The Extent of the Role of Apoptosis in Oral Lichen Planus – A morphometric study

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November 2016

A mini thesis submitted in partial fulfilment of the requirements

for the degree of Master of Science in Dental Sciences in

Oral & Maxillofacial Pathology at the Faculty of Dentistry

University of the Western Cape.

DECLARATION

I declare that the thesis entitled "***The Extent of the Role of Apoptosis in Oral Lichen Planus – A morphometric study***" is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Dr. Marwa Zwet

ACKNOWLEDGEMENTS

I am very grateful to my supervisor for his continuous support, encouragement and patience during the completion of this dissertation. I am very thankful to him because he was very patient with me and provided me with unlimited support.

I am also grateful to every member of staff at the Department of Oral Pathology at the University of Western Cape as they were very cooperative and supportive. I would like to acknowledge the following people for their support in this project: Professor Jos Hille for his expertise, instructive guidance, and useful direction; Dr Henry Adeola for his invaluable suggestions and his assistance in making this study possible; Professor D. Govender, Dr Amir Afrogheh, Dr Khadija Abrahams, Mr Wessel Kleinhans, for their unlimited help during the phase of data collection for the study; and Professor Stefan Maritz for his assistance with the data analysis and his expert statistical advice.

I would also like to express my thanks to my family and friends who made this journey an enjoyable one. My family back home inspired me all the way and also gave the moral support I needed while I have been away from home.

DEDICATION

This work is dedicated to my parents, my brothers, and my husband, Dr Issadig Rhoma.

ABSTRACT

Oral lichen planus (OLP) is a T-cell mediated chronic inflammatory disease with different clinical types that remains inscrutable in respect of its pathogenetic mechanisms and effective therapy. Increased apoptosis may influence the histopathological criteria of oral lichen planus (decrease in thickness of the epithelium and band of inflammatory infiltrate).

Null hypothesis

The apoptotic rate does not correlate with a decrease in the epithelial thickness as well as the thickness of the band of inflammatory infiltrate in OLP.

Aim

The present study aims to quantify apoptotic activity and to correlate the apoptotic rate with epithelial thickness as well as thickness of the inflammatory infiltrate of OLP cases diagnosed at Tygerberg Hospital from 2006 – 2015. Further, the epithelial thickness and thickness of the inflammatory infiltrate were also assessed for their association, if any.

Materials and Methods

The study sample comprised 17 diagnostically verified cases of OLP. Sections stained with Haematoxylin and Eosin (H&E) were used to identify and count the number of apoptotic cells as well as measure the thickness of epithelium and the thickness of the lymphocytic inflammatory infiltrate by using software morphometric analysis (Zen Blue lite 2012). Statistical analysis was applied to analyse the correlation between apoptotic cells and histopathological features of OLP.

Results

The present study's results showed no statistically significant association between the apoptotic rate, the epithelial thickness and the thickness of the lymphocytic inflammatory infiltrate.

Keywords*: Apoptosis, Oral Lichen Planus, Epithelial thickness, Inflammatory infiltrate*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

The table below describes the various abbreviations used throughout the thesis.

ABBREVIATION	MEANING
APC	Antigen presenting cells
CD4+ T-cells	T-helper cells
CD8+ T-cells	Cytotoxic-T lymphocyte
HCV	Hepatitis C Virus
H&E	Haematoxylin and Eosin
HLA	Human leukocyte antigens
HPV	Human papilloma virus
IFN-α	Interferon gamma
IL-2	Interleukin2
LCs	Langerhans cells
MMPs	Matrix Metalloproteinase
MHC-I	Major histocompatibility complex class I
MHC -2	Major histocompatibility complex class II
m.Eithel	Epithelium thickness
m.apop	Number of apoptosis
m. infl	Thickness of inflammatory infiltrate
mm.Eithel	The mean of the epithelium thickness values
mm. infl	The mean of the inflammatory infiltrate values
mm. apop	The mean number of apoptosis values
OLL	Oral Lichenoid Lesion
OLP	Oral lichen planus
RANTES	Regulated on Activation, Normal T-Cell Expressed and secreted
TNFR-1	Tumour necrosis factor receptor 1
TUNEL	Transferase –mediated deoxyuridine triphosphate –digoxigenin
WHO	World Health Organization
UWC	University of the Western Cape

CHAPTER 1

INTRODUCTION

The term “lichen planus” is derived from the Latin word *lichen,* meaning “ringworm”, and the Greek word *planus* meaning “flat”, and refers to the clinical appearance of the skin papulae which appear depressed, flattened and smooth (Wilson, 1869). OLP is a commonly encountered disease in clinical dental practice, affecting approximately 0.5% to 2.0% of the entire population (McCreary and McCartan, 1999). Lichen planus is thus a chronic inflammatory disease which can affect both the mucosal and cutaneous tissues. The cutaneous form of OLP is less common than the mucosal form with reduced resistance to treatment in comparison to the mucosal form (Mollaoglu, 2000). Furthermore, OLP has been defined by the World Health Organization (WHO) as a potentially pre-cancerous disorder (WHO 1978; Georgakopoulou *et al.,* 2012). OLP is present in a variety of clinical forms (Andreasen, 1968) and it is histologically characterised by a band-like lympho-histiocytic infiltrate that occupies the superficial layer of the lamina propria (interface mucositis). In addition, vacuolar degeneration of the basal keratinocytes with absence of epithelial dysplasia are other important characteristics of OLP (Van der Meij and Van der Waal, 2003).

Although the key mechanisms involved in OLP pathogenesis are not fully understood, the role of a dysregulated immune response has been implicated in the condition. It is hypothesised that, due to the abnormal immune response, the antigenicity of the surface epithelial cells is altered resulting in the recognition of self-epithelial cells as foreign cells and subsequently their destruction by T-lymphocytes (Patel *et al.,* 2005; Sugerman and Savage, 2002). Further, apoptosis has also been reported as the key mechanism by which

epidermal cells die, leading to the destruction of epithelium and reduction of its thickness (Brant *et al*., 2012; Doddawad, 2014). However, the role of apoptosis in the inflammatory infiltrate of OLP is poorly understood as, to date, most of the studies on apoptosis in OLP have repeatedly focused on the epithelium and not on the combinatorial effect of apoptosis on the epithelium and the inflammatory cells. Keeping the above facts in mind, the present study aims to investigate the link between the apoptotic rate with epithelial thickness and intensity of the inflammatory infiltrate and to correlate between the thickness of the epithelium and the thickness of the band of inflammatory infiltrate in OLP samples. This retrospective study was conducted on OLP lesions in patients registered at the Oral Health Centre of the Tygerberg Hospital in Cape Town from 2006 to 2015. Morphometric analysis software (Zen Blue Lite 2012) was used to identify and count the number of apoptosis and to detect whether apoptosis should be an important component of the histopathological criteria in OLP. It is expected that the findings from this study will improve our understanding of the role of apoptosis in OLP in relation to the other histopathological features.

CHAPTER 2

LITERATURE REVIEW

2.1 STRUCTURE OF THE HEALTHY MUCOSA

The oral cavity is lined by a mucous membrane comprising epithelium and underlying supporting tissue. It is broadly classified into keratinised and non-keratinised types of oral mucosa depending on its location and functions. While the keratinised oral mucosa is located in areas that are subjected to masticatory force, such as the hard palate and the marginal gingival (resembling the epidermis of the skin), the non-keratinised epithelium is located in the alveolar mucosa, buccal mucosa, floor of the mouth, ventral tongue, lips and soft palate. Some areas of the mouth consist of both keratinised and non-keratinised types of epithelium, like the vermilion border of the lip and the dorsal surface of the tongue *(*Dale *et al*., 1990; Presland and Dale, 2000; Presland and Jurevic, 2002*).*

The lining epithelium of oral mucosa is of the stratified squamous type (Mackenzie and Fusenig, 1983), and the underlying supporting tissue (lamina propria) contains blood vessels, nerves, fat and muscles. These two layers (oral epithelium and supporting tissue) are separated by dense connective tissue material called the basement membrane comprising collagen Type 4 (Ten Cate, 1998).

2.2 EPIDEMIOLOGY OF ORAL LICHEN PLANUS

OLP is considered to be a common condition which affects about 0.5 – 2.2% of the world population (McCreary and McCartan, 1999). The disease mainly appears in the middle-aged population, in particular in individuals between 30 and 50 years of age and is

more common in females than in males (Brown *et al.*, 1993). OLP is uncommon in children and if it affects children, it usually presents as a cutaneous form of lichen planus (Alam and Hamburger, 2001). The rate of recovery from OLP is only about 17% of affected people (Thorn *et al.*, 1988).

2.3 CLINICAL FEATURES

OLP is clinically represented and recognised in multiple forms including reticular, papular, plaque, atrophic, ulcerative (erosive) and a rare bullous form (Andreasen, 1968; Ismail *et al*., 2007*).* The disease tends to be bilateral and occurs commonly on the buccal mucosa area of the oral cavity (Bagan-Sebastián *et al*., 1992). Other areas that can be affected include the palate, gingiva, tongue and alveolar ridge.

OLP always affects the oral mucosa bilaterally. The most common form of OLP lesions is the reticular type which typically appears with an erythematous border and interlacing white hyperkeratosis lines referred to as Wickham's striae (Ingafou *et al.*, 2006). The second most common type of OLP is the atrophic type which appears as diffuse, erythematous patches (Thorn *et al.*, 1988). The bullous form is commonly situated in the buccal mucosa or at the lateral border of the tongue. This form ruptures easily and presents as an erosive type of OLP (Thorn *et al*., 1988). The most destructive forms are the ulcerative/erosive types. These are associated with more pain and oral discomfort compared to the white reticular forms which usually present fewer symptoms (Brant *et al.*, 2012). The erosive form appears clinically as a central ulceration covered by a pseudo-membrane or a fibrin plaque and is often surrounded by fine radiant keratinised striae with a network appearance (Neville and Damm, 1998).

The papular form appears as small papules of approximately 1.0 mm in size that are present in conjunction with other clinical variants, whereas the plaque form resembles multifocal leucoplakia which vary from irregular elevated areas to smooth and flat areas

(Bricker, 1994). The latter is more common in smokers and most frequently affects the tongue and buccal mucosa (Thorn *et al.*, 1988).

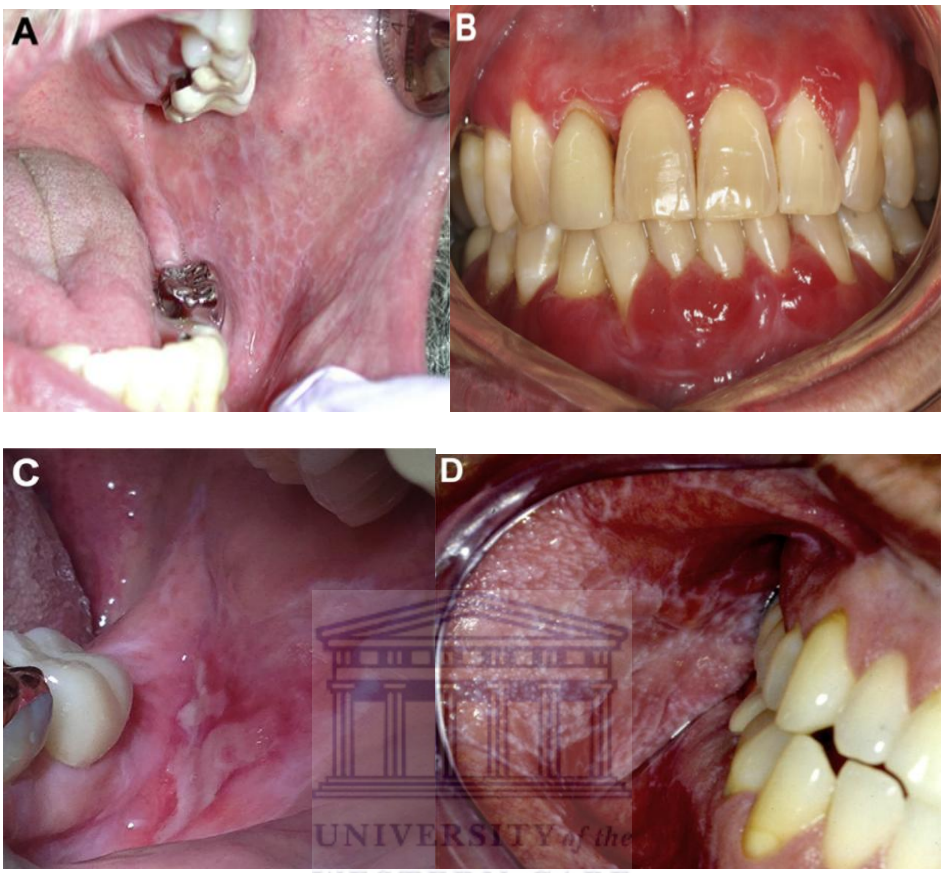

Figure 1: Clinical features of OLP showing (A) reticular, (B) atrophic (C) erosive, and (D) papular variants (Cheng et al., 2016).

2.4 HISTOPATHOLOGY

The histopathological features of OLP are highly similar to the cutaneous lichen planus. According to the WHO (1978), these features largely include: (1) a dense band-like layer of lymphocytic infiltrate within the underlying connective tissue, (2) liquefactive degeneration of the basal keratinocytes and (3) absence of epithelial dysplasia. From a histopathological view the lesion is considered to be OLP if the lesion includes all of these three criteria. In the absence of any one of these three criteria, the lesion is considered to be 'histologically compatible with OLP' (Fernández *et al.*, 2011). Other histopathological

features of the lesion include hyperkeratosis (hyper-parakeratosis, hyper-orthokeratosis or a combination) and eosinophilic apoptotic keratinocytes also known as Civatte bodies (Burgdorf and Plewig, 2014). The mingling of the lymphocytes with the basal cell area is referred to as "interface mucositis" and can also be seen in other oral lesions like oral lichenoid lesions and Lupus erythromatosus (Jorizzo *et al.*, 1992; Khudhur *et al.*, 2014). In addition, other features reported in the literature include the presence of basement membrane alterations such as branches, breaks, and thickenings (Jungell *et al.*, 1988). These abnormal changes in the basement membrane and basal cells create clefts between the epithelium and the underlying lamina propria called the Max-Joseph spaces. Cleft formation can also result in the formation of a blister of the oral mucosa as seen in bullous OLP (Sugerman *et al.*, 2002). Additional findings reported a thinner epithelium in comparison to normal mucosa with "saw-tooth" shaped rete pegs (Karatsaidis *et al.*, 2003; Aminzadeh *et al.*, 2013).

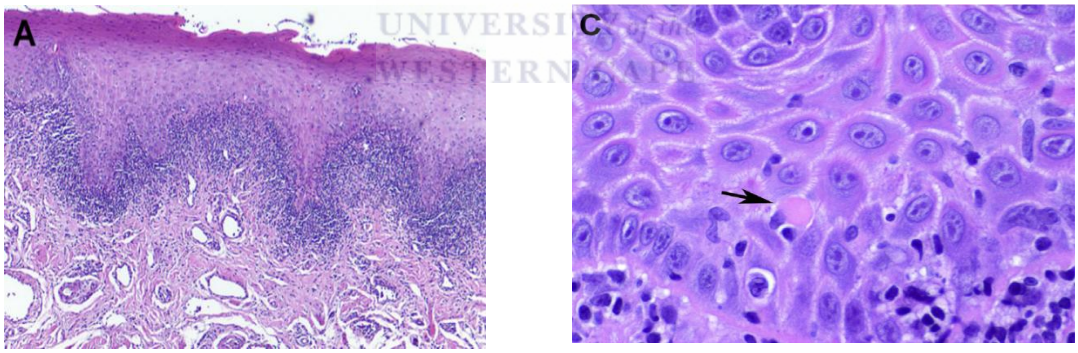

Figure 2: (A) Typical histopathologic features of OLP. (C) Lymphocytic- mediated injury of oral epithelium with keratinocyte apoptosis (arrow) (Cheng et al., 2016).

2.5 INFLAMMATORY INFILTRATE IN ORAL LICHEN PLANUS

The band-like inflammatory infiltrate in the sub-epithelial layer of OLP is principally composed of T-Cells (CD3+) including T-Helper cells (CD4+) and Cytotoxic T-lymphocytes (CD8+) (Hasseus *et al.*, 2001). Previous studies have shown that this inflammatory cell

infiltrate is variable in different forms of the disease. CD4+ T-cells predominate in the initial lesion of reticular OLP, whereas the numbers of CD8+ T-cells are increased in more advanced atrophic or erosive lesions (Sugerman *et al*., 2002; Charazinska-Carewicz *et al*., 2008).

Langerhans cells (LCs) and mast cells are increased in OLP lesions when compared to normal oral mucosa (Zhao *et al*., 2001; Hasseus *et al*., 2001). The proportions of B-lymphocyte and plasma cells in oral lichen planus are relatively lower and represent only about 5% of all inflammatory cells (Sugerman *et al*., 2002).

2.6 AETIOLOGY OF ORAL LICHEN PLANUS

Although the exact cause of OLP is not completely understood, several studies have proposed a number of factors; for example, genetic predisposition, dysregulated immune functions, systemic diseases, dental materials, etc, that trigger the development of this lesion. While the role of genetic predisposition has been considered in disease pathogenesis, only a few familial cases of OLP have been reported. Watanabe and co-workers, among others, have stated the role of human leukocyte antigens (HLA) A3, A11, A26, A28, B3, B5, B7, B8, DR1 in the pathogenesis of oral lichen planus (Watanabe *et al*., 1986; McCartan and Lamey, 1997; Ognjenovie *et al*., 1998).

Recently, it was found that pathogenic microorganisms of periodontal origin were found to be associated with OLP patients (Ertugrul *et al*., 2013). Viral agents such as human papilloma virus (HPV) were also found to be implicated with OLP lesions (Campisi *et al*., 2004). Furthermore, recent studies have also shown a strong connection between Hepatitis C Virus (HCV) and OLP (Lodi and Porter, 1997; Prabhu *et al.*, 2002; Gimenez and Perez, 2003; Lodi *et al.*, 2004).

An association between autoimmune disorders such as ulcerative colitis, chronic active hepatitis, myasthenia gravis, and OLP lesions has also been reported (Abbate *et al.*, 2006). Similarly, OLP has been seen in patients affected by neoplasms such as metastatic adenocarcinoma as well as breast cancer (Scully *et al*., 1998).

Certain systemic diseases like hypertension, Diabetes mellitus, Myasthenia gravis, Lupus erythematous, and Ulcerative colitis were also considered to be associated with OLP (Lozada and Miranda, 1997). Restoration materials used in dental clinics, such as amalgam, silver, cobalt, gold, chromium and composite are believed to stimulate the progression of OLP (Issa *et al.*, 2004).

Other causes like anxiety, stress and food allergies are also considered to be factors responsible for the development of OLP (Chaudhary, 2004). Stress has long been implicated in the dysregulation of innate and adaptive immune functions through neuro-endocrine mediators secreted from the hypothalamus-pituitary-adrenal axis as well as the sympathetic-adrenal axis (Kemeny and Schedlowski, 2007). Therefore, its influence in OLP pathogenesis, most likely as a secondary mechanism, cannot be ruled out. Food allergies with ingredients like cinnamon have also been found to trigger the development of OLP (Scully *et al*., 1998).

2.7 PATHOGENESIS OF ORAL LICHEN PLANUS

The exact pathogenesis of OLP disease is still unclear, but a large body of evidence supports the role of the dysregulated immune system (Scully *et al.*, 1998; Sugerman *et al.*, 2002; Lodi *et al.*, 2005). Sugerman believes that both the antigen-specific cell mediated immune responses as well as non-specific mechanisms are involved in the pathogenesis of OLP. He surmised that other mechanisms like humoral and autoimmune response are also involved (Sugerman *et al*., 2002).

2.7.1 ANTIGEN SPECIFIC MECHANISM

Although OLP has been recognised as a T-cell mediated inflammatory disease the antigen in OLP lesions is yet to be identified (Porter *et al*., 1997; Sugerman and Savage, 2002). Moreover, since it is hypothesised that the body's own self-peptide or a heat-shock protein can act as an antigen, OLP can also be considered as an autoimmune disease. These self-peptides are induced by many factors such as contact with allergens (e.g. toothpaste or dental restorative materials), mechanical trauma, infections (such as bacterial/viral infection), or an unidentified agent (Sugerman and Savage, 2002).

Heat shock proteins as antigens are over-expressed in OLP lesions and could be linked to a variety of exogenous agents, such as systemic drugs, contact allergens, mechanical trauma, bacterial or viral infections (Sugerman *et al*., 1995). An over-expression of the heat shock protein gene by stressed oral keratinocytes, or failure to suppress the response of an immune system after self-HSP recognition due to decreased immune response was hypothesised in the OLP predisposition (Sugerman and Savage, 2002).

The early stages of the disease include expression or unmasking of the keratinocyte antigen (Eversole, 1997; Zhou *et al*., 2002). The response of the immune system to this undetermined antigen (heat shock or self-peptide) includes the subsequent stages:

1. Migration of the T-cells, mainly CD8+ T-cells and a few CD4+ T-cells to the epithelium (Sugerman *et al*., 2002).
2. Activation of CD8+ T-cells either directly or indirectly. The direct mechanism involves the binding of antigen to the MHC-I on basal keratinocytes. The indirect mechanism occurs through binding of antigen to MHC -2 on antigen presenting cells (APC). This binding leads to the sequential activation of CD4+ T-cells and CD8+ T-cells followed by the release of mediators like interferon gamma and interleukin2 (IL-

2 and IFN) which in turn facilitates the binding of the CD4+ T-cells RCA receptor to the RCA present on the surface of CD8+ T-cells (Zhou *et al*., 2002; Sugerman *et al*., 2002).

3. Killing of basal keratinocytes - occurs by one of three mechanisms:

- The activated CD8+ T-cell secrete Tumour Necrosis Factor alpha (TNF-α) which binds to the TNFR-1 on the surface of the basal keratinocytes thereby killing these cells.
- *FasL* receptors located on the T-cells' surface binds to Fas receptors on the cell membrane of the keratinocytes.
- The secretion of granenzyme B via T-cells which enters the keratinocytes through membrane pores.

All of these mechanisms subsequently activate the Caspase cascade, leading to apoptosis of keratinocytes (Sugerman *et al*., 2002; Ammar *et al*., 2008).

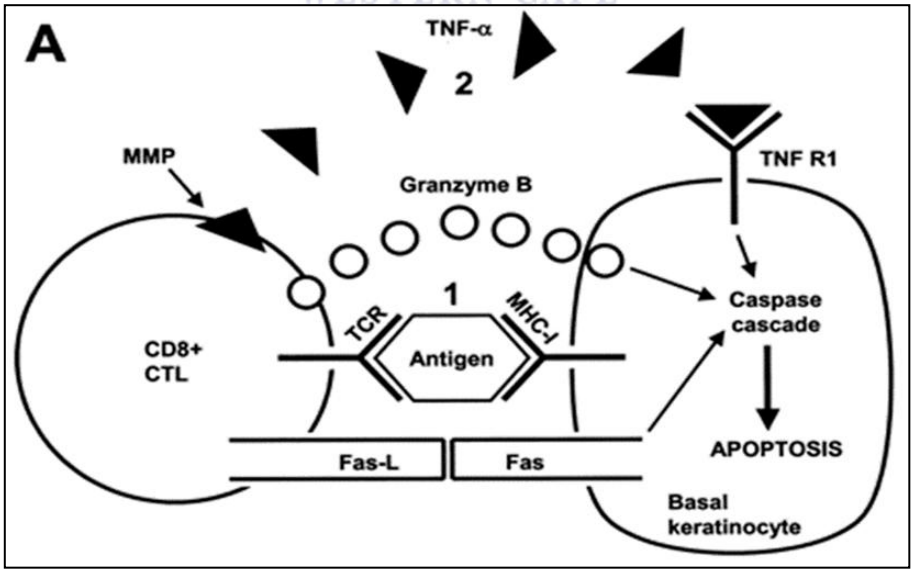

Figure 3: Diagram showing the Hypothesis of pathogenesis of OLP (the antigen of lichen planus is expressed on the basal keratinocyte in association with MHC class I molecules on basal keratinocytes. Antigen-specific CD8+ T-cell after activation trigger apoptosis of keratinocyte via secreted TNF that bind to the receptor of TNF (TNF-R1) (Sugerman et al ., 2002).

2.7.2 NON-SPECIFIC MECHANISMS

Some of the T-cells' lymphocytes infiltrated in OLP have been attracted to the superficial layer of lamina propria by numerous non-specific mechanisms. Sugerman *et al.* (2002) suggested that these mechanisms aim to destroy basal keratinocytes by migration of the lymphocytes to the epithelium and include the following:

2.7.2.1 EPITHELIAL BASEMENT MEMBRANE DESTRUCTION

Basement membrane alteration like breaking, branching, duplication, or thickening is common in OLP disease (Jungell *et al.,* 1988). Basal keratinocyte cells are essentially required for the maintenance of epithelial basement membrane structure integrity as it secretes Laminin V and Collagen IV (Marinkovich *et al*., 1993). However, due to the presence of apoptosis in keratinocytic cells these cells will no longer be able to perform this function resulting in the disruption of the basement membrane as is observed in OLP. Conversely, some evidence indicates that keratinocyte cells themselves require the basement membrane for maintenance and send the survival signal for termination of apoptosis (Pullan *et al.,* 1996).

Nevertheless, both apoptosis of keratinocytes as well as disruption of the epithelial basement membrane are implicated in the pathogenesis of OLP. The two mechanisms may indeed have occurred as a cyclic event where keratinocyte apoptosis might have been triggered by the disruption of the basement membrane, and apoptotic keratinocytes may not be capable of repairing the basement membrane disruption. Such cyclical events may be responsible for the chronicity of the lesion (Sugerman and Savage, 2002).

2.7.2.2 MATRIX METALLOPROTEINASES

Matrix MetalloProteinases (MMPs) belong to the zinc-containing endo-proteinases family. The main function of this enzyme is the proteolytic degradation of connective tissue matrix proteins. Activation of this enzyme leads to the disruption of the basement membrane through the release of the MMP's activator from T-cells (Zhao *et al*., 2001; Sugerman and Savage, 2002).

Keratinocyte survival signals cannot pass through the disrupted basement membrane thereby triggering the apoptosis of keratinocytes. Moreover, the passage of antigen-specific CD8+ T-cells into the OLP epithelium may facilitate the disruption of the basement membrane which can trigger more apoptosis of the keratinocytes (Moss *et al.*, 1997; Itai *et al.*, 2001).

2.7.2.3 MAST CELLS

Mast cell density was reported to be increased in OLP (Zhao *et al*., 1997; Zhao *et al*., 2001). Approximately 60% of such mast cells were degranulated in OLP, as compared with 20% of degranulated mast cells found in the normal buccal mucosa (Zhao *et al*., 2001). These degranulated mast cells release a number of inflammatory mediators such as TNF-α, Chymase and Tryptase, which may up-regulate endothelial cell adhesion molecule expression. This in turn is required for lymphocyte adhesion to the luminal surface of blood vessels and subsequent extravasation (Klein *et al.*, 1989; Zhao *et al*., 1997).

In addition, clusters of mast cells and intra-epithelial CD8+ T-cells have been found at sites of basement membrane disruption in OLP (Zhao *et al*., 2002). Analysis of this data suggests that mast cells and CD8+ T-cell migration may play a role in epithelial basement membrane disruption in OLP.

2.7.2.4 CHEMOKINES

Chemokines are cytokines that are produced by virtually all somatic cells. RANTES (Regulated on Activation, Normal T-Cell Expressed and Secreted) is a member of the chemokines family which plays an important role in the recruitment of lymphocytes and mast cells in OLP.

RANTES receptors like CCR1, CCR3, CCR4, CCR5, CCR9 have been identified in OLP (Zhao *et al*., 2001; Sugerman *et al*., 2002). The recruited mast cells undergo degranulation under the influence of RANTES, which releases Chymase and TNF-α, resulting in further up-regulation of RANTES secretion by OLP lesion T-cells. This cyclical activity may underlie the chronicity of the lesion (Sugerman *et al.*, 2002).

2.7.3 AUTOIMMUNITY

It has been hypothesised that OLP disease is an autoimmune lesion. This hypothesis is supported by numerous autoimmune features of oral lichen planus like adult onset, the chronicity of the disease, being more common in females, and its association with other autoimmune lesions (Sugerman *et al.*, 2002).

2.7.4 HUMORAL IMMUNITY

The presence of circulating antibodies including auto-antibodies reacting against Desmogleins 1 and 3 has been implicated in OLP patients. This finding suggests a role of humoral immunity in the pathogenesis of oral lichen planus (Lukač *et al*., 2006).

2.8 DIAGNOSIS OF ORAL LICHEN PLANUS

Due to the challenges in the correct diagnosis of OLP lesions, it is obligatory to include clinico-pathological correlation. In 1987, the WHO defined criteria for the diagnosis of OLP lesions which were modified in 2003 by Van der Meij and Van der Waal. According

to the clinical criteria, the lesion must be bilateral with the presence of interlacing white hyperkeratosis lines displaying a reticular pattern.

The other variants, which include erosive, plaque bullous and atrophic types, are accepted as subtypes in the presence of reticular lesions elsewhere in the mucosa of the oral cavity. The histopathological criteria involve the presence of the band of cellular infiltration composed mainly of lymphocytes in the superficial part of connective tissue, vacuolar or hydropic degeneration of the basal layer, and absence of epithelial dysplasia. They suggest the use of the term "histopathologically compatible" whenever the criteria are less obvious (Van der Meij and Van der Waal, 2003).

2.8.1 DIRECT IMMUNOFLUORESCENCE

This is a useful technique to support the diagnosis. A characteristic of OLP lesions is the presence of a shaggy pattern of fibrinogen deposits along the basement membrane with absence of Complement and Immunoglobulin deposits (Crincoli *et al*., 2011).

2.9 DIFFERENTIAL DIAGNOSIS OF ORAL LICHEN PLANUS

These include Leukoplakia, cheek biting, Mucous membrane pemphigoid, lichenoid reactions, Candidiasis, Graft versus Host Disease (GVHD), frictional keratosis, Lupus erythematous, Pemphigus, Chronic ulcerative stomatitis and para-neoplastic Pemphigus (Lavanya *et al*., 2011).

2.10 TREATMENT OF ORAL LICHEN PLANUS

Due to the uncertainty of the pathogenesis of OLP many clinicians use immune-suppressant corticosteroids as the first line of treatment for the disease (Scully *et al.,* 2000; Lavanya *et al*., 2011).

OLP cases are occasionally resistant to corticosteroids due to genetic variability in lymphocyte sensitivity potential (Hearing *et al*., 1999; Creed *et al.,* 2009; Nicolaides *et al*., 2014). Such patients were administered with other options like calcineurin inhibitors which target T-cell activation and proliferation, or cytokine receptor inhibitors (Cheng *et al.,* 2012; O'Neill and Scully, 2013). Furthermore, natural remedies such as green tea consumption have also been found to be useful in OLP treatment. Green tea is reported to prevent T-cells proliferation, antigen presentation, migration, activation and the apoptosis of keratinocytes (Zhang and Zhou, 2012).

2.11 ORAL LICHENOID LESIONS (OLL)

These lesions resemble OLP both histopathologically and clinically. While OLP does not have a specific aetiology, OLL's have a defined or suspected cause in patients. Drugs are considered one of the major causes of lichenoid reactions in the oral mucosa and skin (McCartan and McCreary, 1997). Lichenoid lesions can also be caused by direct contact of the oral mucosa with dental restoration materials such as mercury, amalgam and resin materials (Östman *et al*., 1994; Blomgren *et al*., 1996). OLL could also be caused by flavouring agents in foods such as cinnamon. Some investigators described a focal type and diffuse type presentation of OLL on consumption of cinnamon-based candies and mouth rinses, respectively (Miller *et al.,* 1992).

One histopathological study aimed to differentiate OLP from OLL by correlating epithelium thickness and thickness of inflammatory infiltrate in other lesions. This study found that in lichenoid reactions, the relationship was positive between epithelial and inflammatory band thickness, whereas it was negatively correlated in cases of Lichen Planus (Usha *et al.,* 2012).

2.12 APOPTOSIS

Apoptosis is a Greek word described by Kerr *et al.* which means falling as in the case of the falling of leaves from trees in autumn (Kerr *et al*., 1972). It is one of the mechanisms that maintain balance between dying cells and normal cells and is considered as programmed cell death (PCD) (Hanahan and Weinberg, 2011). While cell death involves several mechanisms like apoptosis, necrosis, autophagy, anoikis, necroptosis, entosis and cornification (Roychowdhury *et al*., 2013), the two main mechanisms are necrosis and apoptosis (Shuh *et al.,* 2013).

The microscopic alterations of apoptosis occur in two separate stages. The first stage comprises the nucleus and cytoplasm condensation and splitting of the cell into membranous fragments, whereas the second stage involves either shacking of the apoptotic body fragments from the cell's surface or being phagocytosed by the phagocytic cells, which are subsequently degraded by a mixture of lysosome enzymes inside the phagosomes (Kerr *et al*., 1972).

2.12.1 MECHANISMS OF APOPTOSIS

Apoptosis usually occurs through two major pathways – the intrinsic (or mitochondrial) pathway and the extrinsic pathway (Fig 2.4). Another mechanism was also reported to initiate apoptosis which occurs by releasing perforin/granzyme from cytotoxic T-lymphocytes and NK cells (Martinvalet *et al.*, 2005).

2.12.1.1 INTRINSIC PATHWAY OF APOPTOSIS (MITOCHONDRIAL PATHWAY)

The mitochondrial pathway of apoptosis is mediated by pro-apoptotic members causing an increase in the permeability of the outer mitochondrial membrane which leads to Caspase activation (Green and Kroemer, 2004). Many initiators and stimuli such as; ultraviolet radiation, DNA damage, heat, the actions of tumour suppressor genes (i.e. p53),

viral virulence factors, chemotherapeutic agents and stress can initiate the intrinsic pathway of apoptosis (Kroemer, 2003). These cytotoxic stimuli trigger the permeability of the mitochondrial outer membrane and the mechanism is regulated by mitochondrial lipids and Bcl-2 family proteins (Decaudin *et al.*, 1998; Green and Kroemer, 2004). This leads to the disruption of the outer mitochondrial membrane with consequent disintegration of numerous proteins located between the outer and inner mitochondrial membrane which is released into the cytosol where they trigger cell death by stimulating caspase-dependent and -independent pathways (Saelens *et al.*, 2004).

2.12.1.2 EXTRINSIC PATHWAY OF APOPTOSIS

This is one of the major pathways and is also called receptor-mediated apoptosis. It is mediated through activation of the pro-apoptotic receptors located on the surface of cells. Some examples of these death receptors are the TNF-1 receptor (p55 or CD120a), Fas (CD95/APO-1), DR4 (TRAIL-R1) and DR5 (TRAIL-R2) which transmit apoptotic signals after their activation upon binding to molecules known as pro-apoptotic ligands (Elmore, 2007; Henseleit *et al.,* 1997). Activation of these receptors leads to the recruitment of adaptor proteins namely TNF receptor-associated protein with Death Domain (TRADD) and Fas-associated protein with Death Domain (FADD) (Guicciardi and Gores, 2009). These adaptor proteins recruit and activate caspase 8 and caspase 10 (initiator caspases) through Death Effector Domains (DEDs) thereby leading to the formation of a complex known as the death-inducing signalling complex (DISC) (Boatright and Salvesen, 2003; Guicciardi and Gores, 2009). The activation of these initiator caspases subsequently activates effector caspases causing degradation of cellular targets and apoptosis induction (Scaffidi *et al.*, 1998).

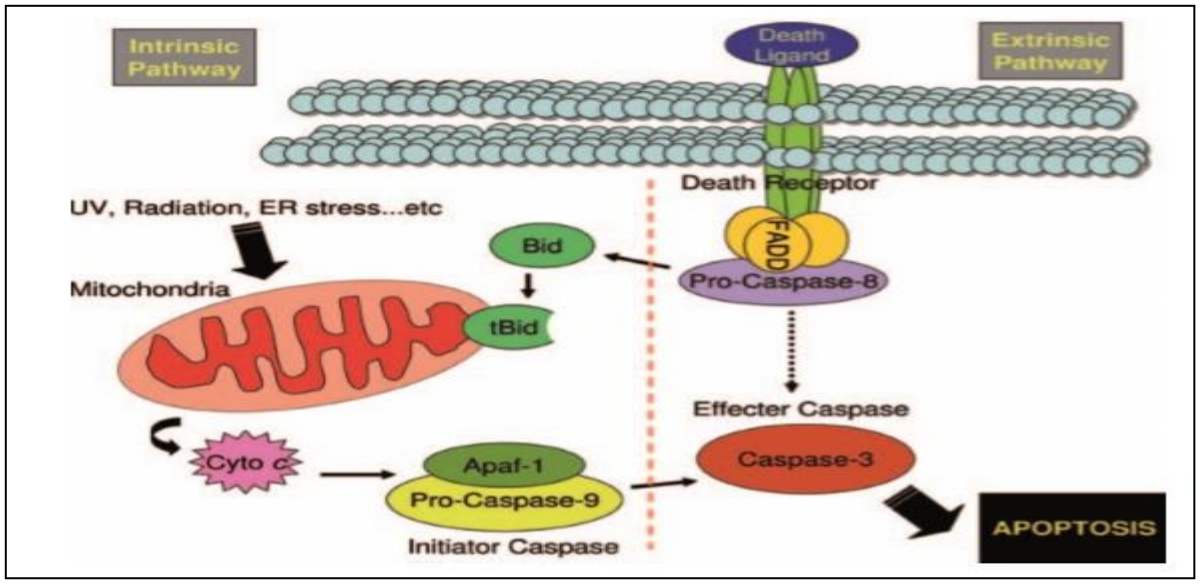

Figure 4: Diagram showing the intrinsic and extrinsic apoptotic pathway (Anas and Roya, 2008).

2.12.2 SIGNIFICANCE OF APOPTOSIS IN BIOLOGICAL SYSTEMS

Apoptosis plays a key role during embryogenesis where it contributes in shaping tissues and organs in the body and maintaining normal homeostasis. During development, most cells are produced in excess and thereafter undergo apoptosis or PCD to develop in requisite form, as noticed in the development of the immune system, nervous system and reproductive organs (Meier *et al*., 2000). Apoptosis is also important in adult organism cells where it continues to maintain a balance between proliferation and cell death in order to maintain homeostasis of constant cell numbers (Rathmell and Thompson, 2002).

2.12.3 MALFUNCTIONING OF APOPTOSIS AND PATHOGENESIS

Malfunctioning and dysregulation of apoptosis occurs either due to its down- or up-regulation thereby causing many diseases in the body. Most prominent diseases like cancer, autoimmunity and other persistent infections largely occur due to insufficient apoptosis. Recently it was found that tumour formation occurs not only as a result of excessive proliferation of cells due to the activation of oncogenes, but also depends on the impairment of apoptosis checkpoints (Hanahan and Weinberg, 2000; Wang, 1998). On the contrary, diseases like neurodegenerative diseases (Parkinson's disease, Alzheimer's disease) and AIDS (caused by depletion of T -lymphocytes) largely occur due to excessive apoptosis (Reed, 2002).

2.13 APOPTOSIS AND OLP

Apoptosis in OLP has been hypothesised as one of the major underlying mechanisms involved in basal keratinocyte destruction (Lavanya *et al.,* 2011). Occurrence of apoptosis in the keratinocytes of OLP lesions was first suggested by Hashimoto and it was assumed that the cytotoxic T-cells are involved in the apoptosis of basal keratinocytes (Hashimoto, 1976; Weedon, 1980; Roopashree, *et al.*, 2010). Furthermore, it was reported that apoptosis increases significantly in OLP patients compared to the normal oral mucosa (Karatsaides, 2003; Nelperg, 2007). Although apoptosis serves as an important histopathological feature of the OLP lesion (Burgdorf and Plewig, 2014), only a few studies are available to validate its relations with other histopathological features of OLP. It was suggested by Neppelberg *et al*., (2001) that increased apoptotic rates could be responsible for the decreased thickness of the oral epithelium. A couple of studies indicate that the increase in apoptotic rate is linked to an enhanced lymphocyte infiltration in OLP (Bloor *et al.,* 1999; Doddawad, 2014). Other studies

revealed that the chronic inflammatory infiltrate is responsible for inhibition of the occurrence of apoptosis in the corresponding epithelium (O'Byrne and Dalgleish, 2001).

Results from studies conducted to determine the significance of apoptosis in the inflammatory infiltrate of OLP showed that the absence or low rate of apoptosis noticed in inflammatory cells in OLP seems to contribute to the persistence of the inflammatory infiltrate. This also heightens the influence of the onset of molecular disorders in epithelium and favour malignant transformation (Bascones *et al*., 2006).

Further, the relationship between apoptosis and epithelial thickness was studied in the most common types of OLP i.e. the reticular and erosive forms by Brant *et al.* (2008). In apoptotic analysis performed through immunohistochemistry like TUNEL and M30 CytoDEATH, apoptosis was found to be significantly increased in both reticular and erosive OLP cases in comparison to normal mucosa. However, the apoptotic intensity was much higher in cases of erosive OLP. Similar results were obtained regarding epithelial thickness where thickness was found to be greatest in normal mucosa followed by reticular and erosive OLP cases, respectively. Moreover, the study reported a significant negative correlation between the epithelial thickness and the apoptosis index (AI). Hence, the erosive OLP showed the thinnest epithelia and the highest AI (Brant *et al.,* 2008). In an extended study, Brant *et al.* (2012) evaluated the role of apoptosis in reticular and erosive OLP, particularly in the epithelium and sub-epithelia inflammatory cell infiltrates. While the initial findings for an inverse correlation between the epithelium thickness and amount of apoptosis was reported similar to a previous study, different apoptotic levels were reported in the erosive and reticular forms of OLP, determining different clinical presentations. Regarding apoptotic activity in the inflammatory cell infiltrates, while the apoptosis index was found to be higher in reticular OLP in comparison to erosive OLP, the number of lymphocytes was less in reticular OLP, suggesting a negative correlation between apoptosis and lymphocytic content

of infiltrates. Overall, that study concluded that a higher apoptosis index and a lower lymphocytic content in reticular OLP results in increased elimination of inflammatory cells, less inflammation, less aggression to epithelial cells, and reduced epithelial apoptosis. On the contrary, in case of erosive OLP, less apoptosis and high lymphocytic content in the inflammatory infiltrate cause persistent lymphocyte recruitment with continuous inflammation and aggression towards epithelium (Brant *et al*., 2012).

Although involvement of apoptosis in basal cell degeneration in OLP has been reported, research findings regarding the correlation between apoptosis and histopathological characteristics of OLP are highly sparse. Hence, the present study aims to investigate the association of apoptosis with epithelial thickness and the inflammatory infiltrate in OLP. It is expected that findings from this study will aid in enhancing the in-depth understanding of the role of apoptosis in OLP, as well as its association with other histopathological features.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 AIM

- To determine and correlate the apoptotic rate with the epithelial thickness and band-like lymphocytic infiltration in H & E sections of OLP samples diagnosed at Tygerberg hospital from 2006 – 2015.
- To gauge the possible association of the sub-epithelial chronic inflammatory infiltrate with the thickness of the overlying epithelium.

3.2 OBJECTIVES

A) To determine the apoptotic rate of histologically diagnosed cases of OLP.

B) To evaluate the thickness of the epithelium and band-like lymphocytic infiltrate in OLP.

C) To correlate the findings in "A" and "B."

D) To determine the correlation between the thickness of epithelium and band-like lymphocytic infiltrate.

3.3 MATERIALS AND METHODS

3.3.1 STUDY DESIGN

The study was performed as a retrospective study where the data for the patients diagnosed clinically as well as histologically with OLP were retrieved from the Department of Oral Medicine & Periodontics at Tygerberg Oral Health Centre, Cape Town, South Africa during the period between 2006 and 2015. Prior to the study, ethical approval was acquired from the Human Health Research Ethics Committee of UWC and in lieu of research ethics, the

patient's data was displayed as a registration code number rather than by their name, for identification purposes. All archived files of patients with OLP diagnosed in the period between 2006 and 2015 were retrospectively reviewed, and a total of 29 cases diagnosed as OLP were selected from the aforementioned period. The corresponding histopathology slides were accessioned from the Anatomical Pathology archives of the National Health Laboratory Service at Tygerberg hospital. The clinical files and histological material were further screened based on the following inclusion and exclusion criteria based on the modified WHO clinical and histopathological diagnostic criteria, proposed by Van der Meij and Van der Waal (2003).

3.3.1.1 CLINICAL CRITERIA

The clinical criteria stipulate that the lesions must be bilateral with the presence of interlacing white hyperkeratosis lines (reticular pattern). The other variants include erosive, plaque, bullous and atrophic types which are accepted subtypes in the presence of reticular lesions elsewhere in the mucosa of the oral cavity. Patients receiving drug treatment or with non-symmetrical or unilateral lesions were excluded in order to avoid possible diagnostic confusion with lichenoid reactions.

3.3.1.2 HISTOPATHOLOGICAL CRITERIA

Only specimens obtained from the buccal mucosa were used for morphometric analysis in order to avoid histological differences due to the differing anatomical origins of the samples. The inclusion criteria comprised the presence of sufficient epithelium and a band-like inflammatory infiltration in the submucosa with deeper connective tissue. The presence of the band of cellular infiltration had to be composed mainly of lymphocytes in the superficial part of connective tissue intermingling with the epithelial basal layer (interface mucositis) resulting in vacuolar or hydropic degeneration of the basal layer. There had to be

an absence of fungal infection or epithelial atypia in order to avoid possible or other processes that can develop immune reactions in the corium. Cases with any tissue artefact or lack of inflammation were also not considered for inclusion in the study.

3.3.2 HISTOPATHOLOGY ANALYSIS

Based on the above stringent screening measures, 17 paraffin block samples were selected out of these 29 cases and were recut for histopathological analysis as mentioned below: Paraffin blocks, containing fixed OLP tissues in 10% buffered neutral formalin solution, of each of these 17 samples were sectioned. The 4 μm sections of each sample were stained with H & E staining using an automated stainer. All slides were further examined for determination of apoptotic cells as well as sub-epithelial and epithelial band thickness as described below.

Figure 5: Diagram showing wax block of OLP specimen.

Figure 6: Photograph showing a rotary microtome and a water bath.

Figure 7: Photograph showing an automated H&E stainer (LEICA).

3.3.2.1 DETECTION OF APOPTOTIC CELLS

Using a camera attached to a digital imaging microscope, representative images of H & E sections of OLP cases were taken. Apoptotic cells were identified in the basal and intermediate layers based on the following criteria put forward by Bloor *et al.*, (1999):

- Cell shrinkage.
- Chromatin condensation at the periphery of the nucleus.
- Uniform eosinophilic cytoplasm.
- Nuclear and/or cytoplasmic fragmentation.
- Nuclear pyknosis.
- Nucleolar disintegration.

The total number of apoptotic cells were counted and recorded in 131 adjacent 250 μm wide fields of the section; the same areas were used for measuring the other histopathological parameters (thickness of the epithelium and corresponding inflammatory infiltrate).

Figure 8: Photograph showing a digital imaging microscope.

3.3.2.2 MEASUREMENT OF EPITHELIAL AND SUB-EPITHELIAL INFLAMMATORY BAND THICKNESS

The epithelial thickness and the thickness of the sub-epithelial band of inflammatory infiltrate was measured in 131 adjacent 250 μm wide fields of the sections in the same areas where the apoptotic bodies were counted. The epithelium was measured from the superficial keratotic area to the basal layer of the epithelium. The band of inflammatory infiltrate was measured in sub-epithelial connective tissue. Five measurements of the inflammatory infiltrate thickness were taken from each region (each 250 μm wide) at 12 x magnification with a 20 x plane achromatic objective. The average of five measurements of epithelium and inflammatory infiltrate was calculated and correlated with the number of apoptotic bodies. All measurements were made by using a free modular image-processing and analysis software for digital microscopy (ZEN Blue Lite, 2012). The results were entered into an Excel® spreadsheet (Microsoft 2010, USA) for statistical analysis. The data was analysed statistically by using R statistical software program (R Core Team®, 2013).

CHAPTER 4

RESULTS

4.1 SOCIODEMOGRAPHIC FEATURES OF THE SAMPLE

The selected patients' gender and age of onset of OLP was recorded. An almost equivalent gender ratio was observed in OLP patients, with 52.94% females and 47.05% males (9 females and 8 males), as illustrated in figure 9. Furthermore, the average age of patients with OLP was 52 years with an overall age range of 29 to 69 years.

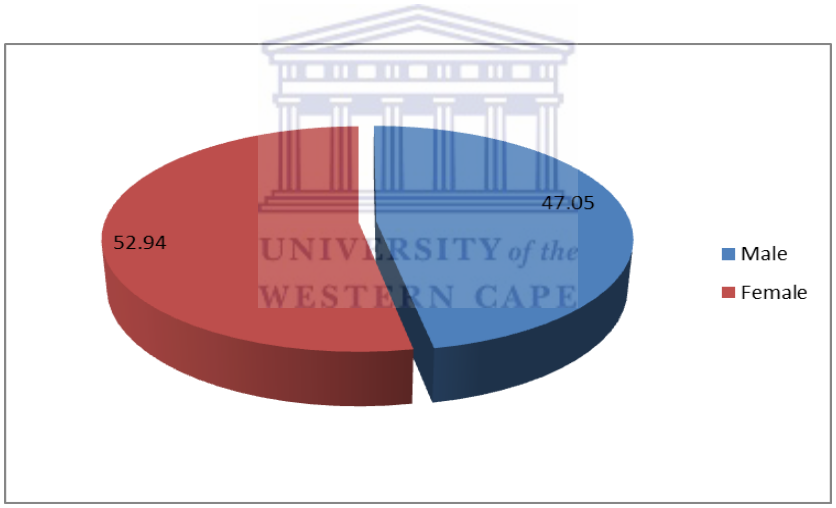

Figure 9: Gender ratio among OLP patients**.**

4.2 HISTOPATHOLOGICAL FEATURES

The histopathological features comprised interface mucositis with vacuolar degeneration of the basal epithelial layer and a sub-epithelial band of lymphocytic inflammatory infiltrate with absence of epithelial dysplasia in all the included cases. Furthermore, epithelial hyperkeratosis with spherical eosinophilic hyaline bodies (apoptotic bodies) was also noticed in the study samples.

4.3 RELATIONSHIP BETWEEN EPITHELIUM THICKNESS OR INFLAMMATORY INFILTRATE AND NUMBER OF APOPTOSIS

Correlation between epithelium thickness and number of apoptosis can be interpreted as a tendency for epithelium thickness to increase or decrease systematically with apoptosis and *vice versa*. There are several regions for every case which makes it possible to examine the relation between the two variables in two ways: viz. by examining the correlation between the two variables within cases and between cases. The statements above also apply to the thickness of inflammatory infiltrate.

4.3.1 RELATIONSHIP BETWEEN EPITHELIUM THICKNESS AND NUMBER OF APOPTOSIS (WITHIN CASE ASSOCIATION)

The association between epithelium thickness and number of apoptosis within cases was assessed separately for each case using the Poisson regression analysis method with the intercept equal to zero. The dependent variable was number of apoptosis and the independent variable was epithelium thickness. The results of the Poisson regression equation for the 17 cases are given in Table 1. The findings indicate that there are no significant differences between the thickness of epithelium and number of apoptosis within the 17 cases.

Figure 10 demonstrates a plot of number of apoptosis versus epithelium thickness for all cases, each case represented by a different symbol. The results expressed in this figure are in agreement with the results of the regression analyses, as no upward or downward trend in epithelium thickness was found with the number of apoptotic bodies.

Case	Epithelial slopes	p-values	Significance
1	0.004	0.291	No
2	-0.005	0.148	No
3	-0.01	0.319	No
4	0.015	0.091	No
5	0.005	0.337	No
6	-0.004	0.559	No
7	-0.003	0.614	No
8	-0.001	0.864	No
9	0.042	0.026	No
10	-0.033	0.368	No
11	-0.005	0.499	No
12	0	1	No
13	-0.008	0.399	No
14	0.001	0.746	No
15	-0.005	0.586	No
16	-0.002	0.231	No
17	0.003	0.352	No

Table 1: Showing the epithelial slopes and the epithelial p-values.

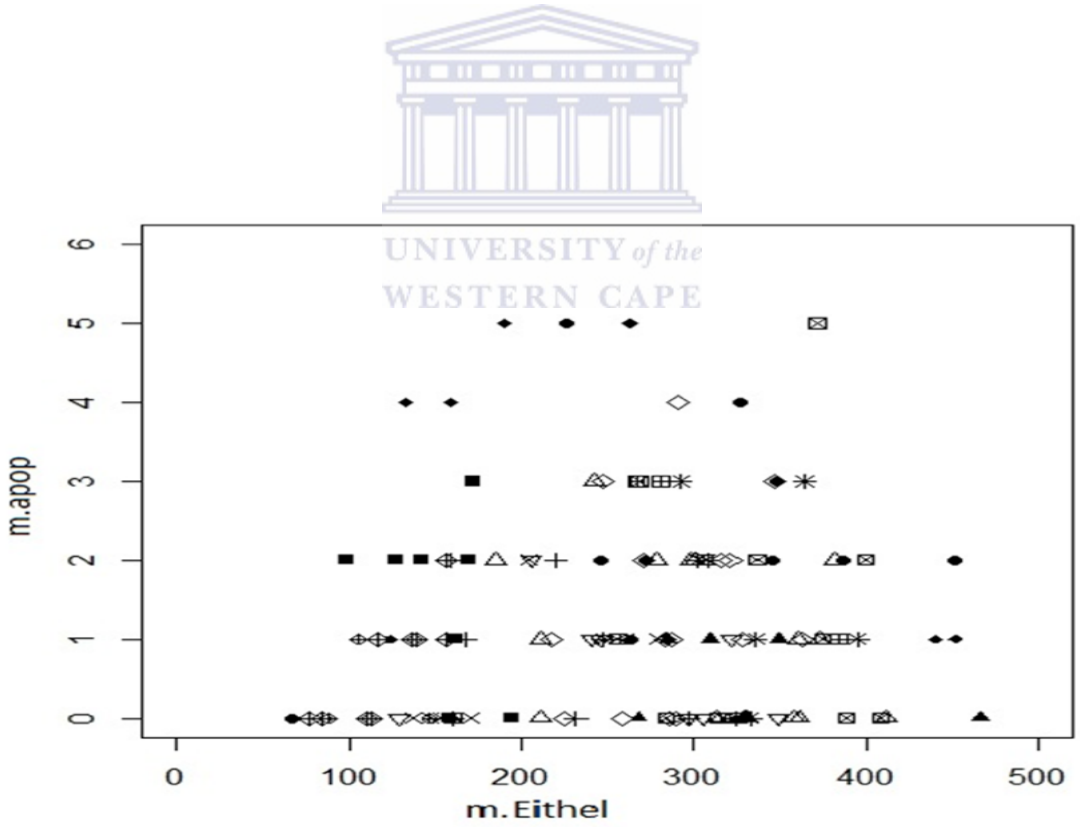

Figure 10: Diagram showing the relationship between epithelium thickness (m. Epithel) and number of apoptosis (m. apop) (The different symbols represent different cases).

4.3.2 RELATIONSHIP BETWEEN INFLAMMATORY INFILTRATE AND NUMBER OF APOPTOTIC BODIES (WITHIN CASE ASSOCIATION)

The correlation between the inflammatory infiltrate thickness and rate of apoptosis for each case was calculated as in section 4.3.1. The columns named inflammatory slopes and inflammatory p-values in Table 2 show results similar to epithelium thickness results for the variable inflammatory infiltrate. No statistically significant results were shown in the 17 cases, none of the p-values were less than 0.05 and the slopes of 10 cases were positive. The conclusion is as for epithelium thickness, namely that there is no within case correlation between the thickness of inflammatory infiltrate and the number of apoptotic bodies (Figure 11).

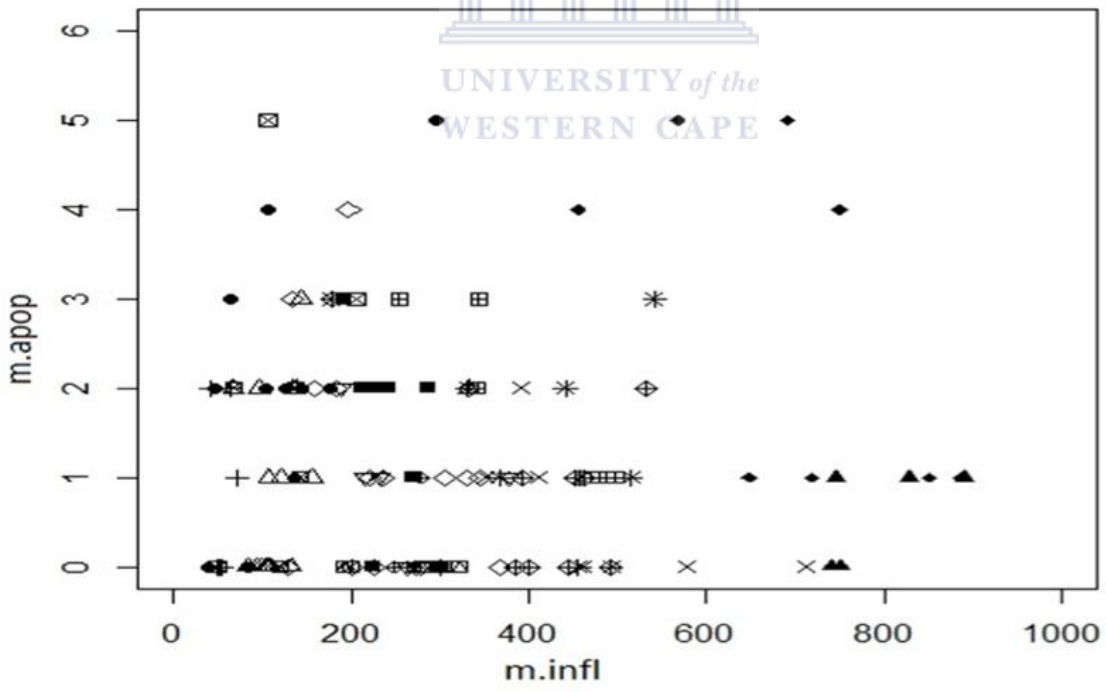

Figure 11: Diagram showing the relationship between thickness of inflammatory infiltrate (m.infl)and number of apoptosis (m. apop).
(The different symbols represent different cases)

Case	Inflammatory infiltrate slopes	Inflammatory infiltrate p-values
1	0.007	0.291
2	0.004	0.211
3	0.012	0.776
4	-0.006	0.091
5	-0.004	0.194
6	-0.009	0.34
7	0.001	0.876
8	-0.001	0.721
9	0.011	0.192
10	0.017	0.742
11	-0.005	0.187
12	0	1
13	-0.011	0.211
14	0.002	0.707
15	0.008	0.392
16	-0.003	0.083
17	0.01	0.022

Table 2: Showing the inflammatory slopes and the inflammatory p-values

4.4 RELATIONSHIP BETWEEN EPITHELIUM THICKNESS AND NUMBER OF APOPTOSIS (BETWEEN CASE ASSOCIATION)

In this study, the mean values of the three variables: epithelial thickness, inflammatory infiltrate and rate of apoptosis calculated for each case are represented in Table 3 (see below).

Figure 12 demonstrates a plot of the mean number of apoptotic counts *versus* the mean epithelial thickness values. It indicates no significant correlation between the two variables. The correlation coefficient (Pearson) is 0.136, non-significant p-value = 0.602. Similarly, Figure 13 represents a plot of the values for the mean number of apoptosis *vs* the values for the mean inflammatory infiltrate. No correlation was found between the two variables; the Pearson correlation coefficient is 0.000; non-significant p-value >0.999.

When the between case correlation coefficients (Pearson coefficient) were calculated for mean epithelial thickness and inflammatory infiltrate, no statistically significant results were obtained, the mean Pearson correlation coefficient was 0.011, p-value= 0.968, not significant. (r = 0.011, p = 0.968) (Figure 14).

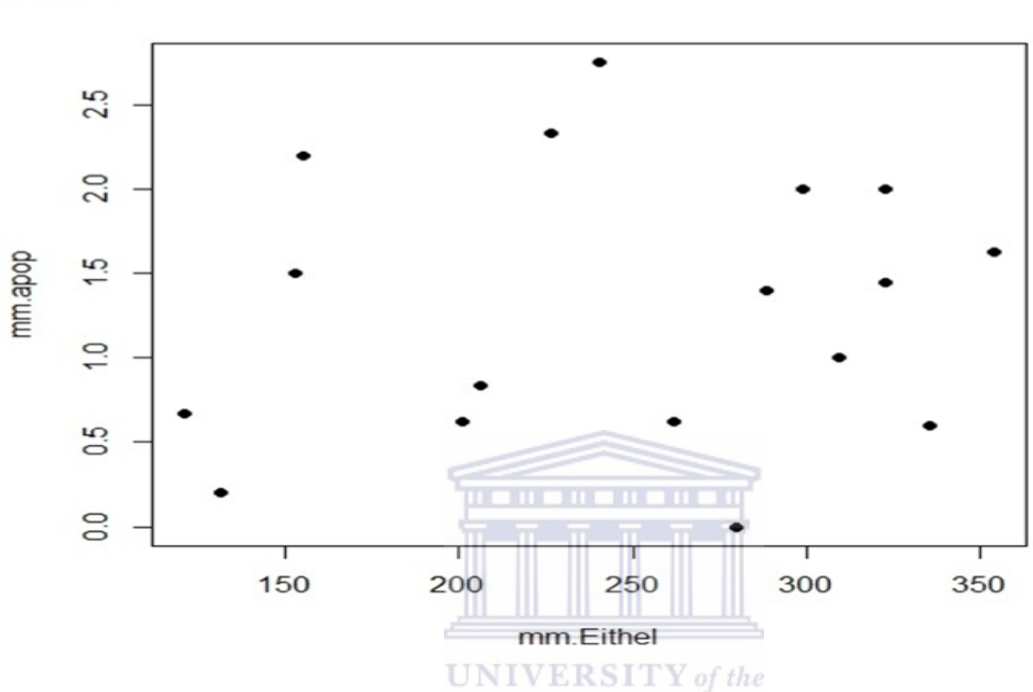

Figure 12: Plot Diagram showing the lack of relationship between the mean epithelium thickness values (mm.Epithel) and the mean apoptotic counts (mm.apop).

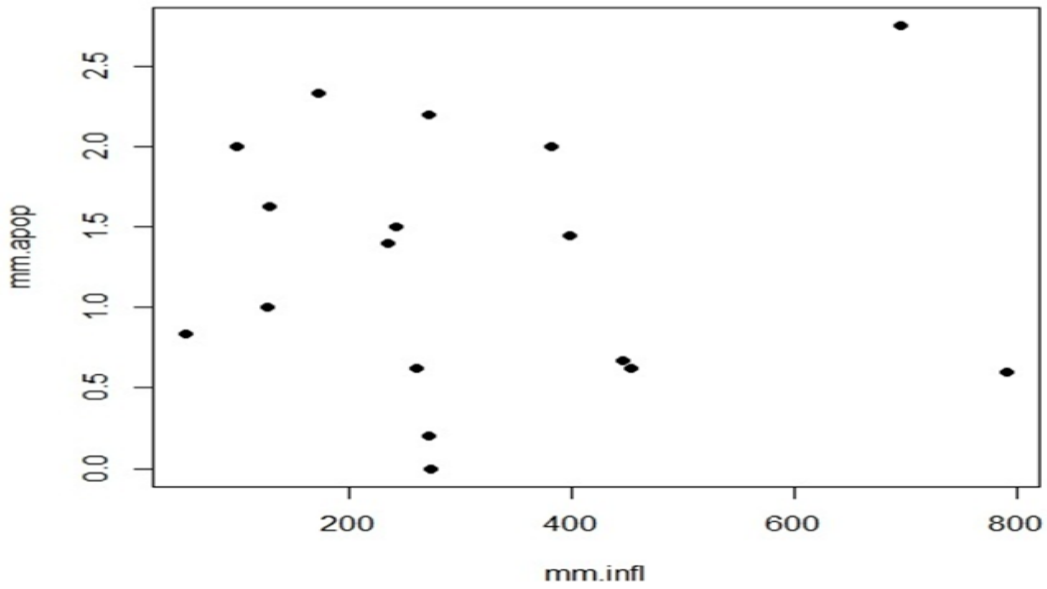

Figure 13: Plot Diagram showing the lack of relationship between the mean of the inflammatory infiltrate values (mm. infl) and the mean number of apoptosis values (mm. apop).

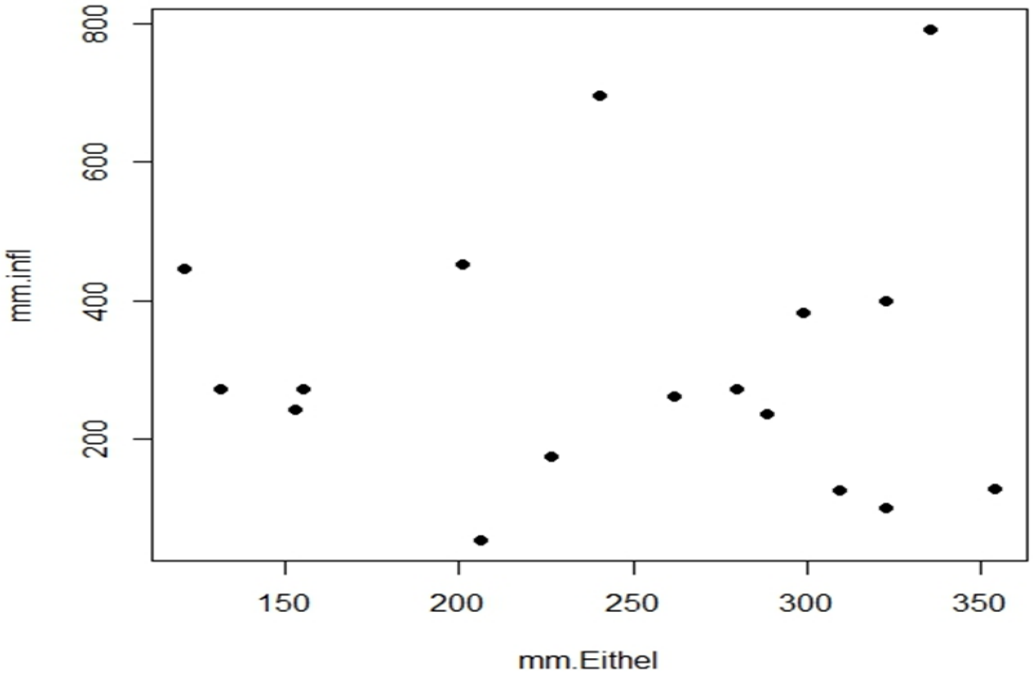

Figure 14: Plot Diagram showing the lack of relationship between the mean epithelium thickness values (mm. Epithel) and the mean of the inflammatory infiltrate values (mm. infl).

Case	mm. Epithelial thickness	mm. inflammatory infiltrate	mm. apoptosis
1	155.32 µm	272.36 µm	2.2
2	309.096 µm	126.679 µm	1
3	205.9 µm	54.667 µm	0.833
4	201.05 µm	453.275 µm	0.625
5	288.707 µm	236.053 µm	1.4
6	261.85 µm	261.675 µm	0.625
7	354 µm	128.6 µm	1.625
8	322.644 µm	398.667 µm	1.444
9	121.15 µm	445.872 µm	0.667
10	131.44 µm	272.72 µm	0.2
11	298.96 µm	383.08 µm	2
12	279.8 µm	273.25 µm	0
13	153 µm	243.6 µm	1.5
14	322.6 µm	99.688 µm	2
15	335.56 µm	791.44 µm	0.6
16	240.575µm	696.463 µm	2.75
17	226.2 µm	174.133 µm	2.333

Table 3: Represents the mean values of the three variables.

CHAPTER 5

5.1 DISCUSSION

OLP is a chronic inflammatory disease mainly affecting the mucosal membranes of the oral cavity and the skin. It may also affect skin appendages (for example, nails) and other mucosal membranes (for example, the genitalia and oesophagus) (Le Cleach and Chosidow, 2012). Since the pathogenesis of OLP has been poorly understood and it is unclear exactly which molecular mechanisms play key roles in the underlying clinical manifestations and presentations, the present study aimed to investigated the possible role of apoptosis in OLP.

In the current study, an immense effort was invested in the selection of suitable cases. The majority of the cases registered in the department of Oral Medicine and Periodontics were lichenoid type reactions, rather than OLP and were therefore excluded from the study. For the diagnosis of OLP cases, clinico-pathological correlation was performed using the modified WHO criteria (Van der Meij and Van der Waal, 2003). Furthermore, stringent selection criteria were applied to ensure that patients included in the study did not suffer from any other diseases or used medications, or their lesions had non-typical histopathological features. This exercise resulted in relatively small study sample (17 cases), however this was compensated by histologically measuring numerous small adjacent fields of 250 μm wide.

In the present study, the incidence of OLP was found at the mean age of 52 years. Additionally, the prevalence of OLP was observed to be slightly higher in females when compared to males. These observations are in agreement with previous published reports which revealed that OLP is more frequent in females than in males with a higher incidence in older patients (Ingafou *et al.*, 2006; Pakfetrat *et al.*, 2009).

Microscopically, the study observed three histopathological criteria of OLP in all the study samples, namely: vacuolar degeneration of the basal epithelial layer; a sub-epithelial

band of lymphocytic inflammatory infiltrate consisting mainly of T-lymphocytes with absence of epithelial dysplasia; and epithelial hyperkeratosis. These findings are also in accordance with other studies (Van der Meij and Van der Waal, 2003; Batra *et al*., 2008; Hall *et al.,* 2008). In a study conducted by Juliana *et al* (2008), the presence of spherical eosinophilic hyaline bodies (apoptotic bodies) was reported in the basal and parabasal epithelial layers. It has been reported that such structures occur due to the programmed destruction of basal keratinocytes by apoptosis. The present study expressed similar findings thereby providing further evidence for the apoptosis-mediated basal keratinocytes cell death.

In the present study, considerable amounts of apoptotic cells were noticed in the basal and intermediate layers despite the fact that the identification and counting of apoptotic cells was a difficult and tedious task, often due to the presence of the highly intense interface mucositis. Furthermore, measuring the thickness of the epithelium proved difficult due to the degeneration of cells and variable density of lymphocytes in side-to-side assessment of lymphocyte infiltration. Further, the restricted occurrence of apoptosis in the basal / parabasal layers was only explained by the fact that these layers are the most prone to damage by the associated sub-epithelial lymphocytic infiltrate. Results regarding the presence and role of apoptosis in OLP cases are highly debatable. Several studies including our study supported the impact of apoptosis in oral lichen planus progression (Brant *et al.,* 2008 & 2012; Doddawad, 2014). Studies conducted by Brant *et al.,* had detected apoptotic cells using sensitive immunohistochemistry assays like TUNEL and M30 CytoDeath. Further, the authors reported an overall increase in apoptosis in both erosive and reticular subtypes as well as in both epithelia and inflammatory infiltrate in comparison to normal mucosa, although the intensity was higher in epithelia (Brant *et. al.,* 2008 & 2012). Similar results were revealed in another study where apoptosis within the epithelium was significantly increased in OLP, compared to normal oral mucosa, and seems to be related to the decreased thickness of the

oral epithelium (Neppelberg *et al.*, 2001). Although the present study was not able to conduct the immunohistochemical analysis for the apoptotic index due to facility and antibody-related constraints, the study by Neppelberg *et al* (2001) strongly supports the findings of the present study. In addition, similar results were also reported in another recent study where an increased apoptotic count was reported in 40 OLP sections stained through H & E staining (Doddawad, 2014). Moreover, the current research findings regarding the presence of high numbers of apoptotic bodies in the epithelium was in corroboration with another study where higher numbers of apoptotic cells were reported in hematoxylin and eosin stained sections than in situ end labelling (ISEL), in OLP and normal buccal epithelium (Bloor *et. al.,* 1999). On the contrary, a few authors observed no significant impact of apoptosis in the pathogenesis of OLP (Karatsaides *et al.,* 2003; Bascones *et al.,* 2007). They believe that the cells which are seen as shrunken and condensed cells in the epithelium are intra-epithelial lymphocytes but not apoptotic bodies. In addition, the deviations may also be attributed to the use of samples at different disease stages as the presence of intense and ulcerated lesions during the advanced disease stages may not be ideal to accurately identify and quantify programmed cell death.

Although apoptosis has been implicated in OLP pathogenesis, its association with other clinical and pathological features has not been extensively explored. The present study aimed to determine the existence of any correlation between the apoptotic index and epithelial or inflammatory infiltrate thickness but found no significant association. These outcomes are not in corroboration with the majority of the studies which reported a negative correlation between increased apoptotic rates and decreased thickness of the oral epithelium in OLP (Neppelberg *et al.,* 2001; Brant *et al.,* 2012; Doddawad, 2014). However, a study conducted by Karatsaides and his co-workers is consistent with our study, as they were not able to observe any reduction in epithelial thickness with increased apoptosis using the

TUNEL marker. Their study proposed that the decrease in the oral epithelial thickness could be due to abnormal or premature terminal differentiation of the keratinocytes (Karatsaides *et. al.*, 2003).

Similarly, no correlation was observed between the apoptotic index and the thickness of the subepithelial inflammatory infiltrate, although a trend for a positive correlation was noticed (the apoptotic rate increased with increased thickness of the inflammatory infiltrate). Multiple studies revealed a positive correlation between the number of apoptotic bodies and the thickness of the inflammatory infiltrate (Bloor *et al.*, 1999; Doddawad, 2014). The presence of the band-like lymphocyte infiltrate in the superficial part of the lamina propria has long been implicated as one of the main histopathological features of OLP.

The present study also assessed the association between the thickness of the sub-epithelial chronic inflammatory infiltrate and the squamous epithelial thickness. The evidence regarding the relationship between the two variables is highly sparse. One of the studies undertaken by Usha and co-workers (2012) determined the histopathological differentiation between OLP and lichenoid type reaction by using micrometry to gauge if the sub-epithelial chronic inflammatory infiltrate had any impact on the thickness of overlying epithelium. Their study included 30 cases of OLP and 10 cases of lichenoid reactions, studied by using an eye-piece graticule. The measurements of epithelial thickness and sub-epithelial inflammatory band thickness were taken in these lesions. Their results showed a negative correlation between epithelial thickness and band of inflammatory infiltrate in lichen planus cases; and a positive relationship in lichenoid lesions (Usha *et al.,* 2012). However, the results of a recent study conducted by Ramya *et al., (*2014) were in disagreement with the former study. While evaluating the correlation between epithelium and inflammatory cell infiltrate thickness measured by using image analysis, the authors reported a direct influence of inflammatory cell infiltrate and epithelium thickness. They stated that the thickness of the

overlying epithelium decreases as the thickness of inflammatory infiltrate increases (Ramya *et al.,* 2014). However, these study outcomes were not reproduced in the present study as no significant correlation was found between the two variables (Usha *et al*., 2012; Ramya *et al.*, 2014). This may possibly be due to the differences in the methodological approach. While the previous reports used highly sensitive immunohistochemical apoptotic markers like TUNEL, CASPASE 3, M30 CYTODEATH where it is easy to detect more apoptotic bodies, the present study detected the same using H&E staining. Other possible reasons for the non-accordance of the present study outcomes with previous reports were the study in a different population group (Western Cape, South Africa) and the small sample size meeting the stringent diagnostic criteria for true OLP.

5.2 LIMITATIONS

- Very few studies have been conducted on the relationship between apoptosis, epithelial thickness and corresponding inflammatory infiltrate in OLP. This limits the scope for comparison of our research findings with published results and therefore no firm conclusions can be derived from this study.
- The storage conditions of the wax blocks might have affected the identification and detection rate of apoptotic bodies.
- Detection of the apoptotic bodies was performed by using H&E staining instead of using immunohistochemical markers; hence limiting the ability to identify and count apoptotic bodies.

5.3 CONCLUSION

Based on the present study outcomes, it is suggested that the apoptotic rate might not have a significant influence on the thickness of overlying epithelium and does not correlate with the thickness of the corresponding inflammatory infiltrate in OLP. Also, the inflammatory infiltrate thickness does not impact on the thickness of the overlying epithelium and further immunological and molecular studies are required for stronger evidence of non-correlation of apoptotic activity with the key histological parameters of OLP.

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APPENDIX – Table showing raw data

REGIONS	CASE	M.EITHE1 (μm)	M.INFL(μm)	M.APOP
1	1	108	240.8	2
2	1	96	233.6	3
3	1	85.8	226.2	0
4	1	245.8	337	3
5	1	241	324.2	3
1	2	330.6	83.8	0
2	2	211.2	107	0
3	2	381.4	67.4	2
4	2	411.33	132.6667	0
5	2	313.6	99.4	0
6	2	242.6	144	3
7	2	357.8	108.8	0
8	2	300.2	331.6	2
9	2	278.6	137.6	2
10	2	360.6	155.2	1
11	2	211.4	121.4	1
12	2	185.4	97	2
13	2	329	104.4	0
14	2	372.6	107.4	1
15	2	361.2	94.6	0
16	2	298	134.6	2
1	3	168	72.4	1
2	3	231.6	50.6	0
3	3	159	63.2	2
4	3	159.8	48	0
5	3	219.8	40.6	2
6	3	297.2	53.2	0
1	4	260.6	350.8	1
2	4	279.2	227.2	1
3	4	256	411.6	1
4	4	136.8	461	0
5	4	153.4	493	0
6	4	171	713.2	0
7	4	146.6	578.6	0
8	4	204.8	390.8	2
1	5	290.6	197	4
2	5	247.4	134	3
3	5	258	128.4	0
4	5	271.2	182.6	2
5	5	346.4	177.6	3
REGIONS	**CASE**	**M.EITHE1(μm)**	**M.INFL(μm)**	**M.APOP**
6	5	363.2	234.8	1

7	5	316.2	159.4	2
8	5	286.4	200.4	0
9	5	287	304.8	1
10	5	283.4	221.4	1
11	5	217.4	344.8	1
12	5	224.4	368	0
13	5	321.2	332.6	2
14	5	328.2	330	1
15	5	289.6	225	0
1	6	348.8	262.4	0
2	6	305.2	278.6	0
3	6	240.6	232.2	1
4	6	246.4	216	1
5	6	297.2	263.6	0
6	6	129.4	274	0
7	6	205.6	188.6	2
8	6	321.6	378	1
1	7	388.6	49	0
2	7	336.8	67.8	2
3	7	371.6	106.4	5
4	7	408.2	120.4	0
5	7	268.4	205.6	3
6	7	284	199.6	0
7	7	374.6	143.2	1
8	7	399.8	136.8	2
1	8	308	441.2	2
2	8	325	300.6	0
3	8	302.4	331.2	2
4	8	336	367.6	1
5	8	332.8	455.8	0
6	8	395.2	515	1
7	8	292.4	541	3
8	8	247.8	457.8	1
9	8	364.2	177.8	3
1	9	83.75	492.25	0
2	9	117.2	462.8	1
3	9	156.6	452.8	1
4	9	137	451.2	1
5	9	112.8	400.4	0
6	9	110.2	444.6	0
7	9	77.4	384.4	0
REGIONS	**CASE**	**M.EPITHE1(μm)**	**M.INFL(μm)**	**M.APOP**
8	9	156.6	531.8	2
9	9	138.8	392.6	1

1	10	158.2	304.2	0
2	10	158.8	262.6	0
3	10	147	269.8	0
4	10	105	279	1
5	10	88.2	248	0
1	11	257	497.2	1
2	11	384.2	479	1
3	11	280.8	253.6	3
4	11	306.2	341.8	2
5	11	266.6	343.8	3
1	12	319.8	299.2	0
2	12	325.6	322.2	0
3	12	160.2	279.6	0
4	12	313.6	192	0
1	13	194.6	225	0
2	13	158.4	298	0
3	13	142.2	287	2
4	13	169.2	212.8	2
5	13	161.4	269.6	1
6	13	98.6	241	2
7	13	127.2	223.8	2
8	13	172.4	191.6	3
1	14	326.4	107.2	4
2	14	451.6	47.2	2
3	14	264	135	1
4	14	328.4	38.5	0
5	14	347.8	64	3
6	14	345.6	104.2	2
7	14	271.6	126	2
8	14	245.4	175.4	2
1	15	349.4	828.6	1
2	15	285	889.8	1
3	15	309.4	746.4	1
4	15	267.8	740.6	0
5	15	466.2	751.8	0
1	16	124.6	648.8	1
2	16	159.4	456.2	4
3	16	190.4	691.4	5
4	16	133.4	751	4
5	16	160.8	851.4	1
6	16	263.4	568.5	5
7	16	452	718.8	1
8	16	440.6	885.6	1
1	17	66.4	84	0
REGIONS	**CASE**	**M.EPITHE1(μm)**	**M.INFL (μm)**	**M.APOP**
2	17	386.2	143.4	2
3	17	226	295	5