DIFFERENTIAL EXPRESSION OF BMI1 AND PARP1 IN RETICULAR, EROSIVE AND ATROPHIC ORAL LICHEN PLANUS

by

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Abstract

Background: Oral lichen planus (OLP) is an oral potentially malignant disorder (OPMD) with a risk of progression to oral squamous cell carcinoma (OSCC). The erosive and atrophic variants appear to have an increased malignant transformation risk compared to the reticular variant. Cancer stem cell (CSC) marker B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and DNA repair enzyme Poly (ADP-Ribose) Polymerase 1 (PARP1) have been shown to be upregulated in OSCC.

Objectives: The aims of this study were to identify BMI1 and PARP1 expression patterns in erosive and atrophic OLP compared with reticular OLP. Furthermore we aimed to determine if BMI1 and PARP1 could be used as potential biomarkers for identification of high risk OLP lesions.

Materials and Methods: This was a cross-sectional, observational, pilot study. Patients were prospectively recruited from the Oral Medicine clinic and underwent oral tissue biopsy. 63 paraffin-embedded tissue blocks of histologically confirmed OLP were stained for BMI1 and PARP1 using immunohistochemistry (IHC). BMI1 and PARP1 IHC expression in both the full thickness (FT) and basal half (BH) of the epithelium was analysed using the Immunoreactive Scoring system (IRS).

Results: PARP1 expression was higher than BMI1 expression in both reticular and erosive/atrophic OLP samples. No significant differences were identified between the OLP variants and PARP1 IRS scores for either the FT ($p = .858$) or the BH of the epithelium ($p = .681$) or BMI1 scores for either the FT ($p = .492$) or the BH of the epithelium ($p = .649$). However there was a small increase in the number of erosive/atrophic OLP samples that had strong PARP1 staining compared to reticular. There was a significant positive correlation seen

between BMI1 and PARP1 expression in both the FT (r (61) = .432, p <.001) and the BH of the epithelium (r (61) = .530, p <.001).

Conclusions: Although not significant, PARP1 expression was slightly increased in erosive/atrophic OLP compared to reticular, suggesting a possible association with high risk lesions. Additionally, the findings demonstrated a positive correlation between BMI1 and PARP1 indicating co-expression of the proteins in OLP. Further studies with larger sample sizes, negative tissue controls and follow-up data are indicated to determine if BMI1 and PARP1 could be used as potential biomarkers for identification of OLP lesions at high risk for malignant transformation.

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List of abbreviations

ALDH1 = Aldehyde dehydrogenase 1

AP = Alexandra Perks

BER = Base excision repair

BH = Basal half

BSA = Bovine serum albumin

BMI1 = B cell-specific Moloney murine leukemia virus integration site 1

CSC = Cancer stem cell

DAB = 3,3'-Diaminobenzidine

DNA = Deoxyribonucleic acid

DRTB = Dentistry Research Tissue Bank

DSB = Double strand break

EGFR = Epidermal growth factor receptor

EMT = Epithelial-mesenchymal transition

FT = Full thickness

H&E = Haematoxylin and Eosin

HARM = 'High at-risk mucosa'

HCV = Hepatitis C Virus

HNC = Head and neck cancer

HNSCC = Head and neck squamous cell carcinoma

HPV = Human papilloma virus

HR = Homologous recombination

IHC = Immunohistochemistry

IL-6 = Interleukin-6

IL-8 = Interleukin-8

IRS = Immunoreactive Score

LOC = Lip and oral cavity cancer

MHC = Major histocompatibility complex

MMF = Mycophenolate Mofetil

MT = Malignant transformation

NHEJ = Non-homologous end joining

NRES = National Research Ethics Service

NSAID = Non-steroidal anti-inflammtory

OE = Oral erythoplakia

OED = Oral epithelial dysplasia

OL = Oral leukoplakia

OLL = Oral lichenoid lesion

OLP = Oral lichen planus

OR = Odds ratio

OPMD = Oral potentially malignant disorder

OPSCC = Oropharyngeal squamous cell carcinoma

OSCC = Oral squamous cell carcinoma

PBS = Phosphate buffered saline

RCA = Request cytotoxic activity

REC = Research Ethics Committee

ROS = Reactive oxygen species

RT-PCR = Real time polymerase chain reaction

RT-qPCR = Quantitative reverse transcription polymerase chain reaction

PARP1 = Poly (ADP-Ribose) Polymerase 1

SOX2 = Sex determining region Y-box 2

SP = Side population

SSB = Single strand break

TB = Expert rater

TNF-α = Tumour necrosis factor alpha

UoB = University of Birmingham

WHO = World Health Organisation

CHAPTER ONE

LITERATURE REVIEW

1.1. Oral lichen planus

Lichen planus is a chronic inflammatory mucocutaneous disease that can affect the oral mucosa, skin, genitalia, nails and scalp. The oral variant, oral lichen planus (OLP), is one of the most common oral mucosal diseases with an estimated prevalence of 0.5 to 2% (Alrashdan, Cirillo and McCullough 2016). Age of onset is characteristically between 30-60 years and the female to male ratio is 2 to 1 (Alrashdan, Cirillo and McCullough 2016). Despite its prevalence, the precise aetiology remains unknown. OLP is considered an oral potentially malignant disorder by the World Health Organisation (WHO) (Warnakulasuriya et al., 2020) and malignant transformation rates vary in the literature, on average between 1 to 2% (Landini et al., 2014; Iocca *et al.,* 2019; Gonzalez-Moles *et al.,* 2019; Idrees *et al.,* 2020).

1.1.1. Aetiology and pathogenesis

OLP is considered to be a cell-mediated autoimmune disease, however there is no definitive evidence for this and target antigens are yet to be identified (Cheng *et al*., 2016). Therefore the precise aetiology of OLP remains unknown. A few potential predisposing factors have been postulated, including stress and psychological factors; De Porras-Carrique *et al*. (2021) performed a systematic review and meta-analysis and found that OLP patients have significantly higher frequency of depression, anxiety and stress than control groups. However, it cannot be ascertained whether these psychological factors actually contribute to the aetiology of OLP or whether they are an effect of the OLP symptomatology. Chronic liver disease is thought to play an aetiological role in OLP and the association between Hepatitis C Virus (HCV) and OLP is frequently reported in the literature (Alaizari *et al.,* 2016; Farhi and Dupin 2010; Carrozzo *et al*., 1996) . A systematic review (Alaizari *et al.,* 2016) found a strong

link between the two entities; the summary estimate odds ratio (OR) for all studies was 6.07 (95% CI: 2.73–3.48), showing a statistically significant difference in the proportion of HCV seropositivity in OLP patients compared with controls.

Some systemic medications and dental materials play a specific role in the aetiology of oral lichenoid lesions (OLL). OLL share comparable clinical presentation and certain histological similarities with OLP, but are a separate entity and thought to be type IV delayed hypersensitivity reaction (Ismail, Kumar and Zain 2007). Numerous medications have been suspected to be involved in the causation of OLL including anti-hypertensives, non-steroidal anti-inflammatory drugs (NSAIDs) and anti-diuretics (Alrashdan, Cirillo and McCullough 2016). OLL can clinically and histologically mimic OLP but lacks the characteristic symmetry of OLP, often presenting unilaterally, in direct contact with dental restorations or secondary to drug intake (Warnakulasuriya *et al.*, 2020).

The pathogenesis of OLP is characterised by a T-lymphocyte mediated immunological response, whereby cytotoxic CD8+ T cells are mistakenly directed against basal keratinocytes in skin and mucosae resulting in liquefactive degeneration and apoptosis. Sugerman *et al*. (2002) postulated a hypothesis based on interaction between CD8+ and CD4+ T cells. Activated CD8+ T cells trigger keratinocyte apoptosis via tumour necrosis factor alpha (TNF-α) or Fas-Fas ligand mechanism. There is subsequent upregulated major histocompatibility complex (MHC) Class II expression in the keratinocytes which promotes a CD4+ T cell response (Alrashdan, Cirillo and McCullough, 2016). CD4+ T cells then interact with CD8+ T cells through a “request cytotoxic activity” (RCA) surface molecule on CD8+ T cells and a RCA receptor on CD4+ T cells (Alrashdan, Cirillo and McCullough, 2016).

This results in a set of characteristic histological features including irregular acanthosis, localised epithelial atrophy and hyperparakeratinisation, distorted (triangular-shaped) rete ridges, basal cell liquefaction, keratinocyte apoptosis (referred to as Civatte bodies) and a typical band-like lymphohistiocytic infiltrate of the lamina propria (Alrashdan, Cirillo and McCullough 2016; Cheng *et al*., 2016).

1.1.2. Clinical features and variants

The WHO definition of OLP summarises the clinical features, featuring "…characteristic relapses and remissions, displaying white reticular lesions, accompanied or not by atrophic, erosive and ulcerative and/or plaque type areas. Lesions are frequently bilaterally symmetrical. Desquamative gingivitis may also be a feature" (Warnakulasuriya *et al.*, 2020).

There are six clinical variants of OLP; reticular, atrophic, erosive, plaque-like, papular and bullous. Reticular is the most common variant and is often asymptomatic (Alrashdan, Cirillo and McCullough 2016). The atrophic and erosive variants are generally associated with discomfort and pain, that can be exacerbated by certain foods, toothpastes and environmental triggers such as stress and illness. The symptoms of OLP are commonly relapsing and remitting. (Alrashdan, Cirillo and McCullough 2016; Warnakulasuriya *et al.*, 2020).

Figures 1.1, 1.2 and 1.3 show examples of reticular, atrophic and erosive OLP, respectively.

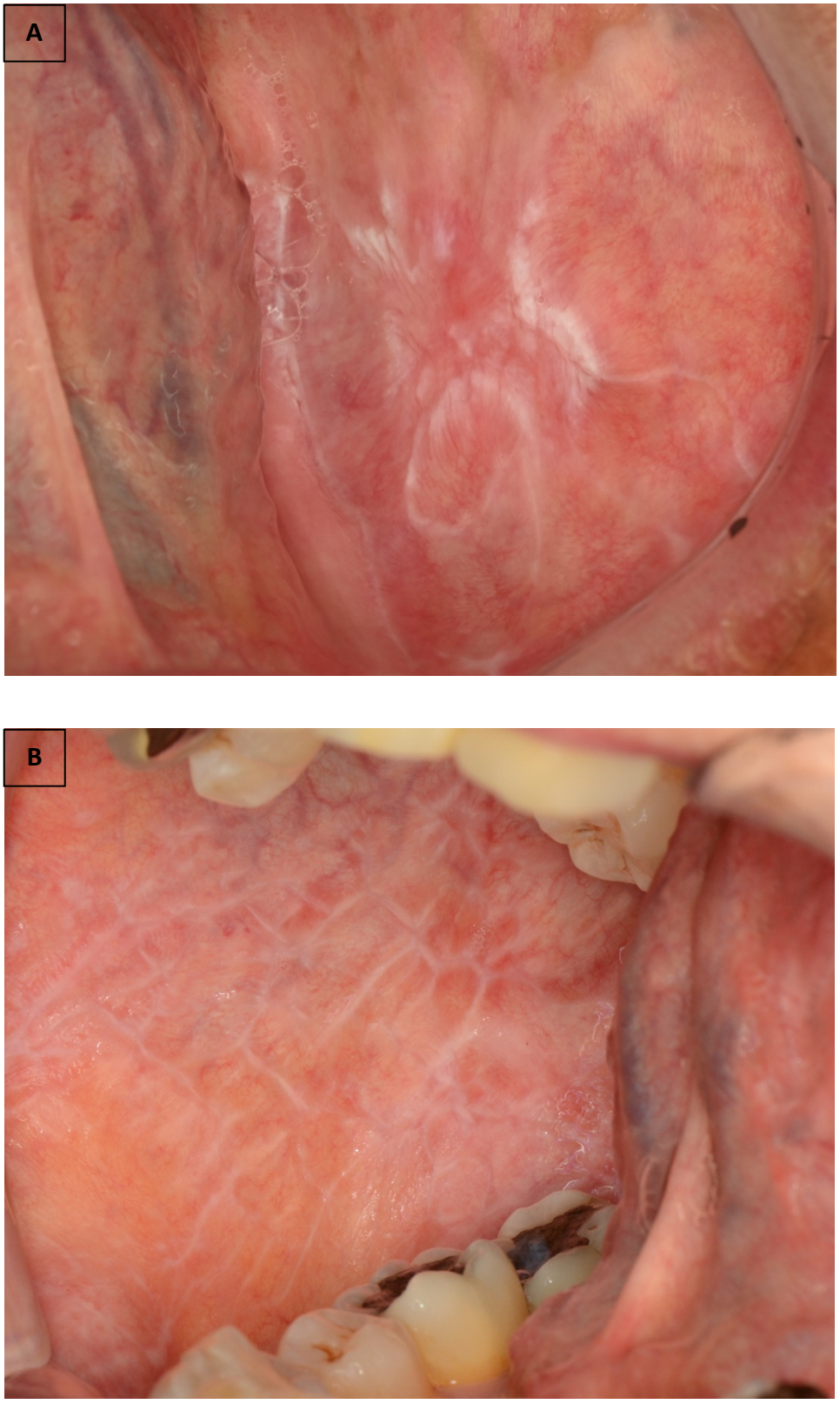

Figure 1.1. Reticular OLP in (A) left buccal mucosa (B) right buccal mucosa

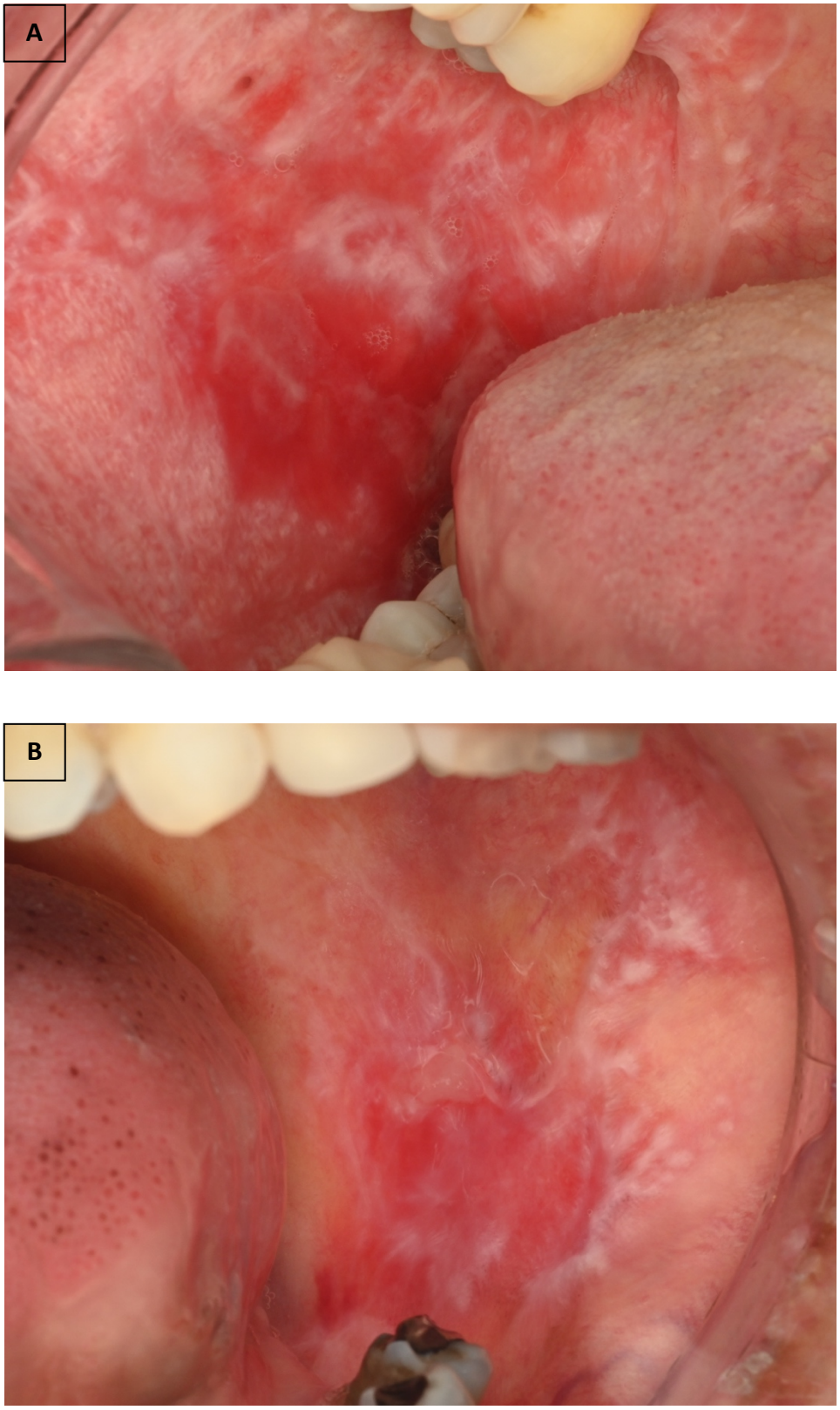

Figure 1.2. Atrophic OLP in (A) right buccal mucosa (B) left buccal mucosa

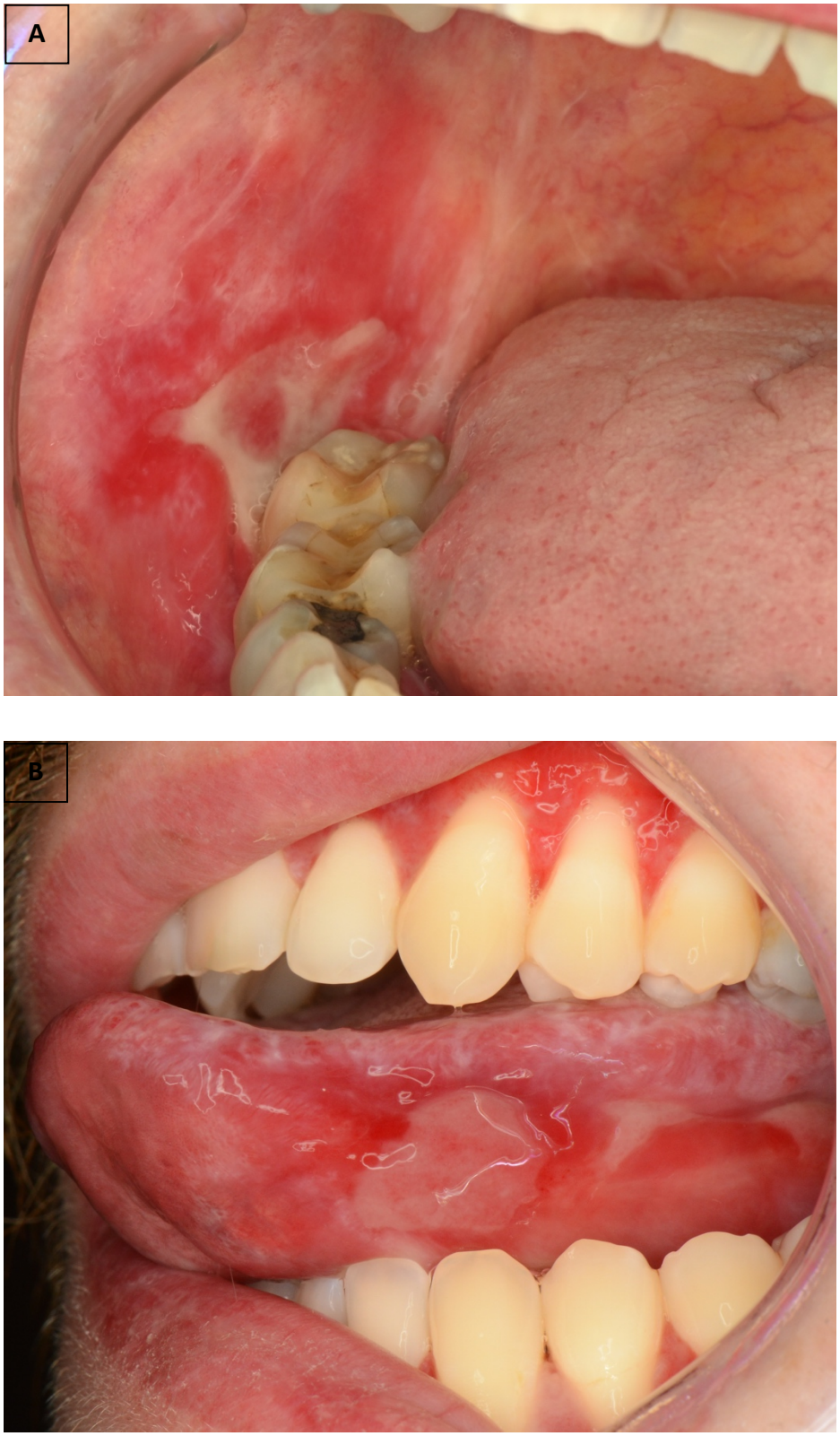

Figure 1.3. Erosive OLP in (A) right buccal mucosa (B) left lateral tongue

1.1.3. Management

OLP is currently not a curable disease, therefore its management is aimed at alleviating symptoms and, if possible, reducing the potential risk of malignant transformation (Lodi *et al*., 2020). Patients are advised to maintain optimal oral hygiene and avoid exacerbating factors such as spicy foods or sodium-lauryl-sulphate containing toothpastes. Benzydamine Hydrochloride 0.15% oral rinse or spray is a locally acting topical NSAID that is commonly prescribed for simple symptomatic relief in OLP (Al-Hashimi *et al.,* 2007; Alrashdan, Cirillo and McCullough 2016).

Topical corticosteroids (+/- topical antimycotics) are the first-line treatment in OLP (Al-Hashimi *et al.,* 2007). Various formulations, doses and modes of delivery are used. Systemic corticosteroids can be used as first-line treatment for severe OLP or recalcitrant multi-site lichen planus. Topical calcineurin inhibitors such as Tacrolimus ointment can be used as a second-line treatment (Lodi *et al*., 2020; Al Johani *et al*., 2009). Long-term systemic management with 'steroid-sparing' agents are often required for severe cases of OLP. Hydroxychloroquine, Azathioprine and Mycophenolate Mofetil (MMF) are the most commonly used of the systemic agents, although the evidence is limited (Al-Hashimi *et al.,* 2007). Hydroxychloroquine is an antimalarial drug which inhibits toll-like receptors thus reducing levels of TNF-α (Goodfield, 2015) which is an important mediator in OLP pathogenesis (Alrashdan, Cirillo and McCullough, 2016; Sugerman *et al*., 2002). There is proven benefit of Hydroxychloroquine in lupus erythematosus, however in OLP there has only ever been one small open trial (Eisen, 1993), so much of the evidence is based on case reports. Azathioprine is a synthetic purine analogue with antiproliferative effect on lymphocytes (Wakelin, 2015), and has been reported successful in the treatment of cutaneous lichen

planus although there is limited evidence to support its role in OLP (Al-Hashimi *et al.,* 2007; Lear and English, 1996; Verma, Mittal and Manchanda, 2001). MMF inhibits *de novo* purine synthesis of lymphocytes via its active metabolite, mycophenolic acid (Chong and Setterfield, 2015). In a retrospective review of 10 patients with recalcitrant ulcerative OLP treated with MMF, Wee *et al*., (2012) found improvement in oral disease activity scores in all 10 patients, with 6 achieving remission. The data suggested that MMF can take up to 1 year to provide significant symptomatic and clinical benefit (Wee *et al*., 2012). Sin *et al.,* (2022) showed similar findings with 80% of OLP patients showing clinical improvement after MMF treatment for at least 13 months.

1.1.4. Malignant potential

OLP is considered an oral potentially malignant disorder by the WHO (Warnakulasuriya *et al.*, 2020). Varying rates of malignant transformation (MT) are reported in the literature, most commonly between 1 to 2%. A 2014 analysis of the literature showed an average transformation rate of 2.28% (mean cohort size 347.72 patients, mean follow up time 6.36 years) (Landini *et al.,* 2014), however the authors suggested that this could be an over-estimation (publication bias) as the rates did not correlate to oral cancer incidence in the UK at the time. More recently, multiple meta-analyses have been published, reporting lower OLP MT rates. Iocca *et al.* (2019) found a MT rate of 1.4% (total 14,362 patients, follow up time range 12 months to 20 years); Gonzalez-Moles *et al.* (2019) found a MT rate of 1.14% (total 25,848 patients, no mean follow up time data provided); Idrees *et al*. (2020) found a MT rate of 0.44% (total 12,838 patients, follow up time range 1 year to over 10 years). There is an interesting contrast in opinion between the authors in the latter two reviews; Gonzalez-Moles *et al.* (2019) suggested that OLP MT rates are under-estimated due to restrictive diagnostic

criteria and believed that studies should include lesions with the presence of dysplasia. On the other hand, Idrees *et al*. (2020) suggests that OLP MT rates are over-estimated and believes that studies should not include lesions with the presence of dysplasia. Similar controversies surrounding MT in OLP have been discussed for many years; Gonzalez-Moles, Scully and Gil-Montoya (2008) highlighted drawbacks in some studies, including inconsistent diagnostic criteria, the inclusion/exclusion of dysplasia, overlap between OLP and OLL, inadequate follow-up periods and lack of prospective studies. A good quality retrospective study of 565 Japanese patients looked at MT rates in patients with a clinical and histopathological diagnosis of OLP using the The American Academy of Oral and Maxillofacial Pathology (AAOMP) diagnostic criteria (excluded patients with OLL and with a follow ups of less than 6 months). Interestingly, they found a MT rate of 0.7% over a duration of 55.9 ± 45.3 months, which is lower than found elsewhere in the literature (Tsushima *et al.,* 2021). The erosive and atrophic variants of OLP have been reported to have a higher MT risk than other variants (Alrashdan, Cirillo and McCullough 2016; Gonzalez-Moles, Scully and Gil-Montoya, 2008). Gonzalez-Moles *et al.* (2019) found a significantly higher risk of MT in atrophic/erosive lesions compared to reticular lesions with a relative risk of 4.09 (95% CI=2.40–6.98, $p < 0.001$). Idrees *et al*. (2020) and Tsushima *et al.* (2021) also found that 'red type' OLP lesions were more likely to develop into oral squamous cell carcinoma (OSCC) than the 'white type.'

The pathogenetic mechanisms for MT in OLP are not fully understood, but current evidence suggests that chronic inflammation gives rise to a cytokine-rich microenvironment which promotes cell survival, growth and proliferation (Mignogna *et al.,* 2004; Gonzalez-Moles, Scully and Gil-Montoya, 2008). Increased levels of inflammatory molecules, such as the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and

cytokines, such as interleukin-6 (IL-6) and TNF-α, promote cell survival, angiogenesis and invasion, whilst inhibiting apoptosis (Mignogna *et al.,* 2004; Georgaki *et al*., 2021; Mantovani *et al.,* 2008). Furthermore, increased oxidative stress can induce Deoxyribonucleic acid (DNA) damage and promote carcinogenesis. (Mignogna *et al.,* 2004; Gonzalez-Moles, Scully and Gil-Montoya, 2008; Ergun *et al.,* 2011). Additionally, it has been hypothesised that chronic tissue stress, in the form of repeated or persistent ulceration or erosion, can promote carcinogenesis in susceptible stem cell populations. The chronic stress affects the tissue architecture and encourage tumour promotion, however only in the presence of pre-existing somatic mutations (Goldberg, Eisen & Bauer, 1988). The combined impact of chronic inflammation and tissue stress from ulceration or erosion may explain the reason for the increased MT risk of atrophic and erosive OLP, however the exact mechanism is unknown and the identification of quantitative biomarkers for high risk lesions is warranted.

A focus of this study will be on identification of OLP at risk of MT, therefore OSCC will now be discussed in more detail.

1.2. Oral squamous cell carcinoma

1.2.1. Epidemiology

OSCC is a malignant tumour arising from oral stratified squamous epithelium and accounts for more than 90% of oral cavity cancers (Chamoli *et al*., 2021). OSCC is categorised under the broader heading of head and neck cancer (HNC) which encompasses a large number of malignancies from various anatomical locations. HNC is the sixth most common cancer worldwide (Johnson *et al*., 2020). The incidence is rising and there is a predicted 30% increase

annually by 2030 (Gormley *et al*., 2022). The Global Cancer Observatory (GLOBOCAN) 2020 data ranks lip and oral cavity cancer (LOC) as the sixteenth most common cancer globally (Sung *et al*., 2021). Asia has the highest incidence of LOC, accounting for 65.8% of the worldwide incidence, and the male to female incidence ratio is approximately 3 to 1. In 2020 there were 177,757 deaths from LOC globally, giving an age standardised (world) mortality of 1.9 per 100,000 (Sung *et al*., 2021).

HNC incidence rates in the United Kingdom (UK) also continue to rise, showing a 16% increase over the last decade and a total 34% incidence increase since the early 1990s (Cancer Research UK 2023). In 2018, there were 4,078 deaths from HNC in the UK. Since the early 1970s, the combined HNC mortality for men and women has fallen by 11% overall, however in the last decade there has been a 15% increase in mortality rates (Cancer Research UK 2023). These figures are projected to rise by 12% between 2023-2025 and 2038-2040 (Cancer Research UK 2023; Gormley *et al.,* 2022).

1.2.2. Risk factors

Over 75% of oral cancers are attributable to tobacco and alcohol consumption (Perks *et al.,* 2019; Hashibe *et al.,* 2007). Tobacco contains over 60 carcinogens and smokers are 8.4 times more likely to develop oral cancer compared to non-smokers (Chamoli *et al.,* 2021). Lin *et al.* (2011) demonstrated an approximately 5-fold increased risk of oral cancer in smokers compared to non-smokers (OR=5.13, 95% CI = 3.17-8.32, $p<0.001$). Khan, Tönnies and Müller (2014) performed a systematic review and meta-analysis regarding the association between smokeless tobacco and oral cancer in South Asia and found the pooled OR for chewing tobacco and risk of oral cancer was 4.7 [3.1–7.1] and 7.1 [4.5-11.1] for paan with tobacco.

Paan (also known as betel quid) is a mixture of areca nut, betel leaf and slaked lime. Areca nut contains carcinogenic alkaloids and Lin *et al.* (2011) found a 12-fold increased risk of oral cancer in betel quid chewers. Consumption of alcoholic beverages is known to be carcinogenic based on human carcinogenicity studies (U.S. Department of Health and Human Services, 2021). The main ingredient in alcoholic beverages is ethanol, which is metabolised into acetaldehyde which is a carcinogen (Baan *et al.,* 2007). Excess alcohol consumption independently increases the risk of oral cancer by 3-5 fold. Data from the International Head and Neck Cancer Epidemiology (INHANCE) consortium shows that when tobacco is combined with excess alcohol consumption, the two risk factors act synergistically and increase the risk of oral cancer by 35-fold (Chamoli *et al.,* 2021; Hashibe *et al.,* 2009).

It is well established that infection with high risk Human Papillomavirus (HPV) types (mainly HPV16 and HPV18) is strongly associated with the incidence of oropharyngeal squamous cell carcinoma (OPSCC), with up to 70% of OPSCC being caused by HPV (Mehanna *et al.,* 2023; Syrjänen, 2018; Perks *et al*., 2019). HPV-positive OPSCC are more often found in younger, healthy, non-smoking individuals compared to HPV-negative cases (Syrjänen, 2018; Perks *et al*., 2019). Furthermore, the prognosis and treatment responsiveness of HPV-positive OPSCC is better than HPV-negative OPSCC (Syrjänen, 2018; Ang *et al*., 2010). According to the WHO, 3% of OSCC are HPV-positive, although the aetiological role is less well understood (El-Nagger *et al*., 2017). A recently published multicentre, multinational, individual patient data analysis collated a cohort of 7654 patients with oropharyngeal cancer with HPV/p16 data available (Mehanna *et al.,* 2023). P16 is the most widely used surrogate marker for HPV in oropharyngeal cancer however this study revealed high level of HPV/p16 discordance.

Furthermore, discordant patients had significantly worse prognosis than p16+/HPV+ patients but better than p16–/HPV– patients (Mehanna *et al.,* 2023).

1.2.3. Pathogenesis

The pathogenesis of OSCC is a complex and multifactorial process that is the result of an accumulation of genetic mutations, chromosomal abnormalities and epigenetic alterations. These changes can either result in stablisation of oncogenes that produce a dominant gain of function, or destabilisation/malfunction of tumour suppressor genes that result in a reduction of tumour suppression activity. Collectively these alterations can initiate dysplastic transformation within normal oral epithelium and subsequent progression to OSCC. Exogenous (e.g. tobacco, alcohol) and endogenous (e.g. genetic predisposition) risk factors also play a role in this process (Georgaki *et al*., 2021).

OSCCs display cellular heterogeneity, which can be partly explained by the ongoing genetic instability as described above. However, there is now increasing evidence to support a different theory for this heterogeneity; the cancer stem cell (CSC) hypothesis proposes that not all tumour cells are equal, but a cell hierarchy exists with distinct self-sustaining subpopulations involved in tumourigenesis (Clarke *et al.,* 2006; Bhaijee *et al.,* 2012; Prince *et al.,* 2007; Krishnamurthy and Nor, 2012). Al-Hajj *et al.* (2003) was the first to demonstrate the presence of CSCs in solid tumours and since then the hypothesis has been studied in numerous solid human malignancies, including head and neck squamous cell carcinoma (HNSCC) (Sayed *et al.,* 2011; Elkashty, Ashry and Tran, 2019). A landmark study on CSCs in HNSCC by Prince *et al.* (2007) demonstrated that CD44+ cells can initiate tumour

development but CD44- cells cannot. CSCs and the CSC hypothesis will be discussed in more detail later in this review.

Chromosomal abnormalities (including DNA aneuploidy and loss of heterozygosity), dysregulation of various molecular signaling pathways, epigenetic alterations, the role of inflammation and evasion of immune surveillance are all involved in the pathogenesis of oral cancer (Odell, 2020; Georgaki *et al*., 2021) however discussion of these processes in further detail is beyond the scope of this review.

1.2.4. Management and prognosis

The treatment of OSCC ultimately aims to eradicate the primary lesion, preserve or restore anatomical structures, minimise treatment sequalae and prevent recurrence. Surgical removal of the entire tumour is the gold-standard first line treatment +/- radiotherapy (Johnson *et al*., 2020). Intensity modified radiotherapy (IMRT) was introduced in the mid-1990s to deliver more precise radiation to the tumour and spare other structures (Gutiontov *et al*., 2016). Neck dissection, either elective or therapeutic, is often required to remove cervical lymph nodes (Johnson *et al*., 2020). Adjuvant chemotherapy may improve prognosis when used in combination with other treatment modalities (Kalavrezos & Scully, 2016). Novel targeted cancer therapies are also now in use, such as epidermal growth factor receptor (EGFR) inhibitors and immune checkpoint inhibitors (Perks *et al.,* 2019; Chamoli *et al.,* 2021; Kalavrezos & Scully, 2016).

Globally, the five-year survival rate for HNC averages at 50%. Although there has been a modest improvement in UK survival rates, the five-year survival rate between 2009 and 2013

for patients diagnosed with oral cancer in England remained 55% (Cancer Research UK; Gormley *et al.,* 2022).

1.3. Biomarkers in OSCC and OLP

An important area of research in oral cancer is the identification and development of valid biomarkers. The BEST (Biomarkers, EndpointS, and other Tools) Resource defines a biomarker as "a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions" (FDA-NIH Biomarker Working Group, 2016). Biomarkers have a wide range of clinical applications including diagnosis, predicting treatment outcomes and monitoring disease status or response to treatment. *Prognostic biomarkers* are used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease of interest. This is differentiated from *susceptibility/risk* biomarkers which indicate potential disease development in a patient who does not currently have the disease of interest (Califf, 2018; FDA-NIH Biomarker Working Group, 2016). Susceptibility/risk biomarkers could potentially help to identify oral lesions at high risk of progressing to OSCC.

Oral cancer biomarkers can be identified in blood, bodily fluids or lesional tissue (Pillai *et al.,* 2021; Hussein *et al.,* 2018). Various genomic, proteomic and metabolomic biomarkers have been identified in the literature. A 2018 review published in the British Journal of Cancer identified promising biomarkers for early diagnosis and prognosis prediction of tongue SCC including IL-6, interleukin-8 (IL-8) and prolactin in liquid samples (serum/plasma and saliva) and hypoxia-inducible factor-1α (HIF-1α), sex determining region Y-box 2 (SOX2), epithelial

cadherin (E-cadherin), vimentin, metastasis associated lung adenocarcinoma transcript 1 (MALAT1), p53 and neurogenic locus notch homolog protein 1 (NOTCH1) in tissue samples (Hussein *et al.,* 2018). More recently, Pillai *et al.* (2021) performed a systematic review of proteomic biomarkers in OSCC, identifying 112 relevant studies. Amongst many others, p53 autoantibodies and EGFR were identified as potential diagnostic and prognostic biomarkers for OSCC.

1.3.1. CSC Biomarkers

As discussed in the pathogenesis of OSCC, there is now increasing evidence to support the CSC hypothesis which proposes that not all tumour cells are equal, but a cell hierarchy exists with distinct self-sustaining subpopulations involved in tumourigenesis (Clarke *et al.,* 2006; Bhaijee *et al.,* 2012; Prince *et al.,* 2007; Krishnamurthy and Nor, 2012). The consensus definition of a CSC from the American Association of Cancer Research Workshop is "a cell within a tumour that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour" (Clarke *et al.,* 2006).

CD44 is a surface glycoprotein involved in cell adhesion and migration and is now widely known as a CSC biomarker in HNSCC (Curtarelli *et al.,* 2018). Al-Hajj *et al*., (2003) demonstrated the heterogeneity of breast tumours and found that only around one hundred CD44(+)CD24(-/low) cells were capable of tumour initiation whereas tens of thousands of different cell types were not. They also found that this subpopulation of tumourigenic cells consistently expressed specific cell-surface markers allowing for recognition of CSCs. Since then, the CSC hypothesis has been studied in numerous solid tumours, including HNSCC (Sayed *et al.,* 2011; Elkashty, Ashry and Tran, 2019). In a key study by Prince *et al.* (2007), an

immunodeficient mouse model developed from primary human HNSCC samples demonstrated that CD44+ cells gave rise to new tumours *in vivo*, but CD44- cells did not. The CD44+ cells could be serially passaged demonstrating their ability to self-renew and differentiate. Furthermore, immunohistochemistry and quantitative reverse transcription polymerase chain reaction (RT-qPCR) showed that a protein named B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) was expressed in the nuclei of CD44+ cells (with strongest staining in the basal region), but not CD44- cells, hence could potentially be further biomarker of tumorigenic populations (Prince *et al.,* 2007).

CSC biomarkers are an important area of study as they allow for CSC identification, risk stratification of potentially malignant lesions such as OLP and are prospective therapeutic targets (Herzog, Somayaji and Nör, 2023; Ghazi *et al.,* 2019). As discussed, CD44 and BMI1 are two of the many CSC biomarkers that have been studied in HNSCC. Other biomarkers include aldehyde dehydrogenase 1 (ALDH1), SOX2 and octamer-binding transcription factor 4 (OCT4) (Curtarelli *et al.,* 2018).

1.3.2. Biomarkers in OLP

There is ongoing research aimed to identify susceptibility/risk biomarkers that could help to identify those OLP lesions at high risk of transformation. Ghazi and Khorasanchi *et al.* (2021) and Tampa *et al.* (2018) performed very similar non-systematic reviews looking at biomarkers associated with malignant transformation in OLP and both identified six main categories of markers; apoptosis-related biomarkers, cell cycle regulators, galectins, inflammation-related factors, intercellular adhesion proteins and tissue re-modelling factors. Of relevance to this study, cell cycle regulators will be discussed in more detail.

Cell cycle regulators are proteins that can switch different steps in the cell cycle on and off, hence controlling cell proliferation and survival. They consist of cyclin-dependent kinases (CDKs) and their regulatory subunits called cyclins, CDK inhibitors, the retinoblastoma family members, and E2F transcription factors (Huber *et al.,* 2021; Pecorino, 2016). Among those, $p16^{ink4a}$ is a CDK inhibitor that inhibits the CDK4/CDK6 – Cyclin D complex preventing phosphorylation of the retinoblastoma protein (pRB) causing arrest of the cell cycle. $p16^{ink4a}$ is a tumour suppressor gene that is commonly inactivated in cancer and has been studied as a potential biomarker in OLP (Ghazi and Khorasanchi *et al.,* 2021; Tampa *et al,* 2018). Hadzi-Mihailovic *et al.* (2020) showed that $p16^{ink4a}$ expression was significantly higher in OLP than in OSCC but did not describe any findings in relation to OLP malignant potential. Interestingly, Montebugnoli *et al.* (2011) concluded that positive $p16^{ink4a}$ expression in OLP was related to reactive inflammatory processes rather than risk of malignant transformation. Ki67 is a prominent cellular proliferation marker expressed during all active phases of the cell cycle. Zargaran *et al.* (2013) found that Ki67 expression was significantly higher in OLP than in epithelial hyperplasia and Rosa *et al.* (2018) found Ki67 expression was significantly higher in oral epithelial dysplasia compared to OLP, and OLP compared to oral fibrous hyperplasia.

BMI1, a protein that has already been highlighted as a CSC biomarker, is another cell cycle regulator studied as a potential OLP biomarker. This will now be discussed in more detail below.

1.3.3. B cell-specific Moloney murine leukemia virus integration site 1 (BMI1)

BMI1 is a member of the polycomb group (PcG) proteins and functions as a transcriptional suppressor through chromatin modification. It is directly involved in cell cycle regulation and senescence by suppressing the *INK4a* locus that encodes the tumour suppressors $p16^{ink4a}$ and $p14^{ARF}$ which activate senescence and apoptosis respectively (Herzog, Somayaji and Nör, 2023; Allegra *et al.,* 2014; Siddique and Saleem, 2012). BMI1 has been implicated in several signaling pathways involved in cell renewal and proliferation including Hedgehog and SOX2 pathways (Herzog, Somayaji and Nör, 2023). Studies in human mammary cells have suggested that Hedgehog signaling mediates stem cell self-renewal through Bmi-1 (Liu *et al.,* 2006). BMI1 has also been shown to be essential in promotion of epithelial-mesenchymal transition (EMT), a process where tumour cells transition to a more mesenchymal phenotype, which may facilitate invasion (Herzog, Somayaji and Nör, 2023; Yang *et al.,* 2010; Pecorino, 2016). Yang *et al.* (2010) showed direct regulation of BMI1 by the EMT regulator, Twist1. Furthermore, BMI1 contributes to DNA repair by suppressing the intracellular levels of reactive oxygen species (ROS) through negative regulation of the $p16^{ink42}$ pathway. BMI1 is also recruited to double strand breaks (DSB) and contributes to repairing the lesion through H2A ubiquitination (Herzog, Somayaji and Nör, 2023).

In summary, BMI1 increases self-renewal and proliferation, while decreasing apoptosis and senescence, in both normal and malignant stem cells (Herzog, Somayaji and Nör, 2023; Allegra *et al.,* 2014). Thus, upregulated BMI1 levels can drive tumourigenesis and there is increasing evidence of this in many solid malignancies including HNSCC (Allegra *et al.,* 2014; Curtarelli *et al.,* 2018; Ghazi *et al.,* 2019; Siddique and Saleem, 2012). Various studies have also shown increased BMI1 expression in oral potentially malignant disorders (OPMDs) (Liu *et al.,* 2012;

Klein *et al.,* 2019, Ma *et al*., 2013; Feng *et al.,* 2013) suggesting it could be a potential candidate as a susceptibility/risk biomarker for high risk oral lesions.

An initial literature search of BMI1 expression in OLP using PubMed, Ovid and Web of Science databases, from the start of published records until 26th April 2023, using the search terms 'BMI1' OR 'Bmi-1' OR 'B cell-specific Moloney murine leukemia virus integration site 1' AND ((oral lichen planus) OR (OLP)) only revealed one relevant study by Ma *et al.* (2013) who carried out a retrospective study of 96 OLP patients; immunohistochemical analysis of BMI1 expression showed 0% expression in normal tissue, 36.8% in non-progressing OLP, 88.9% in progressing OLP and 100% in OSCC. A multivariate analysis, revealed that the risk of malignant progression in BMI1-positive patients was significantly higher than in BMI1-negative patients (adjusted OR, 20.75; 95% CI, 2.21-194.57; P = .008). Although the P value suggests the results are statistically significant, the confidence interval range is extremely wide. However, the findings suggest that BMI1 could be used as a marker of lesions at high risk of malignant change and opens potential for further research (Ma *et al.,* 2013).

A further literature search was performed to explore the relationship between BMI1 expression and OSCC. PubMed, Ovid and Web of Science databases were searched, from the start of published records until 26th April 2023, using the search terms 'BMI1' OR 'Bmi-1' OR 'B cell-specific Moloney murine leukemia virus integration site 1' AND 'oral squamous cell carcinoma' OR 'oral cancer'. The inclusion criteria were any research study or review directly related to the relationship between expression of BMI1 and OSCC and written in English. Exclusion criteria included; not directly related to the relationship between expression of BMI1 and OSCC, related to the effect of a treatment or another molecular marker, not written

in English, related to non-human OSCC, related to non-oral SCC, abstract only or unable to access full text. The literature search across the three databases revealed a total of 394 publications. After excluding studies that did not meet the eligibility criteria, 47 studies were included.

Many of those studies assessed BMI1 expression as part of a wider study on the behaviour of multiple CSC biomarkers in HNSCC. Chen *et al.* (2009) created a highly malignant third generation HNSCC cell line, SASVO3. Using western blot, the study demonstrated notably increased expression of BMI1 in SASVO3 cells compared to the first generation SAS-GFP (green fluorescent protein) cells. BMI1 was also detected in mouse tumour tissue from the SASVO3 xenografts but not the SAS-GFP xenografts (Chen *et al*., 2009). Kaseb *et al.* (2016) demonstrated using immunofluorescence analysis, increased expression of BMI1, CD44, CD133 (also known as prominin-1, a pentaspan membrane glycoprotein) and SOX2 in early HNSCC cell cultures (cells derived from short-term HNSCC CSC cultures) compared with normal oral epithelial cells. Tamatani *et al.* (2018) performed immunohistochemistry on 70 stage I or II OSCC samples and found 47.1% of cases were BMI1 positive. CD44 expression was strongly detected in all OSCC samples compared to normal oral epithelium. Interestingly, they also found a significant positive association between high BMI1 expression and disease-free survival rate. Poi *et al.* (2014) investigated the mRNA expression of cyclin-dependent kinase-4 (CDK4) in HNSCC and 'high at-risk mucosa' (HARM) along with its regulators including BMI1. RT-qPCR showed that BMI1 mRNA was significantly elevated in HNSCC and HARM compared to healthy control specimens ($p < .01$). However the numbers were relatively small with 30 HNSCC, 30 HARM and 16 controls; furthermore the control samples were collected using brush biopsy method which may affect validity. In contrast to the majority of other published

findings, Wu *et al.* (2017) did not find any difference in Bmi-1 expression between normal epithelium, dysplastic epithelium and OSCC groups.

Zhang *et al.* (2009) characterised the features of side population (SP) cells in OSCC and found higher levels of BMI1 in SP cells compared to non-SP cells (p=0.036). SP cells are identified by their ability to efflux a fluorescent dye at a greater rate than the main cell population, and have been suggested to be a source of CSCs (Zhang *et al*., 2009; Wolmarans *et al*., 2018). As previously discussed, Prince *et al.* (2007) demonstrated that CD44+ cells, but not CD44-, initiate HNSCC tumourigenesis and express BMI1. Hence many other studies have since investigated the CD44+ subpopulation in HNSCC. Wu *et al.* (2013) examined expression of various markers in a CD44+ subpopulation of the SCC-9 malignant oral cancer cell line using immunofluorescence and found BMI1 was higher in CD44+ cells than CD44- cells ($p < .05$). Noto *et al.* (2013) demonstrated using real-time PCR (RT-PCR) in the oral cancer cell line HSC-4, that mRNA expression levels of BMI1 in CD44+SSEA-4+ cells were significantly higher than in the other subpopulations (CD44+SSEA-4- and CD44-SSEA-4-) ($p < .01$). The general consensus in the literature is that BMI1 expression is higher in CD44+ cells than CD44- cells, however there are some contrasting findings; immunohistochemical analysis of OSCCs derived from $CD44^{high}$ cells by de Andrade *et al.* (2016) found that these subpopulations had low BMI1 expression. Patel *et al.* (2016) also did not find any significant differences in BMI1 expression between CD44+ cells and CD44- cells.

Other studies have looked more specifically at BMI1 expression in OSCC and oral epithelial dysplasia (OED). Chen *et al.* (2017) showed through lineage tracing and genetic ablation that BMI1+ CSCs mediate invasive growth and cervical lymph node metastasis in a HNSCC mouse

model. Yamazaki *et al.* (2013) found that BMI1 was highly expressed in well-differentiated HNSCCs but declined with progression, which is converse to the findings of de Lima *et al.* (2017) who found that BMI1 was associated with poorly differentiated tumours and poor prognosis. These contrasting findings are interesting as the former suggests BMI1 is involved mainly in early stages of carcinogenesis, whereas the latter suggests a role in late tumour stage, migration and invasion. In regards to BMI1 expression in OPMDs; Liu *et al.* (2012) performed immunohistochemistry on 135 oral leukoplakia (OL) samples and found a statistically significant difference ($p = .002$) between BMI1 positivity in OL without subsequent malignant transformation (25.2%) and OL with subsequent malignant transformation (56.2%). 15.4% of BMI1 negative lesions developed into cancer, compared to 40.9% of BMI1 positive lesions. Klein *et al.* (2019) also used immunohistochemical analysis to compare non-dysplastic OL samples with non-dysplastic OLP samples. They did not find a statistically significant difference in BMI1 expression between these groups, however did find a statistically significant difference ($p < .01$) between OL compared to normal oral mucosa, and in OSCC compared to normal oral mucosa and OL. This study also found a significant positive correlation between BMI1 and Ki-67. Feng *et al*. (2013) used immunohistochemistry on 34 oral erythoplakia (OE) samples and found increased BMI1 expression in OE with subsequent malignant transformation compared to OE without subsequent malignant transformation, however this was not statistically significant ($p = .08$), possibly related to small sample size. The co-expression of both ALDH1 (another potential biomarker in HNSCC) and BMI1 was a strong indicator for malignant transformation ($P < 0.01$). The same research group revisited the same cohort with follow up data and found expression of both markers within an OE lesion correlated with developing multiple and multifocal carcinomas, but there were no significant findings for BMI1 alone (Feng *e al.,* 2020).

A number of studies focused on the expression of BMI1 in tongue SCC. He *et al.* (2015) performed immunohistochemistry on 77 tongue SCC samples and 22 premalignant tongue samples (including OLP). Results showed that BMI1 expression was higher in tongue SCC and correlated with poor overall survival (the difference in survival rates between low and high BMI1 expression was significantly significant $P < 0.05$). In this study, they also found BMI1 overexpression in OLP indicating it may occur at early stages in carcinogenesis. These results are consistent with Kang *et al*. (2007) who found that 100% of pre-neoplastic oral tissues showed elevated BMI1 staining in comparison to normal oral tissue. Rodrigues *et al.* (2022) carried out a prospective study on 24 fresh tongue SCCs using immunohistochemistry and RT-qPCR. They found a statistically significant ($P=0.0001$) overexpression of BMI1 in tongue SCC in relation to non-tumoural margins, although confidence intervals appeared wide. Häyry *et al* (2010) found that in a retrospective cohort of 73 tongue SCC specimens, BMI1 negative tumours showed poor prognosis. This is conflicting with the findings from He *et al*. (2015) and it has been suggested that the variation in results may be because Häyry *et al* (2010) only included pT1 and pT2 specimens whereas He *et al*. (2015) included all tumour stages.

1.3.4. Poly (ADP-Ribose) Polymerase 1 (PARP1)

Another area of current interest in oral cancer research relates to the role of Poly (ADP-Ribose) Polymerase 1 (PARP1), a nuclear enzyme encoded by the *PARP-1* gene, which plays a critical role in DNA repair (Dulaney *et al.,* 2017; Bouchard *et al.,* 2003). It is thought to be involved in single strand break (SSB) repair via its role in base excision repair (BER). BER is the process of removing and replacing small, non-helix distorting base lesions (Pecorino, 2016). The altered DNA base is excised by DNA gycosylase, the apurinic sites are removed by apurinic

endonuclease and the new correct nucleotide is inserted by DNA polymerase. Ligation is mediated by DNA ligase III and X-ray repair cross-complementing protein 1 (XRCC1) (Pecorino, 2016). PARP1 plays a role in this process by binding to the damaged DNA in BER, becoming catalytically activated, and recruiting repair proteins including DNA polymerase and the DNA ligase III-XRCC1 complex (Dulaney *et al.,* 2017; Bouchard *et al.,* 2003; Chaudhuri and Nussenzweig, 2017). DNA DSBs are the more detrimental type of DNA damage which are repaired by via two pathways; homologous recombination (HR) and non-homologous end joining (NHEJ). HR is an 'error-free' repair process whereby the damaged DNA utilises sister chromatids with homologous sequences as a template to re-synthesise the damaged region. This process is initiated by breast cancer type 1 susceptibility protein (BRCA1) and also involves the recruitment of another DNA repair protein, Rad51 (Pecorino, 2016). PARP1 plays an important role in early recruitment of BRCA1 to DSBs. In NHEJ the break ends in DNA are directly ligated without an homologous template which is an error-prone process. PARP1 prevents excessive NHEJ by promoting repair via HR (Dulaney *et al.,* 2017; Bouchard *et al.,* 2003; Chaudhuri and Nussenzweig, 2017).

Given its significant role in DNA repair, it is not surprising that PARP1 has been found to be upregulated in various malignant tumours (Ossovskaya *et al.,* 2010; Wang *et al.,* 2017) including OSCC (Cervigne *et al.,* 2014; Kossatz *et al.,* 2016). It has also been found to be amplified in OLs progressing to OSCC (Cervigne *et al.,* 2014) suggesting it could be another potential candidate as a susceptibility/risk biomarker for high risk oral lesions.

An initial literature search of PARP1 expression in OLP using PubMed, Ovid and Web of Science databases, from the start of published records until 26th April 2023, using the search

terms ((PARP1) OR (PARP-1) OR (Poly (ADP-Ribose) Polymerase 1)) AND ((oral lichen planus) OR (OLP)) did not reveal any studies. A further literature search was performed explore the relationship between PARP1 expression and OSCC. PubMed, Ovid and Web of Science databases were searched, from the start of published records until 26th April 2023, using the search terms ((PARP1) OR (PARP-1) OR (Poly (ADP-Ribose) Polymerase 1)) AND ((oral cancer) OR (oral squamous cell carcinoma)). The inclusion criteria were the same as for the previously described BMI1 literature search. The search across the three databases revealed a total of 991 publications. After excluding studies that did not meet the eligibility criteria, 14 studies were included.

Kossatz and team have published a number of papers on the use of PARPi-FL, a fluorescent PARP1-targeted dye as an optical imaging agent in oral cancer. In 2016, they published an analysis of PARP1 expression in 12 human tongue samples, comparing malignant, premalignant and normal tissue. Using immunohistochemistry, they found that PARP1 expression was markedly increased in malignant and premalignant tissue when compared to normal tissue, however the difference between premalignant and malignant tissue was not statistically significant; mean PARP1-positive area was 3.1 ± 1.4% in normal tissue, 12.6 ± 2.5% ($P < 0.0001$ vs. normal) in premalignant tissue, and 17.4 ± 4.2% ($P < 0.0001$ vs. normal) in malignant tissue (Kossatz *et al.,* 2016). They also published data showing that PARP1 expression was further upregulated in OSCC treated with radiation (Kossatz, Weber and Rainer *et al.,* 2016). The similarly high levels of PARP1 expression in premalignant and malignant tissue could indicate a role for PARP1 in predicting high risk lesions. These findings are supported by Cervigne *et al.* (2014) who also found increased PARP1 expression in premalignant oral lesions. Their study aimed to find prognostic biomarkers to identify OL at

high risk of progression. RT-qPCR performed on 49 samples (21 non-progressive OL, 28 paired progressive OL and OSCCs) and showed *PARP1* expression was amplified in progressive OL and OSCC but not in non-progressive OL (p=0.0112).

PARP1 has also been studied in oral cancer recurrence and metastasis. Mascolo *et al.* (2012) used immunohistochemistry and western blotting on 66 primary OSCC samples. PARP1 was expressed in all tumour samples, however no healthy control tissue was studied for comparison. They found that HPV-negative OSCC with concurrent high expression of PARP1, CAF-1/p60 and nestin had the worst prognosis and increased risk of metastasis. Using immunoblotting on human OSCC cell lines, Wang *et al.* (2022) found that PARP1 expression was higher in recurrent oral tumour cells compared to primary tumours. Interestingly, PARP1 expression was further upregulated by treatment with Cisplatin and 5-fluorouracil in recurrent tumours. The study also showed that over-expression of PARP1 increased primary oral cancer resistance to DNA damage treatment and suggests the utilisation of PARP-1 inhibitors in oral cancer (Wang *et al*., 2022).

1.4. Summary of literature review

OLP is one of the most common oral mucosal diseases. It is an OPMD with an average reported MT rate between 1 to 2% (Landini et al., 2014; Iocca *et al.,* 2019; Gonzalez-Moles *et al.,* 2019; Idrees *et al.,* 2020). The erosive and atrophic variants appear to have a higher MT risk than other variants although the exact mechanism for this remains unknown (Alrashdan, Cirillo and McCullough 2016; Gonzalez-Moles, Scully and Gil-Montoya, 2008). The incidence of OSCC is rising and there is a predicted 30% increase annually by 2030 (Gormley *et al.,* 2022).

The five-year survival rate for patients diagnosed with oral cancer between 2009 and 2013 in England was 55% (Cancer Research UK; Gormley *et al.,* 2022). Therefore, secondary prevention of oral cancer through early identification of high risk oral lesions represents an important research area.

The identification of valid quantitative biomarkers in OLP could help predict those lesions at high risk of progression to OSCC. BMI1 is a member of the polycomb group (PcG) proteins with roles in cell self-renewal and proliferation (Herzog, Somayaji and Nör, 2023; Allegra et al., 2014). It has been identified as a cancer stem cell biomarker as it is expressed in the nuclei of CD44+ cells (Prince *et al.,* 2007). There are numerous studies showing that BMI1 expression is upregulated in OSCC and oral premalignant lesions, however there is only one study on BMI1 expression in OLP (Ma *et al.,* 2013) which found that the risk of malignant progression in BMI1-positive lesions was significantly higher than in BMI1-negative lesions.

PARP1 is a nuclear enzyme with critical roles in DNA repair (Dulaney *et al.,* 2017; Bouchard *et al.,* 2003). It has been shown to be upregulated in various cancers including OSCC (Cervigne *et al.,* 2014; Kossatz *et al.,* 2016). Studies have also shown increased PARP1 expression in premalignant tissue and OL (Cervigne *et al.,* 2014; Kossatz *et al.,* 2016) however, there are currently no reported studies on the association between PARP1 and OLP.

The published literature has identified both BMI1 and PARP1 as potential candidates for susceptibility/risk biomarkers for high risk oral lesions. One study has shown the increased risk of malignant progression in OLP lesions with increased BMI1 expression (Ma *et al.,* 2013), however, there are no published studies relating to BMI1 expression in the different OLP

variants, PARP1 expression in OLP or the potential relationship between PARP1 and BMI1. Therefore, this is an interesting and novel area of research which this study aims to explore.

CHAPTER TWO

AIMS, OBJECTIVES AND NULL HYPOTHESIS

2.1. Aims

It is generally accepted that erosive and atrophic variants of OLP have a higher malignant transformation risk than reticular (Alrashdan, Cirillo and McCullough 2016; Gonzalez-Moles, Scully and Gil-Montoya, 2008). However the exact mechanism for this increased risk remains unknown and this is further complicated by a lack of quantitative markers for high risk lesions.

Both BMI1 and PARP1 have been identified as potential candidates for susceptibility/risk biomarkers for high risk oral lesions, however there is a lack of published studies relating to these markers in OLP. This study aims to fill this current void in the knowledge and potentially discover novel biomarkers which can identify OLP lesions at high risk of malignant transformation, hence improving the management of patients with OLP.

2.2. Objectives

Primary Objective:

Identify any significant differences in BMI1 and PARP1 expression patterns in erosive/atrophic OLP compared with reticular OLP.

Secondary Objectives:

1. Identify any correlation between BMI1 and PARP1 expression in OLP.
2. Determine whether BMI1 and PARP1 can be used as molecular markers for distinguishing OLP lesions at high risk of malignant transformation from those at low risk.

2.3. Null Hypothesis

There are no statistical differences in the levels of expression of BMI1 and PARP1 between reticular OLP and erosive/atrophic OLP types.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Research Design

This is a cross-sectional, observational, pilot *in vitro* laboratory study using immunohistochemistry to identify if any significant difference exists between BMI1 and PARP1 expression patterns in erosive/atrophic OLP compared with reticular OLP.

3.2. Ethics

Ethical approval for this study was granted by London – Hampstead Research Ethics Committee (REC reference number 16/LO/0305, protocol number RG_15-112). Sponsorship was granted by the University of Birmingham.

OLP tissue samples were donated to and requested from the University of Birmingham Dentistry Research Tissue Bank (UoB DRTB) which is authorised by the National Research Ethics Service (NRES) Central Bristol Research Ethics Committee (REC reference number 14/SW/1148) to release samples to researchers.

3.3. Funding

A grant of £6,004 was awarded by the Oral and Dental Research Trust for this study (Oral and Dental Research Trust - GSK Research Award 2019).

3.4. Setting

This is a single centre study. Patients were recruited from the Oral Medicine Department in the Birmingham Dental Hospital, Birmingham, United Kingdom. All laboratory analysis took place in the University of Birmingham School of Dentistry Laboratories in the Birmingham Dental Hospital, Birmingham, United Kingdom.

3.5. Study participants

3.5.1. Population

The target population for this study was any patient attending the Oral Medicine clinic with a clinical diagnosis of either reticular or erosive/atrophic OLP who required a routine biopsy for confirmation of diagnosis. The clinical diagnosis was made based upon the clinical criteria in the modified WHO diagnostic criteria for OLP (van der Meij and van der Waal, 2003). The sample population was defined by the below inclusion and exclusion criteria.

3.5.2. Inclusion criteria

The inclusion criteria for participation in this study were as follows:

a. Patient is 16-years-old or over

b. Patient is willing and able to provide valid informed consent

c. Patient requires routine clinical biopsy taken diagnosis of suspected reticular or erosive/atrophic OLP

3.5.3. Exclusion criteria

The exclusion criteria for participation in this study were as follows:

a. Patient is under 16-years-old

b. Patient is immunocompromised

c. Patients with clinical evidence of other oral diseases

d. Patients with a comorbid autoimmune or inflammatory disease state

e. Patients with a different subtype of OLP other than reticular or erosive/atrophic

3.5.4. Sample size

As this was a pilot study, a conventional sample size calculation was not required. Sample size *rules of thumb* are commonly applied to estimate a pilot study sample size (Whitehead et al., 2016). Various rules of thumb are suggested in the literature; a popular recommended minimum sample size is 30 (Browne et al., 1995), but other suggested rules of thumb include minimum 12 subjects per arm (Julious et al., 2005) and at least 70 subjects (Teare et al., 2014).

Based upon the common rules of thumb, and considering realistic and achievable recruitment numbers, it was decided that the sample size for this study would be a minimum of 50 patients; minimum 25 patients with reticular OLP and minimum 25 patients with erosive/atrophic OLP.

3.5.5. Sampling Methods and Recruitment

Convenience sampling was used as the target population was identified prospectively upon attendance to the Oral Medicine clinic. Once a patient had been identified as being part of the target population by qualified Oral Medicine clinicians (who had previously been informed of the study and inclusion criteria), they were provided with details of the study and invited to participate. Patients who were interested in participating were either recruited on clinic by researcher Alexandra Perks (AP), or given a Participation Information Sheet (Appendix 8.1) and advised they would be contacted by researcher AP by telephone to complete recruitment.

The recruitment process by researcher AP involved confirming that patients met the study inclusion criteria, providing patients with details of the study and a Participation Information

Sheet (Appendix 8.1). Patients were informed that participation was entirely voluntary and they may stop taking part at any time without it effecting their normal care. It is important to note that as part of the inclusion criteria for this study, patients had to require a routine biopsy for clinical diagnostic purposes as to not subject patients to biopsy for purely research purposes. Therefore, if they did not choose to participate in the study, or withdrew their decision to participate at any time, they would still undergo biopsy for diagnostic purposes. A research appointment date with researcher AP was then arranged for the patient.

3.6. Materials

3.6.1. Tissue samples

At the research appointment, the patient completed the informed consent form (Appendix 8.2) and their demographic data and medical history were recorded. The *Chronic Oral Mucosal Diseases Quality of Life Questionnaire (COMDQ)* (Appendix 8.3) and *Oral Health Impact Profile Questionnaire (OHIP-14)* (Appendix 8.4) were completed by the patient. These questionnaires were not analysed as part of this study but provide useful data for future studies. A clinical examination was completed, in which the clinical type of OLP was recorded.

Biopsy procedure

To obtain the tissue sample for the study, a routine clinical biopsy was taken from a representative area of OLP. Informed consent was gained prior to the procedure which included a discussion of the possible risks of the biopsy procedure (pain, swelling, bleeding, infection and numbness). Local anaesthetic was administered into the identified biopsy site. The biopsy was taken with an 8mm punch biopsy device; a circular blade was advanced into

the oral tissue with continuous rotational pressure and the resultant cylinder of tissue was grasped at the base with tissue forceps and a scalpel used to excise it (Perks et al., 2019) (Figure 3.1). Resorbable sutures were placed to close the resultant wound and achieve haemostasis.

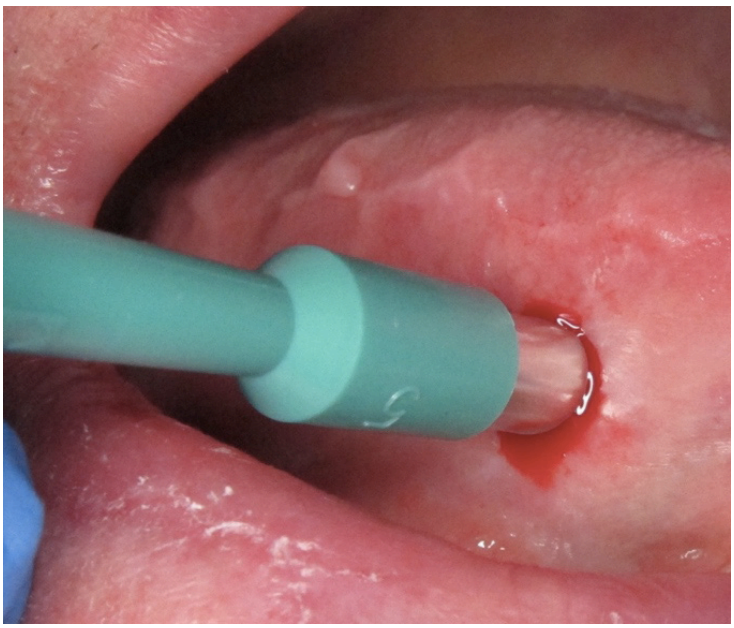

Figure 3.1. Punch biopsy technique. The photograph shows a punch biopsy device being advanced into the lateral tongue.

A number 15 scalpel blade was then used divide the tissue sample; two-thirds of the sample was place in formaldehyde and sent for histopathological diagnosis as part of the patient's routine clinical care. The surplus one-third of the sample was further divided into two equal-sized specimens (approximately 1-2mm); specimen A was placed into 10% neutral buffered formalin (Sigma) for 18-24hrs and specimen B was placed in RNAlater™ (Quiagen). The specimens were labelled with the study ID number, donated to the UoB DRTB using the Sample Donation Form (Appendix 8.5) and taken to the laboratory where they were appropriately processed and stored. After fixation, specimen A was dehydrated through

graded alcohols and embedded in paraffin wax blocks for histological processing. Specimen B was stored in an ultra-low temperature freezer for future molecular analyses.

At the end of the appointment, haemostasis was confirmed and the patient was given post-operative instructions. Any adverse events were recorded. A routine Oral Medicine clinic appointment was arranged to provide the biopsy results and ongoing management. Researcher AP confirmed that the histopathology report for each patient was consistent with a diagnosis of OLP, according to the histopathological criteria in the modified WHO diagnostic criteria for OLP (van der Meij and van der Waal, 2003).

It was not possible to blind researcher AP to the OLP subtype at the time of sample collection due to nature of the procedure.

3.7. Methods

3.7.1. Initial tissue processing

Specimen A samples were fixated for 18-24 hours in 10% Neutral Buffered Formalin, dehydrated through graded alcohols and cleared with xylene (Fisher Scientific) before being embedded into paraffin was blocks. See fixation table for further details (Appendix 8.6).

3.7.2. Immunohistochemistry (IHC)

Preparation of paraffin sections

Four-micrometre thick serial tissue sections were cut from the formalin-fixed, paraffin-embedded tissue blocks (specimen A) using a Leica Rotary Microtome and mounted on

Superfrost positively charged glass slides (VWR) and left for 18 hours (overnight) at room temperature to flatten. Sections were then incubated at 56°C for 1 hour. Prior to staining the sections were de-waxed in xylene (1 x 5 minutes, 1 x 1 minute), rehydrated through graded alcohols (100% x 2 for 1 minute, 90% for 1 minute, 70% for 1 minute, 50% for 1 minute) and placed in PBS (phosphate buffered saline, pH7.6, 7.25g of NaCl, 1.5g of K_2HPO_4 and 0.2g of KH_2PO_4 (Sigma), dissolved in 1L of RO water (reverse osmosis water – 15 ohms - made by 'E-Pod Elix', Millipore)) for immunological staining or water for routine histological staining.

Antigen retrieval – microwave pre-treatment

Antigen retrieval was performed by placing the sections in heat-mediated citrate buffer (1mM (ph6) sodium citrate buffer - 2.94g sodium citrate (Sigma) and 0.05% (0.5ml) Tween 20 (Sigma) made up to 1L with RO water) in heat-proof plastic containers and placing inside a 95°c water bath for 20 minutes. After pre-treatment, sections were washed in PBS for 2 minutes.

Staining

All procedures until 3,3'-Diaminobenzidine (DAB) incubation were carried out in a humidity box to prevent the sections drying out. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. The sections were then washed in PBS for 2 minutes. Non-specific protein binding sites were blocked using 2.5% normal horse serum (VECTASTAIN® Elite® ABC-HRP Kit) for 10 minutes. The excess horse serum was tipped off and the sections were then incubated with the primary antibodies, appropriately diluted with PBS-BSA (Bovine Serum Albumin, 1%), for two hours; anti-BMI1 rabbit monoclonal antibody (1:200 dilution,

ab126783; Abcam, Cambridge, UK) and anti-PARP1 rabbit monoclonal antibody (1:500 dilution, ab191217; Abcam, Cambridge, UK).

After two hours incubation the sections were washed in PBS for 3 x 2 minutes, then incubated with universal biotinylated anti-mouse/rabbit IgG (VECTASTAIN® Elite® ABC-HRP Kit) for 30 minutes. The sections were washed in PBS for 3 x 2 minutes, the incubated with stabilised Elite ABC (Avidin/Biotinylated enzyme Complex) reagent (VECTASTAIN® Elite® ABC-HRP Kit) for 30 minutes. Sections were washed in PBS for 3 x 2 minutes, then incubated with DAB (10mg of DAB (Sigma) in 20ml of PBS, filtered, then 25 microLiters of H_2O_2 (Sigma)) for 10 minutes, washed in distilled water and counterstained with Methyl Green (0.5g of Methyl green (Sigma) and 100ml of 0.1M sodium acetate buffer (1.36g sodium acetate (Sigma) in 100mls water adjusted to pH 4.2 with Glacial acetic acid (Sigma)).

The sections were finally dehydrated through graded alcohols (70% for 1 minute, 90% for 1 minute, 100% x 2 for 1 minute) and xylene (x 2 for 1 minute) , before being mounted with DPX mounting medium (Sigma) and coverslips.

Controls

Negative reagent controls were performed for all samples following the same methods as above but incubating with negative rabbit IgG antibody (1:500 dilution, Recombinant Rabbit IgG, monoclonal [EPR25A] – Isotype control (ab172730)).

For positive tissue controls, sections of rat brain (ten-micrometre thick) were incubated with anti-BMI1 antibody and sections of rat testis (four-micrometre thick) were incubated with

anti-PARP1 antibody, as per manufacturer datasheet recommendations (Abcam Cambridge, UK).

For ethical reasons, no negative tissue controls could be prospectively collected, as this would require control patients with healthy oral mucosa to undergo an invasive surgical procedure for no clinical benefit. This meant the researchers were limited to using previously collected samples from the UoB DRTB where unfortunately no appropriate samples could be identified. Therefore, a limitation of this study is that no negative tissue controls were possible.

3.7.3. Haematoxylin and Eosin (H&E) staining

Routine H&E staining was carried out as described in Bancroft & Stevens (1982). In summary, sections were dewaxed in xylene and rehydrated through graded alcohols. Sections were then stained with Gill's III Haematoxylin (Cell Path), differentiated with 0.3% Acetic acid (Sigma), treated with 0.3% Hydrochloric acid (Sigma) in 70% alcohol, treated with Scott's Tap Water Substitute (Cell Path) and stained with Eosin (Cell Path). Between each stage the sections were rinsed in running tap water. Sections were finally dehydrated through graded alcohols and xylene, before being mounted with DPX mounting medium and coverslips.

3.8. Data analysis

3.8.1. Specimen visualisation

The Microscopy Facility team at UoB created high resolution virtual slides from the immunostained sections. Brightfield images were acquired on the Zeiss AxioScan .Z1 slide

scanner using a 20x objective (Plan-Apochromat 20z/0.8). The virtual slides were then visualised using ZEN 3.1 (blue edition) visualisation software.

3.8.2. IHC scoring

The virtual slides were examined by two raters, TB and AP. TB is a consultant histopathologist specialised in Oral and Maxillofacial pathology while AP has basic microscopy and oral histopathology training but is not a specialist. Both raters were blinded to all clinical data including the OLP subtype in order to reduce observational bias. This was achieved by ensuring the specimens were labelled with study numbers only (LP001, LP002, etc.); however, it is appreciated that it is possible to deduce whether a sample is reticular or erosive/atrophic type according to the histological features present in the section.

Nuclear immunoreactivity localised in the epithelium was considered to indicate either PARP1 or BMI1 positivity (Abcam Cambridge, UK) and the level of expression was scored as discussed below.

3.8.3. Scoring system

Various histopathological scoring systems exist for the evaluation of tissue staining, including IHC. Crissman *et al.* (2004) suggested that scoring systems should be definable, reproducible, and meaningful. IHC is not stoichiometric and uses a series of amplification steps that make it unsuitable for accurate quantification based on chromogen intensity (i.e. darkness of stain). Therefore semi-quantitative scoring approaches have been suggested to characterise the staining patterns found in tissues (Meyerholz and Beck, 2018; Fedchenko and Reifenrath, 2014). There are several types of semi-quantitative approaches but the ordinal method is

most commonly used in clinical and preclinical research (Meyerholz and Beck, 2018). Frequently, scoring systems use multiple ordinal parameters providing a total composite score which can be used for statistical testing (Fedchenko and Reifenrath, 2014). Composite scoring systems are widely accepted and are considered to be "gold-standard" for immunohistochemistry (Fedchenko and Reifenrath, 2014). One such scoring system is the Immunoreactive Score (IRS) (Table 3.1) which was originally used for oestrogen-receptor detection in breast cancer tissue (Remmele and Stegner, 1987). This scoring system has also been used for BMI1 expression analysis in oral tissue (Huber *et al.,* 2011).

The IRS score (Table 3.1) was chosen for BMI1 and PARP1 expression analysis in this study, as it is a semi-quantitative composite score which is commonly used for immunohistochemistry including BMI1 analysis in oral tissues (Huber et al., 2011). Furthermore, in comparison to other composite scoring systems such as the Allred score (Allred et al., 1998), it is also able to convert the composite numerical score into a categorical one, which can be useful for statistical analysis (Fedchenko and Reifenrath, 2014).

Table 3.1. The Immunoreactive Score (IRS)

A (percentage of positive cells)	B (intensity of staining)	IRS score (multiplication of A and B)
0 = no positive cells	0 = no colour reaction	0-1 = negative
1 = < 10% of positive cells	1 = mild reaction	2-3 = mild
2 = 10-50% of positive cells	2 = moderate reaction	4-8 = moderate
3 = 51-80% of positive cells	3 = intense reaction	9 -12 = strong
4 = > 80% of positive cells		
Final IRS score (A x B): 0-12		

3.8.4. Scoring pilot

A scoring pilot exercise of ten OLP samples was carried out separately by the two raters. Both raters were blinded to the other rater's scores until a formal meeting was held to discuss the results. At the meeting, scoring discrepancies were discussed to try and improve rater agreement for the remainder of the scoring. Figure 3.2. shows what was agreed by both raters as representing no colour reaction (0), mild reaction (1), moderate reaction (2) and intense reaction (3) for IRS Score B (intensity of staining). It was identified that both PARP1 and BMI1 staining was predominantly in the basal half of the epithelium. As the raters were initially only scoring the full thickness of the epithelium, this lead to low A Scores (percentage of positive cells). The expert rater (TB) recommended that for the remainder of the scoring, the full thickness and the basal half of the epithelium were scored separately and compared. Meyerholz and Beck (2018) raised this issue of deciding whether scoring should represent the whole tissue section or a defined area, especially when tissues have different functional activity at different locations.

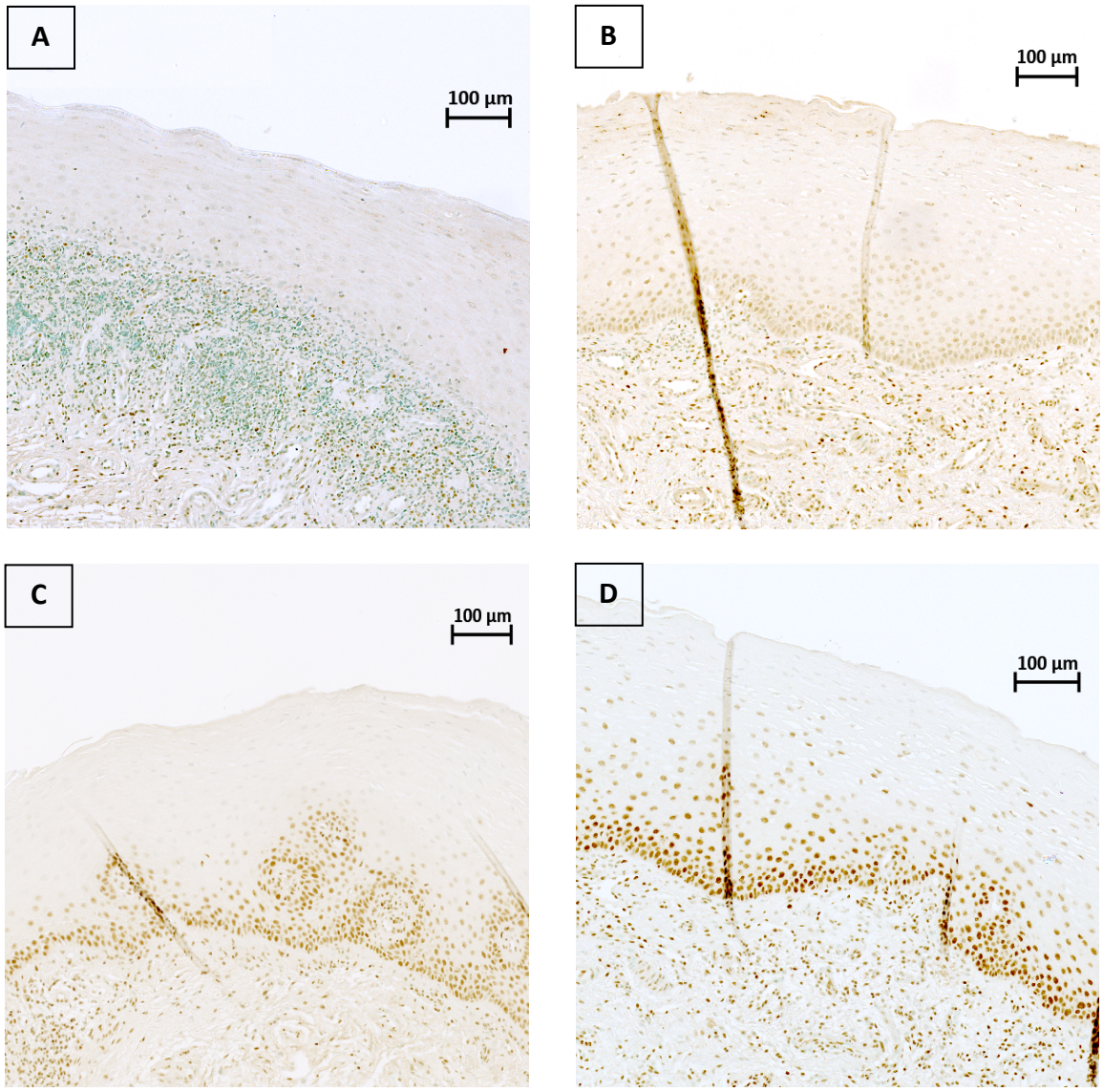

Figure 3.2. Rater agreement on intensity of staining (IRS Score B). (A) 0 = no colour reaction, (B) 1 = mild reaction (C) 2 = moderate reaction (D) 3 = intense reaction

3.8.5. Statistical Analysis

Inter-rater reliability was quantified by the kappa statistic (κ) with 95% confidence intervals. The χ^2 test of independence (when there were five or more observations in all cells) or Fisher's exact test (when there were fewer than five observations in any cell) were used to analyse categorical data (baseline characteristics and categorical IRS scores). A Pearson correlation coefficient (*r)* was computed to assess the linear relationship between numerical IRS scores for BMI1 and PARP1. The significance level was set at $p < .05$ for all tests. Statistical analysis was performed using IBM SPSS Statistics (Version 29.0.1.0).

CHAPTER FOUR

RESULTS

4.1. Immunohistochemical staining

Determining optimum staining conditions

To determine the optimum staining conditions different primary antibody concentrations and pre-treatments were tested. Three different concentrations of the anti-PARP1 antibody (1:1000, 1:500 and 1:250) and of the anti-BMI1 antibody (1:200, 1:100 and 1:50) were tested. All of the described antibody concentrations were tested with three different pre-treatment methods; microwave heat treatment, 0.1% trypsin digestion and no pre-treatment. Anti-PARP1 antibody at a dilution of 1:500 and anti-BMI1 antibody at a dilution of 1:200 both heat treated was determined to be the optimum staining conditions.

Representative examples of IHC staining

Figures 4.1 to 4.4 show a representative example of the whole tissue virtual histology slides for specimen LP001. Figure 4.1 shows the section stained with H&E, Figure 4.2 shows the negative reagent control section, Figure 4.3 shows the section stained with anti-PARP1 antibody and Figure 4.4 shows the section stained with anti-BMI1 antibody.

Figure 4.5 shows representative PARP1 expression in OLP samples with different categorical IRS scores (as scored by the expert observer) alongside the PARP1 positive control and a representative negative reagent control.

Figure 4.6. shows representative BMI1 expression in OLP samples with different categorical IRS scores (as scored by the expert observer) alongside the BMI1 positive control and a representative negative reagent control.

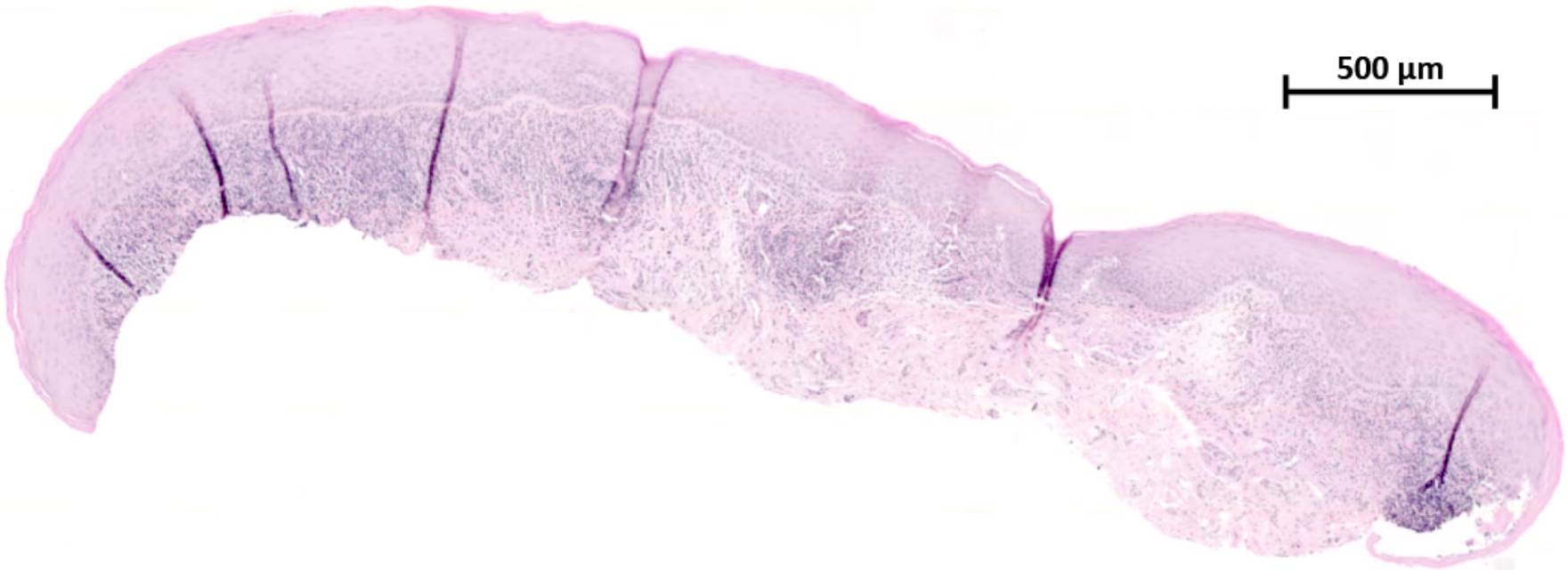

Figure 4.1. LP001 specimen stained with H&E.

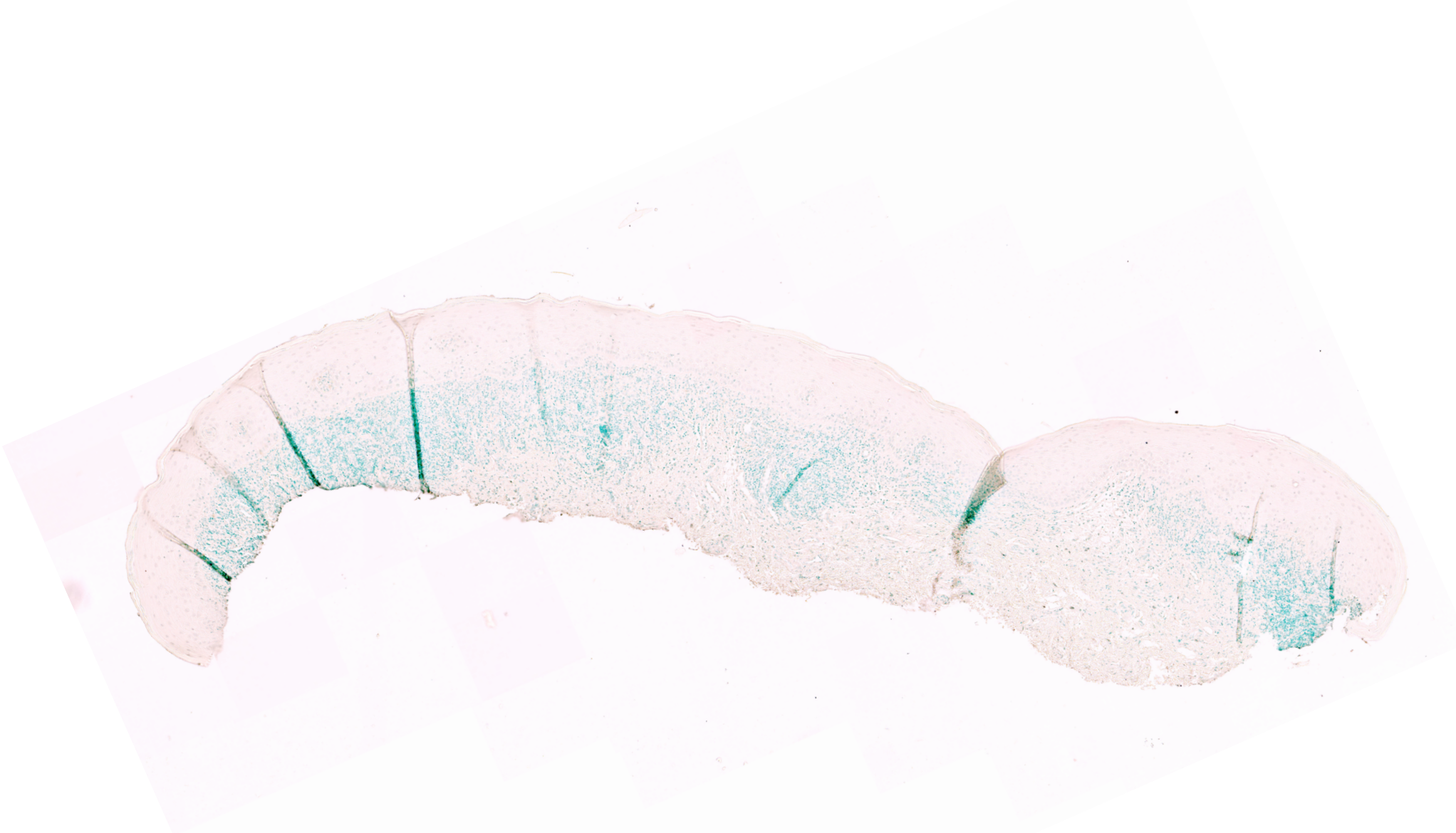

Figure 4.2. LP001 specimen negative reagent control. Stained with negative rabbit IgG antibody (1:500 dilution, Recombinant Rabbit IgG, monoclonal [EPR25A] – Isotype control (ab172730)).

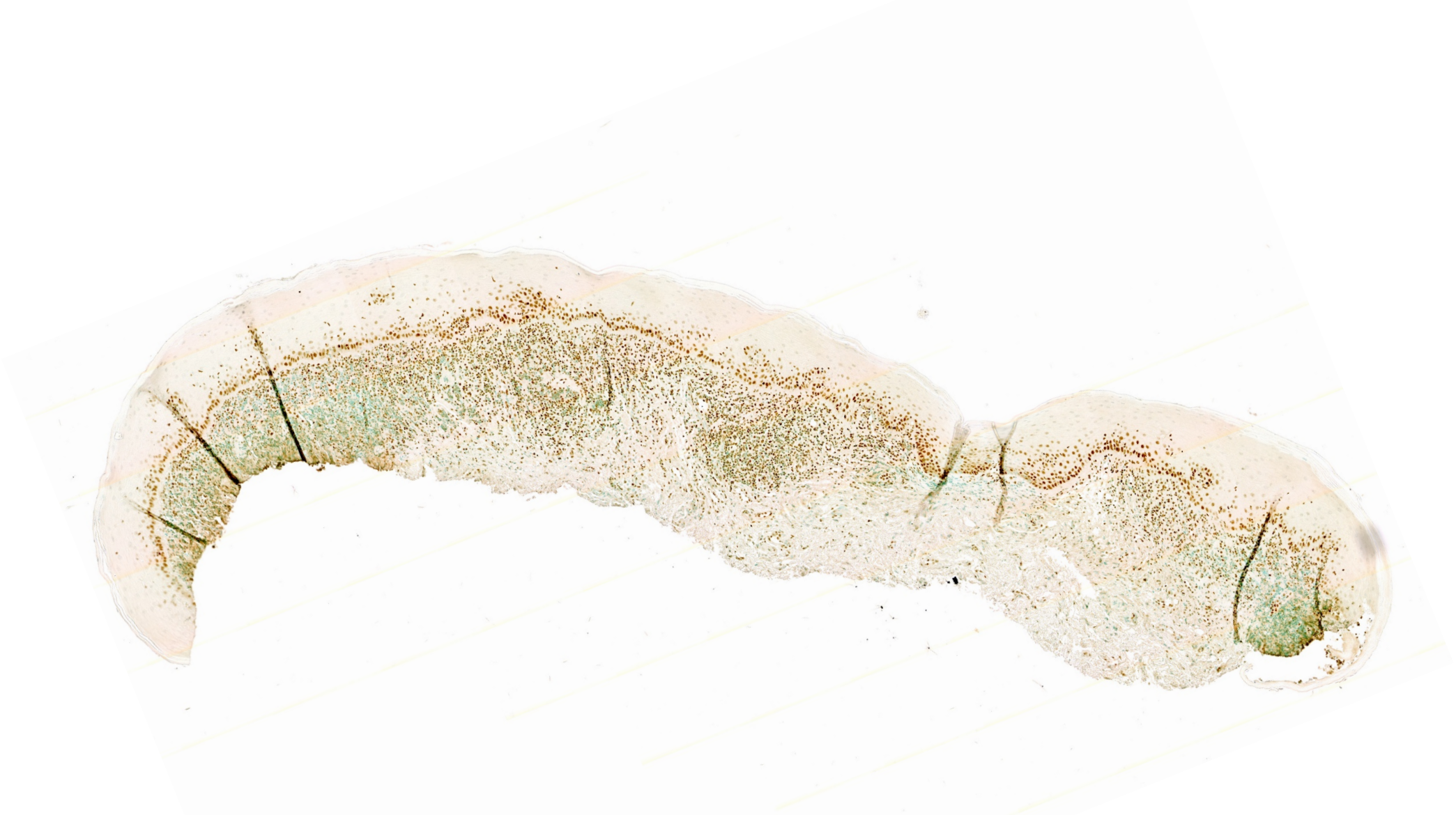

Figure 4.3. LP001 specimen stained with anti-PARP1 antibody (anti-PARP1 rabbit monoclonal antibody (1:500 dilution, ab191217; Abcam, Cambridge, UK))

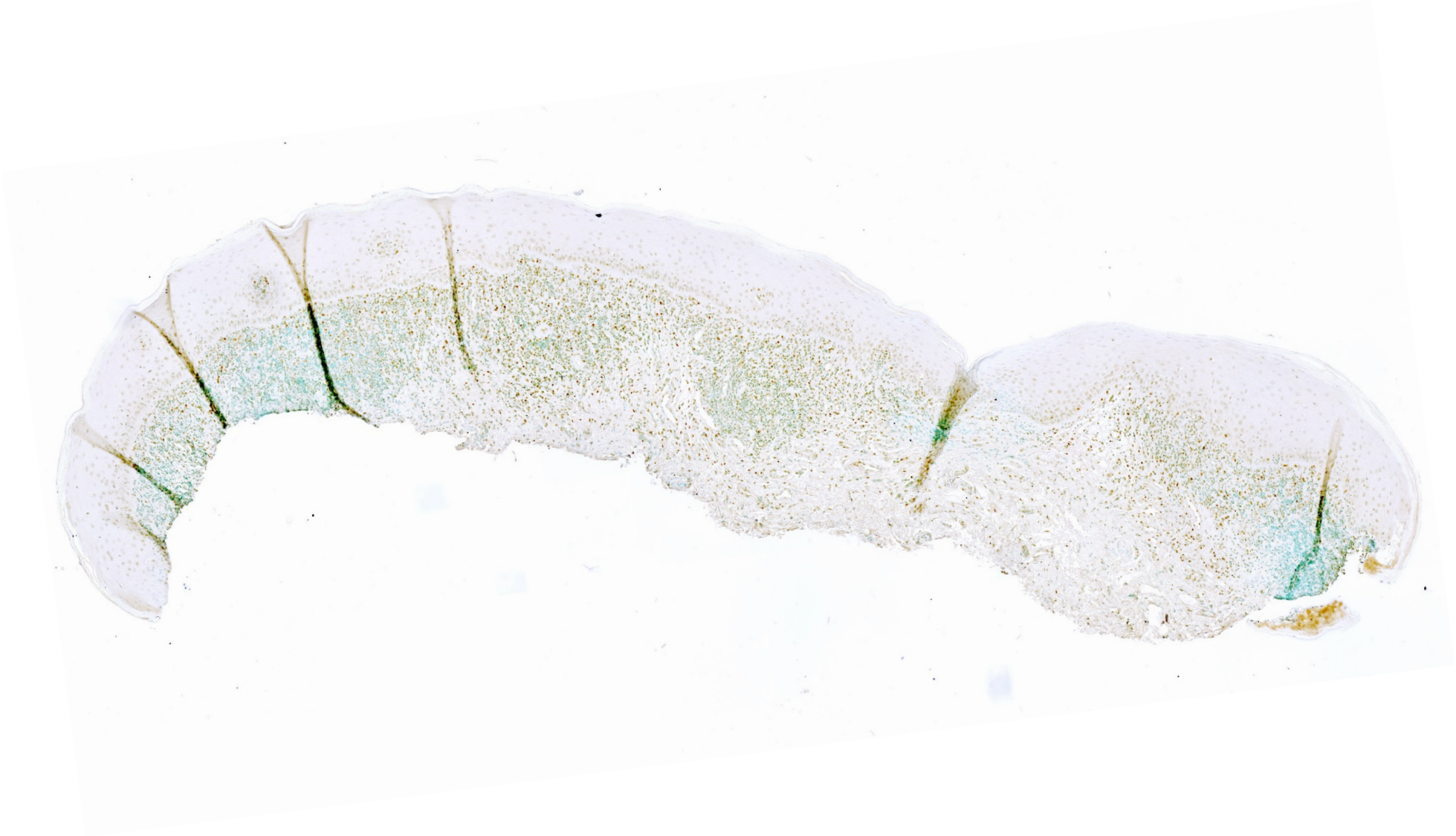

Figure 4.4. LP001 specimen stained with anti-BMI1 antibody (anti-BMI1 rabbit monoclonal antibody (1:200 dilution, ab126783; Abcam, Cambridge, UK))

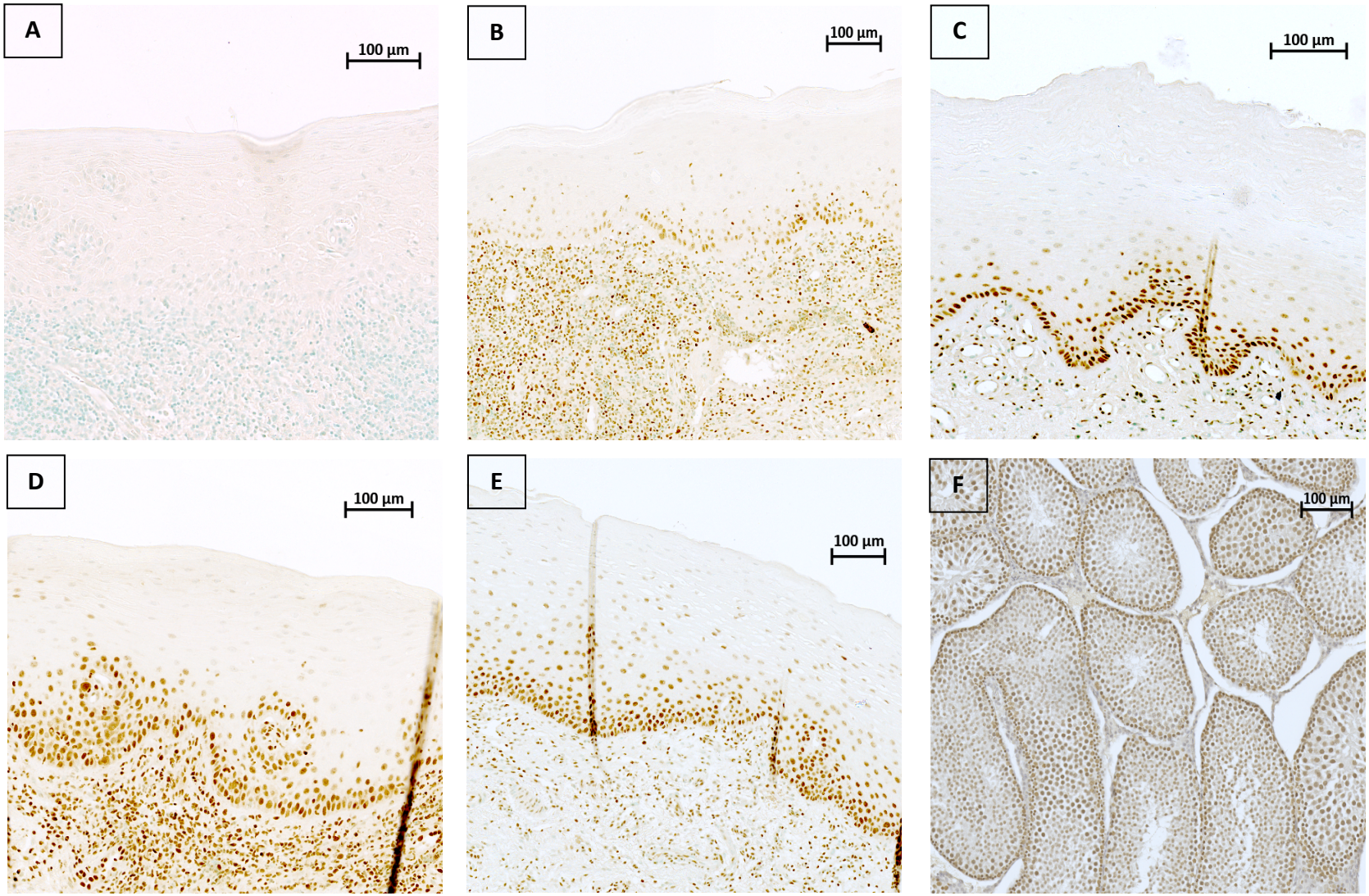

Figure 4.5. Representative PARP1 expression. (A) Negative reagent control (B) IRS score = Negative (C) IRS score = Mild (D) IRS score = Moderate (E) IRS score = Strong (F) Positive tissue control (rat testis). Expert observer IRS scores.

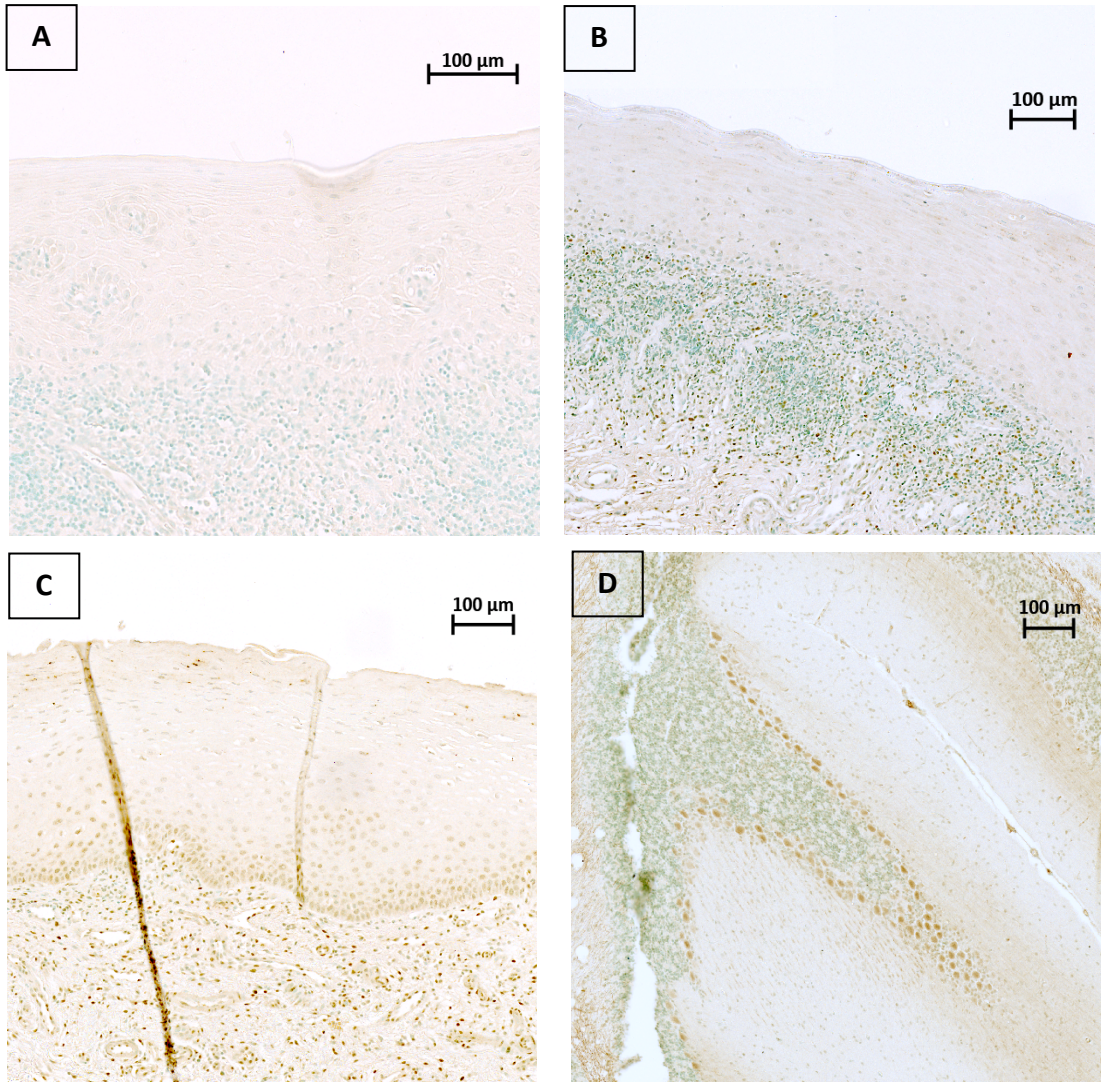

Figure 4.6. Representative BMI1 expression. (A) Negative reagent control (B) IRS score = Negative (C) IRS score = Mild (D) Positive tissue control (rat brain). Expert observer IRS scores.

4.2. Baseline characteristic of the sample

The minimum sample size of 50 subjects was exceeded with 67 participants being recruited into the study. Of these, 63 samples were included in immunohistochemical analysis of BMI1 and PARP1 staining; 4 samples were excluded due to suboptimal tissue sampling or embedding. Of the 63 included samples, 32 were diagnosed as reticular OLP while 31 were atrophic/erosive OLP. Table 4.1 summarises the baseline characteristics of the sample. A χ^2 test of independence or Fisher's exact test was performed to identify if any significant associations between baseline characteristics and OLP subtypes existed. The female to male ratio was higher in the reticular cohort (3.5:1) compared to the atrophic/erosive cohort (2.1:1) however the χ^2 test did not find this to be statistically significant, χ^2 (1, N=63) = .86, p = .353.

The overall age distribution was similar in both cohorts, with the majority of participants aged between 45 and 74 years old. The Fisher's exact test did not reveal any significant association between age and OLP subtype (p = .795). The proportion of smoking and alcohol status were very similar in both cohorts; 68.7% of the reticular and 67.7% of the erosive/atrophic cohort were non-smokers, 75% of the reticular and 67.7% of the erosive/atrophic cohort were current consumers of alcohol. The Fisher's exact test found that neither smoking (p = 1.00) nor alcohol (p = .691) status had a significant association with OLP subtype. The participants who were current consumers of alcohol were sub-divided into those who drank up to 14 units per week and those who drank more than 14 units per week. The Fisher's exact these found that the amount of alcohol consumed did not have a significant association with OLP subtype (p = .826).

Table 4.1. Baseline characteristics of the OLP cohort

Characteristic	OLP subtype				χ^2	*p* value
	Reticular (*n* = 32)		Erosive/Atrophic (*n*=31)			
	N	%	*N*	%		
Gender					.86	.353
Male	7	21.9	10	32.3		
Female	25	78.1	21	67.7		
Age						.795
16-24	0	0	0	0		
25-34	3	9.4	3	9.7		
35-44	5	15.6	3	9.7		
45-54	6	18.8	8	25.8		
55-64	12	37.5	8	25.8		
65-74	4	12.5	7	22.6		
75-84	2	6.2	2	6.4		
85 +	0	0	0	0		
Smoking status						1.00
Never	22	68.7	21	67.7		
Ex	7	21.9	8	25.8		
Current	3	9.4	2	6.4		
Alcohol status						.691
Never	7	21.9	7	22.6		
Ex	1	3.1	3	9.7		
Current	24	75	21	67.7		.826
Up to 14 units/week	20	62.5*	18	58.1*		
More than 14 units/week	4	12.5*	3	9.7*		

* % of reticular or erosive/atrophic cohort

4.3. Inter-rater reliability

An inter-rater reliability analysis using the Kappa (κ) statistic was performed to assess the level of agreement between the two raters, AP and TB in relation to the categorical IRS scores for the whole of the data. The inter-rater reliability for the complete data set was found to be Kappa = .347 (*p* <.001), 95% CI (0.271, 0.423) indicating 'fair' agreement. Furthermore, Table 4.2 shows the separate inter-rater reliability analysis for PARP1 and BMI1, for both the full thickness (FT) and basal half (BH) of the epithelium. For both PARP1 data sets, the Kappa values indicate 'fair' agreement. For both BMI1 data sets, the Kappa values indicate 'slight' agreement.

Table 4.2. Inter-rater reliability analysis for BMI1 and PARP1 categorical IRS scores

	Kappa (κ)	**Kappa interpretation**	***P* value**	**95% CI**
PARP1				
Full thickness of epithelium	.400	Fair	< .001	0.228, 0.572
Basal half of epithelium	.215	Fair	.013	-0.01, 0.441
BMI1				
Full thickness of epithelium	.028	Slight	.329	-0.027, 0.083
Basal half of epithelium	.071	Slight	.156	-0.003, 0.145

4.4. PARP1 and BMI1 expression in reticular and erosive/atrophic OLP

The χ^2 test of independence (when there were five or more observations in all cells) or Fisher's exact test (when there were fewer than five observations in any cell) was performed to evaluate the relationship between OLP variants (reticular and erosive/atrophic) and PARP1 and BMI1 expression levels (categorical IRS scores). As inter-rater reliability was inadequate, the following analysis primarily focuses on the expert observer (TB) scores, although mean scores between the two observers are also presented for categorical IRS scores, for comparison.

4.4.1. Categorical IRS scores

4.4.1.1. PARP1

Expert observer IRS scores

There were no significant differences between the OLP variants and PARP1 IRS scores for either the FT of the epithelium (p = .858) or the BH of the epithelium (p = .681) as per the Fisher's exact test (Table 4.3). The null hypothesis is therefore accepted.

There were no statistical significant differences in the distribution of categorical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP (Figure 4.7). However, there was a small difference between the number of erosive/atrophic samples that have strong staining compared to reticular (Table 4.3 and Figure 4.7). When considering the FT of the epithelium, no reticular samples showed strong PARP1 staining compared to 1 erosive/atrophic sample (3.2%) which did. In the BH of the epithelium, 2 reticular samples showed strong staining

(6.2%) compared to 3 erosive/atrophic samples (9.7%). There was also a very minimal increase in moderate staining in erosive/atrophic samples (FT 29.0%, BH 77.4%) compared to reticular (FT 28.1%, BH 71.9%). Whilst these differences appear very small, it is worth noting that the malignant potential of OLP appears to be approximately 1-2% (Landini et al., 2014; Iocca et al.,2019; Gonzalez-Moles et al,. 2019; Idrees et al., 2020) therefore it seems unlikely that large and consistent differences in marker expression would be observed across the clinical subtypes but the samples showing stronger staining could still represent those at higher risk of malignant transformation.

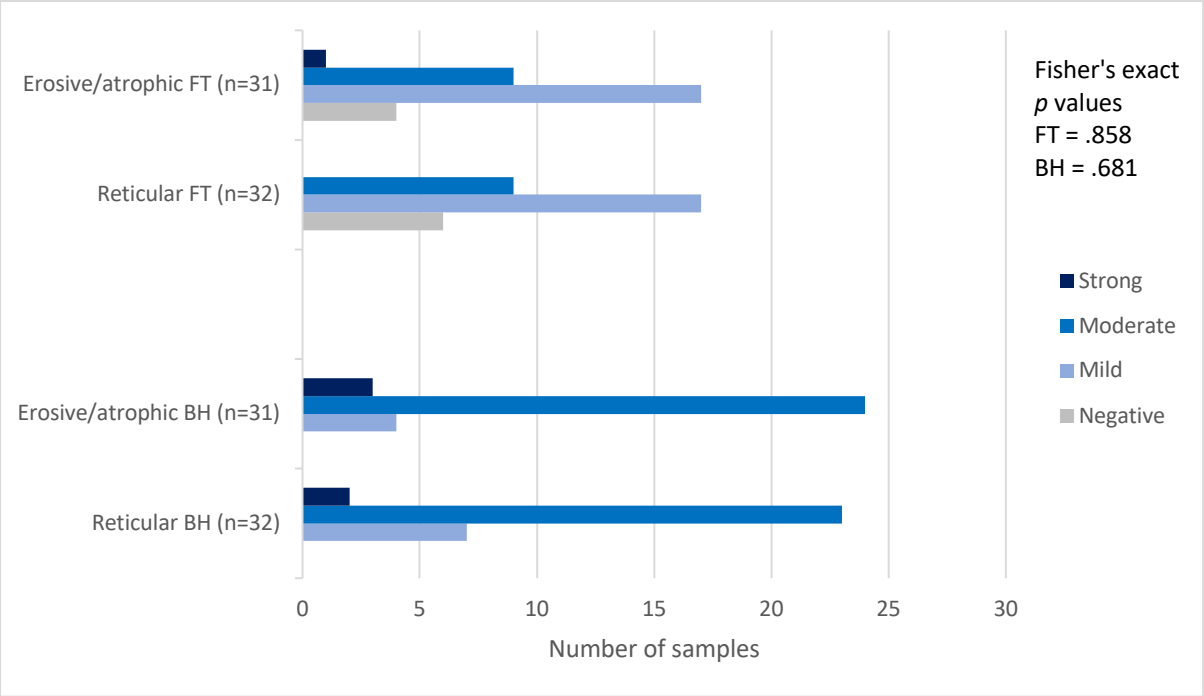

Figure 4.7. PARP1 expert categorical IRS scores. The graph shows the distribution of categorical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP in both FT and BH of epithelium. A Fisher's exact test did not find any statistically significant differences.

Table 4.3. Expert Categorical IRS scores for PARP1

		Categorical IRS scores for PARP1								*p* value
		Negative		Mild		Moderate		Strong		
		N	%	N	%	N	%	N	%	
Full thickness	OLP variant									.858
	Reticular (*n*=32)	6	18.8	17	53.1	9	28.1	0	0	
	Erosive/Atrophic (*n*=31)	4	12.9	17	54.8	9	29.0	1	3.2	
Basal half	OLP variant									.681
	Reticular (*n*=32)	0	0	7	21.9	23	71.9	2	6.2	
	Erosive/Atrophic (*n*=31)	0	0	4	12.9	24	77.4	3	9.7	

P-value calculated using Fisher's exact test

Mean IRS scores

There was no significant relationship between OLP variants and PARP1 IRS scores for either the FT of the epithelium (p = .310) or the BH of the epithelium (p = .726) as per the Fisher's exact test. The mean and expert categorical IRS scores for PARP1 were compared in the FT epithelium and BH epithelium (Figures 4.8 and 4.9 respectively). These results do not show any significant differences between expert and mean IRS scores.

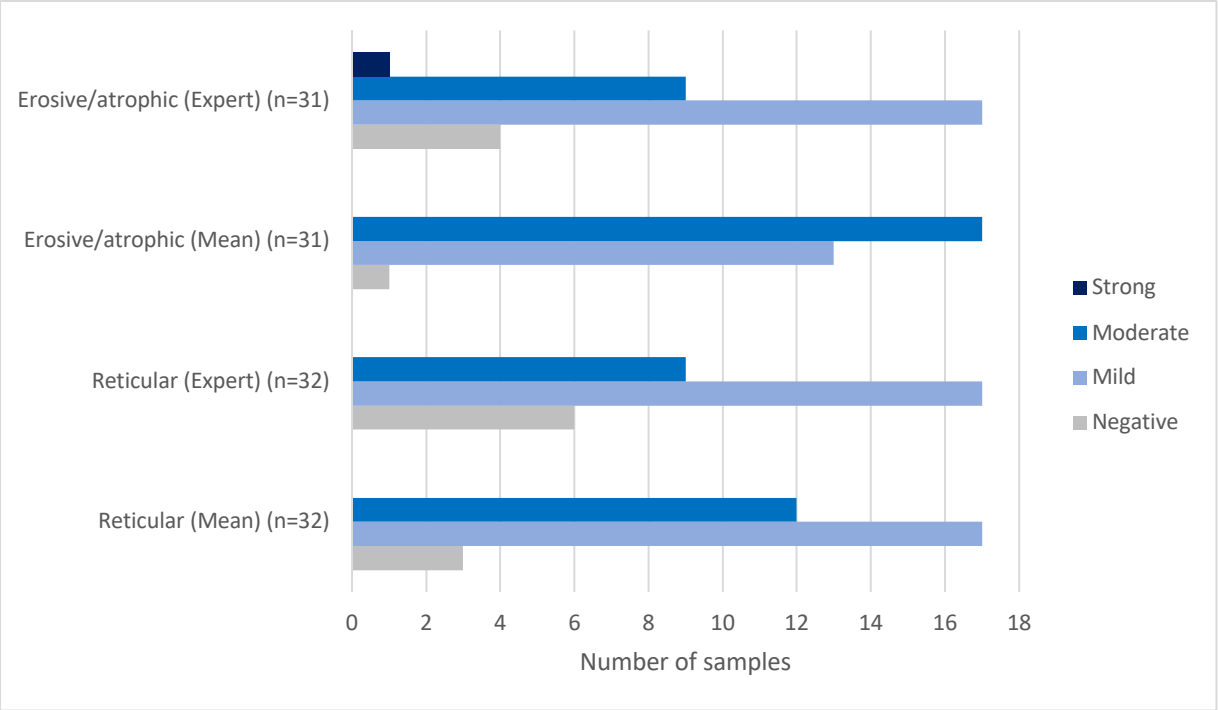

Figure 4.8. Comparison of expert and mean categorical IRS Scores for PARP1 full thickness of epithelium. The graph shows the distribution of expert and mean categorical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP in FT of epithelium. There were no statistically significant differences between expert and mean IRS scores.

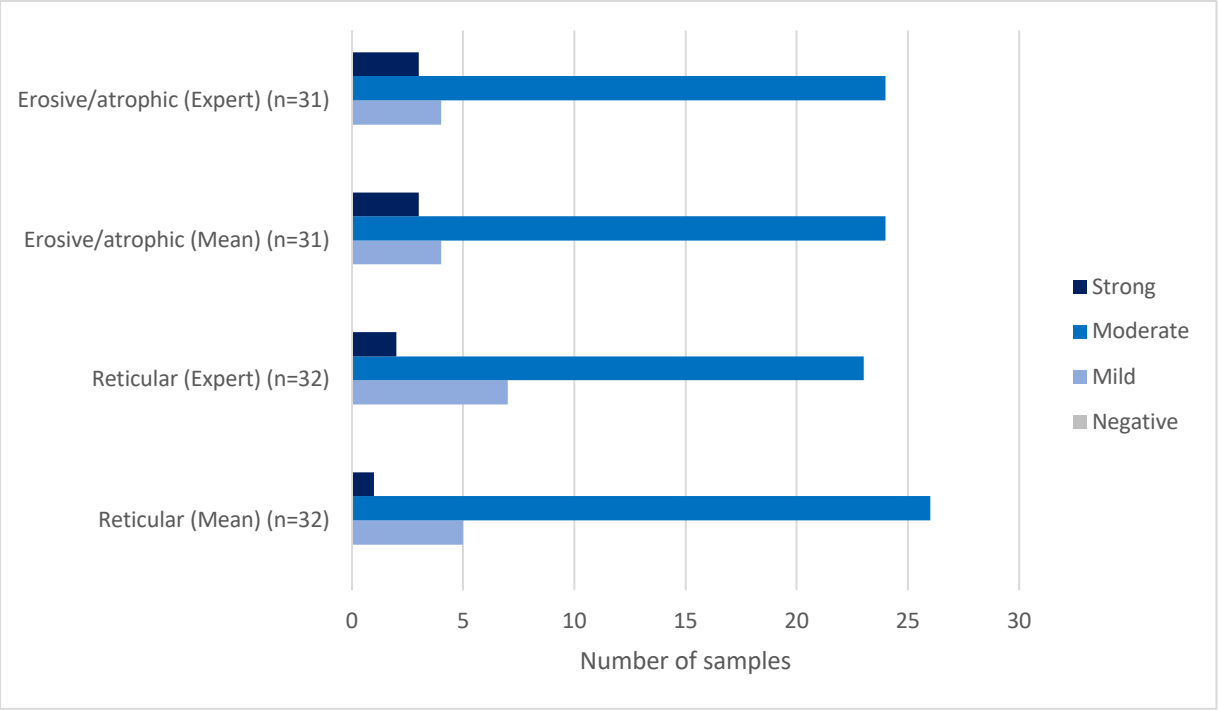

Figure 4.9. Comparison of expert and mean categorical IRS Scores for PARP1 basal half of epithelium. The graph shows the distribution of expert and mean categorical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP in BH of epithelium. There were no statistically significant differences between expert and mean IRS scores.

4.4.1.2. BMI1

Expert observer IRS scores

There were no significant differences between OLP variants and BMI1 IRS scores for either the FT of the epithelium (p = .492) or the BH of the epithelium (p = .649) as per the Fisher's exact test (Table 4.4.). The null hypothesis is therefore accepted.

The categorical IRS scores were much lower for BMI1 compared to PARP1, with most samples being scored as negative and a small number as mild. Similarly to PARP1, although there was no statistical significant differences between the two subtypes, there was small difference between the number of erosive/atrophic samples that have mild BMI1 staining compared to reticular (Table 4.4 and Figure 4.10). In the FT of the epithelium, no reticular samples showed strong staining compared to 1 erosive/atrophic sample (3.2%). In the BH of the epithelium, 3 reticular and 3 atrophic/erosive samples showed mild staining (9.4% and 9.7% respectively). As with PARP1, these small differences could represent those OLP samples at higher risk of malignant transformation.

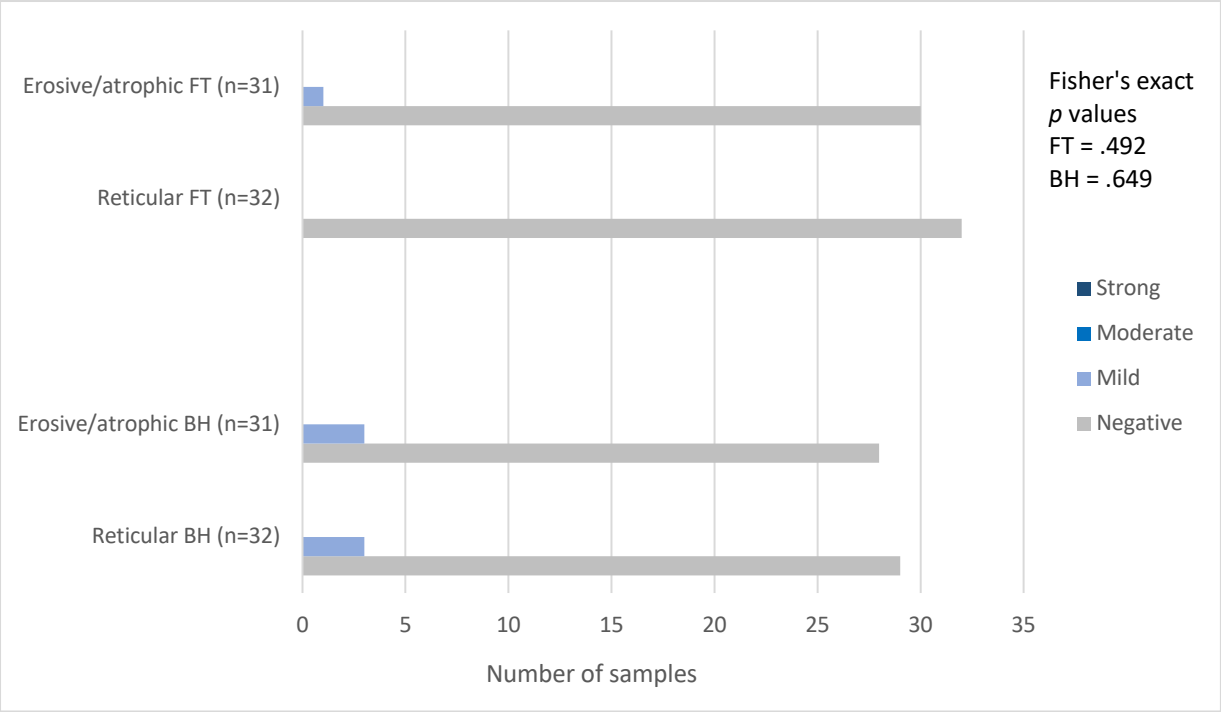

Figure 4.10. BMI1 expert categorical IRS scores. The graph shows the distribution of categorical IRS scores for BMI1 in reticular OLP compared to erosive/atrophic OLP in both FT and BH of epithelium. A The Fisher's exact test did not reveal statistically significant differences between the groups.

Table 4.4. Expert Categorical IRS scores for BMI1.

		Categorical IRS scores for BMI1								*p* value
		Negative		Mild		Moderate		Strong		
		N	%	N	%	N	%	N	%	
Full thickness	OLP variant									.492
	Reticular (*n*=32)	32	100	0	0	0	0	0	0	
	Erosive/Atrophic (*n*=31)	30	96.8	1	3.2	0	0	0	0	
Basal half	OLP variant									.649
	Reticular (*n*=32)	29	90.6	3	9.4	0	0	0	0	
	Erosive/Atrophic (*n*=31)	28	90.3	3	9.7	0	0	0	0	

P-value calculate using Fisher's exact test.

Mean IRS scores

There was no significant difference between OLP variants and BMI1 IRS scores for the FT of the epithelium, χ^2 (1, N=63) = .019, p = .891 (Yates correction, p = 1.00) as per the χ^2 test of independence. There was also no significant difference between OLP variants and BMI1 IRS scores for the BH of the epithelium (p = .221) as per Fisher's exact test. The mean BMI1 IRS scores were generally higher than the expert observer's IRS scores, indicating that the non-expert observer scored the staining higher the expert observer Figures 4.11 and 4.12 compare the mean and expert categorical IRS scores for BMI1, in full thickness epithelium and lower half epithelium, respectively.

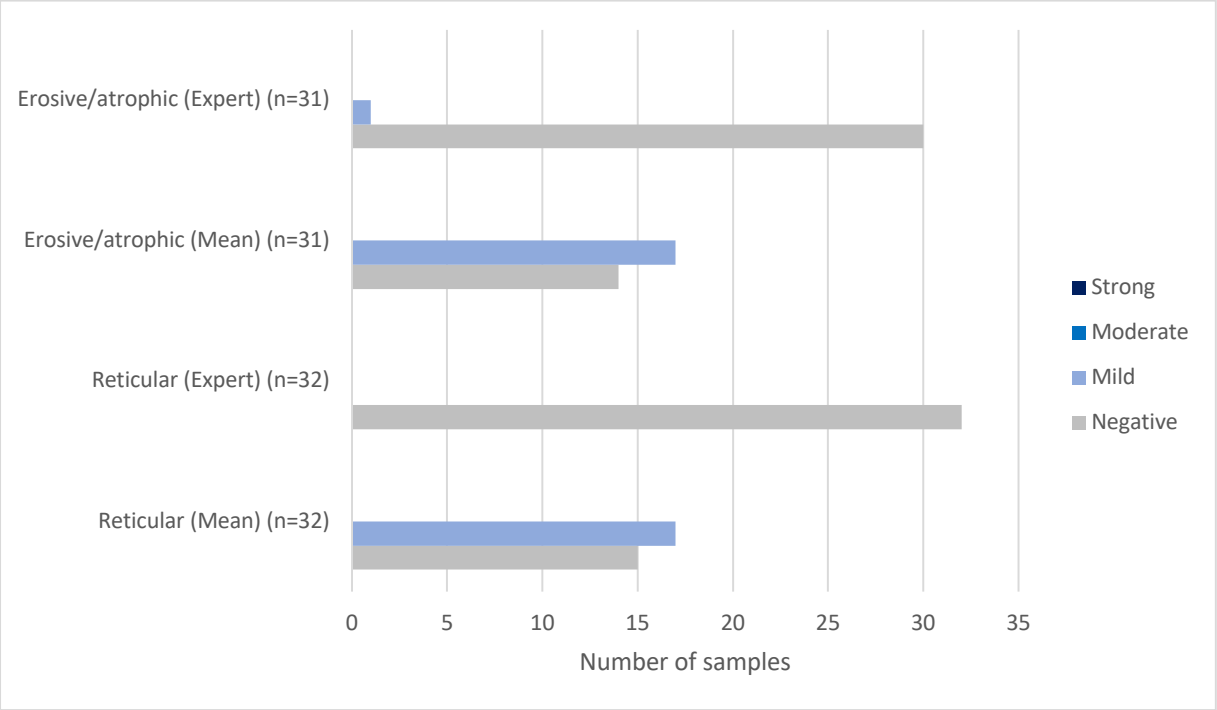

Figure 4.11. Comparison of expert and mean categorical IRS Scores for BMI1 full thickness of epithelium. The graph shows the distribution of expert and mean categorical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP in FT of epithelium. The mean BMI1 IRS scores were generally higher than the expert observer's IRS scores.

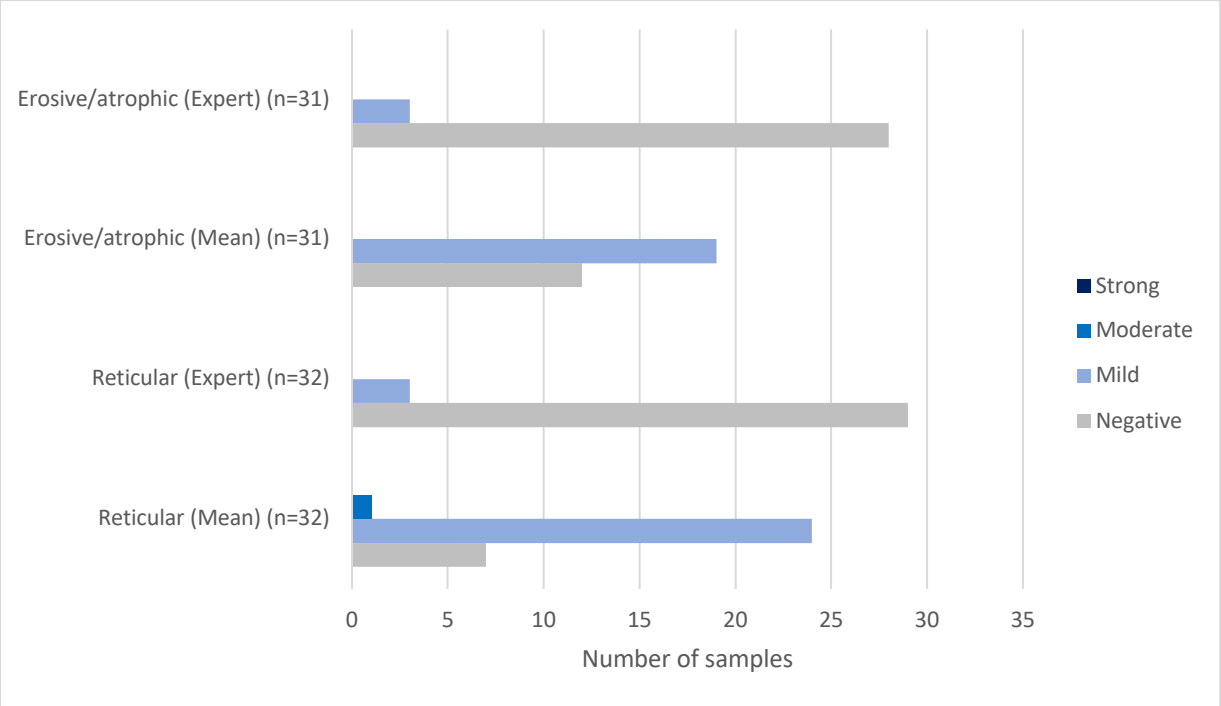

Figure 4.12. Comparison of expert and mean categorical IRS Scores for BMI1 basal half of epithelium. The graph compares the distribution of expert and mean categorical IRS scores for BMI1 in reticular OLP compared to erosive/atrophic OLP in BH of epithelium. the mean BMI1 IRS scores were generally higher than the expert observer's IRS scores.

4.4.2. Distribution of Score A, Score B and numerical IRS scores.

The categorical IRS scores were converted from numerical IRS scores which were calculated by multiplying Score A (percentage of positive cells) and Score B (intensity of staining). Subgroup analysis of the expert observer distribution of scores A and B and the numerical IRS scores (AxB) was performed to try and understand any underlying differences that might exist between reticular OLP and erosive/atrophic OLP. Figures 4.13 to 4.15 depict the distribution of the scores for PARP1, and figures 4.16 to 4.18 depict the distribution of the scores for BMI1. In all three score groups, PARP1 showed higher scores than BMI1, which is in line with the higher categorical IRS scores in PARP1.

PARP1

There were no significant differences found between Score A and Score B patterns although both scores were overall slightly higher in erosive/atrophic OLP than in reticular. For Score A, the highest score of 4 was given for two erosive/atrophic samples in the BH of the epithelium, but not for any reticular samples. A score of 3 was given for more reticular samples then erosive/atrophic in the BH of the epithelium, however in the FT of the epithelium, this score was only given to two atrophic/erosive samples and no reticular. For Score B, a higher proportion of score 3 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

The highest numerical IRS score in the BH of the epithelium was 12 in an atrophic/erosive sample. The highest score in the FT of the epithelium was 9 in an atrophic/erosive sample.

BMI1

Similar to PARP1, there were no significant differences found between Score A and Score B patterns although both scores were overall slightly higher in erosive/atrophic OLP than in reticular. For both Score A and Score B, a higher proportion of score 1 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium. For Score A, the highest score of 2 was given for 3 reticular and 2 erosive/atrophic samples in the BH of the epithelium and 1 erosive/atrophic sample in the FT of the epithelium.

The highest numerical IRS score was 2; this was given for 3 reticular and 3 erosive/atrophic samples in the BH of the epithelium and 1 erosive/atrophic sample in the FT of the epithelium. A higher proportion of score 1 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

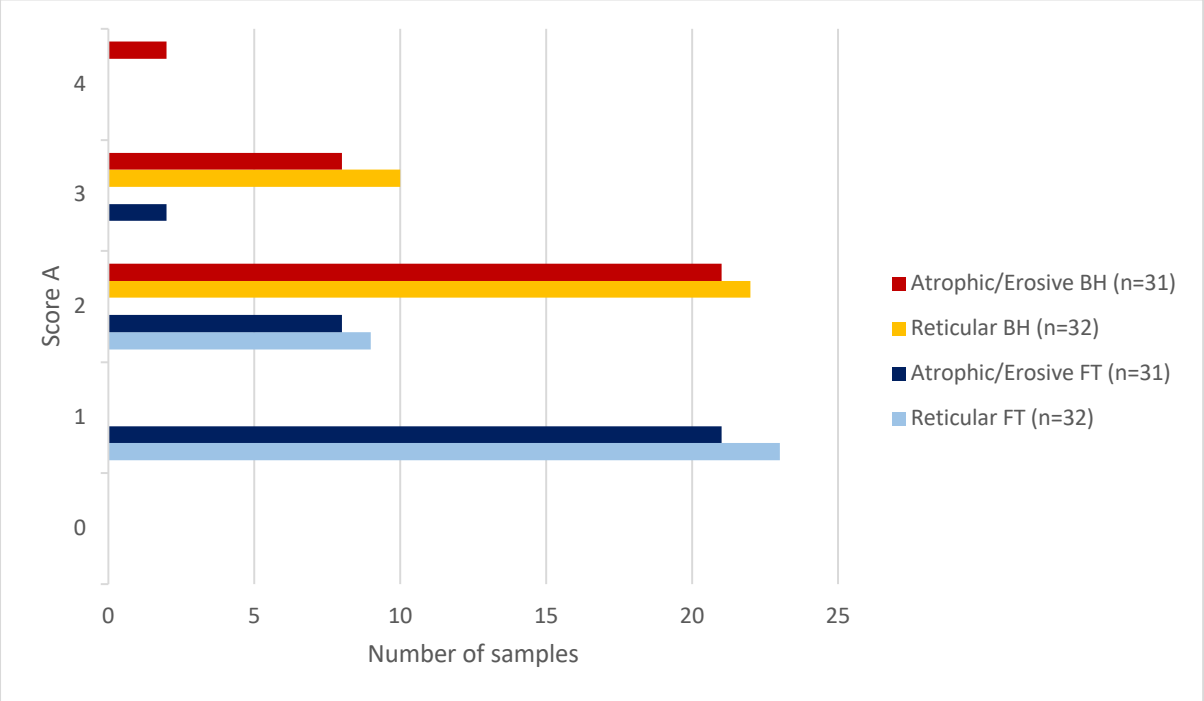

Figure 4.13. Distribution of Score A for PARP1 (expert observer). The graph compares the distribution of Score A for PARP1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. The highest score of 4 was given for two erosive/atrophic samples in the BH of the epithelium.

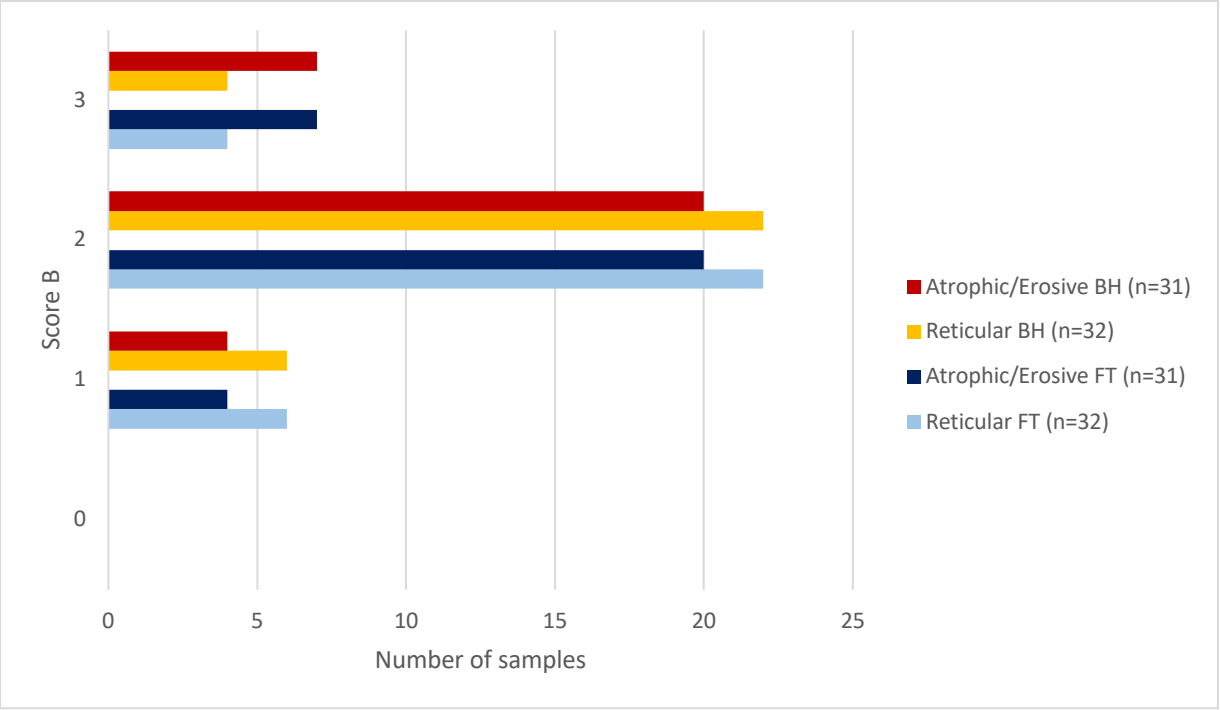

Figure 4.14. Distribution of Score B for PARP1 (expert observer). The graph compares the distribution of Score B for PARP1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. A higher proportion of score 3 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

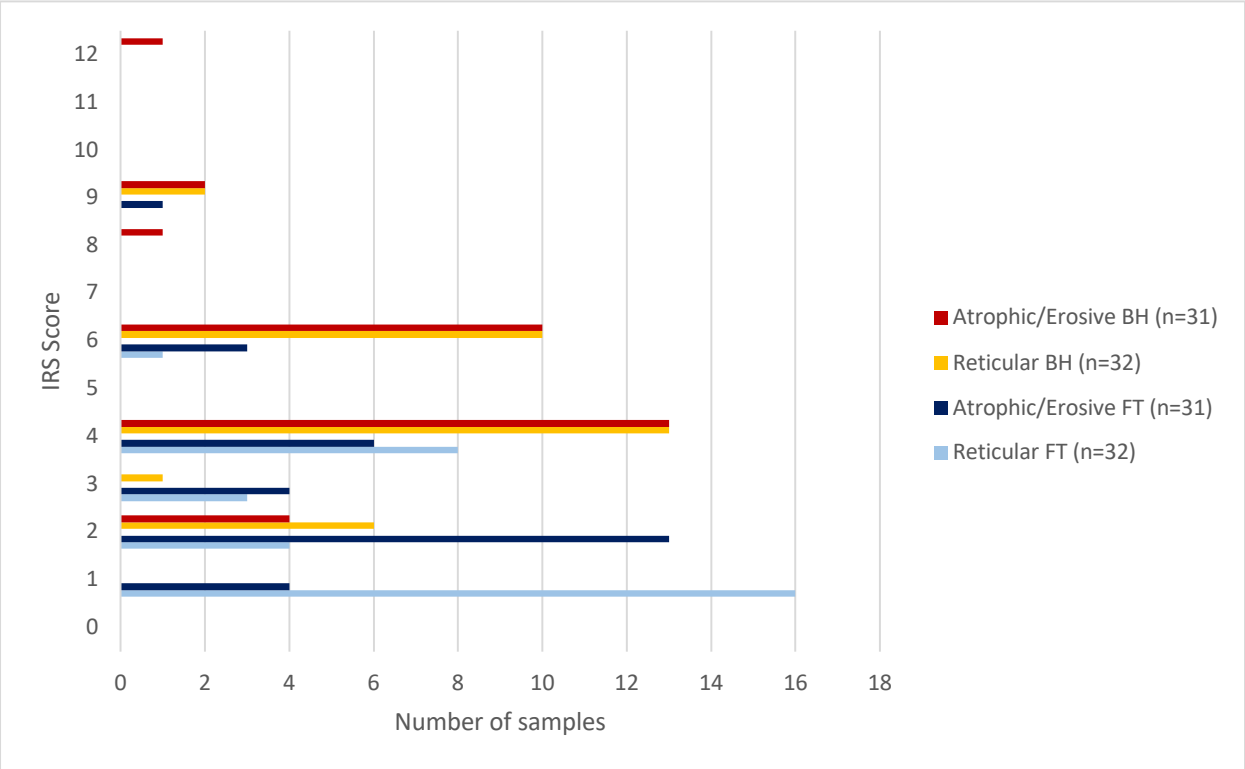

Figure 4.15. Distribution of numerical IRS scores for PARP1 (expert observer). The graph compares the distribution of numerical IRS scores for PARP1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. The highest score in the BH of the epithelium was 12 in an atrophic/erosive sample. The highest score in the FT of the epithelium was 9 in an atrophic/erosive sample.

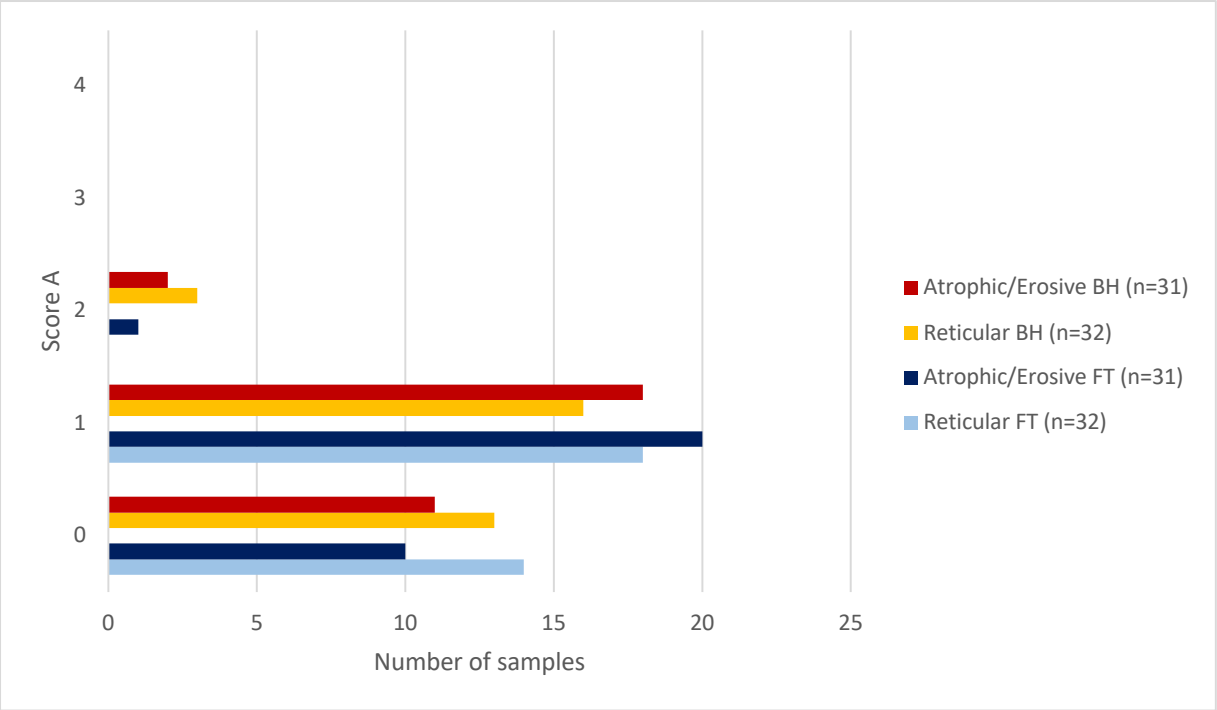

Figure 4.16. Distribution of Score A for BMI1 (expert observer). The graph compares the distribution of Score A for BMI1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. A higher proportion of score 1 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

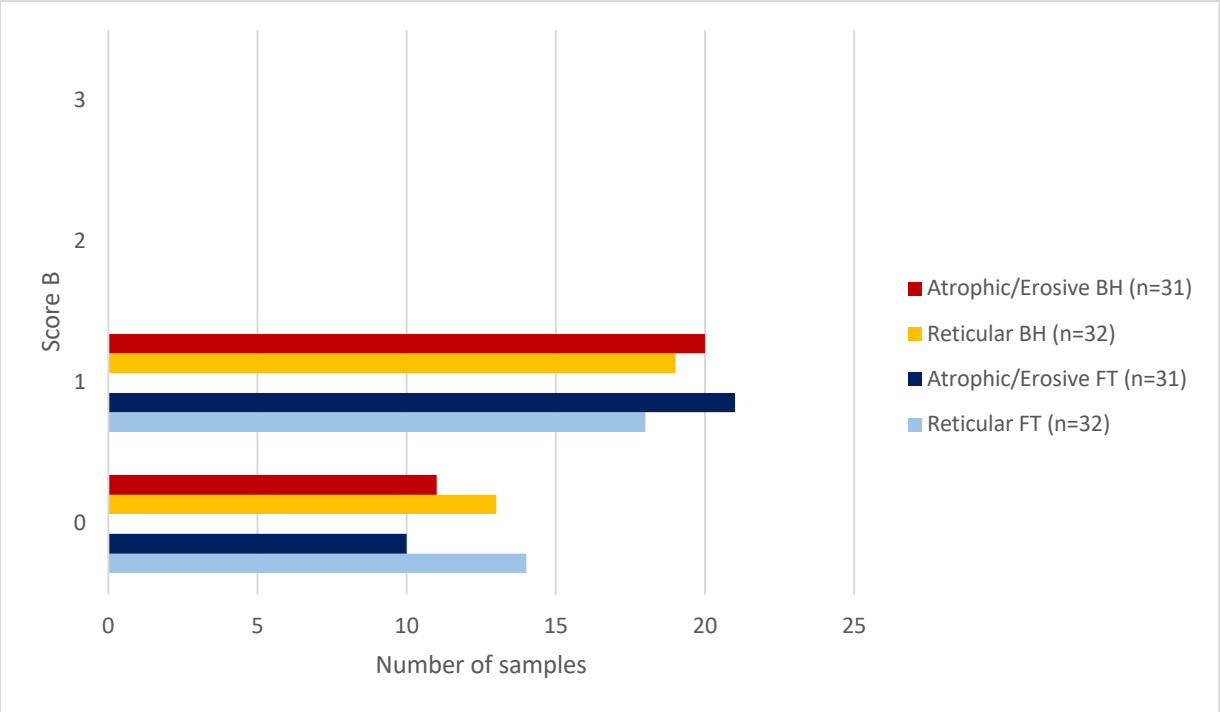

Figure 4.17. Distribution of Score B for BMI1 (expert observer). The graph compares the distribution of Score B for BMI1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. A higher proportion of score 1 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

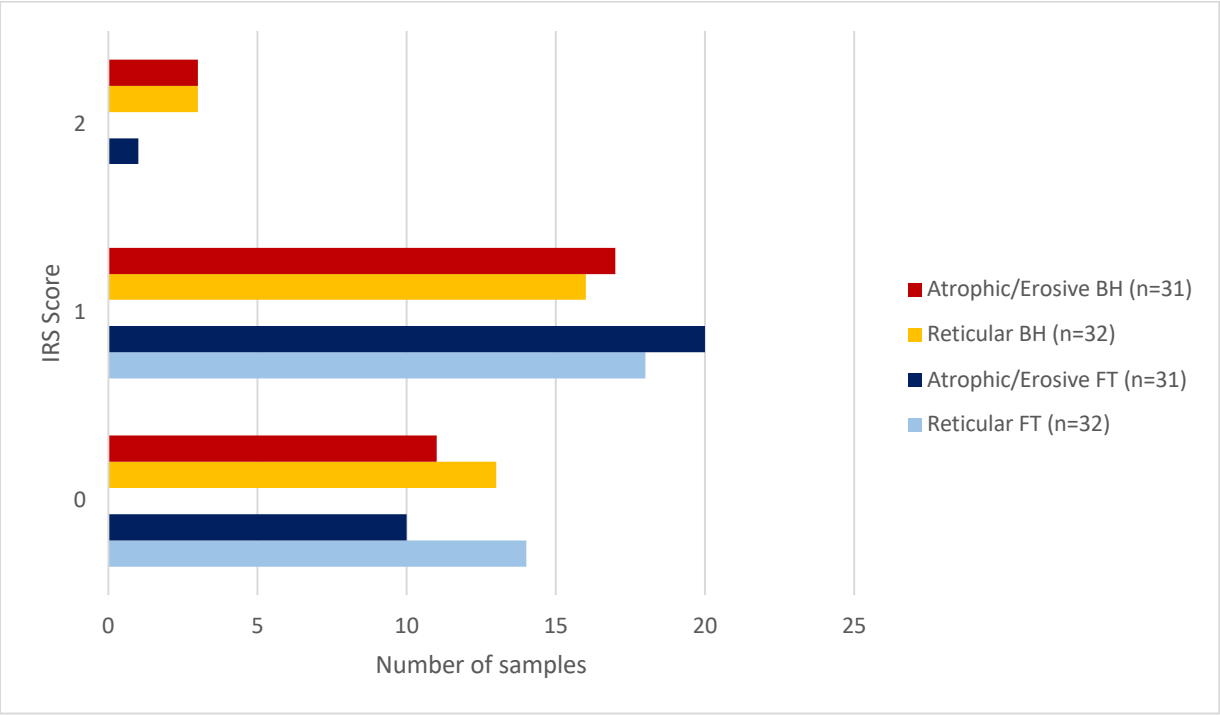

Figure 4.18. Distribution of numerical IRS scores for BMI1 (expert observer). The graph compares the distribution of numerical IRS scores for BMI1 in reticular OLP compared to erosive/atrophic OLP in both the FT and BH of epithelium. A higher proportion of score 1 was seen in erosive/atrophic OLP compared to reticular in both FT and BH of epithelium.

4.5. Correlation between BMI1 and PARP1 expression

A Pearson correlation coefficient (*r)* was computed to assess the linear relationship between numerical IRS scores for BMI1 and PARP1 (expert observer TB only). For FT of the epithelium, there was a significant positive correlation between the BMI1 and PARP1 expression , r (61) = .432, p <.001. There was also a significant positive correlation seen for the BH of the epithelium, r (61) = .530, p <.001 (Figures 4.19 and 4.20). The positive correlation between BMI1 and PARP1 expression suggest a possible association between the two biomarkers in OLP.

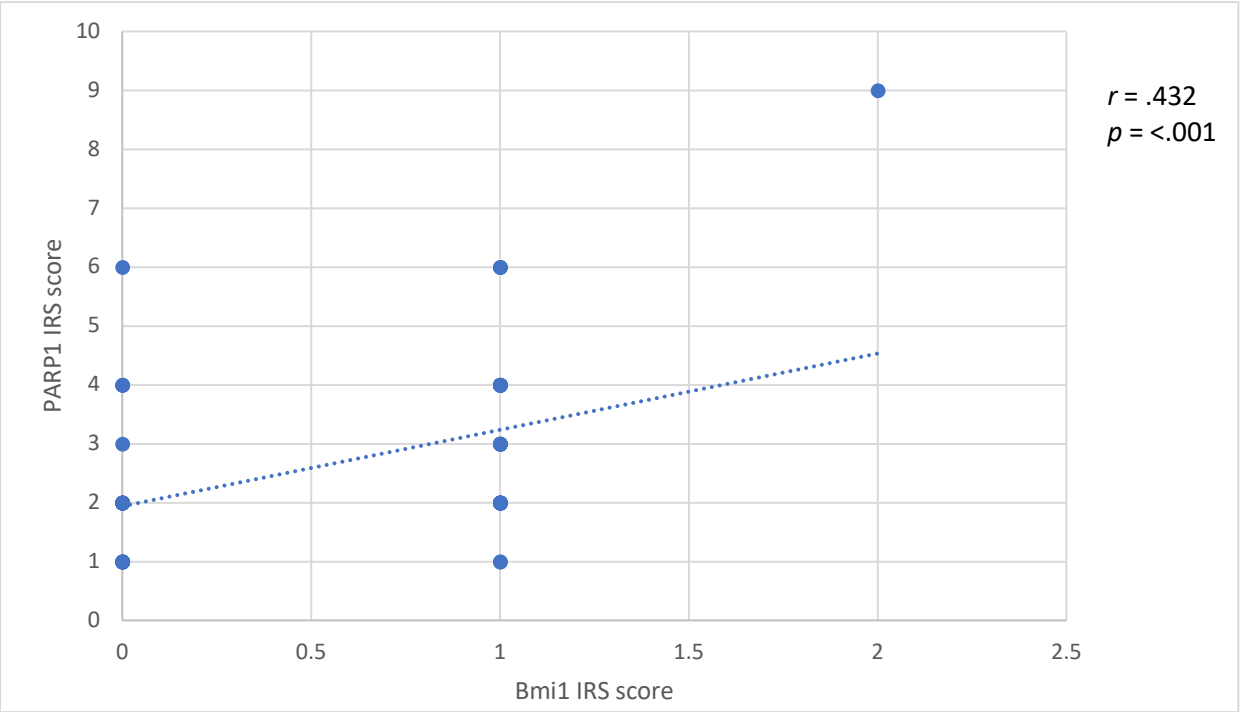

Figure 4.19. Correlation between BMI1 and PARP1 IRS scores in full thickness epithelium.

The scatter graph shows a positive linear association between BMI1 and PARP1 expression, as quantified by the correlation coefficient (*r*).

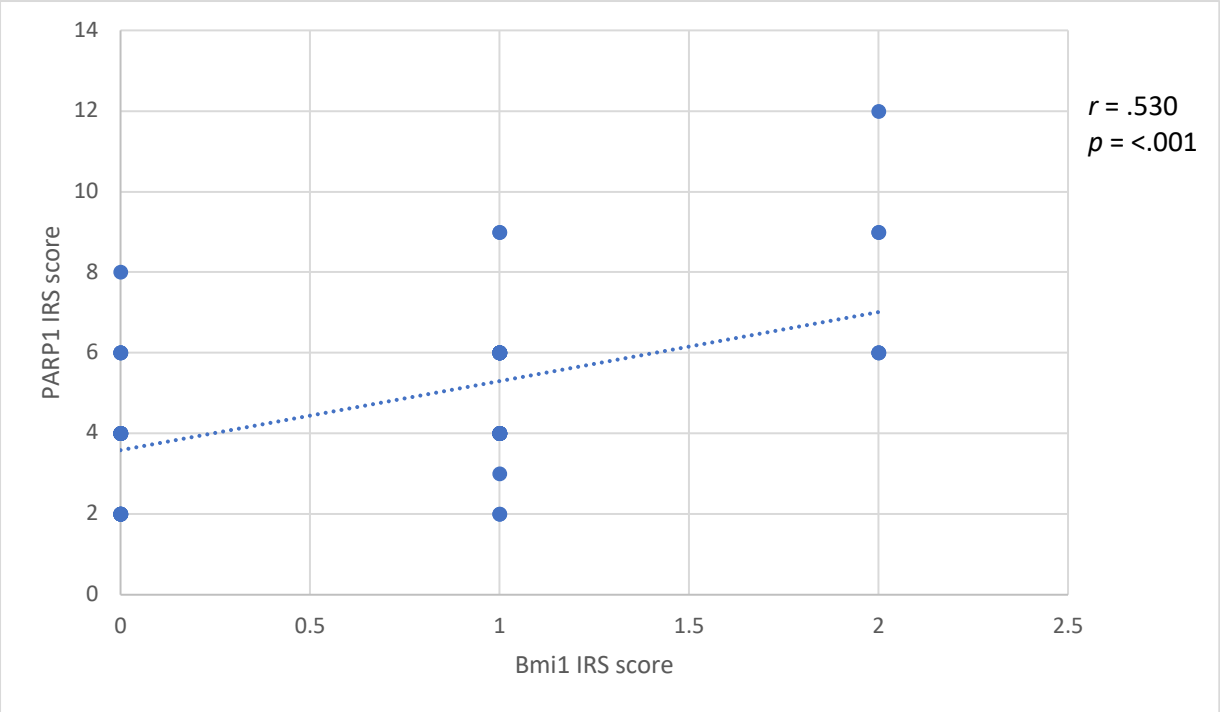

Figure 4.20. Correlation between BMI1 and PARP1 IRS scores in basal half of epithelium.

The scatter graph shows a positive linear association between BMI1 and PARP1 expression, as quantified by the correlation coefficient (*r*).

CHAPTER FIVE

DISCUSSION

5.1. Discussion

BMI1 and PARP1 have been shown to be upregulated in OSCC and identified as potential biomarkers for oral lesions at high risk of malignant transformation. The primary objective of this study was to identify BMI1 and PARP1 expression patterns in erosive and atrophic OLP compared with reticular OLP. Furthermore we aimed to determine if BMI1 and PARP1 could be used as potential biomarkers for identification of high risk OLP lesions.

The study found that PARP1 expression was higher than BMI1 expression in both reticular and erosive/atrophic OLP. No significant differences were identified between the OLP variants and PARP1 IRS scores for either the FT ($p = .858$) or the BH of the epithelium ($p = .681$) or BMI1 scores for either the FT ($p = .492$) or the BH of the epithelium ($p = .649$). However there was a small increase in the number of erosive/atrophic OLP samples that had strong PARP1 staining compared to reticular. There was a significant positive correlation seen between BMI1 and PARP1 expression in both the FT ($r (61) = .432$, $p <.001$) and the BH of the epithelium ($r (61) = .530$, $p <.001$).

This chapter will now interpret these key findings in the context of the existing literature and acknowledging limitations of the study. Possible implications for clinical practice will be discussed alongside recommendations for further research.

5.2. BMI1 and PARP1 expression patterns in erosive/atrophic OLP compared with reticular OLP

The erosive and atrophic variants of OLP have been reported to have a higher malignant transformation risk than other variants (Alrashdan, Cirillo and McCullough 2016; Gonzalez-Moles, Scully and Gil-Montoya, 2008). Gonzalez-Moles *et al.* (2019) found a significantly higher risk of MT in atrophic/erosive lesions compared to reticular lesions with a relative risk of 4.09 ($p< 0.001$). The exact mechanism for this increased risk remains unknown, however epithelial atrophy is thought to increase the permeability of the epithelium and allow for diffusion of carcinogens into the basal cell layer (Squier, 1991). Chronic inflammation in OLP is thought to promote carcinogenesis through increased levels of cytokines and transcription factors promoting cell survival, angiogenesis and invasion, inhibiting apoptosis and causing DNA damage via oxidative stress (Mignogna *et al.,* 2004; Georgaki *et al*., 2021; Mantovani *et al.,* 2008; Ergun *et al.,* 2011). Furthermore, it is hypothesised that chronic tissue stress can promote carcinogenesis in susceptible stem cell populations (Goldberg, Eisen & Bauer, 1988). Although this theory was postulated over a decade before the CSC hypothesis, both suggest distinct stem cell subpopulations involved in tumourigenesis. Previous studies have also considered the association between CSCs and epithelial atrophy. Xie *et al.* (2020) hypothesised that the accumulation of CSCs were accompanied by epithelial atrophy in oral submucous fibrosis (an OPMD primarily caused by the chewing of areca nut). They found Ki67 was upregulated in atrophic epithelium, and BMI1 was reported to be highly expressed when dysplasia was present. The ulceration, erosion and increased levels of inflammation that occur in erosive or atrophic OLP render the epithelium more prone to DNA damage (Goldberg, Eisen & Bauer, 1988; Mignogna *et al.,* 2004). Therefore the authors of this study hypothesised

that BMI1 and PARP1, both involved in DNA repair, would show increased expression in erosive/atrophic OLP compared to reticular.

The present study did not reveal statistically significant differences in either PARP1 or BMI1 IRS scores between reticular and erosive/atrophic OLP types. The results suggest that there is no difference in BMI1 and PARP1 expression patterns between reticular OLP and atrophic/erosive OLP and hence the null hypothesis is accepted. There is no existing literature regarding BMI1 and PARP1 expression in different OLP variants for these findings to be directly compared to. The results showed overall very low BMI1 expression in both reticular and erosive/atrophic OLP samples (> 90% of cases in both full thickness (FT) and basal half (BH) of the epithelium were deemed negative for BMI1). Although a definitive conclusion cannot be made in the absence of a negative tissue control, the findings suggest that BMI1 is not upregulated in either OLP subtype. This is contrasting to the observations by Ma *et al.* (2013) that BMI1 expression is significantly increased in both non-progressing and progressing OLP compared to normal oral mucosa. However, one must be cautious regarding the interpretation and comparison of these results, due to the different scoring systems used for immunohistochemical analysis. Ma *et al.* (2013) used an arbitrary qualitative scoring system of staining intensity to classify BMI1 expression as either negative or positive. Negative (-) or barely detectable (-/+) staining was classified as negative, and weak (+), moderate (+ +) or strong (+++) staining was classified as positive (Kang *et al.,* 2007). As previously mentioned, immunohistochemistry (IHC) is not stoichiometric and is unsuitable for accurate quantification based on the intensity of chromogen (DAB) precipitation used for the visualisation of positive stained regions. Hence the current study used the semi-quantitative immunoreactive score (IRS) which also considers the percentage of positive cells. This could

partially explain the contrasting findings between the two studies, and it is possible that BMI1 expression was over-estimated in the Ma *et al.* (2013) study due to the scoring system used.

BMI1 is a cell-cycle regulator which increases self-renewal and proliferation. It is a CSC marker and implicated in tumourigenesis (Herzog, Somayaji and Nör, 2023; Prince *et al.,* 2007). Although there is a lack of published studies on BMI1 expression in OLP, there is an increasing evidence base for its over-expression in OSCC and other oral premalignant conditions. For example, Liu *et al*. (2012) found that BMI1 positivity was statistically increased in transformed OL compared to un-transformed OL (p= .002) and Feng *et al*. (2013) found increased BMI1 expression in transformed OE compared to non-transformed OE. Both studies used the same IHC scoring system as described by Kang *et al.* (2007). At variance with other studies, Wu *et al*. (2017) did not find any difference in Bmi-1 expression between normal epithelium, dysplastic epithelium and OSCC groups. Interestingly, this study quantitatively analysed the IHC staining using Aperio Quantification software. Computer-assisted quantitative analysis produces more objective and reproducible results (Meyerholz and Beck, 2018; de Matos *et al.,* 2010), so it could be suggested that these results are a more accurate representation of BMI1 expression in oral premalignant and malignant tissue. The findings of the present study also suggest there may not be a difference in BMI1 expression between low and high-risk oral lesions.

Although there were no statistically significant differences found in PARP1 expression between reticular and erosive/atrophic OLP, PARP1 IRS scores were overall higher than BMI1 in both reticular and erosive/atrophic OLP (Tables 4.3 and 4.4) suggesting that PARP1 may be upregulated in OLP. Again, this cannot be definitively concluded in the absence of a negative

tissue control. To our knowledge, this is the first study to investigate PARP1 expression in OLP, while previous studies have shown increased PARP1 expression in other oral premalignant conditions. Cervigne *et al.* (2014) analysed PARP1 expression in OL and found that PARP1 expression was amplified in progressive OL and OSCC but not in non-progressive OL. Kossatz *et al.* (2016) found high levels of PARP1 expression found in both premalignant and malignant tissue and postulated a role for PARP1 in predicting high risk lesions.

Although not statistically significant, the present study did find a small increase in the number of erosive/atrophic OLP samples that had strong PARP1 staining compared to reticular. In the FT of the epithelium, there was one atrophic/erosive sample with strong straining compared to zero reticular samples. In the BH of the epithelium, there were three atrophic/erosive samples with strong staining compared to two reticular samples. Whilst the difference of one sample may initially appear very small, it is worth noting that the malignant potential of OLP is approximately 1-2% (Landini et al., 2014; Iocca et al.,2019; Gonzalez-Moles et al,. 2019; Idrees et al., 2020). Therefore, in this study's cohort of 63 patients, one would expect only 0.63 – 1.26 patients to undergo malignant transformation. Hence, the erosive/atrophic samples showing stronger PARP1 staining could still represent those at higher risk of malignant transformation. PARP1 plays a critical role in DNA repair processes including DSB repair via HR by early recruitment of breast cancer type 1 susceptibility protein (*BRCA1*) (Dulaney *et al.,* 2017; Bouchard *et al.,* 2003; Chaudhuri and Nussenzweig, 2017). In erosive/atrophic OLP, the epithelium undergoes recurrent or continual ulceration and erosion, leading to an exaggerated tissue repair response (Goldberg, Eisen & Bauer, 1988), which could explain the increased PARP1 expression in these variants compared to reticular.

5.3. Correlation between BMI1 expression and PARP1 expression in OLP

The study found a statistically significant positive correlation between levels of BMI1 and PARP1 expression suggesting that OLP lesions with higher BMI1 expression are likely to have higher PARP1 expression and vice-versa. As discussed, PARP1 plays a critical role in DNA repair. BMI1 also plays a role in DNA repair through suppression of intracellular levels of ROS and recruitment to DSBs to help repair the lesion through H2A ubiquitination (Herzog, Somayaji and Nör, 2023).). Given their similar roles in DNA repair, the positive correlation between the two proteins may not be particularly surprising. However to our knowledge, this is the first study to demonstrate this relationship in oral tissues and indicates co-expression of BMI1 and PARP1 in OLP. The relationship between OLP variants and co-expression of BMI1 and PARP1 was not specifically investigated in this study but could be a potential area for further research. Previous studies have shown that co-expression of BMI1 and ALDH1 (another potential biomarker in HNSCC) was a strong indicator for malignant transformation in OE ($p<0.01$) as well as development of multiple and multifocal carcinomas, but BMI1 expression alone was not significant (Feng *et al.,* 2013; Feng *et al.,* 2019). Another study showed that co-expression of PARP1, CAF-1/p60 and nestin in HPV-negative OSCC had the worst prognosis and increased risk of metastasis (Mascolo *et al.,* 2012). An evolving area of research is the analysis of gene co-expression using Weighted Gene Co-expression Network Analysis (WGCNA) which aims to identify functional associations between genes in tumourigenesis (Shi *et al.,* 2020). This has been utilised in various types of cancers, including HNSCC (Jin and Qin, 2020; Zhang *et al.,* 2018; Ge *et al.,* 2019), and could also be applied to OPMDs such as OLP.

5.4. BMI1 and PARP1 as potential molecular markers for distinguishing OLP lesions at high risk of malignant transformation from those at low risk

BMI1 and PARP1 play a role in tumourigenesis and have been shown to be upregulated in OSCC and oral premalignant lesions. Previous studies have put both proteins forward as potential susceptibly/risk biomarkers for malignant transformation in oral lesions. To our knowledge, this is the first study to investigate expression of both BMI1 and PARP1 in OLP, and to specifically look at the difference in expression between atrophic/erosive and reticular variants. As this was a cross-sectional study and no follow-up data is yet available, we are unable to make any accurate conclusions on the utility of BMI1 and PARP1 as biomarkers for identifying OLP lesions at high risk of malignant transformation. Our results showed no significant difference in BMI1 and PARP1 expression patterns between reticular OLP and atrophic/erosive OLP, however even if a difference did exist, this would not alone be able to predict malignant transformation. We have previously discussed the mechanisms behind (Goldberg, Eisen & Bauer, 1988; Squier, 1991), and the literature supporting (Alrashdan, Cirillo and McCullough 2016; Gonzalez-Moles, Scully and Gil-Montoya, 2008), the increased risk of malignant transformation in erosive/atrophic OLP. Hence, the small increase in erosive/atrophic samples showing stronger PARP1 staining could still represent those at higher risk of malignant transformation, although further studies with follow-up data would be needed to confirm this. It is interesting to note that although reticular OLP is still considered at risk of malignant transformation, a meta-analysis by Gonzalez-Moles *et al.* (2019) found that increased malignant transformation risk was exclusively associated with erosive/atrophic lesions whereas reticular lesions showed no risk. If this data is accurate and reticular OLP holds zero risk of malignant transformation, then the increased PARP1

expression in erosive/atrophic OLP in this study may be more informative in regards to malignant transformation risk than initially thought.

The translational and clinical relevance of identifying valid biomarkers for predicting risk of malignant transformation in OLP is significant. If proven in future follow-up studies that PARP1 *is* upregulated in high-risk OLP lesions, PARP1 IHC could be utilised at the time of histopathological diagnosis to aid clinicians in risk stratification of patients. This would allow for more personalised care and tailored clinical follow-up periods, focusing efforts on those with higher risk lesions. Of course many other factors would still need to be taken into consideration including clinical appearance and patient risk factors. Effective clinical follow-up of patients allows for early identification and treatment of OSCC, which has a significant positive impact on prognosis (Rogers *et al.,* 2009; Rusthoven *et al.,* 2010). Furthermore, the identification of biomarkers provides therapeutic targets for OSCC and oral premalignant lesions. PARP-inhibitors are already licensed for ovarian, breast and pancreatic cancers, and there are ongoing clinical trials into their efficacy in oral cancer (Wang *et al.,* 2022).

5.5. Limitations of the study

One of the limitations of this study was the sampling method. Convenience sampling is a type of non-random sampling which introduces the risk of selection bias, therefore the results should be viewed with an element of caution. Although this is not the most ideal sampling method, it was appropriate for this study design as patients needed to be recruited prospectively from their attendance on clinic. It was not possible to blind researcher AP to the OLP subtype at the time of sample collection due to nature of the procedure, which could

introduce an element of observation bias. This could have been improved by an independent operator, who was not involved in scoring the samples, performing the biopsy procedures.

For ethical reasons, no negative tissue controls could be prospectively collected, as this would require control patients with healthy oral mucosa to undergo an invasive surgical procedure for no clinical benefit. This meant the researchers were limited to using previously collected samples from the UoB DRTB where unfortunately no appropriate samples could be identified. Furthermore, comparison of BMI1 and PARP1 expression between OLP and normal tissue could not be performed which slightly limits interpretation of the results. Consideration was given to using fibroepithelial polyps, frictional keratosis or excess gingival tissue from gingival surgery as the negative tissue controls; however these would not have been ideal 'healthy' controls as they are still pathological tissue, likely with hyperplastic epithelium and often inflamed.

The process of IHC has its own limitations and the outcome depends on numerous factors (de Matos *et al,* 2010). Yaziji & Barry (2006) described two types of bias that can occur in IHC; reaction bias, related to tissue handling and tissue staining techniques; and interpretation bias, related to selection of antibody panel and tissue scoring. Quality and consistency of the initial tissue handling (fixation, trimming, processing, embedding and storage) is very important and can affect subsequent tissue staining and interpretation. There are multiple stages involved in tissue staining and each one represents a chance for variation (Meyerholz and Beck, 2018). In this study, we tried to minimise bias by strictly following standard IHC protocols and optimising staining conditions by testing different primary antibody

concentrations and pre-treatments, however it is not possible to completely eliminate risk of reaction bias in IHC.

Visual IHC scoring is an inherently subjective process. Historically, scoring was based on qualitative assessment of staining intensity, but semi-quantitative scoring approaches have been suggested to improve objectivity (Meyerholz and Beck, 2018; Fedchenko and Reifenrath, 2014). A semi-quantitative composite scoring system (IRS) was used in this study however subjectivity and reliability were still an issue. A scoring pilot exercise of ten OLP samples was carried out separately by the two raters, followed by discussion of discrepancies and agreements on staining intensity scores. Despite this, an inter-rater reliability analysis using the Kappa (κ) statistic showed only slight-fair agreement between the raters for categorical IRS scores. However, it is acknowledged that the raters did not have the same level of expertise, which is another limitation. The kappa level of agreement was inadequate to enable reliable conclusions to be drawn from the mean scores, so expert observer scores were used for the primary analysis. To reduce observational bias, both raters were blinded to the clinical data during IHC scoring, however, it is acknowledged that it is possible to deduce whether a sample is reticular or erosive/atrophic type according to the histological features present in the section. Computer-assisted quantitative analysis can produce more objective and reproducible results (Meyerholz and Beck, 2018; de Matos *et al.,* 2010), therefore using image processing and analysis software such as ImageJ could improve this study.

5.6. Recommendations for future research

This study has provided preliminary data to suggest that PARP1, but not BMI1, is upregulated in OLP. In the absence of negative tissue controls, this cannot be definitely concluded, so

future studies comparing against healthy oral mucosa would be beneficial. Although not statistically significant, PARP1 expression was slightly increased in erosive/atrophic OLP compared to reticular OLP, suggesting a possible association of PARP1 with high risk lesions. Further studies with larger sample sizes and follow-up data are indicated to determine if BMI1 and PARP1 could be used as potential biomarkers for identification of OLP lesions at high risk for malignant transformation**.**

Additionally, the findings demonstrated a positive correlation between BMI1 and PARP1 indicating co-expression of the proteins in OLP. Further studies into whether the co-expression of BMI1 and PARP1 indicate an increased risk of malignant transformation in OLP would be recommended. An interesting area for further research would be to study gene co-expression in OLP, or OPMDs as a whole, to observe how different genes may be functionally associated in oral carcinogenesis. ALDH1 is another CSC biomarker which plays a role in regulating stem cells proliferation and differentiation through the retinoid signalling pathway (Huang *et al.,* 2015). It has been significantly associated with malignant transformation in OLP (Xu *et al.,* 2013) and the co-expression of ALDH1 and BMI1 has been shown to be a strong indicator for malignant transformation in OE ($P < 0.01$) (Feng *et al.,* 2013). It would therefore be of interest to explore co-expression of BMI1, PARP1 and ALDH1 in OLP.

Lastly, this study only looked at reticular, erosive and atrophic OLP. Plaque-like OLP is also thought to have increased risk of malignant transformation compared to reticular (Mignogna *et al.,* 2001) however in studies and systematic reviews it is often grouped under 'white type' OLP alongside reticular and popular, hence more studies are needed on this topic. Long-term

follow up studies analysing and comparing biomarkers in all variants of OLP would be beneficial.

CHAPTER SIX

CONCLUSIONS

6.1. Conclusions

The primary objective of this study was to identify any significant differences in BMI1 and PARP1 expression patterns in erosive/atrophic OLP compared with reticular OLP. The results showed no statistical differences in the levels of expression of BMI1 and PARP1 between reticular OLP and erosive/atrophic OLP types and so the null hypothesis is accepted. The study findings suggest, but cannot definitely conclude, that PARP1 is upregulated in OLP but BMI1 is not. Although not statistically significant, PARP1 expression was slightly increased in erosive/atrophic OLP compared to reticular, suggesting a possible association with lesions at high risk of malignant transformation. The upregulation of PARP1 in OLP, particularly the erosive and atrophic types, may be explained by its critical role in DNA repair hence increased activity in exaggerated tissue repair responses. Additionally, the findings demonstrated a positive correlation between BMI1 and PARP1 indicating co-expression in OLP.

To the best of our knowledge, this is the first study to investigate the expression of PARP1 in OLP, the comparison of BMI1 and PARP1 expression in different OLP variants, and the relationship between BMI1 and PARP1 in OLP. This preliminary data begins to fill this current void in the literature and adds to the evidence regarding biomarkers in OLP. However, this study was only a pilot and was limited by sample size and lack of follow-up data. Further studies with larger sample sizes, negative tissue controls and follow-up data are indicated to determine if BMI1 and PARP1 could be used as potential biomarkers for identification of OLP lesions at high risk for malignant transformation. Additionally, future research into co-expression of biomarkers in all variants of OLP is recommended.

CHAPTER SEVEN

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CHAPTER EIGHT

APPENDICES

8.1. Patient Information Sheet

UNIVERSITY OF BIRMINGHAM

Birmingham Community Healthcare NHS
NHS Foundation Trust

PARTICIPANT INFORMATION SHEET
VERSION 3 (9/December/2019)
Molecular & Cellular Characterisation of Oral Lichen Planus, IRAS ID 178800
Chief Investigator: Gabriel Landini

INTRODUCTION

You are being invited to take part in a research study. Before you decide whether or not to take part it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY?

Oral Lichen Planus is a disease affecting the mouth with a small risk of malignant transformation. The most common signs are white patches on the cheeks, tongue and gums. Sometimes this can cause oral soreness and be associated with red patches and ulcers. This observational study is being carried out in order to learn more about the condition and find out how inflammation causes the disease.

WHY HAVE I BEEN INVITED TO TAKE PART?

You have been chosen to take part as you appear to have lichen planus affecting the mouth.

DO I HAVE TO TAKE PART IN THE STUDY?

No, your participation is entirely voluntary and you may stop taking part at any time without it affecting your normal care.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you decide to take part in this study, you will be asked to sign this consent form. This type of research is called an 'Observational Study' and does not affect the care you receive. If you take part in the study, we will record information such as age, gender, medical and social history and a description of your clinical examination. We are also asking that you fill out two short questionnaires about how your lichen planus affects you on a daily basis. These questionnaires will be given to you when you agree to take part in the study and you can complete them in the hospital while you wait. They should each take less than five minutes to complete. We will collect a small blood sample (around 25 ml / 5 teaspoons) and some saliva. We will also examine part of the biopsy sample that will be taken as part of your diagnosis. In the scenario that your biopsy sample does not show lichen planus, it will still be valuable for comparison with samples that show lichen planus

WHAT WILL HAPPEN TO MY SAMPLES AND DATA?

The samples taken will be analysed so we can build up a picture of oral lichen planus. We are asking if you would be willing for any samples that you donate but which are not completely used in this study to be stored for future research in the School of Dentistry Research Tissue

Bank. Your samples and any related information will be anonymised and researchers will not have access to any information that could identify you. Your samples will only be used in future research studies that are ethically approved.

WILL MY DETAILS BE CONFIDENTIAL?
Yes. You will be allocated a study number, so that your name will not be disclosed to anyone except the clinical people treating you. Your responses to the questions on the questionnaires will also be kept confidential.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?
There are no direct benefits to you but you will be helping to advance research into oral lichen planus. We hope that this may lead to more effective treatments in the future.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?
There are no additional risks to taking part in observational research. A biopsy is likely to be carried out in order to obtain a definitive diagnosis whilst you are under the care of the Oral Medicine department regardless of whether you choose to take part in the study or not. Taking part in the study will not mean that we require an additional sample but instead, we will use a small portion of the original biopsy sample taken for use in the study. A biopsy is carried out under local anaesthetic and this procedure carries the usual common risks of bleeding, pain, discomfort, swelling, infection and possible numbness in the region which can be temporary. The clinician involved in your treatment will discuss these with you prior to carrying out the biopsy. Taking part in the study does not result in any increased risk for the factors mentioned above. A small fraction of the biopsy sample will then be used for research purposes.

WHAT IF SOMETHING GOES WRONG?
We do not expect you to experience any problems as a result of this study. However, should you have any concern or if you have any general questions about the study please call Professor Gabriel Landini *on 0121 466 5519.*

If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. 'The Sponsor' will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol of the study. Your right at law to claim compensation for injury where you can prove negligence is not affected.

WHO IS ORGANISING THIS RESEARCH?
The study is organised and sponsored by the University of Birmingham.

WHO HAS REVIEWED THE STUDY?
The study has been reviewed by London - Hampstead Research Ethics Committee (Reference: 16/LO/0305).

CONTACT FOR FURTHER INFORMATION

If you require any further information please telephone 0121 466 5519 and ask to speak to Professor Gabriel Landini. If you have any concerns about the study and wish to contact someone independent, you may contact the Birmingham Community Healthcare Patient Experience Team on 0800 917 2855.

A copy of this information sheet and a signed consent form will be given to you to keep.

Data Protection Essentials

In order to carry out the research project described above, we will need to collect information about you, and some of this information will be your personal data. Under data protection law, we have to provide you with very specific information about what we do with your data and about your rights. We have set out below the key information you need to know about how we will use your personal data.

More information on how the University processes personal data can be found on the University's website on the page called 'Data Protection - How the University Uses Your Data' (https://www.birmingham.ac.uk/privacy/index.aspx).

Who is the Data Controller?

The University of Birmingham, Edgbaston, Birmingham B15 2TT is the data controller for the personal data that we process in relation to you.

What data are we processing and for what purpose will we use it?

We will collect and process your personal data to conduct the research project, as explained in the Participant Information Sheet.

What is our legal basis for processing your data?

The legal justification we have under data protection law for processing your personal data is that it is necessary for our research, which is a task we carry out in the public interest. These data will not be used to make decisions about you.

Who will my personal data be shared with?

We will not share your data with any third party.

Sometimes, external organisations assist us with processing your information, for example, in providing IT support. These organisations act on our behalf in accordance with our instructions and do not process your data for any purpose over and above what we have asked them to do. We make sure we have appropriate contracts in place with them to protect and safeguard your data. If your personal data are transferred outside the European Union (for example, if one of our partners is based outside the EU or we use a cloud-based app with servers based outside the EU), we make sure that appropriate safeguards are in place to ensure the confidentiality and security of your personal data.

How will my personal data be kept secure?

The University takes great care to ensure that personal data is handled, stored and disposed of confidentially and securely. Our staff receive regular data protection training, and the University has put in place organisational and technical measures so that personal data is processed in accordance with the data protection principles set out in data protection law.

The University has an Information Security Management System based on ISO27001 with a range of controls covering the protection of personal information. Annual security awareness training is mandatory for staff and the University is accredited under the NHS Information Governance Toolkit, the Payment Card Industry Data

Security Standard and is in the process of gaining Cyber Essentials Plus for defined services.

In relation to this project, the data we collect will be kept in a locked file cabinet on site or in a locked cabinet in a University of Birmingham office and will not be accessible to anybody other than those involved in the project.

How long will my personal data be kept?
Your data will be retained for 10 years after the publication of the research outcomes. If you withdraw from the project, we will keep the information we have already obtained but, to safeguard your rights, we will use the minimum personally-identifiable information possible.

Your rights in relation to your data
You may have the following rights in respect of your personal data:

- The right to access to your data (often referred to as a Subject Access Request).
- The right to rectification of inaccuracies in your data.
- The right to erasure of your data (in certain circumstances).
- The right to restrict processing of your data (in certain circumstances).
- The right to object to the processing of your data (in certain circumstances).
- The right to ask for your personal data to be transferred electronically to a third party.
- If the research is being done on the legal basis of your consent (see above), the right to withdraw consent.

However, your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate.

If you would like more information on your rights, would like to exercise any right or have any queries relating to our processing of your personal data, please contact:

The Information Compliance Manager, Legal Services, The University of Birmingham, Edgbaston, Birmingham B15 2TT
Email: dataprotection@contacts.bham.ac.uk Telephone: +44 (0)121 414 3916

If you wish to make a complaint about how your data is being or has been processed, please contact our Data Protection Officer.

Mrs Carolyn Pike, OBE, The Data Protection Officer, Legal Services, The University of Birmingham, Edgbaston, Birmingham B15 2TT
Email: dataprotection@contacts.bham.ac.uk Telephone: +44 (0)121 414 3916

You also have a right to complain to the Information Commissioner's Office (ICO) about the way in which we process your personal data. You can make a complaint using the ICO's website.

8.2. Informed Consent Form

UNIVERSITY OF BIRMINGHAM

Birmingham Community Healthcare NHS
NHS Foundation Trust

INFORMED CONSENT FORM

VERSION 3 (9th December 2019)

Lichen Planus Study, IRAS ID 178800

Volunteer Number:	

Please Initial Box

1. I confirm that I have read and understand the information sheet dated 9th December 2019 (Version 3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that representatives from regulatory authorities, the University of Birmingham or from the NHS Trust may look at the data collected during the study, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data and to have the data stored in a locked cabinet at this site or in a University of Birmingham office. ☐
4. I agree to have my blood and saliva taken, stored and analysed for research into oral lichen planus. ☐
5. I agree that part of my oral biopsy sample will be used for research. ☐

The following statement is optional and you can still take part in the main study without consenting for future use of samples. If you do not consent to future use, leave the box blank.

6. I agree that any samples that are left over from this study will be donated to the University of Birmingham Dental Tissue Bank and may be used in future ethically approved research. ☐
7. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of person taking consent	Date	Signature

1 copy for Patient; 1 copy for Site File; original to be kept in medical notes

8.3. COMDQ Questionnaire

QUESTIONNAIRE

Chronic Oral Mucosal Diseases Quality of Life Questionnaire

Pain and functional limitation

1. How much do certain types of food /drink cause you discomfort (spicy food, acidic food)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
2. How much does your oral condition cause you to limit the types of food/drink you consume?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
3. How much do certain food textures cause you discomfort (rough food, crusty food)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
4. How much does your oral condition cause you to limit the textures of the food you consume?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
5. How much does the temperature of certain foods/drinks cause you discomfort?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
6. How much does you oral condition cause you to limit the temperature of the foods/drinks you consume?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
7. How much does your oral condition lead to discomfort when carrying out your daily oral hygiene routine (brushing, flossing, mouthwash usage)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
	Not at all	4

8. How much does your oral condition cause you to limit your daily oral hygiene routine (brushing, flossing, mouthwash usage)?	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0

9. How much does your oral condition lead to discomfort when wearing a denture (false teeth)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0

Medication and treatment (including mouthwashes, gels, creams, ointments, injections, tablets, infusions)

1. How much do you feel you need medication to help you with activities of daily life (talking, eating, etc.)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
2. How satisfied are you with the medication being used to treat your oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
3. How concerned are you about the possible side effects of the medications used to treat you r oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
4. How much does it frustrate you that there is no single standard medication to be used in your oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
5. How much does the use of the medication limit you in your everyday life (routine/the way apply or take your medications)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
6. How much does it bother you that there is no cure for your oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0

Social and emotional

1. How much does your oral condition get you down?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
2. How much does your oral condition cause you anxiety?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
3. How much does your oral condition cause you stress?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
4. How much does the unpredictability of your oral condition bother you?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
5. How much does your oral condition cause you to worry about the future (spread of the condition, possible cancer risk)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
6. How much does your oral condition make you pessimistic about the future?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
7. How much does your oral condition disrupt social activities in your life (social gatherings, eating out, parties)?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0

Patient support

1. How satisfactory do you consider the information available to you regarding your oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
2. How satisfied are you with the level of support and understanding shown to you by family regarding this oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
3. How satisfied are you with the level of support and information shown to you by friends/work colleagues regarding your oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0
4. How isolated do you feel as a result of this oral condition?	Not at all	4
	Slightly	3
	Moderately	2
	Considerably	1
	Extremely	0

8.4. OHIP-14 Questionnaire

QUESTIONNAIRE

Oral Health Impact Profile Questionnaire (Short Version) OHIP-14

Q1. Have you ever had trouble *pronouncing any words* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q2. Have you felt your *sense of taste* has worsened because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q3. Have you have *painful aching* in your mouth? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q4. Have you found it *uncomfortable to eat any foods* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q5. Have you been *self-conscious* because of your teeth, mouth or dentures?
(TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q6. Have you ever *felt tense* because of your teeth, mouth or dentures?
(TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q7. Has your *diet been unsatisfactory* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q8. Have you had to *interrupt meals* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q9. Have you found it *difficult to relax* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q10. Have you been a bit *embarrassed* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q11. Have you been a bit *irritable with other people* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q12. Have you had *difficulty doing your usual jobs* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q13. Have you felt that life in general was *less satisfying* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

Q14. Have you been *totally unable to function* because of problems with your teeth, mouth or dentures? (TICK ONE BOX ONLY)

Never	Hardly Ever	Occasionally	Fairly Often	Very Often

8.5. Sample Donation Form

SAMPLE DONATION FORM

Please complete this form about your project and sample/s. All samples must be signed in by a member of laboratory staff before use. By submitting samples to the lab you have agreed to the terms and conditions of use.

No samples will be accepted without a completed sample donation form

STUDY AND PARTICIPANT INFORMATION

Under HTA Licence only		*Project under Generic Ethics (EDGE)*		*Project under REC ethics*	

Project reference (EDGE/REC)	

If CRT – tick CRT Box and Sign. DO NOT fill participant ID

I can confirm that the participant has given verbal consent to retain waste tissue (CRT) ☐

Participant ID (RIO/research number)		Visit Date		Visit Number	

I can confirm that the participant has received the information sheet (PIS) and has given informed consent (ICF) ☐

Print ***Sign*** ***Date***

Name of Clinician (if different from signee above)	

SAMPLE INFORMATION

enter number (#) of samples for each (leave blank if not required))

Sample		Tubes #	Time taken	DRN issued by lab	Details
Blood	Green top #				
	Red top #				
	Purple top #				
	Other #				
Saliva	Stimulated #				
	Unstimulated #				
GCF					
PTF					
Plaque					
Calculus					
Tissue					
Other					
Any other information or Instructions					

Auxiliary tables can be used if your data does not conform to this format. Please speak to a member of biorepository team about this prior to sample submission.

(Lab staff to check form has been filled correctly) (Lab Manager checked, Initials_____)

8.6. Fixation Table

Fixation	
10% Neutral Buffered Formalin	18-24hr
Dehydration	
IDA99 30%	20min
50%	20min
70%	20min
90%	20min
100%	20min x3
Clearing	
Xylene 1	20min
2	20min
Wax Impregnation	
1	30min
2	30min
3	30min
Wax Embedding	↓