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THE MOLECULAR PROFILE OF ORAL PLASMABLASTIC LYMPHOMAS IN A SOUTH AFRICAN POPULATION SAMPLE

by

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DECLARATION

I, Sonja Catharina Boy, hereby declare that the work on which this thesis is based, is original and that neither the whole work nor any part of it has been, is being, or shall be submitted for another degree at this or any other university, institution for tertiary education or examining body.

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SC Boy

I DEDICATE THIS THESIS TO:

MY TWO CHILDREN, MARINUS AND NATANYA, BOTH BORN DURING THE FOUR YEAR DURATION OF THIS PROJECT, AND WHO HAD TO SHARE MY TIME AND LOVE WITH THE BIGGEST RESEARCH PROJECT OF MY LIFE

MY PARENTS, JOSEPH AND DINA FOR THEIR UNCONDITIONAL LOVE AND SUPPORT TO FULFILL THIS DREAM

MY HEAVENLY FATHER WHO GAVE ME THE IMPOSSIBLE STRENTH TO PERSIST

SUMMARY

Plasmablastic lymphoma (PBL) was originally described in 1997 as an AIDS-associated tumour although cases have been described in individuals not infected with HIV. Due to the high number of people living with HIV in South Africa, a substantial number of cases are diagnosed annually and 45 cases were included in this study. This represented the largest cohort of PBL affecting the oral mucosa published to date. Three main aspects of PBL were investigated: pathological features, viral status and certain genetic characteristics.

The results from the genetic studies were the most important and interesting. These included rearrangements of the *IGH* gene in 63% and *MYC*-rearrangements in 62% of PBL's. Seven of 43 cases (16%) showed rearrangement of both the *IGH* gene alleles, a finding never described before. New genetic findings also included increased *CCND1* gene copy numbers in 17/41 (42%) and increased *IGH* gene copy numbers in 6/41 (15%) of cases.

The exact role of *MYC*-rearrangements in the development of PBL is unclear. Many factors may be responsible for *MYC* deregulation but in the case of PBL of the oral cavity the possible role of Epstein Barr Virus (EBV) infection was considered. All but one of the patients with known HIV-status (32/45) was HIV positive and I supported the proposal that the diagnosis of PBL should serve as a sign of immunodeficiency, either as diagnostic thereof or as a predictor of a progressive state of immunodeficiency in patients with known HIV/AIDS status. The HIV-negative patient in this study was the only one that presented with an EBV-negative PBL on *in situ* hybridisation. The clinico-pathological features of the current study therefore strongly suggested an association between EBV, PBL and HIV/AIDS although the exact nature thereof remains uncertain.

Routine genetic evaluation of tumours diagnosed as PBL should be introduced, as this may have prognostic and eventually treatment implications in the future. The exact panel of genes to be evaluated with a possible diagnosis of PBL

should still be determined but examination of *IGH* and *MYC* for rearrangements should be included.

This study proved the histomorphological features including the degree of plasmacytic differentiation not to have any diagnostic role although its prognostic value should be determined. The results of the immunohistochemical investigations performed in this study confirmed PBL always to be negative for CD20 but proved PBL not to be a morphological or immunohistochemical diagnosis by any means.

In conclusion, it became clear that PBL should never be diagnosed without thorough clinical, systemic, pathological and genetic investigations, especially in the backdrop of HIV/AIDS. No pathologist should make the diagnosis of PBL and no clinician should accept such a diagnosis or decide on the treatment modality for the patient involved unless all other possibilities of systemic plasma cell disease have been excluded.

Key Words:

Lymphoma; HIV/AIDS; plasmablastic lymphoma; Epstein Barr virus (EBV); *MYC* rearrangement;

PUBLICATIONS AND PRESENTATIONS

Publications

- Boy SC, van Heerden MB, Raubenheimer E, van Heerden WFP. Plasmablastic lymphomas with light chain restriction – Plasmablastic extramedullary plasmacytomas? *Journal of Oral Pathology and Medicine*, 39 (5): 435-439, 2010.
- Boy SC, van Heerden MB, Babb C, van Heerden WFP, Willem P. *MYC* aberrations and EBV infection are major role players in the pathogenesis of HIV-related Plasmablastic lymphomas. Accepted for publication in the *Journal of Oral Oncology***,** January 2011.

National Congress presentations:

- Van Heerden M, Boy SC, van Heerden WFP. Necessity of a negative control as well as the stringency of the post hybridization wash in the *in situ* hybridization protocol. *Pathvine IAP South African Division Congress*. Cape Town, South Africa, September 2010.

International Congress presentations

- Boy SC, van Heerden MB, Bapp C, van Heerden WFP. The immunohistochemical and viral profile of plasmablastic lymphomas in a South African population sample. *22nd European Congress of Pathology*, Florence, 2009.

- Boy SC, van Heerden MB, Bapp C, van Heerden WFP, Willem P. Burkittt's translocation is a common finding in plasmablastic lymphomas. *15th International congress of IAOP*, Seoul, Korea, August 2010.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Acquired immune deficiency syndrome	AIDS
Activation-induced cytidine deaminase	AID
Anaplastic lymphoma kinase-1	ALK-1
Alkaline phosphatase substrate buffer	AFSB
B-cell lymphoma-6 protein	BCL6
B-lymphocyte induced maturation protein	BLIMP-1
Break apart	BA
5-bromo-4-chloro-3-indolylphosphate	BCIP
Burkitt's lymphoma	BL
Centromere enumeration probe	CEP
Class-switch recombination	CSR
Cluster of differentiation	CD
Constant regions	C regions
Cyclin D1	CCND1
4', 6-Diamidino-2-phenylindole dihydrochloride	DAPI
Diffuse large B-cell lymphoma	DLBCL
Diversity gene segments	D segment
Deoxyribonucleic acid	DNA
Epithelial membrane antigen	EMA
Epstein Barr virus	EBV
EBV-encoded latent membrane protein-1	LMP-1
EBV-encoded RNA	EBER
Ethylene diamine tetra-acetic acid disodium salt	EDTA
Extra-medullary plasmacytomas	EMPC
Fibroblast growth factor receptor	FGFR
Fluorescein isothiocyanate	FITC

Fluorescent *in situ* hybridisation	FISH
Formalin fixed paraffin embedded	FFPE
Germinal center	GC
Haematoxylin and eosin	H&E
Heat induced epitope retrieval	HIER
Highly active antiretroviral therapy	HAART
Human herpesvirus-8	HHV-8
Human immunodeficiency virus-1	HIV-1
Hydrochloric acid	HCl
In situ hybridisation	ISH
Interferon regulatory factor 4	*IRF*-4
Interleukin 6	IL-6
Immunoglobulins	Ig
Immunoglobulin heavy chain gene	*IGH*
Junctional gene segments	J segment
Kappa light chain	κ
Lambda light chain	λ
Major histocompatibility complex	MHC
Monoclonal gammopathy of undetermined significance	MGUS
Mucosa associated lymphoid tissue	MALT
Multiple myeloma	MM
Multiple Myeloma oncogene-1	MUM-1
Nitroblue tetrazolium	NBT
Non-Hodgkin's lymphoma	NHL
Not otherwise specified	NOS

Phosphate buffered saline buffer	PBS
Plasmablastic lymphoma	PBL
Polymerase chain reaction	PCR
Primary effusion lymphoma	PEL
Recombination activating enzyme 1/2	RAG1/2
Revised European American Classification of Lymphoid Neoplasms	REAL
Saline sodium citrate	SSC
Sodium thiocyanate	NaSCN
Somatic hypermutation	SHM
Tris Buffered Saline	TBS
Variable regions	V regions
World Health Organisation	WHO
X-box binding protein-1	*XBP-1*

LIST OF ANNEXURES

INTRODUCTION AND AIM

South Africa is the country with the highest number of people living with human immunodeficiency virus (HIV) in the world. By mid 2006 it was estimated that more than half a million adults in South Africa were ill with acquired immune deficiency syndrome (AIDS)[1]. The majority of HIV positive patients in South Africa present to the public health care sector only when they reach end-stage HIV disease.

Non-Hodgkin's lymphoma (NHL) was included as an AIDS-defining illness in 1985 and the incidence of all subtypes thereof shown to increase 60-200 times in the presence of HIV-related immunosuppression[2]. The vast majority of HIV-associated lymphomas are aggressive, high-grade B-cell lymphomas such as Burkitt's lymphoma (BL), diffuse large B-cell lymphoma (DLBCL) and its variants, primary effusion lymphoma (PEL) and plasmablastic lymphoma (PBL)[2]. Lymphomas as AIDS-defining diseases are increasing[3, 4].

PBL as an entity was initially described in 1997 by Delecluse and co-workers as a lymphoma affecting the oral cavity of HIV-infected individuals[5]. Even though cases of PBL in immune competent individuals have been described, the stronger association thereof with HIV infection and advanced immunosuppression has been confirmed by many. It was recently even suggested that immunosuppression should be suspected and excluded when the diagnosis of PBL is made in an individual with otherwise unknown immune status[6-8].

The clinico-pathological features, association with viruses and genetic features of PBL have been described by many, both as single case reports and case series. Although initially described as an oral tumour, substantial evidence has proven its existence in anatomical locations outside of this confined cavity. The oral mucosa however still seems to be the preferred site of involvement.

PBL is currently recognised as a highly aggressive neoplasm that, with or without treatment, has a median survival of less than 12 months. A better

understanding of the pathogenesis and biology of this neoplasm is therefore essential for development of more effective treatment strategies which will enable oncologists to extend the survival of patients afflicted by this malignancy.

The pathogenesis of PBL has not been elucidated and the normal cell counterpart from which this lymphoma takes origin is still to be determined. After its initial inclusion in the 2001 World Health Organisation (WHO) classification of tumours of haematopoietic and lymphoid tissues as a subtype of DLBCL, it was reclassified as a separate diagnostic entity in the 2008 classification[9]. Even after its reclassification, it remains difficult to distinguish PBL from extra-medullary plasmacytomas or multiple myeloma (MM) with plasmablastic morphology and the possibility that PBL may be related to these neoplasms has been proposed[10-12].

PBL is variably associated with the Epstein Barr virus (EBV)[5, 11, 13, 14], but the role of this virus in the pathogenesis of PBL remains unexplained. A possible aetiological role of the human herpesvirus-8 (HHV-8) has been proposed by some[15-20] but the presence of HHV-8 is currently accepted to rule against the diagnosis of PBL[21, 22].

Evidence for the role of genetic aberrations in the pathogenesis and biological nature of lymphomas has grown substantially since its incorporation into the diagnostic armamentarium of the WHO classification of tumours of haematopoietic and lymphoid tissues in 2001[23]. Certain genetic rearrangements have become diagnostic for some lymphomas and its role as indicators of prognosis and long term survival as well as the influence thereof on the choice of treatment has been demonstrated. Recently, reports on some genetic features of PBL's have shown the frequency of *MYC* gene rearrangements, mostly with the immunoglobulin heavy chain gene (*IGH*) as a partner[13] and suggested a possible role for *MYC* in the pathogenesis of these tumours[11, 13, 24-28]. Despite this, there is a significant shortage of genetic data relating to this neoplasm, most likely due to its overall rarity apart from countries with high incidences of HIV/AIDS. As a result of the high incidence of HIV-associated pathology encountered in South Africa, a substantial

number of PBL's are diagnosed annually and therefore available to study these neoplasms.

The aim of this study was to examine various aspects of the molecular features of PBL in a South African population sample, known for its high incidence of HIV/AIDS and to compare these with what has been published in the literature. A thorough morphological description of the cellular features was done in order to evaluate the validity of the morphological classifications proposed by several groups over a period of time. Evaluation and detailed description of the immunophenotypic features through various selected antibodies was done in order to evaluate its diagnostic role in PBL affecting the oral cavity. The possible role of the EBV in the pathogenesis and pathology of PBL was evaluated in this study and compared to the variable incidence reported in the literature. A possible role for HHV-8 in the pathogenesis of PBL is postulated in the world literature which included work in South Africa. It was decided to examine all the cases included in the current study for the presence of this virus. Due to the increase in genetic studies on PBL recently published in case reports and small series, it was also decided to evaluate our cases for certain genetic alterations of the *MYC* and *IGH* genes and compare the results with what has been found by others.

The results of this study may help to set the diagnostic criteria of PBL affecting the oral cavity and explain its aggressive clinical behaviour and poor prognosis. Knowledge of the molecular nature of PBL may eventually assist oncologists to develop more appropriate treatment regimes for patients afflicted by this neoplasm.

CHAPTER I

LITERATURE REVIEW

1.1 CLASSIFICATION OF PLASMABLASTIC LYMPHOMAS

The 2001 WHO Classification of tumours of the haematopoietic and lymphoid tissues was the first true world-wide consensus classification of haematological malignancies[23]. This classification was based on the 1994 'Revised European American Classification of Lymphoid Neoplasms' (REAL) classification which uses morphological, immunophenotypic and genetic features as well as clinical features to define an entity. The importance of each of these features varies among different lymphomas and a single "gold standard" for diagnosis does not exist. The WHO Classification of haematological neoplasms "groups" neoplasms mainly according to their cell lineage after which distinct diseases within each category are further defined using a combination of morphology, immunophenotype, genetic features and clinical syndromes. It seems relevant to base the classification of lymphoproliferative neoplasms on the corresponding cell lineage in a normal state but several entities in this group of malignancies do not have a normal differentiation stage such as hairy cell leukemia. Some lymphoid neoplasms also have immunophenotypic heterogeneity and the normal counterpart of the neoplastic cell can therefore not be the sole basis for classification[29]. The 2008 WHO Classification has refined the 2001 classification. More attention is now given to genetic features, clinical information and to the immunophenotype of neoplasms rather than being based merely on morphology[30].

The name 'oral plasmablastic lymphoma' was proposed by Delecluse in 1997 after describing a high grade neoplasm with a very specific immunophenotype in the oral cavities of 16 immunosuppressed patients[5]. In the last decade numerous additional cases were published which exhibited morphological and immunophenotypic features similar to that of 'oral plasmablastic lymphoma'. These included both oral and extra-oral cases in both immunosuppressed and immune competent individuals. Less than 200 cases of PBL affecting the oral

cavity have been published in the literature[6, 8, 14, 16, 17, 20, 25, 26, 31-71]. Up until 2008[9] the WHO classified 'oral plasmablastic lymphoma' as a subtype of DLBCL[23]. Since the 2001 classification[23], various researchers however proposed the term 'oral plasmablastic lymphoma' to be changed to 'plasmablastic lymphoma' (PBL). The neoplasm was subsequently reclassified as a separate diagnostic entity, distinct from DLBCL in the 2008 WHO classification of tumours of the haematopoietic and lymphoid tissues (Table 1)[9].

Table 1: *Diffuse large B-cell lymphoma: variants, subgroups and subtypes/ entities (Adapted from the 2008 WHO classification[9])*

Diffuse large B-cell lymphoma not otherwise specified (NOS)

- Common morphologic variants
  - Centroblastic
  - Immunoblastic
  - Anaplastic
- Rare morphologic variants
- Molecular subgroups
  - Germinal centre B-cell-like
  - Activated B-cell-like
- Immunohistochemical subgroups
  - CD5-positive DLBCL
  - Germinal centre B-cell-like
  - Non-germinal centre B-cell-like

Diffuse large B-cell lymphoma subtypes

- T-cell/histiocyte-rich large B-cell lymphoma
- Primary DLBCL of the central nervous system
- Primary cutaneous DLBCL, leg type
- EBV positive DLBCL of the elderly

Other lymphomas of large B-cells

- Primary mediastinal (thymic) large B-cell lymphoma\DLBCL associated with chronic inflammation
- Lymphomatoid granulomatosis
- ALK-positive large B-cell lymphoma
- Plasmablastic lymphoma
- Large B-cell lymphoma arising in HHV-8-associated muticentric Castleman disease
- Primary effusion lymphoma

Borderline cases

- B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma
- B-cell-lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

1.2 CLINICO-PATHOLOGIC FEATURES OF PLASMABLASTIC LYMPHOMAS

1.2.1 Clinical features

Since its initial description in 1997, the published literature on PBL has increased substantially over the last 13 years[5]. It was confirmed to be strongly associated with immunodeficiency, particularly with HIV infection. A recent review reported 79% of PBL cases to be HIV related and the rest to be associated with some other form of immunosuppression[8] such as organ recipients[54] and lengthy immunosuppressive therapy[61]. In this setting, PBL is commonly reported to be associated with advanced immunosuppression as demonstrated by CD4+ counts of patients well below 200 cells per microliter[7, 8]. PBL have however also been described in HIV negative individuals with other forms of immunosuppression[53, 58, 61, 69].

The gingiva and palatal mucosa are described as the predominant intra-oral sites affected by this tumour and account for more than half of the reported cases, occasionally with adjacent bone infiltration[5, 6, 8, 14, 20, 25, 26, 35-38, 40-42, 44-47, 72-74]. The lesions are typically fleshy masses on the gingiva or palate with a history of rapid growth, sometimes following dental extraction or complaints of tooth ache representing a possible tooth abscess[6].

According to the literature approximately 34% of PBL's occur in extra-oral locations either alone or in association with an oral mass[8]. The gastrointestinal tract and skin were the most common extra-oral sites reported[8]. Other extra-oral sites include the lung and bone marrow[24, 25, 32], spleen[75], upper airway tract[53, 56, 59, 72, 76, 77], orbit[78, 79], scapula[80], lymph nodes[25, 56, 58], esophagus[81], cranium and cervix[69], female breast[82], cardial muscle[83], gastrointestinal tract[17, 49, 56, 61, 77], spinal cord[70], para-vertebral area[71], mediastinum[25, 51, 56], testes[17, 59, 84], central nervous system[85, 86], rectum[56], anus[17, 25, 43, 52, 55, 56, 61] and skin[25].

All PBL's display an aggressive clinical course and extremely poor prognosis[5, 36, 37, 87]. Reports on the median survival of the afflicted patients vary from a

few months to three years. In some HIV-positive individuals the neoplasms have been described to regress when the patients received antiretroviral therapy[38, 51]. In these cases the median and the overall survival appears to be similar to that of previously reported AIDS-related lymphomas when the patient is well-controlled on highly active antiretroviral therapy (HAART)[61, 88, 89].

The median age of patients diagnosed with PBL is almost always around 39 years[5, 8, 14, 25, 61] with a male predominance confirmed by many[6, 8, 14, 90]. Interestingly the 2008 WHO classification states that PBL occur in patients with a median age of 50 years[9].

1.2.2 Microscopic features

Microscopically the neoplastic cells of PBL are described as large blastic cells with abundant cytoplasm, more or less eccentrically placed, slightly irregular round to oval nuclei with little, and fine chromatin. Plasmablasts were initially defined by Delecluse as cells that still have the blastoid features of immunoblasts but which have otherwise already acquired the antigen profile of plasma cells[5]. All tumour cells usually have prominently visible nucleoli. Some cells have immunoblastic features with a single prominent, centrally located nucleolus and others exhibit several peripherally located nucleoli. Both cell types with regard to the number and location of nucleoli have been described in the literature with the latter being more common[5, 14, 36, 37, 41, 73, 91] than the former[41, 56, 72, 73]. The cells of PBL have abundant cytoplasm with a paranuclear hof and demonstrate a proliferation index of more than 90% as determined by proliferation markers such as Ki-67 (MIB-1). Numerous interspersed tingible body macrophages result in a starry sky appearance. Necrosis and ulceration of the mucosal epithelium are common features in PBL of the oral cavity[5, 8, 14, 36].

Morphological classifications based on various parameters, but especially certain cellular features were proposed by some authors. These classifications divided PBL's into 'PBL of the oral mucosa' type, 'PBL with plasmacytic differentiation' and 'extra-medullary plasmablastic tumours

secondary to plasma cell neoplasms'[56, 92, 93]. 'PBL of the oral mucosa type' was defined as a monotonous proliferation of large lymphoid cells with immunoblastic features including abundant basophilic cytoplasm with occasional paranuclear hofs, little or no plasmacytic differentiation as well as EBV and HIV positivity in most cases[56]. In contrast, 'PBL with plasmacytoid differentiation' was defined as immunoblasts and plasmablasts with more differentiation towards mature plasma cells and less EBV and HIV positivity[56]. The third group defined as 'extra-medullary plasmablastic tumours secondary to plasma cell neoplasms' consisted predominantly of large immunoblasts and plasmablasts with a variable number of smaller cells with more mature plasma cell features intermingled with the former[56]. The latter group can only be considered when the patient has a clinical history of other plasma cell dyscrasias such as MM. Other morphological classification systems proposed for PBL include classifying these lesions as 'immunoblastic', 'Burkitt's-like' and 'plasmacytic' PBL's[91]. The latter classification did not get wide acceptance in the literature. The classification of these neoplasms into 'PBL of the oral mucosa type' and 'PBL with plasmacytic differentiation' received most attention and is also utilised by the 2008 WHO classification[22].

1.2.3 Immunophenotype

Immunophenotypically PBL displays a characteristic late or terminally differentiated B-cell type, negative for B-cell antigens such as CD20 but with variable positivity for CD79a and epithelial membrane antigen (EMA). Variable staining patterns for CD5, CD7, CD8, CD10, CD30, CD45, and anaplastic lymphoma kinase (ALK-1) has been reported. CD3 positivity was reported by one group[71] and CD4 expression by some others[25, 92, 94], the meaning of which is uncertain. Variable positivity for plasma cell markers such as CD38 and CD138 are described by many as well as cytoplasmic immunoglobulin light chain restriction (kappa or lambda) suggestive of clonality[5, 14, 37, 40, 43, 52, 56, 69]. The use of CD56 to distinguish between PBL and extra-medullary MM is controversial. Some authors found it to be positive in MM rather than PBL[56] but others found CD56 expression in a large proportion of their PBL cases rendering it inappropriate for differentiation between these neoplastic entities[92].

1.2.4 The differential diagnosis of PBL: A diagnostic dilemma

Many lymphoproliferative and plasma cell neoplasias display plasmablastic features which results in difficulty when aiming to diagnose PBL's using solely morphologic criteria. Plasmablastic features are often encountered in aggressive B-cell lymphomas. These include several varieties of DLBCL such as the immunoblastic variant of DLBCL not otherwise specified (NOS)[95], DLBCL associated with chronic inflammation[96], ALK-positive DLBCL[97], HHV-8-associated DLBCL associated with multicentric Castleman's disease (also known as HHV-8-positive PBL)[98], extracavitary solid forms of PEL[99], and extra-medullary plasmablastic tumours secondary to MM or plasmacytomas[90, 92]. The diagnostic dilemma was underlined by Dong and co-workers who elaborated on how pathologists have variably diagnosed PBL's as 'immunoblastic lymphoma with plasmacytic features', 'plasmacytomas with anaplastic features' or even 'unclassifiable neoplasms with features intermediate between plasmacytoma and immunoblastic lymphoma'[17].

Of all these entities, extramedullary tumours secondary to plasma cell neoplasias such as MM and plasmacytomas with plasmablastic morphology are still the most difficult or even impossible and controversial to differentiate from PBL[17, 56, 90, 92]. These neoplasms have nearly identical immunophenotypic profiles, histomorphological[92] and very importantly, clinical features. Some are of the opinion that pre-existing or concurrent clinical evidence of MM as defined by the International Myeloma Working Group[100] favours the diagnosis of plasmablastic MM over that of PBL[56, 92]. These include the presence of serum monoclonal para-proteins, bone involvement with radiographically evident lytic lesions and proliferation of plasma cells in the bone marrow, peripheral blood, or extra-medullary sites. Disseminated bone involvement with hyper-metabolic disease[61] as well as bone marrow infiltration by neoplastic plasmablasts have however been reported in cases of PBL in the literature[24, 56, 61, 101, 102]. The absence of serum monoclonal proteins and/ or bone marrow involvement, the presence of EBV in the tumour cells, HIV/AIDS-related immunodeficiency, and the aggressive clinical course with poor survival are accepted by some to favour the diagnosis of PBL above other plasma cell neoplasias[11, 17, 92, 103]. But MM has also been described in

the setting of AIDS, with and without EBV involvement[104, 105] and EBV-positive MM has also been described in immunocompetent patients[106]. MM in these patients may for instance present without the monoclonal spike on serum electrophoreses. This could either be due to non-secretory tumours or to the common presence of polyclonal gammopathy which can obscure a small monoclonal spike[107].

The minimum diagnostic criteria for PBL of the oral and sinonasal regions was recently suggested by Kane and co-workers[72]. The authors made an interesting remark that PBL with plasmacytic differentiation actually represents a rare plasmacytoma with plasmablastic component that should be excluded from the homogenous group of PBL. This concept was further underlined by Taddesse-Heath *et al.*, who reported three cases of PBL that showed clinical, morphological, phenotypical as well as genetic features that overlapped with MM[11]. In their study, all patients presented with disseminated disease with nodal as well as extra-nodal involvement such as extensive infiltration of small bowel and colon wall. Two of the patients had monoclonal serum immunoglobulin and lytic bone lesions without bone marrow involvement either morphologically or by flow cytometry. The third patient had monoclonal serum immunoglobulin, diffuse infiltration of the bone marrow but no lytic bone lesions. None of the aforementioned patients had hepatosplenomegaly or peripheral blood involvement[11]. The morphological and immunohistochemical features in all cases were consistent with those of a plasma cell neoplasm with plasmablastic morphology but because the patients did not have all the criteria consistent for the diagnosis of MM[108], the tumours were diagnosed as PBL[11]. Most studies that report cases of PBL describe the tumours as 'microscopically consistent with PBL' even though the patients involved never had a proper clinical workup for MM.

The concept that PBL may actually represent an aggressive form of MM or plasmacytoma with plasmablastic morphology, has also been proposed by others[11, 17, 56, 72, 90, 92, 109]. Both MM and plasmacytoma can contain plasma cells that vary from mature plasma cells to immature plasmablasts, pleomorphic or sometimes anaplastic cells[109]. A recent study proposed a classification of plasmacytomas in mice which basically mirrors these stages

of development and classify plasmacytomas in mice as anaplastic, plasmablastic, and plasmacytic[110]. The diagnosis of plasmacytomas may become very difficult when cells are poorly differentiated and exhibits plasmablastic or anaplastic features[109]. A biological continuum between plasmacytomas and MM has been demonstrated by the eventual transformation of approximately two thirds of cases of solitary plasmacytomas to MM[111-113]. Extra-medullary plasmacytomas have unfortunately not been investigated or reported extensively in the literature and data on this neoplasm is scarce. Transformation seems to occur at a much lower rate than solitary plasmacytomas and is reported to occur only in about 15% of all such cases[114]. Plasmacytomas and MM with plasmablastic morphology are reported to be immunophenotypically and genetically similar[109] which complicates matters even further.

1.2.5 Plasma cell tumours and HIV/AIDS

Non-Hodgkin's lymphoma represents the second most common group of neoplasms with a 60-200 fold increased risk in HIV-patients when compared to the general population[2, 115, 116]. Patients are usually younger than those diagnosed in non-HIV patients and present with advanced disease and a high tumour burden[2]. Involvement of extranodal sites is common, in particular the gastrointestinal tract, central nervous system, liver and bone marrow while tumours are usually EBV positive, have a prominent plasmablastic morphology and are clinically aggressive[104, 105, 107, 117, 118]. The lymphomas most commonly encountered in HIV-AIDS patients include PEL[119], BL, DLBCL, PBL and HHV-8-positive PBL associated with Castleman's disease[2, 120].

The reasons why HIV-infected patients have an increased risk for the development of lymphomas, particularly of mature and terminally differentiated B-cells/ plasma cells, appear to be multifactorial. This is supported by the fact that lymphomas in HIV/AIDS patients are heterogeneous reflecting different pathogenetic mechanisms. These include the transforming properties of the retrovirus itself[121], the high incidence of infection with opportunistic lymphotrophic viruses such as EBV and HHV-8[122,

[123] as well as the polyclonal B-cell activation through chronic and excessive stimulation of a compromised immune system and cytokine dysregulation. The idea that chronic B-cell stimulation plays a role is supported by the presence of hypergammaglobulinemia and persistent generalised lymphadenopathy often preceding the development of these lymphomas[2, 124] as well as increased para-proteins directed against HIV-specific antigens such as p24[125, 126]. It has been confirmed that HIV-infected macrophages and other cells up-regulate the expression of Interleukin 6 (IL-6), an important plasma cell growth factor[127]. EBV and/or HHV-8 infection, common in HIV-infected patients, are also associated with high serum levels of IL-6[2] which may further explain the common association of plasma cell neoplasms in HIV-patients.

MM is a non-AIDS defining plasma cell neoplasm which shows an increased incidence in HIV/AIDS patients[115, 116]. In this setting MM and even plasmacytomas are associated with a younger age (mean age of 38.8 years), presentation in multiple and unusual extra-medullary sites[107, 116, 128-130] and a more aggressive clinical course with poor prognosis. The median survival for patients with MM in immune competent hosts is 33 months[131] but it is reduced to only a few months in HIV-positive patients[107]. The adverse clinical prognostic factors in HIV-positive individuals diagnosed with any lymphoma include age over 35 years, intravenous drug usage, stage of the disease at presentation and the CD4 count which, in itself, has been shown to be linked with disease progression[7, 8]. These features are similar to those described in PBL.

The role of HIV infection in the diagnosis of PBL versus that of other plasma cell neoplasms such as MM is uncertain. It is unknown if PBL represents a unique entity or when it is associated with disseminated disease in an HIV-positive patient does not rather represent a solid plasmablastic extra-medullary plasmacytoma in the context of MM. It should also be determined whether PBL diagnosed in an HIV-negative or immune competent patient does not actually represent some form of extra-medullary plasmablastic plasmacytoma.

1.3 VIRUSES AND PLASMABLASTIC LYMPHOMA

1.3.1 Epstein Barr Virus (EBV)

EBV is a member of the human herpes virus family and has been associated with the development of various B-cell lymphoproliferative diseases including lymphoma[132-134]. EBV is present in 40-50% of lymphomas including BL, DLBCL with immunoblastic morphology, primary central nervous system lymphoma, PEL and PBL[9, 87, 135]. The virus therefore seems to play a definitive role in the pathogenesis of HIV/AIDS-related lymphomas, although the incidence of EBV varies considerably with the site of presentation and the histological type of lymphoma.

The strong association of EBV with PBL has been confirmed by various authors with an incidence that ranges from 60-100%[5, 8, 11, 14]. The EBV association is especially prominent in PBL classified as 'PBL of the oral mucosa type'[56]. Lower incidences have however been reported in 'PBL with plasmacytic differentiation'[22, 136].

The close association between PBL, HIV and EBV infection, although evident, is not clearly understood. Most HIV-associated lymphoproliferative diseases display a plasma cell phenotype and are linked to EBV infection[134]. It is still speculated whether EBV infection is pathogenic or merely coincidental in PBL.

1.3.2 Human Herpes Virus-8 (HHV-8)

HHV-8 is frequently associated with certain lymphoproliferative disorders such as PEL occurring in association with HIV infection[137, 138] and large B-cell lymphoma arising in HHV-8-associated multicentric Castleman's disease, also known as HHV-8-positive PBL. The pathogenetic role of HHV-8 in lymphoproliferative diseases is partly explained by its role in cytokine upregulation with increased production of IL-6 which acts as a B-cell stimulant and results in increased incidences of B-cell lymphomas[139].

Although some groups reported the presence of HHV-8 in their cases of PBL[15-18, 20], most authors, including the WHO, currently accept HHV-8 not to play any role in the pathogenesis of PBL[14, 22, 25, 36, 37, 41, 140-142].

1.4 GENETIC PROFILE OF PLASMABLASTIC LYMPHOMA

The genetic features form an important part of the diagnostic algorithms in the 2008 WHO classification of lymphoid tumours[9] and additional genetic data continue to inform scientists on the pathogenesis of these diseases. There is still a significant shortage of genetic studies on PBL likely due to its overall rarity, apart from countries with high incidences of HIV/AIDS. Since its first description by Delecluse in 1997, less than 300 cases of PBL affecting the oral cavity have been published in the literature[6, 8, 14, 16, 17, 20, 25, 26, 31-47, 49-71]. Knowledge on the cellular origin of this tumour is therefore still based on the expression of some immunohistochemical markers.

1.4.1 General aspects of chromosomal abnormalities in lymphoid malignancies

Acquired chromosome abnormalities as causal factors in the origin of cancer have been investigated extensively over the last fifty years. The first to suggest such a relationship was Boveri in 1914[143] cited in a recent review[144]. Only when chromosomes could be visualised and studied more accurately could this hypothesis be evaluated and ultimately proven. It has been shown that the consequences of chromosome aberrations are what defines cancer as 'a genetic disease at the cellular level'[145]. The genetic pathogenesis of malignant neoplasia is mainly dominated by two types of initiating events namely the inactivation of genes or their dysregulation. The genes involved in tumour development and therefore prone to be affected in such a manner can be divided in three broad categories: oncogenes, tumour-suppressor genes and stability genes or also known as caretaker genes.

Proto-oncogenes are genes that, when mutated in any way, will result in a oncogene which is constitutively active, that is, active under conditions when the wild type gene is not[145]. Tumour suppressor genes or anti-oncogenes are genes that encode proteins which are responsible for repressing cell proliferation or promoting apoptosis, sometimes both. When activated, oncogenes and tumour suppressor genes may lead to increased net growth of the neoplastic cells. Genetic alterations of tumour suppressor genes on the

other hand often inhibit normal cell-cycle arrest or apoptosis[145]. Stability or caretaker genes, a third group of cancer genes are generally responsible for the repair of genetic alterations that happen during normal cell proliferation or exposure to mutagens. When the function of caretaker genes are inactivated, mutations in other genes such as proto-oncogenes and tumour suppressor genes occur more frequently[146].

Mutations are changes in the DNA sequence of a cell's genome and may be caused by many factors including viruses, different forms of radiation, chemicals and many more. Genetic alterations in the DNA of oncogenes, tumour suppressor genes and DNA repair genes, amongst others, have the potential to lead to the formation of various forms of malignant tumours. Alterations to these genes include gains and losses of parts of or whole chromosomes, amplifications as well as chromosomal translocations. Mutations of oncogenes, tumour suppressor genes and caretaker genes can occur in the germ line or, more commonly in single somatic cells which results in sporadic tumours.

Chromosomal translocations are considered as the primary cause for many malignancies but have been proven to be the most common class of mutations found in haematological and lymphoproliferative malignancies[147]. Paired double strand DNA breaks on separate chromosomes with proximity of the broken ends and then joining of the heterologous DNA ends are required in order to result in a chromosomal translocations[148]. These translocations can have various results. Sometimes fusion of two genes can produce a chimeric protein with oncogenic activity such as the Philadelphia chromosome found in subtypes of acute lymphoblastic leukemia and chronic myeloid leukemia[147]. Chromosomal translocations can also result in bringing transcriptional regulatory sequences from one gene in apposition with a proto-oncogene such as *MYC*, leading to its distorted expression[148].

Approximately 95% of lymphomas diagnosed in humans are of B-cell origin[147]. B-cell lymphomas are a very heterogeneous group of malignancies and reflect all stages of B-cell development: from early B-cells such as seen in acute lymphoblastic leukemia to mature B-cells in DLBCL or even terminally

differentiated B-cells (plasma cells) in MM. The position of PBL in the B-cell repertoire has not been elucidated yet.

The high incidence of B-cell lymphomas seems surprising when one thinks about the ratio of B- to T-cells in the normal human body. Considering the very complex process of B-cell maturation though[149-151] it becomes easier to understand why this population of cells is more prone to malignant change. The same key factors needed for normal B-cell differentiation and survival are also required for malignant growth of most B-cell lymphomas[147]. The programmed DNA damage that the B-cell encounters during normal B-cell differentiation, especially during the germinal center (GC) reaction, makes these cells very prone for somatic mutations as previously defined. For a B-lymphocyte to acquire the capacity to secrete antibodies for normal immunity, the cell must undergo extensive differentiation driven by changes in gene expression, also known as genetic reprogramming, prone to mistakes.

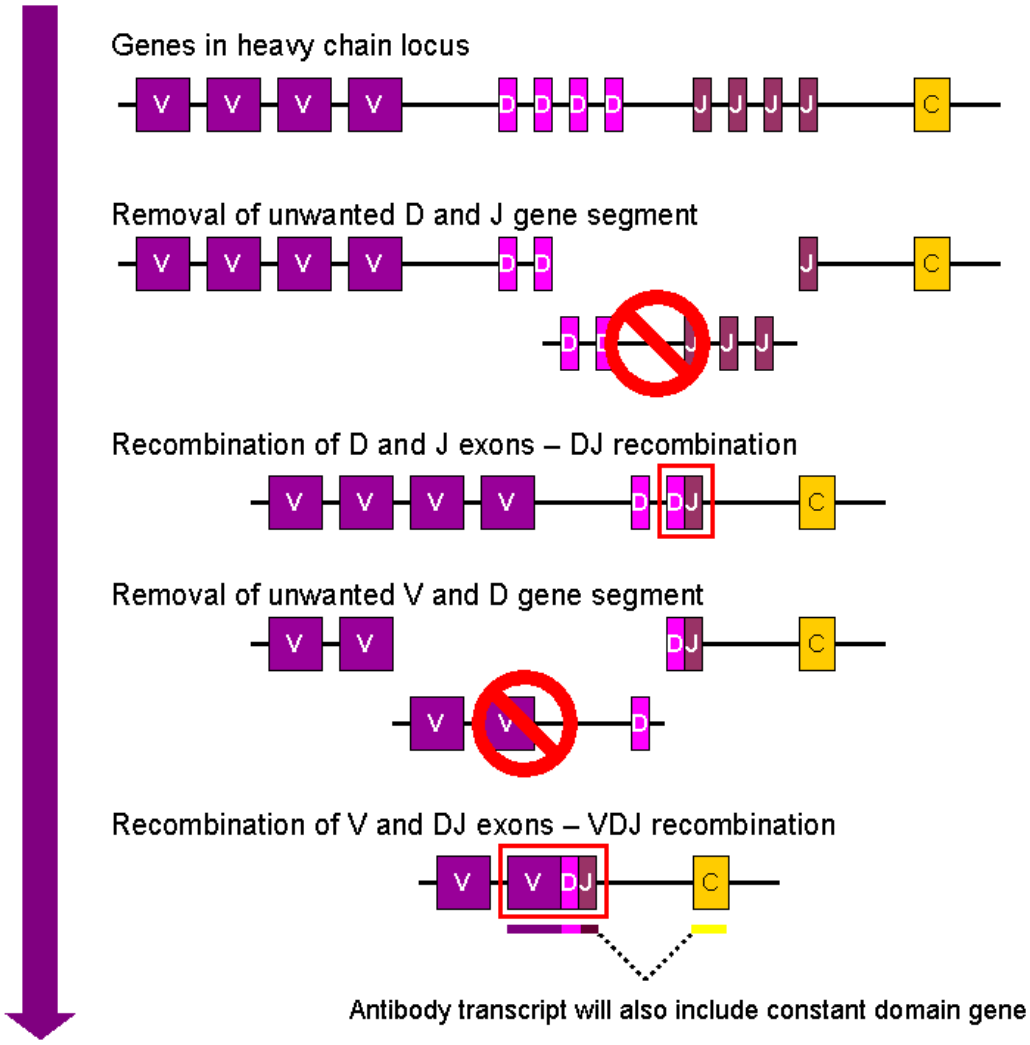
1.4.2 Normal B-cell development

B-cells are responsible for immune surveillance against a wide variety of pathogens. For a B-cell to be able to perform its functions, it has to go through a complex process of maturation which has tight check points aimed at eliminating B-cells abnormal in any way. When B-cells are mature they produce antibodies in the form of immunoglobulins (Ig) composed of two distinct chains, a heavy and a light chain, encoded by separate chromosomes. Both the heavy and light chains contain variable (V) regions for antigen recognition. The heavy chains also contain constant (C) regions which influence the fate of the encountered antigen. B-cell development takes origin from haematopoietic stem cells in the primary lymphoid organs represented by the fetal liver and the adult bone marrow. The earliest B-cells are represented by the progenitor or pro-B-cells which express the B-cell antigens CD19 and CD79a. The first Ig recombination event takes place between one of 27 diversity (D), and one of 6 junctional (J) gene segments present on the *IGH* on chromosome 14q32[152]. Any unwanted DNA between these two sequences is deleted (Fig.1). Once the D and J gene segments are recombined, i.e. D_H – J_H, the pro-B-cell will differentiate into an early pre-B-

cell. At this stage, further rearrangement in IGH with removal of unwanted V and D gene segments result in one of the 123 available V genes to be attached to the newly formed D-J segment giving rise to a late pre-B-cell (Fig.1). These cells are recognised by expression of a rearranged VDJ-Cμ heavy chain or pre-B-cell receptor (pre-BCR) on their cell surface in which [μ]M provides the first crucial check-point for B-cell development[152].

Light chain rearrangement will now take place in a similar fashion except the light chains lack a D segment. The first step of recombination for the light chains involves the joining of a V segment (which can be either kappa (κ) on chromosome 2 or lambda (λ) on chromosome 22) and J segment to give a VJ complex before the addition of the constant chain gene during primary transcription. Translation of the spliced mRNA for either the κ or λ chains results in formation of the Ig κ or Ig λ light chain protein. This results in pairing a μHC with λ or κ light chain and the expression of a complete IgM molecule[149].

Figure 1 *The figure gives a simplistic overview of V(D)J recombination of immunoglobulin heavy chains.*



V = variable genes; D = diversity genes; J – junctional genes; C = constant genes

During the process of V(D)J somatic recombination in developing B-cells of the bone marrow, the recombination activating enzyme, RAG1/2 is responsible for DNA breaks at the recombination signal sequences and it catalyzes a *trans*-esterification reaction resulting in two DNA ends[148]. A DNA damage response is triggered[153] to repair the 'damaged or broken DNA by the non-homologous end-joining DNA repair machinery[154]. These cellular repair pathways play a major role in the maintenance of genomic integrity and stability. Such pathways, along with DNA-damage checkpoints, ensure that either the damage is properly repaired or cells with damaged DNA are eliminated completely.

The presence of surface IgM is required to pass the second check-point, which then allows for negative selection of abnormal B-cells to occur[152]. The B-cell is now known as an immature B-cell which soon gives rise to a mature B-cell that has the ability to leave the primary lymphoid tissue[149].

Mature but still antigen naive B-cells represent approximately 60-70% of circulating B-cells. These cells migrate to the spleen, lymph nodes, Peyer's patches, tonsils and mucosa associated lymphoid tissue (MALT), also referred to as the secondary lymphoid tissues, where they form the primary B-cell follicles. Should an antigen be encountered by the B-cells of secondary lymphoid tissue, the cells will undergo complex processes collectively known as the GC reaction and ultimately produce plasma cells and memory B-cells[149]. The GC reaction includes clonal expansion, somatic hypermutation (SHM) and class-switch recombination (CSR).

The GC reaction is not the only pathway for plasma cell differentiation and it is now well documented that two subsets of peripheral B-cells, the B1 cells (without antigen encounter) and the marginal zone B-cells (with antigen encounter) can proliferate and differentiate into plasma cells outside the GC microenvironment[149]. According to this, B-cells are classified by some authors as antigen-insensitive B1 cells and the more common antigen-sensitive B2 cells further represented by the GC B-cells and the marginal zone B-cells[149].

Antigen activated B-cells transform into large B-blasts which will either proliferate and differentiate into short lived plasma cells responsible for rapid release of low-affinity antibodies or will migrate to primary B-cell follicles where they will proliferate and differentiate into long-lived plasma cells producing high-affinity antibodies and memory B-cells[149]. During the GC reaction in the follicles of secondary lymphoid organs some very important changes take place, in particular the antibody diversification processes of centroblasts that will result in the generation of high affinity antigen-specific antibodies. Morphologically, centroblasts are large blast-like cells with round, vesicular nuclei, one to three prominent nucleoli and narrow rim of basophilic cytoplasm. The antibody diversification processes involves the introduction of point mutations, or non-templated nucleotide substitutions in the rearranged *IgV* gene region that encodes for the antigen binding site. This process is

known as SHM[155, 156]. Unlike germ line mutations, SHM affects only individual cells, and the mutations are not transmitted to offspring. Mistakes during somatic hypermutation are a likely mechanism in the development of B-cell lymphomas.

Centroblasts express no Ig on their surfaces. The B-cell lymphoma-6 protein (BCL6) is a nuclear transcription factor which is strongly up regulated in GC B-cells[157]. Evidence confirms BCL6 as a major regulator of B-cell development, required for GC formation[158] and the persistence of the germinal center[159]. BCL6 down regulates the *BCL2* and *BCLX* genes resulting in suppression of apoptosis and promotion of cell proliferation. BCL6 also down regulates the expression of B-lymphocyte induced maturation protein (BLIMP-1)[160], necessary for plasma cell differentiation which further enhances rapid expansion of the specific GC B-cell population[161].

Apart from SHM the cells also undergo CSR during which DNA are deleted between repeated sequences called switch regions located upstream of the C region of the *IGH*[162]. This ultimately results in IgM and IgD to be switched to either IgG, IgA or IgE[157, 162]. Class switching occurs after activation of a mature B-cell *via* its membrane-bound antibody molecule, or B-cell receptor. This leads to the generation of different classes of antibody, all with the same variable domains as the original antibody generated in the immature B-cell during V(D)J recombination, but with distinct constant domains in their heavy chains. Thus CSR allows production of different Ig isotypes or antibody classes which are determined by the different *IGH* constant region genes with maintenance of the same variable region antigen-binding specificity. CSR leaves the V(D)J regions unaffected but only changes the effector function of the specific antibody molecule[149].

After SHM and CSR, centroblasts differentiate into centrocytes. Centrocytes are cells that vary in size from little larger than mature lymphocytes to a just smaller size than centroblasts. They have deeply indented nuclei, inconspicuous nucleoli and scant cytoplasm[149]. Centrocytes re-express Ig on their cell surfaces, which now contains the same V(D)J rearrangements as the naive B-cells but due to SHM have different antibody binding sites and due to CSR have altered C regions[149]. Centrocytes have the ability to bind the

antigens in its vicinity and to present it to helper-T-cells, numerously present in the GC. When unfavorable mutations occurred during V(D)J recombination, SHM or CSR, the resulting centrocytes are usually unable to bind with high affinity to antigens trapped by the presenting follicular dendritic cells or to appropriately interact with the GC T-cells. Because of this anomaly, the specific 'abnormal' centrocytes are negatively selected and do not receive any survival signals from these cells, there is down-regulation of the anti-apoptotic molecules BCL2 and BCLX which ultimately results in death of these B-cells[149]. Positively selected centrocytes however activate adjacent T-cells to express CD40-ligand and to secrete IL-4 and IL-10 which ultimately induces B-cell clonal proliferation. GC B-cells may undergo repeated rounds of SHM and CSR or may differentiate into post-GC B-cells which includes plasma cells and memory B-cells[149, 163]. The reason why some GC B-cells become plasma cells and others memory B-cells is not yet clear. The germinal center reaches its maximum size in about two weeks and then slowly involutes to disappear again in a few weeks[163].

1.4.3 General aspects of the genetic basis of B-cell lymphoma pathogenesis

Molecular aspects of lymphomagenesis, especially of the B-cell lineage, have been extensively investigated. As described earlier, specific stages of B-cell development and differentiation are characterised by the particular structure of the B-cell receptor and expression of differentiation markers at every stage. Malignant B-cells are the clonal expansion of B-cells that seems to be 'frozen' in a specific stage of B-cell development and differentiation as characterised by its B-cell receptor structure and or differentiation markers. This concept forms the basis for the classification of B-cell lymphomas[9]. It has been shown that all B-cell NHL's, apart from lymphoblastic and mantle cell lymphoma display somatically mutated *IgV* genes indicating that almost all forms of B-cell lymphoma are derived from B-cells either blocked within or B-cells that have just passed through the GC[163-165].

1.4.3.1 *IGH* gene rearrangements

Approximately 50% of B-cell lymphomas are characterised by recurrent translocations involving the *IGH*[166, 167]. The *IGH* locus is situated on chromosome 14, at band 14q32.3 spanning 1,250kb. At least one partner of almost all translocations documented in lymphoid cancers is one of the *Ig* gene variable or switch regions[148]. Reciprocal translocations usually involve one of the *Ig* loci and a proto-oncogene resulting in the oncogene being deregulated by the very active *Ig* promoter sequences with constitutive expression of the associated oncogene[147]. Examples include the t(14;18) translocation that joins the *IGH* promoter to the *BCL2* gene at 18q21, resulting in constitutive expression of the anti-apoptotic BCL2 protein and the t(8;14) translocation where the *IGH* promoter is joined with the *MYC* gene at 8q24 resulting in the constitutive expression of *MYC*.

As eluded to before, translocations involving *IGH* may occur as a consequence of illegitimate V(D)J recombination during early B-cell development or during SHM and/or CSR in the later stages of B-cell development. SHM and CSR share an important common B-cell specific

enzyme namely activation-induced cytidine deaminase (AID)[168]. Many researchers have shown AID to be responsible for the lesions in the *IGH* and *MYC* loci that eventually lead to translocations[169-172]. Some have even demonstrated AID to be essential for the *MYC/IGH* / t(8;14) translocation[171].

Aberrant joining of heterologous chromosomes after paired DNA breaks is necessary for translocations to occur and double strand breaks in the *IGH* alone would therefore be insufficient for translocations to occur[173]. Translocation partner genes must physically encounter each other in the nucleus to undergo a translocation. Researchers have shown that interphase chromosomes are non-randomly organized in nuclei in tissue-specific configuration so that the chromosomal position and juxtaposition of certain genes may result in the formation of non-random translocations[174]. Chromosomes 12 and 15 in mice represent human chromosomes 14 and 8 respectively. They carry the frequent B-cell translocation partner *IGH* on chromosomes 12 (mice) or 14 (human) and the proto-oncogene *MYC* on chromosomes 15 (mice) or 8 (human). Chromosomes 12 and 15 have been shown to be preferred neighbours in mouse spleen lymphocytes[175] and *MYC* and *IGH* are found in the same vicinity in a third of human nuclei[176]. Any process that brings these genes together would obviously increase the risk of a translocations between them[177].

Three types of *Ig* breakpoints have been identified. They can be classified according to various stages of B-cell development: 1) breakpoint directly adjacent to *Ig* J_H or adjacent to D_HJ_H joining region – likely to represent translocations which takes place at the time of V(D)J recombination during early B-cell development; 2) breakpoint within or adjacent to rearranged and somatically mutated V(D)J genes – likely to represent translocations as the by-products of double DNA strand breaks during SHM; 3) breakpoints in the *IGH* constant switch regions – most probably represent breaks during CSR[147].

1.4.3.2 Other factors thought to be involved in lymphoma pathogenesis

The AID protein is found in GC B-cells responding to antigen in the periphery[178]. Generally speaking, AID drives antibody diversification by deaminating cytosine in the DNA to uracil during the processes of SHM and CSR. During these processes AID introduces double DNA strand breaks[168]. The genome is usually protected from AID-induced *IGH* translocations by the ATM-, p53- and p19Arf-dependant pathways[179].

Unlike the previously mentioned RAG1/2 protein, which is sequence specific, AID, a nucleotide editing enzyme, can deaminate cytosines in nearly any sequence context[156] which includes *Ig* and certain oncogenes such as *MYC*[180]. The deregulated expression of AID in B-cells leads to chromatid breaks and translocations involving almost every chromosome[178]. Recent evidence shows that deregulated AID expression leads to B-cell lymphoma when combined with deletion of p53[178]. Interestingly, AID does not however seem to play a role in deletions or duplications observed in *MYC* or other oncogenes[181-183]. AID-induced lesions in non-*IgH* genes are repaired by different pathways and seems to be less error prone than AID-induced *IGH* lesions[182].

Technically speaking, RAG1/2-induced double strand breaks (during the initial V(D)J rearrangements in the bone marrow) and AID breaks (during SHM and CSR) should not coexist and should therefore not be concomitants in chromosome translocations[148]. RAG-induced breaks can persist under at least two circumstances: 1) if the DNA damage checkpoint ataxia telangiectasia mutated kinase is defective for any reason, it will result in cells with breaks at the *IGH* locus which could be paired with AID breaks later[184]; 2) loss of the non-homologous end-joining factor XRCC4 in mature B-cells[185] with deficient p53 that will lead to B-cell lymphomas with *c-MYC/IGH* translocations[186].

Other non-Ig gene targets involved in B-cell lymphoma genesis include inactivating mutations of the genes that encode for CD95 (FAS)[187] and *BCL6*[180, 181].

1.4.3.3 *MYC* gene

The *MYC* gene was initially described in avian retroviruses as the oncogene that induces ***my***elo***c***ytomatosis in birds, from there the abbreviation *MYC*[188]. *c-MYC* is the cellular homologue and represents the most widely studied proto-oncogene. *MYCN* and *MYCL* are homologues of the gene found amplified in neuroblastoma and small cell cancer of the lung respectively[189].

The human *c-MYC* is localised on chromosome 8q24.21[190] and encodes two proteins, the p67 (MYC-1) and the p64 (MYC-2) each with biologically distinct functions[191]. The MYC protein is a basic Helix-Loop-Helix Leucine Zipper (bHLHZip) protein which heterodimerises with another small bHLHZip protein called Max resulting in dimers having a DNA-binding ability for specific DNA sequences[192]. This dimerization is critical for all known *MYC* functions[193].

MYC regulates various cellular activities such as aspects of the cell cycle, cell growth and metabolism, cell differentiation, apoptosis but it is also involved in transformation, genomic instability and angiogenesis[194]. In fact, *c-MYC* and *max* are essential for survival[189]. *c-MYC* deficiency results, amongst other effects, in the accumulation of defective haematopoietic stem cells[195]. In line with its biological functions, *c-MYC* expression is high during early embryogenesis[196] but low or undetectable in differentiated tissue, consistent with the low to complete absence of cell proliferation in fully differentiated cells[192].

MYC expression and activity are regulated at multiple levels through transcriptional, post-transcriptional, translational and post-translational mechanisms[197]. The diverse functions of *MYC* include both proliferation and apoptosis and a model was brought forward that proposes *MYC* to promote apoptosis as the preferred physiological response[198]. In the presence of excessive amounts of survival factors or mutations in the apoptotic pathway

however, the cellular *MYC* response would instead be uncontrolled proliferation, the so-called "dual signal model"[198, 199]. It was shown that elevated *c-MYC* levels accelerate the progression of cells through the G1 phase[200]. It was also soon discovered that *c-MYC* itself can acquire mutations that potentiate neoplastic transformation by affecting apoptosis independently of cell cycle progression[193].

A large number of human malignancies exhibit deregulated *MYC* activity[201] which include chromosomal translocations such as the *MYC/IGH* fusion gene in BL[202] and increased *MYC* expression due to gene amplification[203] and protein stabilisation[204]. Oncogenic features of *MYC* also include genomic destabilisation[205], increased vascularisation and angiogenesis[206]. Due to its diverse functions, recent data demonstrated the targeting of *MYC* as an effective, efficient and tumour-specific cancer therapy[207].

BL is a high-grade B-cell lymphoma invariably associated with chromosomal translocations with a resultant *MYC* deregulation[202]. In this setting, the chromosomal translocations always result in the juxtaposition of *MYC* with enhancer sequences from immunoglobulin genes, usually the *IGH*. The Ig enhancers are particularly active in mature B-cells and their juxtaposition to *MYC* drives abnormally high levels of MYC mRNA and protein expression[208]. Constitutive *MYC* expression results in cell cycle deregulation with uncontrolled cell proliferation[209]. This contributes to the increased *MYC* activity and excessive cell proliferation seen in cases of BL. In 80% of BL's, the translocation partner for *MYC* is the *IGH* locus, resulting in the t(8;14)(q24;q32) abnormality[202]. In approximately 15% of cases the partner is the Ig light chain *κ* locus at chromosome 2p11 [t(2;8)] and in approximately 5% the Ig light chain *λ* locus on chromosome 22q11 [t(8:22)] is involved[202] both forms known as variants Burkitt translocations. Although *MYC* rearrangements are sensitive markers for BL, they are not specific and are also seen in DLBCL where it is associated with poorer prognostic implications[209-213]. A new category in the 2008 WHO Classification of lymphoid neoplasms known as 'B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL'[11, 213] were created in order to accommodate 'atypical' cases of both BL and DLBCL.

It has previously been shown that plasmablastic cell transformation is highly associated with *MYC* translocations. This is seen in both PBL *de novo* as well as in plasmablastic transformation of plasma cell neoplasms such as MM[214-216]. The incidence of *MYC* abnormalities and increased *MYC* expression in MM is reported to be more frequent in extra-medullary and more proliferative intra-medullary tumours compared to less aggressive intra-medullary tumours[215]. In murine plasmacytoma, BL and some other B lymphocyte tumours, dysregulation of *MYC* appears to be one of the earliest oncogenic events but *MYC* dysregulation seems to be a late event representative of tumour progression in MM, consistent with the higher proliferative capacity late in the disease[11, 24-26, 31]

The prevalence of *MYC* rearrangement in PBL of the oral cavity *versus* that seen in other anatomical locations is unknown. Recent studies on PBL from various anatomical locations have highlighted the importance of *MYC* gene rearrangements[11, 13, 24-28] and suggest an important role for *MYC* in the pathogenesis of PBL.

1.4.4 Plasma cell differentiation: position of the plasmablast in the B-cell repertoire

Naïve and memory B-cells both have the ability to differentiate into antibody-secreting plasmablasts and eventually plasma cells when activated, either with or without T-cell help and with or without the presence of an antigen[217].

The term 'plasma cell' is defined as either long- or short-lived, non-dividable, immobile, antibody-secreting cells with prominently distended endoplasmic reticulum (seen with light microscopy as the paranuclear hof) and well-developed secretory apparatus[217]. Plasmablasts on the other hand are mobile, migratory, activated B-cells that secrete antibodies while remaining in cell cycle and have up regulated major histocompatibility complex (MHC) expression[217]. These cells still have the blastoid features of immunoblasts but have already acquired the antigen profile of plasma cells[5]. The exact developmental relationship between plasmablasts and plasma cells are unknown but the maturation of plasmablasts into plasma cells is marked by

loss of mobility, down regulation of its MHC-expression and no further proliferation. Plasmablasts can either differentiate into sessile plasma cells that reside in the tissue of origin, that is one of the secondary lymphoid tissues, or are attracted to the bone marrow or inflamed tissue by certain chemokines[218]. The ability of plasmablasts to migrate is crucial in order for them to reach specialized niches that provides them with survival signals[218]. Both the site of induction as well as the Ig isotypes expressed determines the homing potential and final tissue distribution[218]. For instance, earlier studies have already shown the existence of differential mechanisms that target IgA producing plasmablasts to mucosal surfaces[219].

The molecular signals that induce a GC B-cell to differentiate into plasma cells are still unknown. The vast majority of long-lived plasma cells are derived from the GC, as evidenced by somatic mutations in their *V* genes[217]. The importance of various transcription factors in this process however is becoming clearer. Expression of *BLIMP-1*, a transcriptional repressor, promotes plasma cell development by suppressing genes associated with the GC program (*BCL6, PAX-5, Spi-B*) and induces the expression of those essential for plasma cell development such as the *X-box binding protein-1* (*XBP-1*)[149, 152, 157, 163]. *XBP-1* is expressed at high levels in plasma cells, essential for inducing their secretory phenotype[152]. More recently the *interferon regulatory factor* (*IRF*)-4 was also identified as an additional master gene in plasma cell differentiation[220].

In the non-GC or extra-follicular pathway of plasma cell differentiation, a particular subset of naive B-cells in the marginal zone of the spleen can be activated in a T-cell independent manner through contact with bacterial polysaccharides[221]. The resultant plasma cells are mainly of the IgM type and facilitate rapid antibody release for immediate reaction to the blood borne antigen. This is known as the early immune response. The plasma cells generated by the non-GC pathway survive for approximately three days only before undergoing apoptosis. By that time the GC-generated plasma cells which have undergone SHM and CSR are producing high-affinity antigen specific antibodies to increase the immunity, also known as the late immune response[222]. The extra-follicular pathway of plasma cell development

provides an explanation of how B-cell responses are induced at a time when T-cell help is limited.

1.4.5 Plasma cell neoplasia

1.4.5.1 Multiple myeloma

MM is a plasma cell malignancy with clonal proliferation of genetically transformed neoplastic plasma cells in the bone marrow and that results in overproduction of light and heavy chain monoclonal Ig or the so-called M-protein that refers to its monoclonal characteristics[223, 224]. The diagnosis of MM as defined by the International Myeloma Working Group depends on the identification of abnormal monoclonal plasma cells in the bone marrow, the presence of M protein in the serum or urine, the evidence of end-organ damage and a clinical picture consistent with MM[100]. The pathogenesis of MM is poorly understood but due to the advances in molecular research techniques such as fluorescent *in situ* hybridisation (FISH), some advances in the understanding of the genetic basis of this disease have been made.

Genetic aberrations in MM include chromosomal gains and losses as well as specific chromosomal translocations affecting the *Ig* gene locus, most commonly the *IGH* locus at 14q32.3[225] but also the light chains gene loci at 2p12 (kappa) or 22q11 (lambda)[226]. These translocations result in the juxtaposition of *Ig* enhancers to various genes including *cyclins D1 (CCND1*), *D2* and *D3*, *MAF* family members and fibroblast growth factor receptor (*FGFR*)-3 leading to deregulation of their expression[227]. Specific recurrent translocations include the t(11;14)(q13;q32) seen in 15-20%, the t(4;14)(p16.3;q32) seen in 15-20%, the t(14;16)(q32;q23) seen in 5-10% and the t(6;14)(p21;q32) seen in 2-3% of MM patients[228, 229].

Recurrent translocations as a whole are seen in approximately 40% of MM cases and the remaining 60% are characterised by hyperdiploidy with chromosomal duplications[230]. *IGH* translocation and hyperdiploidy are unified by downstream up regulation of *CCND1, D2* or *D3*[231]. These genetic changes are eventually followed by further instability that often includes deletions or

monosomy of 13q14[230] or p53[232] or amplifications of chromosome 8 (*MYC*)[233]. Primary translocations occur early in the pathogenesis of MM but secondary translocations such as those involving the *MYC* gene usually indicate disease progression[215, 234].

Only about 3% of MM cases have an *IGH* translocation that targets *MYC*[216], usually with a t(8;14). Translocations involving *MYC* and *IGH* are therefore accepted as very late progression events. High risk MM signatures were recently shown to include any of the following aberrations: translocations t(4;14), t(14;16) or t(14;20), deletion of 17q13, deletion of 13q, presence of high tumour burden or high proliferation index[235]. Early deaths in patients with MM presumably always reflected advanced age, co-existent morbidity or treatment related toxicities with evidence now demonstrating that the presence of aggressive genetic features are the reason for the clinical outcome of these cases[235]. It was recently proposed that clinical trials should adopt the genetic criteria to define a high-risk patient[224].

1.4.5.2 Plasmacytoma

Plasmacytomas are clonal proliferations of plasma cells, classified as solitary when arising in bone or as extra-medullary when arising in soft tissue. The latter currently represent only 3% of plasma cell neoplasms[236] and most commonly affect the nasal cavity, paranasal sinuses and nasopharynx[114, 237]. Solitary plasmacytoma of bone is defined as a localised bone tumour consisting of monoclonal plasma cells. No other lesions should be present on skeletal radiographs, there should be no evidence of bone marrow plasmacytosis or any other clinical features of MM[9, 100]. Plasmacytomas may be very difficult to distinguish from lymphomas with prominent plasmacytic differentiation but CD20 expression amongst others would always favour the diagnosis of lymphoma[109]. Like MM, plasmacytomas can contain plasma cells that vary from mature to immature plasmablasts, pleomorphic or anaplastic plasma cells[109]. With increasing maturity of plasma cells in these lesions they may be classified as anaplastic, plasmablastic, and plasmacytic[110]. Patients with plasmacytomas are typically in the fifth to seventh decade of life[238].

1.4.5.3 Plasma cell leukaemia

Lymphomas and leukaemia have always been classified separately but this distinction has been shown to be artificial[29]. Plasma cell leukaemia is defined by the WHO as MM in which the malignant plasma cells in the peripheral blood exceeds $2X10^9$/L or represent 20% of the leukocyte differential count[111]. The neoplastic plasma cells are present in the bone marrow, peripheral blood as well as soft tissue collections and in effusion and ascites fluid[109]. This disease may either present as primary disease in 2-5% of cases of MM or evolve as a late feature in the course of MM, described as secondary plasma cell leukaemia[239]. This is an aggressive disease with poor and short survival[239].

The recurrent genetic abnormalities in 14 patients with plasma cell leukemia were reported in 2005[240]. Chromosomal abnormalities were present in 100% of these patients. 13q14 deletions were detected in 11 (78%) and deletions of 17p13.1 (TP53) in six (43%) cases. Translocations t(11;14), t(4;14), and t(14;16) were found in 5 (35%), 2 (14%), and 1(7%) cases, respectively. This study revealed recurrent genetic changes to be more frequent in plasma cell leukemia than in MM. The genetic changes were hypothesised to be the reason for the increased propensity for myeloma cells to migrate from the bone marrow environment and enter the leukemic phase[240].

1.4.5.4 Plasmablastic lymphoma

Due to its rarity in most parts of the world, only single reports on the molecular features of PBL's are found in the literature and its molecular pathogenesis and genetic features remains poorly understood. Delecluse, whom originally described PBL's could not find any rearrangement of the *BCL2* gene in three cases evaluated by polymerase chain reaction (PCR)[5]. In 2002, Gaidano described a single mutation (G756C) of the *BCL6* gene in one of 12 cases using PCR[37]; Vega found positive p53 expression in all of eight cases evaluated by immunohistochemistry with loss of p16 in the same eight cases and loss of p27 in two of four cases tested[92]; p16 hypermethylation was shown in one case of PBL affecting the skin using methylation-specific PCR[94];

Hassan *et al*., performed FISH on two cases of PBL of the head and neck utilising a commercial *MYC* (8q24) break apart (BA) probe set and they found *MYC* chromosomal rearrangement in one case, the first described in the literature[31]. In the same year, t(8;14)(q24;q32) was confirmed in another patient with PBL using conventional cytogenetics on a bone marrow aspirate of an HIV-positive hemophiliac patient with florid systemic relapse and circulating plasmablasts[26].

The exact prevalence of *MYC* rearrangements in PBL of the oral cavity is unknown. Recently, a large study of 42 PBL's from various anatomical locations, has highlighted the importance of *MYC* gene rearrangements (49%) mostly with the *IGH* gene as a partner[13]. This confirms previous case reports and small series and suggest an important role for *MYC* in the pathogenesis of PBL[11, 13, 24-28]. Some of the authors even proposed conventional cytogenetics and FISH studies to be routinely applied to characterise the genetic features of this neoplasm as those with the *MYC* rearrangement seems to occur in severely immunocompromised patients and, more importantly have a more aggressive clinical course[25]. Taddesse-Heath and co-workers found PBL and MM to be cytogenetically very similar with rearrangements of chromosome 1, deletions of 13q and 17p as well as the simultaneous gains of odd-numbered chromosomes postulating a common histogenesis and possible link between the two entities[11].

1.5 General aspects of the FISH technique in the study of lymphomas

The WHO classification of hematolymphoid malignancies includes various parameters needed to classify a lymphoproliferative neoplasm. Cytogenetic data represents one of these[9]. FISH is a technique which allows both fresh and paraffin-embedded materials to be assessed for the presence of cytogenetic anomalies for research, diagnostic as well as prognostic utility[241]. This technique is used to investigate chromosomal aberration in intact cells in any phase of the cell cycle. FISH can therefore be applied to touch smear preparations, peripheral blood or bone marrow samples, single cell suspensions and solid tissue. The aberrations evaluated by the FISH technique include gains and losses of whole chromosomes, specific chromosomal regions as well as chromosomal translocations[242].

FISH overcome the limitations of standard cytogenetics and allows for the detection of numerical and structural chromosomal abnormalities in both metaphase spreads and interphase nuclei[243]. What makes it an excellent investigative tool is the fact that one can directly analyse these aberrations on a tissue section allowing the pathologist to carefully distinguish between tumour cells and normal stromal or inflammatory cells. One set of FISH probes, instead of multiple PCR primers and PCR reactions offers another advantage[243].

To detect gene amplifications, a probe specific for the target gene of interest is combined with a differentially labeled probe as a control probe, usually the corresponding chromosomal centromere (centromere enumeration probes (CEP's). In the case of amplification of the target gene, there will be an increased number of target gene signals but with no change in the control probe for centromeric signals. In the case of polysomy of the entire chromosome, both signals for the target gene and the CEP's will be increased. The ratio of target gene signals to CEP signals therefore allows to distinguish true amplification from polysomy[244]. Gains and losses of whole chromosomes can be evaluated using CEP's for the specific target chromosomes under evaluation. The only chromosomes that can not be uniquely identified in this way, because they can not be distinguished using

CEP's, are chromosomes 13 and 21 and chromosomes 14 and 22 respectively[245].

The probes used to assess cells for the presence of translocations are the most widely used in NHL's. For this purpose, two types of probes have been developed: fusion probes and BA probes, previously known as colocalisation assays and segregation assays respectively[244]. The fusion probe sets utilise two differentially labeled probes, usually one green and one orange/ red labeled probe to mark two separate gene loci normally not in close proximity to one another. The signal pattern in a normal cell will therefore be two green and two red signals but in the case of a translocation between the two gene loci, the red and green signals will be juxtaposed and result in a single yellow fusion signal on one of the two resultant derivative chromosomes[244]. There are three kinds of fusion signal probe sets: double fusion signal if both probes cover all possible breakpoints on both chromosome partners; single fusion probes are designed near the breakpoints on both chromosomes and fusion with a residual signal if one probe only covers all possible breakpoints leaving a residual signal on one of the translocation derivatives. The sensitivity of the probes differs on their designs. The double fusion probes are the most sensitive of these.

The BA probes consist of two differentially labeled probes that hybridise to opposite sides of a breaking point in the same locus. One probe will cover the 5' part of the locus while the second will span the 3' side. In a normal cell, two yellow fusion signals will be seen whilst in the case of a break due to a translocation occurring within the locus under investigation, one fusion signal will be seen for the normal chromosome and one red and one green signal each representing one of the two derivative chromosomes[244].

CHAPTER II

MATERIALS AND METHODS

2.1 Sample collection

Cases diagnosed as PBL and DLBCL were retrieved from the archives of the Departments of Oral Pathology of the Universities of Pretoria and Limpopo, South Africa. Only cases affecting the oral cavity were included in this series. This was a retrospective study on archival material and patient records. Ethics approval was received from the Research Ethics committee of the Faculty of Health Sciences, University of Pretoria: number 104/2008.

2.2 Clinical features

The clinical features of all cases retrieved for this study were noted from the histology request forms. When available the HIV status of the patients was recorded. Many patients presented for the first time with an intra-oral tumour or ulcer without any previous medical work-up. Very little medical history accompanied the histology request forms from where we retrieved clinical information for this study. After diagnosis of the lesions, most patients were lost to us for further study.

2.3 Microscopic features

The microscopic features of all confirmed cases of PBL were evaluated on thin (2µm) haematoxylin and eosin (H&E) stained sections and described in detail. Each case was classified according to its morphology as either 'PBL of the oral mucosa type' or as 'PBL with plasmacytic differentiation' using the criteria as described previously[56, 92, 93] and also referred to in the 2008 WHO classification[9]. 'PBL of the oral mucosa type' was defined as a monotonous proliferation of large lymphoid cells with immunoblastic features with little or no plasmacytic differentiation and 'PBL with plasmacytic differentiation' was defined as immunoblasts and plasmablasts with more differentiation towards

mature plasma cells. HIV and EBV status was not taken into consideration for the morphologic classification in our study.

2.4 Immunohistochemistry

CD45, CD20, CD79 alpha, CD138, CD38, CD3, Ki-67 (MIB-1), MUM1 PROTEIN, IMMUNOGLOBULIN LIGHT CHAINS (KAPPA AND LAMBDA) and ALK protein

Immunohistochemical investigations were performed on three micrometer sections cut from formalin fixed paraffin embedded (FFPE) tissue blocks. Sections were de-paraffinized in two changes of fresh xylene for ten minutes each, re-hydrated with two changes of absolute ethanol, followed by 90%, 70% and 50% graded alcohol solutions respectively and washed with distilled water. Endogenous peroxidase activity was blocked by incubating the sections to undergo heat induced epitope retrieval (HIER) in a high pH buffer of a 3% aqueous solution of hydrogen peroxide for six minutes at 37ºC. Blocking of endogenous peroxidase activity for the sections to undergo low pH HIER were performed after HIER. HIER in citric acid buffer, pH 6, was performed on the sections for CD45, CD20, CD79α, CD138, CD38 and ALK protein and HIER in ethylene diamine tetra-acetic acid disodium salt (EDTA) buffer, pH 8.00 was performed for antibodies CD3, Ki-67, MUM1 protein and immunoglobulin light chains (kappa and lambda). HIER was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120ºC for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 minutes. The sections were rinsed in six changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS) (Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for ten minutes at room temperature.

The sections on which low pH (citric acid) HIER was performed were incubated with the following monoclonal mouse anti human ready to use

(RTU) antisera from Dako (Dako Cytomation Inc. 6392 Via Real, Carpintera, California 93013 USA) i.e. CD45 [Leucocyte Common Antigen Clone 2B11 and PD7/26 (Dako N1514)], CD20cy [clone L26 (Dako N1502)], CD79α [clone JCB117 (Dako N1628)] and a 1:50 dilution of CD138 anti-serum [Clone MI15, (Dako M7228)] for 20 minutes at 37°C. The sections for ALK protein [CD246 Clone ALK1 (Dako IR641)] were incubated for 60 minutes at room temperature. A section of each case was also incubated in a 1:100 dilution of monoclonal mouse anti human CD38 [clone SPC32, (NCL-L-CD38-290) Novocastra Laboratories Ltd., Balliol Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW United Kingdom] for 35 minutes at 37°C.

A section of each case, on which high pH HIER was performed, was incubated with RTU monoclonal mouse anti human CD3 [Clone PS1 (Novocastra RTU-CD3-PS1)] and Ki-67 [Clone MM1 (Novocastra RTU-Ki-67-MM1)], for 40 minutes at 37°C and in a 1:25 dilution of monoclonal mouse anti human MUM1 protein [clone MUM1p (Dako M7259)] for 60 minutes at room temperature. The sections for immunohistochemical investigation of kappa and lambda were first incubated in Protein Block Serum (Dako X0909) for ten minutes at room temperature to quench background staining before incubation with 1:100 monoclonal mouse Kappa light chain [Clone kp-53, (Novocastra NCL-KAP), Leica Microsystems] and 1:200 monoclonal mouse Lambda light chain [Clone HP-6054, (Novocastra NCL-LAM)] antibodies for 60 minutes at room temperature respectively.

All of the abovementioned sections were then incubated in Dako Envision+™ System Labelled Polymer, anti mouse, HRP [(horse radish peroxidase) Dako K4001], for 30 minutes at room temperature and rinsed in three changes of fresh PBS for five minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469) for four minutes at 37ºC, washed in distilled water and counterstained in Mayer's Haematoxylin for two minutes at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025).

Strong red-brown granular staining, either on the cell membrane (CD45, CD3, CD20, CD38, CD138), cell nucleus (Ki-67) or in the cytoplasm of the tumour cells (MUM, ALK, CD79a, kappa and lambda) was regarded as positive. Photomicrographs (400X magnification) were taken of representative areas with a Leica DMD108 digital microimaging microscope. The image is displayed to a monitor. At least 500 tumour cells were then manually counted and evaluated in each case. Positivity was recorded, based on the percentage of positive staining tumour cells as follows:

- Focal: when less than 20% of cells in a given case stained positive,
- Intermediate: when 20-70% of cells stained positive
- Diffuse: when more than 70% of tumour cells stained positive.

Only tumour cells were evaluated in each case. Because the nature of the plasmacytic cells, neoplastic or reactive, was unknown, these cells were discarded from the evaluation of the immunohistochemical analysis.

Light chain restriction was defined as tumour cells staining for one light chain marker only (kappa or lambda) with no positive staining in any tumour cells for the other light chain.

2.5 In Situ Hybridisation for HHV-8 and EBV

2.5.1 HHV-8

Three micrometer sections of FFPE tissue blocks were cut, mounted onto Histobond^{R} slides (Paul Marienfeld GmbH & Co. KG Am Wöllerspfad 4 97922 Lauda-Königshofen, Germany) and incubated at 55 ºC overnight. The sections were dewaxed in two changes of fresh Xylene, hydrated in two changes of absolute alcohol, 70% alcohol and three rinses of distilled water for three minutes each. HIER in citric acid buffer, pH 6, was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvey 42, DK-2600, Glostrup, Denmark). The sections were treated at 120℃ for 14 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for twenty minutes. The sections were rinsed in three changes of fresh distilled water for three minutes each, incubated in 0.01% pepsin (Sigma P6887, Sigma-Aldrich-Chemie. GmbH, P.O. 1120, 89552 Steinheim, Germany) in 0.2M hydrochloric acid for 11 minutes at room temperature and rinsed in three changes of distilled water for five minutes each. The slides were dehydrated in 95% and 100% alcohol for three minutes each and air dried.

Twenty microliter of Human Herpes virus (type 8) fluorescein labelled oligonucleotide cocktail probe solution designed to hybridise with a small transcript, designated T1.1 mRNA (Novocastra, NCL-HHV-8, Leica Biosystems Newcastle Ltd, Balliol Business Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW, UK) was added to each slide, cover slipped and sealed with Fixogum rubber cement (Poseidon^{TM} RF^{TM} Marabuwerke GmbH & Co. KG, Asperger Strabe 4, D 71732 Tamm, Germany). Denaturing was performed at 65℃ for 15 minutes to block endogenous alkaline phosphatase activity followed by hybridisation at 37℃ for two hours using a StatSpin Thermobrite^{TM} (Abbott Molecular, 3100 Woodcreek Dr. Downers Grove, 1L 60515 USA).

The cover slips were drained off in Tris Buffered Saline (TBS) [50mM Tris/HCl, 150mM NaCl pH 7.6) containing 0.1% Titron-X-100 (Bio Rad Laboratory, 2000 Alfred Nobel Drive, Hercules CA 94547)] for three minutes.

A post hybridisation stringency wash in 1:50 dilution of ready to use Dako stringent wash concentrate (Dako S3500) was performed at 55 degree Celsius for twenty minutes. The slides were rinsed in TBS containing 0.1% Titron-X-100 for three minutes.

To reduce background staining slides were placed on an incubation tray and the sections were covered with 100 µl of normal rabbit serum (Novocastra NCL-R-Serum) diluted 1:5 in TBS containing 3% w/v Bovine Serum Albumin (BSA) (Sigma A7906) and 0.1% Titron-X-100 and incubated at room temperature for ten minutes.

Detection was performed using Novocastra's *in situ* hybridisation detection kit (NCL-ISH-D). The blocking solution was tipped off and the slides incubated in 1:200 alkaline phosphatase-conjugated antibody to fluorescein isothiocyanate (FITC) (affinity-isolated rabbit F(ab') in 100mM Tris Buffer, 50mM $MgCl_2$, 0.1 mM $ZnCl_2$, 4% w/v BSA, stabiliser and preservative, pH 7.5) in TBS containing 3% w/v BSA and 0.1% v/v Titron X-100 for 30 minutes at room temperature. The slides were then washed in two rinses of TBS for three minutes each and thereafter in alkaline phosphatase substrate buffer (AFSB) (100mM Tris/HCl, 50mM $MgCl_2$, 100mM NaCl pH 9.0) for five minutes.

The sections were covered with 200 µl Enzyme substrate (5-bromo-4-chloro-3-indolylphosphate (BCIP) and Nitroblue tetrazolium (NBT) in dimethylformamide solution) diluted 1:100 in AFSB containing 1 µl Levamisole inhibitor per millilitre diluted enzyme substrate and incubated in the dark overnight.

The slides were washed in running water for five minutes, counterstained with Mayer's Haematoxylin for ten seconds and mounted with Dako Faramount aqueous mounting.

Black granular nuclear staining was accepted as positive for HHV-8. A Kaposi sarcoma section positive for HHV-8 was used as positive control and a brain section was used as negative control. Positive and negative controls were run with every batch of ISH for HHV-8.

2.5.2 EBV

Three micrometer sections of FFPE tissue blocks were cut, mounted onto Histobond^{R} slides (Paul Marienfeld) and incubated at 55° C overnight. The sections were dewaxed in two changes of fresh xylene, hydrated in two changes of absolute alcohol, 70% alcohol and three rinses of distilled water for three minutes each. The sections were placed on an incubation tray and covered with ready to use Proteinase K (Dako S3020) for three minutes at room temperature. The slides were then washed in two changes of distilled water for five minutes each, followed by 95% and 100% alcohol for three minutes each and air dried.

Twenty microliter of EBV fluorescein labelled oligonucleotide cocktail probe solution for the detection of mRNA (Novocastra, NCL-EBV, Leica Biosystems) sequences in EBV-encoded RNA (EBER) transcripts was added to each slide, cover slipped and sealed with Fixogum rubber cement (Poseidon$^{TM)}$. Denaturing was performed at 65℃ for fifteen minutes to block endogenous alkaline phosphatase activity followed by hybridisation at 37℃ for two hours using a StatSpin Thermobrite^{TM} (Abbott Molecular). The cover slips were drained off in TBS containing 0.1% Titron-X-100 for three minutes and washed in three changes of fresh TBS containing 0.1% Titron-X-100 for three minutes each.

To reduce background staining slides were placed on an incubation tray and the sections were covered with 100µl of normal rabbit serum (Novocastra NCL-R-Serum, Leica Biosystems) diluted 1:5 in TBS containing 3% w/v BSA (Sigma A7906) and 0.1% Titron-X-100 and incubated at room temperature for ten minutes.

Detection was performed using Novocastra's ISH detection kit (NCL-ISH-D Novocastra, Leica Biosystems) according to the manufacturer's guidelines.

The blocking solution was tipped off and the slides incubated in 1:400 alkaline phosphatase-conjugated antibody to FITC (affinity-isolated rabbit F(ab') in 100mM Tris Buffer, 50mM $MgCl_2$, 0.1 mM $ZnCl_2$, 4% w/v BSA, stabiliser and preservative, pH 7.5) diluted 1:400 TBS containing 3% w/v BSA and 0.1% v/v Titron X-100 for thirty minutes at room temperature. The slides were then washed in two rinses of TBS for three minutes each and thereafter in AFSB buffer for five minutes.

The sections were covered with 200µl Enzyme substrate (NBT/BCIP) diluted 1:200 in AFSB containing 1µ Levamisole inhibitor per millilitre diluted enzyme substrate and incubated in the dark overnight.

The slides were washed in running water for five minutes, counterstained with Mayer's Haematoxylin for ten seconds and mounted with Dako Faramount aqueous mounting media.

Black granular nuclear staining was accepted as positive for EBV. A nasopharyngeal carcinoma section positive for EBV was used as positive control and a brain section was used as negative control. Positive and negative controls were stained with every batch of *ISH* done for EBV.

2.6 Fluorescence *in situ* hybridisation

2.6.1 FISH technique

Four micron sections of FFPE tissue blocks were cut, mounted on Super Frost plus slides (Menzel-Gläser, Gerhard Menzel GmbH, Saarbrückener Str.248 D-38116 Braunschweig, Germany), dried overnight at 56℃ and matured at room temperature for two days. The sections were deparaffinized in two changes of fresh xylene for 15 minutes each followed by two rinses in absolute alcohol for five minutes each at room temperature and air dried. The slides were incubated in 0.2 N hydrochloric acid (HCl) for 20 minutes at room temperature, rinsed in pure water for three minutes followed by three minutes in two times saline sodium citrate (SSC) buffer and air dried. Pre-treatment of the slides were done in a 0.1M sodium thiocyanate (NaSCN) for 30 minutes at 80℃ followed by a one minute rinse in pure water and two washes in two times SSC buffer for five minutes each at room temperature. Digestion of the sections was done in a pre-warmed 0.05% Pepsin (Pepsin, Roche Diagnostics GmbH Mannheim, Germany) in 0.1N HCl at 37℃ for 60-90 minutes. This was followed by two washes in two times SSC for five minutes each at room temperature. Slides were fixed in a 1% formaldehyde solution for ten minutes at room temperature, rinsed twice in two times SSC buffer and dried on a slide warmer.

FISH analyses using the following probes from Vysis (Vysis Inc, Downers Grove, Illinois, USA) were done on separate sections of each case: LSI *IGH* dual colour BA rearrangement probe, LSI *MYC* dual colour BA rearrangement probe, LSI *BCL6* dual colour BA probe, LSI *IGH/MYC* CEP 8 tri-colour, dual fusion translocation probe, LSI *CCND1/IGH* dual colour dual fusion translocation probe and LSI *IGH/BCL2* dual colour dual fusion translocation probe. The probes were prepared according to the manufacturer's instructions. The sections were cover slipped with probe solution and sealed with Marabu Fixogum Rubber cement (Marabuwerke GmbH & Co. Tamm Germany). The sections were protected from light from hereon. Denaturation and hybridisation was performed in a thermocycler (Thermobrite, Abbot

Molecular Illinois, USA). Slides were denatured at 75°C for five minutes and then hybridised overnight at 37°C.

The rubber cement was removed and the cover slips washed off in 2 x SSC. A post hybridisation wash was performed on the slides at 76°C for four minutes in 0.05% Tween/2 x SSC and counterstained in 200 ng/ml 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Merck, Darmstadt, Germany) for 15 minutes at room temperature. The sections were rinsed in 0.05% Tween/2 x SSC and cover slipped with Vectashield mounting media for fluorescence (Vector Laboratories, Inc Burlingame, CA 94010), sealed with fixogum rubber cement and evaluated with a Nikon Eclipse E1000 microscope equipped with a spectrum- green, orange, aqua, DAPI and triple band filter.

2.6.2 Criteria for positive FISH results:

IGH-BA rearrangement probe (14q32.3 LSI IGH)

In a normal cell hybridised with the *IGH*-BA probe, the expected signal pattern is two yellow fusion signals per nucleus. In a cell with an *IGH* translocation breakpoint, one spectrum green (LSI 5' flanking probe), one spectrum orange (LSI 3' flanking probe) and one yellow fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts and verified by three individual analysts. The cut-off value for a positive result with the IGH-BA probe in this study was 5%.

***IGH/MYC*, CEP 8 tri-colour, dual fusion translocation probe [t(8;14)(q24;q32)]**

In a normal cell hybridised with this probe, the expected pattern is two spectrum aqua (chromosome 8 CEP), two spectrum orange (*MYC*-gene), and two spectrum green (*IGH*-gene) signals. A cell harboring the reciprocal t(8;14) with a 8q24 breakpoint within the *MYC* locus is expected to produce a pattern of one orange, one green, two orange/green fusions, and two aqua signals. If the cell contains a breakpoint very far 5' of *MYC*, a fusion on the

der(8) may not be visible or may be very weak, as little or no red probe target would remain on the der(8). Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(8;14) probe in this study was 15%.

***MYC* BA rearrangement probe (8q24 LSI MYC)**

In a normal cell hybridised with the *MYC*-BA probe, the expected signal pattern is two orange/green fusion signals. In a cell with a translocation breakpoint, one green, one orange and one fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the *MYC*-BA probe in this study was 5%.

***IGH/CCND1* dual colour, dual fusion translocation probe [t(11;14)(q13;q32)]**

A cell harboring a t(11;14) with breakpoints in the *IGH* locus on 14q32 is expected to have a signal pattern of two yellow fusions, one on each of the translocation derivative chromosomes 11 and 14 as well as one spectrum orange (*CCND1* on chromosome 11) and one spectrum green (*IGH* on chromosome 14) signal from the normal chromosomes 11 and 14. Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(11;14) probe in this study was 15%.

***IGH/BCL2* dual colour, dual fusion translocation probe [t(14;18)(q32;q21)]**

A normal nucleus hybridized with the LSI *IGH/BCL2* probe shows two spectrum orange (*BCL2*) and two spectrum green (*IGH*) signals. In a nucleus harbouring a t(14;18), the most common pattern is one orange signal, one

green signal (representing the normal chromosome 14 homolog) and two yellow fusion signals representing the two derivative chromosomes resulting from the reciprocal translocation. Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(14;18) probe in this study was 15%.

***BCL6* BA rearrangement probe (3q27)**

In a normal cell hybridised with the *BCL6* BA probe, the expected signal pattern is two yellow fusion signals. In a cell with a translocation breakpoint in the BCL6 locus, one spectrum green, one spectrum orange and one fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the *BCL6* BA probe in this study was 5%.

Selected images were captured using the GenusTM CytoVision 3.0 software (Applied Imaging).

2.6.3 General aspects of FISH analysis

Scoring of FISH results in FFPE slides required compensating for several potential artifacts. My approach in this study was to evaluate the signals in 100-200 tumour cells, depending on the availability of tumour cells optimal for evaluation and then to study them in detail in order to work out the percentages of signal patterns encountered in every case. Although this was very time consuming, the method resulted in optimal results which was 100% comparable between all three analysts involved in the study.

The possibility of false positive results was considered in each case. This depended very much on the type of probe used. The two probes of a fusion assay may come to lie accidentally close to one another, producing a false positive yellow-like fusion signal. This also holds true for the BA probe where great care should be taken when interpreting the distance between the orange

and green signals. This varies between different probes. An example of this was the IGH/CCND1 dual colour, dual fusion translocation probe (t(11;14)). Due to the gap between the two probes in the *IGH* probe set, the normal *IGH* loci may sometimes appear as two slightly separated green signals. This gap may in some instances also cause a slight separation of the orange and green signals on the der(11) chromosome. One way to overcome these problems is to have well-tested cut off criteria to mark a case as either positive or negative by doing enough negative control cases. In our study the cut-off value for a positive result with BA probes were 5% and the cut-off value for a positive result with the fusion probes were 15%.

CHAPTER III

RESULTS

3.1 Clinical features

Forty-five cases of PBL were included in the study. The group consisted of 31 males, 12 females and 2 of unknown gender. Two patients were white males, 1 was of unknown race and the rest were black patients. The male to female ratio was 2.6:1. The mean age of the patients was 41 years (29-58 years). The gingiva as well as the buccal vestibule and palate were the most commonly affected intra-oral areas. The HIV-status of 13 patients was unknown, 31 were HIV positive and 1 was HIV negative (Table 2).

Table 2: *The table represents a summary of the clinical features of all PBL cases included in the study*

Case	Age	Race	Sex	HIV status	Anatomical Location
1	47	B	M	+	Gingiva posterior maxilla
2	48	B	M	+	Gingiva posterior mandible & sulcus
3	34	B	F	+	Gingiva posterior mandible
4	52	B	F	+	Buccal sulcus
5	U	B	M	+	Gingiva unknown location
6	U	B	U	+	Gingiva maxilla
7	45	B	F	+	Buccal mucosa
8	48	W	M	U	Palate
9	39	B	F	+	Gingiva posterior mandible
10	33	B	F	+	Swelling of face
11	34	B	M	+	Buccal mucosa & soft palate
12	36	B	F	+	Buccal & palate mucosa
13	39	B	M	+	Gingiva unknown location
14	43	B	M	+	Palate, buccal vestibulum
15	28	B	F	+	Gingiva mandible
16	50	B	M	+	Gingiva maxilla
17	36	B	F	-	Gingiva mandible
18	37	B	M	+	Gingiva mandible
19	44	B	M	+	Gingiva mandible
20	45	B	M	U	Gingiva mandible
21	32	B	F	U	Gingiva maxilla & palate
22	45	B	M	U	Palate
23	35	B	M	U	Sockets of teeth 37-38
24	29	B	M	U	Palate
25	44	B	M	U	Gingiva mandible
26	43	B	M	U	Gingiva maxilla
27	45	B	M	U	Gingiva mandible
28	58	B	F	U	Palate
29	43	B	M	+	Gingiva maxilla
30	35	B	M	U	Gingiva anterior mandible
31	39	B	M	+	Gingiva mandibular molar
32	39	B	M	+	Buccal sulcus left
33	50	B	M	+	Maxilla & buccal mucosa
34	U	U	U	+	Gingiva mandible & buccal mucosa
35	U	B	M	+	Gingiva maxilla
36	43	B	M	+	Gingiva left mandible
37	U	B	M	U	Gingiva mandibular retromolar area
38	27	B	M	+	Buccal mucosa
39	36	B	M	+	Gingiva post maxilla & palate
40	40	B	M	U	Gingiva maxilla
41	32	B	M	+	Gingiva unknown location
42	43	W	M	+	Gingiva unknown location
43	45	B	F	+	Gingiva maxilla
44	U	B	F	+	Gingiva mandible
45	44	B	M	+	Gingiva maxilla

B = black; W = white; U = unknown; M = male; F = female

3.2 Microscopic features

Morphologically, all cases showed dense infiltrates of mostly monomorphic, large blastic cells growing in a cohesive, sheet-like pattern (Figs.2 & 3). Some cases showed discohesion between the tumour cells (Fig.4). Tumour cells had either a single prominent nucleolus (Fig.2) (immunoblastic appearance) or several nucleoli (Fig.3) in round to oval eccentrically placed nuclei (plasmablastic appearance). Binucleate and multinucleated tumour cells were sometimes seen focally (Fig.5). In some cases, the tumour cells exhibited prominent pleomorphism with a mixture of blastic cells and cells with large nuclei, either slightly indented or sometimes binucleate (Fig.5). Tumour cells always had abundant cytoplasm with occasional prominent paranuclear hof. Numerous tingible body macrophages imparting a starry sky appearance on microscopic examination were always present (Fig.6). Using the above criteria, 14/45 (31%) of cases were classified as 'PBL with plasmacytic differentiation' based on the presence of more mature plasmacytic cells intermingled with the blasts (Fig.7). Thirty one cases (69%) showed more of the features defining 'PBL of the oral mucosa type' consisting mostly of large blastic cells with much fewer plasmacytic cells (Fig.2). In all cases however, more mature-appearing plasma cells could be identified although the percentage of these cells varied amongst cases examined.

Figure 2: *A case of PBL where almost all of the tumour cells exhibit prominent immunoblastic morphology with a single prominent nucleolus (H&E, 2µm section, original magnification: 400X).*

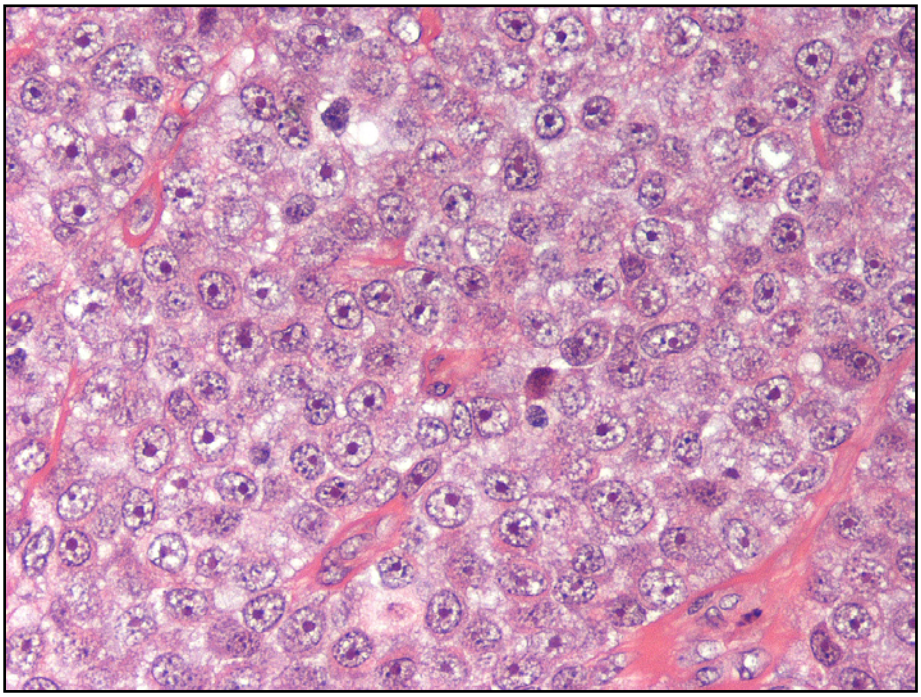


Figure 3: *A case of PBL where all of the tumour cells have a plasmablastic appearance with the tumour cell nuclei exhibiting several nucleoli in large eccentric nuclei (H&E 2µm section, original magnification: 400X).*

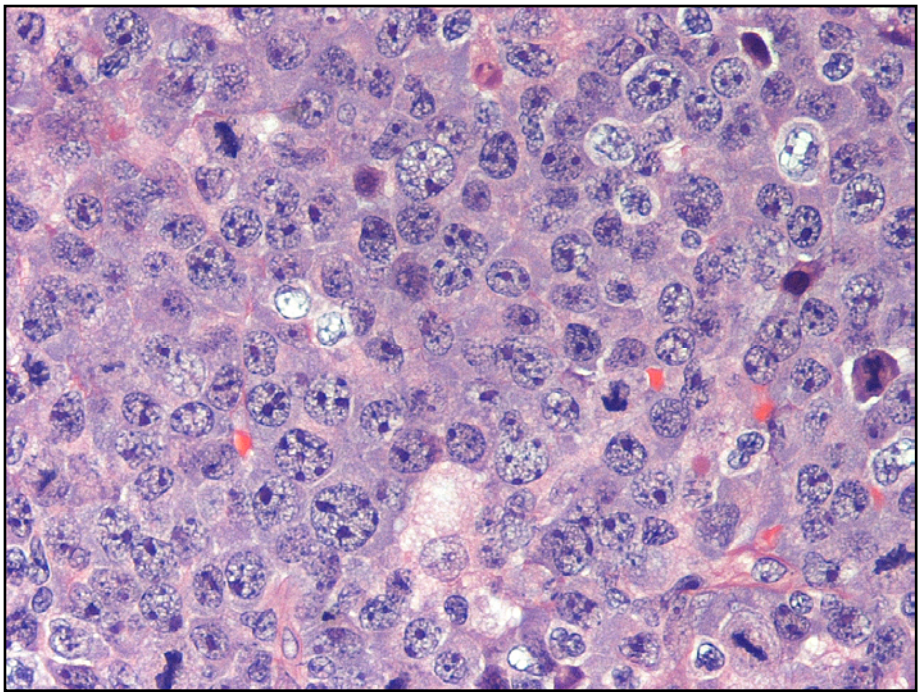


Figure 4: *This photo shows a case of PBL with prominent discohesion (arrows) between the tumour cells. Erythrocytes fill the spaces between the tumour cells (H&E 2µm section; Original magnification: 400X).*

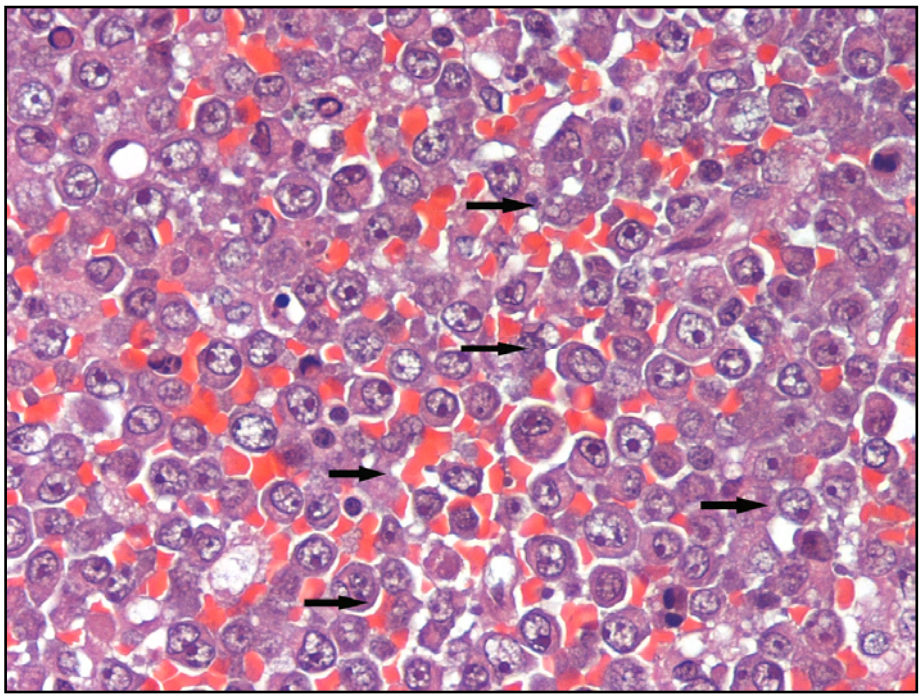


Figure 5: *The micrograph shows a case of PBL with prominent pleomorphism demonstrated by binucleate (thin arrow) and multinucleated (thick arrow) lymphoma cells (H&E 2µm section; Original magnification: 400X).*

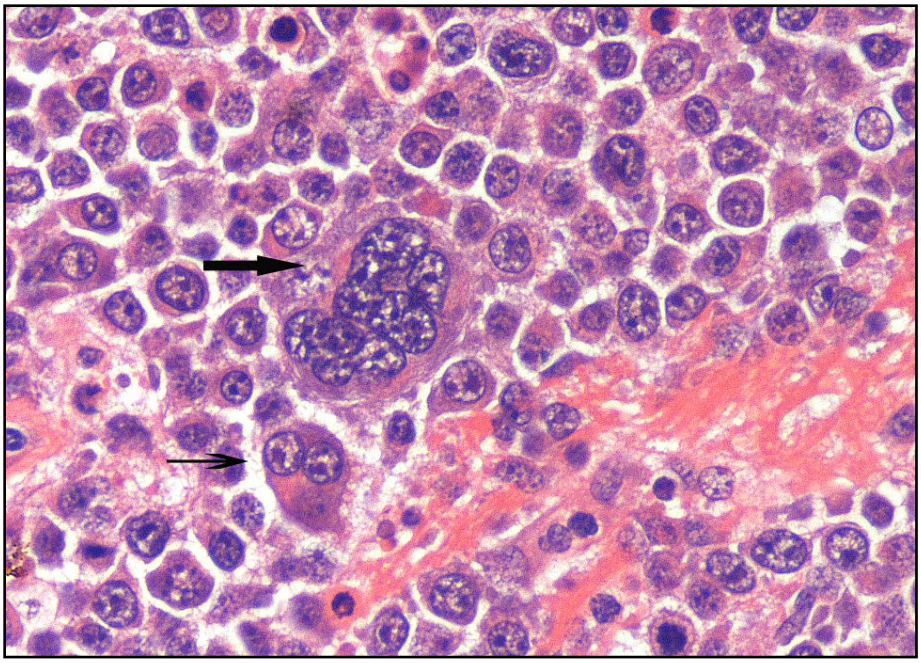


Figure 6: *The micrograph demonstrates the typical 'starry sky' appearance in a case of PBL caused by the presence of numerous tingible body macrophages (arrows) between the tumour cells (H&E 2µm section; Original magnification: 100X).*

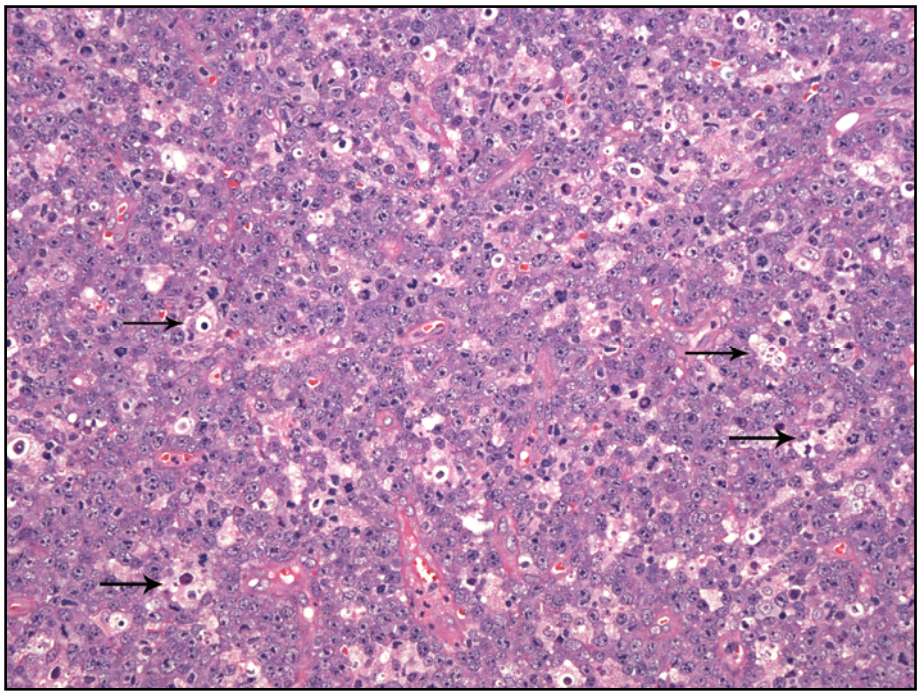
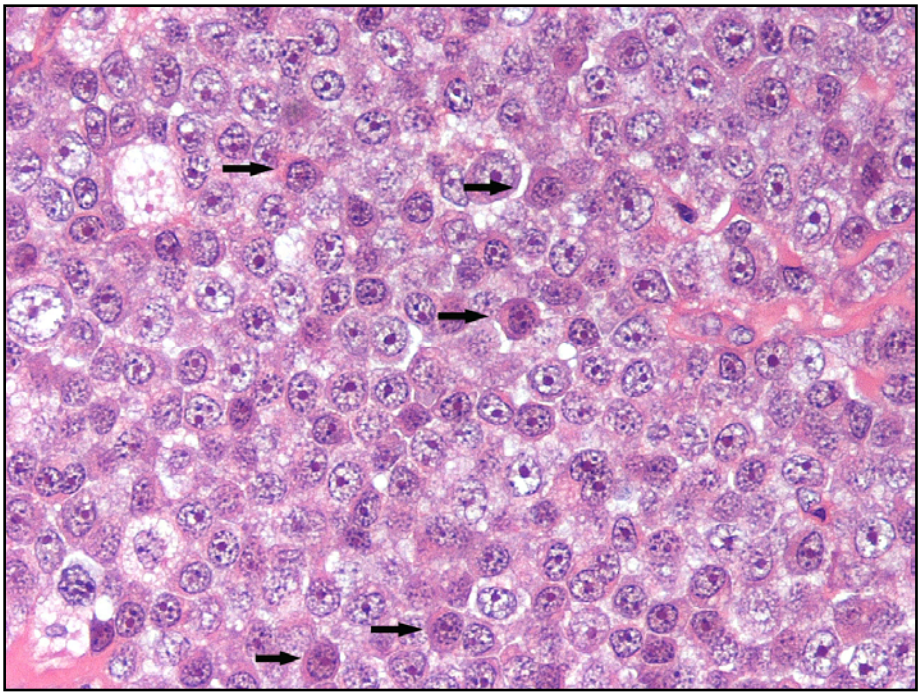


Figure 7: *The micrograph demonstrates a case of PBL with plasmacytic differentiation defined by the presence of more mature plasmacytic cells (arrows) intermingled with the blasts in the background (H&E 2µm section; Original magnification: 400X).*



3.3 Immunohistochemistry

3.3.1 CD20 and CD3

All cases of PBL included in this study were uniformly negative for CD20 although some reactive B-cells were always present to serve as a positive internal control (Fig.8). No tumour cells stained with CD3. All CD3-positive cells were interpreted as reactive T-cells. Some cases showed only a few CD3 positive T-cells (Fig.9) whilst others showed many more (Fig.10).

Figure 8: *The micrograph taken from a case of PBL confirms all tumour cells to be CD20 negative although reactive B-cells served as positive internal control (arrow) (CD20 stain; 3µm; Original magnification: 400X).*

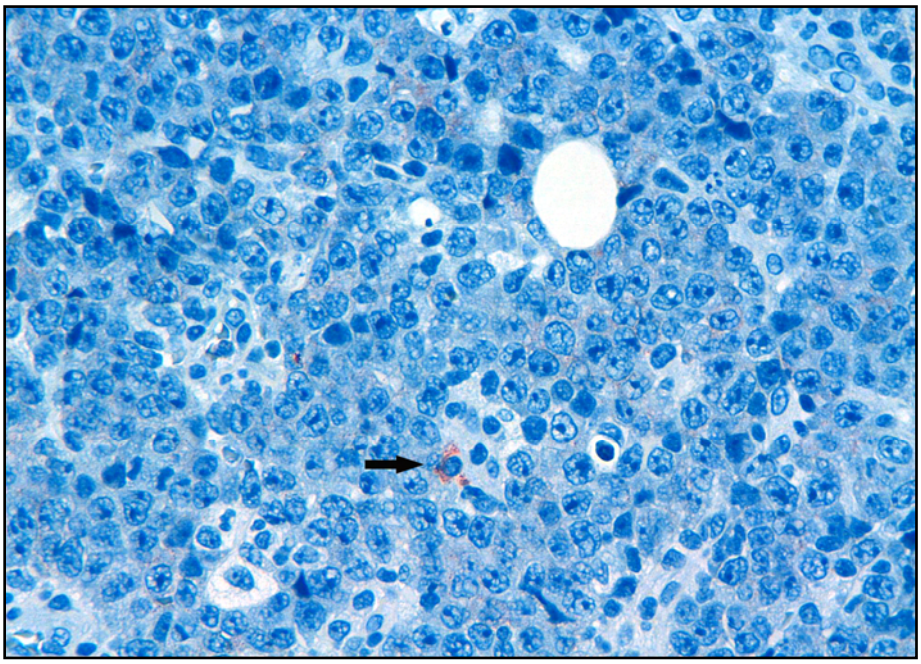


Figure 9: *The micrograph was taken from a case of PBL and confirms all the tumour cells to be CD3 negative. Reactive T-cells (arrows) served as a positive internal control for the CD3 stain (CD3 stain; 3µm; Original magnification: 400X).*

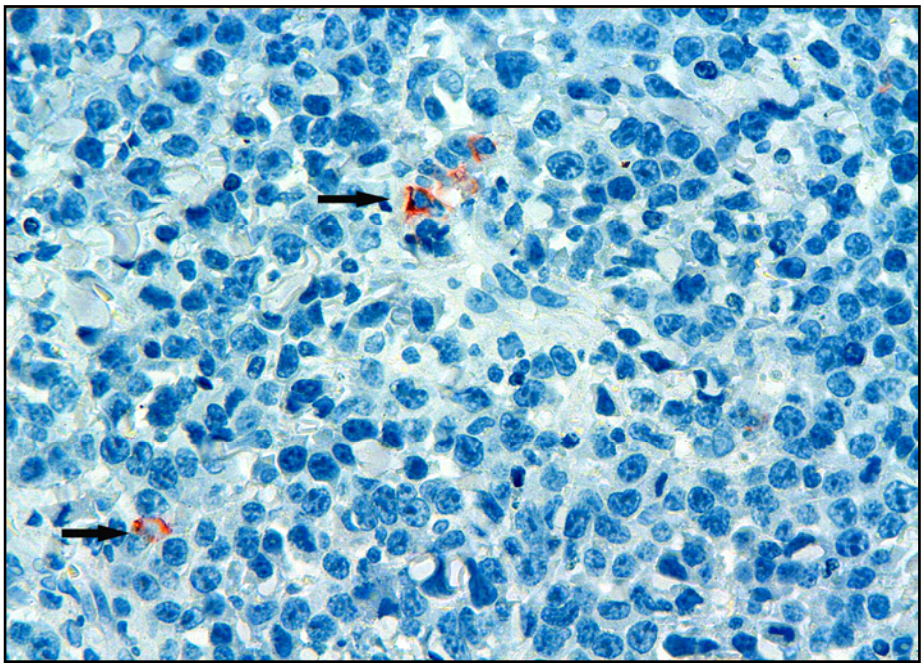
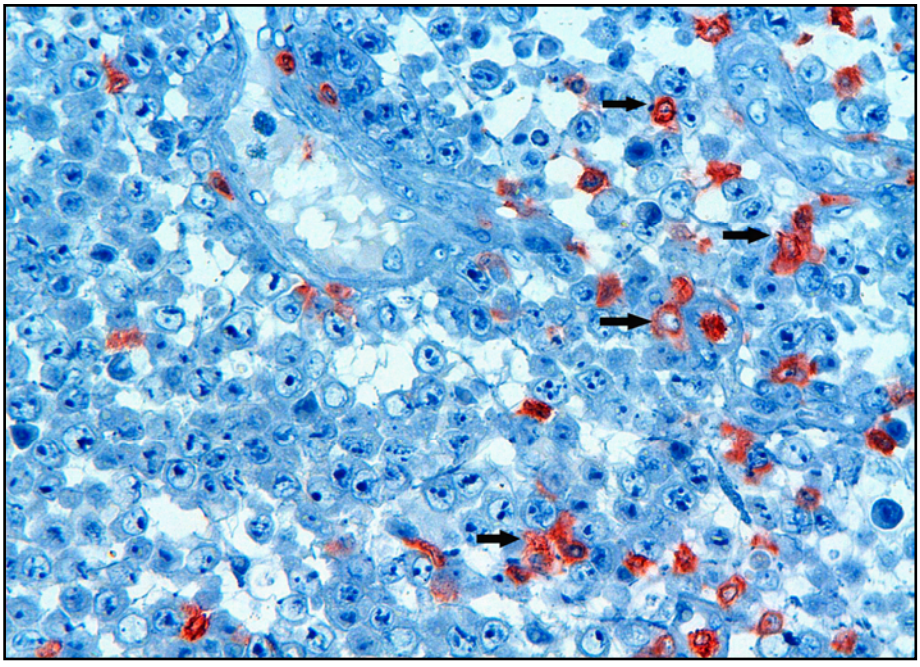


Figure 10: *The micrograph was taken from a case of PBL again confirming the tumour cells to be CD3 negative. More abundant reactive T-cells staining positive with CD3 (arrows) is however present (CD3 stain; 3µm; Original magnification: 400X).*



3.3.2 Ki-67

Diffuse and strong positive staining was found with Ki-67 in 34/45 (76%) of cases (Fig.11), intermediary positivity was found in the tumour cells of 9/45 (20%) and in 2 cases (4%) (Cases 17 and 24) positive staining was only focally present in some of the tumour cells (Fig.12).

Figure 11: *The micrograph represents a PBL case with typical diffuse and strong, red-brown positive nuclear staining for the proliferation marker, Ki-67 (Ki-67 stain; 3µm; Original magnification: 100X).*

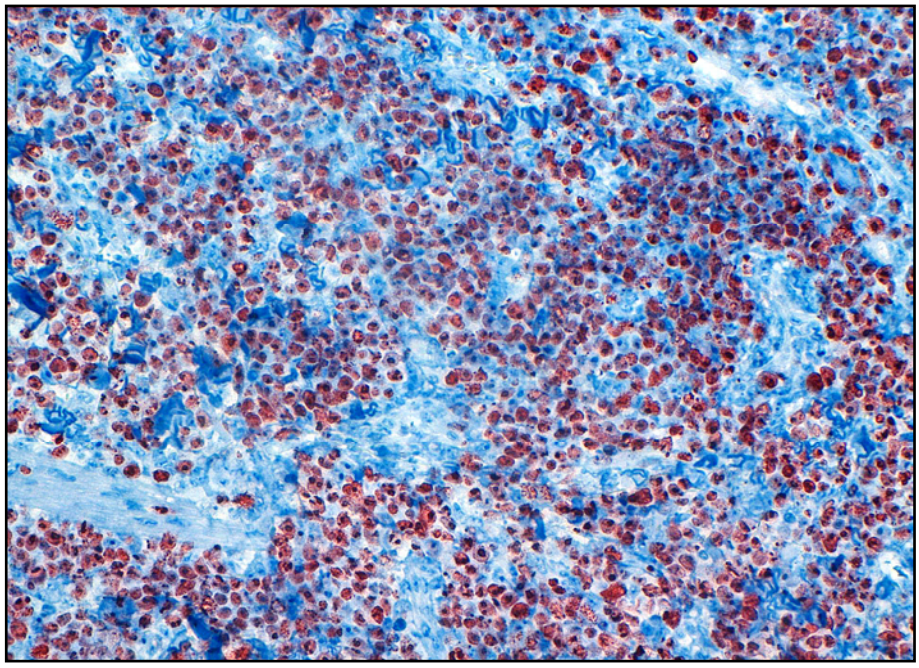
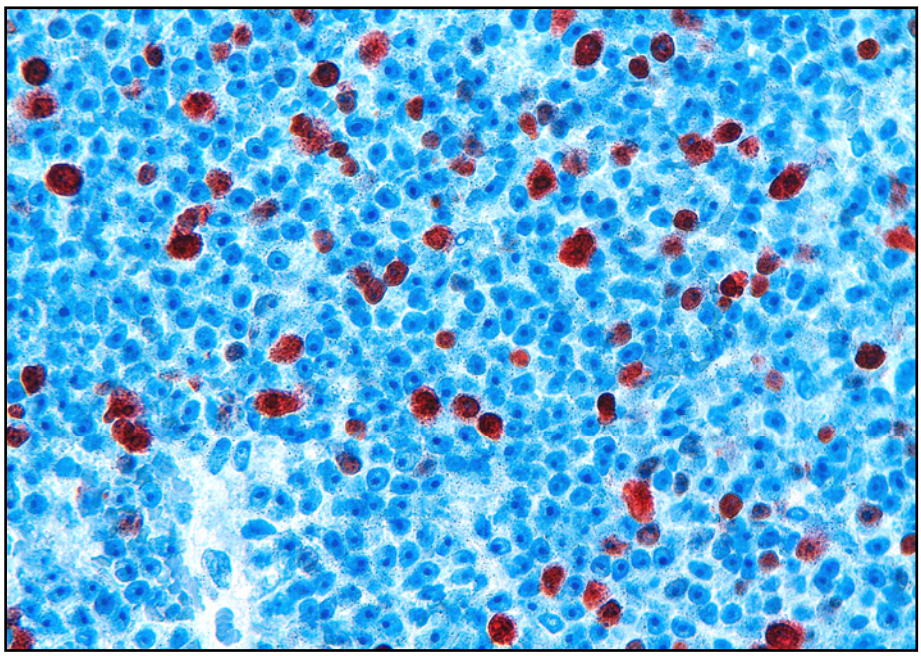


Figure 12: *A micrograph of case 17 showing only focal positivity for Ki-67. (Ki-67 stain; 3µm; Original magnification: 200X).*



3.3.3 Multiple Myeloma oncogene-1 (MUM1)

In the case of MUM1, 39/45 (87%) of cases showed strong and diffuse staining of the tumour cells (Fig.13), 5/45 (11%) showed positivity in only an intermediary number of the cells whilst one case (2%) (Case 43) showed only focal staining with the antibody (Fig.14).

Figure 13: *Strong and diffuse positive cytoplasmic staining with MUM in the tumour cells of a case of PBL (MUM stain; 3µm; Original magnification: 200X).*

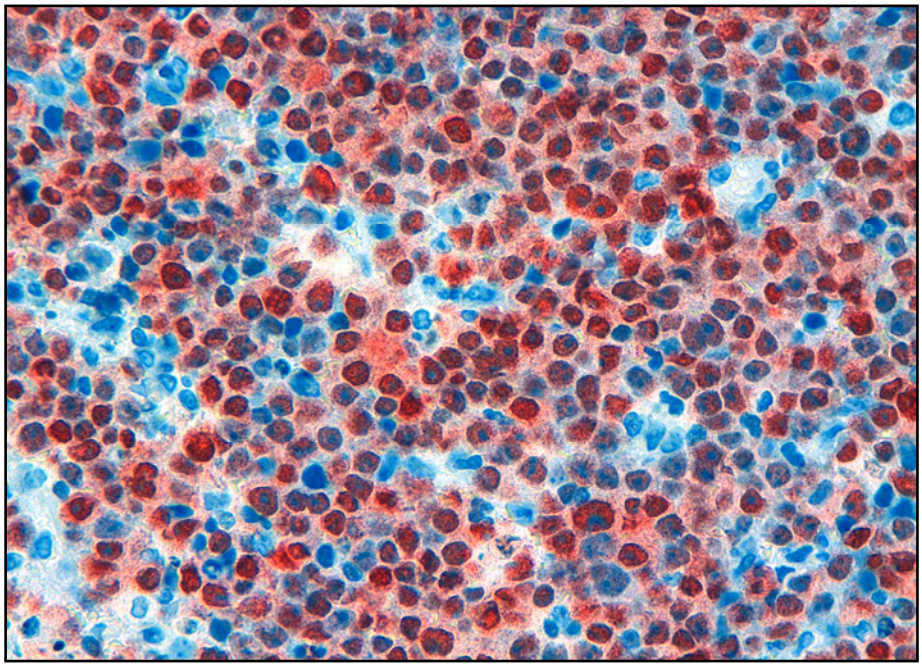
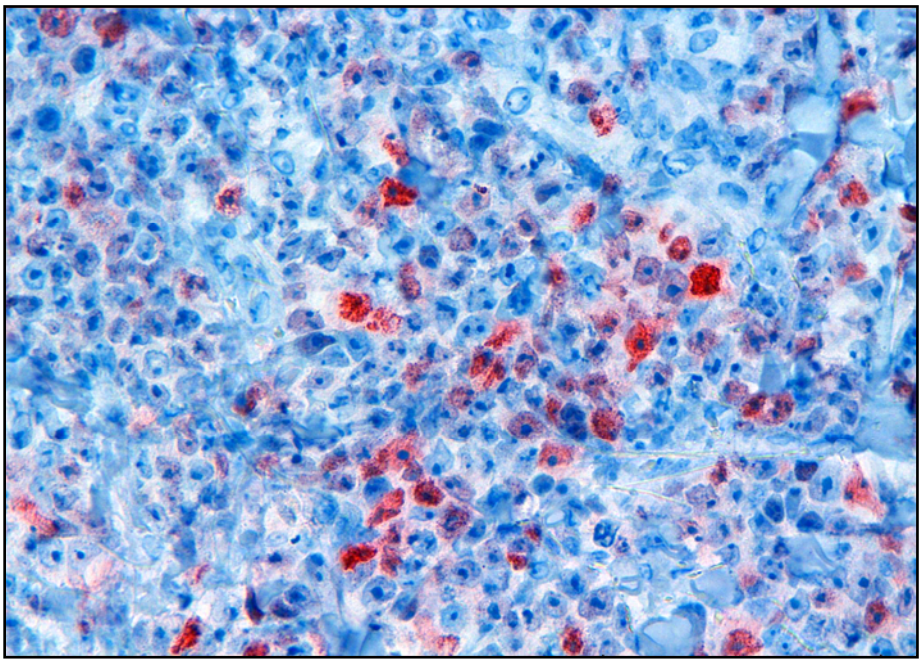


Figure 14: *The micrograph was taken of the MUM stain of case 43. Only focal positivity was present (MUM stain; 3µm; Original magnification: 200X).*

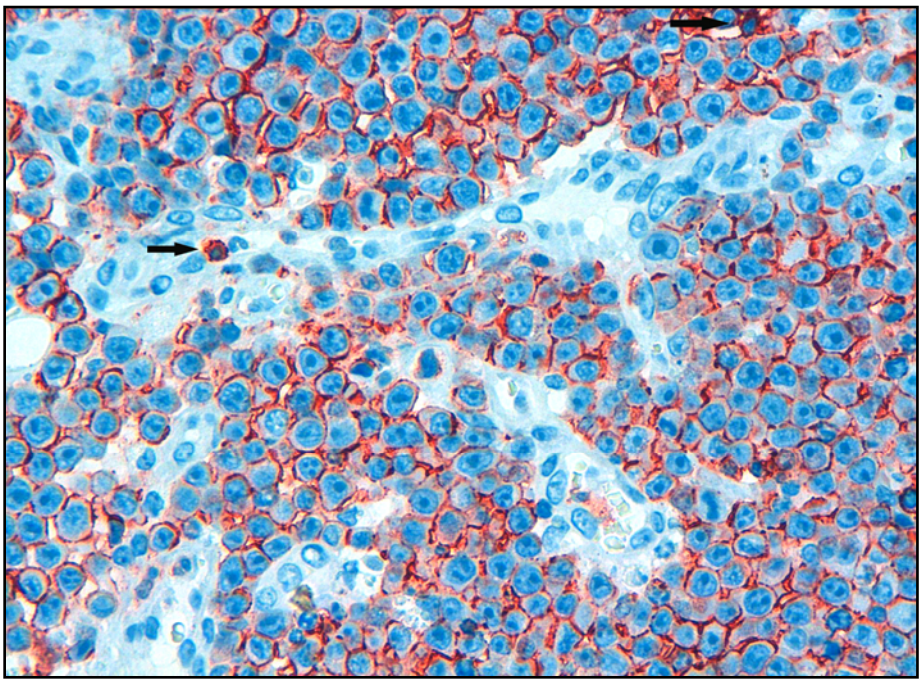


Variable immunoreactivity was found with CD45, CD79a, CD38 and CD138.

3.3.4 CD45

Apart from one case which stained negative for CD45 (Case 42), 44/45 (98%) of cases showed at least some form of positivity with this antibody with 17/45 (38%) showing diffuse and strong positivity (Fig.15).

Figure 15: *Strong positive staining for CD45 is present on the cell membranes of most of the tumour cells in this PBL. Some reactive B- and T-cells are also seen on this micrograph (arrows). (CD45 stain; 3µm; Original magnification: 200X).*



3.3.5 CD79alpha

Not a single case stained diffusely positive for CD79a. Intermediary positivity was seen in only one case (2%) (Case 32) (Fig.16) while 27/45 (60%) of cases showed focal staining only. No staining could be detected in 17/45 (38%) of cases, although strong positivity were present in reactive B-cells on the section (Fig.17) which served as internal control for this immunohistochemical stain. Overall, only 62% of cases showed some positive staining for this B-cell marker.

Figure 16: *Red-brown granular staining for CD79a is seen in the cytoplasm of the tumour cells (arrows) in this case of PBL (CD79a stain; 3µm; Original magnification: 200X).*

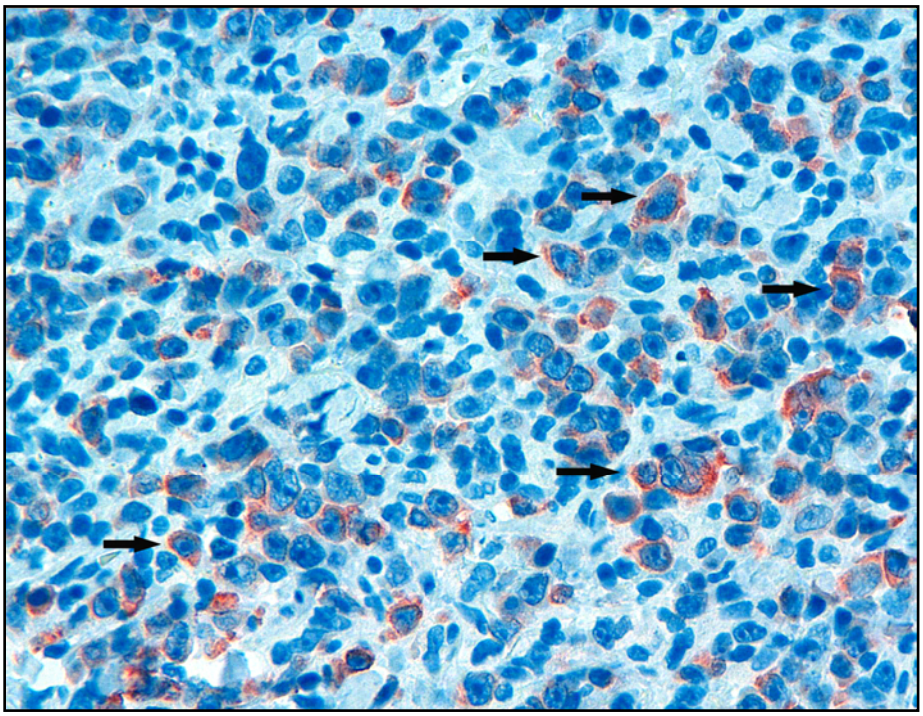
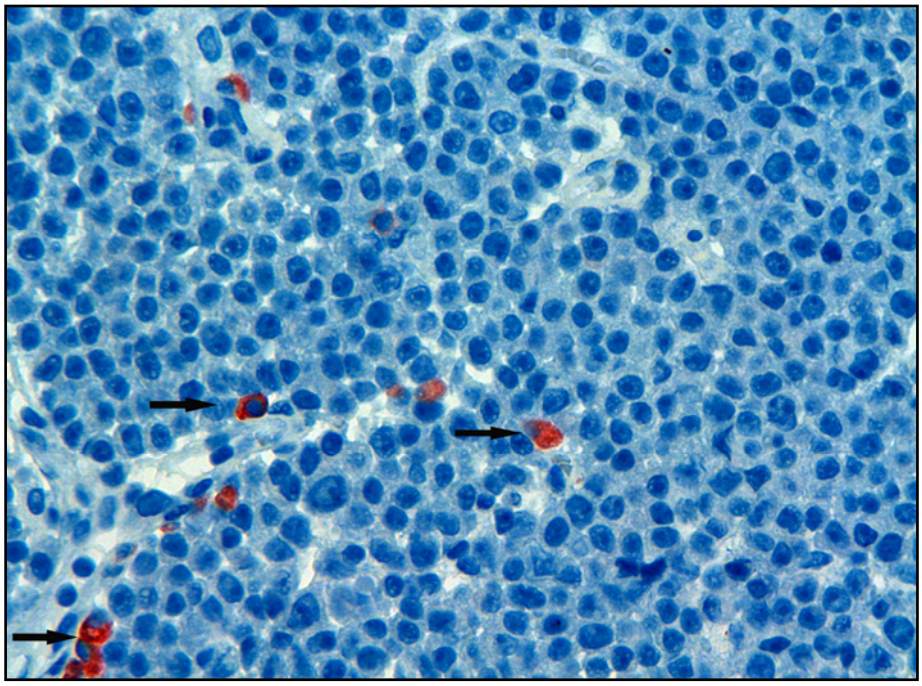


Figure 17: *The micrograph represents a case of PBL where positive staining for CD79a is seen in only the reactive B-cells (arrows) but not in the tumour cells (CD79a stain; 3µm; Original magnification: 200X).*



3.3.6 CD38

The plasma cell marker CD38 showed no staining in 16/45 (36%) of cases while 15/45 (33%) showed focal staining in the tumour cells, 7/45 (16%) showed positive staining in an intermediary number of cells and 7/45 (16%) cases showed diffuse positivity for this marker (Fig.18). Strong positivity was always present in reactive plasma cells which served as positive internal control for CD38 negative cases (Fig.19).

Figure 18: *The micrograph shows diffuse positive CD38 staining on the cell membranes of the tumour cells of this case of PBL. Membranous staining clearly delineates the cell membranes of some of the tumour cells (arrows) (CD38 stain; 3µm; Original magnification: 200X).*

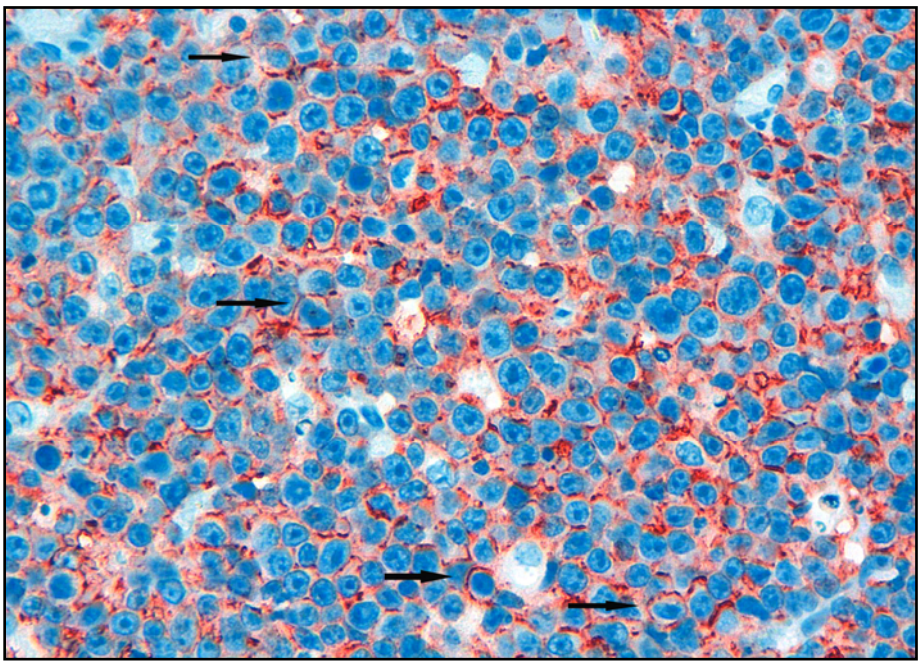
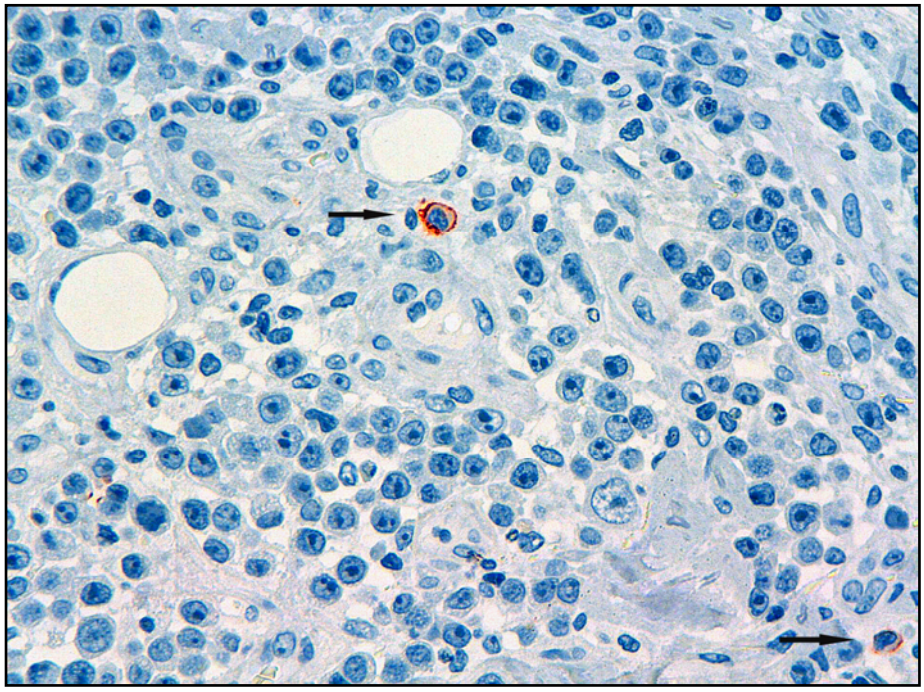


Figure 19: *Positive staining for CD38 is seen as red-brown granular staining in the reactive plasma cells (arrows). None of the larger tumour cells stained positive for CD38 in this case of PBL (CD38 stain; 3µm; Original magnification: 200X).*



3.3.7 CD138

CD138, another plasma cell marker, showed no staining in 7/45 (16%) of cases, focal staining in 22/45 (49%), intermediary number of cells in 7/45 (16%) and diffuse staining in 9/45 (20%) of cases (Fig.20). All cases negative for CD138 always showed strong positivity in reactive plasma cells (Fig.21) as well as overlying squamous epithelial cells which served as positive internal control for all cases (Fig.22).

Figure 20: *Diffuse positive staining for CD138 is present on the cell membranes of most of the tumour cells in this case of PBL. The membranous stain delineates the cytoplasmic rim of the tumour cells (arrows) (CD138 stain; 3µm; Original magnification: 200X).*

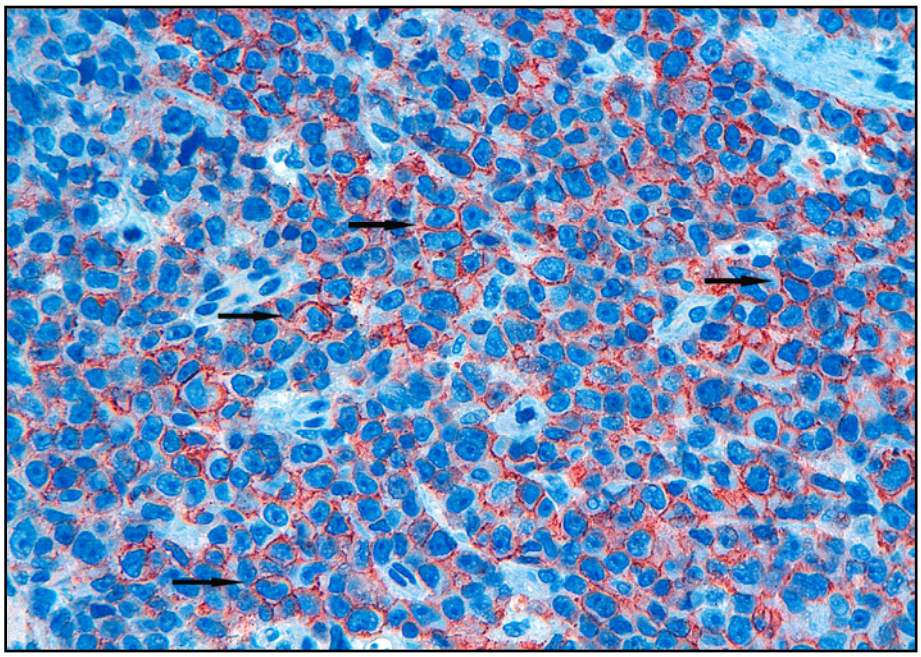


Figure 21: *Positive staining for CD138 is seen only in reactive plasma cells (arrows) of this PBL case. None of the tumour cells stained with this marker (CD138 stain; 3µm; Original magnification: 200X).*

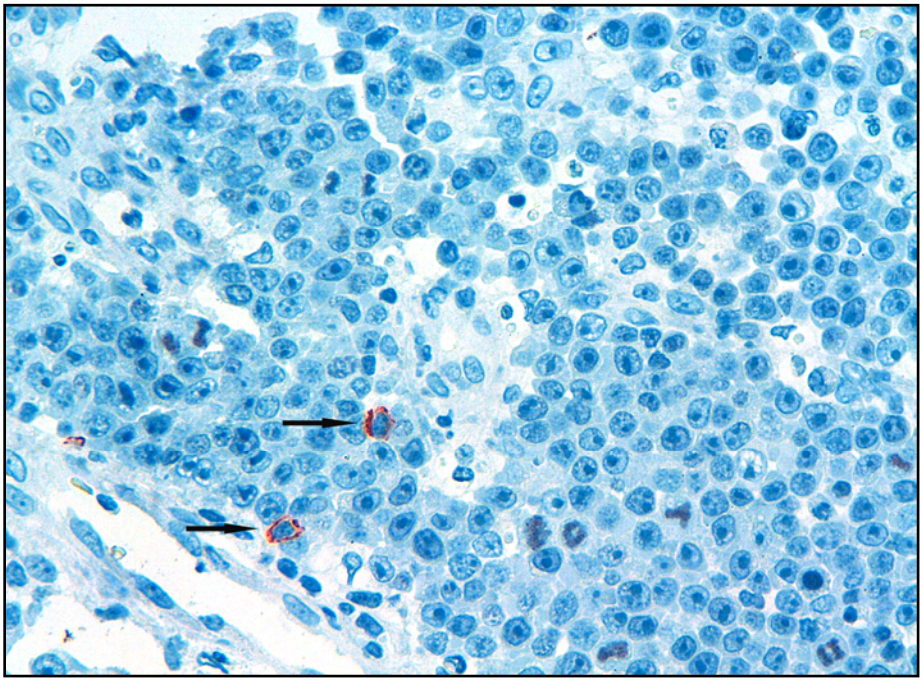
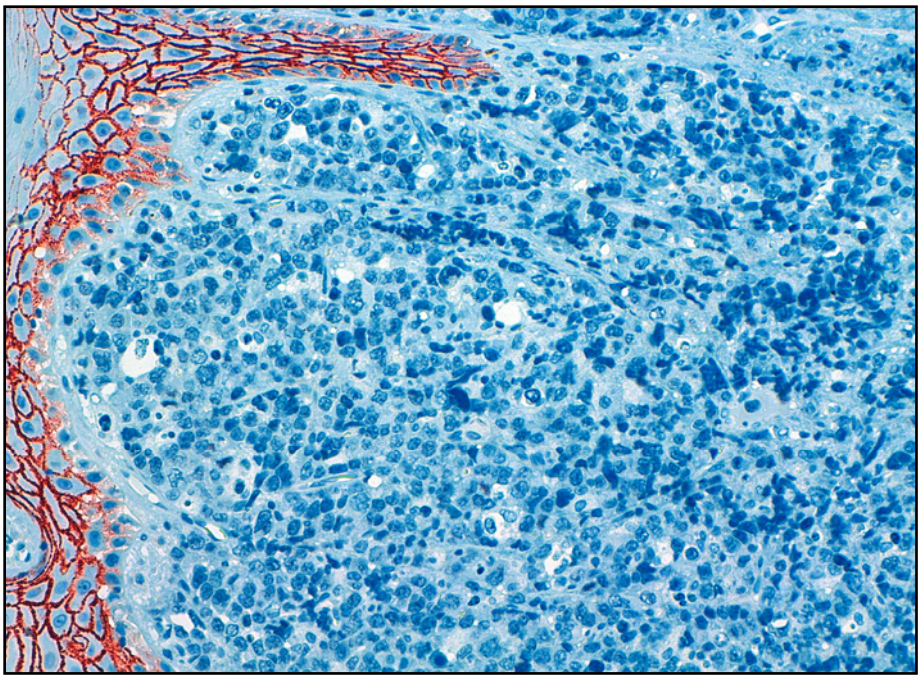


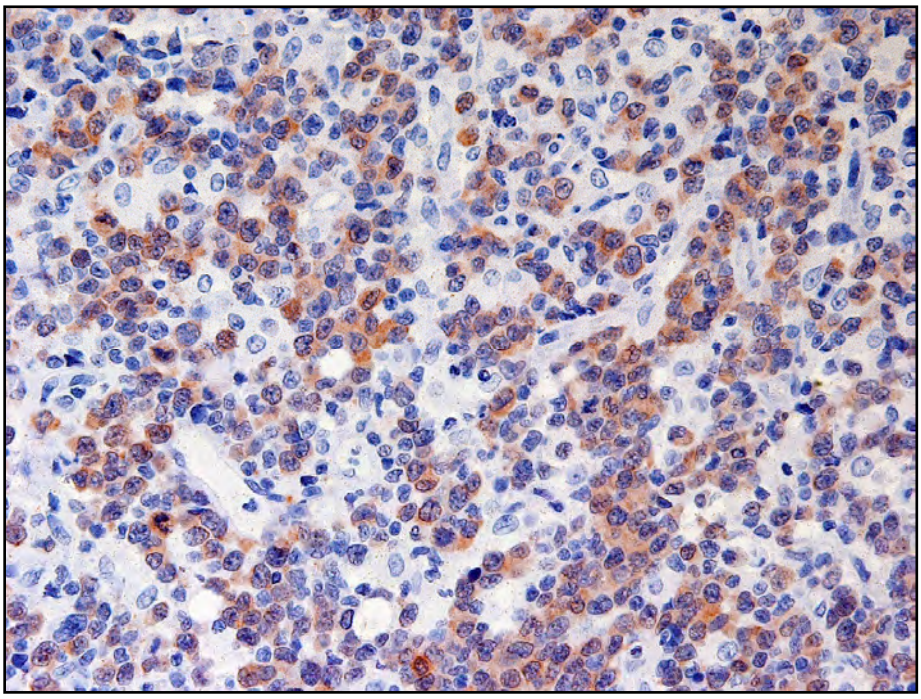
Figure 22: *The micrograph shows a case of PBL that was negative for CD138. Strong positive staining for CD138 is however visible in the basal epithelial cells of the overlying covering epithelium of the oral mucosa (left) (CD138 stain; 3µm; Original magnification: 100X).*



3.3.8 ALK protein

No cytoplasmic ALK protein could be detected in any of the 43 cases of PBL evaluated for the presence of this fusion protein. A case of ALK-positive DLBCL were utilised as positive control and showed strong, red-brown granular staining in the cytoplasm of the tumour cells (Fig.23).

Figure 23: *The micrograph was taken from a case of diffuse large B-cell lymphoma that served as positive control for the ALK protein stain. Red-brown granular staining is seen in the cytoplasm of the tumour cells (ALK stain, 3µm; Original magnification: 200X)*

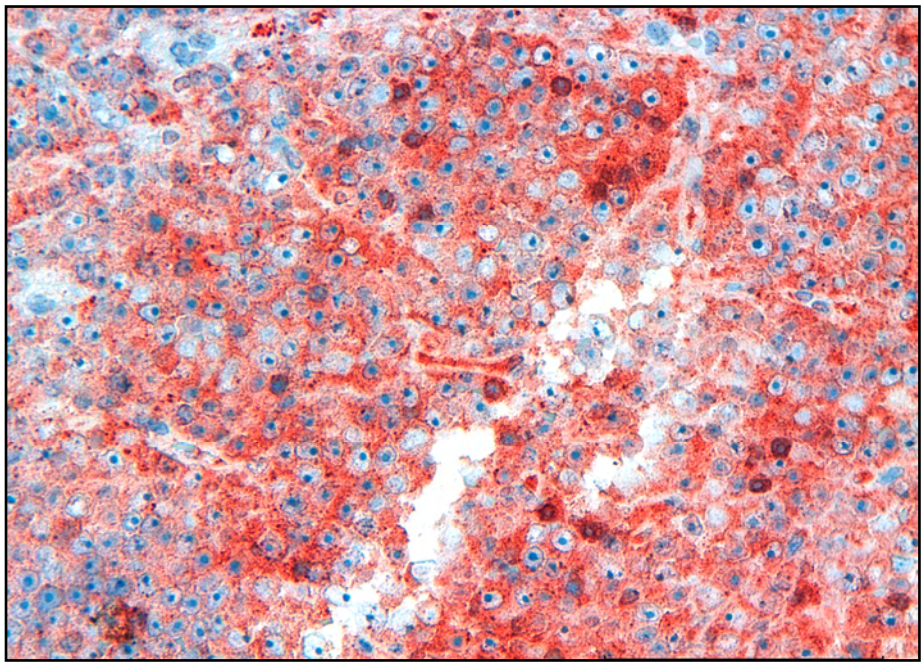


3.3.9 Immunoglobulin light chains

Twenty one of 45 (49%) cases showed light chain restriction with 17/21 (81%) of these cases showing restriction for kappa light chain (Fig.24a) and 4/21 (19%) with lambda light chain restriction. Reactive plasma cells were always present to confirm that negative staining was not aberrantly obtained (Fig.24b).

Figure 24: *This case of PBL shows clear kappa light chain restriction with diffuse red-brown granular cytoplasmic staining for the kappa light chain (a) but with no staining for the lambda light chain (b) in the tumour cells. Reactive plasma cells served at positive internal control in all negative light chain stains (arrow) (kappa (a) and lambda (b) stains; 3µm; Original magnification 200X).*

(a)



(b)

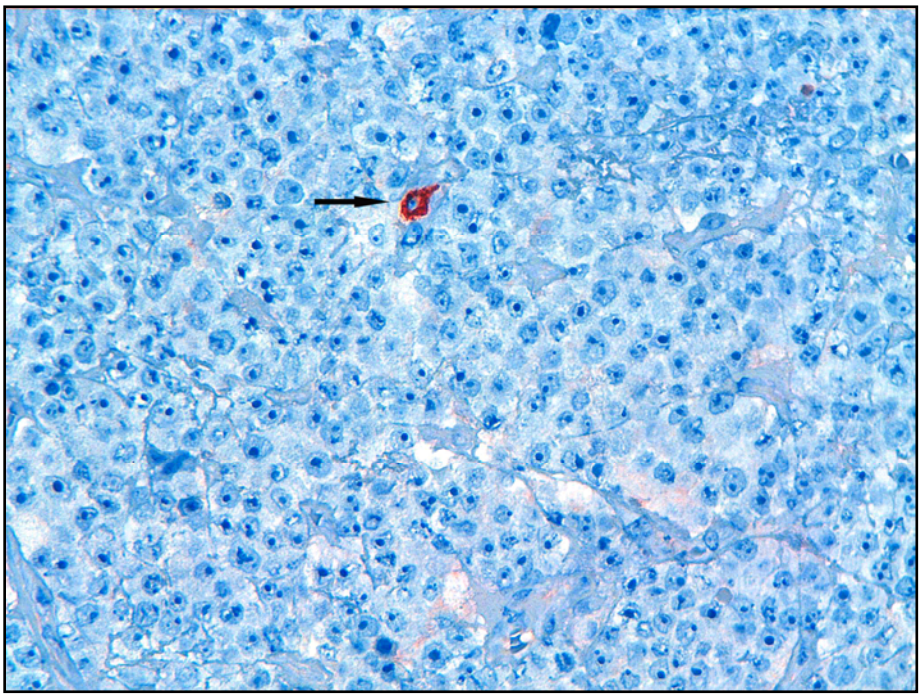


Table 3: *The table represents a summary of the immunophenotypic features of all PBL's included in this study. All cases stained negative for CD3, CD20 and ALK protein (not shown in the table).*

Case	CD45	Ki-67	CD79a	CD38	CD138	MUM-1	K	Λ
1	F	D	F	F	I	D	-	D
2	F	D	F	F	F	I	-	D
3	F	D	F	-	F	D	F	F
4	D	D	-	-	-	D	F	F
5	D	I	-	-	I	D	-	-
6	F	D	-	F	I	I	D	-
7	D	D	-	-	F	D	F	F
8	D	D	F	-	F	D	D	-
9	I	D	F	-	-	D	F	F
10	D	D	F	F	F	I	ND	ND
11	F	D	F	I	F	D	F	F
12	D	D	-	I	D	D	D	F
13	D	I	F	F	I	D	F	F
14	I	D	F	-	-	D	F	-
15	I	D	F	I	F	D	F	F
16	F	D	F	I	D	D	D	F
17	F	F	-	-	D	D	D	-
18	D	D	F	-	F	D	-	F
19	F	D	F	F	-	D	F	F
20	F	I	F	F	D	D	D	F
21	F	I	-	F	I	D	F	F
22	F	I	F	I	F	D	F	-
23	F	I	F	F	D	D	D	-
24	D	F	-	-	F	I	F	D
25	F	D	-	I	F	D	F	-
26	D	D	F	-	F	I	D	F
27	F	D	F	-	F	D	D	-
28	I	D	F	F	I	D	F	-
29	I	D	F	F	-	D	F	F
30	D	D	-	-	-	D	D	-
31	F	D	-	-	F	D	-	F
32	D	I	I	-	F	D	F	-
33	F	D	F	D	I	D	D	F
34	F	D	F	F	F	D	F	D
35	F	D	-	D	D	D	D	-
36	D	D	-	I	-	D	F	-
37	F	I	-	F	F	D	F	-
38	I	D	-	D	F	D	F	-
39	F	D	F	D	D	D	D	F
40	F	I	-	D	D	D	F	F
41	D	D	F	-	F	D	F	F
42	-	D	F	F	F	D	F	-
43	D	D	-	D	F	F	F	-
44	D	D	F	D	D	D	F	F
45	D	D	F	F	F	D	F	F

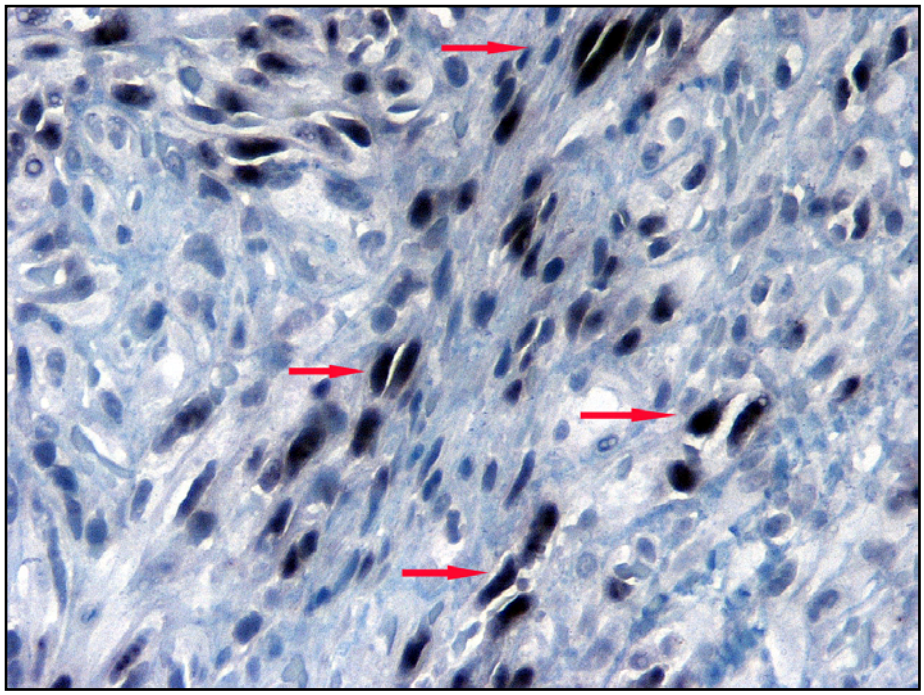
- = negative; ND = not done; F = focal positive staining (<20% of cells); I = intermediate positive staining (20-70% of cells); D = diffuse positive staining (>70% of cells)

3.4 *In Situ* Hybridisation

3.4.1 Human Herpes Virus-8

Prominent positive staining was evidenced in the Kaposi sarcoma used as positive control for HHV-8 (Fig.25). No positive staining could be found in any of the 45 cases of PBL's evaluated for the presence of this virus.

Figure 25: *The micrograph shows positive, black nuclear staining for HHV-8 (arrows) on the Kaposi sarcoma section hybridised with the HHV-8 probe. This served as positive control for the HHV-8 ISH (ISH for EBV; 3µm; Original magnification: 400X).*



3.4.2 EBV

Forty four (98%) cases showed positive staining for EBV (Fig.26 & 27). The brain sections used as negative controls were always negative for the virus (Fig.28)

Figure 26: *Positive, black nuclear staining for EBV can be seen in many of the tumour cell nuclei on the micrograph of this case of PBL (EBV ISH; 3µm; Original magnification: 200X).*

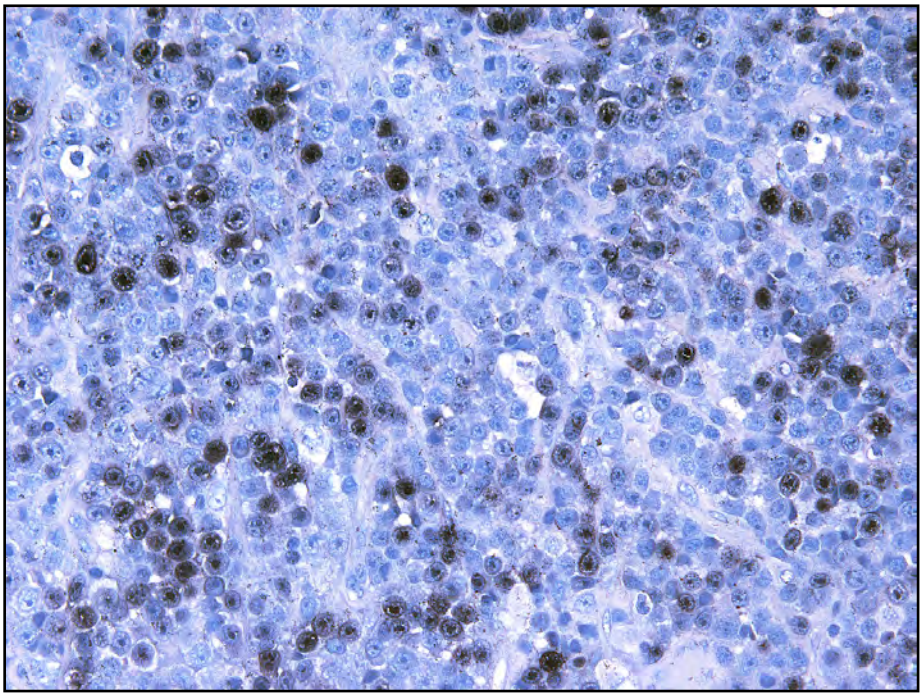


Figure 27: *This is a close-up view to demonstrate the black nuclear staining accepted as positive for EBV ISH (EBV ISH; 3µm; Original magnification: 400X)*

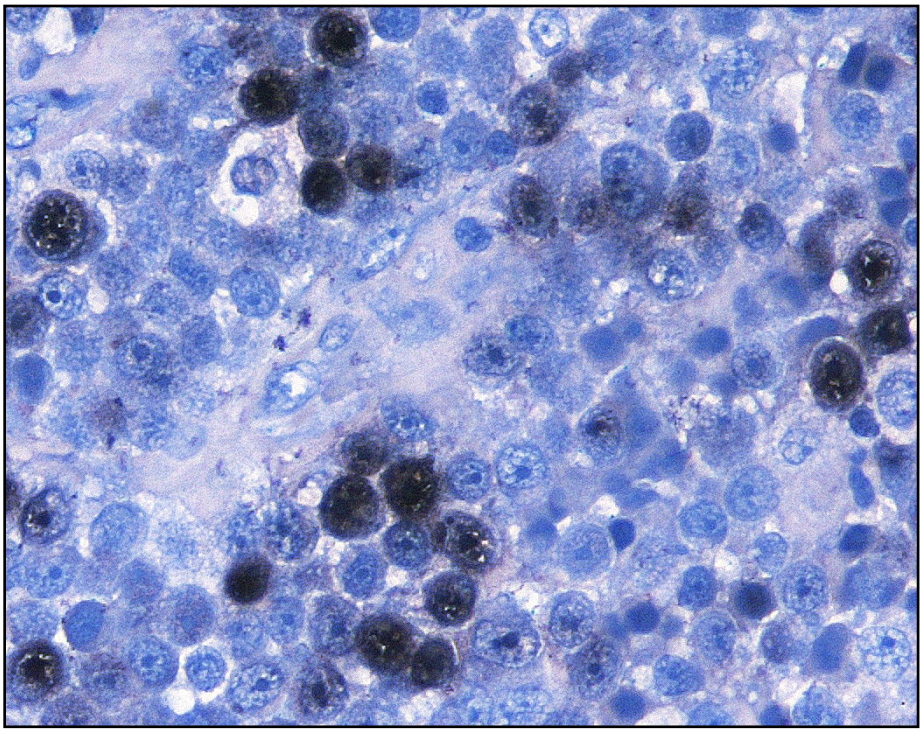
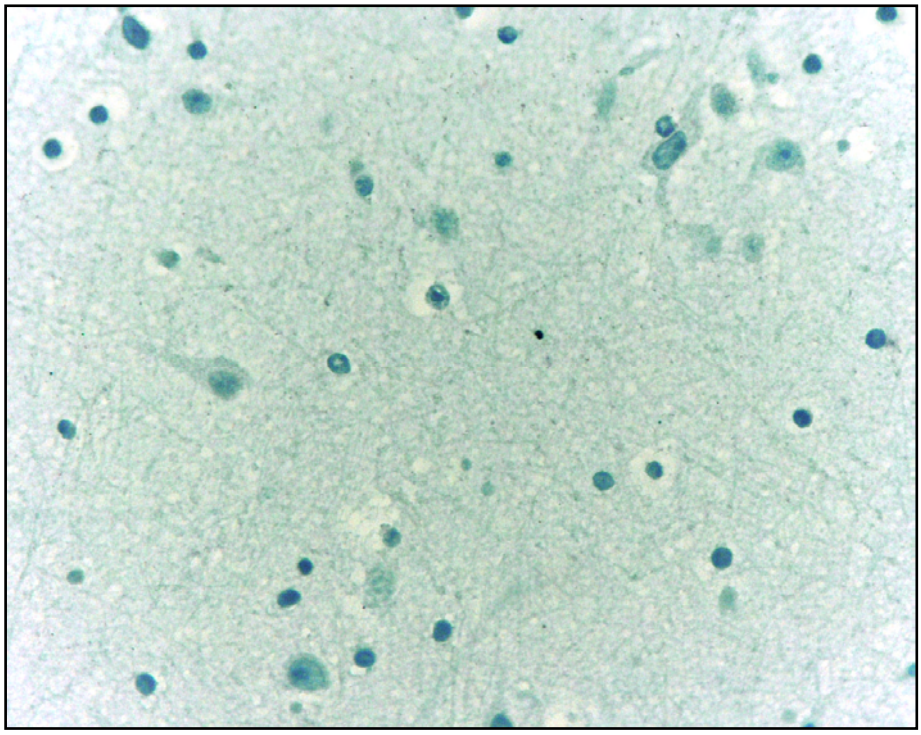


Figure 28: *This micrograph was taken from the brain section that served as negative control for the EBV ISH. No nuclear staining is visible (EBV ISH; 3µm; Original magnification: 400X).*



The relationship of EBV and HIV status was analysed (Table 4). The HIV status of only 32/45 (71%) patients were known. Thirty one of these patients with known HIV status were HIV-positive and of these, all were positive for the presence of EBV. Only one patient had a known HIV negative status (Case 17) and in this case no EBV could be demonstrated with ISH.

Table 4: *The table serves as a summary of the HIV, EBV and HHV-8 status of all PBL cases included in this study.*

Case	Age	Sex	HIV	EBV	HHV-8
1	47	M	+	+	-
2	48	M	+	+	-
3	34	F	+	+	-
4	52	F	+	+	-
5	U	M	+	+	-
6	U	U	+	+	-
7	45	F	+	+	-
8	48	M	U	+	-
9	39	F	+	+	-
10	33	F	+	+	-
11	34	M	+	+	-
12	36	F	+	+	-
13	39	M	+	+	-
14	43	M	+	+	-
15	28	F	+	+	-
16	50	M	+	+	-
17	36	F	-	-	-
18	37	M	+	+	-
19	44	M	+	+	-
20	45	M	U	+	-
21	32	F	U	+	-
22	45	M	U	+	-
23	35	M	U	+	-
24	29	M	U	+	-
25	44	M	U	+	-
26	43	M	U	+	-
27	45	M	U	+	-
28	58	F	U	+	-
29	43	M	+	+	-
30	35	M	U	+	-
31	39	M	+	+	-
32	39	M	+	+	-
33	50	M	+	+	-
34	U	U	+	+	-
35	U	M	+	+	-
36	43	M	+	+	-
37	U	M	U	+	-
38	27	M	+	+	-
39	36	M	+	+	-
40	40	M	U	+	-
41	32	M	+	+	-
42	43	M	+	+	-
43	45	F	+	+	-
44	U	F	+	+	-
45	44	M	+	+	-

U = unknown; M = male; F = female; + = positive; - = negative;

3.5 Fluorescence *in situ* hybridisation (FISH)

3.5.1 *IGH* dual colour break apart rearrangement probe (14q32.3 LSI IGH)

FISH analysis with the *IGH* BA probe was performed on the formalin-fixed, paraffin-embedded (FFPE) tissue sections of 43 of the original 45 cases. Cases 1 and 40 did not have adequate tissue to perform FISH analysis and were therefore excluded.

Results for the *IGH*-BA probe are summarised in Table 5. *IGH* gene rearrangements were found in 27/43 (63%) of PBL cases screened with the *IGH*-BA probe (Fig.31). Seven of these cases were also found to have rearrangements affecting both *IGH* alleles, considered as double hit rearrangements on *IGH* BA (Figs.32 and 33). A high degree of intra-tumour heterogeneity and complexity were seen.

Figure 29: *DAPI stained interphase nuclei of a PBL case hybridised with the LSI IgH dual colour BA rearrangement probe (Vysis®, Abbot Laboratories) showing no BA. Two yellow fusion signals are seen per cell nucleus (yellow arrows). Spectrum orange represents the 3' probe and spectrum green represents the 5' probe, which covers almost the entire variable region of the IGH gene.*

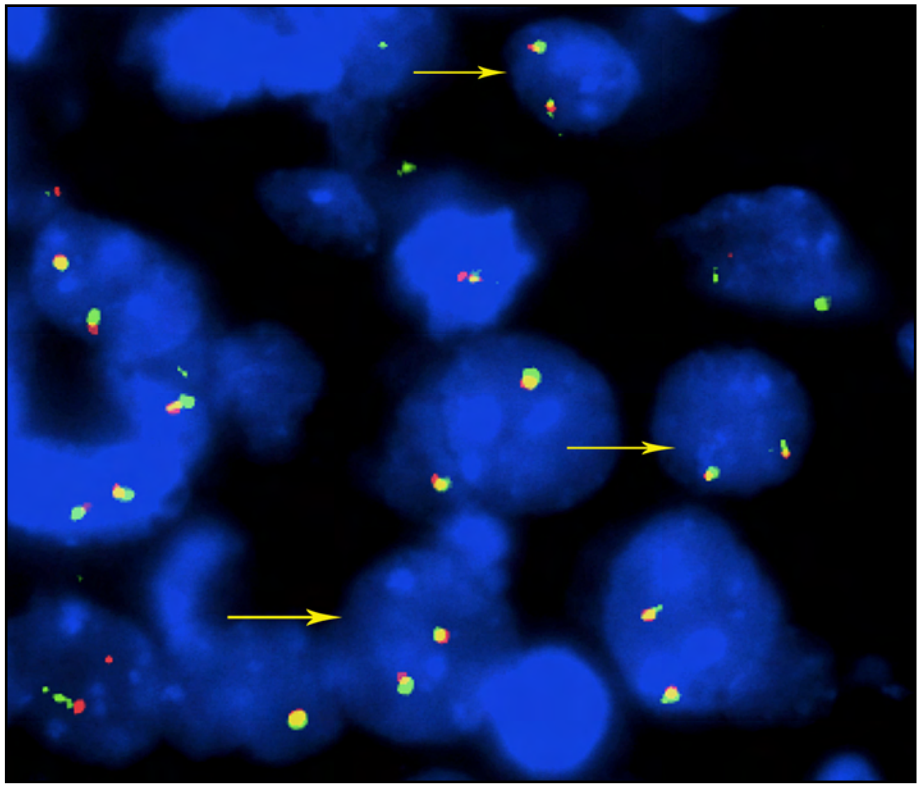


Figure 30: *DAPI stained interphase nuclei of a PBL case hybridised with the LSI IgH dual colour BA rearrangement probe (Vysis®, Abbot Laboratories). Two yellow fusion signals are present in most cell nuclei and no break apart was present in this case. The arrow demonstrates an area of overlapping cell nuclei which created the impression of three fusion signals in one cell. FISH analysis was therefore always performed in single cell nuclei only.*

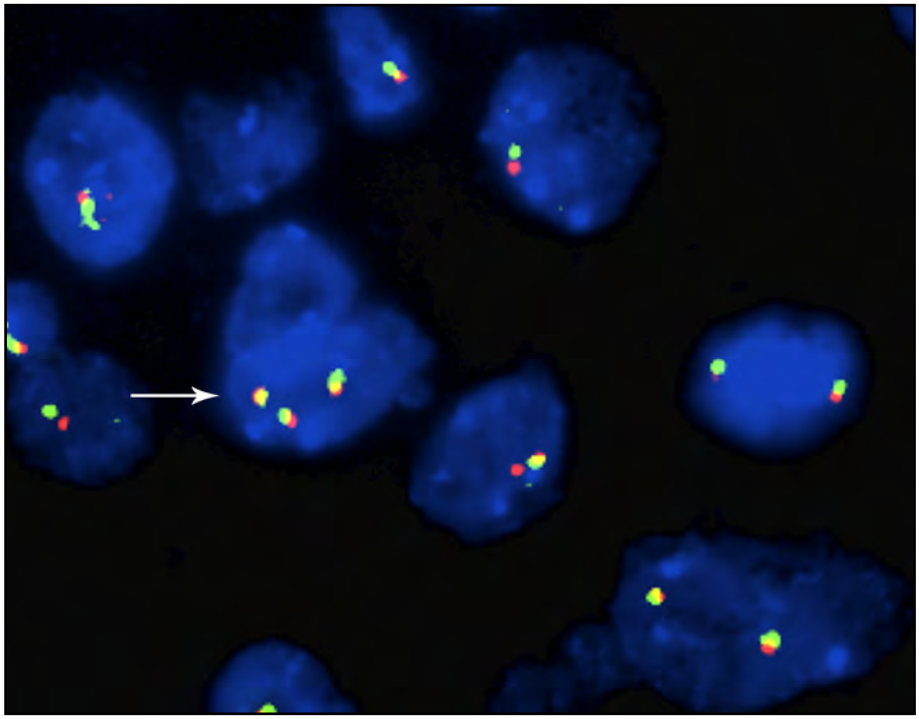


Figure 31: *DAPI stained interphase nuclei of a PBL case hybridised with the LSI IgH dual colour BA rearrangement probe (Vysis®, Abbot Laboratories). IGH rearrangement of one allele is present in some cell nuclei represented as one orange (3') (red arrows) and one green (5') (green arrows) signal apart from each other. The unaffected allele on chromosome 14 is seen as one yellow fusion signal (yellow arrow).*

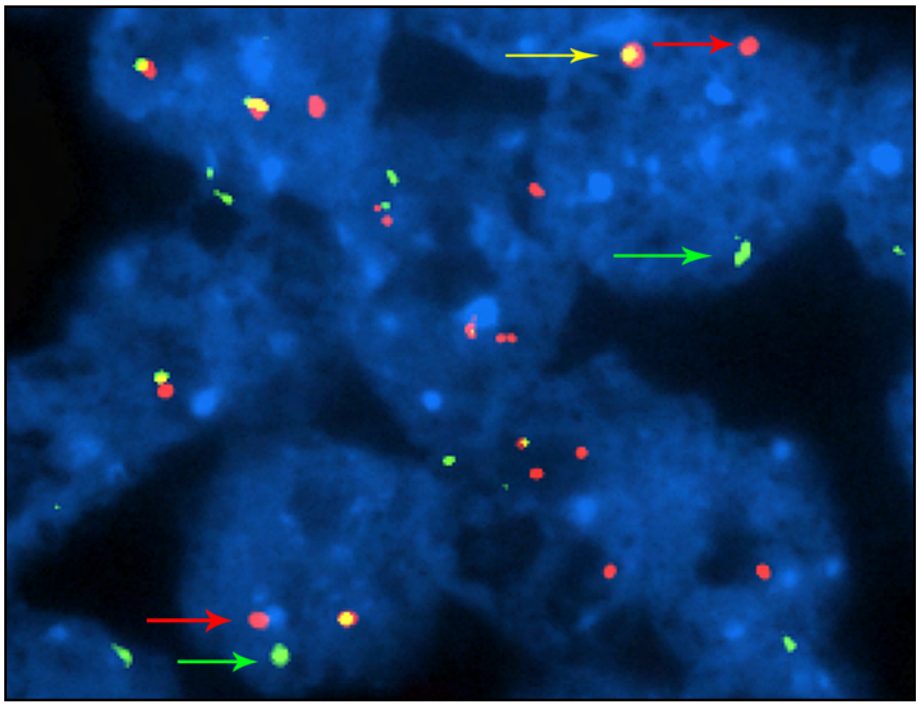


Figure 32: *DAPI stained interphase nuclei of a case of PBL hybridised with the LSI IgH dual colour BA rearrangement probe (Vysis®, Abbot Laboratories) showing IgH rearrangement of chromosomes 14. Two to three copies of orange (3') (red arrow) and one to four copies of green (5') (green arrow) signals are seen in the tumour cell nuclei and there are no normal fusion signals. There are no fusion signals; all IHG copies have a gene rearrangement.*

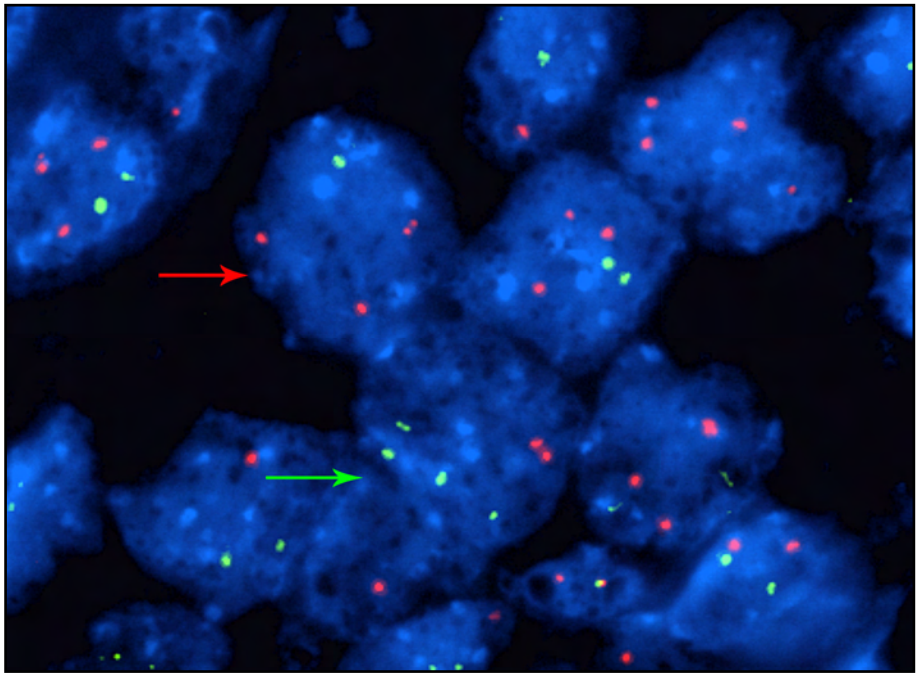
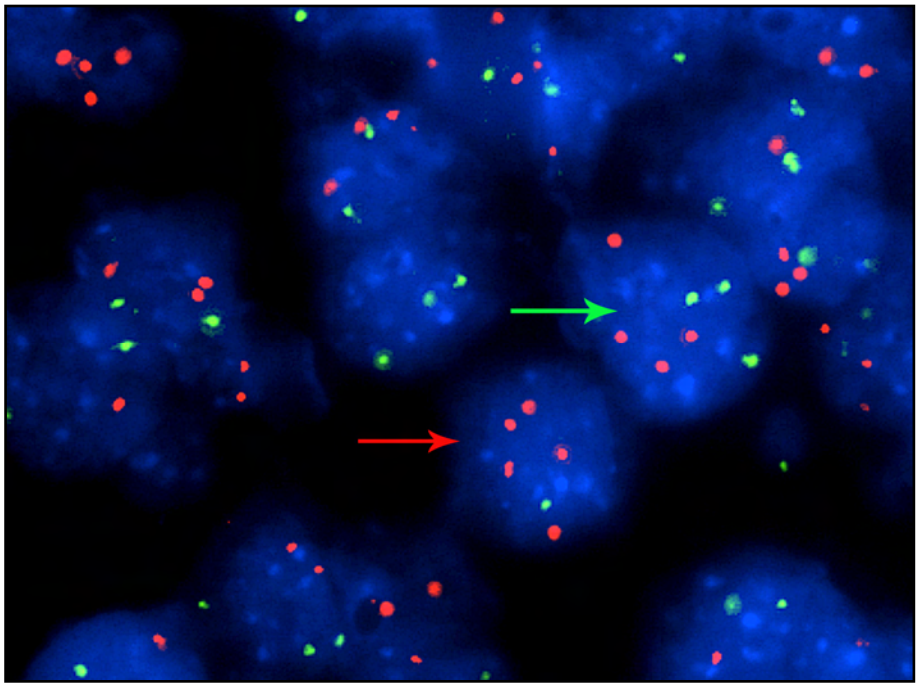


Figure 33: *DAPI stained interphase nuclei of a case of PBL hybridised with the LSI IgH dual colour BA rearrangement probe (Vysis®, Abbot Laboratories) showing IgH rearrangement affecting both alleles on chromosome 14. More than three orange (3') (red arrow) and one to three green (5') (green arrow) signals are seen in the tumour cell nuclei signalling additional copies of IGH. No normal fusion signals are visible in any nucleus.*



3.5.2 *IGH/MYC*, CEP 8 tri-colour, dual fusion translocation probe [t(8;14)(q24;q32)]

Analysis with the *IGH/MYC* tri-colour, dual fusion translocation probe followed the *IGH*-BA probe in order to see if the *MYC* gene (chromosome 8) was a partner for the *IGH* gene (chromosome 14) in cases positive for *IGH*-BA. The probe could also be used to evaluate chromosome 8 aneuploidy with the centromere 8 enumeration probe. The probe was applied to the FFPE tissue sections of the same cases of PBL analysed with the *IGH*-BA probe. Again cases 1 and 40 were excluded from the analysis. The results for the *IGH/MYC* probe are summarised in Table 5. Twenty-two of 43 PBL cases (51%) were positive for the I*GH/MYC* translocation (Fig.35). A high degree of intra-tumour heterogeneity and complexity were seen.

Figure 34: *DAPI stained interphase nuclei of case 6 hybridised with the MYC/IGH dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). The nuclear signals represent a normal pattern with two spectrum aqua (chromosome 8 CEP), two spectrum orange (MYC-gene), and two spectrum green (IGH-gene) signals per cell nucleus (arrows). No yellow fusion signals indicative of a t(8;14) are visible here.*

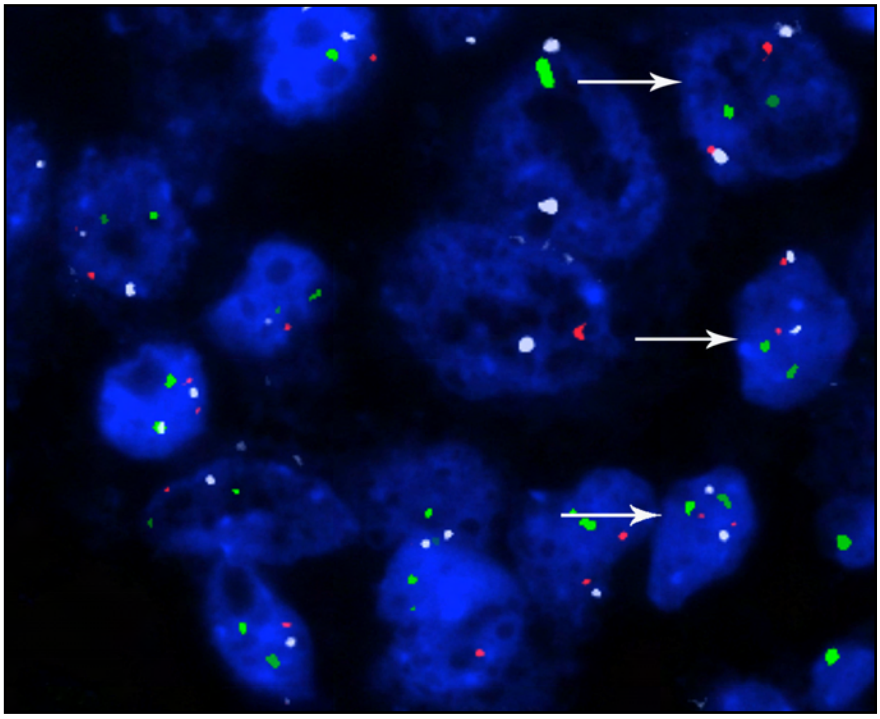
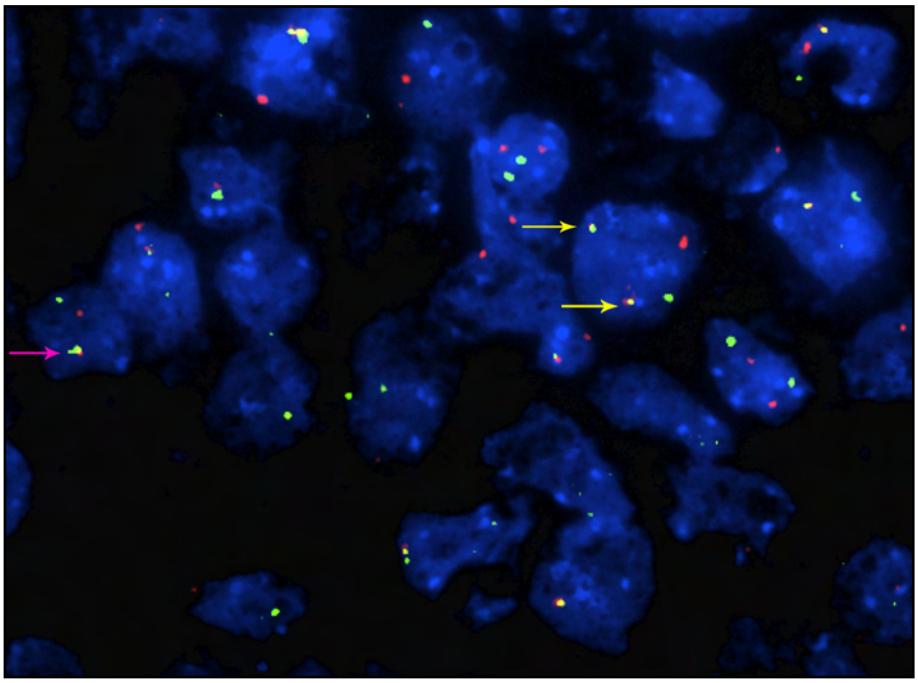


Figure 35: *DAPI stained interphase nuclei of case 2 hybridised with the MYC/IGH dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). Positive t(8:14) translocation is demonstrated by the positive yellow fusion signals (yellow arrows). The presence of one fusion signal in many cells likely reflects the loss of one translocation derivative (pink arrow). Orange signals represent the MYC gene on chromosome 8 and the green signals represent the IGH gene on chromosome 14.*

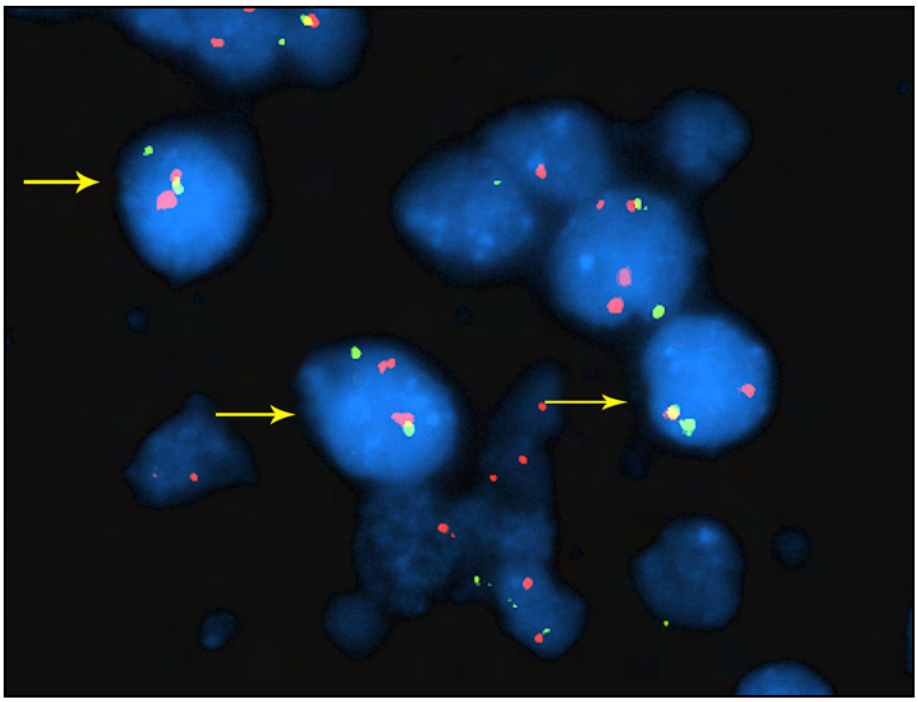


3.5.3 *MYC* dual colour break apart rearrangement probe (8q24 LSI MYC)

The *MYC* dual-colour BA probe was subsequently used to determine if the *MYC* gene was rearranged in cases negative for t(8;14) as the *IgH* BA positive cases would indicate a variant Burkitt translocation. Unfortunately case 13 was negative for *IGH*-BA and t(8;14) but *MYC*-BA was not performed. Twenty cases of PBL were screened with the *MYC*-BA probe. The results for the *MYC* BA probe are summarised in Table 5. Four of 20 (20%) cases (Cases 11, 16, 35 and 37) showed a positive BA signal of the *MYC* probes set (Fig.36). *MYC* was therefore rearranged in 26/42 (62%) of PBL's evaluated in this study (excluding case 13). *IGH* was a partner in 22/42 cases (52%) suggesting that *MYC* was translocated to partners other than *IGH* in four cases.

Interestingly case 16 was positive for both *MYC* and *IGH BA* probes set but were negative for t(8;14).

Figure 36: *DAPI stained interphase nuclei of case 11 hybridised with the LSI MYC dual colour BA rearrangement probe (Vysis®, Abbot Laboratories) showing MYC rearrangement of one allele as one spectrum orange (5') and one spectrum green (3') signal (arrows) apart from each other. The normal yellow fusion signal represents the unaffected allele on chromosome 8.*



3.5.4 *IGH/CCND1* dual colour, dual fusion translocation probe [t(11;14)(q13;q32)]

Forty one of the PBL cases in this study were screened for t(11;14). Cases 1, 23, 40 and 44 were excluded due to inadequate tissue for analysis.

The results for the *IGH/CCND1* probe are summarised in Table 5. The *CCND1* gene was found to be involved in a translocation with *IGH* in only one of 41cases (case 27) (5%) (Fig.37). Increased *CCND1* copy number (orange signals) was however present in 17/41 (41%) cases screened with the t(11;14) probe. This was defined as three or more orange signals per nucleus. Seven cases had more than six copies (cases 4, 8, 14, 21, 29, 38 & 42) (Fig.39) with some having more than 10 signals, noted as "multiple red". Increased *IGH* copy number (green signals) was present in 6/41 (15%) cases screened with this t(11;14) probe set (cases 4, 16, 27, 29, 33 and 38). The high number of green *IGH* signals seen in those cases with *IGH* BA can partly be explained by the fact that there was a split of the green probe spanning

both the 3' and 5' region of *IGH*. In four cases however (cases 4, 16, 27 and 29), cells with more than six copies of *IGH* could be found (Figs. 38 & 39). Cases with more than four signals are representative of a true increase in the copy number of chromosome 14.

Figure 37: *DAPI stained interphase nuclei of complex case 27 hybridised with the IGH/CCND1 dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). Positive t(11;14) translocation is demonstrated by the positive yellow fusion signals . Two fusion signals are visible in some cells (yellow arrows). Orange signals represent the CCND1 gene on chromosome 11 and the green signals represent the IGH gene on chromosome 14. The IGH gene was also shown to be rearranged on the IGH BA analysis.*

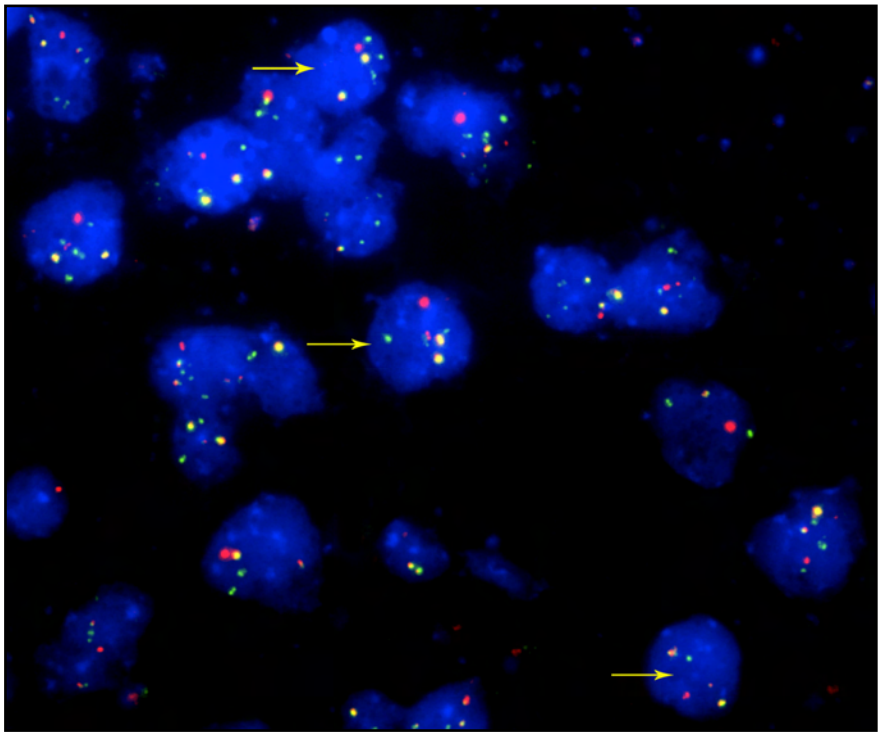


Figure 38: *DAPI stained interphase nuclei of case 7 hybridised with the IGH/CCND1 dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). Three to six copies of the CCND1 gene on chromosome 11 are represented by the spectrum orange signals (red arrows). A cell with seven green signals, representative of three to four IGH signals on chromosome 14 (green arrow) is also shown here. IGH was also rearranged on the IGH BA probe analysis of this case.*

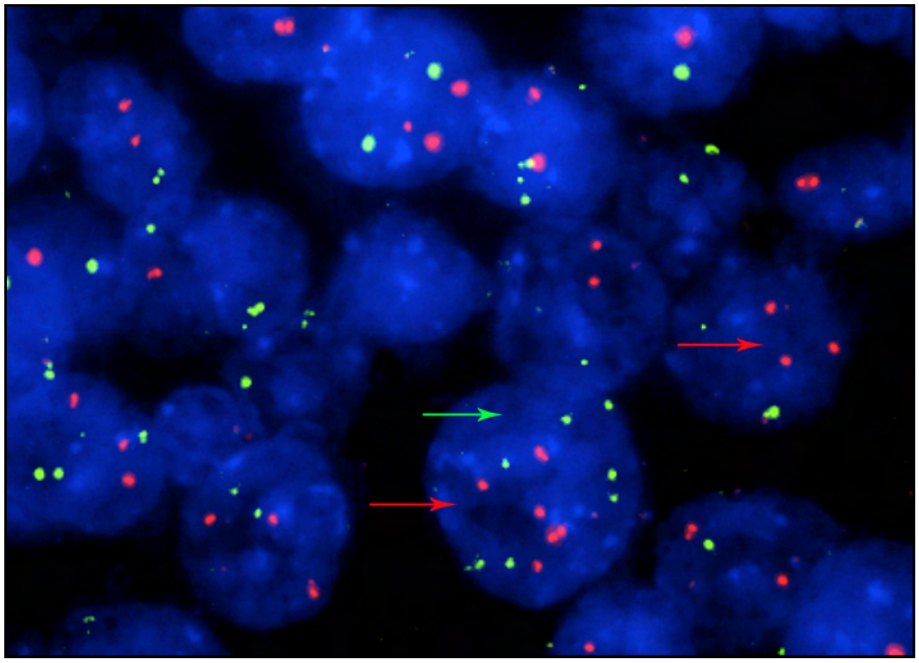
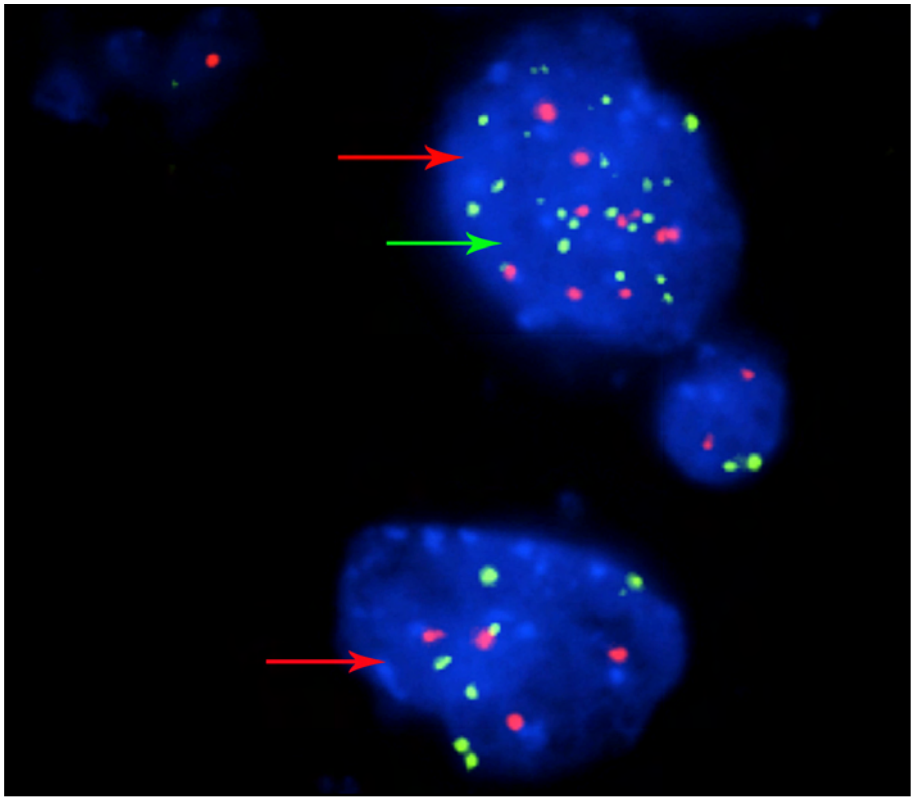


Figure 39: *DAPI stained interphase nuclei of case 38 hybridised with the IGH/CCND1 dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). Four to ten copies of the CCND1 gene on chromosome 11 are represented by the orange signals (red arrows). Green signals represent the IGH gene on chromosome 14 which also shows an increased copy number with up to 10 copies per nucleus (green arrow). The IGH gene was also rearranged on the IGH BA probe analysis of this case.*

.



3.5.5 *IGH/BCL2* dual colour, dual fusion translocation probe [t(14;18)(q32;q21)]

The seven PBL with two *IGH* gene alleles rearranged on *IGH* BA probe analysis were screened with the *IGH/BCL2* dual fusion translocation probe in order to evaluate the *BCL2* gene as a possible translocation partner for *IGH*. The results for the *IGH/BCL2* probe are summarised in Table 5. Only case 45 was positive for the t(14;18) translocation.

3.5.6 Double Hit Lymphomas

Seven of the PBL cases included in the study had two *IGH* gene alleles rearranged on *IGH*-BA, and were therefore evaluated as possible double hit lymphomas (Cases 4, 14, 15, 16, 27, 29 and 45) (summarised in Table 5) (Figs. 32 and 33).

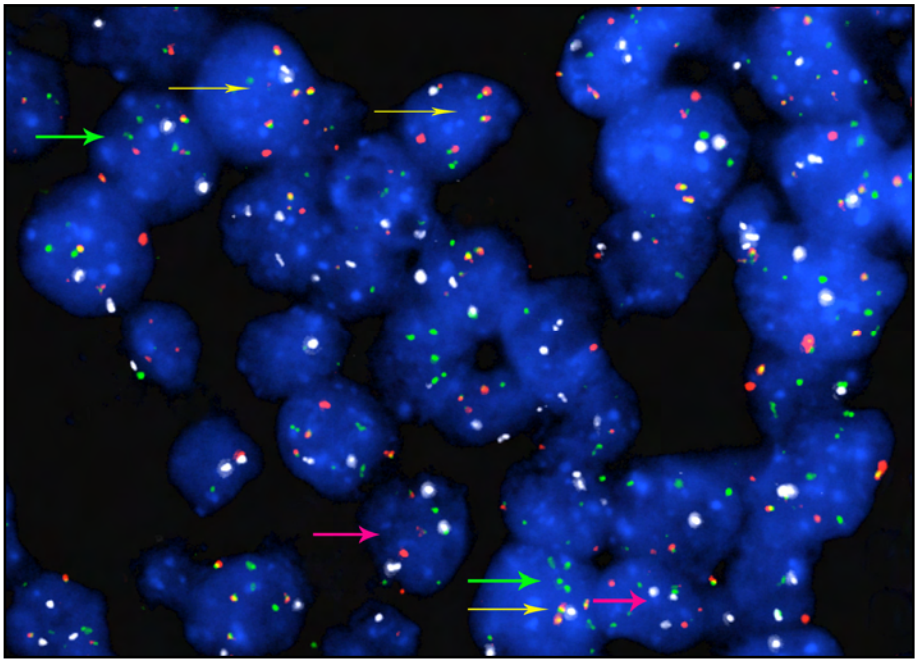
In case 27, both the *MYC* and *CCND1* genes were partners of *IGH* genes and positive for t(8;14) and t(11;14) respectively (Fig.37), therefore a true double hit lymphoma case.

In case 45, both *MYC* and *BCL2* genes were discovered to be partners of the *IGH* gene and positive for t(8;14) and t(14:18) respectively, therefore another true double hit lymphoma case

Three cases (4, 15 and 27) were positive for t(8;14) with complex rearrangements of case 4 on t(8;14) (Fig.40). One partner remained unidentified in these three cases;

In cases 14 and 16, both *IGH* chromosome partners remained unknown.

Figure 40: *DAPI stained interphase nuclei of case 4 hybridised with the MYC/IGH dual colour dual fusion translocation probe (Vysis®, Abbot Laboratories). The nuclear signals have a complex pattern with various copy numbers of the the IGH-gene (represented by spectrum green) (green arrows) and two to four yellow fusion signals (yellow arrows) signaling a MYC-IGH translocation. Three CEP 8 signals (represented by spectrum aqua), are seen in some cell nuclei (pink arrows)*



3.5.7 *BCL6* break apart rearrangement probe (3q27)

Forty of the 45 cases were screened for breaks within the *BCL6* gene loci using Vysis LSI dual colour BA rearrangement probes for the *BCL6* locus on chromosome 3 (Abbott Molecular Inc, IL, USA) (cases 1, 4, 6 and 40 were excluded).

The results for the *BCL6* BA probe are summarised in Table 5. No rearrangements of *BCL6* were detected in any of the 40 PBL's screened with the *BCL6* BA probe but gains with three to seven fusion signals per cell were observed in 11 cases (28%) (Fig.41). Loss of fusion signals were seen in three cases (cases 25, 35 and 43) defined as less than two fusion signals per nucleus in more than 15% of cells screened with the *BCL6* BA probe.

Figure 41: *DAPI stained interphase nuclei of case 38 hybridised with the LSI BCL6 dual colour BA rearrangement probe (Vysis®, Abbot Laboratories) showing no rearrangement of the BCL6 gene but with gains of the BCL6 locus represented by three fusion signals in a significant number of the cell nuclei (arrows). Spectrum orange represents the 5' BCL6 probe and spectrum green represent the 3' probe.*

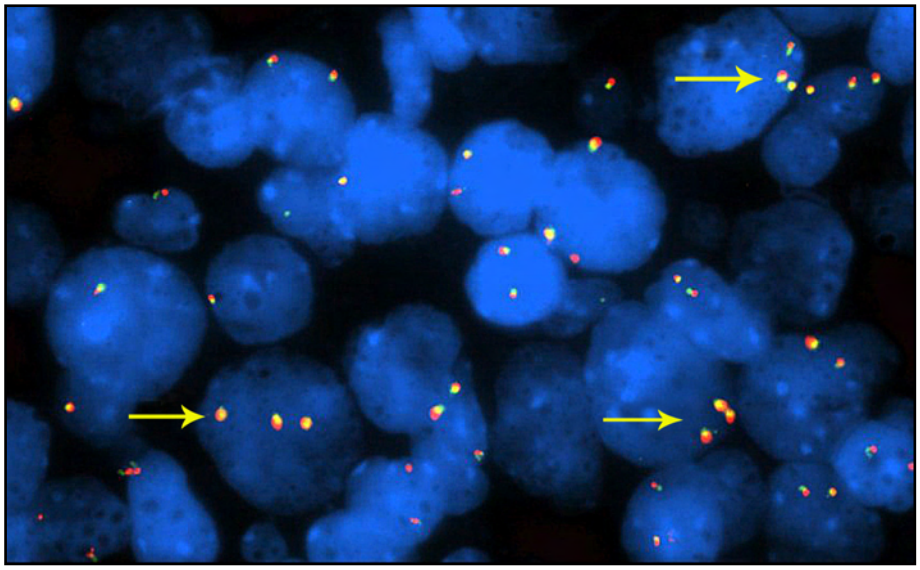


Table 5: *The table provides a summary of the FISH results obtained from this study.*

Case	IGH	MYC/IGH	MYC	t(11;14)	BCL6	t(14;18)
1	ND	ND	ND	ND	ND	ND
2	R	R	ND	-	-	ND
3	R	R	ND	-	-	ND
4	R / DH	R	ND	G (11 & 14)	ND	-
5	R	-	-	-	G	ND
6	-	-	-	-	ND	ND
7	R	R	ND	G (11)	G	ND
8	-	-	-	G (11)	-	ND
9	R	R	ND	G (11)	-	ND
10	R	R	ND	-	-	ND
11	R	-	R	G (11)	-	ND
12	R	R	ND	-	-	ND
13	-	-	ND	-	-	ND
14	R / DH	-	-	G (11)	G	-
15	R / DH	R	ND	-	-	-
16	R / DH	-	R	G (11 & 14)	G	-
17	-	-	-	G (11)	-	ND
18	-	-	-	G (11)	-	ND
19	R	R	ND	-	ND	ND
20	-	-	-	G (11)	-	ND
21	R	R	ND	G (11)	G	ND
22	R	R	ND	-	-	ND
23	-	-	-	ND	-	ND
24	-	-	-	-	-	ND
25	R	R	ND	-	L	ND
26	R	R	ND	-	-	ND
27	R / DH	R	ND	R/G (11 & 14)	-	-
28	R	R	ND	-	-	ND
29	R / DH	R	ND	G (11 & 14)	G	-
30	-	-	-	-	-	ND
31	-	-	-	-	-	ND
32	R	-	-	-	G	ND
33	R	R	ND	G (11 & 14)	-	ND
34	-	-	-	-	-	ND
35	-	-	R	-	L	ND
36	R	R	ND	-	G	ND
37	-	-	R	-	-	ND
38	R	R	ND	G (11 & 14)	G	ND
39	-	-	-	-	G	ND
40	ND	ND	ND	ND	ND	ND
41	-	-	-	G (11)	-	ND
42	-	-	-	G (11)	G	ND
43	R	R	ND	-	L	ND
44	R	R	ND	ND	-	ND
45	R / DH	R	ND	-	-	+

R = positive for rearrangement; - = negative; ND = not done; DH = double hit; G = gain; G (11) = gain of CCND1 on chromosome 11; G (11 & 14) = gain of CCND1 on chromosome 11 and of IGH on chromosome 14; G (14) = gain of IGH on chromosome 14; L = loss

Table 6: *This table provides a summary of the detailed results presented in table 5.*

***IGH*-BA positive**	**Possible double hit on IGH-BA**	**t(8;14) positive**	**t(11;14) positive**	***MYC* BA positive**	**t(14;18) positive**
27/43 screened (63%)	7/43 screened (16%)	22/43 screened (51%)	1/41 screened (2%)	4/20 screened (20%)	1/7 Screened (14%)

Table 7: *The table shows the EBV status of the tumour cells, HIV-status of the patient as well as presence of MYC-rearrangement in every PBL case included in the study.*

Case	HIV	EBV	MYC
1	+	+	ND
2	+	+	R
3	+	+	R
4	+	+	R
5	+	+	-
6	+	+	-
7	+	+	R
8	U	+	-
9	+	+	R
10	+	+	R
11	+	+	R
12	+	+	R
13	+	+	-
14	+	+	-
15	+	+	R
16	+	+	R
17	-	-	-
18	+	+	-
19	+	+	R
20	U	+	-
21	U	+	R
22	U	+	R
23	U	+	-
24	U	+	-
25	U	+	R
26	U	+	R
27	U	+	R
28	U	+	R
29	+	+	R
30	U	+	-
31	+	+	-
32	+	+	-
33	+	+	R
34	+	+	-
35	+	+	R
36	+	+	R
37	U	+	R
38	+	+	R
39	+	+	-
40	U	+	ND
41	+	+	-
42	+	+	-
43	+	+	R
44	+	+	R
45	+	+	-

U = unknown; + = positive; - = negative; ND = not done; R = rearranged

Table 8: *This table represents a summary of the data presented in Table 7 showing the correlation between the EBV statuses and MYC rearrangements in patients with known HIV- status.*

HIV	EBV	*MYC* R	Total patients
+	+	+	18 (58%)
+	+	-	12 (39%)
-	-	-	1 (3%)
			31

+ = positive; - = negative; MYC R = MYC rearranged

CHAPTER IV

DISCUSSION

4.1 Clinicopathological features of plasmablastic lymphoma

The current study represents the largest series of PBL affecting the oral cavity to date. The second largest series was published by Kane *et al.*, in 2009 and included 25 cases affecting mostly the gingiva[72]. The results of this study were in line with others who reported the gingiva and the palatal mucosa as the most common anatomical locations for PBL affecting the oral cavity[8, 25]. The majority of patients in this series presented with a rapidly growing mass, some clinically reminiscent of Kaposi sarcoma, that affected mostly the gingiva, palatal mucosa, buccal sulcus and extraction sockets (Table 2). One patient presented with a swelling of the face due to diffuse infiltration of the facial soft tissues.

The mean patient age was 41 years (29-58 years) with a male predominance. This correlated with the results of previous studies which reported a median age of 39 years[22]. The high median age of 50 years reported by the 2008 WHO classification is difficult to explain but may reflect a lower percentage of HIV-positive compared to HIV-negative patients diagnosed with PBL in WHO source data. In my study the only known HIV-negative patient was 36 years old correlating with the median age found in the rest of the group.

The male to female ratio of 2.6:1 in this study was less than the 5.7:1 reported previously by some authors[246]. This discrepancy might be explained in part by the fact that females represent a larger proportion of the HIV population in South Africa than in other parts of the world. This is likely to be due to HIV transmission being heterosexual rather than homosexual in South Africa[247]. The number of females affected by HIV has either being stable or increased in certain parts of the world[6]. Due to the strong association of PBL with HIV infection in our South African sample, we concurred with others who proposed the diagnosis of PBL to suggest immunosuppression in an individual with otherwise unknown immune status[25]. Recently a large study of 42 PBL's was

published by Valera and co-workers[13]. The 42 cases included only eight cases affecting the mucosa of the oral cavity. Twenty of their 35 patients (57%) with known HIV status and PBL in locations other than the oral cavity were HIV-positive but interestingly seven of the eight patients (88%) with oral PBL were immunosuppressed due to HIV-infection. PBL of the oral cavity therefore seem to have a stronger association with HIV-infection than those occurring in other anatomical locations. As a result of this we proposed that PBL should be included as a group 1 lesion in the EC-Clearinghouse and WHO Collaborating Centre on Oral Manifestations of the Immunodeficiency Virus classification of oral problems related to HIV infection[248] as a specific entity. This is especially relevant to parts of the world with high incidences of HIV infection such as sub-Saharan Africa.

A limitation of my study was the shortage of clinical information with regard to the CD4 counts, HIV viral loads, extent of the disease beyond the oral cavity, the possible presence of other plasma cell dyscrasias or any further follow-up before or after treatment. Others have shown a strong association of PBL with advanced immunosuppression as reflected by low CD4+ counts and high viral loads[21, 56, 93]. The correlation between the diagnosis of PBL, state of immunosuppression as measured by the CD4 counts of the patient and prognosis should be confirmed in large series of PBL such as the current one.

In general, the histomorphologic features of all cases included in this study were similar. The tumour infiltrates consisted mostly of monomorphic, large blastic cells with abundant cytoplasm and a cohesive, sheet-like growth pattern in an extra nodal and extra-medullary location (Fig.2 and Fig.3). In some cases tumour cells exhibited a more discohesive appearance (Fig.4), a feature that eventually made single cell FISH analysis easier, as will be discussed later. This phenomenon may be explained by the inherent character of the tumour cells, being a lymphoproliferative neoplasm, cells are not cohesive by nature or it may be artifactual in some instances with tissue shrinkage and artificial discohesion. An interesting finding not previously described in the literature was the pronounced cell pleomorphism noted in a percentage of the tumour cells of some cases with large nuclei, either slightly indented, binucleate or even multinucleated in some instances. These were

always intermingled with the more monomorphic plasmablasts of the tumour bulk (Fig.5). There was no specific correlation between the presence of cell pleomorphism and specific immunohistochemical or genetic features. Due to the fact that all cases except one were EBV positive, the association between pleomorphism and EBV status was difficult to exclude in this study.

All blastic tumour cells displayed eccentrically placed, slightly irregular round to oval nuclei with little chromatin. Nucleoli were always prominently visible in the cell nucleus. A mixture of tumour cells with a single, centrally located nucleolus and therefore immunoblastic appearance (Fig.2) and others with several peripherally located nucleoli (Fig.3) was always seen in all cases. Occasionally one or the other cell type predominated but this did not have any diagnostic implication in this study. These results suggest that PBL can be defined as having a mixture of blastic tumour cells with either a single prominent nucleolus or several smaller nucleoli distributed throughout the cell nucleus. The results of this study confirmed that these features did not to warrant separate classifications.

The degree of plasmacytic differentiation within each case was evaluated and noted. Plasmacytic differentiation was defined as tumour cells exhibiting more mature plasma cell morphology. This was done to evaluate the theories of previous authors who classified PBL morphologically according to the amount of plasmacytic differentiation. These categories included '*PBL of the oral mucosa type*' where the tumour consisted mostly of blastic cells, '*PBL with plasmacytic differentiation*' where the tumour consisted of a higher percentage of mature plasma cells and '*PBL secondary to plasma cell neoplasms*' when the presence of another plasma cell neoplasia such as MM or plasmacytoma was confirmed[13, 21, 43, 56]. The latter group poses diagnostic difficulty and imply a thorough clinical work-up to exclude MM or plasmacytoma before a diagnosis of PBL can be accepted. All 45 cases of PBL in this study showed the presence of mature plasma cells in the tumour cell infiltrate (Fig.7). It was impossible to differentiate reactive plasma cells from true plasmacytic differentiation unless the 'plasmacytic cells' showed features of neoplasia such as being much larger than reactive cells or exhibited at least some degree of atypia or pleomorphism. Atypia and pleomorphism might be absent

in plasma cell neoplasias such as MM and plasmablastic morphology might be present in aggressive forms of MM as well as various other lymphoproliferative entities. This poses a major diagnostic dilemma[249]. Whether PBL represents a group of plasma cell neoplasias with plasmablastic 'dedifferentiation' or whether one should rather consider them as plasmablastic neoplasms with some plasmacytic 'differentiation' should be considered. Maybe it would be possible to evaluate these plasmablastic and plasmacytic cells through single cell laser microdissection and subsequent comparative genetic analysis thereof in order to determine their relationship with one another. Until such time however, the conclusion of this study was that morphologic classifications based on the amount of plasmacytic differentiation currently has no diagnostic advantage. It would be interesting to determine whether the percentage of blastic cells versus plasmacytic cells has any prognostic implication for a patient with PBL and this should be further investigated.

A prominent feature of PBL was the presence of numerous tingible body macrophages with a prominent starry sky appearance on lower magnifications (Fig.6). This was ascribed to the high proliferation index of more than 75% confirmed in most cases using Ki-67 (MIB1) antibody. Two of the 45 cases (Case 17 and 24) initially only showed focal Ki-67 positivity (Fig.12). The stains were repeated in both cases but similar results were obtained the second time. The reason for this was uncertain but likely related to poor immunoreactivity due to the age of the stored FFPE specimen for case 24. This ten year old paraffin block also showed lower MUM-1 positivity compared to the other cases and negative or focal positivity with other stains. Case 17, a 3 year old paraffin block, showed strong and diffuse reactivity for CD138, MUM and κ light chain and general low immunoreactivity was therefore an unlikely explanation. Case 17 was the only EBV negative case in this series of PBL's. Whether this might aid in explaining the lower proliferation index in this case was uncertain.

Ki-67 on cases 17 and 24 was repeated a third time using a rabbit monoclonal antibody (RabMAbs) against Ki-67 antigen [clone SP6 (Spring Biosciences M3060, 6920 Koll Centre Parkway, Suite 211, Pleasanton, CA 94566]. In

both cases intermediate positivity, defined in this study as positive staining in 20 to 70% of cells, was found. This might be explained by the superior nature of RabMAbs[250]. They are known to provide the combined benefits of superior antigen recognition of the rabbit immune system with the specificity and consistency of a monoclonal antibody[251, 252]. Rabbit monoclonal antibodies also have more diverse epitope recognition and bind with greater affinity to their target antigen[252].

4.2 Immunoprofile of plasmablastic lymphomas

The diagnosis of PBL as an entity has been controversial. Many lymphoma entities display plasmablastic features which results in difficulty when trying to diagnose PBL's using morphologic criteria. However, PBL can be distinguished from most of these entities through immunohistochemical examination with pan B-cell markers such as CD19, CD20, CD22 and CD79a. Immunohistochemically all cases of PBL included in this study were negative for CD20 although reactive B-cells served as positive internal control in all cases (Fig.8). It was concluded that the absence of CD20 immunoreactivity should be regarded as one of the main diagnostic criteria for PBL.

It was previously shown that CD56, frequently expressed in plasma cell neoplasms[249, 253], might also be expressed in PBL[92]. CD56 is known to be negative in reactive plasmacytosis and monoclonal gammopathy of undetermined significance (MGUS), up-regulated substantially in MM and down-regulated again during evolution of MM to aggressive plasmablastic, anaplastic or extra-medullary forms of the disease[254]. CD56 negative MM is characterised by poor prognosis, plasmablastic morphology, lack of osteolysis and extra-medullary disease[249] all features of PBL. Although the presence of CD56 positivity in PBL's with overt plasmacytic differentiation is postulated to be suggestive of MM with plasmablastic morphology[21] it will not assist in distinguishing between PBL or MM with plasmablastic morphology and was therefore not performed in this study.

The CD79a molecule is present on the cell membrane of B-cells for longer than CD20 during B-cell to plasma cell development and maturation[255] and

also during the terminal stages of plasma cells differentiation which is why this marker is frequently used to estimate the fraction of plasma cells in the bone marrow[256]. CD79a forms a complex with CD79b to enable B-cell antigen signal transduction[257]. During the plasma cell stages however, the CD79a molecule is found as an intracellular component and therefore visualised as a cytoplasmic stain. CD79a also plays a significant role in B-cell development, stabilisation and function[258] and plasma cells without membranous or cytoplasmic CD79a expression are therefore seen as abnormal B-cells. Apart from one case that showed ontermediary positivity, focal CD79a staining was seen in the tumour cells of 27/45 (60%) cases but not more than 20% of the cells stained positive in any of the 27 cases (Fig.16). This was in agreement with the aberrant loss of CD79a expression reported in various other malignancies including MM[256], ALK-positive large B-cell lymphoma[97] and HHV-8-positive PBL associated with Castleman's disease[21, 98]. The molecular mechanism responsible for the loss of CD79a expression in neoplastic plasma cell infiltrates is unknown. Tanaka and co-workers indicated that, when CD79a is used in conjunction with other antibodies such as CD138 (usually positive in plasma cells) and cyclin D1 (often over expressed in MM), it may serve as a diagnostic tool in favour of plasma cells[256]. The low expression of CD79a in 60% and complete loss of this molecule in 38% of our cases of PBL were in line with what has been described for PBL in the literature[13, 22, 71]. This was mainly ascribed to the aberration of neoplastic cells found in these neoplasms but does not seem to reflect any lineage specificity of the tumour cells. It was the conclusion of this study that CD79a did not play any role in the diagnostic panel of PBL.

CD3 positivity was reported in cases of PBL by one group[71]. None of our cases showed CD3 positivity in any cells other than reactive T-cells. It is interesting to note that aberrant T-cell expression has been reported in cases of PEL[99] and DLBCL associated with chronic inflammation[96], both morphologically and immunophenotypically similar to PBL. CD3 expression in this study varied from only a few cells (Fig.9) to dense infiltrates of reactive T-cells. The possibility that the number of reactive T-cells amongst the tumour cells could have represented the T-cell count of the patient should be considered. It was postulated that PBL in patients with higher T-cell counts

might have denser infiltrates of reactive T-cells which decreased as the CD4+ counts did, a hypothesis that should be tested in future studies.

MUM-1 plays an important role in the development of normal lymphoid cells[220] and is primarily expressed in B-cells committed to plasmacytic or memory cell differentiation, plasma cells[259] but also in activated T-cells[260]. This marker is not specific for either plasmablasts or plasma cells and is expressed in a wide variety of hematolymphoid neoplasms and even in malignant melanoma[96, 97, 220]. The 2008 WHO classification does not include MUM-1 as useful immunohistochemical marker for PBL and also does not mention the degree of positivity that might be expected with this marker. All but one of the PBL cases in the current study showed MUM-1 positivity in more than 20% of the cells (Fig.13) with 87% showing strong and diffuse positivity. This was also reported by others[13]. There might be several reasons for this association of PBL with MUM-1 expression. Firstly, MUM-1 is a strong marker of plasma cells[259, 261] and MUM-1 expression has been shown in 100% of MM cases[259, 261] which further substantiate the plasma cell nature and possible biological relationship of PBL with other plasma cell dyscrasias. Secondly, a relationship between MUM expression and HIV-infection was demonstrated by Carbone and co-workers in 2001[15]. Based on the expression of MUM1, BCL6, and CD138 the authors separated HIV-related lymphoproliferative neoplasms into three groups: (a) the BCL6+ /MUM1- /CD138- pattern, seen in certain cases of AIDS-associated DLBCL; (b) the BCL6- /MUM1+ /CD138- pattern, associated with a fraction of AIDS-immunoblastic lymphoma; and (c) the BCL6- /MUM1+ /CD138+ pattern, associated with systemic and primary central nervous system immunoblastic lymphoma, PEL, PBL of the oral cavity (7 of 7), and HIV-associated Hodgkin's lymphoma[15]. BCL6 expression was not evaluated in this study. The true relationship between MUM-1 expression and HIV infection should be investigated in future studies. A third explanation for the high MUM-1 expression in our cases of PBL might be related to the presence of EBV which was found in all but one (case 43) of our cases. The expression of EBV-encoded latent membrane protein-1 (LMP-1) has previously been shown to be associated with the BCL6-/MUM1+/CD138+ expression pattern[15]. Increased MUM-1 expression was also proven to be associated with EBV-related transformation of human B-cells in *in vitro*

studies. Xu and co-workers demonstrated that EBV-related LMP-1 stimulates MUM-1 expression in B lymphocytes and suggested that MUM-1 may be a critical factor in EBV transformation and therefore a useful target in the therapy of EBV-mediated neoplasia[262]. This could not be confirmed by others[220]. The single EBV negative case (case 17) in this study displayed diffuse MUM-1 positivity and the results of our study did also not support the proposed hypothesis of Xu and co-workers that EBV might play some role in MUM-1 expression.

Syndecan-1/CD138 is a transmembrane proteoglycan seen in the late stages of B-cell maturation and this marker is generally accepted as a hallmark of mature plasma cells[263, 264]. Apart from its use in haematopoietic neoplasms, it should be noted that CD138 antibody also stains other forms of mature tissue such as stratified squamous epithelium, which served as a positive internal control in all PBL cases in the current study (Fig.22). Only 9/45 cases (20%) of PBL's showed diffuse positive staining for this marker (Fig.20) although 38/45 (84%) of cases showed some degree of positivity in some of the tumour cells.

CD38 (cyclic ADP ribose hydrolase) is a multifunctional ectoenzyme essential for cell adhesion and signalling as well as the regulation of intracellular calcium[265]. For the purpose of this study, CD38 served as another marker of post-germinal B-cell development usually strongly expressed in both normal and myelomatous plasma cells[266]. CD38 proved positive in only 29/45 (65%) of PBL's in this study. Only 7/45 (16%) showed diffuse positivity with this marker (Fig.18).

The exact time of CD138 and CD38 expression during B-cell development into plasmablasts and into plasma cells is still uncertain. Recently Caraux and co-workers reported that circulating plasma cells can be subdivided in $CD138^{+}CD20^{-}CD38^{++}$ and $CD138^{-}CD20^{-}CD38^{++}$ cells using flow cytometry[263]. I interpreted negative staining for either or both of these markers in two ways. Either the plasmablasts were in an earlier stage of development and have not yet gained the CD38 and/ or CD138 receptors on their cell surfaces[263] or alternatively, it might have been due to some form of cellular dedifferentiation

with loss of the normal plasma cell markers. This should be clarified by future genetic and flow-cytometric studies.

There is currently no clarity on the precise antigen expression profile of neoplastic cells in PBL or the interpretation of immunohistochemical markers for post-germinal B-cell development in these neoplasms. It was clear from this study that the expression of MUM-1, CD38 and/or CD138 or the lack thereof played a very small role in confirmation or exclusion of the diagnosis of PBL, unless interpreted in conjunction with the expression of other cell antigens. In other words CD20 negativity in conjunction with a positive CD38 and/ or CD138 might for instance be helpful to confirm the diagnosis of PBL. However this combination may also be encountered in other plasma cell neoplasias such as MM and plasmacytomas, other HIV-related lymphomas such as PEL[99] and the rare ALK-positive DLBCL[97].

An interesting finding in this study was the two cases (Cases 4 and 30) which stained negative for CD79a, CD38 and CD138 although both cases showed diffuse and strong positivity for MUM-1. It would be very interesting to see if the ratios of CD79a, CD38, CD138 and MUM1 positivity might be reflective of a certain stage of maturity in the tumour cells of PBL and therefore have any prognostic implications.

CD45, initially characterised as a leukocyte common antigen, can be found on all hematopoietic cells except platelets and red blood cells[267]. CD45 is a key regulator of antigen-mediated signalling and activation in B- and T-lymphocytes[268]. CD45 expression is seen in plasma cells in the early stages of development but then disappears during normal maturation of plasma cells[269]. The ratio of CD45 positive versus CD45 negative plasma cells in the normal bone marrow seems to be stable but changes in the course of plasma cell disease such as MM. MM was recently classified according to its CD45 expression with the evidence that loss of CD45 expression indicates disease progression with a more immature cell morphology and a less favourable prognosis[266, 269]. In line with many reports in the literature, 44/45 (98%) of the PBL cases in this study showed some degree of CD45 positivity in at least some of the tumour cells. Seventeen of 45 cases (38%) showed diffuse CD45

staining in more than 70% of the tumour cells (Fig.15). But, due to the exceptionally high numbers of reactive T-cells in some cases, the percentage of CD45 positivity was often difficult to interpret. Other PBL studies in the literature reported CD45 positivity to be minimal or absent in all their cases[43, 270]. Although a quantitative analysis was not done by all authors, our CD45 positivity seemed to be one of the highest ever reported. Although I tried to evaluate only tumour cells, reactive lymphoid cells might appear bigger than normal with false positive interpretation of the CD45 stain in at least some cells. The laboratory techniques and antibodies used may also have played a role in the variety of results published by various authors. Even so, CD45 immunoreactivity in PBL did not play any diagnostic role in PBL's. Instead CD45 positivity might be indicative of a plasma cell neoplasm with immature morphology (plasma cells in early stages of development) in line with the low CD38 and CD138 positivity observed in many cases in this study. CD45 negative cases could also be reflective of neoplastic plasma cells that have lost their normal CD45 expression.

No PBL case in this study stained positive for the ALK protein which excluded the rare ALK-positive DLBCL, also known as ALK-positive plasmablastic B-cell lymphoma in the WHO classification[97]. These rare lymphomas show remarkable similarities to PBL. They have been described in less than 50 cases of adult males with a median age of 36 years involving the lymph nodes as well as extra-nodal sites such as the nasopharynx[271]. The median survival is less than 11 months and the tumour cells have the exact immunophenotypic and cytomorphologic features as described for PBL's, with the exception of the ALK-positivity (Fig.23). PBL involving the oral cavity in HIV-positive patients are listed as a very specific differential diagnosis. Genetically, these neoplasms are most frequently associated with the t(2;17) translocation responsible for the Clathrin-ALK fusion protein. Future studies might have to be done on ALK-positive DLBCL's in order to determine if they do not possibly represent a subtype of PBL.

Immunohistochemical analysis for κ and λ light chains was performed in all cases of PBL in this study. Light-chain restriction is used as a diagnostic feature of B-cell lymphomas. Although many authors have previously

reported evaluation of monoclonality in PBL's by analysis of immunoglobulin light chains through immunohistochemistry, none described how they actually did this. Kappa: lambda ratios accepted as 'normal' on flow cytometric examinations varies substantially[272]. To simplify interpretation of the results of the immunohistochemical evaluation used in my study, I did not use ratios to determine the presence or absence of light chain restriction. Only cases completely negative for one of the light chains but positive for the other was accepted and subsequently noted as having light chain restriction. Twenty one of 45 cases (49%) showed clear light chain restriction in the current study, a percentage that would in all probability be higher should I have used ratios (Table 3). Seventeen of the 21 cases (81%) showed κ light chain restriction (Fig.24) and four of these 21 (19%) cases showed λ light chain restriction. The role of light chain restriction in the context of PBL is uncertain at this stage. Although light chain restriction is one of the diagnostic features of MM and was also confirmed as a valuable aid in the diagnostic armamentarium of plasmacytomas[273, 274], the lack thereof is seen in approximately 10% of extra-medullary plasmacytomas and 15% of MM[109]. This lack of light chain restriction has tentatively been explained by acquired mutations of the *Ig* genes resulting in dedifferentiation of the tumours cells to the extent that they no longer secrete Ig[275]. The determination of κ and λ light chain restriction by immunohistochemical methods proved to be very difficult with high background staining in many cases. This was recently explained to be due to non-specific adsorption of the antibodies by collagen, resulting in high background staining and difficulty when interpreting the immunohistochemical results[256]. The presence or absence of light chains in the cell cytoplasm with or without restriction played no significant role in the diagnosis of PBL. There were no correlations between light chain restriction and any other morphological or immunophenotypic features of these neoplasms. The exact incidence of light chain restriction in PBL's should be determined with better methods such as flow cytometry and the implications with regards to prognosis or classification of these neoplasms should be investigated further.

The results of the immunohistochemical investigations of PBL's in this study supported PBL's to either represent a unique form of B- or plasma cell malignancy or an aggressive variant of an existing plasma cell neoplasia such

as MM or plasmacytoma. Neither of these hypotheses has yet been demonstrated. None of the immunohistochemical markers played a major role in the diagnosis of PBL. The combination of immunoblastic or plasmablastic morphology, loss of CD20 expression and simultaneous expression of one or more plasma cell markers such as CD138, CD38 and/ or MUM-1 may be indicative of a diagnosis of PBL. This is further supported by a relevant clinical history such as an aggressive oral tumour in an HIV/AIDS patient without any history of other plasma cell diseases such as MM or plasmacytoma. This study therefore supports the WHO decision to remove PBL as a variant of DLBCL as it was previously classified and to reclassify it as a separate entity[22]. The classification of PBL might change again as soon as its true biological nature is unravelled through genetic studies.

4.3 Viral status of plasmablastic lymphomas

HHV-8, a gamma herpes virus is the oncogenic virus associated with Kaposi's sarcoma and certain lymphoproliferative diseases such as PEL[99] and large B-cell lymphoma arising in HHV-8-associated multicentric Castleman's disease[98]. Its presence is necessary for confirmation of these diagnoses. Africa is the continent with the highest prevalence of HHV-8 which increases with the presence of HIV-1 infection[276]. The genome of HHV-8 harbours an analog of the *Il-6* gene which is responsible for B-cell and plasma cell proliferation and may sometimes result in a lymphoproliferative disorder[139].

The methods used for HHV-8 detection as well as the use of strict positive and negative internal controls are very important when considering the results obtained by any study. When the role of HHV-8 in the pathogenesis of a tumour is in question, detection techniques where cell morphology remains intact are preferred. This ensures confirmation of the intracellular presence of the virus in the tumour cells examined. The HHV-8 virus may be harboured in B-lymphocytes of immunocompromised patients and signals detected on PCR may be from a source different to the tumour cells, therefore a false positive result caused by the presence of 'bystander' virus contamination[277]. Due to the high HHV-8 and HIV-1 infection rates in Africa[278], PCR is not a good choice as detection method. Techniques for HHV-8 detection that fit the

prerequisite of intact cell morphology include immunohistochemistry and *in situ* hybridisation. The latter has been confirmed as a more sensitive method for detection of HHV-8[279] and was therefore the detection method of choice in the current study.

The recommended guidelines from the manufacturer in the protocol of the HHV-8 virus probe and Novocastra detection kit were not followed in this study. Following their guidelines resulted in false positive results in the brain section utilised as negative control (Fig.42). The protocol was adjusted to rectify this, supported by internal control evaluations. Although digestion with Proteinase K according to the procedural notes of the manufacturer was sufficient for the probe to hybridise HHV-8, it was found that a combination of HIER in citric acid buffer (pH6) and pepsin digestion gave superior results. The latter method was in the protocol. False positivity was however seen in the negative control section with both protocols of enzyme digestion. Various test runs were performed to rectify this. The following steps were adjusted: the digestion time of pepsin was decreased in increments as well as the incubation with the probe and enzyme substrate. The incubation time of the probe was increased and that of the enzyme substrate decreased. The dilution of the rabbit F(ab') anti FITC/AP and enzyme substrate was increased. The wash times in buffer were also increased and the use of a shaker included. The post hybridisation wash was increased from nine minutes up to 30 minutes. The temperature was increased from room temperature to 45℃. Only after adjusting the temperature of the post hybridisation wash, however did the positivity in the negative control section disappear. The stringency of the wash buffer in the protocol of the manufacturer [Tris-buffered saline (TBS), 50mM Tris-HCl, 150mM NaCl, pH 7.6 /0.1% Tween] was found insufficient to remove the false positivity in the negative control where after the post hybridisation wash was done using Dako stringent wash concentrate ([saline sodium citrate(SCC), 75mM sodium citrate, 750mM $NaCl_2$ pH7.6] (Dako Corporation, Carpinteria, CA 93013 USA) at a temperature of 55℃ for 20 minutes. This resulted in strong positivity in the nuclei of the vascular endothelial cells in the control Kaposi Sarcoma section (Fig.26) whilst the cells in the negative brain control block did not stain (Fig.43).

Figure 42: *The micrograph shows an example of a false positive HHV-8 (arrows) in the brain section utilised as negative control for HHV-8 ISH (Original magnification: 400x).*

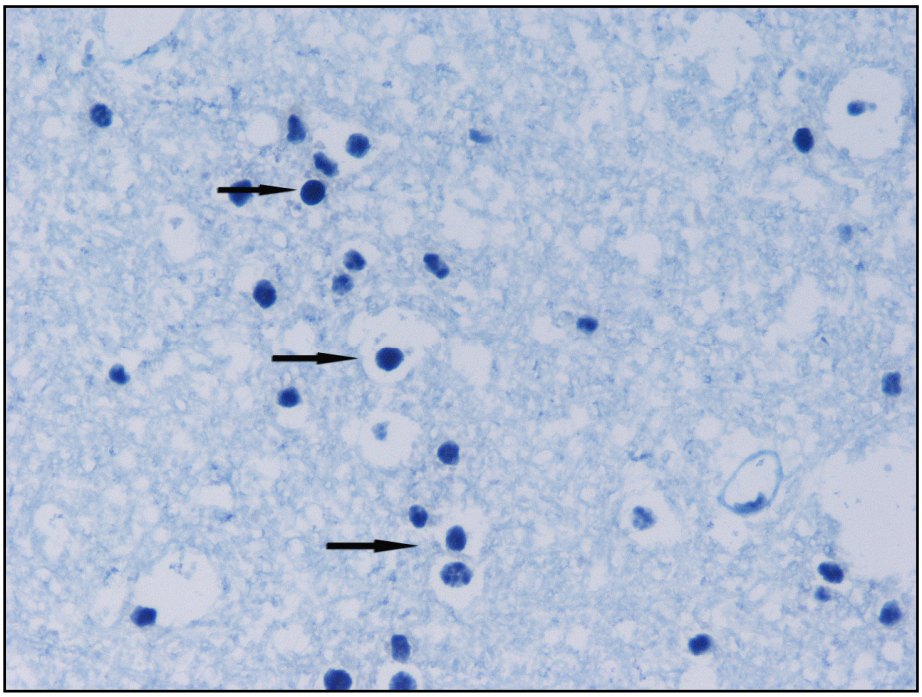
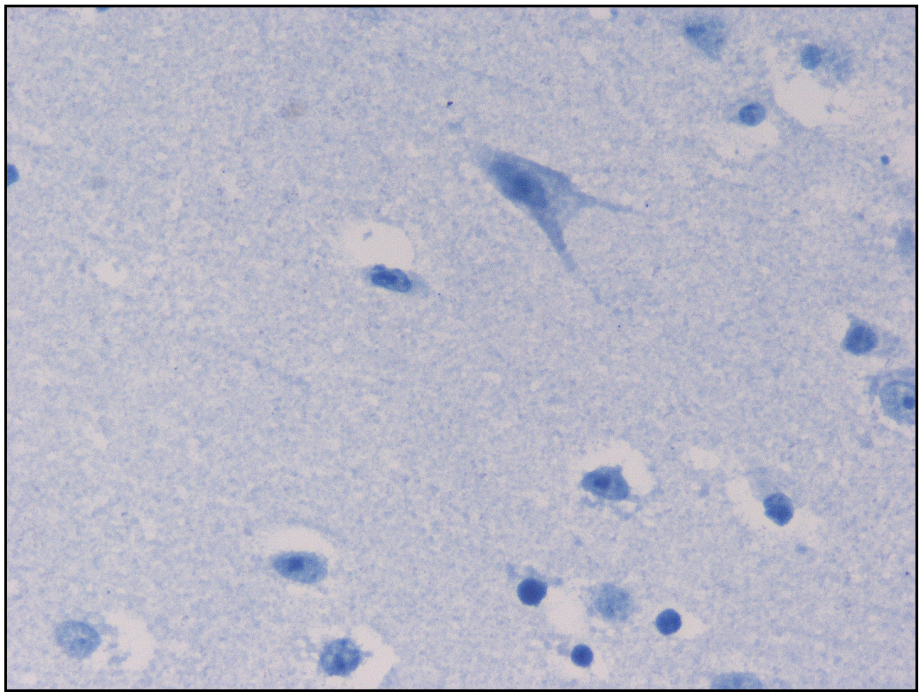


Figure 43: *The micrograph shows the abscence of HHV-8 staining in all of the neural cells of this brain section utilised as negative control for HHV-8 ISH (Original magnification: 400x).*



Several authors reported the presence of HHV-8 in their PBL cases[15-20] The presence of HHV-8 is however currently widely accepted by most, including the WHO, not to form part of the diagnostic criteria for PBL[21, 22]. ISH confirmed the absence of HHV-8 in all cases of PBL in this study. HHV-8 positivity is therefore proposed to be a contraindication for the diagnosis of PBL and should warrant a different diagnosis such as solid extracavitary PEL.

EBV is a lymphotrophic gamma herpes virus implicated in the pathogenesis of various B-cell lymphoproliferative diseases including HIV/AIDS associated NHL such as DLBCL with immunoblastic morphology, primary central nervous system lymphoma and PEL and its solid variants[2, 134]. Two main methods can be used to test for the presence of EBV. These include ISH with probes for EBER's, which represent the most widely used technique, and antibody staining for the LMP1[8]. EBER's 1 and 2 are small, non-coding RNA's expressed in all known forms of EBV latency, although their biological function is only speculative at this stage[280]. We used ISH with probes for EBER-1 and -2 for EBV detection in this study and strict positive and negative internal controls were included in the protocol to ensure optimal results.

The role of EBV in the pathogenesis of PBL is still unclear but it is reported to be associated with 60-100% of these neoplasms[5, 11, 13, 14] All but one (98%) case of PBL (case 17) in this South African-based series was EBV positive (Fig.27). As stated earlier, 31 of 32 patients in this study with known HIV-status was HIV-positive (Table 2). The only patient with a known HIV-negative status (case 17) was also EBV-negative. The reason for the high EBV positivity in this study is uncertain but speculated to be the results of the high incidence of HIV-infection.

EBV alone is insufficient to induce malignant change[280] and its pathogenetic role in AIDS-related lymphomas, although speculative, has been linked to several mechanisms. LMP-1 is a transforming EBV protein that plays a crucial role in the transformation of B-lymphocytes into immortal B-cells[281]. LMP-1 is frequently expressed in DLBCL and might play a pathogenetic role in these lymphomas[282]. EBNA-1, a viral protein required for replication and maintenance of latency is found at a high frequency in BL[280]. EBNA-1 has

strong anti-apoptotic properties and therefore promote malignant proliferation[280]. A decrease of EBV EBNA1-specific memory CD4+ and CD8+ T-cell responses has been shown in HIV infection[283]. The decline in specific immunity in a deregulated and permissive immunological environment of HIV/AIDS as well as the EBV-driven cell proliferation potentially result in the accumulation of genetic alterations with malignant transformation of the lymphocytes as a consequence[123].

Apart from the proliferation induced genetic aberrations, latent EBV infection *per se* has been shown to promote genetic instability[284]. This further enhances the neoplastic drive of EBV infection. Cooperation between the EBV oncoviral proteins such as EBNA-2 and the host *MYC* gene was revealed as a pathogenetic event for lymphoma development[285, 286]. EBNA-2, the key EBV transactivator protein critical for B-cell activation induces *MYC* expression[287]. The possibility that EBV may even protect lymphoma cells from the normally apoptotic effects of over expressed *MYC,* especially secondary to a *MYC* translocation such as the BL translocation t(8;14) has been postulated[134, 280]. Further to this is the possibility that EBV may, through various mechanisms, even re-program B-cells into becoming neoplastic cells with an increased ability to survive oncogenic stress and genomic instability[280].

It is not known which of the above pathogenic mechanisms of EBV-related lymphomagenesis is applicable to PBL and this should be investigated in the future. The plasmacytoid differentiation seen in EBV positive lymphomas, especially in the context of HIV infection in which case it is predominant or almost exclusive[109, 134] does however emphasize the possibility of a role in the pathogenesis of PBL. EBV negative PBL's are occasionally reported in HIV-negative patients in the literature but this should be evaluated carefully. Firstly, the techniques used to demonstrate the presence of the virus should be evaluated. This could furthermore mean that PBL's may be subdivided into PBL associated with HIV/AIDS and PBL not associated with HIV/AIDS, therefore reflecting different pathogenic mechanisms. It may also imply that PBL's diagnosed in an HIV-positive patient may actually represent an atypical presentation of one the plasma cell dyscrasias or that PBL's diagnosed in an

immunocompetent patient actually represent a novel group of malignant lymphoproliferative neoplasms, yet to be defined more clearly. The diagnostic criteria for MM and plasmacytomas with plasmablastic morphology, especially in the setting of HIV/AIDS should be revisited since many neoplasms diagnosed as PBL have clinical features overlapping with the diagnostic criteria for MM.

4.4 Genetic features of plasmablastic lymphoma

Genetic features are an important component of the diagnostic algorithms of lymphomas in the 2008 WHO classification of lymphoid tumours[9] and additional genetic data continue to inform on the pathogenesis of lymphomas. However, there is a significant shortage of genetic studies on PBL apart from single case reports and small series. Recently a large study of 42 PBL's by Valera et al., highlighted the important frequency of gene rearrangements in these neoplasms[13]. The 42 reported cases only included eight PBL affecting the mucosa of the oral cavity. This paper nevertheless confirmed previous reports suggesting an important role for *MYC* rearrangements in the pathogenesis of PBL especially with *IGH* as a partner[11, 13, 24-28]. Valera and co-workers described frequent gains of *MYC*, *IGH* and other loci commonly involved in B-cell lymphoma further suggesting an association with complex karyotypes[13]. It was decided to evaluate all of our cases of PBL by FISH analysis for genes commonly described in lymphoma.

Advantages of FISH are that non-dividing (interphase) nuclei can be evaluated and FISH can be done on archived FFPE specimens[243, 288], allowing retrospective analysis of archival tissue. The technique can also be applied to small biopsies as FISH analysis can be performed effectively on as few as 40 cells[243].

Recurrent translocations of the *IGH* gene (14q32) are found in approximately 50% of B-cell NHL and as such recognised as a hallmark[167, 289]. There is a wide diversity of possible partners for the *IGH* gene in lymphomas. The incidence of *IGH* rearrangements in PBL was evaluated in 43 cases of PBL in this study using the *IGH* BA probe (14q32.3 LSI IGH) which is not dependent

on a specific translocation partner. *IGH* rearrangement were observed in 27/43 (63%) of PBL's in this series. The frequency of *IGH* rearrangement seen in our study was in line with what was expected in a B-cell NHL and other plasma cell neoplasia such as MM, known to have *IGH* rearrangements in more than halve of non-hyperdiploid cases[290].

Substantial evidence has accumulated recently supporting the idea that *MYC* rearrangements do not only characterise BL[213, 291], but also occur in approximately 10% of DLBCL[292] as well as in the new category of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL[213, 291], follicular lymphoma, mantle cell lymphoma, T-cell lymphoma and chronic lymphocytic leukaemia[293]. In 22 of the 27 cases (81%) of PBL with *IGH* rearrangement in this series, *MYC* was shown to be the partner of the translocation involving the *IGH* gene. This was seen using the *IGH/MYC* CEP8 tri-colour, dual fusion translocation probe, t(8;14)(q24;q32). Four cases negative for t(8;14) also showed rearrangement of the *MYC* locus on the *MYC* BA rearrangement probe (8q24 LSI MYC) with partners other than *IGH. MYC* was therefore rearranged in 26/42 (62%) of PBL's affecting the oral cavity in this study.

The incidence of *MYC* rearrangements in PBL has received some attention over the past four years. Reports on the median survival of patients with PBL vary from a few months to 3 years but this seems to be reduced substantially when *MYC* rearrangements, especially t(8;14) are present[24-26]. Recent, case reports on *MYC* rearrangements in PBL include single case reports from different study groups, only some of which were specified to be positive for t(8;14)[24-27]. In some of these reported cases, the patients had a CD4 count of less than 200 cells/mm^3 with an associated median survival of three months, further demonstrating the association of poor survival in PBL cases with *MYC* rearrangements and HIV/AIDS disease. Recently, the largest genetic study on PBL of various anatomical sites was reported by Valera *et al. MYC* gene rearrangement was seen in 7 of 8 cases (88%) of PBL affecting the oral cavity but this genetic alteration was shown in only 13 of 33 (39%) of their cases affecting other anatomical locations. It was previously discussed in this document that PBL affecting the oral cavity mucosa seems to be more

common in the setting of HIV/AIDS than those of other anatomical sites. This present study reinforced the idea that *MYC*-rearrangements seem to be more common in PBL affecting the oral cavity than PBL affecting any other anatomical site.

The mechanism through which *MYC* aberrations in lymphomas other than BL cause clinical aggressiveness is uncertain. Smith and co-workers recently postulated tat *MYC* may modulate a completely different set of target genes in BL than in non-BL[293]. The role of *MYC* in tumour cell growth and survival is a complex process as *MYC* regulates proliferation, apoptosis and cell differentiation in normal as well as in tumour cells[294]. Tumour cells, just like normal cells, respond to the microenvironment which includes cellular stressors such as hypoxia. *MYC* dysregulation, through a variety of mechanisms, provide tumour cells with enhanced growth abilities, even in a microenvironment of hypoxia and other extrinsic stressors. This is achieved by modulating several internal metabolic pathways and by promoting angiogenesis[293, 295-297].

The high incidence of *MYC* rearrangement in PBL affecting the oral cavity in HIV/AIDS patients may tentatively be explained. The cooperation of EBV oncoviral proteins such as EBNA-2 with the products of *MYC* gene aberrations could be the main pathogenic event for the development of PBL and should be investigated further[285-287, 298]. One hypothesis is that EBV may protect the tumour cells from the apoptotic effects of *MYC* over expression driven by a translocation such as the t(8;14)[134, 280]. In the large PBL series reported by Valera *et al.*, the incidence of *MYC* rearrangement were described as frequent among EBV-positive cases although no specific correlation was made between the anatomical sites, EBV-positive cases and *MYC* rearrangement[13]. Forty-four of 45 (98%) of PBL's of the oral cavity in our study were EBV positive on ISH. The only EBV negative case (case 17) was also the only patient known to be negative for HIV amongst the 32 patients with known HIV statuses in this study. *MYC* rearrangements were therefore seen only in EBV-positive tumours and might play a critical role in the initiation of PBL of the oral cavity, especially if one considers the role of the Waldeyer ring lymphoid tissue and posterior oro-pharyngeal and naso-pharyngeal

epithelial cells as possible EBV reservoirs[299, 300]. This HIV and EBV negative case in the current series also showed no rearrangement of *MYC* or *IGH* but did however have an increased gene copy number of *CCND1.* This underlines the possibility that PBL outside of HIV-infection might have a different pathogenesis.

Another new and interesting finding from the results of this study was the increased *CCND1* gene copy number discovered in 17/41 (41%) and increased *IGH* gene copy numbers in 6/41 (15%) cases of PBL cases. More than six *CCND1* gene copies per cell nucleus were seen in seven of these cases and more than six *IGH* gene copies in four of them. While increased gene copy number previously described in PBL tend to be attributed to complex karyotypes[11], cases with multiple copies suggest true amplification of the *CCND1* and less frequently the *IGH* genes in some oral PBL's in our series. *CCND1* gene amplification has been described in aggressive B-cell NHL's[301] as well as MM in which case various mechanisms seems to be responsible for increased *CCND1* amplification and over-expression[302, 303]. Cyclin D1 is a key cell cycle regulatory protein[304] and its increased gene copy number might lead to over expression and contribute to the high proliferation index and aggressive clinical course of PBL.

MYC gene rearrangements in non-BL lymphomas are often associated with additional cytogenetic abnormalities which may further contribute to the aggressive behaviour of the disease and to the poor response to therapy seen in these patients[13, 27]. Patients diagnosed with *MYC* rearranged DLBCL combined with deregulation of *BCL6* or *BCL2,* so-called double-hit lymphomas, have an even worse prognosis[292, 293]. Karyotypic abnormalities in addition to *MYC* rearrangements are currently integrated into the 2008 WHO classification system. Seven PBL's cases in our series demonstrated rearrangement of both *IGH* alleles demonstrating a possible 'double hit' for the *IGH* locus (Figs 32 & 33). The *MYC* and *CCND1* genes were both translocation partners of *IGH* in one case, demonstrated by positive *IGH/CCND1* dual colour, dual fusion translocation probe, t(11:14)(q13q32) (Fig.37). Both *MYC* and *BCL2* genes were discovered to be partners of the *IGH* gene represented by positive t(8;14) and t(14:18) probes in one case

respectively. *MYC* was one of the *IGH* translocation partners in five cases with the other partner unidentified in three of these cases. In two additional cases both *IGH* chromosome partners remained uncharacterised. Double hits have been reported in several DLBCL and particularly in large B cell lymphoma with intermediate features between DLBCL and BL. To the best of our knowledge, the finding of rearrangement of both *IGH* alleles, although of low frequency (16%), was a new observation in PBL.

The findings of various genetic rearrangements in both simple and complex karyotypes may eventually all have an influence on the choice of treatment strategy for the patient diagnosed with PBL, especially those with *MYC* rearrangements[293, 297]. Although we currently lack specific therapeutic approaches for patients with *MYC*-associated lymphomas, it has been proven that bortezomib down regulate *MYC*[297]. This might explain the recent publication by Bose and co-workers who reported dramatic and early response to bortezomib, a proteasome inhibitor widely used in treatment of MM, in a patient diagnose with PBL of the stomach[12]. The authors hypothesised the efficacy of this treatment by suggesting PBL to be related more closely to plasma cell neoplasms than to B-cell lymphomas and therefore the poor performance on other oncotherapy regimes such as CHOP (cyclophophamide, doxorubicin, vincristine and prednisone) and CHOP-like regimens.

Through the results of this study and others, it is recommended that routine genetic analysis for *MYC* rearrangements and others, especially to identify complex karyotypes, should be done and reported in order to identify these aggressive subtypes to the managing clinician[25, 305, 306]. The spectrum of genetic analysis specific for PBL as well as the FISH probes used to determine the scope of genetic rearrangements should still be evaluated by future studies. Currently however, it is advised that a panel of FISH probes including t(8;14), *IGH* BA and investigations of *CCND1* be performed and if double hit rearrangement of both alleles is found on *IGH* BA, *BCL2* and *BCL6* should also be evaluated as possible translocation partners.

It may be important at this point to take note of a recent publication where the authors described *IGH/MYC* rearrangements in three cases of high-grade lymphoma, detectable on *IGH/MYC* dual-fusion FISH probes but which could not be detected using *MYC* BA FISH probes[307]. This highlighted certain limitations of using *MYC* BA probes as a stand-alone test. Explanations why some *MYC* rearrangements escape detection by *MYC* BA probe sets include the position of the breakpoints which varies and sometimes include areas that may leave the BA fusion signal intact[307]. The wide variation of *MYC* breakpoints are well described[308], and breakpoints several hundreds of kilobases upstream of *MYC*[309] or the extreme centromeric region[310] have been reported. Other explanations offered by May and co-workers is that an insertion, rather than a translocation, occurred which makes the translocation partner 'invisible' to the analyst. This may involve insertion of a small *IGH* sequence into the *MYC* gene, which may not be large enough to physically separate the two signals flanking the insertion breakpoints, or insertion of part or the entire *MYC* probe into the *IGH* locus with no disruption of the *MYC* probe. Consequently the presence of *MYC/IGH* may be overlooked[307].

Cytogenetic studies are expensive and not always available. Less expensive and specialised techniques to identify these *MYC*-aberrations are therefore sought after. A novel monoclonal antibody recognizing the c-MYC protein in FFPE tissue by standard immunohistochemistry was recently described[311]. It has been shown that *MYC* expression was exclusively in the nucleus of 88% of BL cases and in both the cytoplasm and nucleus in the other 12% of BL. In cases of DLBCL without *MYC* translocation, the staining with this antibody was cytoplasmic. Nuclear or evenly distributed staining between the nucleus and the cytoplasm were described in 80% of DLBCL that harboured *MYC* rearrangements[311]. It is currently proposed that this monoclonal antibody may be used as a screening tool for a *MYC* rearrangements[293], which would be useful in the light of the clinical implications. It would be interesting to test this antibody in PBL cases in a prospective clinico-pathological study.

BCL6 abnormalities are commonly described in DLBCL and are present in up to 30% of cases[180, 312, 313]. We found no *BCL6* rearrangement in any of the 40 PBL cases screened for this abnormality. Gains in copy number seen as

increased fusion signals per cell nucleus (Fig.41) were however seen in 11/40 (28%) cases screened with the *BCL6* BA probe. Loss of one fusion signal was seen in three cases screened with the same probe.

As discussed throughout this document, it has previously been hypothesised by various groups that PBL may actually represent a plasmablastic form of MM or plasmacytoma[11]. It was shown by this study as well as many others that there is no specific immunophenotypic or morphological feature to differentiate these diseases. Even clinical aspects seem to overlap to the extent that it makes differentiation between these neoplasms impossible. Genetic aberrations in MM include chromosomal gains and losses as well as specific chromosomal translocations affecting the *Ig* gene loci, most commonly *IGH* at 14q32.3[225, 230]. Translocations which result in protooncogenes being misplaced into the *Ig* loci results in the powerful *IGH* enhancers to drive abnormal expression of translocated oncogenes including *cyclin D1*, on chromosome 11q13 leading to dysregulation of its expression[227]. Recurrent translocations are seen in approximately 40% of MM cases and include t(11;14)(q13;q32) encountered in 15-20% of MM patients[228, 229]. *IgH* translocation and hyperdiploidy are unified by downstream up regulation of *cyclin* D1, D2 or D3[231] which are eventually followed by further instability that often includes genetic alterations of chromosome 8 (*MYC*)[233]. Plasmablastic transformation in MM and other malignancies are frequently associated with *MYC* translocations and EBV-positivity[11]. Several reports describe the presence of *MYC* translocations in MM to be associated with tumour progression to high-grade plasmablastic morphology and aggressive clinical behaviour[215, 216, 290, 314]. Very little information on the genetic features of plasmacytomas is available in the literature in order to compare with genetic features of PBL. Bink *et al* recently did the largest study on cytogenetic alterations in 38 cases of extra-medullary plasmacytomas which showed *IGH* breaks in 37%, t(4;14) translocation in 16%, loss of 13q in 40% with chromosomal gains in 82%. No t(11;14) was present in any of their cases[315].

Only the high incidence of *IGH* rearrangements in this study are in line with what is seen in MM[227]. Whereas 15-20% of patients with MM exhibit the t(11;14)[228, 229] only one of 41 (2%) of our cases evaluated was positive for this

translocation. The t(11;14) translocation juxtaposes the *CCND1* gene to the *IGH* locus and is, together with the over expression of *CCND1*, the genetic hallmark of mantle cell lymphomas, considered as the primary genetic event thereof[316]. The role of the increased *CCND1* and *IGH* gene copy numbers in the pathogenesis of PBL's should be investigated. The true role of key genes in the pathogenesis and biology of PBL will be necessary before further comparison with MM and plasmacytomas with plasmablastic morphology will be possible. This should include evaluating rearrangements of chromosome 1, deletions of 13*q* and 17*p* together with possible gains in odd-numbered chromosomes in a large cohort of PBL cases.

The application of FISH to FFPE tissue sections are technique sensitive[288]. Storage conditions and the age of the tissue blocks have shown to influence the rate of successful FISH analysis on paraffin sections[317]. Some of the archival blocks used in this study dated back to the 1990's which could have played a significant role in the loss of certain cases (see tables) for genetic analysis. Tissue fixed in neutral buffered formalin, a cross-linking fixative, is preferred for FISH on FFPE tissue[318]. Fixation of the tissue prior to embedding it into paraffin blocks is known to play a major role in the quality of results obtained. In some PBL cases the overlying epithelial cells as well as those tumour cells present at the periphery of the sample were better exposed to formalin with better fixation and therefore presented with good morphology and sharp signals on FISH analysis. This was in contrast to tumour cells located towards the centre of especially the larger specimens and for which results were at times sub optimal. This was most probably due to suboptimal fixation in the central regions of a larger tissue sample.

Thickness of the histology section for FISH analysis played a major role in the interpretation of the results. Tissue section thickness should be determined by the type of tissue or cells to be analysed. The ratio of cell diameter versus thickness of the tissue section would either result in or prevent truncation artifact, one of the biggest problems encountered in the interpretation of FISH results[241, 245]. In the same regard, overlay or directly adjacent nuclei was also problematic because two overlying cells might be regarded as a single cell and the signals present in these two cells be interpreted as signals from a

single cell. I discovered that the overlying mucosal epithelial cell nuclei of the lower third of the epithelial layer were a good indication of the diameter of the tumour cell nuclei in the PBL cases. The epithelial cells therefore served as internal control for all cases in our study. Due to the fact that all cases of PBL in this study affected the oral mucosa, some overlying epithelial cells were always present on every section. Initially we performed FISH on three micrometer sections. This resulted in truncation of tumour and epithelial cell nuclei and subsequent loss of signals in both the epithelium and the tumour cells. Increase of section thickness to four micrometer resulted in an increased number of signals in both cell types and 4 micrometer sections were therefore performed. To prevent misinterpretation of signals, analysis should strictly be performed on the correct thickness of section determined by the type of cells to be analysed. Analysis should also only be performed in areas where tumour cells are single or loose-lying from other tumour cells with no overlapping between nuclei. When dealing with a small cell neoplasm, thinner sections should be used as too much overlapping of nuclei will be present on thicker sections making interpretation of signal patterns impossible. In the case of larger cells, such as is the case with PBL, a thicker section will be needed as too much truncation artefact will result from thin sections.

Experience in histopathology and tissue morphology in FFPE tissue sections aided the author to distinguish between tumour cells of PBL, reactive T- and B-cells with normal morphology, epithelial cells as well as other bystanders such as fibroblasts. This might be a challenge to the inexperienced interpreter which may wrongly interpret normal bystander tissue as tumour and therefore skew the results when evaluating 100 or 200 nuclei for instance. In some slides, arcades of skewly cut covering epithelium intermingled extensively with the cohesive sheets of similar-sized tumour cells in the underlying lamina propria. Due to the non-discriminating nature of the DAPI stain, it was sometimes almost impossible to distinguish the epithelial cells from the tumour cells. Careful evaluation of the morphological features and intercellular bridging seen between most of the epithelial cells were important to ensure that the FISH analysis was performed on the tumour cells only.

Analysis was always done on tumour cells with well defined borders and cells with ghost-like features were excluded.

Optimal tissue fixation, a clear understanding of the principles of the technique and probe construction as well as thorough knowledge on the morphological features of the tumour cells dealt with in a specific assay is crucial in order identify artefacts, aiding in the successful interpretation of the results.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

This study represented the largest cohort of PBL affecting the oral mucosa published to date. The most important findings of this study were the genetic features, some of which confirmed the findings of others and some of which had never been described before. This study confirmed PBL to be a genetically complex, high-grade lymphoproliferative malignancy for which its current position in the 2008 WHO classification as a separate entity, rather than a subtype of DLBCL is supported.

Rearrangements of the *IGH* gene were found in 27/43 (63%) of cases screened with the *IGH*-BA probe set whilst *MYC*-rearrangements were discovered in 26/42 (62%) of PBL's. *MYC* rearrangements in this study consisted of the t(8;14) translocation which was present in 22/27 (81%) of PBL cases with *IGH* rearrangements but four other cases of PBL, negative for *IGH* BA, showed BA of the *MYC* gene with the *MYC*-BA probe set.

The presence of *MYC* rearrangements is currently accepted as an adverse prognostic factor in cases of DLBCL. *MYC*-rearrangements are more commonly associated with complex genetic karyotypes. In this study, seven of the 43 cases showed rearrangement of both alleles of the *IGH* gene, a finding never described before (Table 5). The *MYC* gene was shown to be one partner in five of these cases, all of which was t(8;14) positive, *CCND1* was one of the partners in one case shown to be t(11;14) and t(8;14) positive and *BCL2* was the one partner in one case shown to be t(14;18) and t(8;14) positive. These two lymphomas were therefore proven to be double hit lymphomas. Both partners of *IGH* remained unknown in two of the seven cases that showed rearrangement of both *IGH* alleles.

Increased *CCND1* gene copy numbers were found in 17/41 (41%) of cases screened with the *CCND1/MYC* dual fusion translocation probe. Seven cases showed more than six copies of *CCND1* per cell nucleus. Six of the cases (15%) screened with the same probe also showed increased copy numbers of

the *IGH* gene, four of which had more than six copies per cell nucleus. These findings, associated with the high incidence of *MYC*-rearrangements, with or without complex karyotypes might all play a significant role in the aggressive clinical behaviour and poor prognosis of patients diagnosed with PBL.

Valera and co-workers recently published a large genetic study on PBL's affecting various anatomical locations. Only eight cases of the oral mucosa were included in their study. Taking their study into consideration, it was interesting that *MYC* gene rearrangements were more common in PBL of the oral cavity than in PBL of different anatomical locations, especially for patients with HIV/AIDS. Fifty-eight percent of PBL in confirmed HIV-positive patients of my study had *MYC* rearrangements and all but one of the patients with known HIV-status in this study was positive for HIV-infection. The only patient known to be HIV-negative in our cohort of patients also showed no *IGH* or *MYC*-rearrangements in the tumour cells although a low level increase in *CCND1* copy number was obvious in the tumour cells of this case.

The exact role of *MYC*-rearrangement in lymphomagenesis is unclear. Factors responsible for the deregulation of *MYC* are numerous but in the case of PBL of the oral cavity the possible role of EBV infection should be considered. All but one of the tumours in the current study was positive for EBV. This was the same PBL case which showed no *IGH* or *MYC* rearrangements on FISH analysis. This supports the findings from other studies that EBV is more commonly associated with PBL of the oral cavity than those occurring in other anatomical locations, and again, more so in the setting of HIV/AIDS.

The association between EBV and HIV/AIDS might be explained by several reasons such as the reduced specific immunity against EBV oncoviral proteins in the setting of HIV/AIDS. The Waldeyer ring lymphoid tissue and oro- and nasopharyngeal epithelium, commonly infected by EBV in HIV/AIDS patients, should also be considered as an explanation for the frequent association between EBV and PBL of the oral mucosa. It is possible that these infected cells play a significant role in the homing of plasmablasts to the oral mucosa. This, in the background of reduced specific and general immunity, and in the

context of EBV associated neoplastic effects on infected B-cells or plasmablasts, might result in the preferred development of these neoplasms in the oral cavity mucosa.

EBV and *MYC* deregulation, either as a consequence of the EBV infection or as consecutive occurring are the most important pathogenetic factors for PBL affecting the oral cavity in HIV-positive individuals. The results from my study further concurred with previous proposals that the diagnosis of PBL should serve as a sign of immunodeficiency, either as diagnostic thereof or as a predictor of a progressive state of immunodeficiency in patients with known HIV/AIDS status. I suggested PBL to be included as a group 1 lesion in the EC Clearinghouse and WHO Collaborating Centre classification of oral problems related to HIV infection, especially in countries with high incidences of HIV such as South Africa.

The absence of *MYC*-rearrangement in some cases of oral PBL in HIV-positive patients warranted a different explanation. This can only be examined in future prospective studies with a wide panel of genetic probes. Through the results gained from the genetic evaluation of PBL cases in this study, I proposed the routine genetic evaluation of tumours diagnosed as PBL, as this may have prognostic and treatment implications in the future. Follow-up studies in order to confirm the clinical impact of the genetic evaluations have become mandatory.

Clinically, the gingiva and palatal mucosa was shown to be the preferred intra-oral location for PBL. Patients in our cohort, just as in most of the published cases, were around 40 years of age.

This study proved that PBL is not a histomorphologic or immunohistochemical diagnosis by any means. The current histomorphologic classifications of PBL according to the degree of plasmacytic differentiation do not have any diagnostic value although plasmacytic features may have prognostic significance and should be evaluated in future studies. Any lymphoproliferative neoplasm that exhibits plasmablastic features with various degrees of plasmacytic differentiation in an extra-medullary location should encourage histopathologists to do a panel of immunohistochemical and

genetic investigations, including viral studies for EBV and HHV-8. This study supported the WHO and other authors that accept HHV-8 to be negative in all cases of PBL. Should a neoplasm with morphological and immunohistochemical features of PBL be HHV-8 positive, a diagnosis of extracavitary PEL should rather be considered. The results of the immunophenotypic investigations performed in this study supported PBL to be negative for the CD20 receptor. Should an extra-medullary neoplasm of this nature be CD20 positive, another diagnosis such as the immunoblastic variant of DLBCL NOS should rather be considered. The role of plasma cell lineage markers such as CD38, CD138 and MUM as well as light chain restriction in the diagnosis of PBL is uncertain at this stage but proposed not to be diagnostic as stand-alone markers or criteria. The results of immunohistochemical investigations with these molecular markers should be interpreted in the context of extensive clinical, pathological and genetic investigations.

From the results of this study and intensive investigation of the literature, I proposed that PBL, as defined by the WHO, should be a single, extramedullary tumour that conforms to all the morphological and immunophenotypic criteria for the diagnosis of PBL as set by the 2008 WHO classification and described above. I proposed that the possibility should be investigated that some of these tumours might actually represent plasmablastic, extramedullary plasmacytomas with aggressive biological nature and poor prognosis. This was underlined by the high percentage of these tumours confirmed to have immunoglobulin light chain restriction. When multiple tumours or disseminated disease with similar criteria in association with multiple radiolytic bone lesions, bone marrow involvement by neoplastic plasmablasts or serological changes are found during the clinical work-up of such a patient, the diagnosis of atypical presentation of MM should rather be considered. The diagnostic criteria of plasma cell neoplasia such as MM should be re-evaluated and validated, especially in the setting of HIV/AIDS. It is well known that HIV/AIDS may influence the clinical presentation, immunophenotype and genetic features of plasma cell neoplasms, pathology commonly associated with immune deficiency.

It became clear that PBL should never be diagnosed without thorough clinical, systemic, pathological and genetic investigations especially in the backdrop of HIV/AIDS. No pathologist should make the diagnosis of PBL and no clinician should accept such a diagnosis or decide on the treatment modality for the patient involved unless all other possibilities of systemic plasma cell disease have been excluded. The role of genetic features in the diagnosis and management of PBL should be investigated further as it might eventually play an important role in the choice of treatment.

Key future research areas that evolved from this study

The true relationship between HIV/AIDS, EBV infection and PBL of the oral cavity should be considered in detailed molecular pathology, genetic and virology studies. It should be investigated if PBL's do not consist of two types of neoplasms, those associated with HIV/AIDS and those not associated with HIV-related immunosuppression, therefore reflecting different pathogenic mechanisms. Further to this, it should be determined if PBL in an HIV-positive patient does not represent an atypical presentation of another plasma cell dyscrasia for which the diagnostic criteria of MM and plasmacytomas in the setting of HIV/AIDS should be revisited.

The spectrum of genetic analysis specific for PBL as well as the FISH probes used to determine the scope of genetic rearrangements should be evaluated. The true role of key genes in the pathogenesis and biology of PBL will be necessary before further comparison with MM and plasmacytomas with plasmablastic morphology will be possible. This should include evaluating rearrangements of chromosome 1, deletions of 13*q* and 17*p* together with possible gains in odd-numbered chromosomes in a large cohort of PBL cases.

Future studies on large cohorts of PBL should evaluate the correlation between the diagnosis of PBL and the clinical state of immunosuppression as measured by the CD4 counts of the patient. From my current study it would also be interesting to see if any correlation exists between the degree of the T-cell infiltrate in the tumour and the CD4 count. The prognostic implications of the CD4 counts at the time of diagnosis should at the same time also be

evaluated through meticulous clinical follow-up of patients diagnosed with this neoplasm. Should there be a correlation between the CD4 count and the degree of T-cell infiltrates in the tumour and eventually between the CD4 count and the prognosis of such a patient, it could serve as a histological prognostic marker for these patients.

The possible prognostic implications of the following microscopic, immunohistochemical and genetic features in PBL should be investigated as part of the prospective clinico-pathologic study of PBL:

- Cellular pleomorphism in the tumour
- The percentage of blasts versus plasmacytic cells in the tumour infiltrate
- Positive versus negative staining for plasma cell markers such as CD38 and CD138
- The ratios of CD79a, CD38, CD138 and MUM1 positivity in the tumour cells
- The presence of specific genetic features such as aberrations of the *MYC, CCND1* and *IGH* genes as well as the influence of complex versus simple karyotypes

The biological nature of PBL as either plasma cell neoplasias with plasmablastic 'dedifferentiation' or as plasmablastic neoplasms with some plasmacytic 'differentiation' should be evaluated through molecular pathology techniques such as single cell laser microdissection and subsequent comparative genetic analysis of the cells in order to determine their relationship with one another.

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