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.

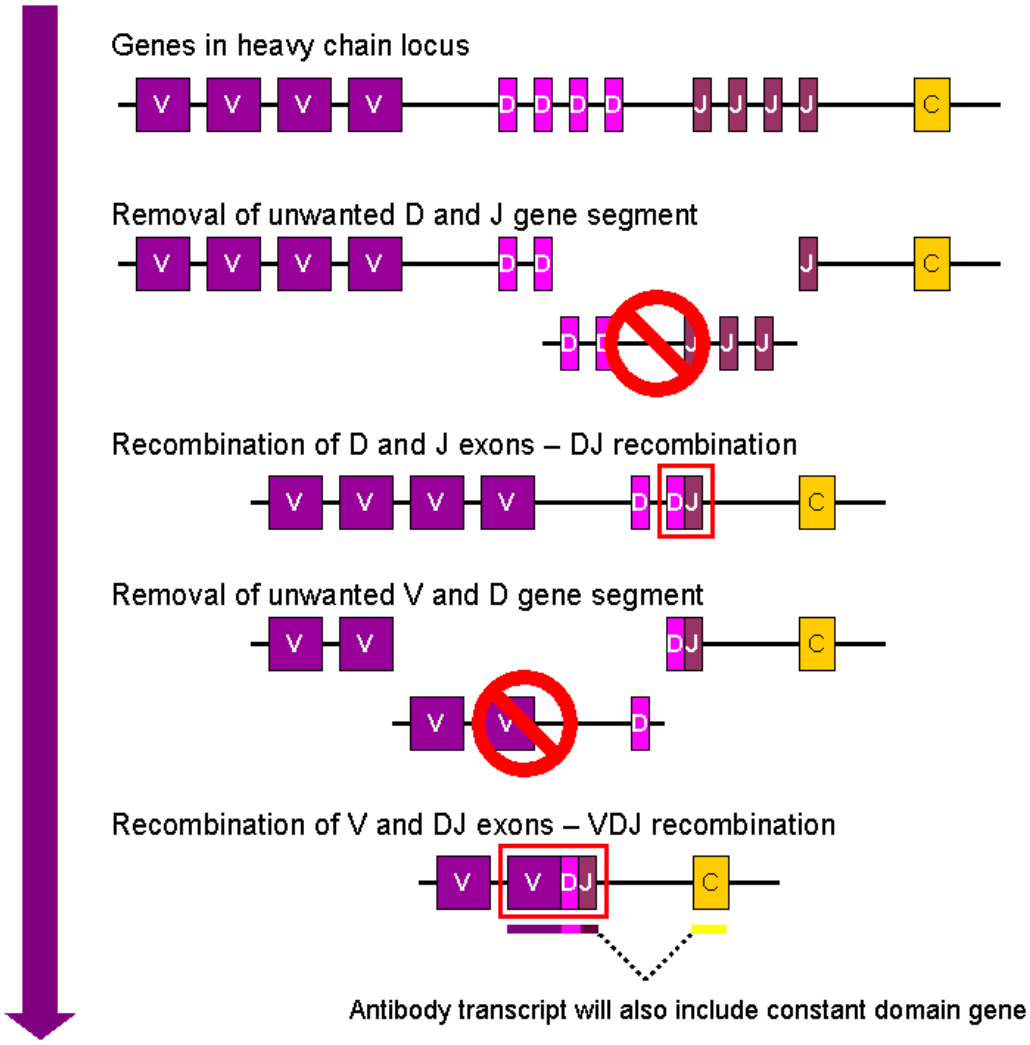
1.4.2 Normal B-cell development

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].