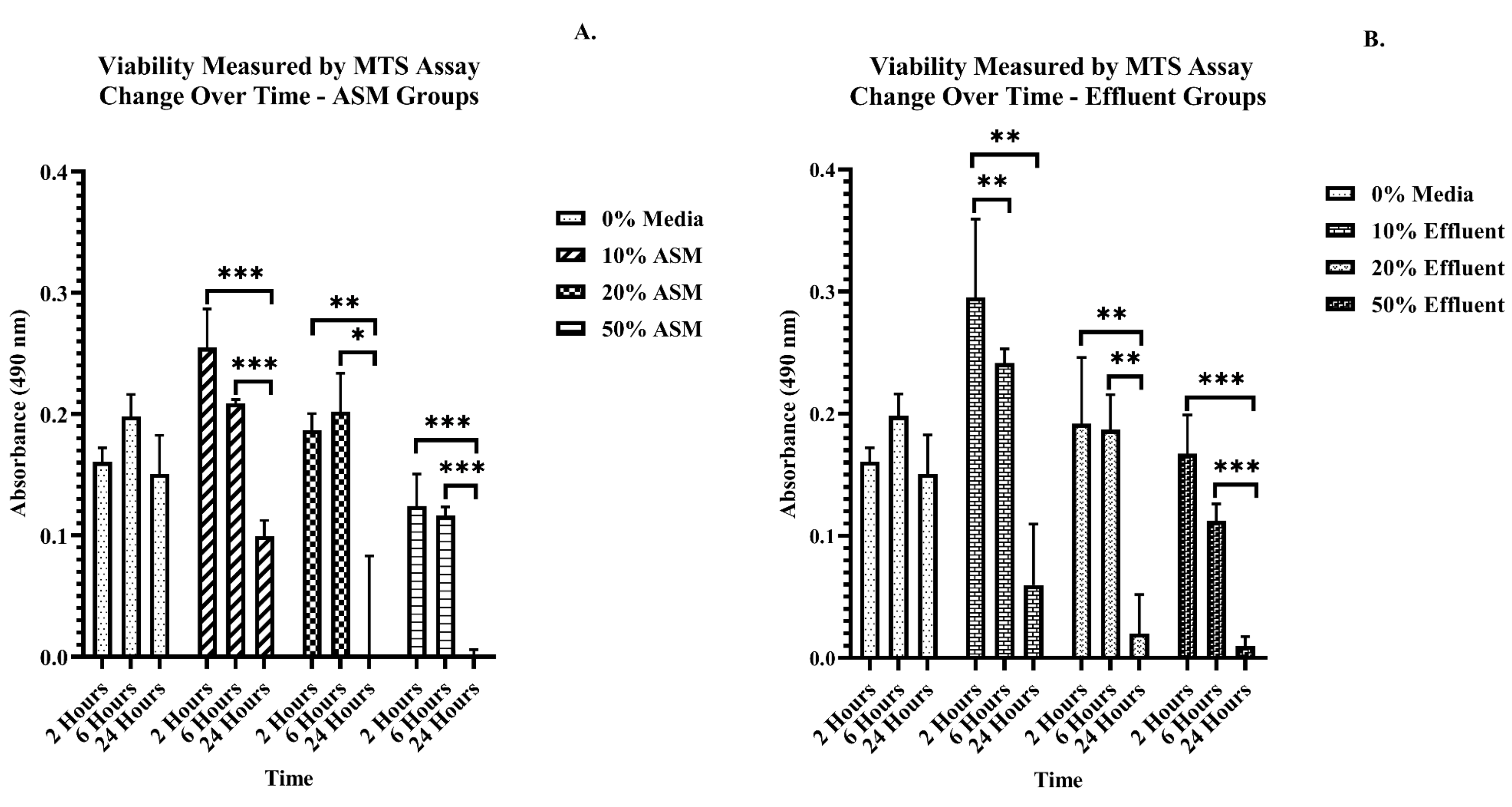

Figure 6.1: *PBMC change in viability over time per group measured by MTS assay. A. ASM media groups, B. Effluent media groups*

*Legend: * significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

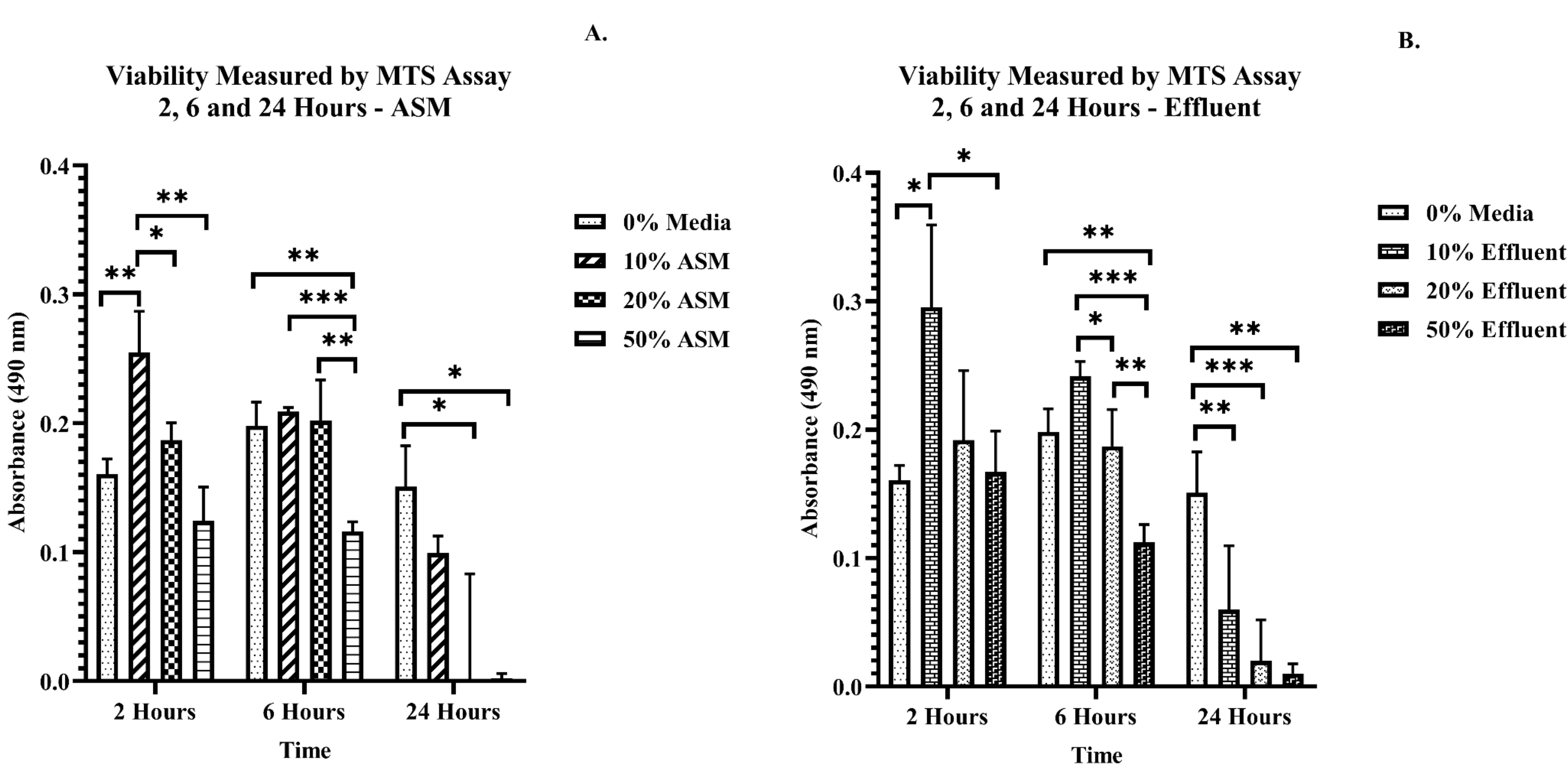

Figure 6.2: *PBMC viability at 2, 6 and 24 hours measured by MTS assay. A. ASM treatment groups, B. Effluent treatment groups*

*Legend: * significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

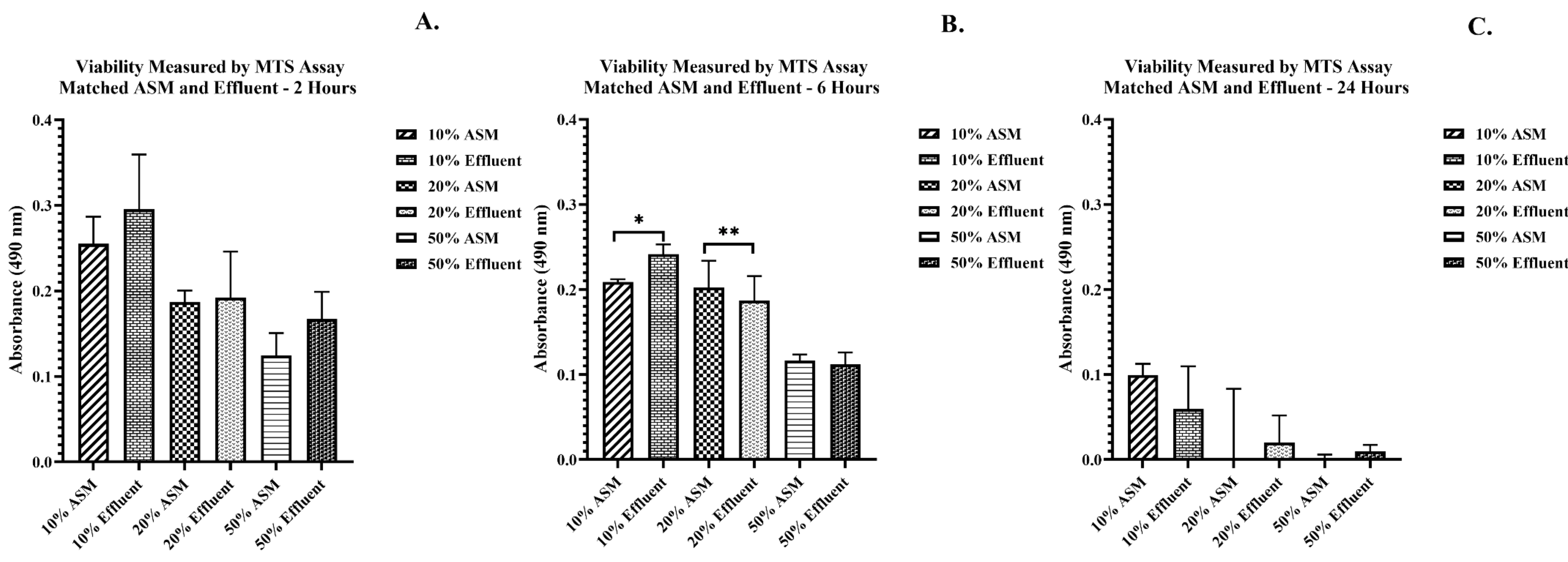

Figure 6.3: *PBMC viability in matched ASM and effluent groups measured by MTS assay. A. 2 hours, B. 6 hours, C. 24 hours*

*Legend: * significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2 BioPlex Assay

PBMC cell media was supplemented with either 10% (v/v) effluent derived from *C. albicans* biofilm (CE) or 10% (v/v) ASM (ASM). In addition, CD28 was added to separate *C. albicans* effluent (CE28) and ASM (ASM28) wells to provide a costimulatory signal for T-cell activation. PMA/Ionomycin was further added to separate *C. albicans* effluent wells with and without CD28 (CEPMA and CE28PMA), as well as ASM with and without CD28 (ASMPMA and ASM28PMA) wells to act as positive controls for cytokine expression.

The BioPlex assays used in this experiment were custom kits and the upper and lower limits of detection for each cytokine was taken as the highest and lowest observed concentration detected. Where possible the concentration in range has been reported; for the concentration to be deemed in range at least two of the three triplicate results must be within the range of the standard curve. If the concentration fell outside the standard curve the observed concentration extrapolated from the standard curve has been reported. For the extrapolated values those still out of range, those lower than the lower limit of detection have been reported as 0 and values above the upper limit of detection (one value for TNF-α in the CE28PMA 24-hour group) have been reported as the highest reported observed concentration for the cytokine.

6.4.2.1 *Interferon Gamma*

The concentrations reported for IFN-γ ranged from 5.1 pg/mL to 2,455.6 pg/mL. IFN-γ was not detected in the ASM, ASM28 and CE groups at any timepoint (Table 6.4).

IFN-γ concentration was significantly lower in the ASMPMA group at 8 hours when compared to both 12 and 24 hours (Fig 6.4 A.). The CEPMA group had significantly lower concentration of IFN-γ at 12 hours when compared to both 8 and 24 hours (Fig. 6.4 B.). The concentration of IFN-γ was also shown to be significantly lower at 8 hours when compared to 24 hours (Fig. 6.4 B.).

At 12 and 24 hours significantly higher expression of IFN-γ was noted in the ASMPMA and ASM28PMA groups when compared both the ASM and ASM28 groups (Fig. 6.5 A.). At both timepoints the ASMPMA group was shown to express significantly higher concentrations of IFN-γ when compared to the ASM28PMA group. (Fig. 6.5 A.). At 8 hours, 12 and 24 hours the IFN-γ concentration was significantly lower in the CE and CE28 groups when compared to the CEPMA and CEPMA28 groups (Fig. 6.5 B.).

IFN-γ was differentially expressed in the ASMPMA and CEPMA groups with the ASMPMA group showing significantly lower IFN-γ concentration when compared to the CEPMA group at 8 hours (Fig 6.6 A.). Differences between the ASMPMA and CEPMA groups approached significance with ASMPMA showing a tendency for increased IFN-γ expression (Fig. 6.6 B.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-values
ASM	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	N/A
ASMPMA	16.6 ± 20.0 [a]	121.0 ± 59.9 [a]	264.6 ± 152.6 [a]	0.007*
ASM28	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	N/A
ASM28PMA	21.7 ± 19.2 [a]	88.8 ± 90.0 [a]	186.1 ± 156.1 [a]	0.173
Change between groups ANOVA p-values	0.044*	0.000*	0.000*	
CE	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	N/A
CEPMA	278.5 ± 63.0 [a]	35.1 ± 13.2 [a]	841.5 ± 162.8 [a]	0.000*
CE28	12.3 ± 10.9 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.079
CE28PMA	181.3 ± 84.5 [a]	125.9 ± 129.3 [a]	974.0 ± 1284 [a]	0.197
Change between groups ANOVA p-values	0.000*	0.000*	0.000*	

Table 6.4: *IFN-γ concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA on log transformed data*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

*N/A depicts one-way ANOVA was unable to be performed as IFN-γ was not detected, * significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

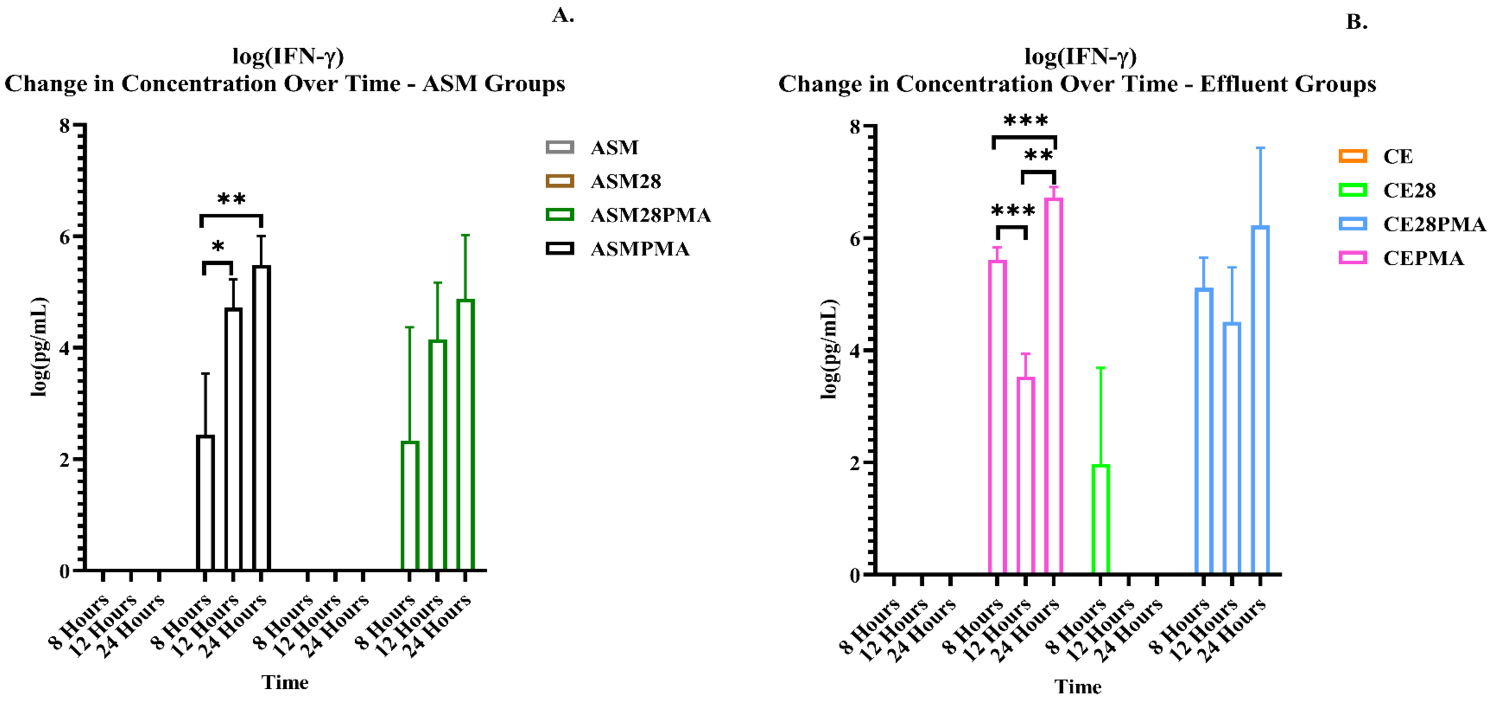

Figure 6.4: *log(IFN-γ) concentration (log(pg/mL)) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

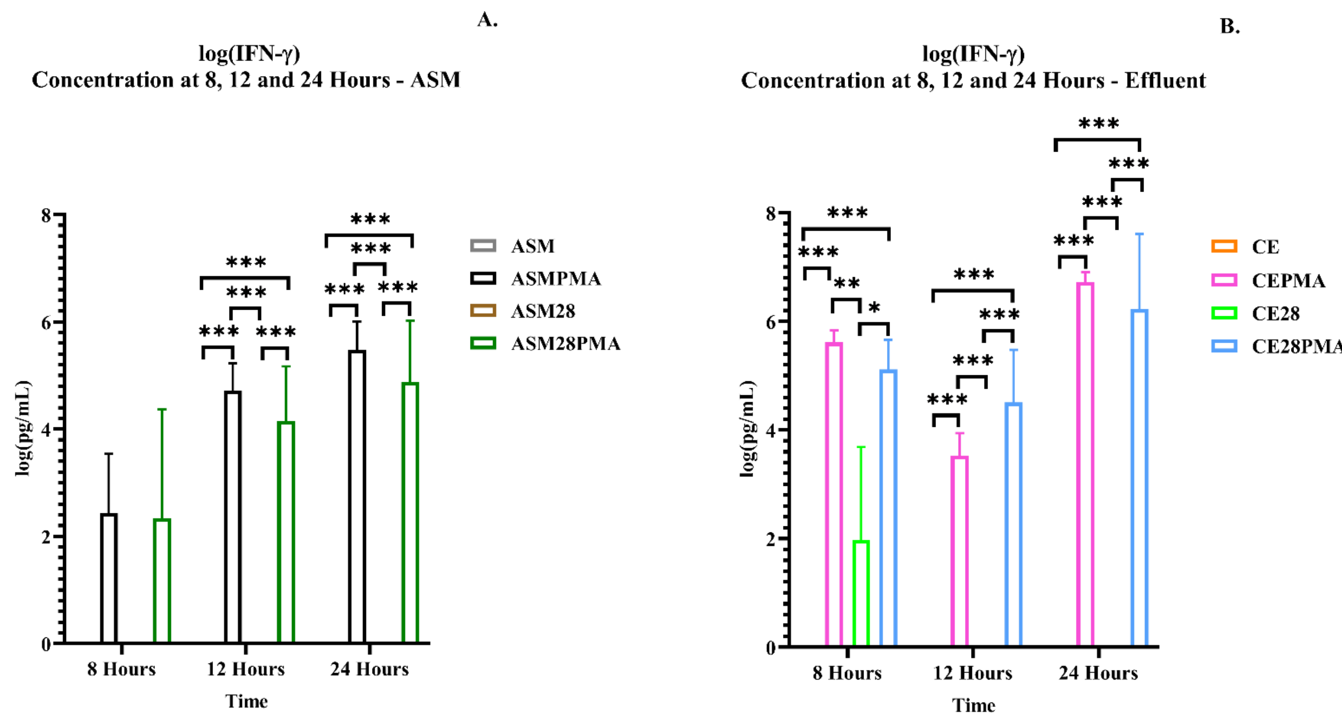

Figure 6.5: *log(IFN-γ) concentration (log(pg/mL)) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

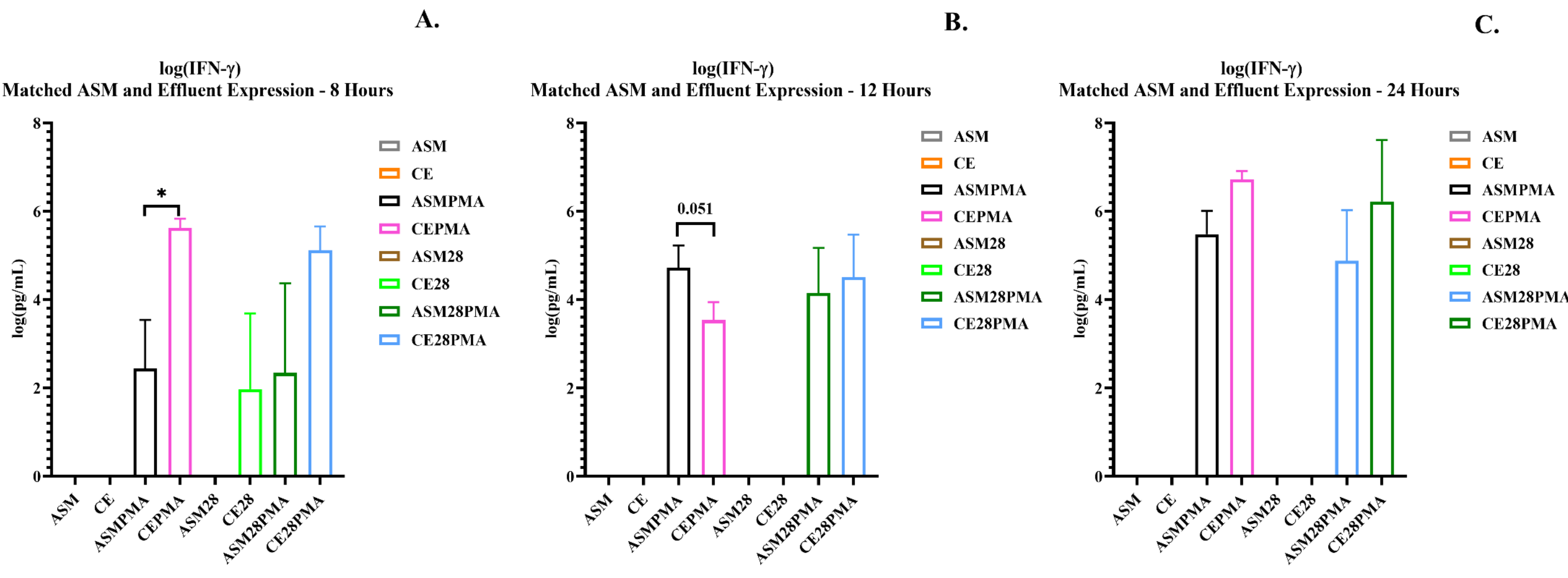

Figure 6.6: *log(IFN-γ) concentration (log(pg/mL)) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.2 Tumour Necrosis Factor Alpha

The concentrations reported for TNF-α ranged from 22,897.3 pg/mL to 211,483.3 pg/mL (Table 6.5).

The concentration of TNF-α was significantly higher at both 12 and 24 hours when compared to 8 hours in the ASM, ASM28, ASM28PMA and ASMPMA groups (Fig. 6.7 A.). The concentration of TNF-α was also noted to be significantly higher at 12 hours when compared to 24 hours in the CEPMA group (Fig. 6.7 A.). Concentration of TNF-α was significantly higher in the CE28 group at 12 hours when compared to both 8 and 24 hours (Fig. 6.7 B.) TNF-α concentration was also shown to be significantly higher at 24 hours when compared 12 hours in the CEPMA group (Fig. 6.7 B.).

Concentration of TNF-α was significantly higher in ASMPMA group at 12 hours when compared to both the ASM and ASM28 groups (Fig. 6.8 A.). At 24 hours, the concentration of TNF-α was shown to be significantly higher in the ASM28PMA group when compared to all other group (Fig. 6.8 A.). At 8 hours it was shown that the both the CE28PMA and CEPMA groups exhibited significantly higher concentrations of TNF-α when compared to the CE group (Fig 6.8 B.). At 24 hours it was noted that the CEPMA exhibited higher TNF-α concentrations when compared to the CE, CE28 and CE28PMA groups (Fig. 6.8 B.).

The concentration of TNF-α was significantly higher at 8 hours in the CE28PMA group when compared to the ASM28PMA group (Fig. 6.9 A.). Concentration of TNF-α was significantly higher in the CE28 group when compared to the ASM28 group at 8, 12 and 24 hours (Fig. 6.9 A., B. and C.). Concentration of TNF-α was significantly higher in the CEPMA group when compared to the ASMPMA group at 8 and 24 hours (Fig. 6.9 A. and C.). The concentration of TNF-α was significantly higher at 24 hours in the CE group when compared to the ASM group (Fig. 6.9 A.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-values
ASM	58,330 ± 4,606 [a]	100,843 ± 4,712 [a]	82,025 ± 7,162 [a]	0.000*
ASMPMA	41,176 ± 11,042	161,065 ± 13,956 [a]	112,698 ± 10,729 [a]	0.000*
ASM28	53,268 ± 12,399 [a]	100,522 ± 5,690 [a]	92,398 ± 4,351 [a]	0.001*
ASM28PMA	27,084 ± 24,763	131,701 ± 27,835 [a]	159,217 ± 30,865 [a]	0.003*
Change between groups ANOVA p-values	0.128	0.005*	0.002*	
CE	81,603 ± 16,558 [a]	88,153 ± 31,520 [a]	108,967 ± 13,959 [a]	0.350
CEPMA	125,266 ± 10,398 [a]	89,508 ± 58919 [a]	203,226 ± 9,485 [a]	0.019*
CE28	110,955 ± 6,473 [a]	136,287 ± 7,073 [a]	114,345 ± 3,473 [a]	0.004*
CE28PMA	115,177 ± 10,885 [a]	153,971 ± 19,377 [a]	140,910 ± 36,485 [a]	0.227
Change between groups ANOVA p-values	0.009*	0.115	0.002*	

Table 6.5: *TNF-α concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration above or below the limit of detection with observed concentration reported*

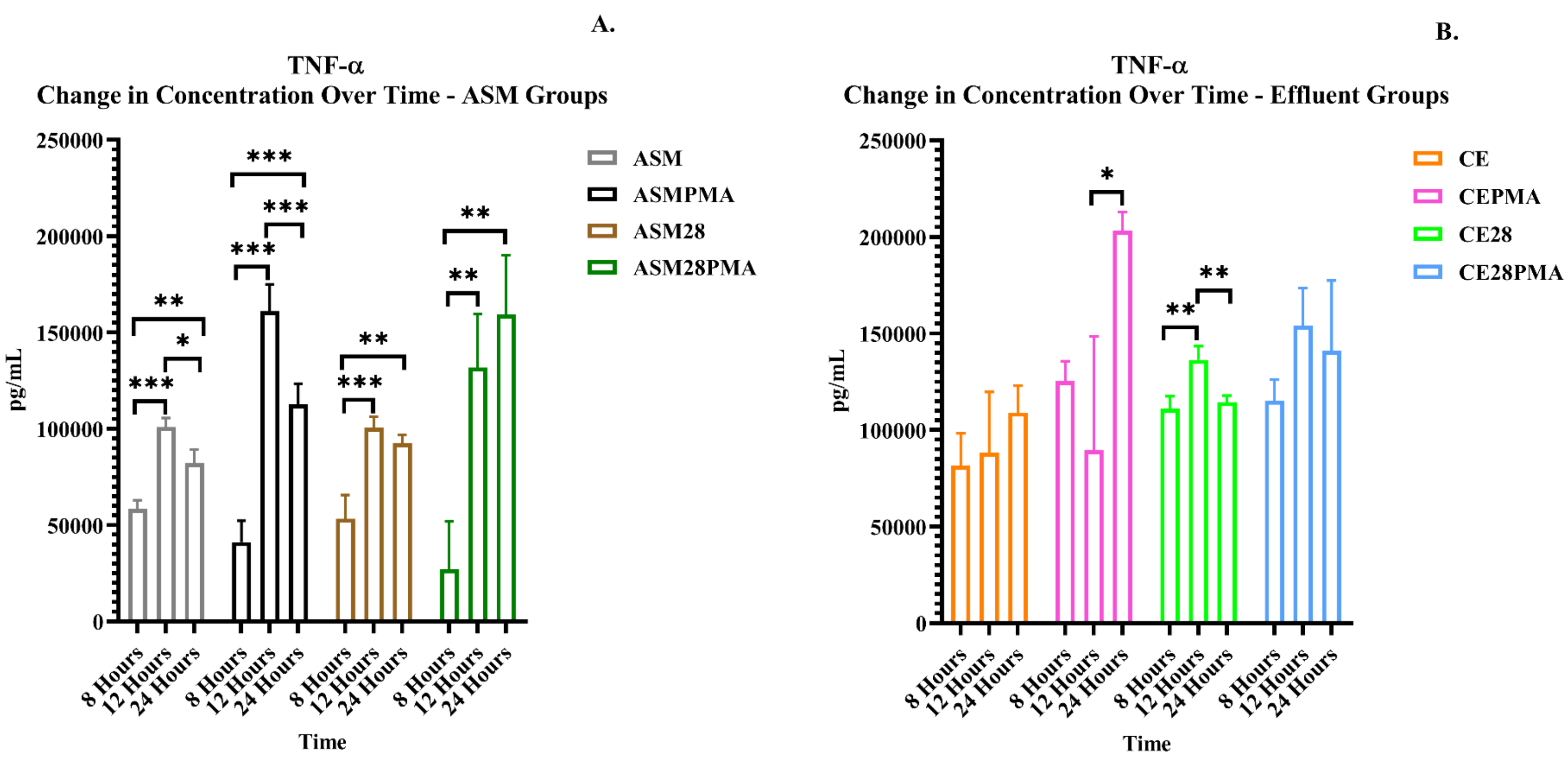

Figure 6.7: *TNF-α concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

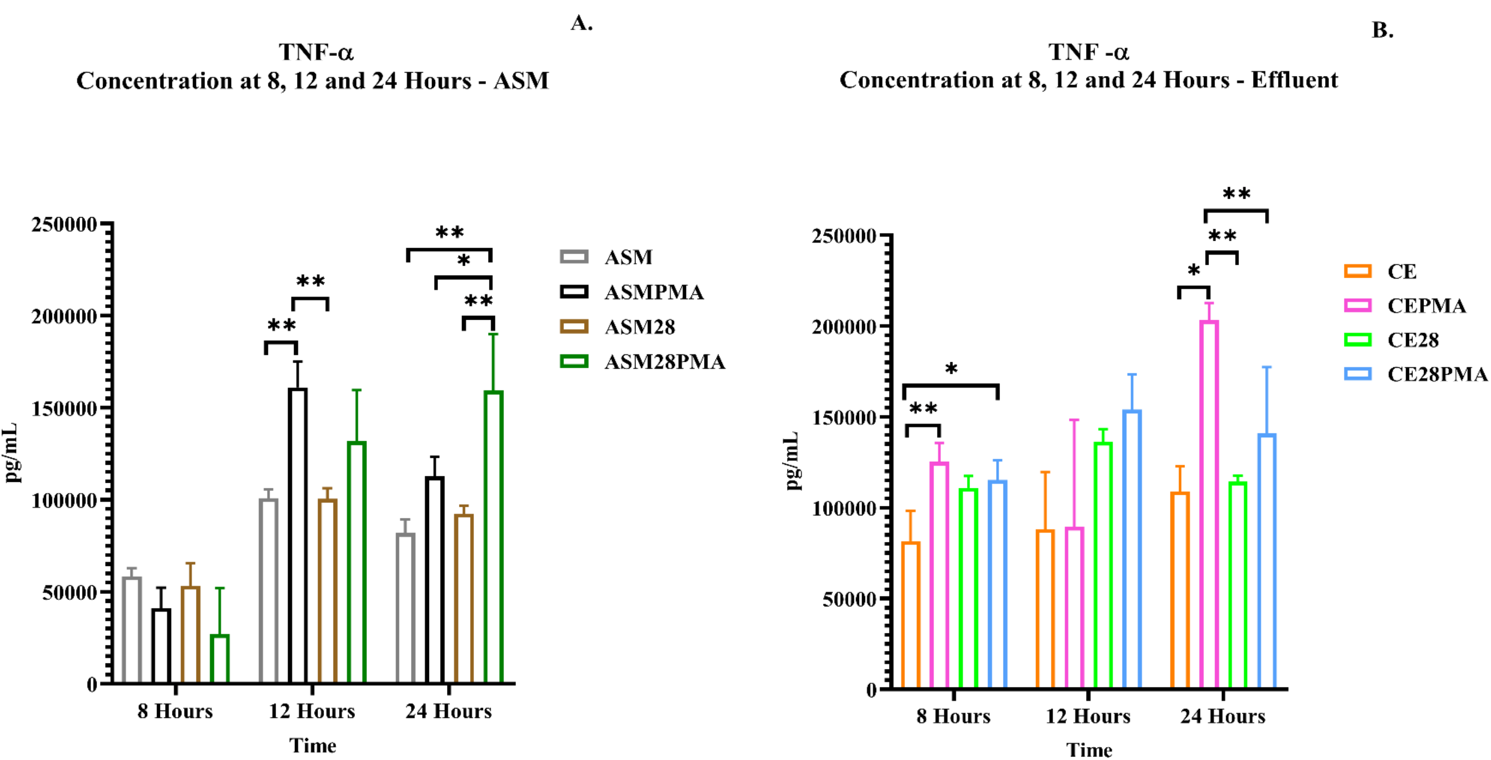

Figure 6.8: *TNF-α concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

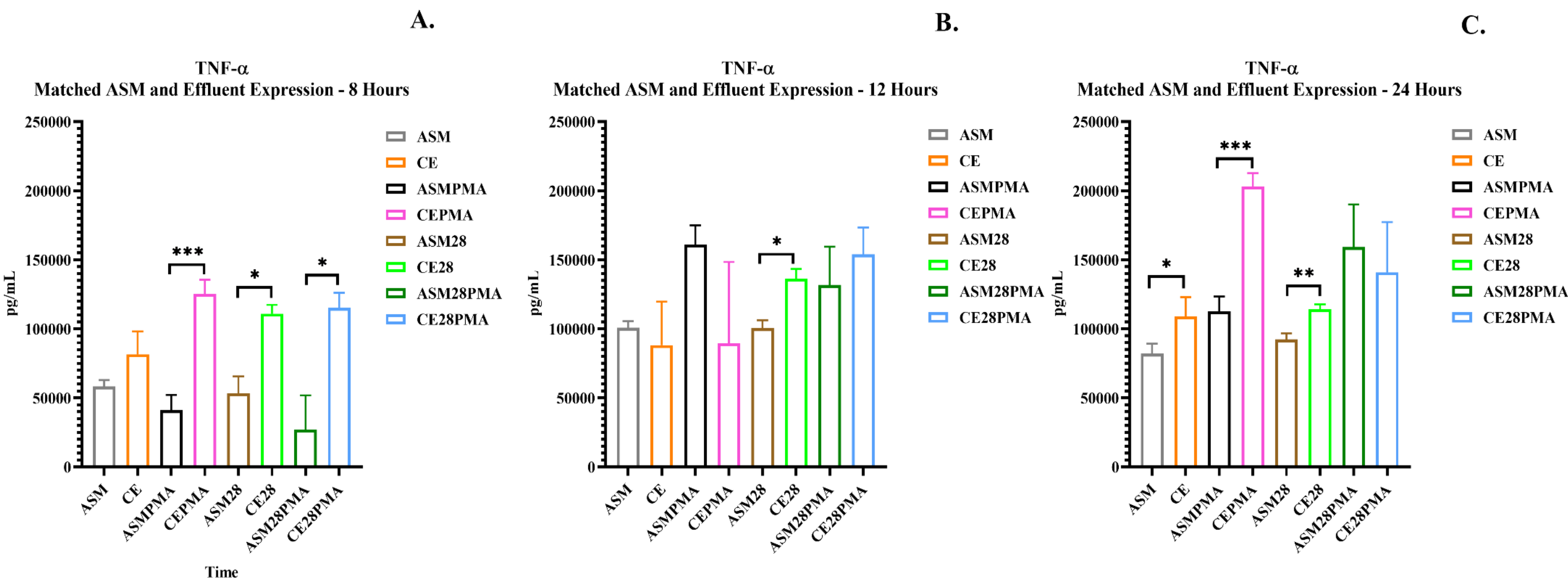

Figure 6.9: *TNF-α concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.3 Interleukin 17A

The concentrations reported for IL-17A ranged from 0.1 pg/mL to 48.8 pg/mL (Table 6.6).

Concentrations of IL-17A was significantly higher in both the ASMPMA and CEPMA groups at 8 hours when compared to both 12 and 24 hours (Fig 6.10 A. and B.). IL-17 A concentration in the ASMPMA was noted to be significantly higher at 12 hours when compared to 24 hours (Fig. 6.10 A.). IL-17A concentration was also noted to be significantly higher in the CEPMA group at 24 hours when compared to 12 hours (Fig. 6.10 B.).

At 8 and 12 hours the ASMPMA exhibited significantly higher concentrations of IL-17A when compared to the ASM, ASM28 and ASM28PMA groups (Fig. 6.11 A.). The CEPMA groups showed significantly higher concentrations of IL-17A at 8 hours when compared to the CE, CE28 and CE28PMA groups (Fig. 6.11 B.) The CE28PMA group also showed significantly higher concentrations of IL-17A at 8 hours when compared to both the CE and CE28 groups (Fig. 6.11 B.).

Concentration of IL-17A was significantly higher in the ASMPMA when compared to the CEPMA group at 12 hours (Fig. 6.12 B.). At 24 hours the concentration IL-17A was significantly higher in the CEPMA group when compared to the ASMPMA group (Fig. 6.112 C.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-values
ASM	0.5 ± 0.8 [a]	0.2 ± 0.3 [a]	0.0 ± 0.0 [a]	0.585
ASMPMA	32.8 ± 5.1	21.7 ± 3.4	5.0 ± 4.0 [a]	0.001*
ASM28	0.4 ± 0.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.091
ASM28PMA	7.3 ± 5.9	3.0 ± 2.5	6.6 ± 5.8	0.560
Change between groups ANOVA p-values	0.000*	0.000*	0.107	
CE	0.0 ± 0.0 [a]	0.8 ± 0.8 [a]	0.16 ± 0.3 [a]	0.195
CEPMA	30.8 ± 1.0	6.9 ± 6.0	18.4 ± 4.2	0.001*
CE28	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.4 ± 0.5 [a]	0.208
CE28PMA	12.0 ± 2.4	3.9 ± 0.9	20.0 ± 25.0	0.448
Change between groups ANOVA p-values	0.000*	0.085	0.162	

Table 6.6: *IL-17A concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

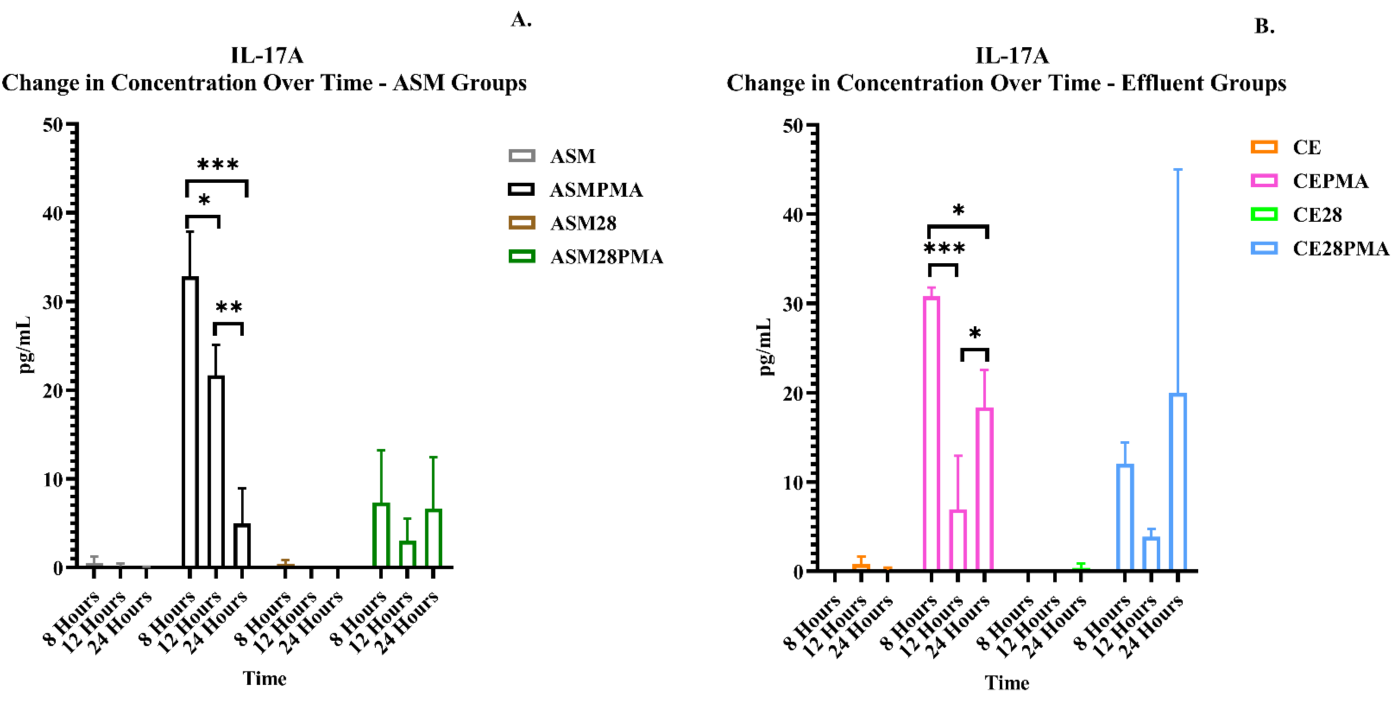

Figure 6.10: *IL-17A concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** Significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

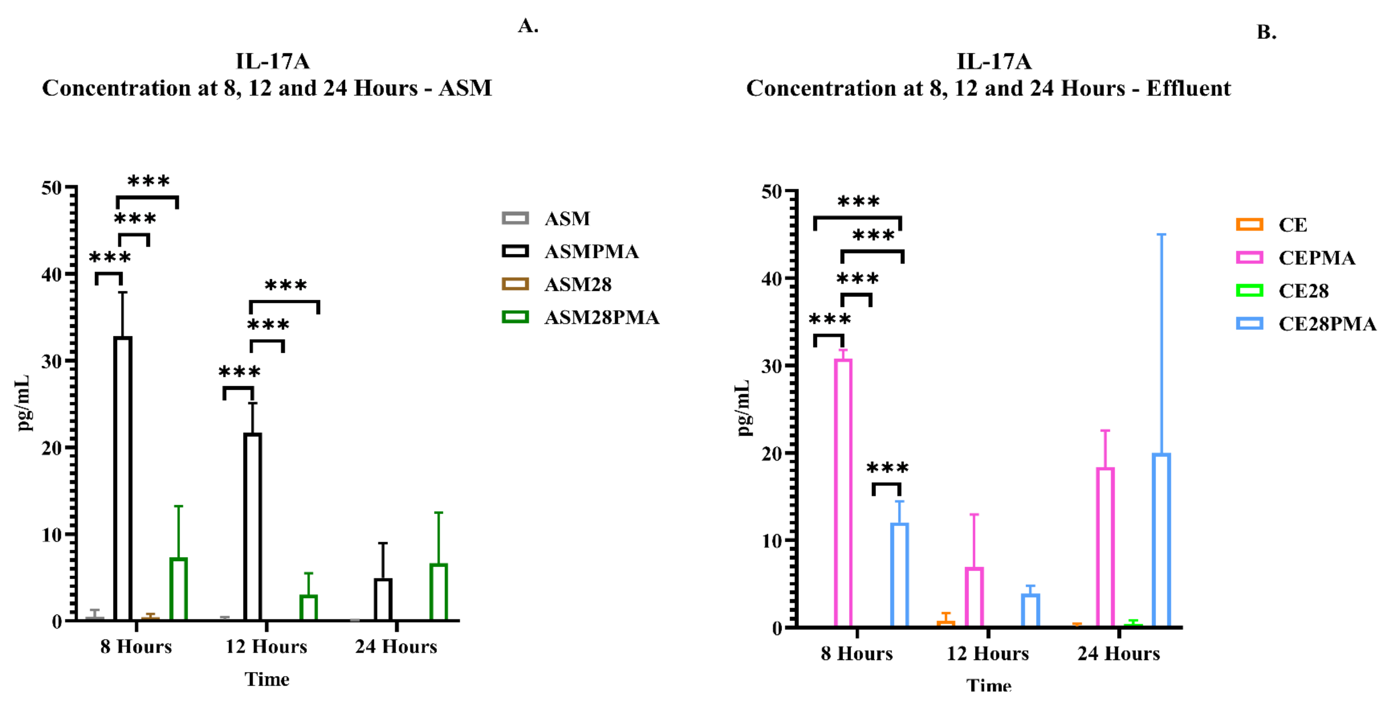

Figure 6.11: *IL-17A concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** Significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

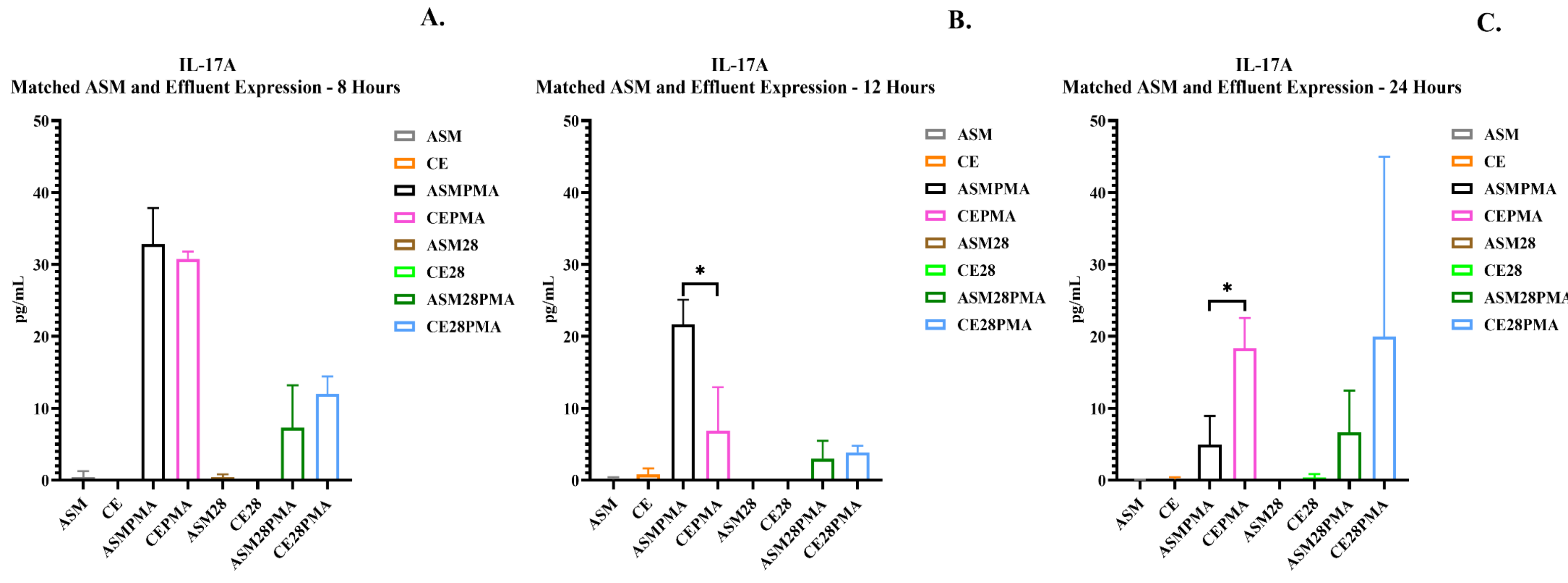

Figure 6.12: *IL-17A concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.4 Interleukin 18

The concentrations reported for IL-18 ranged from 0.2 pg/mL to 2.3 pg/mL (Table 6.7).

IL-18 concentration was significantly higher at 24 hours when compared 8 hours in both the ASM28 and ASM28PMA groups (Fig. 6.13 A.). The concentration of IL-18 was also shown to be significantly higher in the ASMPMA group 12 hours when compared to both 8 and 24 hours (Fig. 6.13 A.).

The ASM group showed significantly higher concentrations of IL-18 at 8 hours when compared to the ASM28PMA and ASMPMA groups (Fig. 6.14 A.). At 12 hours both the ASM and ASMPMA groups showed significantly higher concentrations of IL-18 when compared to the ASM28PMA groups (Fig. 6.14 A.). At 24 hours the ASM28PMA group showed significantly higher IL-18 concentration when compared to the ASMPMA group (Fig. 6.14 A.). One-way ANOVA was suggestive of significant differences between the effluent groups at 8 hours ($p = 0.036$), however, post-hoc testing did not reveal any significant difference. Comparison between the CEPMA and CE28 group approached significance ($p = 0.053$) with the CE28 group showing a trend for increased concentration of IL-18 (Fig. 6.14 B.).

Concentration of IL-18 was significantly lower in ASM28, ASM28PMA and ASMPMA groups when compared to the matched CE28, CE28PMA and CEPMA groups at 8 hours (Fig. 6.15 A.). Concentration of IL-18 was significantly lower in ASM28 group when compared to the CE28 group at 12 hours (Fig 6.15 B.). At 24 hours the ASM28 group exhibited significantly lower concentration of IL-18 when compared to the CE28 group whilst the ASMPMA group exhibited significantly higher concentration of IL-18 when compared to the CEPMA group at 24 hours (Fig. 6.15 C.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-values
ASM	1.2 ± 0.2	1.4 ± 0.1	1.2 ± 0.1	0.125
ASMPMA	0.6 ± 0.1 [a]	1.3 ± 0.0	0.9 ± 0.2	0.001*
ASM28	0.8 ± 0.2	1.0 ± 0.1	1.1 ± 0.0	0.048*
ASM28PMA	0.5 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	0.001*
Change between groups ANOVA p-values	0.005*	0.007*	0.033*	
CE	1.3 ± 0.1	1.0 ± 0.4	1.4 ± 0.2	0.248
CEPMA	1.1 ± 0.1	0.9 ± 0.4	1.6 ± 0.3	0.077
CE28	1.3 ± 0.1	1.6 ± 0.2	1.5 ± 0.0	0.075
CE28PMA	1.2 ± 0.1	1.4 ± 0.1	1.7 ± 0.5	0.165
Change between groups ANOVA p-values	0.036*	0.103	0.648	

Table 6.7: *IL-18 concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

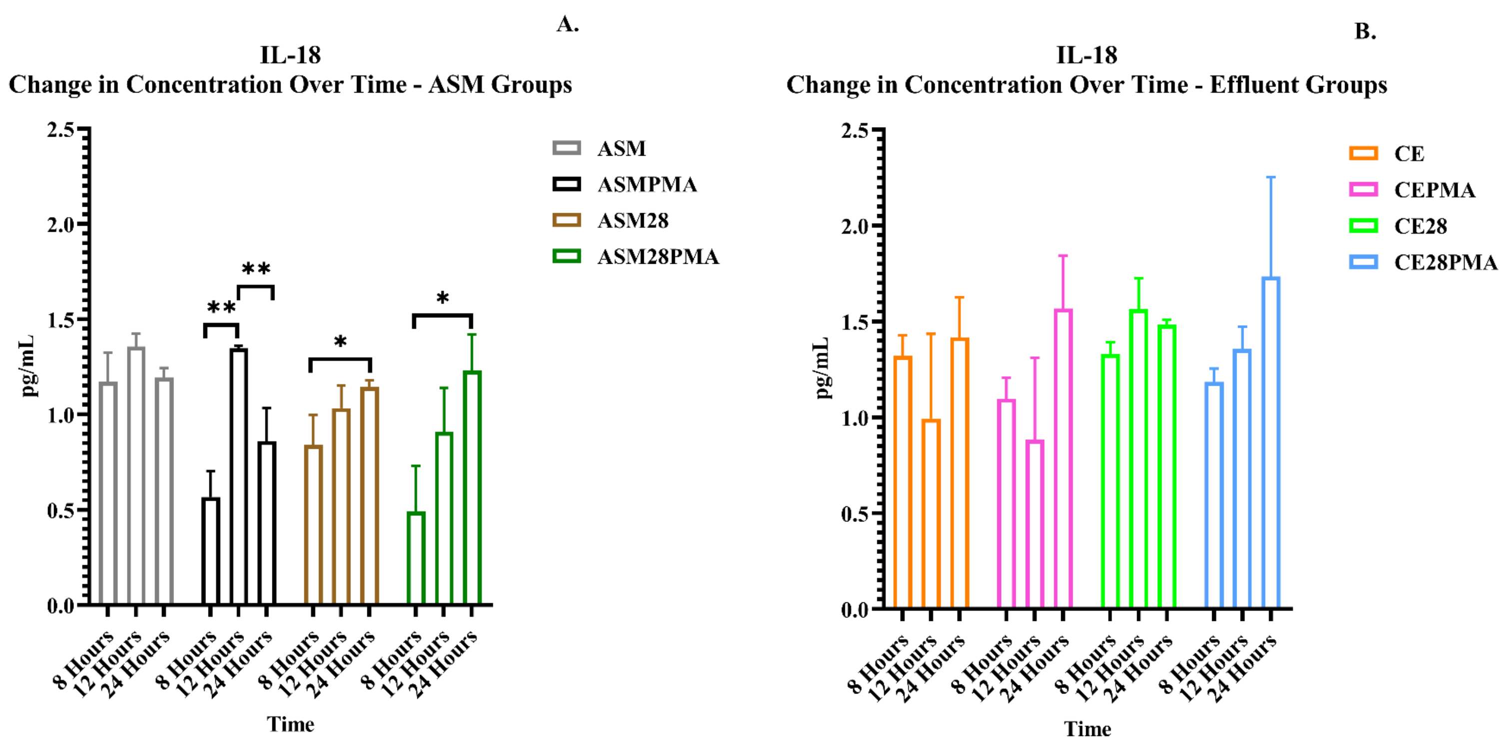

Figure 6.13: *IL-18 concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** Significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

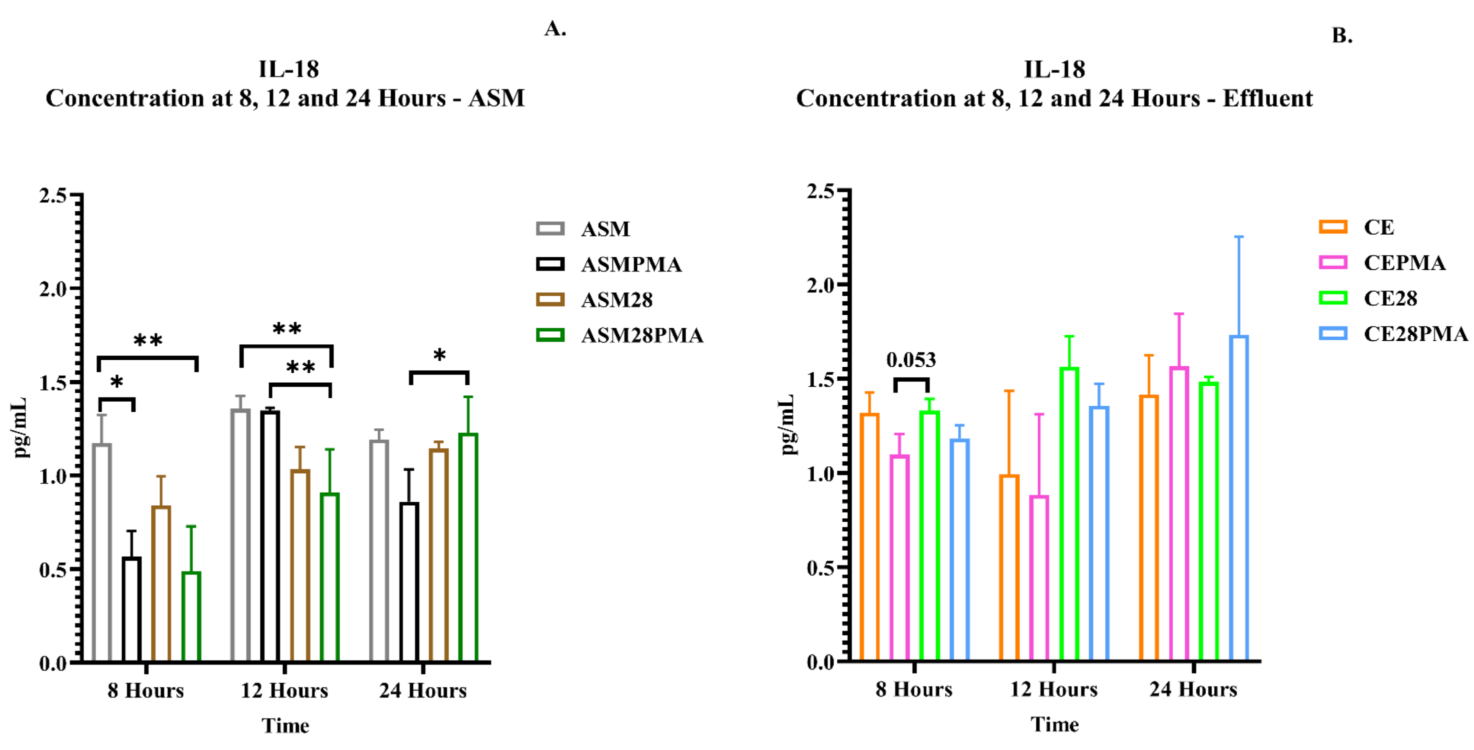

Figure 6.14: *IL-18 concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

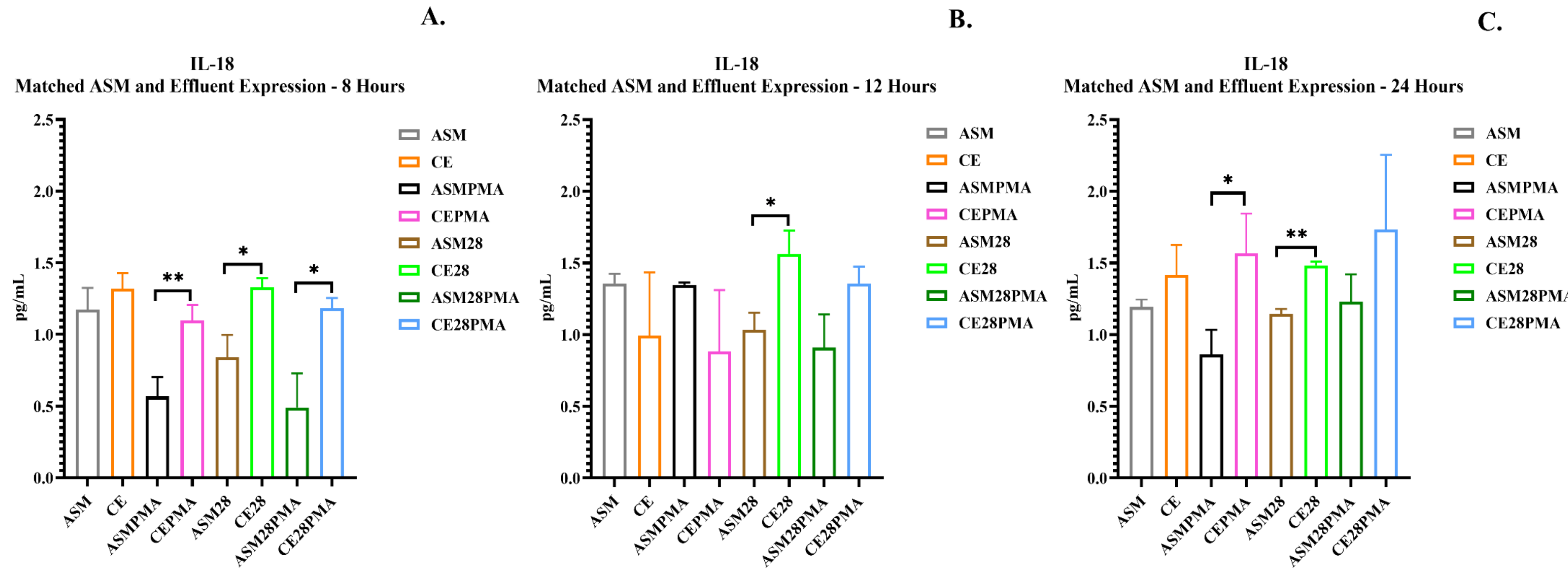

Figure 6.15: *IL-18 concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.4.1 Fold Changes

The CE28PMA groups showed a significant fold changed increase for IL-18 change with respect to the ASM28PMA group at 8 hours (2.41-increase) (Table 6.8).

	8 hours	12 hours	24 hours
CE vs. ASM	1.13	0.73	1.19
CE28 vs. ASM28	1.58	1.51	1.30
CE28PMA vs. ASM28PMA	2.41	1.49	1.41
CEPMA vs. ASMPMA	1.94	0.66	1.82

Table 6.8: *Fold changes in IL-18 concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase

denotes significant fold change decrease

6.4.2.5 Interleukin 12p40

The concentrations reported for IL-12p40 ranged from 0.6 pg/mL to 13.4 pg/mL (Table 6.9).

Significant differences in IL-12p40 concentration over time were noted in the ASM28 group. Concentration of IL-12p40 was significantly elevated at 24 hours when compared to both 8 and 12 hours in the ASM28 group (Fig. 6.16 A.). No significant differences in IL-12p40 concentration were noted between the different ASM or effluent groups at any timepoint (Fig. 6.17 A. and B.). At 24 hours the ASM group exhibited significantly higher concentration of IL-12p40 when compared to the CE group (Fig. 6.18 B.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-value
ASM	2.4 ± 3.7 [a]	1.5 ± 1.4 [a]	3.3 ± 1.9 [a]	0.703
ASMPMA	0.6 ± 0.1 [a]	1.3 ± 0.0 [a]	0.9 ± 0.2 [a]	0.125
ASM28	0.4 ± 0.3 [a]	0.6 ± 1.0 [a]	4.1 ± 2.2 [a]	0.027*
ASM28PMA	2.8 ± 3.0 [a]	0.7 ± 0.9 [a]	6.1 ± 2.8 [a]	0.094
Change between groups ANOVA p-values	0.661	0.127	0.299	
CE	1.5 ± 0.9 [a]	4.4 ± 4.1 [a]	1.5 ± 1.4 [a]	0.331
CEPMA	1.5 ± 1.4 [a]	2.2 ± 3.6 [a]	3.7 ± 2.6 [a]	0.635
CE28	1.7 ± 1.5 [a]	1.0 ± 1.7 [a]	1.5 ± 1.8 [a]	0.847
CE28PMA	0.6 ± 0.0 [a]	2.1 ± 1.8 [a]	2.4 ± 3.7 [a]	0.615
Change between groups ANOVA p-values	0.612	0.587	0.302	

Table 6.9: *IL-12p40 concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

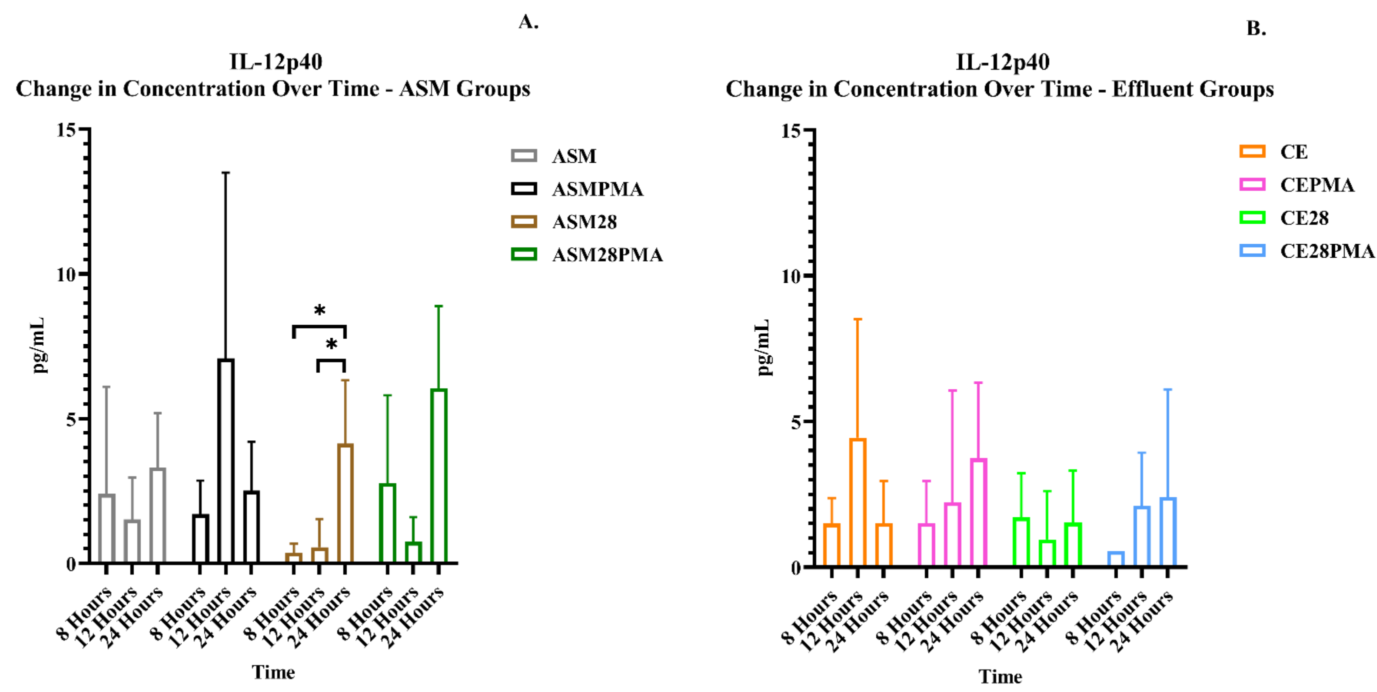

Figure 6.16: *IL-12p40 concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

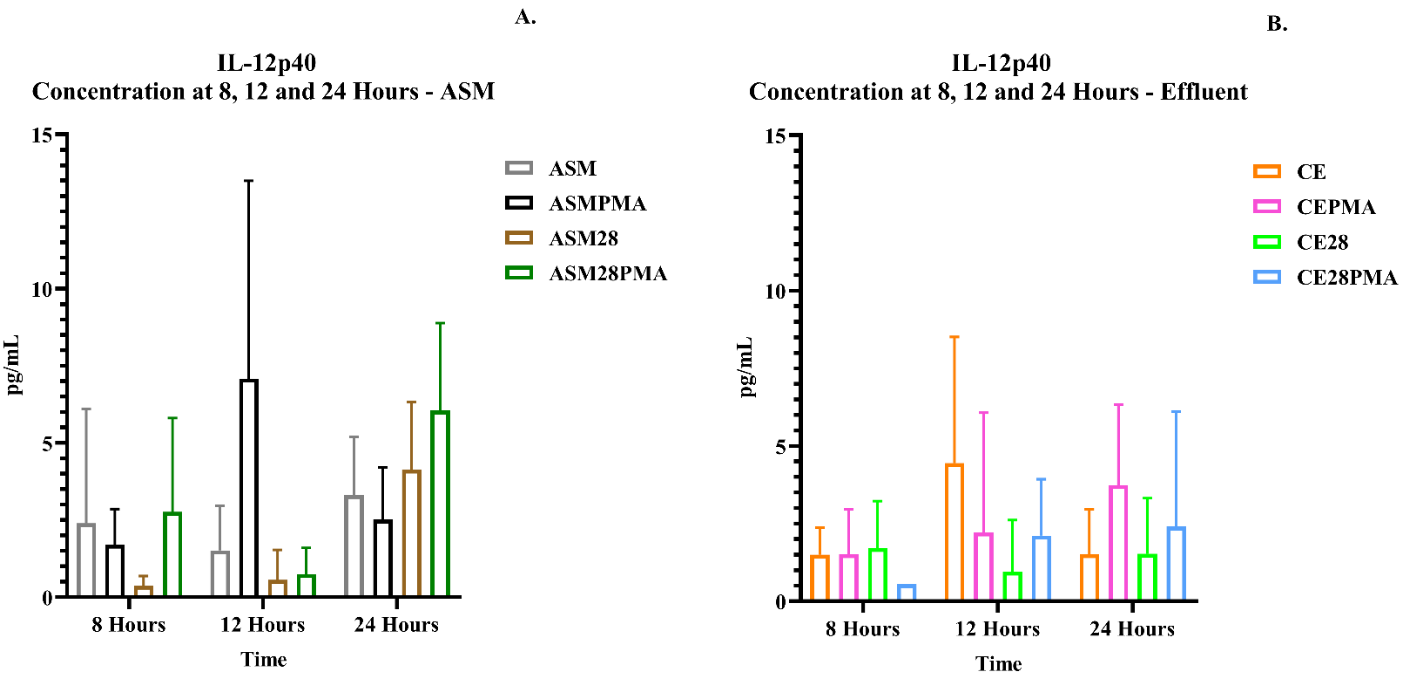

Figure 6.17: *IL-12p40 concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

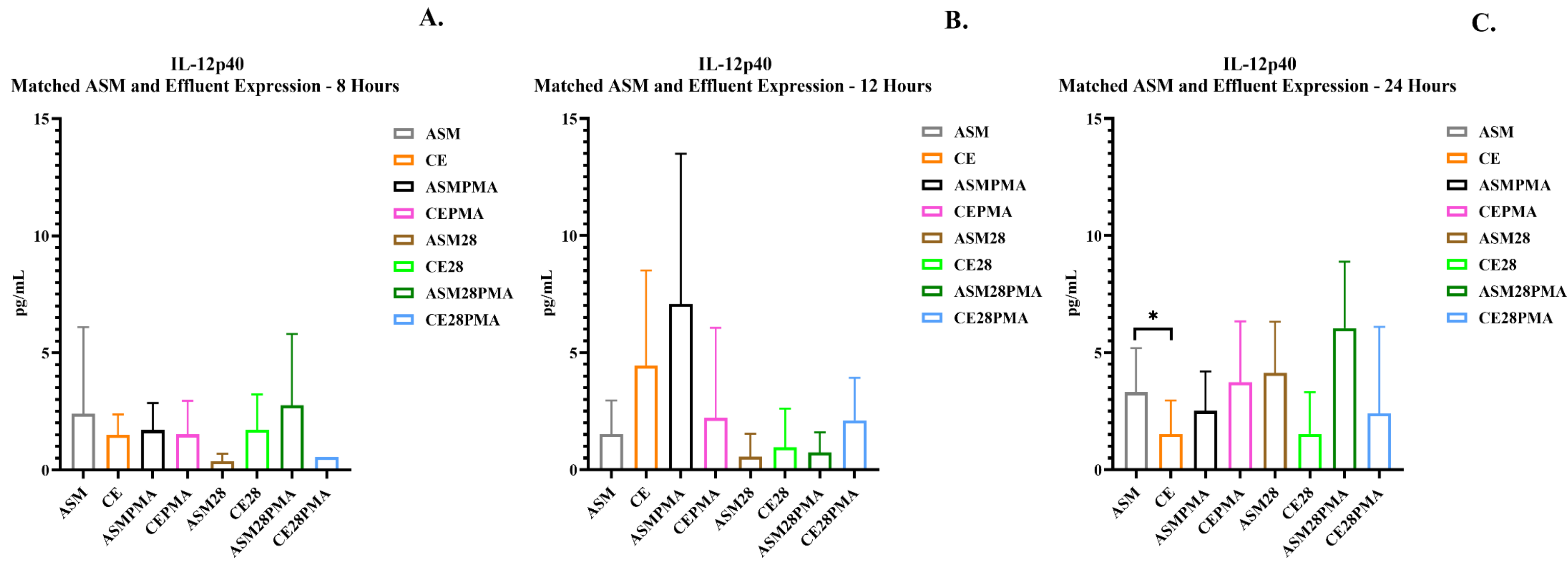

Figure 6.18: *IL-12p40 concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.6 Interleukin 12p70

The concentrations reported for IL-12p70 ranged from 0.5 pg/mL to 1.4 pg/mL (Table 6.10).

No significant in changes in IL-12p40 concentration over time were noted in the ASM or effluent groups (Fig. 6.19 A. and B.) No significant differences in IL-12p40 concentration were noted between the ASM or effluent groups at any timepoint (Fig. 6.20 A. and B.).

No significant differences existed between the effluent and the matched ASM groups at 8, 12 and 24 hours (Fig. 6.21 A., B. and C.). Differences between the CE28 and ASM28 groups at 12 hours approached significance ($p = 0.051$) with CE28 group showing a tendency for increased concentration of IL-12p70.

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-value
ASM	1.0 ± 0.4 [a]	0.8 ± 0.1 [a]	1.0 ± 0.1 [a]	0.454
ASMPMA	1.7 ± 1.2 [a]	7.1 ± 6.4 [a]	2.5 ± 1.7 [a]	0.265
ASM28	0.9 ± 0.4 [a]	1.0 ± 0.1 [a]	0.9 ± 0.1 [a]	0.906
ASM28PMA	0.8 ± 0.2 [a]	1.0 ± 0.1 [a]	1.0 ± 0.0 [a]	0.275
Change between groups ANOVA p-values	0.858	0.456	0.330	
CE	0.8 ± 0.3 [a]	1.1 ± 0.3 [a]	0.8 ± 0.0 [a]	0.320
CEPMA	0.9 ± 0.1 [a]	1.0 ± 0.4 [a]	0.7 ± 0.1 [a]	0.380
CE28	0.9 ± 0.1 [a]	1.1 ± 0.1 [a]	0.9 ± 0.2 [a]	0.315
CE28PMA	0.9 ± 0.2 [a]	0.9 ± 0.3 [a]	0.9 ± 0.2 [a]	0.995
Change between groups ANOVA p-values	0.942	0.832	0.361	

Table 6.10: *IL-12p70 concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

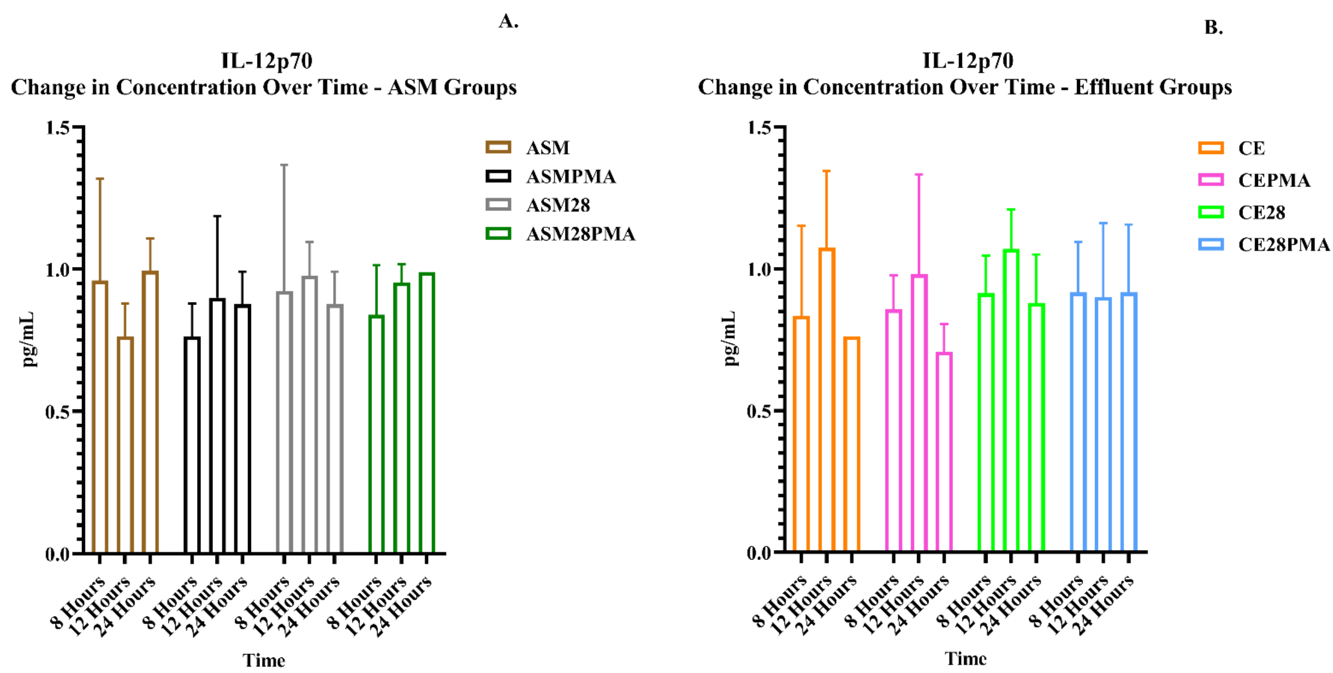

Figure 6.19: *IL-12p70 concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

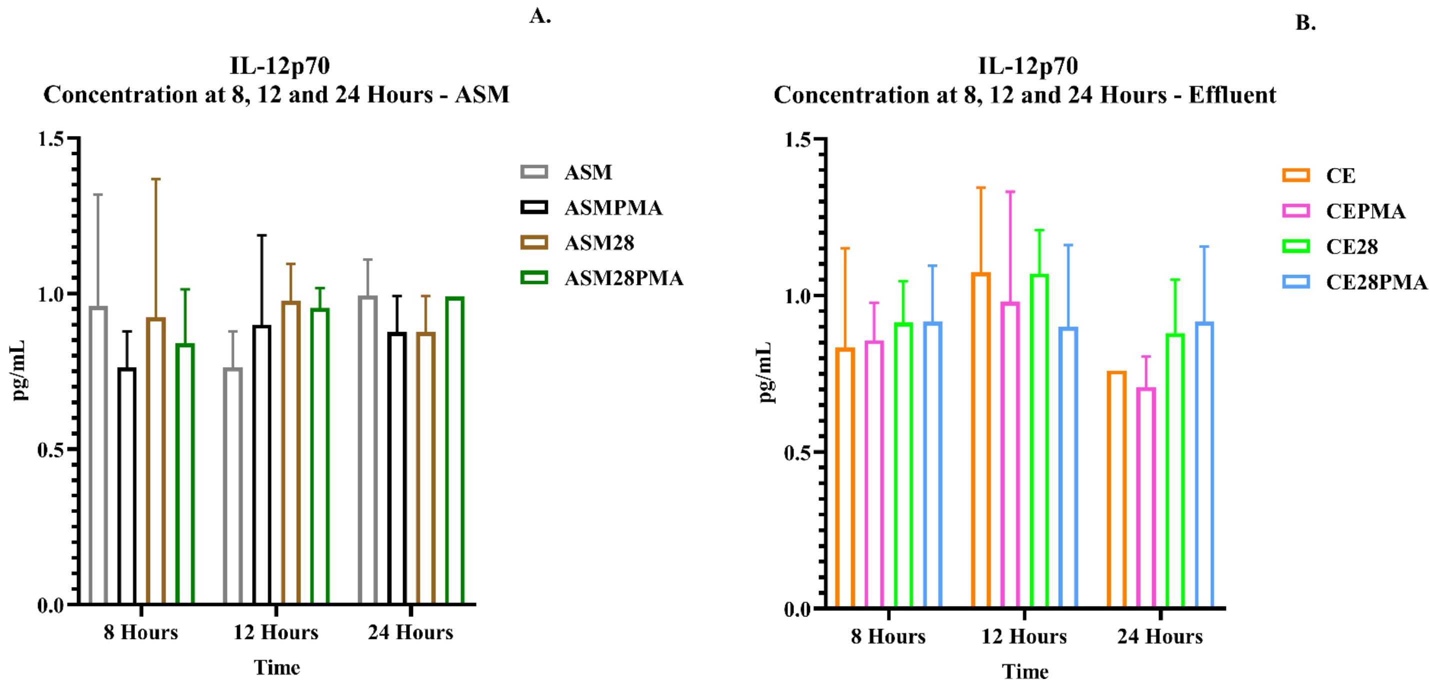

Figure 6.20: *IL-12p70 concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

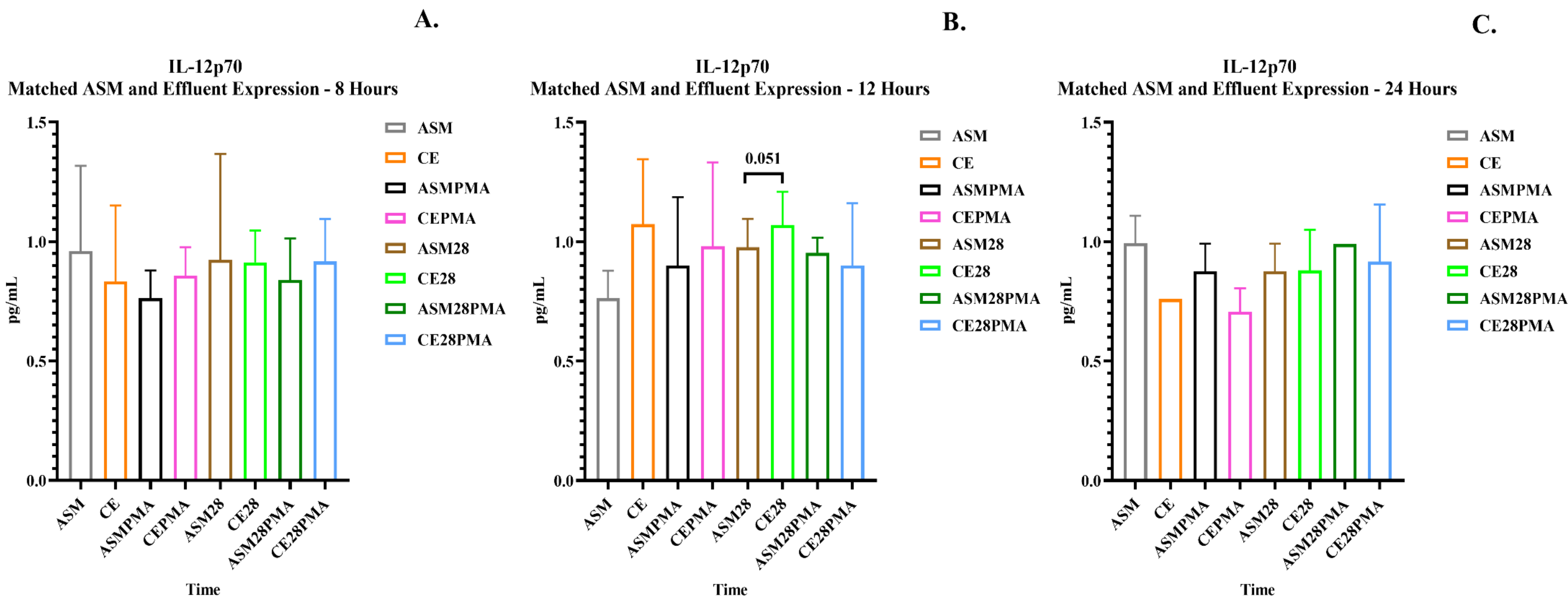

Figure 6.21: *IL-12p70 concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin).

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.7 Interleukin 22

The concentrations reported for Il-22 ranged from 0.1 pg/mL to 16.1 pg/mL (Table 6.11).

Concentration of IL-22 was significantly higher in the ASMPMA group at 8 hours when compared to 24 hours (Fig. 6.22 A.). The ASMPMA group demonstrated significantly higher concentrations of IL-22 when compared to the ASM, ASM28 and ASMPMA28 groups at 8 hours (Fig. 6.23 A.). One-way ANOVA was suggestive of significant differences between effluent groups at 12 hours ($p = 0.046$), however, post-hoc testing did not reveal any significant differences (Fig. 6.23 B.).

The concentration of IL-22 was significantly higher in the CE group when compared to the ASM group at 8 hours (Fig. 6.24 A.). At 12 hours the concentration of IL-22 was significantly higher in the CE28PMA group when compared to the ASM28PMA group (Fig. 6.24 B.).

	8 hours (pg/ mL) μ ± SD	12 hours (pg/ mL) μ ± SD	24 hours (pg/ mL) μ ± SD	Change in concentration over time ANOVA p-value
ASM	0.5 ± 0.8 [a]	1.6 ± 1.9 [a]	1.6 ± 0.6 [a]	0.482
ASMPMA	0.8 ± 0.1	0.9 ± 0.3	0.9 ± 0.1 [a]	0.036*
ASM28	2.0 ± 1.8 [a]	0.7 ± 1.2 [a]	2.2 ± 2.0 [a]	0.525
ASM28PMA	1.0 ± 0.9 [a]	1.2 ± 0.7 [a]	1.2 ± 1.5 [a]	0.937
Change between groups ANOVA p-values	0.002*	0.115	0.904	
CE	3.2 ± 1.4 [a]	1.1 ± 1.3 [a]	2.2 ± 1.2 [a]	0.250
CEPMA	5.6 ± 0.3	4.0 ± 3.1 [a]	4.8 ± 2.0 [a]	0.691
CE28	3.1 ± 2.9 [a]	1.1 ± 1.8 [a]	1.0 ± 0.4 [a]	0.400
CE28PMA	7.3 ± 8.0	6.0 ± 1.4	5.6 ± 9.1 [a]	0.955
Change between groups ANOVA p-values	0.593	0.046*	0.607	

Table 6.11: *IL-22 concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin).

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

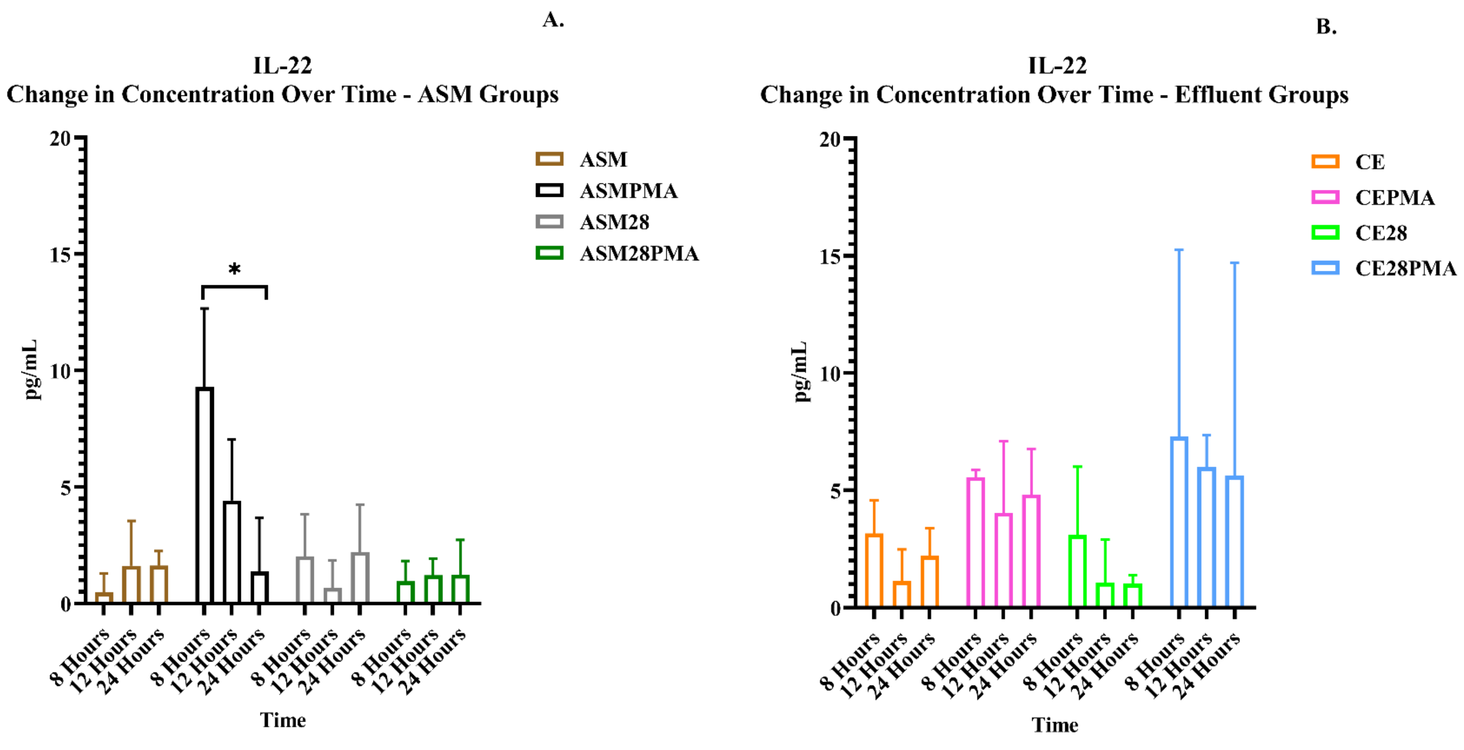

Figure 6.22: *IL-22 concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

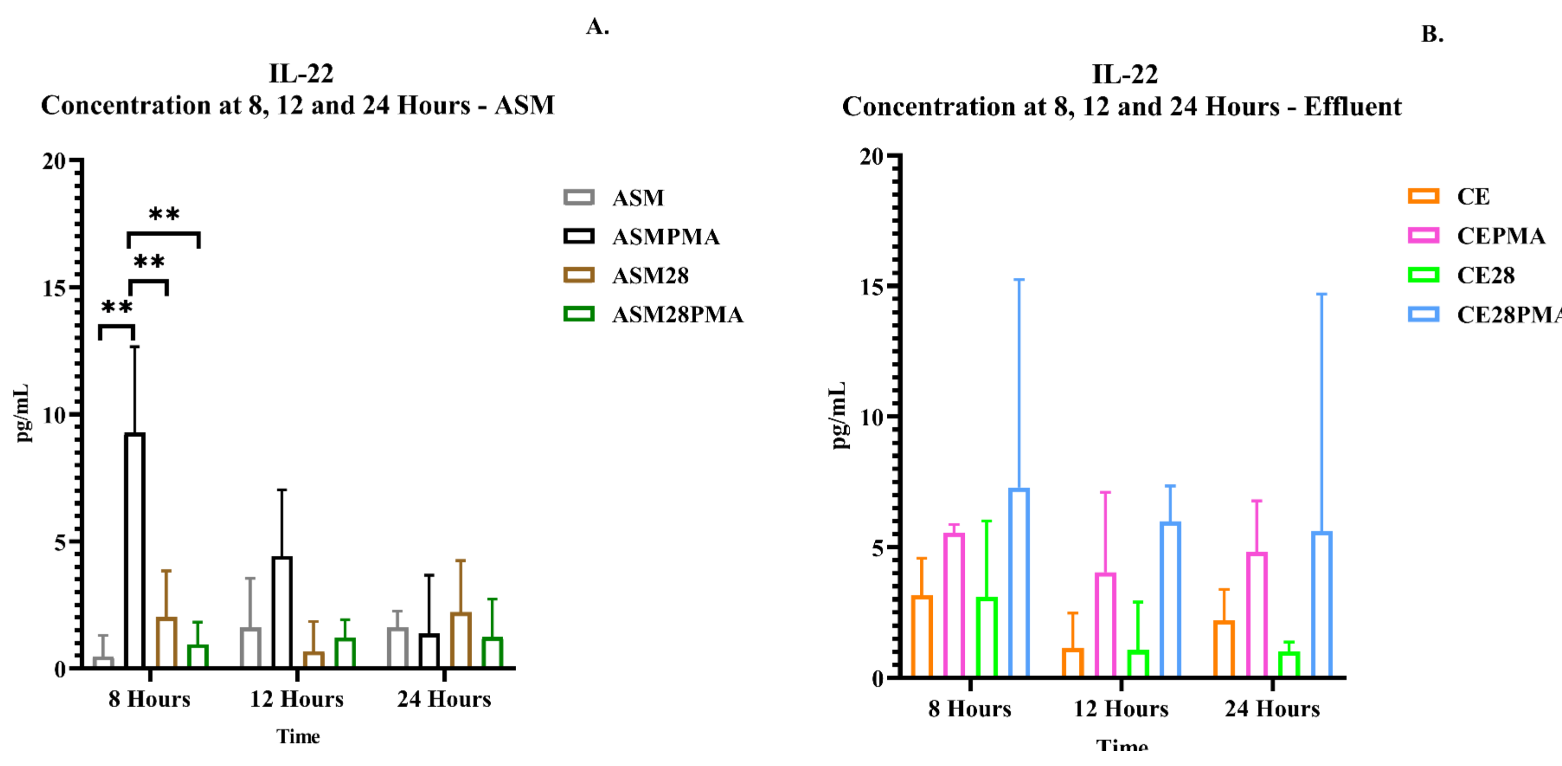

Figure 6.23: *IL-22 concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** Significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

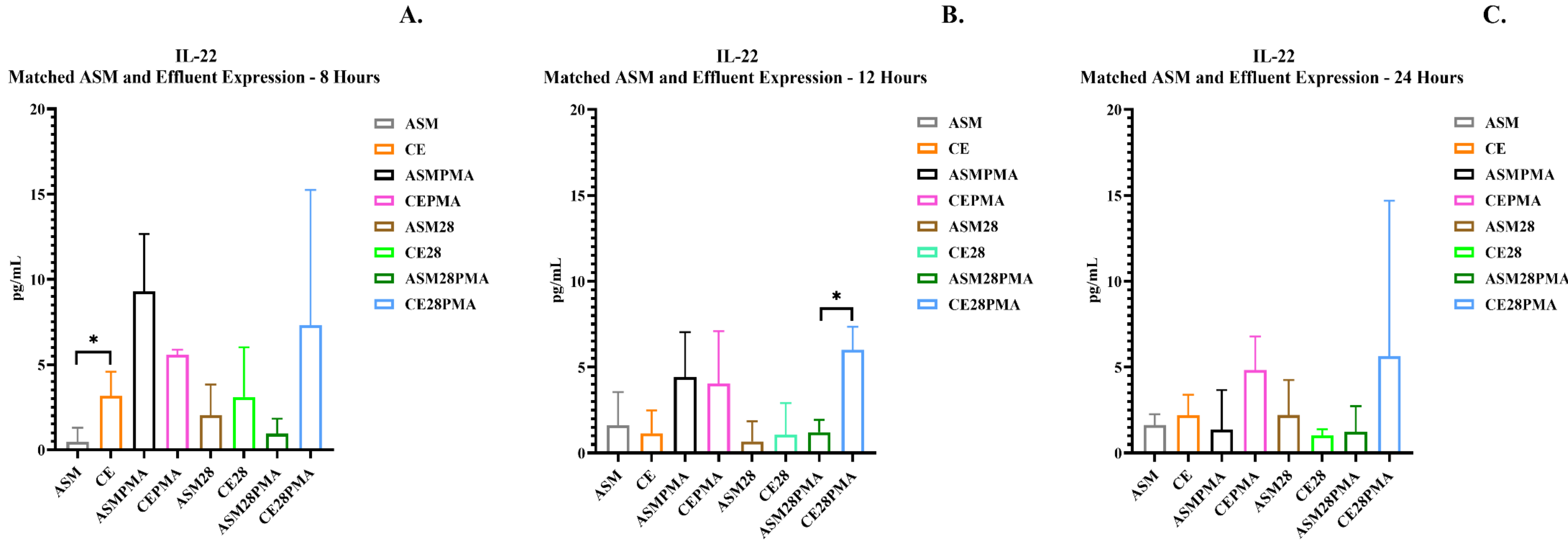

Figure 6.24: *IL-22 concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** Significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.8 Interleukin 23

The concentrations reported for Il-23 ranged from was 3.1 pg/mL to 57.4 pg/mL (Table 6.12).

Concentration of IL-23 was significantly higher in the CE group at 8 hours when compared to both 12 and 24 hours (Fig. 6.25 B.). Concentration of IL-23 was significantly higher in the CEPMA group when compared to the CE, CE28 and CD28PMA groups at 24 hours (Fig. 6.26 B.) Significantly higher concentration of IL-23 were noted in CE group at 8 hours when compared to the ASM group (Fig. 6.27 A.). At 12 hours the CE28PMA group exhibited a significantly higher concentration of IL-23 when compared to the ASM28PMA (Fig. 6.27 B.).

	8 hours (pg/mL) μ ± SD	12 hours (pg/mL) μ ± SD	24 hours (pg/mL) μ ± SD	Change in concentration over time ANOVA p-value
ASM	0.0 ± 0.0 [a]	7.3 ± 6.8 [a]	7.3 ± 6.8 [a]	0.254
ASMPMA	8.5 ± 12.2 [a]	17.8 ± 9.8 [a]	8.8 ± 9.0 [a]	0.508
ASM28	11.3 ± 10.0 [a]	3.8 ± 4.3 [a]	12.1 ± 18.4 [a]	0.680
ASM28PMA	0.0 ± 0.0 [a]	1.0 ± 1.8 [a]	12.1 ± 18.4 [a]	0.367
Change between groups ANOVA p-values	0.255	0.054	0.964	
CE	26.1 ± 4.5 [a]	2.0 ± 3.4 [a]	8.3 ± 5.1 [a]	0.001*
CEPMA	18.0 ± 4.6 [a]	16.5 ± 16.3 [a]	21.7 ± 3.4 [a]	0.811
CE28	13.8 ± 9.4 [a]	13.2 ± 16.1 [a]	1.0 ± 1.8 [a]	0.332
CE28PMA	32.3 ± 29.4	21.8 ± 1.3 [a]	1.0 ± 1.8 [a]	0.152
Change between groups ANOVA p-values	0.514	0.272	0.000*	

Table 6.12: *IL-23 concentration (pg/mL) derived from raw data at 8, 12 and 24 hours with change in concentration over time and change between groups determined by one-way ANOVA*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin).

** significance as determined by one-way ANOVA with p – value less than or equal to 0.05, [a] concentration out of range with observed concentration reported*

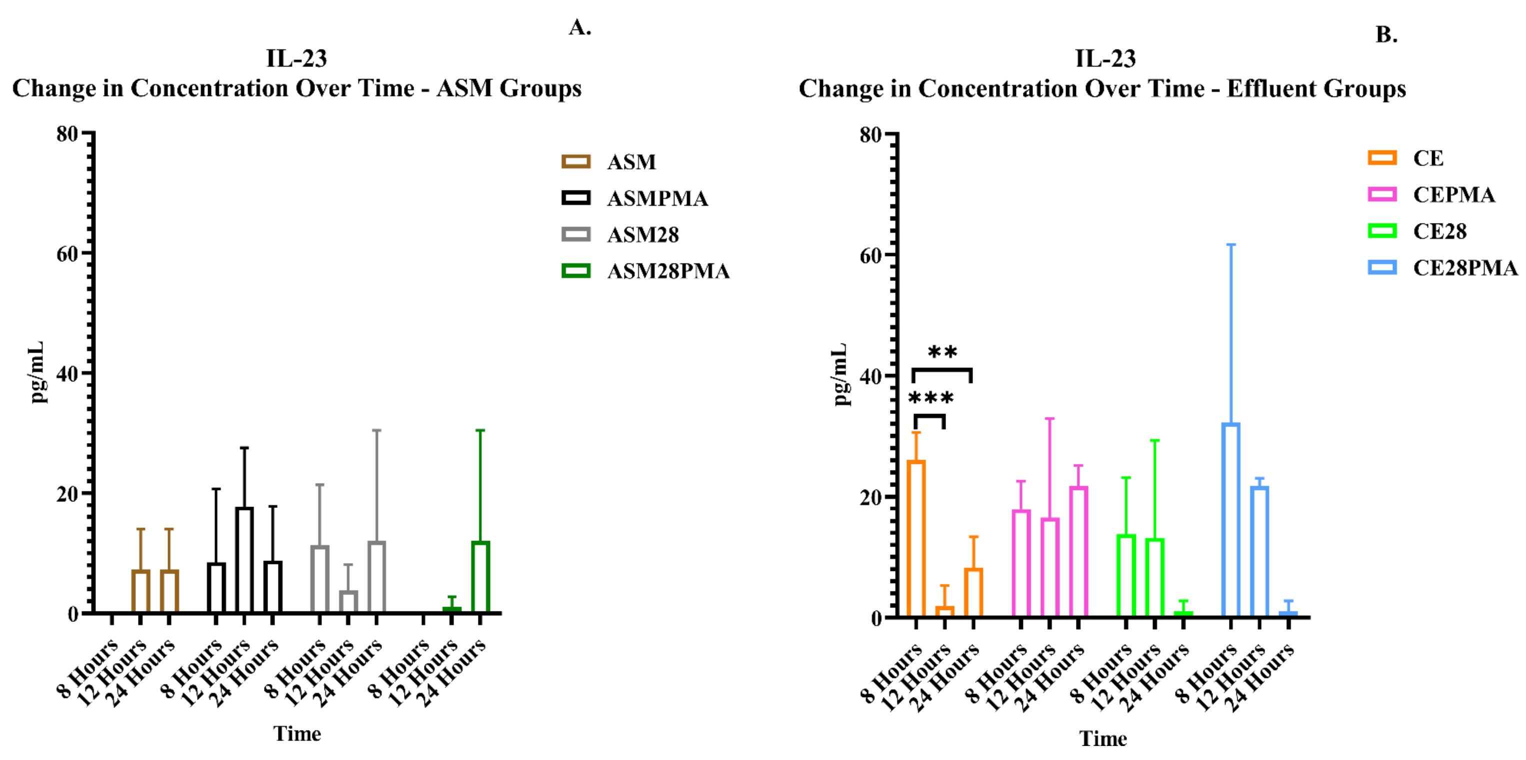

Figure 6.25: *IL-23 concentration (pg/mL) over time per group measured by BioPlex assay. A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

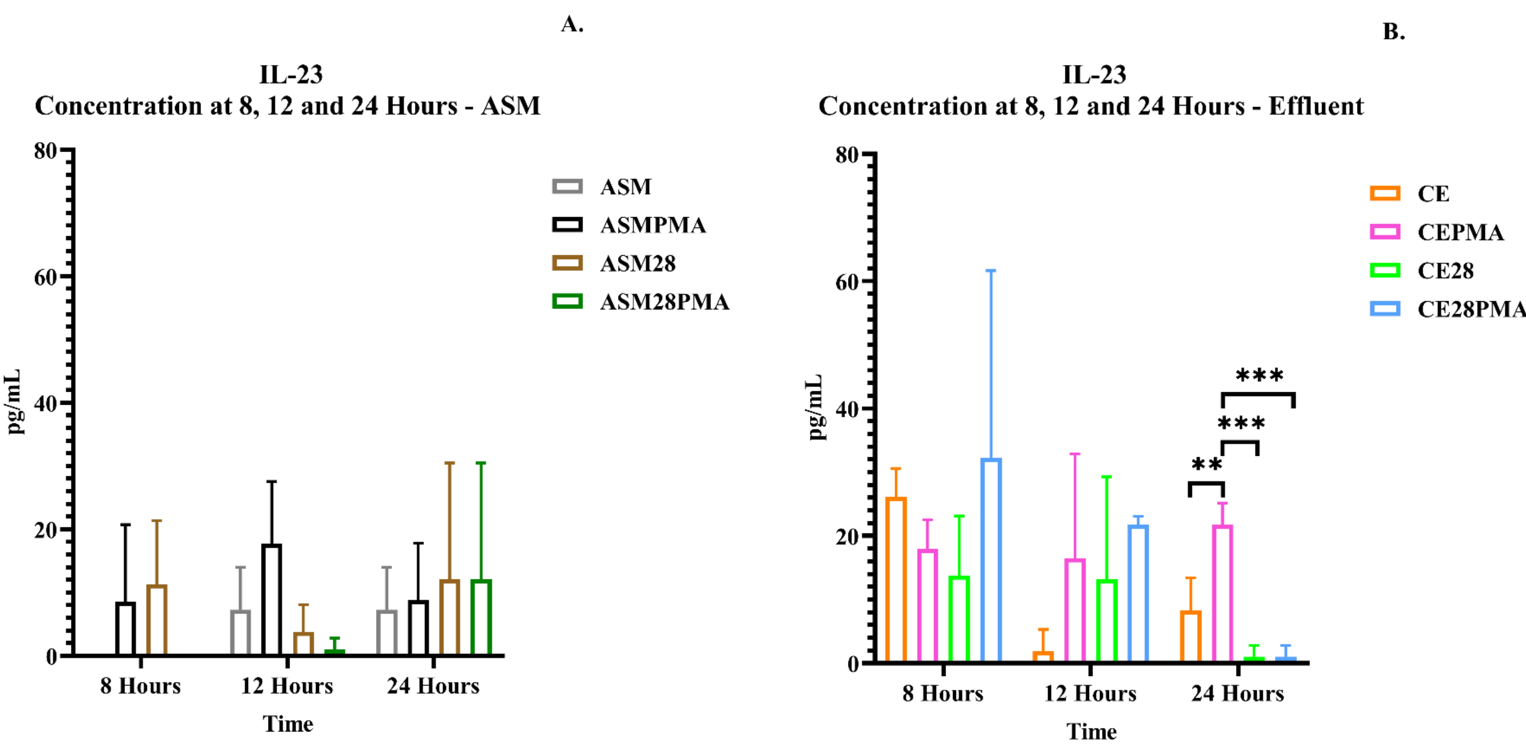

Figure 6.26: *IL-23 concentration (pg/mL) at 2, 6 and 24 hours measured by BioPlex assay A. ASM treatment groups, B. Effluent treatment groups*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

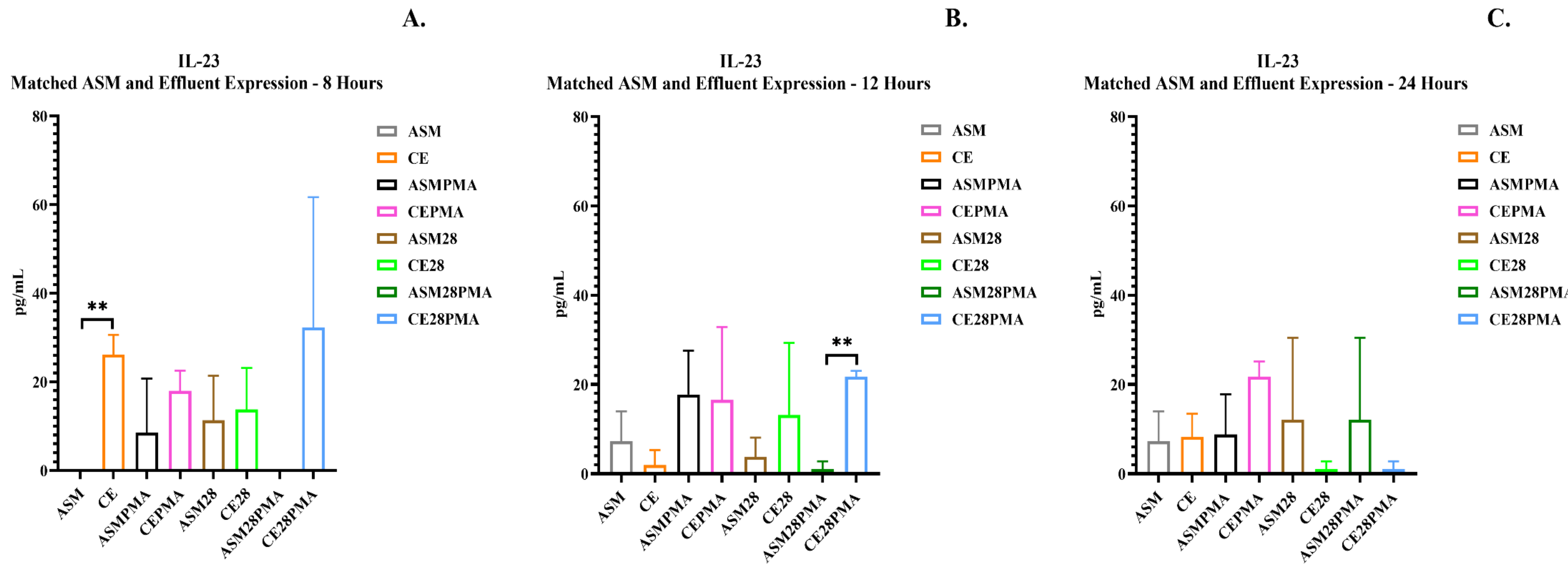

Figure 6.27: *IL-23 concentration (pg/mL) in matched ASM and effluent groups measured by BioPlex assay. A. 2 hours, B. 6 hours, C. 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.2.9 Overall Cytokine Expression

For IFN-γ, TNF-α, IL-12p40, IL-12p70, IL22 and IL-23 the majority of reported results are the observed concentrations due to values being outside of the range of the standard curve. For IL-17A just under half (46%) of the reported results were within the range of the standard curve whilst for IL-18 all but one of the reported results (ASMPMA 8 hours) were within the range of the standard curve.

Taken together the data shows that for IFN-γ, TNF-α, IL-17A, IL-22 and IL-23 the presence of PMA/Ionomycin was a significant factor that increased the concentration of these cytokines. To a lesser extent this was also demonstrated in IL-18. Increased amounts of TNF-α, IL-18, IL-23, IL-22 and to a lesser extent IFN-γ and IL-17A were observed when PBMC were incubated in the presence of effluent derived from *C. albicans* biofilm. IL-18 and TNF-α exhibited increased concentrations when compared to matched controls across all timepoints for PBMC incubated in the presence of both *Candida* effluent and CD28.

6.4.3 Flow Cytometry

PBMC cell media was supplemented with either 10% (v/v) effluent derived from *C. albicans* biofilm (CE) or 10% (v/v) ASM (ASM). In addition to this CD28 was added to some *C. albicans* effluent (CE28) and ASM (ASM28) wells to provide a costimulatory signal for T-cell activation. PMA/Ionomycin was not added to cell media for this experiment as there was no requirement for cytokine stimulation.

6.4.3.1 CD3$^+$CD45$^+$ cells

The percentage of CD3$^+$CD45$^+$ cells within the lymphocyte population and the percentage of CD8 +/- CD4 +/- cells inside the CD3$^+$CD45$^+$ subset was determined using flow cytometry (Table 6.13). The gating strategy for the entire flow cytometry experiment is shown in Figure 6.28.

No significant differences in the percentage of CD3$^+$CD45$^+$ cells were noted between groups (Fig. 6.30). Differences between the CE and CE28 groups approached significance ($p = 0.051$) with the CE28 group exhibiting a trend for higher percentages of CD8$^-$CD4$^+$ cells when compared to the CE group.

Significant differences were noted between the CE and CE28 groups with the CE28 groups exhibiting a significantly higher percentage of CD8$^+$CD4$^+$ cells when compared to the CE group (Fig. 6.30). No significant differences in the percentage of CD8$^-$CD4$^+$ CD8$^+$CD4$^-$ and CD8$^-$CD4$^-$ cells were noted between groups (Fig. 6.29).

Collectively, the data show that addition of CD28 to *Candida* effluent resulted in an increased percentage of CD8$^+$CD4$^+$cells with a tendency for increased percentages of CD8$^-$CD4$^+$ cells also.

	$CD3^+CD45^+$ (%) μ ± SD	$CD8^-CD4^+$ (%) μ ± SD	$CD8^+CD4^+$ (%) μ ± SD	$CD8^+CD4^-$ (%) μ ± SD	$CD8^-CD4^-$ (%) μ ± SD
ASM	37.0 ± 14.7	55.6 ± 13.4	1.9 ± 1.7	30.5 ± 10.0	12.0 ± 2.1
ASM28	40.9 ± 3.5	62.2 ± 0.6	1.9 ± 0.3	26.8 ± 1.1	9.2 ± 0.8
CE	48.6 ± 2.4	64.2 ± 0.6	0.8 ± 0.3	26.3 ± 0.5	8.8 ± 0.6
CE28	40.3 ± 4.9	62.5 ± 0.7	0.2 ± 0.4	26.2 ± 1.0	9.2 ± 0.2

Table 6.13: *Percentage of $CD3^+CD45^+$ cells determined as a percentage of the parent lymphocyte population as well as percentage of $CD8^-CD4^+$, $CD8^+CD4^+$, $CD8^+CD4^-$ and $CD8^-CD4^-$ subsets*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CE28 (10% v/v Candida effluent with CD28)

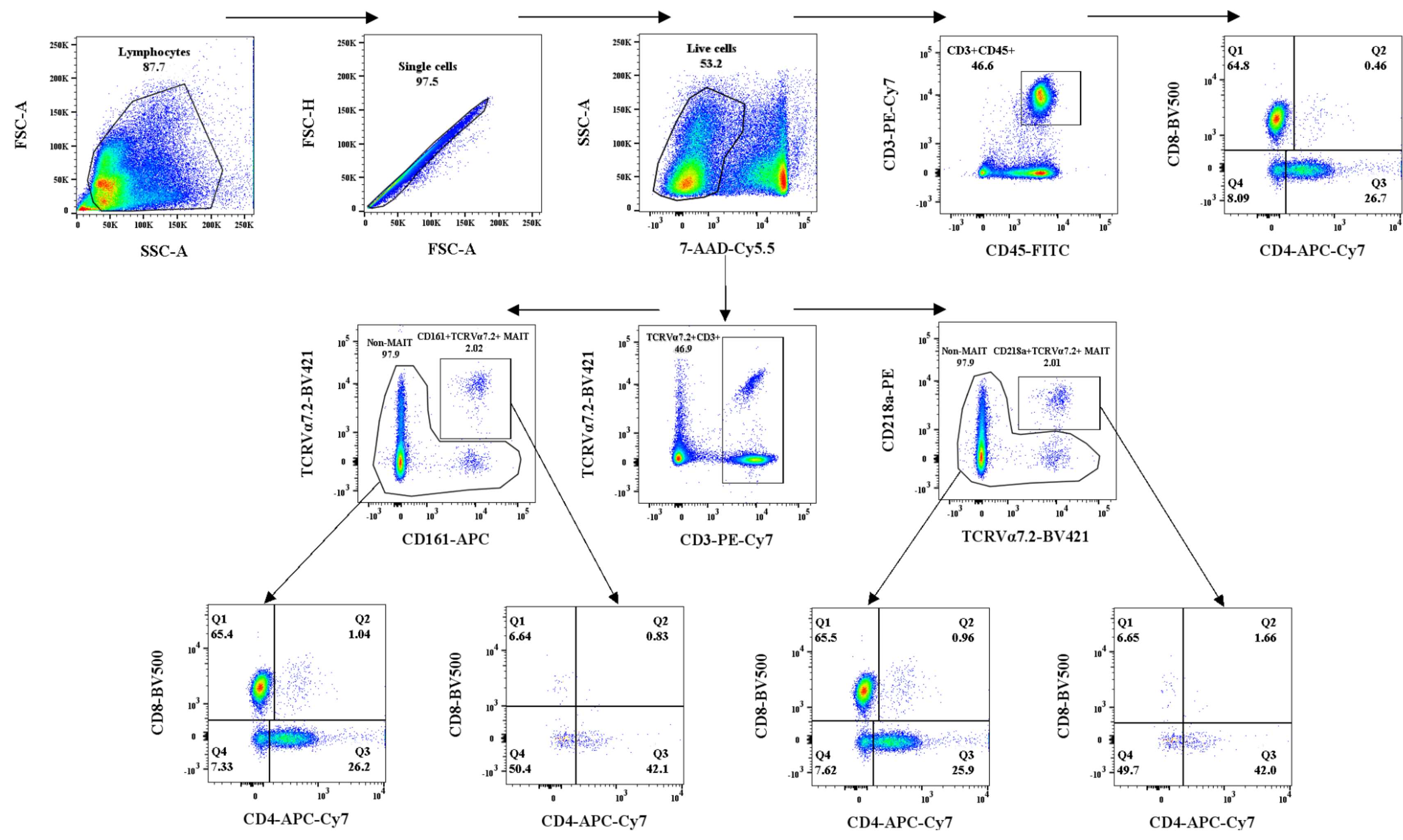

Figure 6.28: *Representative image of gating strategy to identify CD3$^+$CD45$^+$ CD161$^+$ TCRVα7.2$^+$ and CD218a$^+$ TCRVα7.2$^+$ MAIT cells as well as identify CD8 +/- and CD4+/- subsets within these populations using flow cytometry*

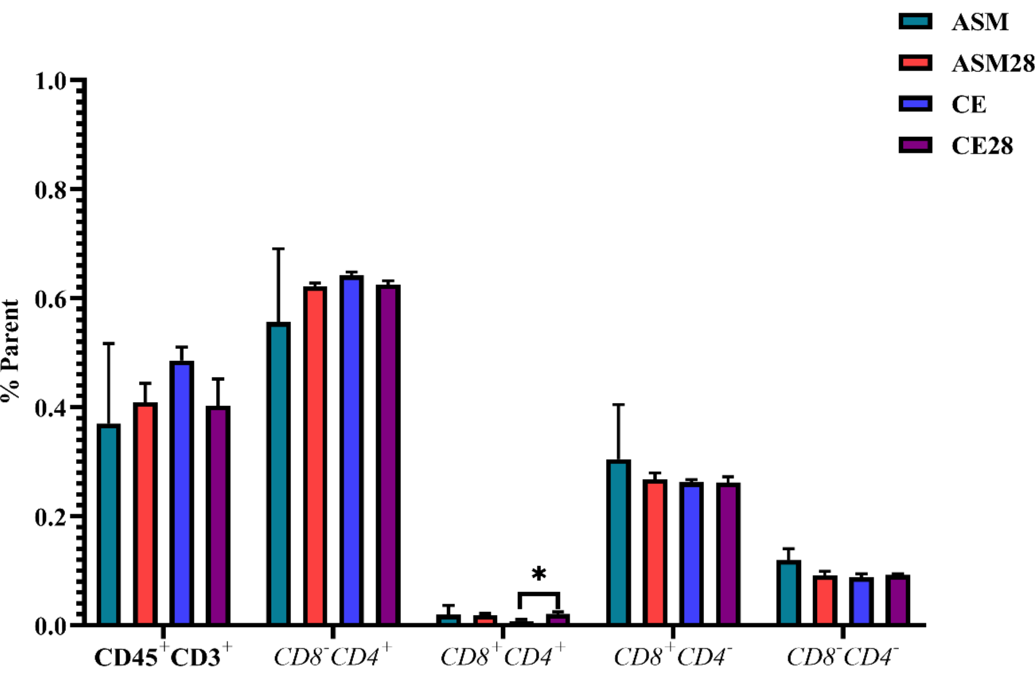

Figure 6.29: *Percentage of $CD3^{+}CD45^{+}$ cells and CD8 +/- CD4+/- subsets as a percentage of the parent population*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.3.2 CD161$^+$ TCRVα7.2$^+$ MAIT cells and non-MAIT cells

The percentage of CD161$^+$TCRVα7.2$^+$ MAIT and non-MAIT cells as a percentage of the parent TCRVα7.2$^+$CD3$^+$ population as well as the percentage of CD8 +/- CD4 +/- subsets were determined using flow cytometry (Tables 6.14 and 6.15). The gating strategy for flow cytometry is show in Figure 6.28.

No significant differences in the percentage of CD161$^+$ TCRVα7.2$^+$ MAIT cells or CD8$^-$CD4$^+$, CD8$^+$CD4$^+$, CD8$^+$CD4$^-$ and CD8$^-$CD4$^-$ subsets were noted between groups (Fig. 6.31 A.). No significant differences in the percentage of non-MAIT cells were noted between groups (Fig. 6.31 B.). The percentage of CD8$^-$CD4$^+$ cells were significantly higher in the CE group when compared to the CE28 group whilst the percentage of CD8$^+$CD4$^+$ cells were significantly higher the CE28 group when compared to the CE group (Fig. 6.30).

Collectively, the data show that addition of CD28 to *Candida* effluent decreased percentages of CD8$^-$CD4$^+$ subsets in the non-MAIT population whilst also increasing the percentage of CD8$^+$CD4$^+$ cells in the non-MAIT population. CD161$^+$TCRVα7.2$^+$ MAIT cells were not significantly affected by the presence of either *Candida* effluent or CD28.

	$CD161^+$ $TCRV\alpha7.2^+$ (%) μ ± SD	$CD8^-CD4^+$ (%) μ ± SD	$CD8^+CD4^+$ (%) μ ± SD	$CD8^+CD4^-$ (%) μ ± SD	$CD8^-CD4^-$ (%) μ ± SD
ASM	3.1 ± 0.9	3.9 ± 1.9	0.9 ± 0.6	33.6 ± 10.2	61.6 ± 8.8
ASM28	2.4 ± 0.2	3.8 ± 1.3	1.0 ± 1.3	36.9 ± 4.0	58.3 ± 3.4
CE	2.2 ± 0.2	5.8 ± 1.0	1.1 ± 0.3	37.7 ± 3.9	38.8 ± 33.1
CE28	2.2 ± 0.1	4.8 ± 1.1	0.6 ± 0.5	35.5 ± 5.6	59.0 ± 4.9

Table 6.14: *Percentage of $CD161^+$ $TCRV\alpha7.2^+$ cells determined as a percentage of the parent $TCRV\alpha7.2^+CD3^+$ T-cell population as well as percentage of $CD8^-CD4^+$, $CD8^+CD4^+$, $CD8^+CD4^-$ and $CD8^-CD4^-$ subsets*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CE28 (10% v/v Candida effluent with CD28)

	Non-MAIT (%) μ ± SD	$CD8^-CD4^+$ (%) μ ± SD	$CD8^+CD4^+$ (%) μ ± SD	$CD8^+CD4^-$ (%) μ ± SD	$CD8^-CD4^-$ (%) μ ± SD
ASM	96.8 ± 1.0	56.7 ± 13.5	2.9 ± 2.5	29.7 ± 9.3	10.7 ± 1.9
ASM28	97.4 ± 0.2	62.8 ± 0.5	3.1 ± 0.6	26.0 ± 1.1	8.1 ± 0.7
CE	97.7 ± 0.2	64.9 ± 0.6	1.5 ± 0.4	25.7 ± 0.5	7.9 ± 0.5
CE28	97.8 ± 0.2	62.9 ± 0.3	3.2 ± 0.6	25.6 ± 1.0	8.4 ± 0.3

Table 6.15: *Percentage of non-MAIT cells determined as a percentage of the parent $TCRV\alpha7.2^+CD3^+$ T-cell population as well as percentage of $CD8^-CD4^+$, $CD8^+CD4^+$, $CD8^+CD4^-$ and $CD8^-CD4^-$ subsets*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CE28 (10% v/v Candida effluent with CD28)

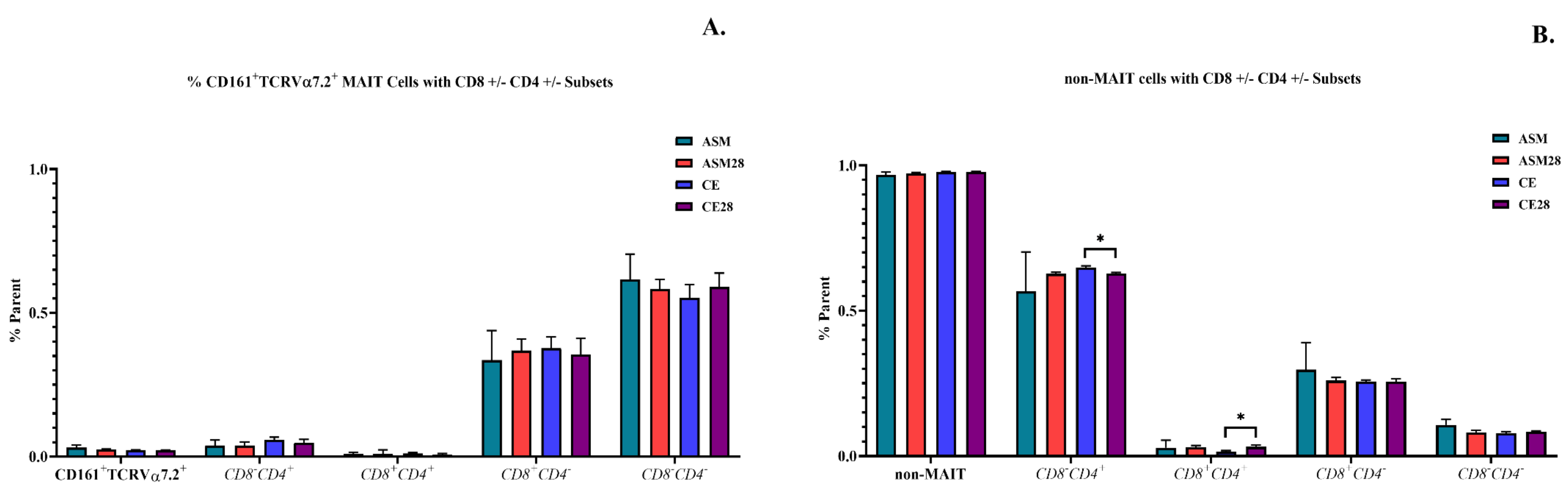

Figure 6.30: *Percentage of CD161⁺TCRVα7.2⁺ MAIT (A.), non-MAIT (B.) and CD8 +/- CD4+/- subsets as a percentage of the parent population*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.3.3 CD218a⁺ TCRVα7.2⁺ MAIT cells and non-MAIT cells

The percentage of $CD218a^{+}TCRV\alpha7.2^{+}$ MAIT and non-MAIT cells as a percentage of the parent $TCRV\alpha7.2^{+}CD3^{+}$ population as well as the percentage of CD8 +/- CD4 +/- subsets were determined using flow cytometry (Tables 6.16 and 6.17). The gating strategy for flow cytometry is show in Figure 6.28.

No significant differences in the percentage of $CD218a^{+}$ $TCRV\alpha7.2^{+}$ MAIT cells or $CD8^{-}CD4^{+}$, $CD8^{+}CD4^{+}$, $CD8^{+}CD4^{-}$ and $CD8^{-}CD4^{-}$ subsets were noted between groups (Fig. 6.31 A.). No significant differences in the percentage of non-MAIT cells were noted between groups (Fig. 6.31). Within the non-MAIT population the percentage of $CD8^{-}CD4^{+}$ cells were significantly higher in the CE group when compared to the CE28 group (Fig. 6.31 B.). A significantly higher percentage of $CD8^{+}CD4^{+}$ cells were noted in the CE28 group when compared to the CE group (Fig. 6.31).

Collectively, the data show that addition of CD28 to *Candida* effluent resulted in decreased percentages of $CD8^{-}CD4^{+}$ subsets in the non-MAIT population whilst also increasing the percentage of $CD8^{+}CD4^{+}$ cells in the non-MAIT population. $CD218a^{+}TCRV\alpha7.2^{+}$ MAIT cells were not significantly affected by the presence of either effluent or CD28.

	CD218a$^+$ TCRVα7.2$^+$ (%) μ ± SD	CD8$^-$CD4$^+$ (%) μ ± SD	CD8$^+$CD4$^+$ (%) μ ± SD	CD8$^+$CD4$^-$ (%) μ ± SD	CD8$^-$CD4$^-$ (%) μ ± SD
ASM	3.4 ± 1.4	5.3 ± 0.8	1.3 ± 0.5	34.3 ± 10.6	59.1 ± 10.1
ASM28	2.5 ± 0.2	5.4 ± 1.5	2.2 ± 0.6	36.8 ± 3.1	55.6 ± 4.3
CE	2.2 ± 0.2	5.9 ± 0.8	1.7 ± 0.1	37.8 ± 3.8	54.6 ± 4.3
CE28	2.2 ± 0.1	6.2 ± 0.5	1.7 ± 0.2	36.6 ± 4.6	55.4 ± 4.3

Table 6.16: *Percentage of CD218a$^+$TCRVα7.2$^+$ MAIT cells determined as a percentage of the parent TCRVα7.2$^+$CD3$^+$ T-cell population as well as percentage of CD8$^-$CD4$^+$, CD8$^+$CD4$^+$, CD8$^+$CD4$^-$ and CD8$^-$CD4$^-$ subsets*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CE28 (10% v/v Candida effluent with CD28)

	Non-MAIT (%) μ ± SD	CD8$^-$CD4$^+$ (%) μ ± SD	CD8$^+$CD4$^+$ (%) μ ± SD	CD8$^+$CD4$^-$ (%) μ ± SD	CD8$^-$CD4$^-$ (%) μ ± SD
ASM	96.6 ± 1.4	56.8 ± 13.4	2.8 ± 2.5	29.3 ± 9.4	11.1 ± 1.8
ASM28	97.4 ± 0.2	62.9 ± 0.5	2.9 ± 0.6	25.7 ± 1.1	8.4 ± 0.7
CE	97.7 ± 0.2	65.0 ± 0.6	1.4 ± 0.4	25.4 ± 0.4	8.2 ± 0.5
CE28	97.7 ± 0.2	62.8 ± 0.5	3.0 ± 0.6	25.3 ± 1.1	8.9 ± 0.4

Table 6.17: *Percentage of non-MAIT cells in determined as a percentage of the parent TCRVα7.2$^+$CD3$^+$ T-cell population as well as percentage of CD8$^-$CD4$^+$, CD8$^+$CD4$^+$, CD8$^+$CD4$^-$ and CD8$^-$CD4$^-$ subsets*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CE28 (10% v/v Candida effluent with CD28)

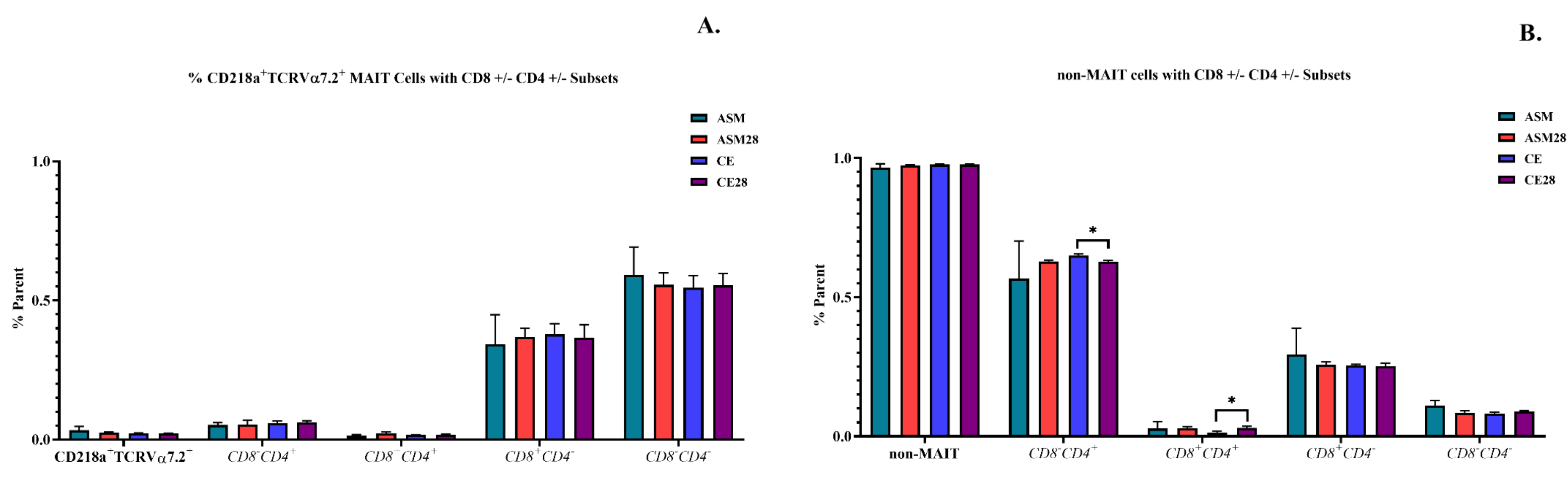

Figure 6.31: *Percentage of CD218a⁺TCRVα7.2⁺ MAIT (A.), non-MAIT (B.) and CD8 +/- CD4+/- subsets as a percentage of the parent population*

Legend: ASM (10% v/v ASM), ASM28 (10% v/v ASM with CD28), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28)

** significance of less than or equal to 0.05 for comparisons, ** significance of less than or equal to 0.01 for comparisons, *** significance of less than or equal to 0.001 for comparisons*

6.4.3.4 Comparison between CD161^{+}TCRVα7.2^{+} and CD218a^{+} TCRVα7.2^{+} MAIT and the non-MAIT cell populations

No significant differences existed between the percentage of CD161^{+} TCRVα7.2^{+} and CD218a^{+}TCRVα7.2^{+} MAIT cells or the percentage of CD8^{-}CD4^{+}, CD8^{+}CD4^{+}, CD8^{+}CD4^{-} and CD8^{-}CD4^{-} subsets. No significant differences existed in the percentage of the CD161^{+}TCRVα7.2^{+} and CD218a^{+}TCRVα7.2^{+} non-MAIT cell populations or the percentage of CD8^{-}CD4^{+}, CD8^{+}CD4^{+}, CD8^{+}CD4^{-} and CD8^{-}CD4^{-} subsets.

6.5 Discussion

In this study we aimed to determine, for the first time, if the production of inflammatory cytokines and percentage of MAIT cells increased or decreased in the presence of effluent derived from *C. albicans* biofilms. In order to facilitate this study, optimisation was performed to determine the optimal concentration of effluent derived from *C. albicans* biofilm to be diluted in PBMC. This study validated optimal concentrations of *Candida* effluent (10% v/v) and ASM (10% v/v) diluted in PBMC media for culturing PMBC. The data shows that PMA/Ionomycin and *Candida* effluent were significant factors that increased the concentration of IFN-γ, TNF-α, IL-17A, IL-18, IL-22 and IL-23 in PBMC. The percentage of $CD161^{+}TCRV\alpha7.2^{+}$ and $CD218a^{+}TCRV\alpha7.2^{+}$ MAIT cells MAIT were shown to be not significantly affected by the presence of either *Candida* effluent or CD28.

Arzmi et al., 2018 used biofilm effluent derived from *C. albicans, Actinomyces naeslundii* and *S. mutans* as well as ASM on malignant (H357) and normal (OFK6) cells to measure cytokine production at 2 and 24 hours. This study confirmed a significant increase in pro-inflammatory cytokine production in all effluent groups at both timepoints when compared to the ASM control (Arzmi et al., 2018), thus confirming that microbial and *Candida* derived effluent has the ability to induce production of cytokines. Effluent derived from *C. albicans* biofilm was used in this study to stimulate production of inflammatory cytokines in PBMC and to our knowledge this is the first study to do so.

Of the cytokines measured, IFN-γ, TNF-α and IL-17A were the cytokines most affected by the presence of PMA/Ionomycin with IL-22 and IL-23 showing some upregulation in the presence of PMA/Ionomycin. Furthermore, IFN-γ and TNF-α showed a general trend for increased concentration at 24 hours in the presence of PMA/Ionomycin. For IL-17A, the presence of PMA/Ionomycin at any timepoint resulted in greater concentration than in samples without PMA/Ionomycin at any timepoint. Interestingly, for IL-18 samples with PMA/Ionomycin in the ASM groups at earlier timepoints inhibited cytokine expression at 8 hours with increased expression noted at later timepoints. Thus, presence of PMA/Ionomycin was a significant factor that increased the concentration of IFN-γ, TNF-α, IL-17A, IL-22 and IL-23. This was not surprising as PMA/Ionomycin is a potent stimulator of cytokines, especially for IL-17 (Olsen and Sollid, 2013) that has been used, in this study and others (Dias et al., 2017b, Gibbs et al., 2017), as a positive control.

TNF-α, IL-18, IL-22, IL-23 and to a lesser extend IFN-γ and IL-17A also demonstrated significant concentration changes in the presence of effluent derived from *C. albicans* biofilm. It should be noted that exceptionally high levels of TNF-α were detected in all control and treatment groups; higher than the levels detected in previous studies assessing the effect *Candida* effluent and ASM on cells (Arzmi et al., 2018). In contrast to this previous study (Arzmi et al., 2018), *Candida* effluent and ASM were used on PBMC rather than epithelial cells. Thus, the contrast in concentration could be explained by the cell type with PBMC potentially being more sensitive to producing TNF-α in the presence of both *Candida* effluent and ASM.

PBMC exposed to heat inactivated *C. albicans* were able to induce production of IFN-γ (Toth et al., 2013). As shown in this study *C. albicans* effluent on its own was able to induce production of IFN-γ with increased concentrations demonstrated with the addition of PMA/Ionomycin. In contrast, *Candida* effluent previously was shown to be unable to induce production of IFN-γ from oral keratinocytes (Arzmi et al., 2018). This could also suggest a greater susceptibility of PBMC to *Candida* effluent for production of IFN-γ that is not demonstrated in oral keratinocytes. The method of cytokine stimulation in the previous study (Arzmi et al., 2018) may also account for why oral keratinocytes were unable to produce IFN-γ and it is possible that heat inactivated *C. albicans* (Toth et al., 2013) may offer a greater stimulatory effect for inducing production of IFN-γ than *Candida* effluent.

IL-18 concentration was shown to be increased in samples with *Candida* effluent and CD28 at all timepoints when compared against matched controls. This same trend was noted for TNF-α. Use of CD28 has been used in multiple MAIT cell studies in the presence of a microbial challenge (Dias et al., 2017a, Dias et al., 2017b, Gibbs et al., 2017) for the likely reason of providing a co-stimulatory signal for T-cell activation. For this study increased concentration of IL-18 and TNF-α in the presence of effluent derived from *C. albicans* biofilm and CD28 together could be explained by the co-stimulatory effect of CD28.

IL-12p70 concentration was not significantly affected by any factor. IL-12p40 showed increased concentration at 24 hours in the ASM28 group with significantly higher concentration noted in the ASM compared to CE group. However, due to low levels of Il-12p40 concentration it is difficult to make assumptions based on the above results.

A study by Toth et al., 2013 measured similar cytokines after exposing PBMC to heat inactivated *C. albicans* and *C. parapslosis* in order to assess for differences between the two strains of *Candida.* Whilst both strains of *Candida* produced similar quantities of TNF-α and IL-6, it was noted that *C. albicans* was able to induce higher levels of IL-22 and IL-17 production in PBMC (Toth et al., 2013). Thus, this study and results demonstrated by Toth et al., 2013 confirm *C. albicans,* both the heat inactivated and effluent form, can induce inflammatory cytokine production in PBMC.

Upregulation of IFN-γ, TNF-α, IL-18, IL-22, IL-23 and IL-17A, especially in the presence of *Candida* effluent could be suggestive of MAIT cell activation (Le Bourhis et al., 2010, Dusseaux et al., 2011, Gibbs et al., 2017). However, for interpretation of the BioPlex results it should be noted that the observed concentration was reported for all cytokines except IL-18, as the concentrations were out of range of the standard curve and in some cases below the limit of detection. These facts need to be considered when interpreting the results of significant changes in concentration and fold changes.

The percentage of $CD8^+CD^+$ subset within the $CD3^+CD45^+$ parent population was significantly higher in the presence of both *Candida* effluent and CD28 than in the presence of *Candida* effluent alone. No significant differences were noted in the MAIT cell populations. This could be in part explained by the low number of MAIT cells detected using flow cytometry or the use of effluent derived from *C. albicans* biofilms as the form of stimulation. Previous studies have been able to induce a MAIT cell response in the in the presence of *Candida* (Le Bourhis et al., 2010). However, previous studies performing functional assays or attempting to induce MAIT cell proliferation have used live fixed microbes (Dias et al., 2017a, Dias et al., 2016, Gibbs et al., 2017). Thus, the form of stimulation used in this study could explain the lack of effect on MAIT cell proliferation and this could be a consideration when planning future experiments. It should also be noted that in line with previous research the majority of $CD161^+TCRV\alpha7.2^+$ and $CD218a^+TCRV\alpha7.2^+$ MAIT cells detected in this study were either $CD8^+CD4^-$ or $CD8^-CD4^-$ (Tilloy et al., 1999, Martin et al., 2009, Gibbs et al., 2017).

Significant differences in percentage the non-MAIT subsets were noted. With respect to the $CD8^+CD4^+$ subsets, both the $CD161^+TCRV\alpha7.2^+$ and $CD218a^+TCRV\alpha7.2^+$ non-MAIT cell populations demonstrated significantly increased percentages in these subsets of cells in the CE28 group when compared to the CE group. For both the $CD161^+TCRV\alpha7.2^+$ and $CD218a^+TCRV\alpha7.2^+$ non-MAIT cell populations significantly

increased percentages of the $CD8^-CD4^+$ subset were noted in the CE group when compared to the CE28 group. This would suggest that for these subsets, CD28 enhanced frequency of $CD8^+CD4^+$ cells in the presence of *Candida* effluent whilst also suppressing $CD8^-CD4^+$ cells in the presence of *Candida* effluent. As discussed in the results with IL-18, CD28 has been used in multiple MAIT cell studies in the presence of a microbial challenge (Dias et al., 2017a, Dias et al., 2017b, Gibbs et al., 2017) with increased $CD8^+CD4^+$ in the CE28 group possibly explained by the T-cell costimulatory effect of CD28.

Identification of MAIT cells with either CD218a or CD161 yielded no differences in the percentage of MAIT cells expressing Vα7.2. The percentage of $CD161^+TCRV\alpha7.2^+$ and $CD218^+$ $TCRV\alpha7.2^+$ MAIT cells detected were not significantly affected by the presence of either effluent or CD28 with no significant differences noted between any of the media treatment groups. This would suggest that neither effluent derived from *C. albicans* biofilms nor CD28 alone or the combination of the two had the ability to induce MAIT cell proliferation.

6.6 Conclusion

This study validated optimal concentrations of *Candida* effluent (10% v/v) and ASM (10% v/v) diluted in PBMC media for culturing PMBC. The presence of PMA/Ionomycin was a significant factor that increased the concentration of IFN-γ, TNF-α, IL-22, IL-23 and IL-17A and to a lesser extent IL-18. *Candida* effluent was shown to be a significant factor for increasing the concentrations of TNF-α, IL-18, IL-22, IL-23 and to a lesser extent IFN-γ and IL-17A. For IL-18 and TNF-α the presence of *Candida* effluent and CD28 together resulted in upregulation of expression when compared to matched control at all timepoints. Whilst upregulation of these cytokines induced by *Candida* effluent is suggestive of a MAIT cell activation, the detected small cytokine concentrations, sometimes under detection limits, need to be considered when assigning significance to the results.

MAIT cells were able to be identified using flow cytometry and in line with previous research the majority of $CD161^{+}TCRV\alpha7.2^{+}$ and $CD218a^{+}TCRV\alpha7.2^{+}$ MAIT cells detected were either $CD8^{+}CD4^{-}$ or $CD8^{-}CD4^{-}$. The frequencies of MAIT cells were not significantly affected by the presence of either *Candida* effluent or CD28 suggesting that neither *Candida* effluent nor CD28 alone or in combination had the ability to induce MAIT cell proliferation. It is possible that the form of stimulation, specifically effluent derived from *C. albicans* biofilm, may not have the inherent ability to induce MAIT cell proliferation or significant activation. Thus, future research is required with consideration to be given to using heat inactivated or fixed forms of *C. albicans* for stimulation.

7 Chapter 7: General Discussion and Future Directions

Oral lichen planus (OLP) OLP is a chronic disease affecting approximately 1-2% of the population and characterised by a cytotoxic T cell response directed at the basal keratinocytes (Axell, 1976, Axell and Rundquist, 1987, Sugerman et al., 2002, Roopashree et al., 2010). What initiates the OLP disease process and maintains the chronic inflammatory state of OLP is currently unknown. The overall aim of this study was to determine whether *Candida* in plays an aetiological role in OLP as well as determine if specific treatment of *Candida* is required in symptomatic patients with OLP. This was investigated by performing a series of experiments including a clinical study, a study on formalin fixed paraffin embedded (FFPE) OLP tissue and control tissue and a cell study utilising peripheral blood mononuclear cells (PBMC). Multiple techniques were employed to analyse patient samples, FFPE tissue, PBMC and PBMC supernatant collected following in vitro cell culture.

7.1 Hypothesis and aim 1 and 2

The first hypothesis was that adjunctive antifungal therapy is required for the symptomatic management of OLP and that inflammation would be reduced in the OLP group treated with adjunctive anti-fungal therapy. The second hypothesis was that *Candida* levels will be lower in OLP patients treated adjunctive antifungal therapy with acetaldehyde production with *Candida* species similar across both test groups. The aim of the study was to assess antifungal therapy effectiveness as an adjunctive treatment in the management of symptomatic patients with OLP. The aim of this study was to also assess observable variation in symptoms and clinical appearance associated with using topical steroids and adjunctive topical antifungals whilst also assessing oral yeast presence, amount, species and acetaldehyde production associated with treatment of symptomatic patients with OLP.

Some studies have shown higher frequencies of *Candida* colonisation in OLP when compared to controls (Zeng et al., 2009) whilst others have postulated presence of *Candida* in potentially malignant lesions such as OLP and malignant lesions is coincidental and reflective of changes to the mucosal environment favouring colonization and overgrowth (McCullough et al., 2002, Hebbar et al., 2013).

Results of the present study showed no significant differences existed between the control group and entire OLP cohort at baseline with respect to colony forming units (CFU) and salivary acetaldehyde levels. However, meaningful statistical analyses could not be performed to assess between the OLP test groups due to the limited number of enrolled patients, specifically 14 control patients and 7 OLP patients, 3 assigned to the placebo group and 4 to the test group. Descriptive statistics showed downward trends in both the OLP test and placebo groups with respect to clinical appearance and subjective analysis of symptoms. With respect to *Candida* and measurements of CFU an upwards trend was noted in the placebo group from 6 to 12 weeks with a downwards trend noted in the antifungal group over 12 weeks. In keeping with the literature (Krogh et al., 1987b), *C. albicans* was the dominant species cultured. Upwards trends in salivary acetaldehyde levels were also noted over 12 weeks in the OLP placebo group.

A previous study has shown that adjunctive antifungal therapy when using topical steroids prevented candidosis but did not improve the efficacy of therapy when managing OLP (Lodi et al., 2007). Systematic review and meta-analysis of the management of OLP

concluded that from the current evidence if cannot be determined if antifungal therapy is truly effective in the management of OLP (Thongprasom et al., 2011, Lodi et al., 2012).

Due to the limited number of patients enrolled in this study and the small samples sizes, meaningful statistical analysis could not be performed, and results of this clinical study need to be viewed with caution. Based on the above results it cannot be conclusively inferred whether adjunctive treatment with a topical antifungal had any significant effect the presence of symptoms, erythema, *Candida, Candida spp.* or production of salivary acetaldehyde.

Low patient numbers and lack of significance means we cannot reject the null hypothesis. Further research is required to assess whether treatment with adjunctive antifungal therapy is required in the symptomatic management of OLP with steroids.

7.2 Hypothesis and aim 3

The hypothesis was that HALO™ (Indica Labs, USA) image analysis software will be more sensitive for single antibody and tissue segmentations analysis of multiplex immunohistochemistry (mIHC) slides than inForm 2.4.1 (PerkinElmer, USA) image analysis software. The aim of this study was to determine variation between HALO™ (Indica Labs, USA) and inForm 2.4.1 (PerkinElmer, USA) for single antibody and tissue segmentation analysis of mIHC slides using an antibody panel for identification of mucosal associated invariant T (MAIT) cells.

MAIT cells are a recently characterised subset of T cells able to be activated by major histocompatibility complex class 1 related protein (MR-1) bound riboflavin derivatives (Tilloy et al., 1999, Gold et al., 2010, Cowley, 2014) and able to be produced by *Candida spp.* Whilst it was shown that adjunctive antifungal therapy is not required in the management of symptomatic OLP (Chapter 3) it was of interest to pursue the role of *Candida* in OLP pathogenesis with respect to identification of MAIT cells and to validate a technique for identification of MAIT cells in OLP FFPE tissue.

Fluorescent mIHC utilises primary antibodies conjugated to fluorophores excited by different wavelengths to allow for detection of multiple makers on one tissue section (Stack et al., 2014). The multispectral image (MSI) generated from this technique can be linearly unmixed for later quantitative image analysis (Dickinson et al., 2001, Mansfield, 2014). To our knowledge, this is the first study to compare between two quantitative image analysis platforms, inForm 2.4.1 (PerkinElmer, USA) and HALO™ (Indica Labs, USA), for mIHC performed on oral mucosa. To our knowledge this is also the first mIHC panel performed on oral mucosa using a panel suitable for identification of MAIT cells consisting of CD3, Interleukin 18 receptor 1 (IL18R1), CD161, MR-1, CD8 and T cell receptor (TCR) Vα7.2 (Sugerman et al., 2000a, Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016, Gibbs et al., 2017).

The results of the present study indicated that analysis with HALO™ (Indica Labs, USA) showed less variability than inForm 2.4.1 (PerkinElmer, USA) for single antibody identification. Assessment of variability for tissue segmentation was only slightly less using inForm 2.4.1 (PerkinElmer, USA) when compared to HALO™ (Indica Labs, USA). Inter-platform and inter-operator reproducibility for digital platforms as well as inter-operator reproducibility has been assessed in other studies (Acs et al., 2019). Whilst this study showed high inter-platform and inter-operator reproducibility (Acs et al., 2019) it

is important to note this previous study assessed one marker only. Hence, the variabilities between programs noted in the presented study is expected due to the increased complexity of analysis expected with analyses of 7 markers.

Overall the findings from this study indicated HALO™ (Indica Labs, USA) was the more reliable program for mIHC quantitative cell analysis and this validated use of HALO™ (Indica Labs, USA) for subsequent experiments (Chapter 5).

The null hypothesis is therefore rejected as HALO™ (Indica Labs, USA) was shown to be the overall more reliable program for quantitative mIHC analysis in OLP.

7.3 Hypothesis and aim 4

The hypothesis was that that OLP affected biopsy tissue will have higher numbers of MAIT cells than control tissue and that OLP biopsy tissue with concurrent presence of *Candida* will have higher numbers of MAIT cells than OLP biopsy tissue without *Candida*. The aim of this study was to determine the number of MAIT present in OLP tissue and control tissue, as well as determine if OLP tissue with *Candida* exhibited higher numbers of MAIT cells than OLP tissue without *Candida*. Whilst it was shown that adjunctive antifungal therapy is not required in the management of symptomatic OLP (Chapter 3) it was of interest to determine if the presence of *Candida* or symptoms in OLP could be a driving factor in the chronic T cell infiltrate of OLP.

To date, this is the first study to characterise the presence of MAIT cells in OLP using mIHC and a panel of antibodies (Chapter 4) to identify MAIT cells. Previously validated phenotypes used for MAIT cell identification included triple positive CD3 + IL18R1 + TCRVα7.2 and CD3 + CD161+ TCRVα7.2 (Dusseaux et al., 2011, Hiejima et al., 2015, Li et al., 2016) and double positive CD3 + TCRVα7.2 and TCRVα7.2 + IL18R1 phenotypes (Gibbs et al., 2017). Seven markers were available for use with phenotyping in this study. Phenotyping was performed using CD3 and a combination of at least two of the following markers CD161, IL18R1 or TCRVa7.2. This allowed for identification of MAIT cells using double, triple or quadruple positive phenotypes plus nuclear counterstain.

MAIT cells were shown to be present within OLP tissue and when present congregated within the OLP inflammatory infiltrate. MR-1 expression did not correlate with variations in MAIT cell phenotype expression within the OLP or control groups. MR-1 expression was also not significantly affected by the presence of *Candida* or symptoms in OLP.

Significant differences existed between the different treatment groups and the expression of MAIT cell phenotypes in tissue. Specifically, CD161 phenotypes were decreased in symptomatic OLP whilst TCRVα7.2 phenotypes were decreased in OLP with *Candida*. *Candida* and symptoms together were significantly associated with DAPI + CD3 expression, specifically with a decrease in this phenotype noted in the presence of *Candida.*

Systemic lupus erythematosus (SLE) mimics OLP both clinically and histologically, studies in SLE have also shown decreased numbers of MAIT cells in SLE correlating

with disease activity (Cho et al., 2014, Chiba et al., 2017). Based on the results it could be hypothesised that either *Candida* or symptoms in OLP downregulates MAIT cell activation, or that decreased MAIT cell expression in OLP patients results in those patients being more likely to be symptomatic with an inherent susceptibility for colonisation of *Candida.*

Whilst the number of MAIT cells were not higher in OLP or OLP with concurrent *Candia* significant differences did existed in the number of MAIT cells expressed between groups. Hence the null hypothesis is rejected as these results demonstrated that patients with poorer MAIT cell expression are more likely to be symptomatic with an inherent susceptibility for candidal infection.

7.4 Hypothesis and aim 5

The hypothesis was that that both the number of MAIT cells present and the production of inflammatory cytokines will increase in PBMC after exposure to *C. albicans* biofilm effluent. The aim of this study was to determine the production of inflammatory cytokines and the percentage of MAIT cells after PBMC are exposed to *C. albicans* biofilm effluent. As it was shown that MAIT cell expression is suppressed in the presence of *Candida* (Chapter 5) it was of interest to explore MAIT cell activation and proliferation in the presence of *Candida* in the form of *C. albicans* effluent.

A panel of cytokines consisting of IL-17A, IL-22, IL-23, IL-18 IL-12p40, IL-12p70, TNF-α and IFN-γ were analysed. IL-17, IL-22, TNF-α and IFN-γ are produced by activated MAIT cells with IL-18 and IL-12 involved in MAIT cell activation (Manigold et al., 2000, Martin et al., 2009, Dusseaux et al., 2011). IL-22, IL-23, IL-17 and IL-18 upregulation has been demonstrated in OLP along with IL-12A gene polymorphisms (Chen et al., 2013, Wang et al., 2013, Lu and Zeng, 2014, Shen et al., 2016, Piccinni et al., 2014, Jiang et al., 2015, Wang et al., 2015).

This study validated optimal concentrations (10% v/v) of *Candida* effluent and (10% v/v) ASM diluted in PBMC media for culturing PMBC. A recent study validated culture of malignant (H357) and normal (OFK6) cells in the presence of *C. albicans, Actinomyces naeslundii* and *S. mutans* effluent and ASM at 80% v/v to measure cytokine production at 2 and 24 hours (Arzmi et al., 2018). This study showed that microbes and *Candida* derived effluent had the ability to induce and increase pro-inflammatory cytokine production at both timepoints in all effluent groups (Arzmi et al., 2018).

In the present study the data showed that PMA/Ionomycin was a significant factor that increased the concentrations of IFN-γ, TNF-α, IL-17A, IL-22 and IL-23 and to a lesser extent IL-18. Incubation in the presence of *Candida* effluent increased the concentration of TNF-α, IL-18, IL-23, IL-22 and to a lesser extent IFN-γ and IL-17A. When compared to matched controls at 8, 12- and 24-hours, IL-18 and TNF-α exhibited significantly higher concentrations in the presence of both *Candida* effluent and CD28.

Whilst increased cytokine concentration is suggestive of a MAIT cell activation it should be noted that for all cytokines except IL-18 concentrations fells outside of the standard curve and the observed concentrations were reported and in some cases concentrations

observed were either below or above the limit of detection. These facts need to be considered when interpreting these findings.

MAIT cells were able to be identified using flow cytometry with the majority of $CD161^{+}TCRV\alpha7.2^{+}$ and $CD218a^{+}TCRV\alpha7.2^{+}$ MAIT cells being either $CD8^{+}CD4^{-}$ or $CD8^{-}CD4^{-}$ as previously shown (Tilloy et al., 1999, Martin et al., 2009, Gibbs et al., 2017). MAIT cells that were detected were not significantly affected by the presence of either effluent or CD28. This is suggestive of the fact that neither *Candida* effluent, CD28 or the combination of the two had the ability to induce MAIT cell proliferation.

Whilst *Candida* effluent was not able to induce MAIT cell proliferation the null hypothesis is still rejected as production of inflammatory cytokines was significantly increased in the presence of effluent derived from *C. albicans* biofilms for TNF-α, IL-18, IL-23, IL-22, IFN-γ and IL-17A.

7.5 Conclusions and Future Directions

This research has shown that adjunctive treatment of symptomatic OLP with a topical antifungal had no significant effect the presence of symptoms, erythema, CFU, *Candida spp.* or production of salivary acetaldehyde. This research also showed that HALO™ (Indica Labs, USA) was a reliable tool for mIHC quantitative cell analysis in OLP. Using this analysis software, it was shown that MAIT cells were present within the OLP inflammatory infiltrate and that patients with poorer MAIT cell expression are more likely to be symptomatic with an inherent susceptibility for candidal infection. Finally, whilst *Candida* effluent did not induce proliferation of MAIT cells in PBMC, cytokines association with both OLP and MAIT cell activation, specifically IFN-γ, TNF-α, IL-18, IL-23, IL-22 and IL-17A, were upregulated in the presence of effluent derived from *C. albicans*.

Further research is required to validate these findings. Future clinical studies are needed to explore the need for adjunctive antifungal therapy in the management of symptomatic OLP and a multicentre study design should be considered as enrolling patients from multiple centres will assist in maximising enrolment. This will allow for meaningful statistical analyses to be performed. Stratifying the patients into groups of 10, 5 placebo and 5 tests as with the above study (Chapter 3) will assist in ensuring even group allocation during enrolment.

For future mIHC studies assessing MAIT cells in OLP tissue use of fresh frozen rather than FFPE tissue samples may help to eliminate issues with background staining whilst also maximising on antigen retrieval and biomarker expression. Use of an autostainer during the mIHC process will allow for larger numbers of slides to be stained per batch whilst also reducing variability of staining between batches.

For future analyses of MAIT cells in OLP researchers should assess blood from OLP and non-OLP patients and separate the PBMC to assess the variabilities in MAIT number and cytokine production between OLP and non-OLP patients. Blood could also be taken from OLP patients before and after treatment to assess for changes in MAIT cell number and cytokine production associated with treatment. For stimulation of PBMC heat inactivated forms of *Candida* may provide greater stimulation of PBMC in culture and further insight into MAIT cell proliferation and cytokines associated with activation.

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9 Appendices

Appendix I

	8 hours	12 hours	24 hours
CE vs. ASM	N/A	N/A	N/A
CE28 vs. ASM28	N/A	N/A	N/A
CE28PMA vs. ASM28PMA	8.36	1.42	5.23
CEPMA vs. ASMPMA	16.73	0.29	3.18

Table 9.1: *Fold changes in log(IFN-γ) concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

N/A depicts fold changes were unable to be determined

denotes significant fold change increase (orange)

denotes significant fold change decrease (blue)

	8 hours	12 hours	24 hours
CE vs. ASM	1.40	0.87	1.33
CE28 vs. ASM28	2.08	1.36	1.24
CE28PMA vs. ASM28PMA	4.25	1.17	0.89
CEPMA vs. ASMPMA	3.04	0.56	1.80

Table 9.2: *Fold changes in TNF-α concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase

denotes significant fold change decrease

	8 hours	12 hours	24 hours
CE vs. ASM	0	5.13	4.00
CE28 vs. ASM28	0	N/A	N/A
CE28PMA vs. ASM28PMA	1.65	1.28	3.01
CEPMA vs. ASMPMA	0.94	0.32	3.69

Table 9.3: *Fold changes in IL-17A concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

N/A depicts fold changes were unable to be determined

denotes significant fold change increase (orange)

denotes significant fold change decrease (blue)

	8 hours	12 hours	24 hours
CE vs. ASM	0.62	2.93	0.46
CE28 vs. ASM28	4.67	1.71	0.37
CE28PMA vs. ASM28PMA	0.20	2.84	0.40
CEPMA vs. ASMPMA	0.89	0.31	1.49

Table 9.4: *Fold changes in IL-12p40 concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase (orange)

denotes significant fold change decrease (blue)

	8 hours	12 hours	24 hours
CE vs. ASM	1.41	0.77	0.62
CE28 vs. ASM28	0.99	1.10	1.00
CE28PMA vs. ASM28PMA	1.09	0.94	0.93
CEPMA vs. ASMPMA	1.12	1.09	0.81

Table 9.5: *Fold changes in IL-12p70 concentration at 8, 12 and 24 hours.*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase

denotes significant fold change decrease

	8 hours	12 hours	24 hours
CE vs. ASM	6.64	0.70	1.36
CE28 vs. ASM28	1.53	1.57	0.46
CE28PMA vs. ASM28PMA	7.68	4.93	4.56
CEPMA vs. ASMPMA	0.60	0.91	3.50

Table 9.6: *Fold changes in IL-22 concentration at 8, 12 and 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase (orange)

denotes significant fold change decrease (blue)

	8 hours	12 hours	24 hours
CE vs. ASM	N/A	0.27	1.14
CE28 vs. ASM28	1.22	3.44	0.08
CE28PMA vs. ASM28PMA	N/A	21.33	0.08
CEPMA vs. ASMPMA	2.11	0.93	2.47

Table 9.7: *Fold changes in IL-23 concentration at 8, 12 and 24 hours.*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

denotes significant fold change increase (orange)

denotes significant fold change decrease (blue)

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	OOR <	[b] 39.78
ASMPMA 2	OOR <	[b] 5.08
ASMPMA 3	OOR <	[b] 5.08
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	[b] 36.26
ASM28PMA 3	OOR <	[b] 28.83
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	OOR <
CEPMA 1	OOR <	[b] 233.44
CEPMA 2	OOR <	[b] 350.44
CEPMA 3	OOR <	[b] 251.55
CE28 1	OOR <	OOR <
CE28 2	OOR <	[b] 16.17
CE28 3	OOR <	[b] 20.68
CE28PMA 1	OOR <	[b] 257.51
CE28PMA 2	OOR <	[b] 90.44
CE28PMA 3	OOR <	[b] 195.98

Table 9.8: *Raw triplicate data for IFN-γ at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	OOR <	[b] 66.68
ASMPMA 2	OOR <	[b] 111.15
ASMPMA 3	OOR <	[b] 185.23
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	[b] 191.7
ASM28PMA 2	OOR <	[b] 49.8
ASM28PMA 3	OOR <	[b] 24.87
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	OOR <
CEPMA 1	OOR <	[b] 20.68
CEPMA 2	OOR <	[b] 38.03
CEPMA 3	OOR <	[b] 46.54
CE28 1	OOR <	OOR <
CE28 2	OOR <	OOR <
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	[b] 275.17
CE28PMA 2	OOR <	[b] 46.54
CE28PMA 3	OOR <	[b] 56.12

Table 9.9: *Raw triplicate data for IFN-γ at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	OOR <	[b] 440.81
ASMPMA 2	OOR <	[b] 178.7
ASMPMA 3	OOR <	[b] 174.3
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	[b] 347.7
ASM28PMA 2	OOR <	[b] 174.3
ASM28PMA 3	OOR <	[b] 36.26
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	OOR <
CEPMA 1	OOR <	[b] 1,028.39
CEPMA 2	OOR <	[b] 730.51
CEPMA 3	OOR <	[b] 765.53
CE28 1	OOR <	OOR <
CE28 2	OOR <	OOR <
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	[b] 2,455.55
CE28PMA 2	OOR <	[b] 183.06
CE28PMA 3	OOR <	[b] 282.92

Table 9.10: *Raw triplicate data for IFN-γ at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR >	[b] 58,359.68
ASM 2	53,708.79	53,708.79
ASM 3	OOR >	[b] 62,920.15
ASMPMA 1	53,925.85	53,925.85
ASMPMA 2	34,890.45	34,890.45
ASMPMA 3	34,711.3	34,711.3
ASM28 1	OOR >	[b] 63,508.10
ASM28 2	39,482.85	39,482.85
ASM28 3	OOR >	[b] 56,813.34
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	48,567.69	48,567.69
ASM28PMA 3	32,683.15	32,683.15
CE 1	OOR >	[b] 64,182.03
CE 2	OOR >	[b] 97,137.15
CE 3	OOR >	[b] 83,490.84
CEPMA 1	OOR >	[b] 136,819.64
CEPMA 2	OOR >	[b] 116,661.99
CEPMA 3	OOR >	[b] 122,316.32
CE28 1	OOR >	[b] 105,747.95
CE28 2	OOR >	[b] 118,202.86
CE28 3	OOR >	[b] 108,913.90
CE28PMA 1	OOR >	[b] 126,722.97
CE28PMA 2	OOR >	[b] 105,101.28
CE28PMA 3	OOR >	[b] 113,708.14

Table 9.11: *Raw triplicate data for TNF-α at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR >	[b] 104,852.23
ASM 2	OOR >	[b] 102,024.87
ASM 3	OOR >	[b] 95,652.95
ASMPMA 1	OOR >	[b] 154,713.73
ASMPMA 2	OOR >	[b] 151,413.19
ASMPMA 3	OOR >	[b] 177,067.04
ASM28 1	OOR >	[b] 98,741.09
ASM28 2	OOR >	[b] 95,935.00
ASM28 3	OOR >	[b] 106,889.05
ASM28PMA 1	OOR >	[b] 161,603.01
ASM28PMA 2	OOR >	[b] 126,958.76
ASM28PMA 3	OOR >	[b] 106,542.16
CE 1	OOR >	[b] 73,668.22
CE 2	OOR >	[b] 66,479.28
CE 3	OOR >	[b] 124,311.48
CEPMA 1	22,987.31	22,987.31
CEPMA 2	OOR >	[b] 110,412.72
CEPMA 3	OOR >	[b] 135,125.05
CE28 1	OOR >	[b] 144,298.64
CE28 2	OOR >	[b] 133,659.18
CE28 3	OOR >	[b] 130,904.29
CE28PMA 1	OOR >	[b] 175,212.33
CE28PMA 2	OOR >	[b] 137,260.04
CE28PMA 3	OOR >	[b] 149,441.62

Table 9.12: *Raw triplicate data for TNF-α at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR >	[b] 90,084.27
ASM 2	OOR >	[b] 76,390.44
ASM 3	OOR >	[b] 79,599.14
ASMPMA 1	OOR >	[b] 123,599.91
ASMPMA 2	OOR >	[b] 102,150.49
ASMPMA 3	OOR >	[b] 112,344.68
ASM28 1	OOR >	[b] 97,392.31
ASM28 2	OOR >	[b] 89,429.55
ASM28 3	OOR >	[b] 90,371.85
ASM28PMA 1	OOR >	[b] 191,188.28
ASM28PMA 2	OOR >	[b] 156,869.36
ASM28PMA 3	OOR >	[b] 129,592.31
CE 1	OOR >	[b] 124,974.65
CE 2	OOR >	[b] 102,602.33
CE 3	OOR >	[b] 99,324.57
CEPMA 1	OOR >	[b] 211,483.34
CEPMA 2	OOR >	[b] 192,865.92
CEPMA 3	OOR >	[b] 205,329.65
CE28 1	OOR >	[b] 117,914.39
CE28 2	OOR >	[b] 110,976.24
CE28 3	OOR >	[b] 114,145.36
CE28PMA 1	OOR >	OOR >
CE28PMA 2	OOR >	[b] 167,544.96
CE28PMA 3	OOR >	[b] 155,860.13

Table 9.13: *Raw triplicate data for TNF-α at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 1.38
ASMPMA 1	38.18	38.18
ASMPMA 2	32.13	32.13
ASMPMA 3	28.14	28.14
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	[b] 0.55
ASM28 3	OOR <	[b] 0.78
ASM28PMA 1	OOR <	[b] 0.48
ASM28PMA 2	10.81	10.81
ASM28PMA 3	10.64	10.64
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	OOR <
CEPMA 1	30.31	30.31
CEPMA 2	30.13	30.13
CEPMA 3	31.95	31.95
CE28 1	OOR <	OOR <
CE28 2	OOR <	OOR <
CE28 3	OOR <	OOR <
CE28PMA 1	14.56	14.56
CE28PMA 2	11.74	11.74
CE28PMA 3	9.8	9.8

***Table 9.14**: Raw triplicate data for IL-17A at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 0.48
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	18.73	18.73
ASMPMA 2	20.83	20.83
ASMPMA 3	25.45	25.45
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	4.53	4.53
ASM28PMA 2	4.36	4.36
ASM28PMA 3	OOR <	[b] 0.19
CE 1	OOR <	[b] 1.68
CE 2	OOR <	[b] 0.78
CE 3	OOR <	OOR <
CEPMA 1	OOR <	OOR <
CEPMA 2	9.97	9.97
CEPMA 3	10.81	10.81
CE28 1	OOR <	OOR <
CE28 2	OOR <	OOR <
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	[b] 3.09
CE28PMA 2	4.85	4.85
CE28PMA 3	3.72	3.72

Table 9.15: *Raw triplicate data for IL-17A at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 0.12
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	9.55	9.55
ASMPMA 2	OOR <	[b] 2.77
ASMPMA 3	OOR <	[b] 2.77
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	10.81	10.81
ASM28PMA 2	9.13	9.13
ASM28PMA 3	OOR <	OOR <
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	[b] 0.48
CEPMA 1	23.22	23.22
CEPMA 2	15.77	15.77
CEPMA 3	16.12	16.12
CE28 1	OOR <	[b] 0.26
CE28 2	OOR <	[b] 0.92
CE28 3	OOR <	OOR <
CE28PMA 1	48.84	48.84
CE28PMA 2	4.69	4.69
CE28PMA 3	6.4	6.4

Table 9.16: *Raw triplicate data for IL-17A at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	1.2	1.2
ASM 2	1.01	1.01
ASM 3	1.31	1.31
ASMPMA 1	0.72	0.72
ASMPMA 2	OOR <	[b] 0.52
ASMPMA 3	OOR <	[b] 0.46
ASM28 1	0.95	0.95
ASM28 2	0.66	0.66
ASM28 3	0.91	0.91
ASM28PMA 1	OOR <	[b] 0.23
ASM28PMA 2	0.7	0.7
ASM28PMA 3	0.54	0.54
CE 1	1.2	1.2
CE 2	1.35	1.35
CE 3	1.41	1.41
CEPMA 1	1.22	1.22
CEPMA 2	1.01	1.01
CEPMA 3	1.06	1.06
CE28 1	1.35	1.35
CE28 2	1.26	1.26
CE28 3	1.38	1.38
CE28PMA 1	1.17	1.17
CE28PMA 2	1.12	1.12
CE28PMA 3	1.26	1.26

***Table 9.17**: Raw triplicate data for IL-18 at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	**Concentration in Range pg/mL**	**Observed Concentration pg/mL**
ASM 1	1.38	1.38
ASM 2	1.41	1.41
ASM 3	1.28	1.28
ASMPMA 1	1.33	1.33
ASMPMA 2	1.36	1.36
ASMPMA 3	1.35	1.35
ASM28 1	1.15	1.15
ASM28 2	0.91	0.91
ASM28 3	1.04	1.04
ASM28PMA 1	1.15	1.15
ASM28PMA 2	0.89	0.89
ASM28PMA 3	0.69	0.69
CE 1	0.88	0.88
CE 2	0.62	0.62
CE 3	1.48	1.48
CEPMA 1	OOR <	[b] 0.39
CEPMA 2	1.12	1.12
CEPMA 3	1.14	1.14
CE28 1	1.75	1.75
CE28 2	1.48	1.48
CE28 3	1.46	1.46
CE28PMA 1	1.23	1.23
CE28PMA 2	1.46	1.46
CE28PMA 3	1.38	1.38

Table 9.18: *Raw triplicate data for IL-18 at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	**Concentration in Range pg/mL**	**Observed Concentration pg/mL**
ASM 1	1.15	1.15
ASM 2	1.18	1.18
ASM 3	1.25	1.25
ASMPMA 1	1.06	1.06
ASMPMA 2	0.76	0.76
ASMPMA 3	0.76	0.76
ASM28 1	1.18	1.18
ASM28 2	1.14	1.14
ASM28 3	1.11	1.11
ASM28PMA 1	1.25	1.25
ASM28PMA 2	1.03	1.03
ASM28PMA 3	1.41	1.41
CE 1	1.35	1.35
CE 2	1.35	1.35
CE 3	1.25	1.25
CEPMA 1	1.86	1.86
CEPMA 2	1.31	1.31
CEPMA 3	1.53	1.53
CE28 1	1.51	1.51
CE28 2	1.46	1.46
CE28 3	1.48	1.48
CE28PMA 1	2.33	2.33
CE28PMA 2	1.38	1.38
CE28PMA 3	1.49	1.49

Table 9.19: *Raw triplicate data for IL-18 at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 0.55
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 6.66
ASMPMA 1	OOR <	[b] 1.68
ASMPMA 2	OOR <	[b] 2.87
ASMPMA 3	OOR <	[b] 0.55
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	[b] 0.55
ASM28 3	OOR <	[b] 0.55
ASM28PMA 1	OOR <	[b] 6.02
ASM28PMA 2	OOR <	[b] 2.27
ASM28PMA 3	OOR <	OOR <
CE 1	OOR <	[b] 0.55
CE 2	OOR <	[b] 2.27
CE 3	OOR <	[b] 1.68
CEPMA 1	OOR <	[b] 2.87
CEPMA 2	OOR <	[b] 1.68
CEPMA 3	OOR <	OOR <
CE28 1	OOR <	OOR <
CE28 2	OOR <	[b] 2.87
CE28 3	OOR <	[b] 2.27
CE28PMA 1	OOR <	[b] 0.55
CE28PMA 2	OOR <	[b] 0.55
CE28PMA 3	OOR <	0.55

***Table 9.20**: Raw triplicate data for IL-12p40 at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 2.87
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 1.68
ASMPMA 1	OOR <	[b] 13.37
ASMPMA 2	OOR <	[b] 0.55
ASMPMA 3	OOR <	[b] 7.32
ASM28 1	OOR <	[b] 1.68
ASM28 2	OOR <	OOR <
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	[b] 0.55
ASM28PMA 2	OOR <	[b] 1.68
ASM28PMA 3	OOR <	OOR <
CE 1	OOR <	[b] 7.97
CE 2	OOR <	[b] 5.37
CE 3	OOR <	OOR <
CEPMA 1	OOR <	OOR <
CEPMA 2	OOR <	[b] 6.66
CEPMA 3	OOR <	OOR <
CE28 1	OOR <	OOR <
CE28 2	OOR <	[b] 2.87
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	[b] 4.11
CE28PMA 2	OOR <	[b] 1.68
CE28PMA 3	OOR <	[b] 0.55

Table 9.21: *Raw triplicate data for IL-12p40 at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	**Concentration in Range pg/mL**	**Observed Concentration pg/mL**
ASM 1	OOR <	[b] 5.37
ASM 2	OOR <	[b] 2.87
ASM 3	OOR <	[b] 1.68
ASMPMA 1	OOR <	[b] 3.49
ASMPMA 2	OOR <	[b] 0.55
ASMPMA 3	OOR <	[b] 3.49
ASM28 1	OOR <	[b] 6.66
ASM28 2	OOR <	[b] 2.87
ASM28 3	OOR <	[b] 2.87
ASM28PMA 1	OOR <	[b] 4.11
ASM28PMA 2	OOR <	[b] 4.74
ASM28PMA 3	OOR <	[b] 9.30
CE 1	OOR <	[b] 2.87
CE 2	OOR <	[b] 1.68
CE 3	OOR <	OOR <
CEPMA 1	OOR <	[b] 6.66
CEPMA 2	OOR <	[b] 1.68
CEPMA 3	OOR <	[b] 2.87
CE28 1	OOR <	OOR <
CE28 2	OOR <	[b] 3.49
CE28 3	OOR <	[b] 1.10
CE28PMA 1	OOR <	[b] 6.66
CE28PMA 2	OOR <	[b] 0.55
CE28PMA 3	OOR <	OOR <

Table 9.22: *Raw triplicate data for IL-12p40 at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 0.65
ASM 2	OOR <	[b] 1.35
ASM 3	OOR <	[b] 0.88
ASMPMA 1	OOR <	[b] 0.88
ASMPMA 2	OOR <	[b] 0.76
ASMPMA 3	OOR <	[b] 0.65
ASM28 1	OOR <	[b] 0.54
ASM28 2	OOR <	[b] 0.82
ASM28 3	OOR <	[b] 1.41
ASM28PMA 1	OOR <	[b] 0.88
ASM28PMA 2	OOR <	[b] 0.65
ASM28PMA 3	OOR <	[b] 0.99
CE 1	OOR <	[b] 1.23
CE 2	OOR <	[b] 0.65
CE 3	OOR <	[b] 0.65
CEPMA 1	OOR <	[b] 0.99
CEPMA 2	OOR <	[b] 0.76
CEPMA 3	OOR <	[b] 0.82
CE28 1	OOR <	[b] 0.99
CE28 2	OOR <	[b] 0.76
CE28 3	OOR <	[b] 0.99
CE28PMA 1	OOR <	[b] 1.11
CE28PMA 2	OOR <	[b] 0.76
CE28PMA 3	OOR <	[b] 0.88

***Table 9.23**: Raw triplicate data for IL-12p70 at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 0.76
ASM 2	OOR <	[b] 0.65
ASM 3	OOR <	[b] 0.88
ASMPMA 1	OOR <	[b] 1.23
ASMPMA 2	OOR <	[b] 0.76
ASMPMA 3	OOR <	[b] 0.71
ASM28 1	OOR <	[b] 0.94
ASM28 2	OOR <	[b] 1.11
ASM28 3	OOR <	[b] 0.88
ASM28PMA 1	OOR <	[b] 0.99
ASM28PMA 2	OOR <	[b] 0.99
ASM28PMA 3	OOR <	[b] 0.88
CE 1	OOR <	[b] 1.23
CE 2	OOR <	[b] 1.23
CE 3	OOR <	[b] 0.76
CEPMA 1	OOR <	[b] 0.65
CEPMA 2	OOR <	[b] 1.35
CEPMA 3	OOR <	[b] 0.94
CE28 1	OOR <	[b] 0.99
CE28 2	OOR <	[b] 1.23
CE28 3	OOR <	[b] 0.99
CE28PMA 1	OOR <	[b] 1.17
CE28PMA 2	OOR <	[b] 0.65
CE28PMA 3	OOR <	[b] 0.88

Table 9.24: *Raw triplicate data for IL-12p70 at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 1.11
ASM 2	OOR <	[b] 0.88
ASM 3	OOR <	[b] 0.99
ASMPMA 1	OOR <	[b] 0.99
ASMPMA 2	OOR <	[b] 0.88
ASMPMA 3	OOR <	[b] 0.76
ASM28 1	OOR <	[b] 0.88
ASM28 2	OOR <	[b] 0.76
ASM28 3	OOR <	[b] 0.99
ASM28PMA 1	OOR <	[b] 0.99
ASM28PMA 2	OOR <	[b] 0.99
ASM28PMA 3	OOR <	[b] 0.99
CE 1	OOR <	[b] 0.76
CE 2	OOR <	[b] 0.76
CE 3	OOR <	[b] 0.76
CEPMA 1	OOR <	[b] 0.65
CEPMA 2	OOR <	[b] 0.82
CEPMA 3	OOR <	[b] 0.65
CE28 1	OOR <	[b] 0.71
CE28 2	OOR <	[b] 1.05
CE28 3	OOR <	[b] 0.88
CE28PMA 1	OOR <	[b] 0.99
CE28PMA 2	OOR <	[b] 0.65
CE28PMA 3	OOR <	[b] 1.11

Table 9.25: *Raw triplicate data for IL-12p70 at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	**Concentration in Range pg/mL**	**Observed Concentration pg/mL**
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 1.43
ASMPMA 1	5.39	5.39
ASMPMA 2	11.11	11.11
ASMPMA 3	11.36	11.36
ASM28 1	OOR <	[b] 3.47
ASM28 2	OOR <	[b] 2.62
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	[b] 1.73
ASM28PMA 3	OOR <	[b] 1.12
CE 1	OOR <	[b] 3.19
CE 2	OOR <	[b] 1.73
CE 3	OOR <	[b] 3.19
CEPMA 1	OOR <	[b] 0.12
CEPMA 2	5.93	5.93
CEPMA 3	5.39	5.39
CE28 1	OOR <	[b] 4.57
CE28 2	OOR <	[b] 1.43
CE28 3	OOR <	[b] 1.43
CE28PMA 1	6.46	6.46
CE28PMA 2	5.93	5.93
CE28PMA 3	15.84	15.84

***Table 9.26**: Raw triplicate data for IL-22 at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	**Concentration in Range pg/mL**	**Observed Concentration pg/mL**
ASM 1	OOR <	[b] 1.12
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 3.75
ASMPMA 1	OOR <	[b] 1.43
ASMPMA 2	6.19	6.19
ASMPMA 3	5.66	5.66
ASM28 1	OOR <	OOR <
ASM28 2	OOR <	[b] 2.03
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	[b] 2.03
ASM28PMA 2	OOR <	[b] 0.81
ASM28PMA 3	OOR <	[b] 0.81
CE 1	OOR <	[b] 0.81
CE 2	OOR <	OOR <
CE 3	OOR <	[b] 2.62
CEPMA 1	7.51	7.51
CEPMA 2	OOR <	[b] 1.73
CEPMA 3	OOR <	[b] 2.90
CE28 1	OOR <	[b] 3.19
CE28 2	OOR <	OOR <
CE28 3	OOR <	OOR <
CE28PMA 1	7.25	7.25
CE28PMA 2	6.19	6.19
CE28PMA 3	OOR <	[b] 4.57

Table 9.27: *Raw triplicate data for IL-22 at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 2.33
ASM 2	OOR <	[b] 1.12
ASM 3	OOR <	[b] 1.43
ASMPMA 1	OOR <	[b] 0.12
ASMPMA 2	OOR <	[b] 4.02
ASMPMA 3	OOR <	OOR <
ASM28 1	OOR <	[b] 4.02
ASM28 2	OOR <	[b] 2.62
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	[b] 0.81
ASM28PMA 3	OOR <	[b] 2.90
CE 1	OOR <	[b] 3.47
CE 2	OOR <	[b] 2.03
CE 3	OOR <	[b] 1.12
CEPMA 1	6.99	6.99
CEPMA 2	OOR <	[b] 3.19
CEPMA 3	OOR <	[b] 4.30
CE28 1	OOR <	[b] 0.81
CE28 2	OOR <	[b] 1.43
CE28 3	OOR <	[b] 0.81
CE28PMA 1	16.09	16.09
CE28PMA 2	OOR <	[b] 0.81
CE28PMA 3	OOR <	OOR <

Table 9.28: *Raw triplicate data for IL-22 at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	OOR <
ASM 3	OOR <	OOR <
ASMPMA 1	OOR <	OOR <
ASMPMA 2	OOR <	[b] 22.50
ASMPMA 3	OOR <	[b] 3.06
ASM28 1	OOR <	[b] 3.06
ASM28 2	OOR <	[b] 8.44
ASM28 3	OOR <	[b] 22.50
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	OOR <
ASM28PMA 3	OOR <	OOR <
CE 1	OOR <	[b] 24.69
CE 2	OOR <	[b] 22.50
CE 3	31.14	31.14
CEPMA 1	OOR <	[b] 13.34
CEPMA 2	OOR <	[b] 22.50
CEPMA 3	OOR <	[b] 18.00
CE28 1	OOR <	[b] 18.00
CE28 2	OOR <	[b] 3.06
CE28 3	OOR <	[b] 20.27
CE28PMA 1	39.46	39.46
CE28PMA 2	57.41	57.41
CE28PMA 3	OOR <	OOR <

Table 9.29: *Raw triplicate data for IL-23 at 8 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	[b] 8.44
ASM 2	OOR <	OOR <
ASM 3	OOR <	[b] 13.34
ASMPMA 1	OOR <	[b] 13.34
ASMPMA 2	29.01	29.01
ASMPMA 3	OOR <	[b] 10.93
ASM28 1	OOR <	[b] 8.44
ASM28 2	OOR <	[b] 3.06
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	[b] 3.06
ASM28PMA 3	OOR <	OOR <
CE 1	OOR <	OOR <
CE 2	OOR <	OOR <
CE 3	OOR <	[b] 5.84
CEPMA 1	OOR <	[b] 5.84
CEPMA 2	OOR <	[b] 8.44
CEPMA 3	35.34	35.34
CE28 1	OOR <	[b] 8.44
CE28 2	31.14	31.14
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	[b] 22.50
CE28PMA 2	OOR <	[b] 20.27
CE28PMA 3	OOR <	[b] 22.50

Table 9.30: *Raw triplicate data for IL-23 at 12 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] indicates data that has been extrapolated from the standard curve

Sample	Concentration in Range pg/mL	Observed Concentration pg/mL
ASM 1	OOR <	OOR <
ASM 2	OOR <	[b] 8.44
ASM 3	OOR <	[b] 13.34
ASMPMA 1	OOR <	[b] 8.44
ASMPMA 2	OOR <	OOR <
ASMPMA 3	OOR <	[b] 18.00
ASM28 1	33.25	33.25
ASM28 2	OOR <	[b] 3.06
ASM28 3	OOR <	OOR <
ASM28PMA 1	OOR <	OOR <
ASM28PMA 2	OOR <	[b] 3.06
ASM28PMA 3	33.25	33.25
CE 1	OOR <	[b] 8.44
CE 2	OOR <	[b] 3.06
CE 3	OOR <	[b] 13.34
CEPMA 1	OOR <	[b] 24.69
CEPMA 2	OOR <	[b] 22.50
CEPMA 3	OOR <	[b] 18.00
CE28 1	OOR <	OOR <
CE28 2	OOR <	[b] 3.06
CE28 3	OOR <	OOR <
CE28PMA 1	OOR <	OOR <
CE28PMA 2	OOR <	OOR <
CE28PMA 3	OOR <	[b] 3.06

Table 9.31: *Raw triplicate data for IL-23 at 24 hours*

Legend: ASM (10% v/v ASM), ASMPMA (10% v/v ASM with PMA/Ionomycin), ASM28 (10% v/v ASM with CD28), ASM28PMA (10% v/v ASM with CD28 and PMA/Ionomycin), CE (10% v/v Candida effluent), CEPMA (10% v/v Candida effluent with PMA/Ionomycin), CE28 (10% v/v Candida effluent with CD28), CE28PMA (10% v/v Candida effluent with CD28 and PMA/Ionomycin)

OOR indicates data that is out of range either above (>) or below (<) the standard curve

[b] *indicates data that has been extrapolated from the standard curve*