EBV STATUS IN EXTRA-ORAL PLASMABLASTIC LYMPHOMAS

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DECLARATION

I, Yvonne Perner declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

Signature of candidate

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Date: 05 February 2016

DEDICATION

I dedicate this work to my father, Hans-Klaus Perner (01.01.1933 – 16.12.2004) and to my teachers.

"To hold my teacher in this art equal to my own parents; to consider his family as my brothers; to impart precept, oral instruction, and all other instruction to my own sons, the sons of my teacher, and to pupils who have taken the Physician's oath".

~The Oath of Hippocrates~

PRESENTATIONS/PUBLICATIONS ARISING FROM THIS STUDY

- Perner Y, Naidoo S, Meer S, du Toit H, Suleman MC & Willem P. EBV status in extra-oral plasmablastic lymphoma. *Histopathology*, 2012; 61(s1):123. (Abstract).

ABSTRACT

Introduction: Plasmablastic lymphoma (PBL) is an uncommon variant of aggressive B-cell non-Hodgkin lymphoma that occurs in immune-compromised individuals, most commonly secondary to HIV infection. This tumour classically occurs in the oral cavity but has also been described in a variety of extra-oral locations. This is a clinicopathological study of 45 cases of extra-oral PBL (EPBL).

Aim: To define the clinical parameters, histology and immunophenotypic features of extra-oral plasmablastic lymphoma (EPBL) and assess the extent to which Epstein–Barr virus (EBV) is associated with this tumour.

Materials and Methods: This retrospective study on archival cases of EPBL included the patients' age, gender, race, HIV status where available and the site of tumour presentation. Of 49 archival cases retrieved, 4 were discounted owing to reclassification as diffuse large B-cell lymphoma (1 case), multiple myeloma or extramedullary plasma cell tumour (3 cases). The remaining 45 cases were reviewed histologically and classified according to whether they displayed a pure plasmablastic (PBm) morphology or a plasmablastic morphology with plasmacytic differentiation (PBm+PCd), and assessed immunohistochemically with CD45 (LCA), CD20, CD79a, PAX5, CD138, MUM1, BLIMP1, VS38c, Ki-67, BCL6, CD10, HHV8 and CyclinD1 using standard automated procedures. The presence of EBV was assessed by chromogenic in-situ hybridisation. Ethical clearance was obtained (M10750 and M120993).

Results: 27of the 45 cases had a pure plasmablastic morphology. The remaining 18 cases showed plasmacytic differentiation. There was no site predilection according to histological pattern. 60% of the tumours were reported in males and 40% in females and all were black African patients. The anus was the favoured

extra-oral site of presentation (13 of 45 cases, 28%), followed by soft tissue (11 of 45 cases, 24%). There was no significant difference in the age of presentation between males (38.5 years) and females (35.4 years). Of the 18 patients of known HIV status, 17 were HIV positive (94%). The immunohistochemical profile of EPBL recapitulated that found for both oral and extra-oral PBL in the literature, except for CD45 (leucocyte common antigen) which signalled positively in a higher percentage of cases. 36 of 42 cases (85.7%) were positive for CD45. The positive membrane signal for CD45 was of variable intensity, between 5 and 100% of tumour cells. EBV was positive by in situ hybridisation in 37 of 40 cases tested (92.5%).

Conclusion: EPBL is identical to its oral counterpart in gender and age distribution, HIV status, morphological appearances, immunophenotypic profile and association with EBV. The high association with EBV as assessed by in-situ hybridisation studies mirrors that of oral-based PBL reported in the literature. EPBL should be regarded as the same tumour as that arising within the oral cavity. A peculiarity observed within this case cohort is the high level of expression of CD45 (leucocyte common antigen). This has been reported to be of low or near absent expression in most cases of PBL, as defined by the 2008 WHO Classification of Tumours of the Haematopoietic and Lymphoid Tissues.

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Finally, I thank my family for their support, which has allowed me to bring this study to completion.

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ABBREVIATIONS

ABC……...Activation status B-cell

AIDS……..Acquired immune deficiency syndrome

ALK……Anaplastic lymphoma kinase

ALK LBL……..Anaplastic lymphoma kinase positive large B-cell lymphoma

BL……..Burkitt lymphoma

BLIMP1…….B-lymphocyte-induced maturation protein 1

CALLA……..Common acute lymphoblastic leukaemia antigen

CD……..Cluster of differentiation

cHL……Classic Hodgkin lymphoma

CLL……Chronic lymphocytic leukaemia

DLBL…….Diffuse large B-cell lymphoma

DNA……...Deoxyribonucleic acid

EBER……Epstein–Barr virus encoded small ribonucleic acid

EBER mRNA……...Epstein–Barr virus encoded messenger ribonucleic acid

EBNA……Epstein–Barr virus nuclear antigen

EBV……..Epstein–Barr virus

EPBL……Extra-oral plasmablastic lymphoma

EPCT……Extramedullary plasma cell tumour

EPCT/PCM……..Extramedullary plasma cell tumour/plasma cell myeloma

FFPET…….Formalin-fixed paraffin-embedded tissue

FISH…….Fluorescence in-situ hybridisation

GCB…….Germinal centre B-cell

HAART……Highly active antiretroviral treatment

H&E……..Haematoxylin and eosin

HIV……...Human immunodeficiency virus

HHV8……Human herpes virus 8

IBL…….Immunoblastic lymphoma

IgHC……..Immunoglobulin heavy chain

IgH_V.....Immunoglobulin variable heavy chain

IHC……Immunohistochemistry

IRF4……..Interferon Regulatory Factor4

ISH…….In-situ hybridisation

LANA…….Latency associated nuclear antigen

LC……..Light chain

LCA……Leucocyte common antigen

LCR……...Light chain restriction

LMP……...Latent membrane protein

MCD……..Multicentric Castleman disease

MM……Multiple myeloma

MUM1……Multiple myeloma oncogene-1

NA.....Not assessed

ND.....Not done

NFκB…….Nuclear factor kappa B

NHL……Non-Hodgkin lymphoma

NHLS…….National Health Laboratory Service

OS……..Overall survival

PBL……Plasmablastic lymphoma

PBm……Plasmablastic morphology

PBm+PCd……Plasmablastic morphology with plasmacytic differentiation

PBS……Phosphate buffered saline

PCM……Plasma cell myeloma

PCNSL……Primary central nervous system lymphoma

PCR……Polymerase chain reaction

PEL……..Primary effusion lymphoma

PMBL……..Primary mediastinal B-cell lymphoma

PRDM1……Positive regulatory domain zinc finger protein1

PTCL……Peripheral T-cell lymphoma

PTLD..……Post-transplant lymphoproliferative disorder

RNA……..Ribonucleic acid

SNP……..Single nucleotide polymorphism

TMA…….....Tissue microarray

XBP1……X-box binding protein1

CHAPTER 1

Introduction

Plasmablastic lymphoma (PBL) was first described in 1997 as an aggressive subtype of diffuse large B-cell lymphoma (DLBL) presenting in the oral cavity of immunocompromised individuals, usually in the setting of human immunodeficiency virus (HIV) infection (Delecluse et al 1997). It has since been documented to occur in a variety of extra-oral, often mucosal, sites including the skin and anorectal region, spermatic cord, gastrointestinal tract, lung, nasopharynx and paranasal sinuses, salivary glands, heart, central nervous system, retroperitoneum and soft tissue (Chetty et al 2003; Rafaniello-Raviele et al 2009; Takahashi et al 2009; Bishop & Westra 2010; Urrego et al 2011). It has also been reported to occur in immune competent individuals (Kim et al 2009; Qing et al 2011). This aggressive B-cell lymphoma is characterised phenotypically by plasmablastic features, an immunophenotypic profile which approximates that of a plasma cell and an association with Epstein–Barr virus (EBV) and *MYC* gene dysregulation (Taddesse-Heath et al 2010).

The relationship between PBL, plasmablastic extramedullary plasmacytoma (EPCT)/multiple myeloma (MM) and DLBL showing terminal plasma cell differentiation remains an area of contention and diagnostic uncertainty. This is particularly relevant in immunosuppressed states related to acquired immune deficiency, notably infection with HIV and organ transplant recipients on long-term immunosuppressive therapy, where this differential diagnosis is not infrequent.

The distinction between PBL and DLBL with terminal plasmacytic differentiation on the one hand, and EPCT/PCM is of significance as the diseases have a different clinical course and prognosis, with different management requirements. There have been several reports in the literature (Colomo et al 2004; Kremer et al 2005; Vega et al 2005; Lorsbach et al 2011) highlighting their similarities in clinical presentation, morphological appearances, immunophenotype, gene expression profile and EBV status. The presence of EBV-encoded RNA was formerly considered of particular significance in favouring the diagnosis of PBL. It has since been shown by several authors (Colomo et al 2004; Kremer et al 2005; Vega et al 2005; Chang et al 2007; Yan et al 2011) that both immune competent and immune compromised individuals may develop EBV-driven EPCT/PCM, although the latter is much more common.

In this regard investigating the role of EBV in extra-oral PBLs would facilitate our ability to aid in the diagnostic distinction between PBL and EPCT/PCM.

CHAPTER 2

Aims and objectives

The aim of this study was to determine the demographics, morphology, immunohistochemical profile and EBV status of patients with extra-oral PBL in a clinical setting with a high prevalence of HIV infection.

The objectives were to determine:

1. the age, gender, race, extra-oral site and frequency of site distribution;
2. the frequency of HIV infection;
3. the morphology;
4. the EBV status;
5. the immunohistochemical profile

CHAPTER 3

3.0 Literature review

3.1 Definition of EPBL

PBL is defined as an aggressive, high-grade B-cell non-Hodgkin lymphoma (NHL) occurring classically within the oral cavity of immune compromised individuals, most notably in the setting of acquired immune deficiency syndrome (AIDS) (Stein et al 2008; Gogia et al 2010). It also occurs, albeit less commonly, in a variety of extra-oral sites. The tumour is characterised by terminal B-cell or plasma cell differentiation and the acquisition of a variety of plasma cell markers, with the loss of conventional B-cell markers (Carbone & Gloghini 2008; Hsi et al 2011).

3.2 History and present status of extra-oral PBL

Following the original description of oral-cavity-based PBL in immunocompromised individuals by Delecluse et al (1997), the tumour was discovered to occur in a variety of extraoral sites. While it shows a predilection for the oral cavity, it is also readily described to occur in other mucocutaneous sites, particularly within the anorectal region. Additional sites reported to date include the skin, spermatic cord, gastrointestinal tract, lung, nasopharynx and paranasal sinuses, salivary glands, heart, retroperitoneum, central nervous system and soft tissues (Chetty et al 2003; Rafaniello-Raviele et al 2009; Takahashi et al 2009; Bishop & Westra 2010;Urrego et al 2011).

There is a considerable body of literature pertaining to oral PBL. Less has been reported on the extra-oral variety of this tumour. The question arises as to

whether these should be regarded as identical tumours occurring in different sites, or as two different tumours. In 2010, Hansra et al reported similarities and differences in clinicopathological characteristics of oral and extra-oral PBL. Oral PBL was associated with HIV infection and a plasmablastic morphology without plasmacytic differentiation. Extra-oral PBL showed a tendency to occur in patients with non-HIV-related immune suppression and universally demonstrated plasmacytic differentiation.

The pathogenesis of this tumour is not fully understood. It is believed to derive from post-germinal centre, terminally differentiated and activated B-cells, probably in transition from an immunoblast to a plasma cell. As described by Castillo and Reagan (2011), the majority of PBL have undergone somatic hypermutation and class switching. A subset of these lymphomas are devoid of heavy chain (IgV_H) mutations and appear to originate from naïve B-cells that have undergone pre-terminal differentiation independent of transit through the germinal centre (Gaidano et al 2002). They have sustained chromosomal aberrations associated with the development of malignancy. Rearrangements involving *MYC* and the immunoglobulin gene have been described in several studies (Dawson et al 2007; Bogusz et al 2009) and a strong association is described with EBV infection.

3.3 HIV association

In the non-HIV infected population, Burkitt lymphoma (BL) and immunoblastic lymphoma (IBL) comprised approximately 10% of all lymphoma cases in the United States and Europe (Carbone 2002). In the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (2008), immunoblastic lymphoma is regarded as a morphological variant of DLBL. It is defined as a large B-cell lymphoma in which >90% of tumour cells have an immunoblastic morphology, with a prominent central nucleolus. In the pre-HAART era, BL accounted for approximately 40% of NHL in HIV positive individuals, with the remainder accounted for by DLBL, half of which showed an immunoblastic morphology (Carbone 2002).

There is a strong alliance between HIV infection and PBL. Other forms of immune suppression are reported to be associated with PBL, albeit much less frequently, such as PBL occurring in iatrogenic immunosuppression in the context of organ transplantation and in the elderly in the setting of immune senescence (Carbone & Gloghini 2008; Kim et al 2009; Castillo & Reagan 2011). PBL accounts for 2.6% of all NHLs in HIV-positive individuals (Folk et al 2006; Nasta et al 2002). HIV infection and AIDS are associated with an increased risk for the development of lymphoproliferative disorders, and BL, DLBL, including primary central nervous system lymphoma (PCNSL) , primary effusion lymphoma and plasmablastic lymphoma are seen much more commonly in AIDS than other lymphoma subtypes (Raphael et al 2008; Castillo et al 2008).

PBL was originally described to arise within the oral cavity of HIV-positive individuals (Delecluse et al 1997), but since the original description, extra-oral sites have been well documented and it would appear that collectively, these occur with a similar frequency to oral tumours. Data regarding the overall frequency of intra- and extra-oral sites of PBL in immune compromised individuals is sparsely reported in the literature. There are individual reports for which the frequency of oral and extra-oral locations is listed, such as that by Dong et al (2005), where 6 of 13 cases involved the oral cavity followed by bone and soft tissue (4 of 13) and then the gastrointestinal tract (3 of 13). Castillo et al (2010) identified 228 cases of PBL from the literature, 157 of which were in HIV-positive individuals and 71 in HIV-negative. The HIV-positive patients tended to be younger, were more frequently men, often presented with oral-based disease and expressed CD20, CD56 and EBV-encoded mRNA more than HIV-negative individuals.

Tumours with plasmablastic differentiation are reported to occur typically in individuals with abnormal immunity (Carbone & Gloghini 2008). Table 3.1 adapted from an article by these authors indicates the tumours involved.

Table 3.1. Lymphomas specifically occurring in HIV+ patients and showing a phenotype related to plasma cells (Adapted from Carbone & Gloghini 2008).

BL plasmacytoid
Systemic IBL plasmacytoid
PCNSL (IBL plasmacytoid)
PEL and its solid variant
PBL of the oral cavity and extra-oral sites
MCD-associated PBL

BL – Burkitt lymphoma; IBL – immunoblastic lymphoma; PCNSL – primary central nervous system lymphoma; PEL – primary effusion lymphoma; PBL – plasmablastic lymphoma; MCD – multicentric Castleman disease

3.4 EBV status

EBV encodes several proteins that exhibit homology to a variety of anti-apoptotic molecules, cytokines and signal transducers, which promote EBV infection, immortalisation and transformation (Klapproth & Wirth 2010). Three types of latent gene expression are described based on the patterns of expression of the EBV genome: latency I, II and III (Carbone; Gloghini & Dotti 2008):

Latency I: Epstein–Barr virus nuclear antigen 1 (*EBNA1*) and the two small non-coding EBV RNAs (*EBERs*) are expressed. This is the latency pattern associated with EBV-associated BL.

Latency II: There is EBV gene expression of *EBNA1*, the *EBERs* and latent membrane proteins (LMP) 1, 2A and 2B. Latency II is associated with classic Hodgkin lymphoma (cHL) and certain types of peripheral T-cell lymphoma.

Latency III: Expression of all six EBNAs (EBNA1, 2, 3A, 3B, 3C, and LP), EBERs and 3 LMPs (LMP1, 2A, 2B). This occurs mainly in infectious mononucleosis, immunocompromised individuals with post-transplant lymphoproliferative disorders (PTLPD) and HIV-associated lymphoproliferative disorders.

EBV infection occurs in 40% of AIDS-associated DLBL and in 90% of AIDS-associated IBLs (Carbone, 2002). HIV-associated PBL is frequently associated with latent EBV infection. This was originally described in the seminal article by Delecluse (1997) where nine of 15 cases showed positivity of EBER and five of the nine cases demonstrated expression of latent membrane protein (LMP-1) implying a latency 2 pattern in distinction to that reported by others (Gaidano et al 2002; Colomo et al 2004; Dong et al 2005). The WHO Classification (Stein et al 2008) cites EBER ISH as positive in 60–75% of cases. A high association (86% of 157 cases) between PBL and EBV was also reported by Castillo et al, (2010) and in most cases, this has been seen with a type-I latency pattern (Gaidano et al 2002; Colomo et al 2004; Dong et al 2005).

EBV is generally believed to be uncommonly associated with plasma cell myeloma even in the context of immune dysregulation. Chang et al (2007) showed an association between plasmablastic cytomorphology and EBV by EBV mRNA in-situ hybridisation (ISH) in immune competent individuals, but in very small case numbers. In that paper, 1 of 4 extramedullary plasma cell tumours (EPCTs) and 3 of 14 plasma cell myelomas (PCMs) displaying plasmablastic morphology were shown to be EBV associated. EBV has been detected in 15% of cases of EPCT of

the head and neck in immune competent patients, highlighting that EBV status alone is insufficient to distinguish between PBL and other plasma cell neoplasms (Colomo et al 2004). EBER positivity has also been described in HIV-negative plasmablastic post-transplant lymphoproliferative disorders (Borenstein et al 2007) and has been rarely reported in post-transplant plasmacytomas, although the latency pattern of EBV in these tumours has not yet been characterised (Weh et al 1995).

Hsi et al (2011) proposed a diagnostic algorithm of B-cell neoplasms displaying plasmablastic differentiation or immunophenotype (Fig.3.1). The first decision-making step of this algorithm is to ascertain the presence or absence of EBV infection. This pivotal decision makes no allowance for plasma cell neoplasms (either plasmacytoma or PCM) associated with EBV infection and therefore fails to address the specific diagnostic challenge of distinguishing PBL from EPCT/PCM.

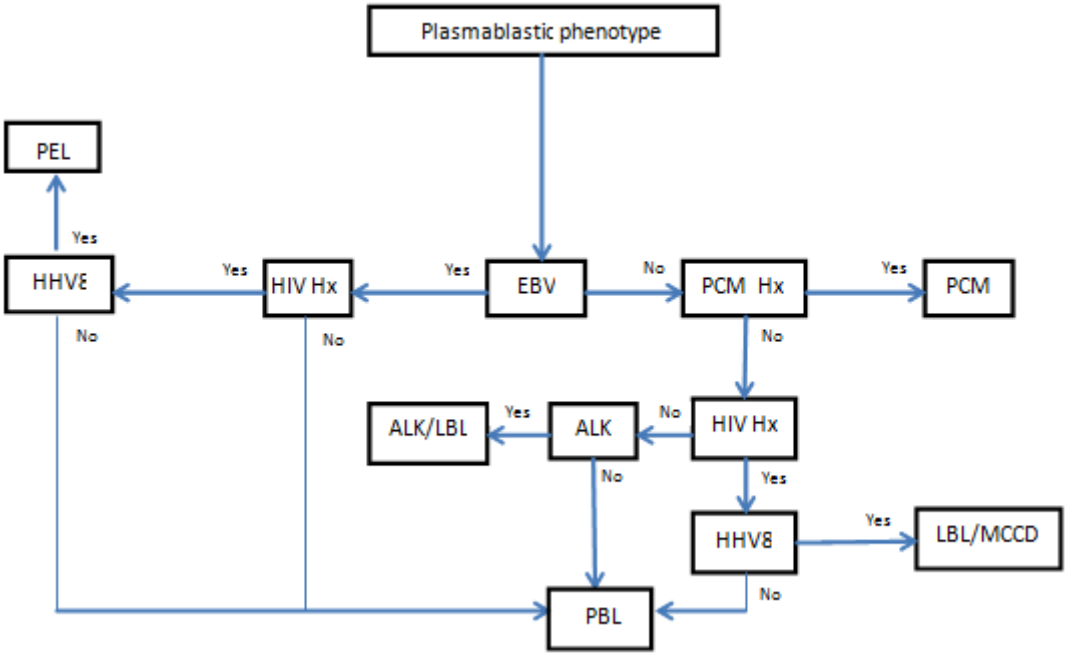

Fig.3.1 Algorithmic approach for the diagnosis of B-lineage malignant lymphomas with plasmablastic differentiation or immunophenotype (Hsi et al, 2011)

ALK – anaplastic large cell lymphoma kinase; ALK/LBL – ALK+ large B-cell lymphoma; EBV – Epstein–Barr virus; HHV8 – human herpes virus 8; HIV – HIV infection; Hx – history; LBL/MCCD – large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease; PBL-plasmablastic lymphoma; PCM – plasma cell myeloma; PEL – primary effusion lymphoma

3.5 Human herpes virus 8 (HHV8) status

HHV8 has been documented by immunohistochemistry and reverse transcriptase in-situ polymerase chain reaction (PCR) in a report of five oral lymphomas, four of which were PBL (Cioc et al 2004). This report was challenged by, amongst others, Goedhals, Beukes & Hardie (2008), who in a letter cited 41% co-infection with HHV8 amongst South African HIV-infected individuals. They reported 8 PBL cases which were negative for HHV8 by immunohistochemistry, 1 of which was positive for HHV8 by PCR. This provides some evidence that HHV8 does not play a significant role in the aetiopathogenesis of PBL.

Dong et al (2005) reported 14 cases of PBL in HIV-positive patients, all of which were positive for EBV encoded RNA (EBER) but lacked EBV-latent membrane proteins. In their cohort, HHV8 DNA was detected in 6 of 10 cases by nested PCR, but HHV8 latent nuclear antigen (LANA) was absent.

Carbone, Gloghini & Gaidano (2008) similarly supported that PBL is not an HHV8-associated tumour. They proposed that PBL expressing HHV8 positivity by immunohistochemistry be considered solid variants of PEL, on the basis of homology with the PEL-specific gene expression profile in 3 cases studied. More recently, Yamada et al (2014) identified HHV8 latent viral protein expression by immunohistochemistry in a single case of PBL in an HIV-positive individual.

A further scenario in which HHV8 positive PBL may arise within the context of multicentric plasma cell variant Castleman disease was described by Isaacson, Campo & Harris (2008). In 80% cases, this condition occurs in HIV-positive individuals, and co-infection of plasmablasts with EBV and HHV8 is documented by in-situ hybridisation (EBV) and immunohistochemistry (HHV8) in almost all cases. PBL in this context is different both pathogenetically and biologically. It is a node-based lymphoma and the plasmablasts are arranged at the interface between mantle and marginal zones of involved germinal centres, and within the interfollicular zone. They show IgM clonality and lambda light chain restriction. When clustered in small groups in these locations, they have been described as microlymphomas. Microlymphomas are purported to be the forerunners of bona fide node-based PBL occurring in the context of Castleman disease. This is the

only other setting in which PBL has consistently been shown to be co-infected with HHV8 and EBV.

3.6 Clinical features

PBL occurs most frequently in the oral cavity of HIV-infected individuals where it presents as a gingival mass, involving soft tissue and sometimes bone. There may be displacement of the teeth. The tumour presents with extra-nodal disease although regional lymphadenopathy is not usual. The tumour mass may ulcerate through gingival mucosa, and may infiltrate the facial soft tissues and present as unilateral facial swelling. It may have a haemorrhagic appearance and mimic Kaposi sarcoma (Flaitz et al 2002). A literature review by Scheper et al (2005) reported oral-cavity-based PBL to occurr in the gingiva (11%), on the palate (10%), in the floor of the mouth (8%) and in the tonsillar area (3%), while one case occurred in the retromolar fossa. In many reports a specific site within the oral cavity is not stated. Boy et al (2010) described 45 cases of oral PBL and the gingiva, buccal vestibule and palate were the most commonly affected areas.

Scheper et al (2005) recorded HIV PBL as occurring at a mean age of 39.6 years, with a range of 23 – 75 years, as compared to NHL in immune competent individuals, which typically occur at approximately 50 years of age. Castillo et al (2008), in a review of 112 published cases of HIV PBL, reported a mean patient age of 38 years (range 7 – 65 years), with a male preponderance of 7:1.

HIV PBL has been consistently reported to occur more frequently in males. In the review of PBL by Scheper et al (2005), a male to female ratio was reported to range from 1.76-1.2:1.

In extra-oral sites, PBL presents similarly, most commonly as a mucocutaneous extranodal mass, which is of variable size and may ulcerate or bleed. The review by Castillo et al (2008) of 112 published cases found the nasal and paranasal regions, gastrointestinal tract, skin, soft tissue and lymph nodes to be the most common extra-oral sites. Marrow involvement occurs early in the disease process and is indicative of advanced-stage disease. Leukaemic spill over of tumour cells into the peripheral blood is unusual. Generalised lymphadenopathy is an unusual clinical presentation (Stein et al 2008).

3.7 Morphology

Colomo et al (2004) described PBL to assume one of several morphological patterns, which can be divided into 3 major groups:

1. firstly, classical PBL with a monotonous population of large tumour cells with immunoblastic features (abundant cytoplasm, eccentric nucleus, a prominent central nucleolus and a paranuclear hof);

2. secondly, a subtype of PBL composed predominantly of immunoblastic/ plasmablastic cells but containing a subset of cells manifesting plasmacytic differentiation; and

3. a third group, morphologically indistinguishable from the second group and including tumours from patients with an antecedent history of PCM.

Several other types of B-cell neoplasms have been described to show plasmacytic or plasmablastic differentiation. The former appears confined to the group of small B-cell NHLs and includes lymphoplasmacytic lymphoma, marginal zone lymphoma, small lymphocytic lymphoma/chronic lymphocytic leukaemia (CLL) and follicular lymphoma (Lin et al 2011).

Those tumours displaying plasmablastic morphology are recognised to be high-grade neoplasms, or alternately, tumours that are undergoing high-grade transformation. This applies to PBL, PEL, PBL (or DLBL) arising in HHV8-associated multicentric Castleman disease, anaplastic lymphoma kinase (ALK)-positive DLBL, plasmablastic PCM/EPCT, PCNSL and BL (Castillo & Reagan 2011).

The WHO Classification recognises the morphological diversity of PBL and describes this tumour as a diffuse proliferation of large neoplastic cells morphologically resembling immunoblasts, sometimes with plasmacytic differentiation (Stein et al 2008). PBL has been shown to encompass tumours with a broader range of plasmacytic differentiation than was initially appreciated. Hansra et al (2010) found that oral PBL commonly showed a monomorphic plasmablastic phenotype without plasmacytic differentiation, whereas extraoral PBLs universally displayed plasmacytic differentiation. Similarities and differences between 13 cases of PBL prompted the proposal that two distinct histological subtypes correlating to oral and extra-oral sites be recognised, and that oral (not extra-oral) PBLs show a strong association with HIV (Hansra et al 2010).

3.8 Immunophenotype

The tumour cells of PBL express plasma cell immunophenotypic markers such as CD138, CD38, VS38c and IRF4/MUM1, but not markers expressed by mature B-cells such as CD45, CD20 and PAX5. CD138 expression may be weak or absent in rare cases (Table 3.2). The significance of this is uncertain, but it has been suggested that in the context of PCM/EPCT, weak or absent CD138 may exhibit more aggressive clinical disease (Yan et al 2011).

Both PBL and EPCT/PCM display weak or absent CD20, CD79a and CD45 and an immunophenotype indicating terminal B-cell differentiation, with MUM1(multiple myeloma oncogene-1), CD38, CD138 (syndecan-1), VS38c and EMA positivity. HIV-associated PBL shows greater expression of CD20 and CD56 compared with PBL in HIV-negative individuals (Castillo et al 2010). Ki67 expression in PBL is variably reported in the literature, ranging from >75% (Boy et al 2010) – 90% positivity (Delecluse et al 1997; Chetty et al 2003). The WHO Classification ascribes a proliferation index of >90% to the category of PBL (Stein et al 2008). A high Ki67 proliferation index of >65% occurs in EPCT/PCM showing plasmablastic transformation (Kremer et al 2005; Vega et al 2005). PAX5 and BCL6 have been shown to be weakly positive in small case numbers of PBL. CD56, CD4 and CD10 positivity is similarly described in small case numbers in PBL (Delecluse et al 1997).

Positive regulatory domain 1 (PRDM1/BLIMP1) protein and activated transcription factor X-box binding protein 1 (XBP1) are proteins involved in terminal B-

lymphocyte differentiation. A study by Montes-Moreno et al (2010) showed strong expression of antibodies against these two proteins and weak or absent CD20 and PAX5 to be reliable markers in identifying cases of PBL. This staining profile was reported to occur in <5% of DLBL.

Table 3.2 Immunophenotypic comparison between PBL, DLBL and EPCT/PCM

Antibody	Type of lymphoma		
	PBL	**DLBL**	**EPCT/PCM**
CD45	-	+	+/-
CD20	-	+	-
CD79a	-/+	+	-/+
PAX5	-/+	+	-
CD38	+/-	-	+
CD138	+	-	+
MUM-1	+	+/-	+
VS38c	+	-	+
EMA	+	+/-	+
Ki-67	>75%	40 – 70%	<40%*
Blimp-1	+	-	+
XBP1	+	-	+
CD10	-/+	+/-	+/-
CD4	-/+	-	-/+
CD56	-/+	-	+/-
BCL6	-/+	+/-	-

***This applies to usual variant EPCT/PCM. Plasmablastic variant may show 40 – 80% positivity.

+ All/almost all positive; -All/almost all negative; +/- >50% positive; -/+ <50% positive

(Adapted from: Hsi et al, 2011; Hansra et al, 2010; Castillo et al, 2008)

3.9 Diagnosis

The diagnosis of both oral and extra-oral PBL usually requires an appropriate clinical setting of AIDS, or less commonly, iatrogenic immune suppression in the

context of organ transplantation. The tumour has the histological appearances of a large cell neoplasm displaying an eccentric nucleus with a prominent, centrally placed acidophilic nucleolus and moderate amounts of amphophilic cytoplasm, sometimes with a paranuclear hof. This is an aggressive tumour and the tumour cells are mitotically active. There may be areas of apoptosis and coagulative tumour necrosis (Boy et al 2010; Qing et al 2011). Immunophenotypic analysis reveals a low level of expression or loss of conventional B-cell markers CD45, CD20, CD79a and PAX5, together with acquisition of markers associated with plasma cell differentiation including MUM-1, BLIMP1, XBP1, CD38, CD138, VS38c and EMA (Montes-Moreno et al 2010).

3.10 Differential diagnosis – EPCT/PCM, DLBL

EPCT/PCM can be associated with EBV in the setting of HIV infection (Lorsbach et al, 2011). Some cases of PBL have been reported to show clinical features that overlap with MM, such as an M-protein and lytic bone lesions, providing an additional challenge to their distinction.

EPCT/PCMs may display a morphology that ranges from mature, atypical to plasmablastic or anaplastic plasma cells. A plasmablastic cytomorphology in bone marrow aspirates from patients with a plasma cell neoplasm is recognised to impart a poor prognosis with clinically aggressive disease. The 2% blast count in aspirate smears as defined by Greipp et al (1998) to denote a plasmablastic subtype of EPCT/PCM is widely used. While there are no currently accepted consensus criteria for plasmablastic PCM in tissue sections, Chang et al (2007)

used a 30% blast count on haematoxylin and eosin (H&E)-stained tissue sections and this has been shown to be an appropriate and reproducible cut-off.
The extent to which cytomorphologic features correlate with other established risk factors such as the international staging system for myeloma and high-risk cytogenetic abnormalities is largely unestablished (Weh et al 1995). Given the similarities in morphological appearances and identification of light chain restriction (LCR) in 47% of 45 cases, a study undertaken at the University of Pretoria, South Africa, proposed that PBL with LCR be reclassified as “plasmablastic extramedullary plasmacytoma” (Boy et al 2010).

PAX5 and BCL6 have been shown to be weakly positive in small case numbers of PBL and are uniformly negative in PCM. CD56, CD4 and CD10 positivity is similarly described in small case numbers in PBL (Delecluse et al 1997). The expression of CD56 should prompt serious consideration for a plasma cell neoplasm given the stronger association of this marker with PCM (Stein et al 2008).

There are a variety of techniques which enable the comparison between DLBL, PBL and EPCT/PCM at a genomic level, as reviewed by Tirado et al (2012) and discussed in point 3.11**.**

3.11 Specialised laboratory investigative techniques

3.11.1 Array-based comparative genomic hybridisation – single nucleotide polymorphisms (SNP) or oligonucleotide arrays

This technique provides a high-resolution, genome-wide measurement of DNA copy number alterations. It highlights patterns of deletions and amplifications. It also allows for the assessment of allelic ratio or loss of heterozygosity. One limitation of this technique is the inability to detect balanced chromosomal translocations.

In 2009, Chang et al undertook comparative genomic hybridisation analysis on 16 PBLs, 26 DLBLs and 8 PCMs. The most frequent segmental gains common to all 3 tumour types (>40%) were 1p36.11-1p36.33, 1p34.1 –1p36.13, 1q21.1-1q23.1, 7p21.3-7p23, 7q11.2-7q11.23, 8q24.3, 10p12, 11q12-11q13.2, 14q32, 16p13.2-p13.3, 16q24, 17p13, 20q11.1-q11.23 and 22q12.2-22q13.3, which corresponded with segmental gains occurring with high frequency in HIV- and non-HIV-associated DLBL.

In addition, they found some segmental gains and losses peculiar to PBL (1p35.1-1p36.12, 1q21.1-1q23.1 and 1p36.11-1p36.33) while others occurred in PBL and HIV-associated DLBL, suggesting these particular foci may be associated with HIV infection. Some segmental gains and losses occurred only in PBL and PCM, suggesting a relationship with plasmacytic differentiation. PBL has been shown by array-based comparative genomic hybridisation studies to align itself with an activation status phenotype (ABC phenotype) analogous to ABC-DLBL (Chang et al 2009). Please refer to point 3.11.2 below.

3.11.2 Gene expression profiling

This technique provides for the assessment of RNA status. DLBL has been stratified into good and poor prognostic subtypes utilising this array-based technique. The germinal centre B-cell phenotype (GCB) is considered a favourable phenotype, with a 59% 5-year survival. The activation status phenotype (ABC) expresses genes that are distinctive of activated B-cells and plasma cells and is associated with a poor prognosis, with a 30% 5-year survival. Amplifications of the *REL* loci, *BCL-2* translocations and hypermutations of the immunoglobulin loci are typical of GCB-DLBL. Constitutive activation of the nuclear factor kappa B (NFκB) pathway is a feature of both ABC-DLBL and primary mediastinal B-cell lymphoma (PMBL) (Gaulard et al 2008).

In 2005, Vega et al showed that PBL and PCM have similar gene profiles, as assessed immunophenotypically in 16 cases: nine PBLs and seven PCMs. Both diseases showed frequent loss of expression of *p16* and *p27*, and positive staining for *p53*. They concluded that most AIDS related PBL have an immunohistochemical and tumour suppressor gene profile virtually identical to PCM, and unlike DLBL.

3.11.3 EBER ISH

In fluorescence in-situ hybridisation, fluorescein labelled DNA or RNA sequences are used as probes to identify a target sequence of interest. The detection of EBV genome within tumour cells by fluorescent or chromogenic ISH is considered standard best practice. Castillo et al (2010) showed expression of EBER by ISH in 82% of PBL in HIV-positive individuals and 46% of PBL in HIV-negative patients.

3.11.4 Cytogenetics and *MYC* rearrangements in PBL

There is limited information on the cytogenetic distinction between PBL and EPCT/PCM. Extramedullary involvement and dissemination of PCM reflects biological independence from growth factors derived from bone marrow stroma, such as IL-6 (Lorsbach et al 2011). This acquisition of stromal independence is accompanied by progressive accumulation of secondary genetic alterations, such as *NRAS, KRAS* and *TP53* mutations and deletions, chromosome 1p deletions and amplifications of 1q21 involving *CKS1B*, and translocations involving *MYC*. The high frequency of *MYC* rearrangements in aggressive plasma cell neoplasms, 50% in the report by Lorsbach et al (2011), which is significantly higher than the 15% observed for typical PCM, indicates that translocations involving *MYC* may be associated with more aggressive clinical behaviour and extramedullary disease. FISH analysis in their series revealed t(8;14) and t(8;22) with involvement of *IgHC* or *LC* loci in 25% cases. The impact of other translocation partners may be different.

The detection of *MYC* alterations in 50% of end-stage PCM and >90% of human PCM cell lines, which are commonly derived from extramedullary sites in patients with terminal disease, further suggests *MYC* expression is associated with aggressive clinical behaviour (Lorsbach et al 2011). While the high frequency of *MYC* alterations in PCM with extramedullary extension may aid in the distinction from primary EPCT, the distinction between PBL and plasmablastic EPCT remains difficult.

MYC/IgH rearrangements were reported in 3 of 9 cases of PBL by Bogusz et al (2009). Four cases of systemic EBV-associated plasmablastic neoplasms in HIV-positive individuals reported by Taddesse-Heath et al (2010) had overlapping features between PCM and systemic PBL, and all showed *MYC* translocation. Cases of PBL in HIV-positive individuals with clinical features reminiscent of PCM such as osteolytic lesions or high levels of M-protein have been documented. The best approach for the classification of these aggressive systemic neoplasms with plasmablastic differentiation in immunocompromised individuals and the roles of EBV and *MYC* alterations requires further study (Colomo et al 2004).

PBLs have been assessed for *MYC* dysregulation, but on relatively few case numbers (Bogusz et al 2009; Boy et al 2011). Valera et al (2010) reported *MYC* translocations in approximately 50% of PBL studied by FISH, usually involving the immunoglobulin locus. In contrast, *MYC* translocations are reported to be relatively infrequent (0-15%) in MM, which increases to 45% in advanced MM, and 40% of these *MYC* translocations involve a non-immunoglobulin partner. The proposal has been made that dysregulation of *MYC* may represent a common genetic mechanism that imparts plasmablastic morphology and an aggressive clinical course to terminally differentiated B-cell neoplasms (Castillo & Reagan 2010).

MYC dysregulation is assessed by FISH in different ways. The most widely used approach is a break-apart or split assay using two differently coloured probes that flank the *MYC* locus at 8q24 (Kluin & Schuuring 2011). Utilising a red and green fluorophore, the two probes are seen as a yellow or red/green fusion signals in

normal interphase nuclei. In a mono-allelic break, one normal signal derived from the normal 8q24 allele is seen, together with a split signal with separate green and red signals. Using this *MYC* segregation assay, only a *MYC* breakpoint is detected and there is no information regarding the partner juxtaposed to *MYC*. It is of interest to assess the relationship of *MYC* to possible partner chromosomes, (*IgH* chain gene locus, *Cyclin D1* gene locus, *BCL-2* gene locus and *BCL-6* gene locus) and to assess for the presence of additional chromosomal aberrations [t(14;18)] by FISH. This chromosomal analysis falls beyond the scope of this research report.

3.12 Current status

From the literature published to date on the distinction between PBL, EPCT and plasmablastic variant PCM in the context of retroviral disease, it would appear that these tumours form part of a disease spectrum. The challenge is with the clinical management of these individuals, where the differences in treatment between PBL and PCM/EPCT are significant, as are the differences in prognosis.

CHAPTER 4

4.0 Materials and methods

4.1 Hospital/ Laboratory setting

This study took place in the laboratories of the Department of Anatomical Pathology of the National Health Laboratory Service (NHLS) and of the University of the Witwatersrand. The cases were derived from the departmental archives and routine diagnostic service and were identified using the NHLS DISA LAB-SNOMED classification system. The following SNOMED codes were utilised:
Topography codes: T-C4000 (lymph node); T-C1000 (bone marrow)
Morphological diagnosis codes: M-95903 (malignant lymphoma); M-96803 (malignant lymphoma, large cell, diffuse, NOS); M-96833 (malignant lymphoma, centroblastic, diffuse); M-96843 (malignant lymphoma, immunoblastic, NOS).

4.2 Study sample and selection of cases

This retrospective study included cases diagnosed as extra-oral PBL between the years 2006 and 2012 selected from the Anatomical Pathology archives, 46 cases from the Charlotte Maxeke Johannesburg Academic Hospital and 3 cases from the Chris Hani Baragwanath Hospital. On histological and immunohistochemical review, three cases were re-classifed as EMPT/PCM and one case as DLBL. These four cases were immediately excluded. The study sample then comprised 45 cases of extra-oral PBL.

Demographic data extracted from the specimen labels and histopathology reports of the patients included their age, gender, race, HIV status, and the extra-oral

biopsy site on which the primary diagnosis of extra-oral PBL was made. CD4 counts and viral load assessments were not obtained as they were not available. The histomorphologic review aimed both to confirm the diagnosis of PBL, but also to distinguish cases with a pure plasmablastic morphology (PBm) from those tumours showing features of PBL with plasmacytic differentiation (PBm+PCd). The intention of making this distinction was to ascertain whether extra-oral PBLs show a propensity for pure plasmablastic morphology, or whether they readily show plasmacytic differentiation.

4.3 Histomorphology

The tumours were divided into two groups according to whether there was a pure plasmablastic morphology (PBm) or whether there was plasmablastic morphology with plasmacytic differentiation (PBm+PCd). A pure plasmablastic morphology was ascribed to tumours that exclusively comprised large cells having eccentric amphophilic cytoplasm and usually a single, prominent acidophilic nucleolus (plasmablasts). Those showing plasmacytic differentiation comprised a dominant population of plasmablasts, but with an admixed population of small plasma cells containing a clock-face chromatin pattern, amphophilic cytoplasm and a paranuclear hof.

The inclusion criteria were those cases that had a morphological and immunophenotypic diagnosis of PBL and that appeared to have occurred at an extra-oral site as ascertained from the demographic data available on the pathology report. Cases were required to have sufficient material in the paraffin-embedded tissue block for immunohistochemical stains and ISH studies.

4.4 Tissue microarrays (TMA)

In order to make optimal use of immunoantibodies for IHC and probes for EBV chromogenic ISH, it was decided to create a tissue microarray (TMA). A single 1mm core TMA block was created using a tissue arrayer (Beecher Instruments, Silver Spring, MD) on which 30 cases were represented. These 30 cases were thought to be of sufficient size for inclusion in the TMA. Cores from areas with characteristic and best-preserved plasmablastic morpho logy were chosen from the paraffin block. In some cases a single core was used, while in others up to four cores were used in constructing the TMA block (Table 4.1).

Of the 30 cases represented in the TMA block, 5 cases, represented by 7 cores (H2 & I2; D4; A4; I4; H4; and I3), were excluded from the TMA cohort due to insufficient tissue, (Table 4.1). In total then 25 (30-5 = 25) cases represented by 37 cores (50-7-6, the 6 being blanks) on the TMA and 20 cases represented by freshly cut whole mount sections and archived sections were used in the IHC and ISH parts of this study.

It must be emphasised that not all of the 45 cases were subjected to the identical panel of antisera at the same time. Cases were selected on individual criteria pertaining to each specific case, the quantity of tissue that remained in the tissue block and the availability of archived sections being the major determinants. The staining was not repeated for the archived sections. Although we recognise that the use of archived IHC sections is not ideal, in that not all cases would have been stained in a single run resulting in lack of standardisation in the antibody used, in the clonality, in dilutions and in antigen retrieval, the large number of antibodies

tested meant serious financial constraints had to be overcome, thus preventing us from repeating IHC stains which already formed a part of the diagnostic record.

Table 4.1 Grid reference for the TMA block

	1	2	3	4	5
A	X	A2	A3	*A4* *Same case as I4: deleted*	A5
B	X	B2	B3	B4	B5 Same case as J4
C	C1	C2	C3	C4	C5
D	D1	D2 Same as E2, F2, G2	D3	*D4* *deleted*	D5
E	E1	E2 Same as D2, F2, G2	E3	E4	E5
F	F1	F2 Same as D2, E2, G2	F3	F4	F5
G	G1	G2 Same as D2,E2, F2	G3	G4	X
H	H1	H2 Same case as I2: deleted	H3	*H4* *Deleted*	X
I	I1	*I2* *Same case as H2, deleted*	*I3* *deleted*	*I4* *Same case as A4: deleted*	X
J	J1	J2	J3	J4 Same case as B5	X

KEY to TMA grid (Table 4.1)
E1, H1, I1, J1, C2, J2, E3, F3, G3, H3, J3, G4, A5, D5, D5 X15 single core cases

C1&D1, F1&G1, A2&B2, A3&B3, C3&D3, B4&C4, E4&F4, E5&F5, J4&B5 X9 two core cases (18)
D2, E2, F2 &G2 X1 four core case
Total cores on TMA = 37 representing 25 cases
X - blank grid spaces (6)
Italicised grid cells - cores excluded due to insufficient tissue (7)
Originally there were 50 core grid spaces less (6+7 = 13)

In constructing the TMA the best preserved areas on the original H&E section of the selected cases were ringed with a permanent marker. These areas were compared to the tissue block and then cored. The cores were made with a manual coring device and inserted into a recipient paraffin block which was then converted into a paraffin embedded tissue block by placing the recipient block face down into an embedding mould and adding molten paraffin wax (56˚C) (Pathwax,

Johannesburg, S.Africa). The block was cooled, removed from the mould and sectioned at 4µm. The 4µm sections were placed on adhesive coated Superfrost glass slides (Superfrost, Labotec, S.Africa) which were then subjected to immunohistochemistry. For each immunohistochemical stain performed on the TMA, (CD138, MUM1, Ki-67, BCL6, CD10, BLIMP1, CyclinD1 and PAX5) a control of a known positive case was run simultaneously on the autostainer.

Visualisation of the TMA required correlation with a grid reference, such that 10 rows and 5 columns were represented, the rows labelled A – J and the columns 1 - 5. Each cell within the grid reference was ascribed a particular case number (Table 4.1). Positive controls were not included in the TMA, but run as whole mount sections in the same automated immunohistochemistry run. Tissue samples to which no primary antibody had been added were used as negative controls. These were also not included in the TMA, but run concurrently as whole mount sections. The archived sections were also run with positive and negative controls, as per departmental standard operating procedures at that time.

4.5 Immunohistochemistry

4.5.1 Choice of antibodies for diagnosis of PBL

Where immunohistochemical stains were available from the time of original diagnosis, these were reviewed, assessed and recorded. Immunohistochemical stains which were not performed at the time of diagnosis or were not available for review, were undertaken on the 4µm sections which constituted the IHC case cohort (TMA and whole mount sections), using the following mouse monoclonal antibodies directed against PAX5, CD138, MUM-1, BLIMP1, BCL6, CD10 and CyclinD1. The proliferation index was assessed with Ki-67 (Table 4.2). CD45,

CD20, CD79a and VS38c were not repeated, as most cases had already been assessed at the time of the original diagnostic work-up and thus archived sections were available. For HHV8, archived sections were available in 16 of 45 cases. The decision not to perform this antibody on the remaining 29 cases was based on the premise that this would have added limited value to the overall diagnostic panel.

Table 4.2 List of immunoantibodies utilised

Antibody	Clone	Dilution	Antigen Retrieval	Control	Supplier
CD45 (LCA)	2B11þPD7/26	1:100	High pH	Lymph node	DakoCytomation, Denmark
CD20	L26	1:700	High pH	Lymph node	DakoCytomation, Denmark
CD79a	JCB117	1:300	High pH	Lymph node	DakoCytomation, Denmark
PAX5	DAK-PAX5	Neat	High pH	Lymph node	DakoCytomation, Denmark
CD138	Syndecan-1	1:25	High pH	Lymph node	DakoCytomation, Denmark
MUM1	MUM1p	1:50	High pH	Lymph node	DakoCytomation, Denmark
BLIMP1	3H2-E8	1:300	High pH+linker	Lymph node	Novus Biologicals, USA
VS38c		1:100	High pH	Lymph node	DakoCytomation, Denmark
Ki-67	MIB-1	1:50	High pH	Lymph node	DakoCytomation, Denmark
BCL-6	PG-B6p	1:50	High pH+linker	Lymph node	DakoCytomation, Denmark
CD10	56C6	1:100	High pH+linker	Lymph node	Leica, UK
HHV8	13B10	1:100	High pH+linker	Skin with Kaposi sarcoma	Leica, UK
CyclinD1	SP4	1:50	High pH	Lymph node with mantle cell lymphoma	Cell Marque, USA

4.5.2 Rationale for antibody choice for diagnosis of PBL

CD45 (LCA): This antibody preferentially marks lymphocytes, but also marks plasma cells, albeit less consistently.

CD20: A pan B-cell marker that is negative in plasma cells. The marker is dropped as B-lymphocytes show plasma cell differentiation.

CD79a: A second line B-cell marker that also signals in plasma cells and B-lymphocytes showing plasma cell differentiation.

PAX5: A pan B-cell marker that shows positivity in primitive B-lymphoblasts through the entire spectrum of B-cell differentiation to plasma cells. It is lost in late (mature) plasma cell differentiation.

CD138/Syndecan-1: A marker of pre-terminal B-cell (plasma cell) differentiation, negative in B-lymphocytes.

MUM1/IRF4 (interferon regulatory factor-4): A lymphocyte-specific transcription factor that signals in B-lymphocytes showing transition from germinal centre B-cells to immunoblasts and plasma cells.

BLIMP1: A marker that is positive in plasma cells and B-lymphocytes committed to plasma cell differentiation.

VS38c: A non-lineage specific antibody that is reactive to rough endoplasmic reticulum, and is expressed by plasma cells, plasmablasts, lymphoplasmacytoid cells and B-immunoblasts. It also stains melanocytic tumours, neuroendocrine tumours and several epithelial tumours. It is considered a very sensitive marker for plasma cells but requires careful interpretation in the correct context because of its reactivity with other tumours (Turley et al, 1994; Banjeree et al, 1997; Shanks et al, 1996).

Ki-67: A proliferation marker. PBL should have a high proliferation index in comparison to conventional myeloma, which has a low PI.

BCL6: To demonstrate origin from the germinal centre. This is negative in PBL and MM, and positive in a proportion of DLBL (those of germinal centre origin).

CD10: Common acute lymphoblastic leukaemia antigen (CALLA), positive in primitive lymphoblasts, B-lymphocytes of germinal centre origin and in plasma cells.

HHV8: To demonstrate latent protein expression of the HHV8 virus within tumour cell nuclei. This is described in the literature to be negative in all high-grade B-cell NHL, except PEL and the DLBL which arises in the setting of plasmablastic variant multicentric Castleman disease (MCD).

CyclinD1: This is not expressed in normal lymphoid cells. The *CCND1* gene is over expressed following the t(11;14) which defines mantle cell lymphoma. The same translocation is present in a proportion of cases of MM.

4.5.3 Immunohistochemical procedures for diagnostic panel

For each immunohistochemistry test, two sections from each paraffin-embedded tissue block (TMA and whole-mount) were cut at 4µ and mounted onto commercial-adhesive-coated slides (Superfrost, Labotec, S. Africa). One slide was used as a test and the other as a negative control. The slides were labelled accordingly. A positive control for each immunohistochemistry test was included in the run.

All sections were exposed to an antigen retrieval buffer for 20 minutes in the DAKO PT Link Automation System using the EnVision FLEX Target Retrieval

Solution (DAKO, Denmark), which is designed to simultaneously deparaffinise tissue sections embedded in most commercially available forms of paraffin. Slides were incubated in PBS (phosphate-buffered saline) buffer for 10 minutes. Endogenous peroxidase was blocked with EnVision FLEX Peroxidase-blocking reagent, a phosphate buffer containing hydrogen peroxide, for five minutes at room temperature. After a short rinse in PBS buffer, test sections were incubated for 20 minutes at room temperature with antibodies listed in Table 4.2. The antibodies were used as per dilutions indicated in Table 4.2.

The negative control slides were allowed to remain in PBS buffer throughout the antibody incubation of the test slides. After the incubation of test slides with the relevant antibodies, slides were rinsed in PBS buffer. All slides, including the negative control slides, were incubated with the EnVision FLEX HRP-detection system (DAKO, Denmark) for 20 minutes followed by several rinses in PBS buffer.

Staining was developed in liquid DAB+ substrate chromogen buffer (3,3'-diaminobenzidine in chromogen solution in Imidazole-HCl buffer pH 7.5) containing hydrogen peroxide and an anti-microbial agent (DAKO,Denmark) and then washed well in running tap water. Sections were counterstained with EnVision FLEX Haematoxylin for 20 seconds, dehydrated with ethanol, cleared in xylene and mounted with Entellan (Merck, Germany) under a coverslip.

4.5.4 IHC data analysis

All the IHC sections were assessed by a single experienced pathologist using light microscopy. Staining was evaluated using the following semi-quantitative

protocol; negative <5% cells stained, focal 5-50 % cells stained, diffuse 50-100% of cells stained. In addition the intensity of staining was assessed as being weak, moderate or strong. Ki-67 was assessed by counting positive and negative cells in 3 high power fields, averaging and then converting to a percentage.

4.6 EBER Chromogenic in-situ hybridisation (CISH) studies

Where the EBER assessment already formed a part of the diagnostic case record, staining was not repeated. CISH was carried out, using the same single TMA block and whole-mount sections as previously described. In total, CISH EBER results were available for 43 cases (TMA + whole mount + archived). The method used is described hereunder:

The INFORM EBER cocktail probe (Ventana Medical Systems, Roche, USA) and the Ventana Benchmark Automated Staining System were used. All slides including a positive control slide (EBV-positive lymph node with Hodgkin lymphoma as control) were stained with the EBER ISH staining protocol using the INFORM EBER Probe (a fluorescein-labelled oligonucleotide probe that has an affinity to the early RNA transcript of Epstein–Barr virus) and the ISH iVIEW Blue Plus Detection Kit using the Ventana Benchmark Automated Staining System according to manufacturer's instructions. The ISH iVIEW Blue Plus Detection Kit utilises an alkaline phosphatase enzyme and NBT/BCIP substrate chromogen reaction, which yields an intense blue permanent colour deposit at the site of probe-target interaction that is readily visualised by light microscopy. Red Counterstain II (which provides pink background staining in nuclei and the cytoplasm) was used for background staining of the tissue sections to aid

microscopic bright field observation for CISH reactions with Ventana DNP-labelled probes.

On completion of the stain run on the Ventana Benchmark Automated Staining System, the slides were washed with a mild detergent solution to remove any greasy residues from the LCS wash buffers used in the staining run, they were rinsed in running tap water, dehydrated through graded alcohol, cleared in xylene and mounted in Entellan mounting medium using glass coverslips.

4.6.1 CISH data collection and analysis

All sections were assessed by a single experienced pathologist. A positive signal appeared as an intense blue density of the cell nucleus. The result was recorded as either positive or negative

4.7 Controls

Positive control: known positive controls were included in each antibody run for the immunohistochemistry and the CISH studies. These were predominantly lymph nodes, and 1 cutaneous biopsy with Kaposi sarcoma.

Negative control: for immunohistochemistry, each antibody run was accompanied by a slide in which the primary antibody had not been applied.

4.8 Ethics

Ethics clearance (M120993) specific for this project was granted by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand. Furthermore, the ethics code M10750 used for this project is a blanket code

allocated to the Department of Anatomical Pathology for use of archival block material obtained from human tissues. Patient slides were given study numbers in order to maintain patient confidentiality (Appendix 1).

4.9 Statistical analysis

The study design was that of a laboratory based (non-interventional) retrospective cross-sectional study that was based on descriptive demographics, histomorphological, immunohistochemical and ISH data. The data was descriptive and variably continuous and categorical, consisting of frequencies that were expressed in percentages, ratios, tables and graphs. Statistical analysis of the data was performed using appropriate statistical analysis techniques and software. Microsoft Excel 2010® was used to store data as a spread sheet and the statistical analysis software R by R Core Team (2013) [http://www.R-project.org] was used for statistical calculations.

Descriptive statistics included calculations of means ± standard deviations. Non-parametric data was assessed using medians as a measure of central tendency and mean absolute deviation as a measure of statistical dispersion. Differences in means between groups were evaluated using two sample, two-tailed t-tests and one-way ANOVA (analysis of variance), the p value of <0.05 indicating statistical significance.

Fisher's exact tests were used to test the differences in categorical data (i.e. data whose value is one of a fixed number of nominal categories). A p value of <0.05 was used to indicate statistical significance.

CHAPTER 5

5.0 Results

5.1 Study sample

A total of 49 cases diagnosed as extra-oral PBL were retrieved from the archives of the Department of Anatomical Pathology, University of the Witwatersrand, Johannesburg over the seven-year period between 2006 and 2012. One case was reclassified as DLBL and excluded. Three cases were reclassified as EPCT/PCM and also excluded, leaving 45 cases that made up the study cohort.

5.2 Patient demographics

5.2.1 Age

Extra-oral PBL occurred over a wide age range between the ages of 9 and 59 years (mean 37.28 years, 95% CI = 34.41 - 40.14); the age of two patients was unknown. The mean age of females in the study population was 35.41 years (95% CI = 32.12 to 38.7) and the mean age of males was 38.5 years (95% CI = 34.39 – 42.61). This difference was not statistically significant (two sample t-test, p = 0.27) (Fig.5.1).

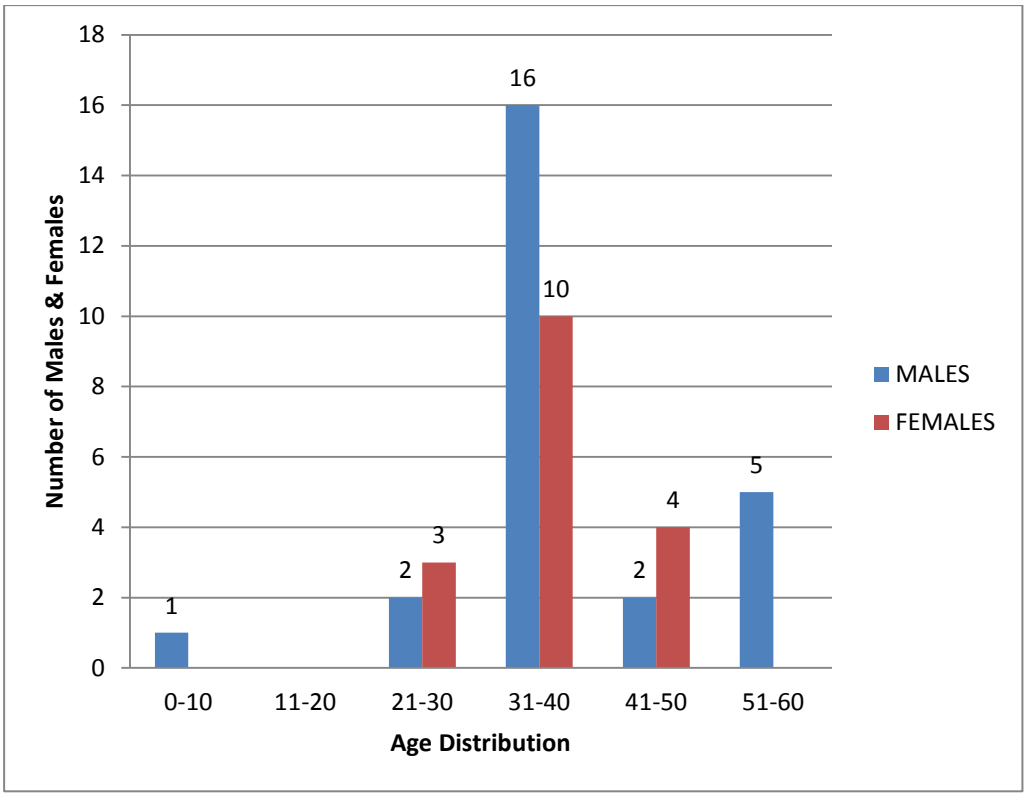

Fig. 5.1 Age distribution in males and females with extra-oral PBL

5.2.2 Gender

Of the 45 extra-oral PBLs, 27 (60%) occurred in males and 18 (40%) in females. There was a male bias with a M:F ratio of 1.5:1.

5.2.3 Race

In all instances the patients were black Africans.

5.2.4 Site

The extra-oral sites of tumour presentation on which the primary biopsy diagnosis was made are listed in Table 5.1.

Table 5.1 Sites of occurrence of extra-oral PBL

BIOPSY SITE	NUMBER OF CASES
Abdominal cavity	1
Anus	13
Bone marrow	2
Breast	1
Lymph node	5
Nasopharynx	1
Ovary	1
Penile foreskin	1
Rectum	1
Skin	1
Small intestine	3
Soft tissue	11
Stomach	2
Urethra	1
Vulva	1
TOTAL:	**45**

Analysis of Table 5.1 shows that extra-oral PBL occurred most commonly in the anus (28.8%), followed by soft tissue (24.4%), then gastrointestinal tract – stomach, small intestine and rectum (13.3%), lymph node (11.1%), and bone marrow (4%). Single cases occurred in the abdominal cavity, breast, nasopharynx, ovary, penile foreskin, skin, urethra and vulva. The soft tissue sites were often anatomical areas in which lymph nodes would ordinarily be found, but where on histological examination residual lymph node was not identified. These were the soft tissue of the axilla, neck and inguinal regions. Other soft tissue sites were the buttock and the orbit. A solitary inguinal lymph node was included in the data but the remaining four lymph nodes were of unknown site. The nasolabial region was the single cutaneous primary. Excluding the anus as a mucocutaneous site, there were two mucocutaneous tumours involving the vulva and urethra.

5.2.5 HIV status

Twenty seven of the 45 cases were of unknown HIV status. Of the 18 cases with known HIV status, 1 was negative and the remaining 17 (94%) were positive. The single negative case was a 59 year old adult male with an abdominal cavity lesion.

5.3 Histomorphology

Typically, PBLs comprise a monomorphic infiltrate of large lymphoid cells which have a plasmablastic morphology in that the cells have abundant amphophilic cytoplasm with an eccentric nucleus and a prominent single acidophilic nucleolus. Most of the cases (27/45, 60%) had this typical appearance. These cases were designated PBm (Fig. 5.2 & 5.3). The remaining cases (18/45, 40%) comprised a mixed population of cells consisting of a predominant plasmablastic component with a variable population of smaller plasmacytic cells. These were designated PBm+PCd (Fig. 5.4).

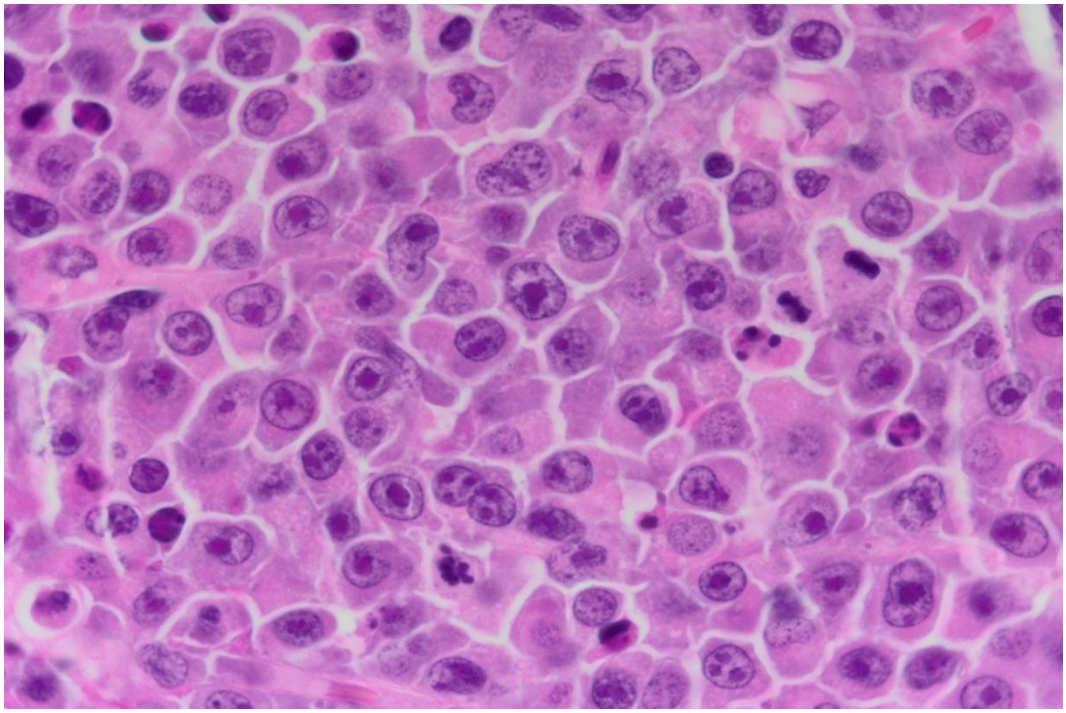

Fig. 5.2 PBL showing a pure plasmablastic morphology (H&E, magnification X100)

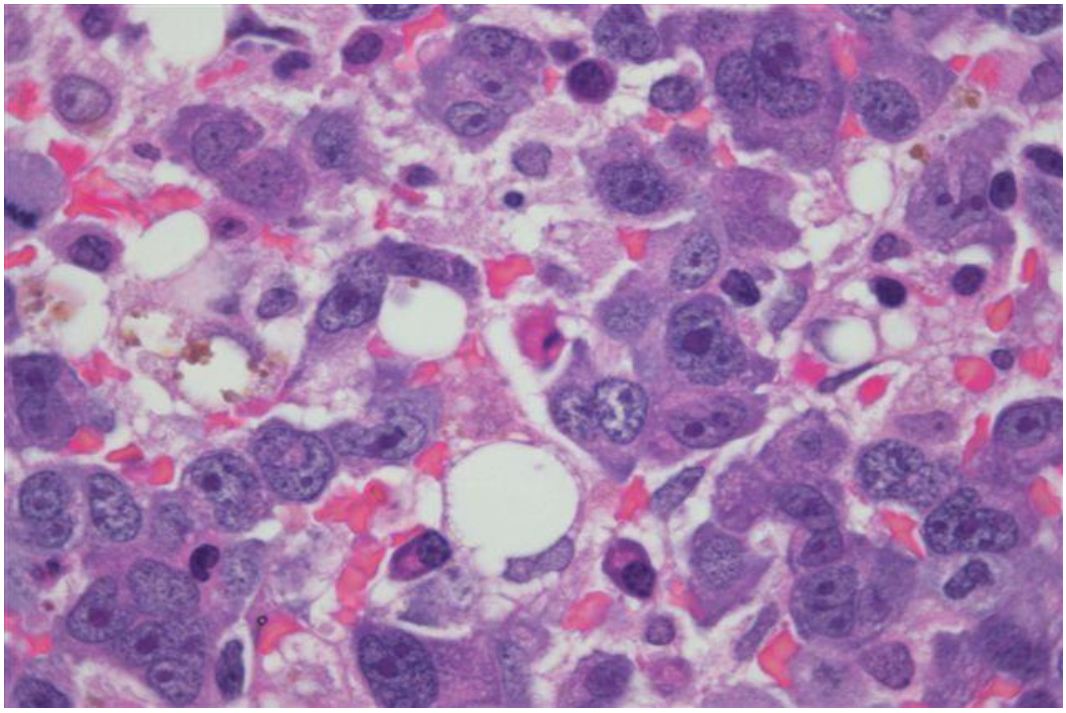

Fig. 5.3 PBL in bone marrow showing pure plasmablastic morphology (H&E, magnification X100)

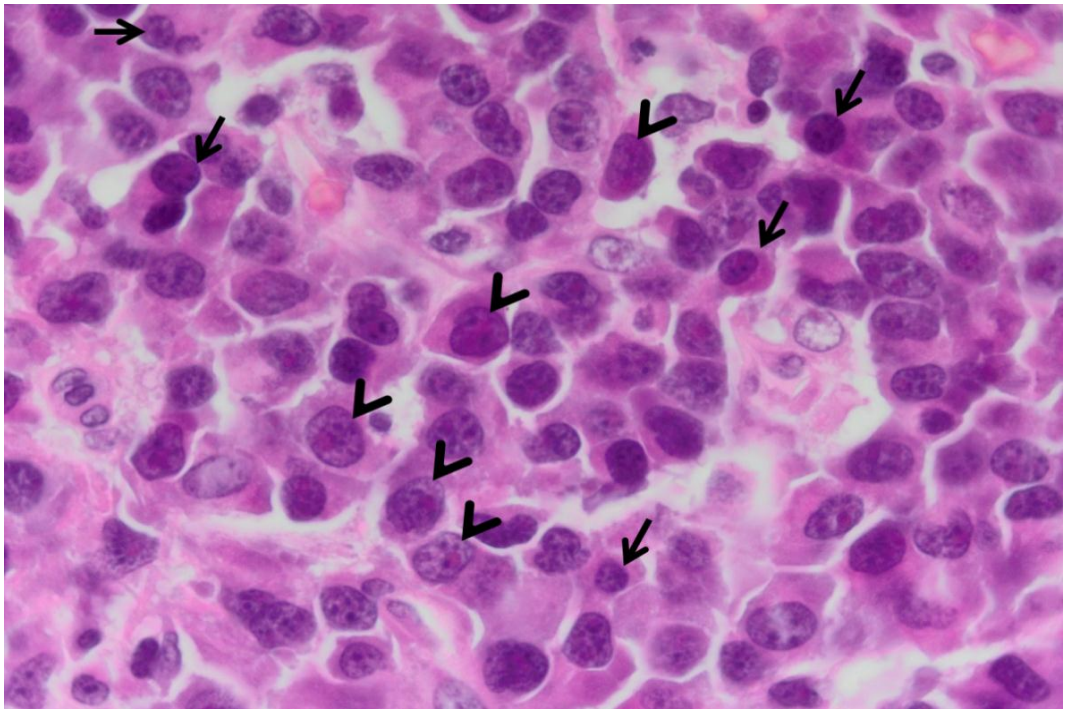

Fig. 5.4 PBL with plasmablastic phenotype (arrow heads), and plasmacytic differentiation (arrows) (PBm+PCd) (H&E, magnification X100)

5.3.1 Confirmation of histological subtypes of PBL

When the demographic characteristics were compared, there was no significant difference between the histological subtype of PBL and age (p=0.98) (Fig. 5.5).

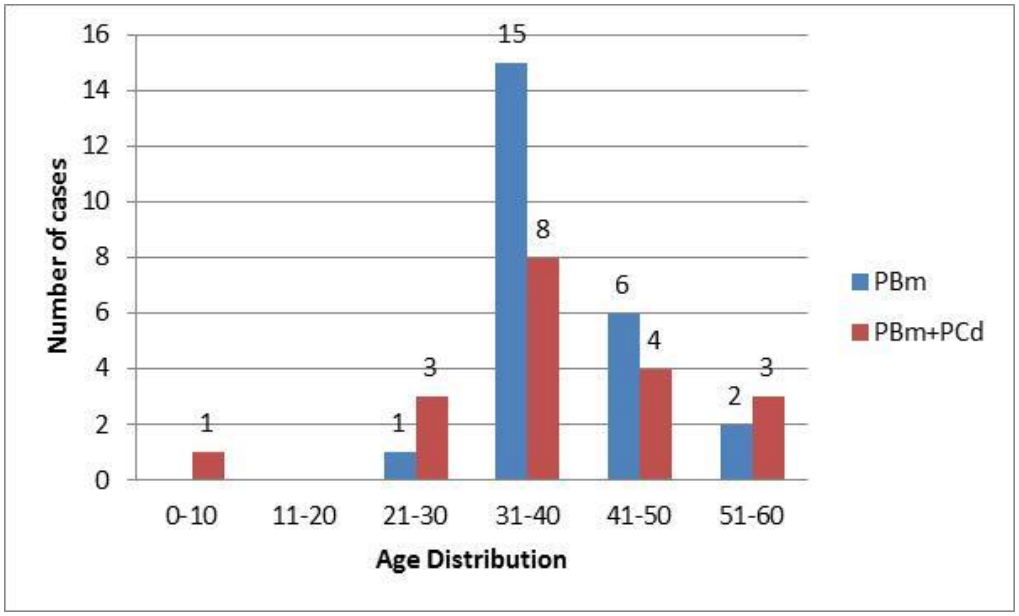

Fig.5.5 Age distribution of histological subtypes in extra-oral PBL

Similarly, there was no significant difference between histological subtype of PBL and gender (Fisher's exact test, p=0.31) (Fig. 5.6).

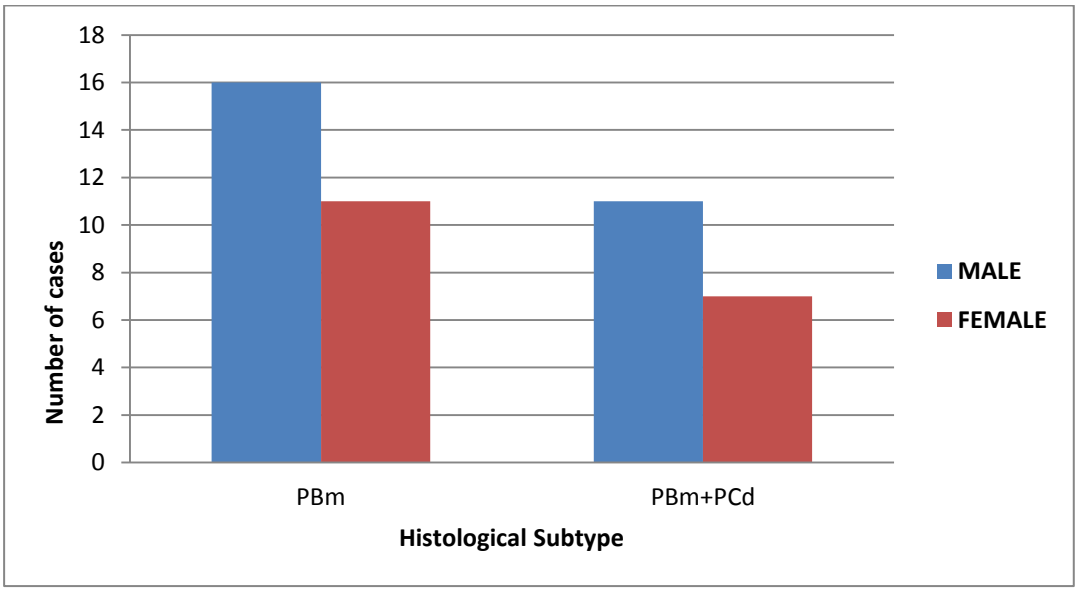

Fig. 5.6 Histological subtype and gender

The relationship between the histological subtypes, age and gender is further illustrated in the box-and-whisker plot (Fig. 5.7). This confirms that there was no significant difference between the histological subtypes as regards age and gender (for both, p=0.05).

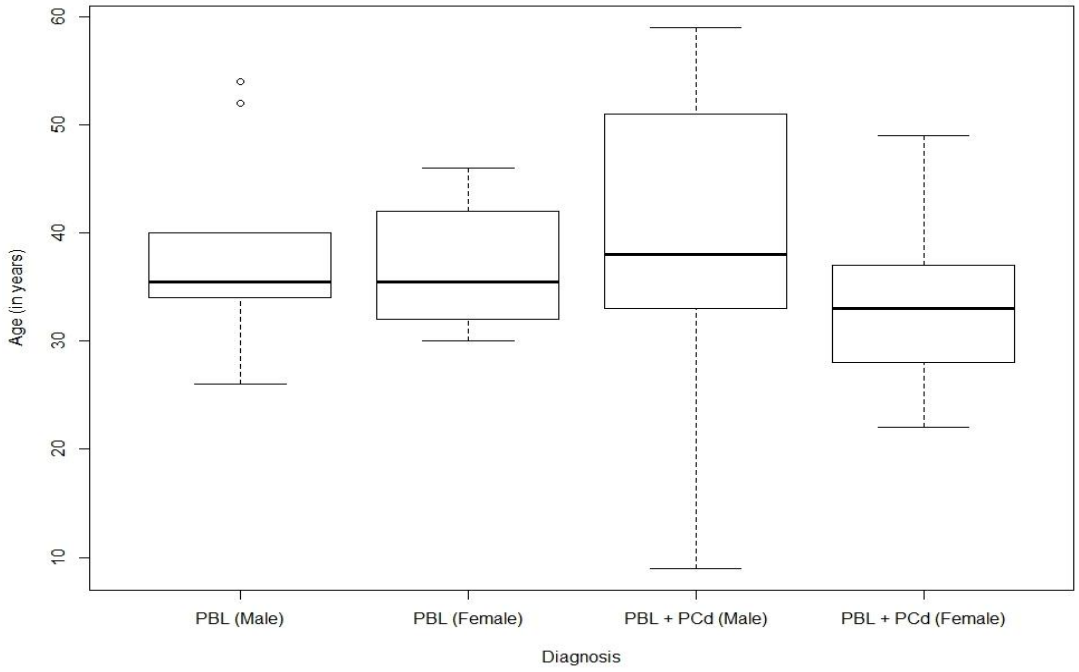

Fig. 5.7 Box-and-whisker plot of mean age (in years) and gender according to histological subtype.

Table 5.2 lists the site of primary diagnosis against tumours showing a pure plasmablastic morphology (PBm) and those with plasmablastic morphology showing plasmacytic differentiation (PBm+PCd). Analysis shows that the histological variant of PBL does not appear to be influenced by site (p=0.05).

Table 5.2 Primary sites and histological subtypes of PBL

Extra-oral PBL site	PBm	PBm+PCd
Abdominal cavity		1
Anus	8	5
Bone marrow	1	1
Breast	1	
Lymph node	1	4
Nasopharynx		1
Ovary	1	
Penile foreskin		1
Rectum	1	
Skin	1	
Small intestine	2	1
Soft tissue	7	4
Stomach	2	
Urethra	1	
Vulva	1	
TOTAL:	**27**	**18**

5.4 Immunohistochemical findings

The raw data of the immunohistochemical stains undertaken on each case are provided in Appendix 2. The results are summarised in Table 5.3 and are detailed hereunder.

Table 5.3 Summary of immunohistochemical and EBER-ISH findings

ANTIBODY	NUMBER OF CASES STAINED (*n*)	POSITIVE		NEGATIVE		NOT DONE	NOT ASSESS	EQUIVOCAL
		N	%	*n*	%			
CD45	42	36	85.7	6	14.3	3		
CD20	45	8	17.7	37	82.3			
CD3	45	2	4.4	43	95.6			
CD79a	41	22	53.6	19	46.3	4		
CD138	34	22	64.7	12	35.2	4	7	
VS38c	36	33	91.6	3	8.3	9		
MUM1	40	40	100	0	0	5		
BCL6	37	4	10.8	33	89.1	4	3	1
HHV8	16	1	6.3	15	93.7	30		
CD10	34	12	35.3	22	64.7	8	2	1
BLIMP1	22	21	95.5	1	4.5	7		16
CYCD1	25	1	4	24	96	9	11	
PAX5	28	5	17.8	23	82.1	7	10	
EBER	40	37	92.5	3	7.5	3	2	

Key: ND=stain not done. NA=stain done but could not be assessed. Equivocal=stain done but signal too ambivalent to categorise.

CD45 (Leucocyte common antigen): CD45 stained positively in 85.7% of the cases (36/42) and was negative in 14.3% (6/42). Staining was always membranous but varied from weak to strong (Fig. 5.8 & 5.9).

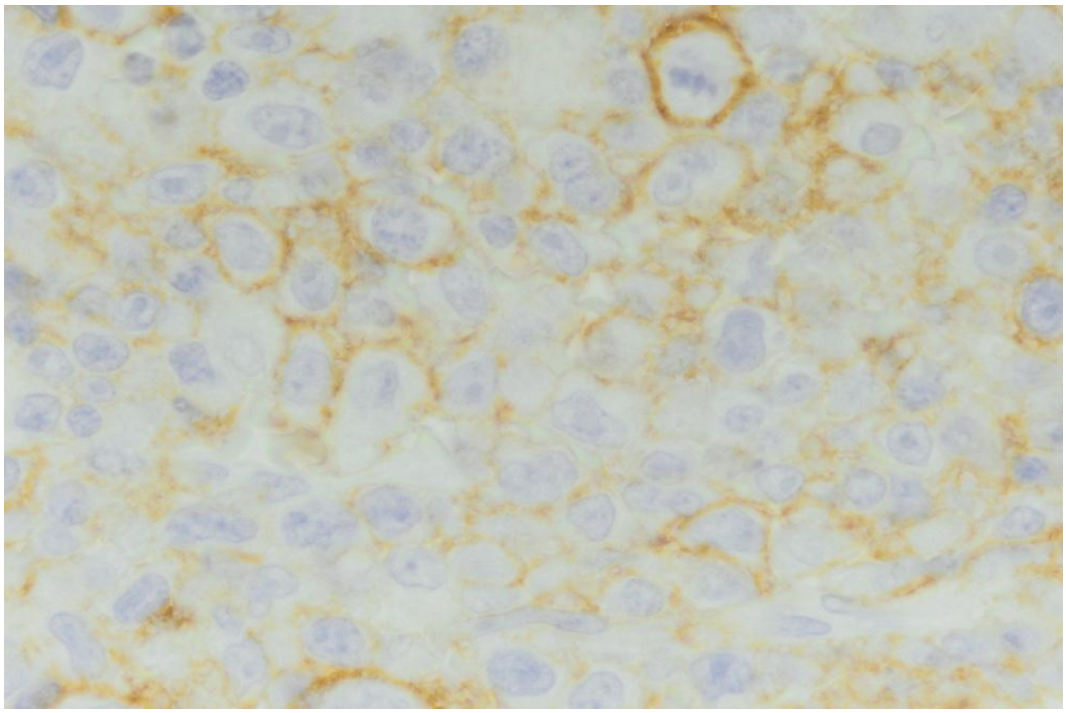

Fig. 5.8 PBL with weak, incomplete membrane signal (CD45, magnification X100)

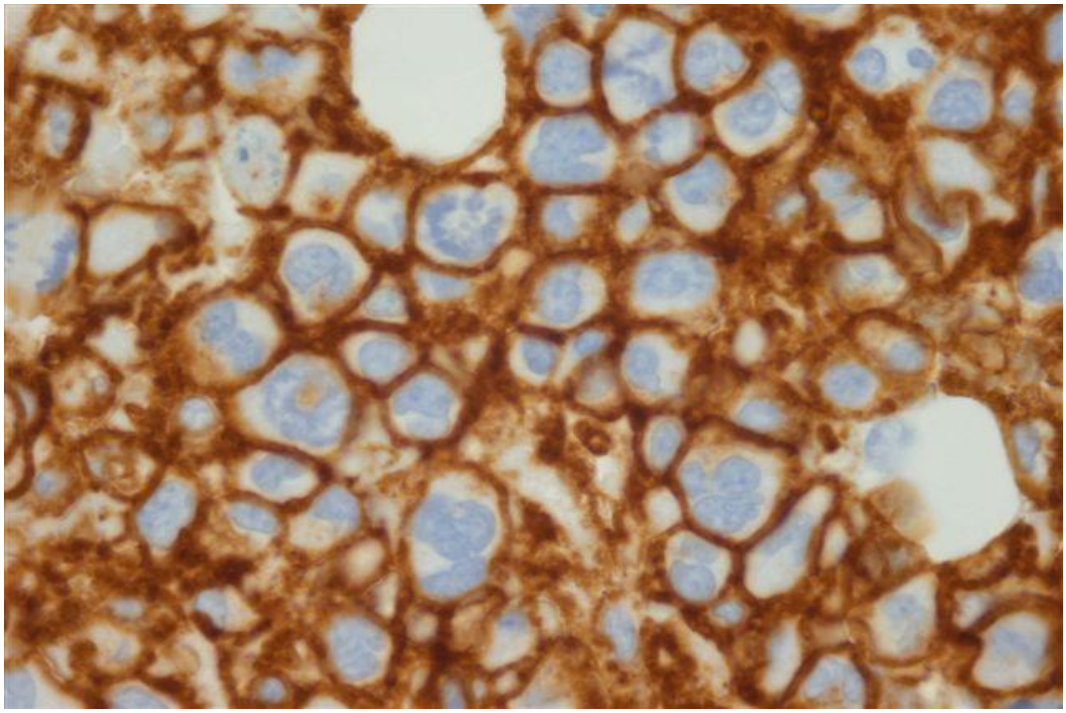

Fig. 5.9 PBL in bone marrow showing diffuse, strong membrane expression (CD45, magnification X100)

CD20: Staining was positive in only 17.7% (8/45) of the cases and was negative in 82.3% (37/45). Where present, staining was membranous, mostly weak in intensity and focal in distribution (Figure 5.10).

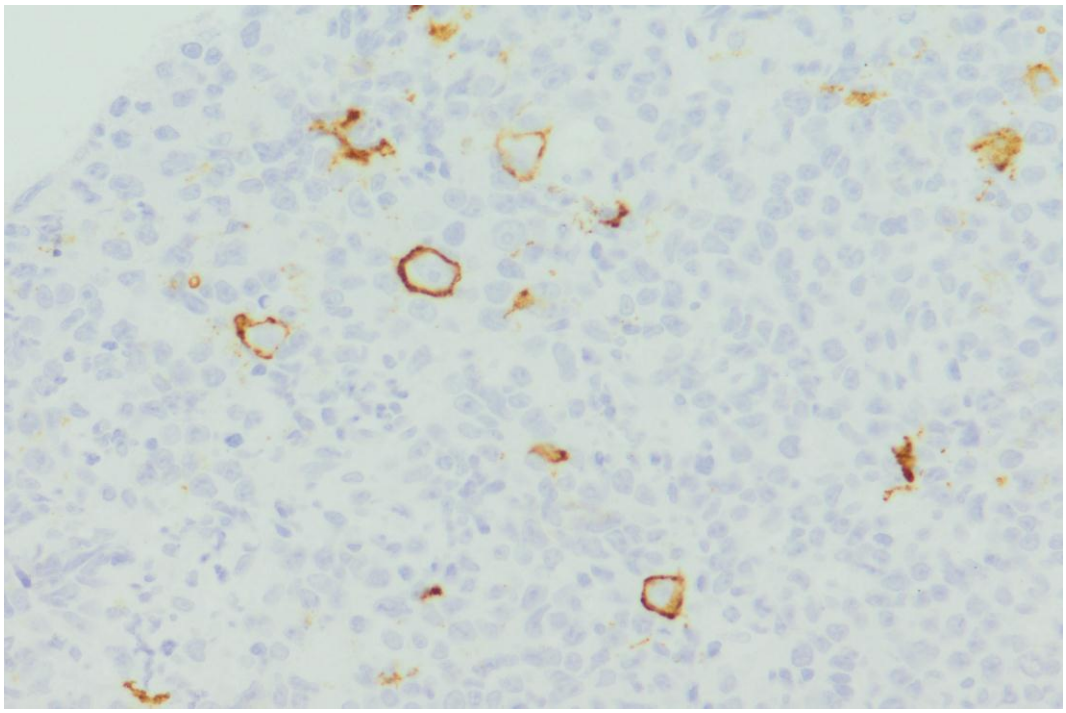

Fig. 5.10 PBL demonstrating focal positive membrane signal in tumour cells (CD20, magnification X40)

CD3: Staining was usually negative (43/45, 95.6%), being positive in only 2/45 (4.4%) of the cases.

CD79a: Approximately half of the cases (22/41, 53.6%) stained positively and half were negative (19/41, 46.3%). Staining was membranous, moderate in intensity and diffuse in distribution.

CD138: Staining was positive in nearly two thirds of cases (22/34, 64.7%), was membranous in location, diffuse in distribution and strong in intensity.

VS38c: 33 of 36 cases (91.6%) stained positively for VS38c and 3 cases (8.4%) were negative. Staining was cytoplasmic in location, diffuse in distribution and strong in intensity.

MUM1: 40 of 40 cases (100%) were positive with this marker. Staining was nuclear in location, diffuse and moderate to strong in intensity (Fig. 5.11).

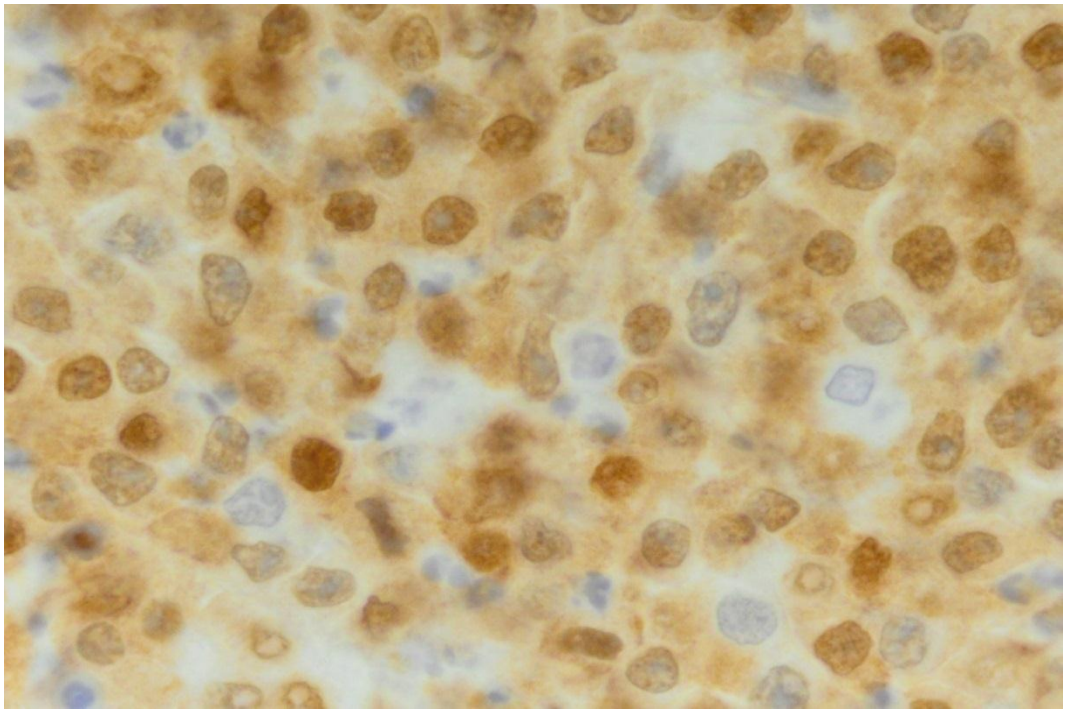

Fig. 5.11 PBL demonstrating positive nuclear signal in tumour cells (MUM1, magnification X100)

BCL6: 4/37 cases (10.8%) were positive with BCL6, 33/37 (89.2%) were negative.

HHV8: 1/16 cases (6.3%) was positive, 15/16 (93.7%) cases were negative. Staining was localised to plasmablasts within the mantle and marginal zones of several lymphoid follicles. It was nuclear in location and of moderate intensity.

CD10: Just over one third of cases (12/34, 35.3%) stained positively with CD10, while 22/34 cases (64.7%) were negative. Staining was membranous, focal to diffuse and weak to moderate in intensity (Figure 5.12)

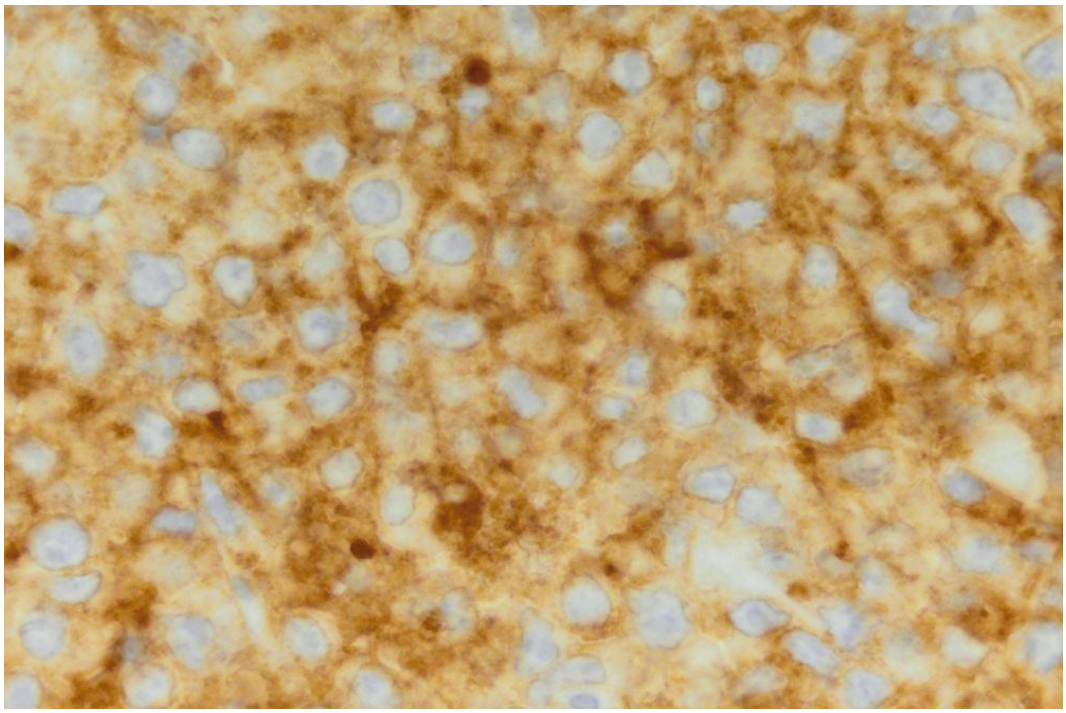

Fig. 5.12 PBL with positive membrane signal in majority of tumour cells (CD10, magnification X100)

BLIMP1: 21/ 22 cases (95.5%) were positive for BLIMP1. Only 1/22 (4.5%) was negative. Staining was focal, of weak to moderate intensity and located in the cytoplasm.

CyclinD1: 1/25 cases (4%) was positive, 24/25 (96%) were negative. The single positive case stained focally and weakly. The staining was nuclear.

PAX5: 5/28 cases (17.8%) were positive and 23/28 (82.1%) were negative. When positive the staining was focal, nuclear and of weak to moderate intensity.

Ki-67: 35/45 cases were assessed for Ki-67. The positive signal was nuclear in location, of moderate to strong intensity and ranged from 40-100% of tumour cells.

In the majority of cases, the proliferation index was ≥90% (19 of 35 cases). In 14 cases, the PI ranged from 60-80% and in the remaining 2 cases (of 35) the PI≤50%.

5.4.1 Comparison of immunohistochemical staining between morphological subtypes.

Selected antibodies were assessed in relation to histologic subtype and significant differences were not observed (Table 5.4).

Table 5.4 PBm and PBm+PCd against selected antibodies

	PBm					PBm+PCd						
ANTIBODY	**POSITIVE**		**NEGATIVE**		**TOTAL**	**POSITIVE**		**NEGATIVE**		**TOTAL**	***p*-value**	***n***
	n	**%**	***n***	**%**		***n***	**%**	***n***	**%**			
CD45	**23**	**88.5**	**3**	**11.5**	**26**	**13**	**81.3**	**3**	**18.7**	**16**	**0.65**	**42**
CD20	**4**	**14.8**	**23**	**85.2**	**27**	**4**	**22.2**	**14**	**77.8**	**18**	**0.69**	**45**
CD10	**7**	**36.8**	**12**	**63.2**	**19**	**5**	**33.3**	**10**	**66.7**	**15**	**1**	**34**
BCL6	**3**	**12**	**22**	**88**	**25**	**1**	**8.3**	**11**	**91.7**	**12**	**1**	**37**
BLIMP1	**14**	**93.3**	**1**	**6.7**	**15**	**7**	**100**	**0**	**0**	**7**	**1**	**22**
MUM1	**24**	**100**	**0**	**0**	**24**	**16**	**100**	**0**	**0**	**16**	**1**	**40**

CD45: Of the PBm cases 23/26 (88.5%) were positive and 13/16 (86.7%) of the PBm+PCd were positive. The difference in CD45 positivity between the two morphological subtypes (PBm and PBm+PCd) was not significant (Fisher's exact test, p=0.66).

CD20: Four of 27 PBm cases (14.8%) were positive and 4 of 18 PBm+PCd cases (22.2%) were positive. The difference in CD20 expression between the two

morphological subtypes (PBm and PBm+PCd) was not significant (Fisher's exact test, p=0.69)

CD10: Of the PBm cases 7/19 were positive (36.8%). Five of 15 PBm+PCd cases (33.3%) were positive. The difference in CD10 expression between the two morphological subgroups (PBm and PBm+PCd) was not significant (Fisher's exact test, p=1).

BCL6: Three of 25 PBm cases (12%) were positive and 1/12 PBm+PCd cases (8%) was positive. The difference in positivity for BCL6 expression was not significant (Fisher's exact test, p=1).

BLIMP1: Fourteen of 15 PBm cases (93.3%) were positive and 7/7 PBm+PCd cases (100%) were positive. There was no significant difference for BLIMP1 expression between these two groups (Fisher's exact test, p=1).

MUM1: 100% (40/40) of cases were positive in both morphological groups.

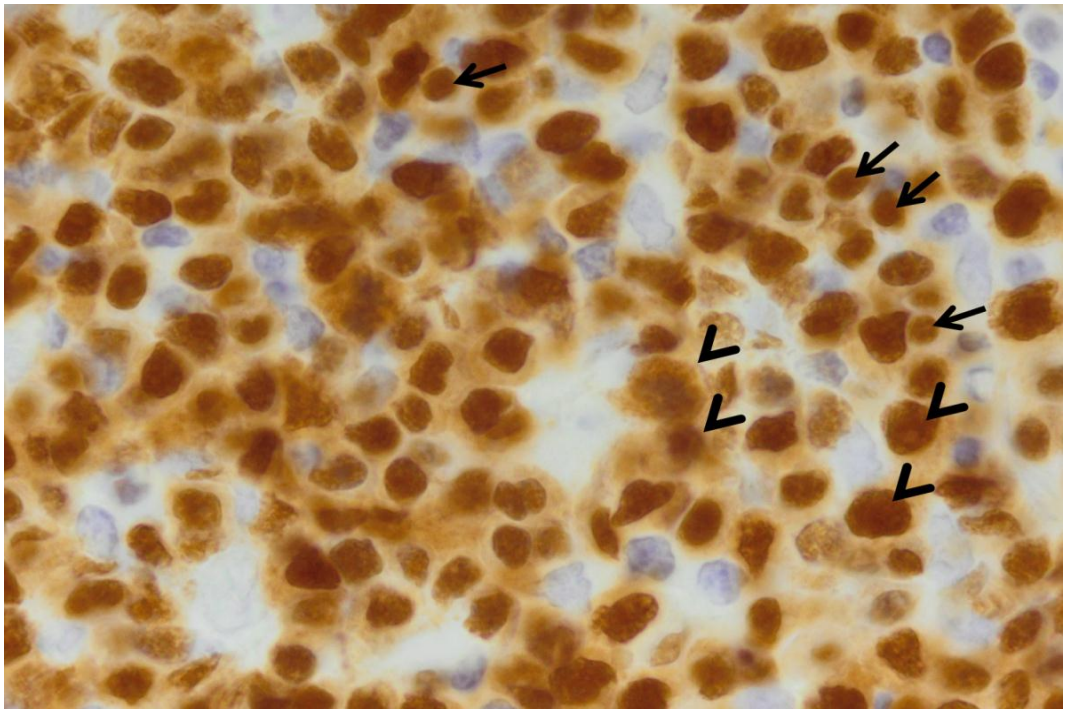

Fig. 5.13 Plasmablastic morphology (arrow heads) with plasmacytic differentiation (arrows) in PBm+PCd (MUM1, magnification X100)

Further detailed analysis of the CD45 and CD20 staining in each of the positive cases is presented in Tables 5.5 and 5.6 respectively. Table 5.5 indicates the 36 positive cases for CD45, the percentage positivity of tumour cells and the intensity of the signal against the morphological subtype. Half of the cases showed focal staining while the other half stained diffusely.

Table 5.5. CD45: Percentage positivity, intensity & morphology

CASE	%POS	INTENSE	MORPH
4	D	S	PBm+PCd
5	F	M	PBm
6	F	M	PBm
7	F	M	PBm
8	F	W	PBm+PCd
9	F	W	PBm
10	F	W	PBm+PCd
11	D	S	PBm+PCd
12	F	W	PBm
14	F	W	PBm+PCd
15	D	M	PBm+PCd
17	D	S	PBm
18	D	S	PBm
19	F	M	PBm
20	F	W	PBm
21	F	W	PBm+PCd
22	D	S	PBm+PCd
23	D	S	PBm
24	F	W	PBm
27	D	M	PBm
28	D	S	PBm
29	D	W	PBm
30	D	W	PBm+PCd
31	F	W	PBm+PCd
32	F	W	PBm+PCd
34	D	S	PBm
35	D	S	PBm+PCd
36	F	W	PBm
37	D	S	PBm+PCd
38	F	W	PBm
39	D	S	PBm
40	D	M	PBm+PCd
41	D	S	PBm
43	D	S	PBm
44	F	W	PBm
45	F	W	PBm

Key:
%Positivity **N= <5%, F= 5-50%, D= >50%**
Intense **W= weak, M= moderate, S= strong**

Table 5.6 denotes the percentage positivity of tumour cells with the CD20 antibody in each of the 8 positive cases and the intensity of the signal against the morphological subtype. All but one case showed focal staining and this was of weak to moderate intensity.

Table 5.6. CD20: Percentage positivity, intensity & morphology

CASE	%POS	INTENSE	MORPH
14	F	W	PBm+PCd
16	F	W	PBm
17	F	W	PBm
29	F	W	PBm
30	D	W	PBm+PCd
33	F	M	PBm+PCd
38	F	M	PBm
40	F	M	PBm+PCd

Key:
%Positivity **N= <5%, F= 5-50%, D= >50%**
Intense **W= weak, M= moderate, S= strong**

5.5 EBV status

There were results for 40 cases, 37 (92.5%) of which were positive, giving a deep blue nuclear signal (Fig. 5.14 & 5.15).

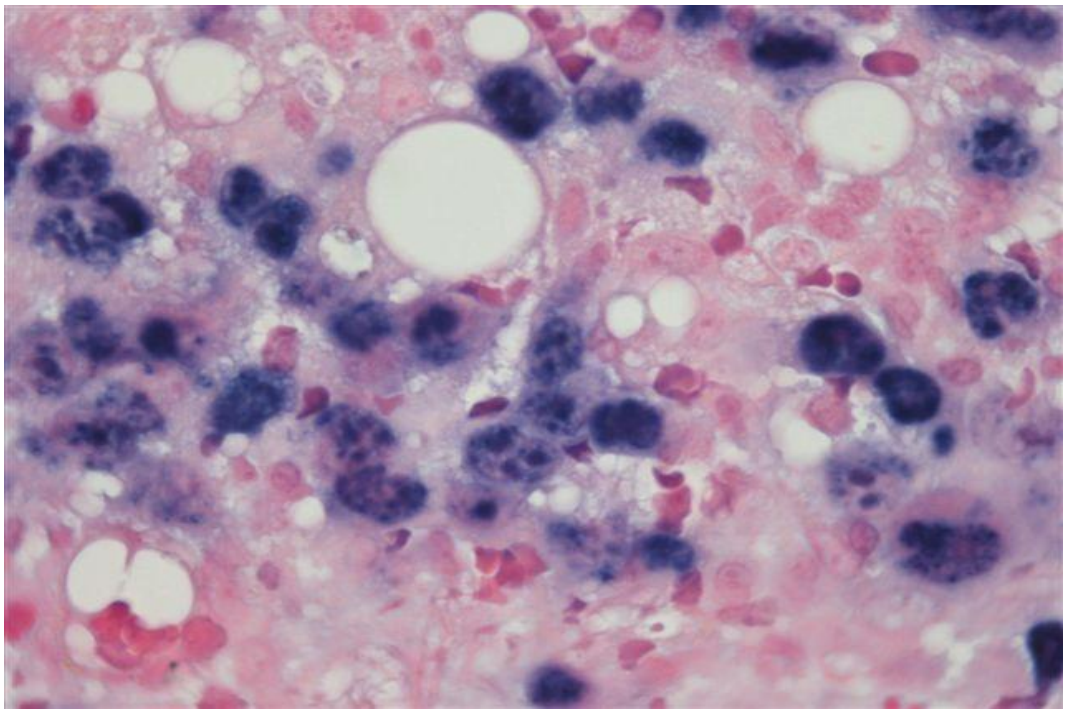

Fig. 5.14 PBL in bone marrow showing positive nuclear expression in tumour cells (EBER ISH, magnification X100)

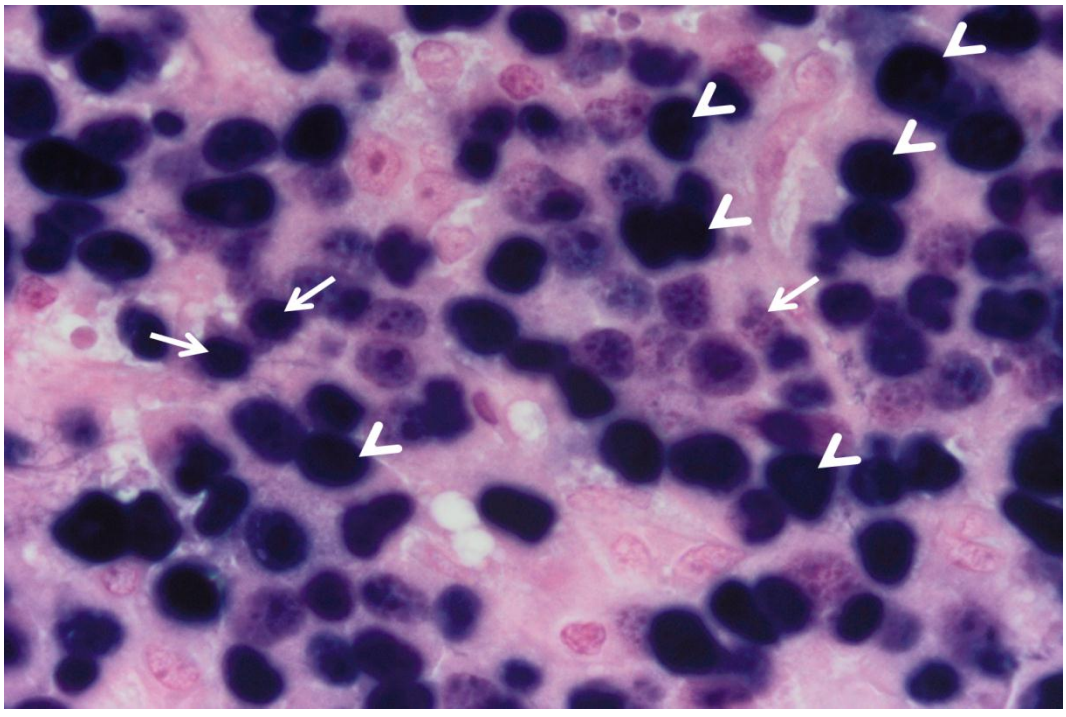

Fig. 5.15 Plasmablastic morphology (arrow heads) with plasmacytic differentiation (arrows) in PBL (EBER ISH, magnification X100)

Fisher's exact test was used to assess for a statistically significant difference in EBER positivity between those tumours with a pure plasmablastic morphology (PBm) and those plasmablastic lymphomas showing plasmacytic differentiation (PBm+PCd). There was no significant difference in positivity for EBV messenger RNA (EBER) between these two morphological groups (p=0.63) (Fig. 5.16).

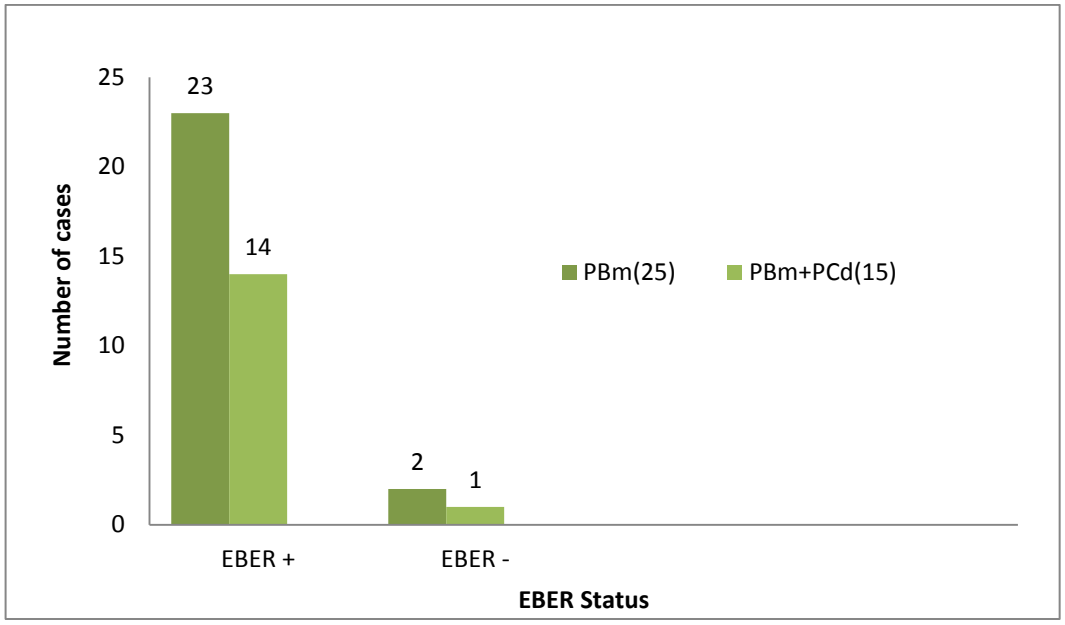

Fig. 5.16 EBER positivity according to histological subtype

CHAPTER 6

6.0 Discussion

PBL is defined as an aggressive, high-grade B-cell non-Hodgkin lymphoma (NHL) occurring classically within the oral cavity of immune compromised individuals, most notably in the setting of acquired immune deficiency syndrome (AIDS) (Stein et al 2008; Gogia et al 2010). The tumour was first described in the oral cavity by Delecluse et al in 1997 and has subsequently been reported to occur, albeit less commonly in a variety of extra-oral sites (Castillo & Reagan 2010). The tumour is characterised by terminal B-cell or plasma cell differentiation and the acquisition of a variety of plasma cell markers, with the loss of conventional B-cell markers (Carbone & Gloghini 2008; Hsi et al 2011). In a high prevalence HIV setting, it is the most frequently diagnosed oral lymphoma where it occurs more commonly than in extra-oral locations (Francischini et al 2010).

The aim of this study was to determine the demographics, morphology, immunohistochemical profile and EBV status of patients with extra-oral PBL in a clinical setting with a high prevalence of HIV infection. The aims and objectives of this study have been achieved but there are a number of results which are worthy of further comment.

The age range in our series was similar to that quoted in the literature, the majority of cases occurring in adults in the 4thdecade (Dong et al 2005; Folk et al 2006; Stein et al 2008), but there was a single paediatric case which was an HIV positive 9 year old male patient who presented with a tumour in the nasopharynx, the only case to arise in this site. It showed a plasmablastic morphology with plasmacytic

differentiation (PBm+PCd) and was negative for both CD45 and CD20, positive for VS38c, MUM1 and BLIMP1 and positive for EBER.

There are infrequent reports of PBL occurring in the paediatric age group. Pather et al (2013) reported 3 cases between the ages of 9 and 11 years, all in HIV positive children presenting with extra-oral disease (orbital, nasal and scalp). The 3 tumours were positive for EBER mRNA by in-situ hybridisation.

All patients were black Africans. This needs to be interpreted with caution as the laboratory from which the material was obtained serves a predominantly black population.

Unfortunately not all patients in our series had HIV results but of those patients for whom the HIV status was known, all but one patient was HIV positive (94%). The HIV negative case was a 59 year old male with an intra-abdominal tumour. All case reports and series of cases of PBL, both oral and extra-oral, show a strong association with HIV infection, documented at 97% by Boy et al (2011), and 100% by Dong et al (2005). In fact, PBL, together with primary effusion lymphoma and PBL arising in the setting of multicentric plasma cell variant of Castleman disease are recognised as occurring specifically in HIV seropositive patients (Stein et al 2008). However, the literature does describe single case reports of PBL occurring in immune competent individuals and in immune compromised settings other than HIV infection, such that the occurrence in our series of a single case, in an HIV-negative individual is not surprising (Kim et al 2009; Rafaniello-Raviele et al 2009).

There is no evidence that HIV by itself is capable of causing neoplastic transformation. There is however little doubt that with prolonged survival the number of AIDS patients who develop NHL has increased steadily. Registry links in North America and Western Europe show the incidence of NHL in HIV positive individuals in the pre-HAART era was 60-200-fold higher than in a matched HIV negative population (Palmieri et al 2006), with an even greater risk for primary cerebral lymphomas. This risk does not appear to have reduced since the introduction of highly active antiretroviral therapy in South Africa (Patel et al 2015). The pathogenesis of AIDS associated B-cell lymphomas probably involves sustained B-cell proliferation and during this frenzy of proliferation some clones undergo mutations or translocations involving oncogenes such as *MYC* or tumour suppressor genes. Neoplastic transformation ensues with clonal proliferation and addition of subsequent mutations (Dong et al 2005; Stricker et al 2010).

The cases in this series occurred in many diverse anatomical locations, most commonly the anus. As far as one could tell, origin was predominantly extranodal because of the absence of recognisable lymph node in the biopsy material at our disposal.

Two morphological subtypes of PBL were recognised. Distinction between the two morphological variants proved of no importance as this did not appear to be influenced by patient age or gender; nor was there a significant difference between the histological subtype and primary site of tumour. This is at variance with the findings of Hansra et al (2010) where a pure plasmablastic morphology occurred preferentially in oral based PBL, and PBL showing plasmacytic differentiation

occurred more commonly in extra-oral sites. This observation has not been endorsed by others, (Boy et al 2010; Dong et al 2005; Colomo et al 2004).

Lack of clinical correlation pertaining to clinical stage of disease and the possibility of concurrent oral involvement by PBL cannot be discounted. In all instances, the biopsy site provided on the specimen label was taken as representative of the site of presentation of the disease but it cannot be stated with certainty that oral PBL was not present at the time of biopsy of the extra-oral sites.

Before attempting to interpret our immunohistochemical results, cognisance needs to be taken of two major shortcomings of the study. Firstly, we experienced considerable difficulties with the use of the TMA. Whilst the assessment of immunohistochemistry on TMA has been validated in numerous studies (Jawhar 2009), we found that mainly owing to lack of experience on our part its use in this study caused many difficulties. It would have been preferable to retain all specimens as whole mount sections to assure optimal tumour representation. The implementation of the TMA allowed for curtailment in cost of antibodies, but the 1mm-diameter cores did not provide sufficient tumour representation for the assessment of some antibodies, particularly CyclinD1 (11 cases not assessable), PAX5 (10 cases), CD138 (7 cases) and Ki67 (7 cases). In some instances, the tumour tissue was replaced by necrotic tumour or uninvolved soft tissue on deeper sections. PBL is a high grade tumour that frequently displays large areas of tumour necrosis, such that there often was a limited quantity of viable, optimally preserved tumour from which to obtain additional cores. This contributed to the decision to perform a single core on 15 of the 25 cases. In other cases, following

extensive immunohistochemical work-up at the time of diagnosis, most of the tissue block had been cut through and the core obtained for TMA floated out of the recipient TMA block. This difficulty in core preservation resulted in five cores being cut through or lost before a full antibody panel was completed and contributed to the total number of cases not being assessed with the full immunohistochemical panel.

Secondly, was the use of archive sections that had been stained at the time of the original diagnostic work-up, rather than in a single fresh run. Thus, three types of sections were included for the immunohistochemistry results: a) TMA b) newly cut whole mount sections c) archived immunohistochemistry sections.

The differences in immunohistochemical expression might reflect differences in fixation or storage of tissues, antigen retrieval methods and laboratory procedures. Immunohistochemical stains on original sections at the time of diagnosis were clearly not done together in a single run under the same laboratory conditions. The period of 6 years during which the cases included in the study sample were diagnosed introduces the possibility that previous laboratory conditions at the time of diagnosis were not identical to those implemented at the time of the study. This could give rise to some variation in the results recorded.

In attempting to establish the immunophenotypic profile of PBL, a number of unusual findings were encountered. These consisted of:

1) **CD45.** Eighty-five percent of our cases stained positively with this antibody, with staining being diffuse and intense in some cases. The WHO

Classification reports that CD45 staining is usually negative or weak and focal (Stein et al 2008) while others have reported positivity rates ranging from 0-78% (Boy et al 2010) and 40-67% (Urrego et al 2011).

2) **CD20.** We found CD20 positivity in 8/45 (17.7%) of cases. However positivity was always focal and weak. The WHO reports that CD20 may be either positive or negative (Stein et al 2008), while Boy et al (2010) found no positive staining in their series.
3) **CD3** was positive in 2 of 45 (4.4%) cases. This is regarded as an aberrant phenotype that is described to occur in rare cases of DLBL and PBL (Wang et al 2009; Suzuki et al 2010). Several mechanisms have been proposed to account for the expression of CD3 in B-cell neoplasms. De-repression of genetic material as a result of neoplastic transformation may account for this phenomenon. The transformation of a progenitor cell prior to full divergence of the B-lymphocyte pathway may be a further explanation for the co-expression of CD3 in B-cell tumours (Wang et al 2009) Lineage ambiguity, infidelity and promiscuity are well recognised in immunophenotypically complex haemopoietic neoplasms, but this pertains more to immature tumours such as acute leukaemias (Borowitz & Chan 2008) Terminally differentiated tumours, of which PBL could be considered an example, may show a tendency to lineage transdifferentiation. Cobaleda et al (2007) showed that mature B-cells are able to transform into functional T-lymphocytes in the absence of PAX5, suggesting that true transdifferentiation may occur in terminally differentiated cell types. In the series by Boy et al (2010), CD3 was reported as negative in the tumour cells, being present only in the reactive T-lymphocytes.

4) **BCL6** was positive in 10.8% (4/37) cases in the study. This was at variance with the postulated origin of PBL, namely that it is a tumour of post-germinal centre phenotype aligned to the activation status of DLBL (Castillo and Reagan 2011). BCL6 protein expression implies germinal centre origin. Chromosomal translocations may cause constitutive expression of BCL6 resulting in DLBL (Saito et al 2007). It is possible that a similar mechanism resulting in constitutive expression of BCL6 may occur in PBL, which would account for the positive signal observed with the BCL6 antibody in our study.

5) **CD10.** Of the 34 cases assessed for CD10, 12 (35.3%) were positive in our cohort. CD10 is known to be aberrantly expressed in cases of plasma cell myeloma (Vega et al, 2005), and this may reflect the extent of plasmacytic differentiation in these cases. Six of the 12 cases co-expressed BLIMP1 which is a marker expressed in B-cells that are dedicated to the pathway of plasma cell differentiation. By definition, BLIMP1 expression contradicts any marker of germinal centre phenotype, although a measure of 'overlap' is conceivable in a minority of cases as a lymphocyte acquires BLIMP1 expression but loses CD10 expression. CD10 and BLIMP1 should be mutually exclusive. All 12 CD10-expressing cases also expressed either VS38c or MUM1, or both. As with BLIMP1, VS38c and MUM1 are markers of plasma cell differentiation. Beyond the possibility of 'overlap' co-expression of CD10 and these plasma cell markers, the biological programme underlying these markers and their co-expression is unclear and can only be attributed to an aberrant phenotype.

6) The **Ki67** proliferation index for PBL is generally reported as being very high (75-100%). Most of our cases were within this range but in occasional cases the PI was of the order of 40-60%. This is undoubtedly a reflection of the difficulties we experienced with the use of the TMA.
7) The plasma cell markers **CD138** (64.7% positive), **VS38c** (91.6% positive), **BLIMP1** (95.5% positive) and **MUM1** (100% positive) indicated plasma cell differentiation in variable case numbers, with MUM1 being the most consistently positive. Of the 38 cases tested for BLIMP1, 16 cases were equivocal and one was negative. The high rate of equivocal results for BLIMP1 (16 of 38 cases tested, 42.1%) was thought to be a result of technical and TMA difficulties. The antibody is designed for assessment on frozen tissue and optimisation for formalin fixed paraffin embedded tissue proved difficult, such that the positive nuclear signal obtained in 21 of the 38 cases tested was often weak and focal, both on the TMA and on the whole mount sections. In view of this, the positivity rate of all cases tested was considered to be an under-representation of the actual BLIMP1 status in this study. Of the four plasma cell markers, MUM1 was found to be the most consistently positive in this cohort.
8) One of 16 cases assessed for **HHV8** was positive for this viral marker. Review of the histology revealed this to be a PBL arising in the context of plasma cell variant Castleman disease in a lymph node of a 35-year-old HIV positive male. In the Boy et al (2010) series, no cases of HHV8 positivity occurred.

The role of EBV which is known to be a polyclonal mitogen for B-cells together with certain cytokines is to stimulate proliferation of B-cells. A variety of potent cytokines and chemokines are induced by EBV infected B- and T-lymphocytes. Viral IL-10 stimulates B-cell replication, inhibits antigen presentation and T-cell growth, protects T-cells from death and suppresses IFN-γ secretion. The induction of cellular cytokine expression by EBV infected cells leads to a pattern of autocrine and paracrine stimulation which likely promotes the development of B-cell lymphomas (Cohen et al 2009). About half of all lymphomas are latently infected with EBV (Kumar et al 2010). Although EBV is believed to be important its exact role in the aetiopathogenesis of PBL remains poorly understood. In our study, 92.5% (37/40) cases were positive for EBV. This compares favourably with the 74% positivity reported by Castillo et al (2008) and 100% positivity in the study by Dong et al (2005).

In their series of oral PBL, Boy et al (2010) demonstrated light chain restriction in 47% of the cases leading them to conclude that such cases were not “true” PBLs but should be regarded and managed as extramedullary plasmacytoma. This conclusion has not been substantiated by other authors. Lorsbach et al (2011) recognise the difficulty in the distinction between plasma cell neoplasms (medullary and extra-medullary), particularly those with plasmablastic features as defined by Greipp et al (1998), and PBL. Lorsbach et al (2011) concluded that the best approach for the classification of these aggressive neoplasms with plasmacytic and plasmablastic differentiation in immune compromised hosts and the roles of EBV and c-*MYC* alterations in their pathogenesis require further study. We did not test for light chain restriction in our series but nevertheless urge a “wait

and see" approach until the publication of larger series of cases with excellent clinical and radiographic correlation and determination of patient outcomes, or until the emergence of new molecular and genetic evidence.

We have already alluded to the very poor prognosis suffered by patients with PBL. Accurate diagnosis is essential so that appropriate treatment can be offered. In the differential diagnosis of PBL both DLBL and EPCT/PCM need to be carefully considered. Clinicopathologic correlation is essential since these lesions show considerable overlap. The clinical characteristics of age, gender and site may give some indication as to the correct diagnosis since PBLs occur in a somewhat younger age group, are more common in males and occur most frequently in the oral cavity and in other extra-nodal sites. Lytic bone lesions, hypercalcaemia and a serum M-protein are often features of EPCT/PCM occurring uncommonly in the other two lesions. Patients with PBL are invariably immunosuppressed as a result of HIV infection, while those with DLBL are less commonly HIV seropositive and those with EPCT/PCM are invariably immune competent. Morphologically PBL tumour cells take on a plasmablastic or an admixed plasmablastic/plasmacytic cellular appearance. DLBL cells usually have a centroblastic morphology but may show the presence of admixed plasmablastic cells while in EPCT/PCM the tumour cells have the appearance of plasma cells. Immunophenotypically, PBL and EPCT/PCM are virtually always MUM1+, CD138+ and BLIMP1+ while DLBL is either MUM1 positive or negative but invariably CD138 negative. CD45 and CD20 are always positive in DLBL. CD20 is always negative in EPCT/PCM and CD45 and CD20 are variable in PBL with CD20 staining being very faint and focal when positive. The Ki67 proliferation index is usually >80% in PBL but may be as low as

40% in a few cases while both EPCT/PCM and DLBL in immune competent individuals show a much lower proliferation index, of less than 40%. The proliferation index in DLBL in HIV positive individuals may be particularly high, often exceeding 80% (Pantanowitz et al 2013) EBER ISH is invariably positive in PBL, is rarely positive in EPCT/PCM and may be either positive or negative in DLBL. Both PBL and EPCT/PCM usually show light chain restriction while this is not described in DLBL (Stein et al 2008).

CHAPTER 7

7.0 CONCLUSION

Extra-oral plasmablastic lymphoma has mimicked the clinical, pathological and immunophenotypic manifestations of oral plasmablastic lymphoma, in that it has shown a similar age and gender distribution, morphology and immunophenotype. As with oral plasmablastic lymphoma, it has shown a strong association with HIV infection and positive EBV status.

- This study has provided evidence that oral and extra-oral PBL should not be classified separately. These are manifestations of the same basic tumour which occur at different sites.
- The reasons as to why PBL occurs more frequently in the oral cavity than in extra-oral sites remains unknown.
- We have provided strong, albeit circumstantial evidence of the pivotal role played by EBV in the pathogenesis of this tumour. Further research at elucidating the molecular oncogenic pathway is recommended.
- A diagnostic and morphologic framework within which molecular studies can take place has been provided.
- A considerable degree of overlap exists between PBL, DLBL and EPCT/PCM and clinicopathologic correlation is essential in order to render an accurate diagnosis.
- Such correlation includes radiologic, histomorphologic, immunophenotypic parameters, and the assessment of EBV positivity and HIV seropositivity.

- From a diagnostic point of view an essential panel of antibodies is recommended and this should include CD45, CD20, MUM1, CD138 and Ki67. In situ hybridisation assessment for EBER should form part of this panel.
- Patients presenting with either primary oral or extra-oral manifestations of PBL should be evaluated for multicentric lesions.

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Appendix 1: Ethics clearance letter

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Yvonne Perner

CLEARANCE CERTIFICATE	**M120993**
PROJECT	EBV status in Extra-Oral Plasmablastic Lymphoma
INVESTIGATORS	Dr Yvonne Perner.
DEPARTMENT	School of Anatomical Pathology
DATE CONSIDERED	Ad hoc
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 28/09/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 2: Raw data

N	YEAR	AGE	GENDER	RACE	HIV STATUS	SITE	DIAGNOSIS
1	2006		M	B	N/S	SOFT TISSUE	PBm
2	2007	9	M	B	P	NASOPHARYNX	PBm+PCd
3	2007	35	F	B	N/S	ANUS	PBm+PCd
4	2008	39	F	B	N/S	SOFT TISSUE	PBm+PCd
5	2008		F	B	P	BREAST	PBm
6	2008	36	F	B	N/S	VULVA	PBm
7	2008	35	M	B	P	SKIN	PBm
8	2008	27	M	B	N/S	ANUS	PBm+PCd
9	2009	43	F	B	P	SOFT TISSUE	PBm
10	2009	25	F	B	N/S	SOFT TISSUE	PBm+PCd
11	2009	43	M	B	P	SOFT TISSUE	PBm+PCd
12	2009	30	F	B	N/S	ANUS	PBm
13	2009	42	F	B	P	S.I.	PBm
14	2009	31	F	B	N/S	LYMPH NODE	PBm+PCd
15	2009	49	F	B	N/S	SOFT TISSUE	PBm+PCd
16	2009	38	M	B	N/S	URETHRA	PBm
17	2009	33	M	B	N/S	ANUS	PBm
18	2009	33	F	B	P	RECTUM	PBm
19	2009	33	M	B	N/S	ANUS	PBm
20	2009	40	M	B	P	LYMPH NODE	PBm
21	2009	31	M	B	P	BONE MARROW	PBm+PCd
22	2009	54	M	B	N/S	ANUS	PBm
23	2010	26	M	B	N/S	SOFT TISSUE	PBm
24	2010	32	F	B	N/S	STOMACH	PBm
25	2010	46	F	B	N/S	SOFT TISSUE	PBm
26	2010	40	M	B	N/S	ANUS	PBm+PCd
27	2010	36	M	B	N/S	SOFT TISSUE	PBm
28	2010	34	M	B	P	SOFT TISSUE	PBm
29	2010	40	M	B	N/S	S.I.	PBm
30	2010	22	F	B	N/S	LYMPH NODE	PBm+PCd
31	2010	35	M	B	N/S	ANUS	PBm+PCd
32	2010	35	M	B	P	LYMPH NODE	PBm+PCd
33	2010	33	F	B	P	S.I.	PBm+PCd
34	2010	54	M	B	N/S	ANUS	PBm
35	2010	36	M	B	N/S	LYMPH NODE	PBm+PCd
36	2011	35	F	B	N/S	ANUS	PBm
37	2011	58	M	B	N/S	ANUS	PBm+PCd
38	2011	36	M	B	P	STOMACH	PBm
39	2011	31	F	B	N/S	SOFT TISSUE	PBm
40	2011	48	M	B	P	FORESKIN	PBm+PCd
41	2011	34	M	B	P	BONE MARROW	PBm
42	2011	59	M	B	N	ABDOMINAL CAV	PBm+PCd
43	2011	35	M	B	N/S	ANUS	PBm
44	2011	40	F	B	P	OVARY	PBm
45	2012	52	M	B	P	ANUS	PBm

N	YEAR	DX	CD45	CD20	CD3	CD79a	CD138	VS38c	MUM1	Ki67	BCL6	HHV8	CD10	BLIMP1	CYCLIND1	PAX5	EBERISH
1	2006	PBm	N	N	N	N	P	P	N/D	70%	N	N	N/D	EQUI	P	N	N
2	2007	PBm+PCd	N	N	N	N	N	P	P	N/A	N	N/D	N	P	N	N	P
3	2007	PBm+PCd	N/D	N	N	P	N/D	P	N/D	N/D	EQUI	N/D	N	P	N/D	N/D	N/D
4	2008	PBm+PCd	P	N	N	N/D	P	N/D	P	40%	N/A	N	P	EQUI	N	N	P
5	2008	PBm	P	N	N	P	P	N/D	P	90%	N/A	N/D	N/A	P	N	N	P
6	2008	PBm	P	N	P	N	N	P	P	60%	N	N/D	P	P	N	N	P
7	2008	PBm	P	N	N	P	N/D	N/D	N/D	80%	N	N/D	N/D	P	N/D	N/D	P
8	2008	PBm+PCd	P	N	N	P	N/A	P	P	80%	N/D	N/D	N	P	N/A	N/A	N/A
9	2009	PBm	P	N	N	P	N	N/D	N/D	95%	P	N/D	N	P	N	P	P
10	2009	PBm+PCd	P	N	N	P	N/A	P	P	95%	N	N/D	N	EQUI	N/A	N/A	P
11	2009	PBm+PCd	P	N	N	N	P	P	N/D	80%	N/D	N/D	N/D	N/D	N	N	P
12	2009	PBm	P	N	N	N	N/A	P	P	80%	N	N/D	P	EQUI	N/A	N/A	P
13	2009	PBm	N	N	N	N	P	N	P	N/D	N	N/D	N	EQUI	N	N	N
14	2009	PBm+PCd	P	P	N	P	P	P	P	90%	N/D	N/D	N/D	N/D	N	P	P
15	2009	PBm+PCd	P	N	N	P	N	N/D	P	60%	N/A	N/D	N	EQUI	N	N	P
16	2009	PBm	N/D	P	N	N	N/A	P	P	N/A	N	N/D	N	P	N/A	N/A	P
17	2009	PBm	P	P	N	P	N	P	P	N/A	N	N/D	N	EQUI	N	P	P
18	2009	PBm	P	N	N	P	N/D	P	P	95%	N	N	N/D	N/D	N/D	N/D	N/D
19	2009	PBm	P	N	N	N/D	N/A	P	P	N/A	N	N/D	EQUI	EQUI	N/A	N/A	P
20	2009	PBm	P	N	N	N	N	P	P	N/D	N	P	N	N/D	N/D	N/D	P
21	2009	PBm+PCd	P	N	N	N	P	P	P	95%	N	N/D	P	P	N	N	P
22	2009	PBm	P	N	N	P	N	P	P	80%	N	N	P	EQUI	N	N	P
23	2010	PBm	P	N	P	P	N/A	P	P	N/A	N	N/D	P	P	N/A	N/A	P
24	2010	PBm	P	N	N	N	P	P	P	95%	N	N/D	N	P	N	N	P
25	2010	PBm	N	N	N	N	N	P	P	90%	N	N/D	N	P	N	N/D	P
26	2010	PBm+PCd	N	N	N	N	P	P	P	95%	N	N	P	P	N/D	N	P
27	2010	PBm	P	N	N	N	N	P	P	95%	P	N/D	N	N	N	N	P
28	2010	PBm	P	N	N	N	P	N/D	P	100%	N	N/D	P	P	N	N	P
29	2010	PBm	P	P	N	P	P	P	P	N/A	N	N/D	P	EQUI	N/A	N/A	P
30	2010	PBm+PCd	P	P	N	P	N/A	P	P	90%	P	N/D	N	EQUI	N/A	N/A	P
31	2010	PBm+PCd	P	N	N	P	P	P	P	80%	N	N	P	EQUI	N	N	P
32	2010	PBm+PCd	P	N	N	N	P	N/D	P	80%	N	N	N	N/D	N	N	N
33	2010	PBm+PCd	N/D	P	N	P	N	P	P	70%	N	N	P	EQUI	N	N	P
34	2010	PBm	P	N	N	N	N/D	P	P	95%	N	N	N	N/D	N/D	N/D	N/D
35	2010	PBm+PCd	P	N	N	P	P	P	P	90%	N	N	N	P	N	N	P
36	2011	PBm	P	N	N	P	P	P	P	95%	N	N	N/D	P	N	P	P
37	2011	PBm+PCd	P	N	N	P	P	P	P	50%	N	N/D	N	EQUI	N	N	P
38	2011	PBm	P	P	N	P	P	P	P	N/A	P	N	N/A	P	N/A	N/A	P
39	2011	PBm	P	N	N	N	N	N/D	P	80%	N	N/D	N	P	N/D	N/D	P
40	2011	PBm+PCd	P	P	N	P	P	P	P	70%	N	N	N/D	P	N/D	N	P
41	2011	PBm	P	N	N	N	N	N	P	80%	N	N	N	N/D	N/A	N	P
42	2011	PBm+PCd	N	N	N	N/D	P	N/D	P	70%	N	N/D	N	EQUI	N/A	N/A	N/A
43	2011	PBm	P	N	N	P	P	P	P	95%	N	N/D	P	P	N	N	P
44	2011	PBm	P	N	N	N/D	P	P	P	80%	N	N/D	N	EQUI	N	P	P
45	2012	PBm	P	N	N	N	P	N	P	90%	N/D	N/D	N/D	P	N/D	N	P

Appendix 3: Turnitin report