BURKITT LYMPHOMA IN UGANDA

A study of some biological and epidemiological aspects of endemic Burkitt lymphoma

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Kampala, January 2011 Sam Kalungi

List of Articles

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Kalungi S, Wabinga H, Molven A, Bostad L. Lymphomas diagnosed in Uganda during the HIV/AIDS pandemic. *East Afr. Med. J. 2009;86:226-32*

II. Kalungi S, Wabinga H, Bostad L. Reactive lymphadenopathy in Ugandan patients and its relationship to EBV and HIV infection. *APMIS 2009; 11:302-7*

III. Kalungi S, Steine SJ, Wabinga H, Bostad L, Molven A. pRb2/p130 protein expression and *RBL2* mutation analysis in Burkitt lymphoma from Uganda. *BMC Clin. Pathol. 2009; 9:6*

IV. Kalungi S, Wabinga H, Bostad L. The RB (pRb2/p16) and p53 (p14/p53/p21) tumor-suppressor pathways in endemic Burkitt lymphoma. *J Pediatr Hematol Oncol 2011;33:e54–e59*

V. Kalungi S, Wabinga H, Bostad L. Expression of apoptosis associated proteins SURVIVIN, LIVIN and THROMBOSPONDIN-1 in Burkitt lymphoma. *Manuscript*

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
ATLL	Adult T-cell leukemia/lymphoma
BL	Burkitt lymphoma
CDKs	Cyclin-dependent kinases
CDKIs	Cyclin-dependent kinase inhibitors
CNS	Central nervous system
CSR	Class switch recombination
DLBCL	Diffuse large B-cell lymphoma
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
FDC	Follicular dendritic cell
FFPE	Formalin-fixed, paraffin-embedded
FISH	Fluorescent in-situ hybridisation
GC	Germinal centre
HCV	Hepatitis C virus
HHV-8	Human herpes virus type 8
HIV	Human immunodeficiency virus
HTLV-1	Human T-cell lymphotropic virus 1

HL	Hodgkin lymphoma
IARC	International Agency for Research on Cancer
ILSG	International Lymphoma Study Group
ISH	In-situ hybridization
KCR	Kampala Cancer Registry
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpes virus
LMP	Latent membrane protein
LP	Lymphocyte predominant
MALT	Mucosa-associated lymphoid tissue
MZ	Marginal zone
NK	Natural killer
NHL	Non-Hodgkin lymphoma
PAS	Periodic acid schiff
PCR	Polymerase chain reaction
PGL	Persistent generalised lymphadenopathy
RLH	Reactive lymphoid hyperplasia
SLL	Small lymphocytic lymphoma
WHO	World Health Organisation

1. Introduction

1.1 Lymphomas in general

1.1.1 Definition and classification

The lymphomas, a diverse group of neoplastic diseases arising from the lymphoid system, vary from highly proliferative and rapidly fatal neoplasms to indolent malignancies. Scientific progress has increased our understanding of the biology of lymphomas and brought forth advanced technology in molecular genetics and immunology. During the last 30 years lymphoma classification has evolved from a purely morphologic and descriptive system to a complex multidisciplinary approach incorporating morphologic, immunophenotypic and molecular genetic features. The methods necessary for modern lymphoma classification may, however, not be available in some regions of the world, especially in the developing countries.

The two main groups of lymphomas are Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), each of which has various subtypes (Table 1). The majority of HL and NHL arise in lymph nodes (nodal lymphomas) [1, 2]. Lymphomas arising from lymphoid cells and tissue outside lymph nodes are called extra-nodal lymphomas. This distinction can be ambiguous for sites such as blood, bone marrow and spleen [3] The proportion of extra-nodal disease varies geographically with the highest reported incidence in developing countries [1, 4]. The gastrointestinal tract represents the most common site of extra-nodal lymphoma whereas the second most frequent localization is the head and neck [4]. In the latter site tonsils are most commonly affected followed by nasopharynx, oral cavity, salivary glands, paranasal sinuses and base of tongue [5].

Table 1: Examples of lymphoma subtypes

Lymphoma group	Sub group	Types (examples)
NON-HODGKIN LYMPHOMA	B-CELL	B lymphoblastic lymphoma/leukaemia
		Pre-GC: Mantle cell
		GC: BL, DLBCL, Follicular
		Post GC: MZ, MALT, SLL, DLBCL, Plasma cell myeloma, lymphoplasmacytic
	T-CELL	T-lymphoblastic lymphoma/leukaemia
		Peripheral T-cell, Natural killer cell
HODGKIN LYMPHOMA	Classical	Nodular sclerosis, Mixed cellularity, Lymphocyte-rich, Lymphocyte-depleted
	Nodular lymphocyte predominant	

GC, germinal centre; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; MZ, marginal zone; MALT, mucosa-associated lymphoid tissue; SLL, small lymphocytic lymphoma

1.1.2 Lymphoma epidemiology

In many parts of the world, there has been a reported increase in lymphomas attributed to improved cancer registration, greater awareness of the disease, changes in the understanding and classification of lymphoproliferative diseases, more sensitive diagnostic methods and the AIDS epidemic [6, 7]. Particularly in the United States and Western Europe, an increased incidence of NHL and a decline of HL have been registered [8]. In general, in countries where the incidence of extra-nodal lymphomas is high, the incidence of all other lymphomas tends to be high and the incidence of HL lower than that of NHL [3, 9].

HL is relatively rare, has a higher incidence in men than in women and is more common in whites than in blacks in the United States [10]. The age distribution of HL is bimodal with the first peak in the 15-34 year age-group and the second peak in

persons older than 55 years [11]. In Africa, however, the number of children affected is high compared to Europe and North America [12].

NHL is the 11th most common cancer world-wide [13]. It is more frequent in males compared to females, and is more common in the elderly in the Western countries [14]. Table 2 shows the prevalence of lymphomas in different regions of the world. The prevalence is higher in males compared to females and is higher in the more developed regions compared to the less developed regions.

Table 2: NHL 5-year prevalence of lymphomas in males and females in different world regions to the nearest thousand; information sourced from GLOBOCAN [15].

Region	Males (Number of cases)	Females (Number of cases)
World	427000	324000
More developed regions	254000	214000
Less developed regions	173000	109000
East Africa	13000	8600
Northern Africa	6000	3500
Southern Africa	1600	1100
Western Africa	9300	5900
South America	19000	14000
North America	111000	93000
East Asia	54000	32000
Western Europe	45000	40000

There has been reported an overall increase in incidence of NHL world-wide not related to age or sex [16]. The incidence of NHL is highest in North America, Europe and central Africa, and lowest in Eastern and Southern Asia [17]. The incidence is low in adults in Africa [12]. The distribution of histologic subtypes of NHL differs across geographic regions [18] (Table 3). This has been attributed to differences in risk factors

in these regions [14]. In Europe and North America, diffuse large B-cell lymphoma (DLBCL) and follicular lymphomas are the most common subtypes, with follicular lymphoma more prevalent in North America compared to Europe [18, 19]. In South America, BL are more prevalent in the tropical parts and T-cell lymphomas are more frequent in the temperate areas [20, 21].

Table 3: Prevalence of BL and DLBCL in different countries

Country	BL	DLBCL	NHL cases (Total)
Uganda [22]	95 (80%)	19 (16%)	119
Kenya [23]	21 (35%)	11 (18.3%)	60
Nigeria [24]	211 (51.1%)	44 (10.7%)	413
Croatia [25]	4 (3.9%)	33 (32.5%)	120
ILSG [19]	10 (< 1%)	422 (30.6%)	1403
USA [26]	1102	24246	114,548†

ILSG, International Lymphoma Study Group combined data from nine study sites, † Total of all lymphoid neoplasms

In the Asian countries, T-cell lymphomas are more prevalent while in the Middle East and North Africa small intestinal lymphoma is the most common subtype [14, 27].

In Africa, about 25000 new cases of NHL were reported in the sub-Saharan region in 2002 and it ranked 5th in relative frequency among cancers, although the incidence rate is low compared to that of Europe and North America. Most NHL in Africa are of B-cell type and clinical series show predominance of high-grade lymphomas and a low prevalence of nodular neoplasms. In tropical East, Central and West Africa, Burkitt lymphoma (BL) accounts for between one quarter and one half of all paediatric cancers [12]. In Uganda, reports from the Kampala cancer registry (KCR) indicate an annual increase in NHL of 6.7% in males and 11% in females during the period 1991-2006 [28]. BL is the third most common childhood cancer in Uganda and childhood BL accounts for about one third of the NHL cases [22, 29].

1.2 Lymphoma pathogenesis

1.2.1 Etiological and pathogenetic factors

Development of lymphomas is considered to be multifactorial. Several factors such as primary and acquired immunosuppression, infectious agents like EBV, KSHV, HTLV-1, HCV, HIV, *H. pylori*, autoimmune disorders, radiation, occupational exposures, lifestyle factors, diet and genetic alterations (Figure 1) are all likely to contribute to the development of several lymphoma subtypes. [9, 30].

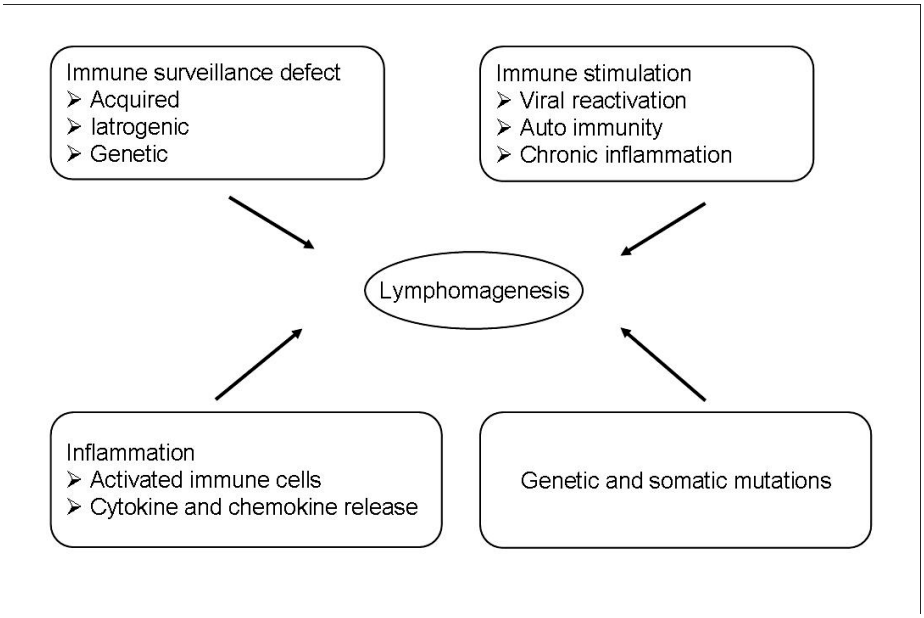

Figure 1: Factors related to lymphomagenesis. Modified from Goldin and Landgren [31].

Epstein-Barr virus (EBV), a member of the herpes virus family, infects over 90% of the adult population. EBV has tropism for B-lymphocytes (and epithelial cells) and maintains latency in memory B-cells. Conditions leading to immunosuppression allow EBV to promote upregulated B-lymphocyte proliferation and transformation mediated by various proteins [32]. EBV is reported to be present in nearly 100% of endemic BL and is associated with B-lymphoproliferative disease in the

immunocompromised host, DLBCL, Hodgkin lymphoma, infectious mononucleosis, tumors derived from T-cells and natural killer (NK) cells, nasopharyngeal carcinoma and stomach cancer [2, 33]. The differences in prevalence, geographical and age distribution of the EBV-associated diseases have been attributed to social-economic conditions, the age at primary EBV infection, EBV strain or cellular tropism [34, 35]. Geographical and age distribution of EBV-related HL has been reported to be similar to that of EBV-related BL [36]. The different EBV-associated diseases may share expression of some latent genes as shown in Table 4. Risk factors for development of lymphoma may be high viral load, defective immune response or chronic infection with progression from a polyclonal to a monoclonal and malignant lymphoid response.

Table 4: EBV latent genes expressed in EBV-associated conditions. Modified from Cohen [37]

Disease	EBNA-1	EBNA-2	EBNA-3	LMP-1	LMP-2	EBER
Burkitt lymphoma	+	-	-	-	-	+
Nasopharyngeal carcinoma, Hodgkin lymphoma	+	-	-	+	+	+
Lymphoproliferative disease, Infectious mononucleosis	+	+	+	+	+	+
Healthy carrier	-/+	-	-	-	+	+

NHL is a well-recognized complication of immunosuppression following organ transplantation, and is the second most common malignancy associated with HIV infection after Kaposi's sarcoma [38]. HIV-related lymphomas are categorized as those also occurring in immunocompetent patients, those occurring specifically in HIV-positive patients and those occurring in other immunodeficiency states [39]. Examples of HIV-associated lymphomas include immunoblastic, BL, CNS lymphoma, and body cavity based lymphomas [40]. The different categories of AIDS-related lymphomas are related to the degree of immunodeficiency of the patient [41]. Human herpes virus type 8 (HHV8/KSHV) which is found in nearly all Kaposi's sarcoma lesions [42] is another infectious agent associated with some lymphomas. KSHV

seropositivity is rare in non-HIV infected patients in most American, Asian and European countries but is common in some Mediterranean and Central African countries with infection rates in Uganda of about 46% [43, 44]. KSHV is associated with HIV-associated body cavity lymphomas and plasmablastic lymphomas [45].

Human T-cell lymphotropic virus type 1 (HTLV-1) is endemic in Japan, the Caribbean, Africa, South America and is transmitted by breast-feeding, through blood and blood products and through sexual intercourse [30]. HTLV-1 is the aetiological agent of adult T-cell leukaemia/lymphoma (ATLL) and the African continent constitutes the largest reservoir of this infectious agent. T-cell lymphomas, however, are rare in the most HTLV-1 endemic regions of Africa like Gabon [46, 47].

Hepatitis C (HCV) infection is involved in a subset of NHL mainly in regions where high prevalence of HCV is found in the general population [48]. The prevalence of chronic HCV infection varies in different continents with the highest prevalence in Africa but HCV infection among HIV-infected pregnant women in Uganda is less than 1% [49, 50]. HCV has been found in NHL in Egypt but not in USA, further emphasizing the regional variation of the association of HCV with NHL [51-53].

Among other carcinogenic agents which have been found to contribute to the development of lymphomas, are bacteria including *H. pylori, B. burgdoriferi, C. trachomatis and C. Jejuni* associated with the pathogenesis of mucosa-associated lymphomas (MALT lymphomas) [54]. There are epidemiological studies suggesting that exposure to herbicides and pesticides are a risk factor for the development of lymphoma [55].

Immune disorders associated with lymphomas include among others; rheumatoid arthritis, Sjögrens syndrome, systemic lupus erythematosus, gliadin allergy, inflammatory bowel disorders and psoriasis [56]. Somatic hypermutations, chronic B-cell stimulation and antigenic drive as well as increased resistance to apoptosis are suggested as possible mechanisms in autoimmune-related lymphomagenesis [57]. Many

B-cell NHL are derived from B-cells whose maturation in passaging through the GC has been blocked, disrupting the different phases of normal B-cell development [58].

1.2.2 Germinal centre formation

Germinal centres (GCs) are formed within follicles of secondary lymphoid organs as the main site of antigen-driven B-cell proliferation [59]. When naïve B-cells encounter antigen, they become activated and may undergo clonal expansion and GC reaction, or clonal expansion and differentiation generating memory B-cells [60]. Follicular dendritic cells (FDC) and T-cells play important roles in GC formation. Maintenance of the GC is associated with intact dendritic cell network and persistence of antigen [61-63].

Mature GCs are divided into dark and light zones. The centroblasts occupy the dark zone and give rise to centrocytes, while the light zone contains predominantly centrocytes and a rich network of follicular dendritic cells [64]. Regulation of the GC reaction involves interaction of activation, proliferation, differentiation and death of B-lymphocytes [65]. Germinal centre termination shows different patterns including progressive transformation, regression and fragmentation, each with distinct morphologic and immunophenotypic features although some overlapping may occur [62]. Many of the B-cells in the GC are clonally related and multiple somatic mutations are present in the cells. Abnormal B-cell differentiation and death in the GC, prolonged survival of the centrocytes coupled with hypermutation predispose to genetic mutations and development of a malignant phenotype [65]. In the immunocompromised individuals, the pool of B-lymphocytes that are the target of translocations expands. The opportunity for spontaneous translocations are increased, some cells are endowed with survival advantages and lymphoma develops [66].

1.2.3 Relationship between the GC and lymphomagenesis

Many B-cell NHL (Figure 2) are derived from B-cells whose maturation in passaging through the GC has been blocked, disrupting the different phases of normal B-cell development [58]. This may be associated with expression of genes not normally expressed in a particular developmental stage of mature B-cells, resulting from single-base changes introduced by somatic hypermutation, class-switch recombinations, and VDJ recombination [67].

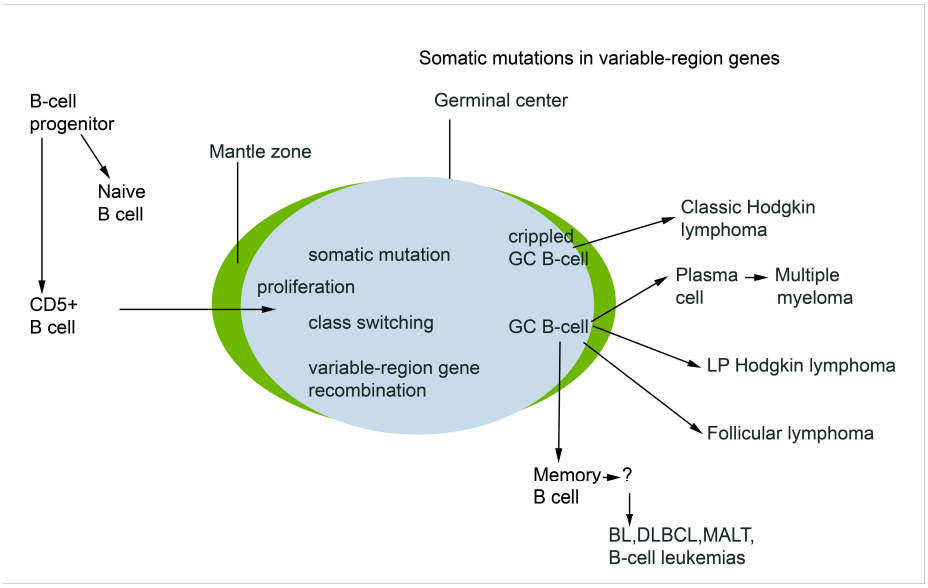

Figure 2: Lymphomas with GC origin. Modified from Kuppers et al [68].

BL is derived from early centroblasts, late GC B-cells or memory B-cells and is known to express GC B-cell signature genes and GC cell markers [69-72]. BL has been reported to arise from follicular lymphoma as well as reactive lymph node hyperplasia [73-75]. Progression from reactive lymphoid hyperplasia (follicular hyperplasia) to high grade lymphoma has been reported and infections like EBV may lead to development of lymphoma in benign lymphadenopathy [76-78]. In patients with

lymphoid malignancies, the transformation from an indolent to a histologic pattern with a more aggressive course is well known [79].

1.2.4 EBV, HIV and lymphoid proliferation

EBV gains entry into the B-cell via interaction of the viral envelope protein gp350/220 with the B-cell surface molecule CD21, adsorption takes place and the virus is internalized [80]. EBV infection of the B-cells causes them to become proliferating B-blasts, the blasts then enter the follicles, expand and form GCs [81]. Latent infection is due to expression of viral proteins including Epstein-Barr virus nuclear antigens (EBNAs), three latent membrane proteins (LMPs) and two Epstein-Barr virus-encoded small RNAs (EBERs) [80]. These viral proteins act as oncogenes and have growth-promoting activity. [82].

The HIV virus primarily infects lymphocytes, and lymph nodes are commonly involved during all stages of the infection. Persistent generalized lymphadenopathy (PGL), is a common finding in many HIV patients. Follicular hyperplasia is the most common finding although different architectural patterns may be seen depending on individual variations and disease progression [83, 84]. Similar to findings concerning other viral infections, HIV particles have been found localized in the GC [85].

1.3 Burkitt lymphoma

The first case of BL in Uganda was probably reported in 1904 by Sir Albert Cook who described it as one of the most malignant forms of cancer [86]. The first definitive description of what is now known as BL, was made by Dennis Burkitt in 1958, who also observed that although the tumors were found in different parts of the body, they were part of a single disease entity [87]. In 1963, it was agreed that this tumor should be given the name Burkitt's tumor as a recognition of Burkitt's work [86].

1.3.1 Description, epidemiology and risk factors

There are three subtypes of BL: endemic subtype found in tropical areas (Figure 3), sporadic subtype in Western countries, and immunodeficiency-associated subtype [88, 89]. Some authors, however, classify BL into classical BL and atypical/Burkitt-like lymphoma [90]. The definition of endemic BL remains controversial especially when defined by geography alone or if occurring in HIV high-prevalence regions [91, 92]. Burkitt lymphoma is the third most common paediatric cancer in Uganda and accounts for about 90% of childhood lymphomas in Uganda [29, 93].

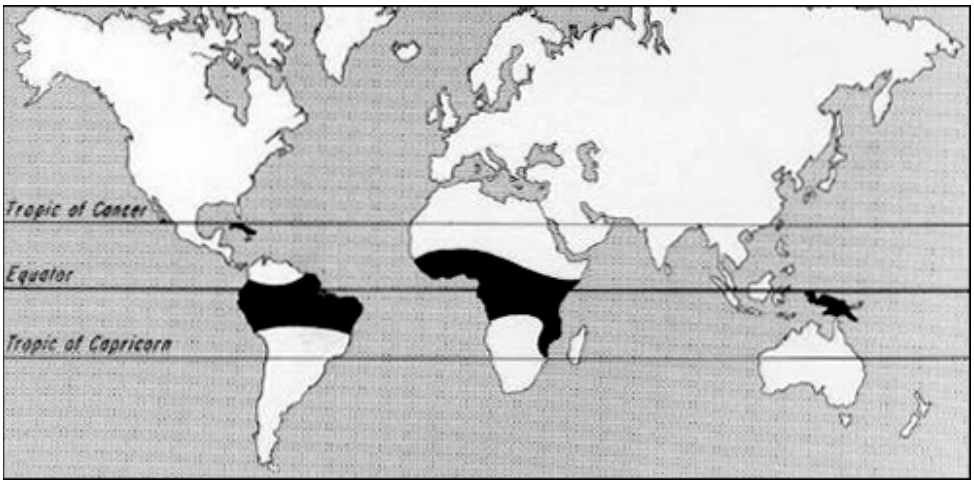

Figure 3: Map showing the distribution of BL in the tropics. From Palmer and Reeder, The Imaging of Tropical Diseases [94]

The etiology of BL has been associated with EBV found in the majority of endemic cases, malaria, humidity, and latitude [95]. Other associated etiologic agents include immunosuppressant therapy, exposure to the plant *Euphorbia tirucalli*, Chikungunya fever and other arbovirus infections, HIV infection and poor socioeconomic conditions [87, 96-98]. Figure 4 shows the interaction between different factors in the etiology of BL.

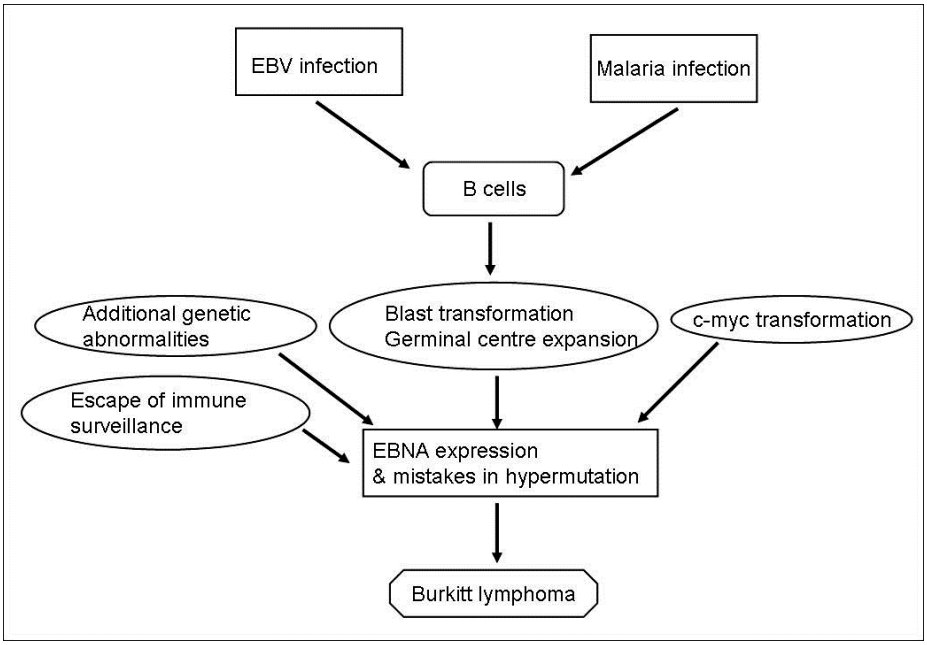

Figure 4: Interaction between different factors in etiology of BL. Modified from Brady et al [99]

1.3.2 Clinical presentation and treatment

Endemic BL typically occurs in children 2-14 years old with a male preponderance. Sporadic and HIV-associated BL are reported in all age groups with the adult sporadic cases generally being more prevalent in males compared to females [88, 98].

Endemic BL shows a characteristic anatomical distribution (Figure 5). Oral lesions may present with loosening of teeth or jaw swelling while tumors originating in the maxilla may present as orbital tumors (Figure 6). Abdominal tumors may present as retroperitoneal masses, ovarian masses, liver enlargement, or gastrointestinal lesions. There may also be involvement of the abdominal lymph nodes, long bones, endocrine system, testis, salivary glands or spinal/intracranial involvement [86]. Some reports indicate that jaw tumors predominate in endemic BL while others report that abdominal tumors either predominate or the percentage of jaw tumors falls after 3 years of age [22, 86, 100, 101].

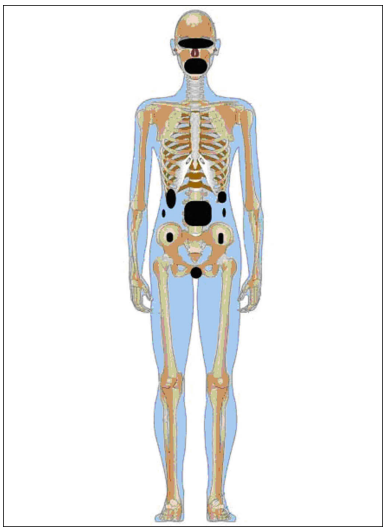

Figure 5: The most common sites of BL presentation are shown. They are: orbit, oral cavity, liver, spleen, intestines, kidneys, ovaries and testes.

Abdominal tumors are the most common initial presentation in non-endemic BL [88]. BL is highly responsive to chemotherapy (Figure 6) with treatment response dependent on disease stage, drug used and the strength of the patient's biological defence against the tumor [86]. Chemotherapeutic agents used include vincristine, cyclophosphamide, doxorubicin, methotrexate, cytarabine, cisplatin, etoposide and ifosfamide [102].

In addition, monoclonal antibodies targeting specific cell surface antigens on malignant hematopoietic cells have also been used for treatment [103]. Rituximab, an anti-CD20 antibody is used in conjunction with chemotherapy, and is also used for treatment of other B-cell lymphomas [104]. The monoclonal antibodies, particularly rituximab may, among other mechanisms, function via induction of apoptosis or cell cycle arrest [105]. An overall cure rate of 90% for Burkitt lymphoma and some other B-cell lymphomas has been achieved in Europe [106]. Chemoresistant or refractory disease is poorly understood but may be due to anti-apoptotic mechanisms in BL pathogenesis or complex chromosomal aberrations [107]. Many children in Africa who develop BL, however, lack access to treatment, leading to a poor outcome [108].

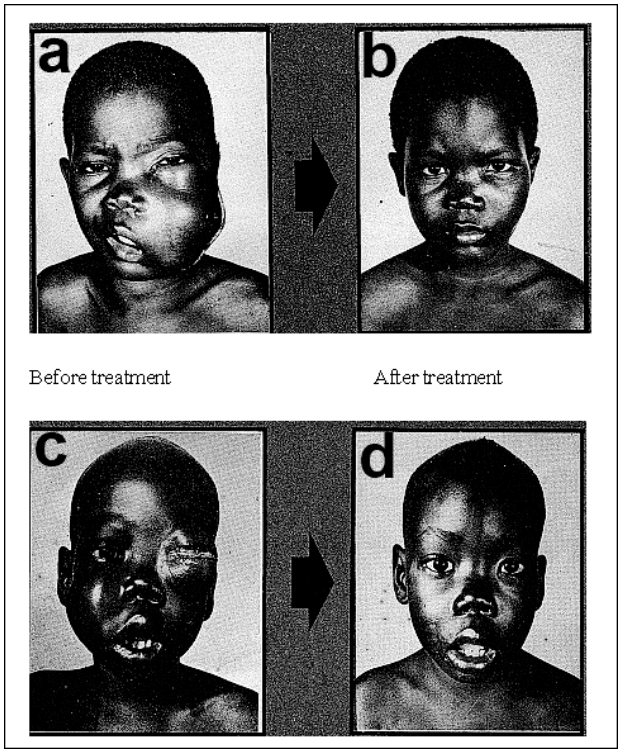

Figure 6: This picture serves to illustrate three typical features of BL: the malignancy frequently occurs in children (a,c), it frequently presents as jaw (a) or orbital tumors (c) and is responsive to chemotherapy (b,d). The pictures are taken from a Uganda Cancer Institute calendar for the year 1976 and had the caption "BURKITT`S LYMPHOMA CAN BE TREATED".

In a cohort of children with BL in Malawi, only 25% of those recruited (n= 44) completed therapy, 15% died and 7% of children treated were confirmed to be disease-free after 2 years [109]. In a Ugandan BL cohort comprising of both HIV-positive and -negative children, HIV seemed to have minimal effect on the treatment outcome following chemotherapy [110]. Extreme poverty, difficulty to understand the disease process or adverse effects of treatment, the role of traditional healers, imprecise physical address, and inability to apply modern diagnostic techniques make both treatment and follow up in Africa difficult [109].

1.3.3 Morphology and markers

BL is one of the most rapidly growing malignant tumors. Histologically, the typical endemic BL is characterized by a diffuse monomorphic infiltrate of small to medium-sized lymphoid cells with sparse basophilic cytoplasm and round nuclei with dispersed chromatin and multiple medium-sized nucleoli. The proliferation rate is extremely high, and is reflected in many mitotic figures [107]. Kinetic studies show a very short doubling time [111]. The typical "starry sky" pattern is due to macrophages phagocytosing nuclear debris from apoptotic tumor cells (Figure 7) [112]. This pattern, however, is not pathognomonic and may be seen in other rapidly dividing tumors with a high cell turnover [113]. Imprint preparations and examination of Romanowsky-stained cells from centrifuged body fluids also aid in diagnosis [86,114]. The majority of BL are negative for PAS and positive for RNA (stained with methyl green-pyronin Y) [86].

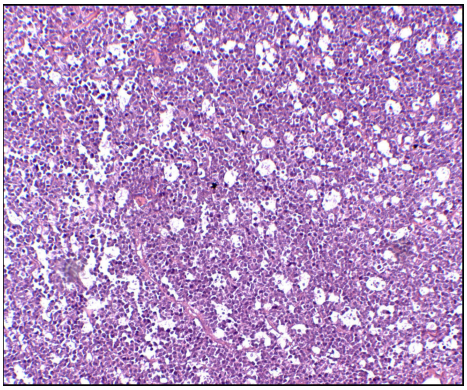

Figure 7: H&E-stained slide showing the characteristic starry sky appearance of BL. Original magnification is 250X.

BL express pan-B-cell and germinal centre markers [40]. The typical tumor cell expresses surface IgM, CD19, CD20, CD22, CD79a, CD10, CD38, CD45, CD43, Bcl-6 but not CD5, CD23, Bcl2 or TdT [40, 89]. There are, however, differences in expression of the different markers within and between the BL subtypes [115, 116].

1.3.4 Genes and molecular mechanisms in BL

DNA mutations that alter the function of normal genes involved in cellular processes such as cell division, apoptosis and differentiation may lead to cancer formation [117]. Thus, the essential hallmarks of neoplastic transformation include growth signal autonomy, evasion of apoptosis, evasion of growth inhibitory signals, angiogenesis, unlimited replicative potential and invasion and metastasis [118]. Alterations in oncogenes, tumor suppressor genes and stability genes are the main genetic events leading to cancer [119]. Genes that have been reported to be somatically mutated in BL include *TP53* (p53), *CDKN2A* (p16), *TP73* and *RB2* (p130) [120].

Genetics of BL

In the majority of BL cases, there is a translocation between the *MYC* gene on chromosome 8 and the *IgH* gene on chromosome 14 [t(8;14)]. This translocation is common to both endemic and sporadic BL although differences in common site of involvement as well as differences in other gene mutations have been reported [121]. In other cases, there is translocation in the kappa light chain on chromosome 2 and the lambda light chain (*IgL*) on chromosome 8 or 22 [t(2;8) or t(8;22)]. The MYC protein is involved in a variety of cellular processes such as cell cycle regulation, apoptosis, cell growth, and cell differentiation [122]. Expression of MYC is positively regulated by factors such as BCL6, epidermal growth factor and platelet-derived growth factor, while it is negatively controlled by Blimp-1 or p21 [123]. MYC is also required for survival and normal differentiation of immature B-cells [122]. MYC abrogates growth arrest caused by p27, p130 and p21 and down-regulates p15, p16 and p21 [124]. *MYC* gene deregulation in BL leads to high levels of MYC protein expression, removal of negative regulatory signals, cooperation with other genetic aberrations such as *TP53* mutations and cooperation with viral genes such as EBV genes [125]. Other lymphoma-associated translocations, such as *IgH*/*BCL2* and translocations involving *BCL6* are not usually detected [89].

Tumor suppressor genes

Tumor suppressor genes code for proteins that play a role in inhibiting growth and tumor formation. They are involved in the control of normal and abnormal cell proliferation and their loss or inactivation is associated with development of malignancy. The processes of cell cycle progression and apoptosis are linked through tumor suppressor genes such as *TP53*, *RB*, *MYC* and *CDKN2A* [126].

The central tumor suppressor gene *TP53* encodes the p53 protein, which integrates signals from different pathways that become activated as a result of DNA damage or oncogene activation, like *MYC* translocation, and triggers responses that lead to cell-cycle arrest, DNA repair, and apoptosis, among others [127]. *TP53* is inactivated by mutations in 50% of human cancers, but also through binding to viral proteins or as a result of alterations in genes whose products interact with p53 [128]. Rb/p105, p107 and pRb2/p130 are members of the retinoblastoma protein family and participate in cell proliferation and differentiation [129]. pRb2 acts as a tumor suppressor in a variety of cell lines [130-132]. Down-regulation of pRb2 is also postulated to lead to the high proliferation rate in high grade NHL [133].

The cell cycle

The cell cycle, a process that ensures that there is complete and accurate replication of the cell before division, is controlled by three main groups of proteins; the cyclin-dependent kinases (CDKs), cyclins and cyclin-dependent kinase inhibitors (CDKIs) [134]. There are two known families of CDKIs, the INK4 family (p16/INK4A, p15/INK4B, p18/INK4C and p19/INK4D) and the CIP/KIP family consisting of p21, p27 and p57 [135]. These proteins are closely linked and play specific roles during specific parts of the cycle (Figure 8) after appropriate stimulation. The active cell cycle is divided into four phases: gap 1 (G1), synthesis (S phase), gap 2 (G2) and mitosis (M phase), whereas a fifth phase (G0) occurs when a viable cell is not involved in the active cell cycle.

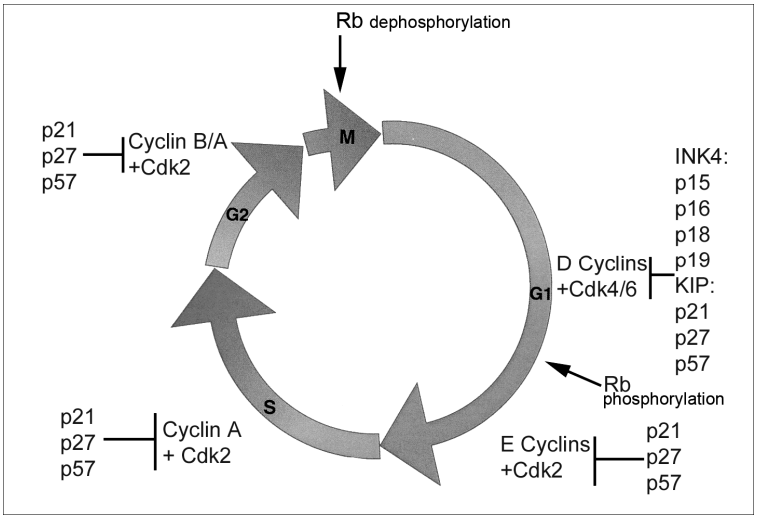

Figure 8: Schematic representation of the cell cycle. Adapted from Slingerland and Pagano [136]

There are checkpoints as the cell progresses from one phase of the cycle to the next and loss of checkpoint control may lead to genomic instability, inappropriate survival of the genetically damaged cells and the evolution of cells to malignancy [137]. There is a "point of no return" during the cell cycle when the cell is committed to proliferation, called the "restriction point". It occurs in the G1 phase whose role is preventing cells from excessive cycling [138]. Oncogenic processes exert their greatest effect by targeting particular regulators of G1 progression and genetic lesions that disable key regulators of G1 phase progression in mammalian cells are present in most human cancers [135, 139]. Mutation of *RB* or genes affecting its function disables the G1 checkpoint and leads to loss of cell cycle control. Inactivation of several cell cycle regulators has been reported in lymphomas, and it has been postulated that the development of BL derives from changes that lead to cell cycle progression [120, 123]. Translocation of *MYC* promotes tumorigenesis by activating CDKs and repressing CDKIs.

Apoptosis

The term apoptosis (or programmed cell death) is used to describe the situation in which a cell actively pursues a course toward death upon receiving certain stimuli.

This process plays a role in the regulation of tissue homeostasis especially in cell systems with a high turnover rate [140]. Deregulation of apoptosis is implicated in pathogenesis of human neoplasms where the balance of apoptosis and proliferation is shifted towards proliferation either by increased mitosis and/or reduced apoptosis [141]. Cell death by apoptosis is exerted by the coordinated action of a number of gene products. The relative importance of p53, MYC, Rb and apoptosis inhibitors (Bcl-2 and its homologs) in the regulation of apoptosis in different human cancers is not fully understood, but p53 and Bcl-2 gene alterations have been found in many lymphoma subtypes [140, 142]. Normal human GC cells also undergo apoptosis and express a number of apoptosis-inducing genes [143]. Aggressive lymphomas are characterized by deregulation of oncogenes or tumor suppressor genes with cell cycle regulatory functions [123]. BL as well as most non-neoplastic GC cells are usually negative for Bcl-2 [144]. The high expression of apoptosis inhibitor proteins in NHL, including in BL, may indicate that resistance to apoptotic stimuli is involved in NHL pathogenesis [145]. The proteins that function in proliferative pathways may sensitize cells to apoptosis and their interplay could determine cell proliferative potential [126].

2. Aims of the study

The pathogenesis of lymphomas is complex, involving interactions between factors such as infection, immunodeficiency and genetic alterations. These factors may be associated with altered cytokine and oncogene expression in the tumor tissue. The advances in immunohistochemical and molecular genetic methods have not been widely applied in the African setting in general, and in Uganda in particular, to characterize the lymphomas and other disorders of lymphoid tissue. Employing these methods would improve the diagnostic quality and could provide information regarding the biology of lymphomas and follicular hyperplasia from Ugandan patients. These ancillary diagnostic methods could also be useful in documenting common viral infections leading to lymphoproliferative conditions in these patients.

The main aim of the study was to examine and characterize endemic BL with special emphasis on the expression profile of cell cycle-associated proteins and to compare the findings with the situation in hyperplastic GC. We wanted to focus on the expression profile of the RB (pRb2/p16) and p53 (p14/p53/p21) tumor suppressor pathways, and to search for tumor-associated *RBL2* mutations that could be involved in deregulated cell cycle control. We also aimed to examine a possible role of some recently described apoptosis-related proteins in BL.

Additional specific aims of the study were to evaluate a possible change in the epidemiology of lymphomas in Uganda in the HIV/AIDS era, as reflected in the diagnostic profile of biopsy-verified lymphomas, and to describe the spectrum of reactive lymphoid hyperplasia (RLH) in Ugandan patients and their possible association with HIV and EBV infection

Our study was accomplished by light-microscopic, immunohistochemical as well as molecular genetic investigations on archival tissue retrieved from the Department of Pathology, Makerere University College of Health Sciences.

3. Materials, methods and methodological considerations

The materials and methods used are outlined in the papers included in the thesis. This section will summarize some aspects of the methods used and their relevance. It will serve to discuss the challenge of conducting retrospective studies using archival material in Uganda.

This study was carried out as part of the research collaboration between the Pathology Department, Makerere University College of Health Sciences (formerly Makerere University Faculty of Medicine), Uganda and the Section for Pathology, The Gade Institute, University of Bergen, Norway. Basic data collection, linked to the histopathology report, and acquisition of paraffin-embedded biopsy blocks were done at Makerere University. Laboratory analysis and writing were carried out at the Gade Institute.

The Department of Pathology, Makerere University College of Health Sciences (MUCHS) is a research and teaching centre for both undergraduate and postgraduate students. The department offers a specialist course in pathology, with the main emphasis on histopathology. It offers diagnostic histopathology services for the main national referral hospital and other hospitals. The majority of Ugandan pathologists are based in this department, which also serves as the main national referral centre. Other services provided besides histopathology and teaching, include cytology and forensic medicine. It was the sole provider of diagnostic histopathology services for the entire country of Uganda up to 1990.

The department houses the KCR, one of the oldest population-based cancer registries in Africa. KCR was established in 1951 with the aim of monitoring the incidence of cancers in the population of Kyadondo county, an area covering the capital city Kampala and surrounding peri-urban areas.

The main national referral hospital that the Department of Pathology serves has a capacity of 1500 beds, and is found within the same campus. The national referral hospital has a lymphoma treatment centre where some histologically diagnosed cases receive chemotherapy.

3.1 Specimen handling

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were retrieved from the archives of the Department of Pathology, MUCHS. The laboratory receives formalin-fixed biopsies from health facilities all over the country, thus, the quality of fixation and handling of the specimens from the time of biopsy to the time the tissue is processed in the laboratory is likely to be very variable. Size and quality of the biopsy, specimen description, clinical information and patient data also vary considerably between the different health facilities.

Preservation of tissue by fixation in formalin solution, followed by dehydration and paraffin wax embedding, is the main method used for preparation of histological sections in Uganda. The concentrations and standard of formalin used, initiation and duration of fixation vary. Buffered formalin is normally not employed. The 10% formal saline provided by the Department of Pathology is sometimes diluted further in the health facilities, and some health facilities use alcohol, normal saline or no fixatives at all. Some health facilities use the commercially available 40% formalin. Manual methods are used in the pathology laboratory where specimens are processed by different technical staff with different levels of training. There are logistical problems faced within the laboratory regarding specimen handling, processing and storage of paraffin blocks. Open, direct flames are sometimes employed in the embedding processes. Haematoxylin and eosin (H&E) is the most commonly used staining procedure, followed by light microscopy examination of the histology tissues. The use of archival tissue has problems like wear and tear due to poor storage, improper fixation, or improper handling prior to or during embedding in

paraffin wax, all of which may have an effect on immunohistochemical and molecular genetics studies [146,147]. The tissues, nevertheless, normally retain antigens that can be unmasked for immunohistochemical studies, and DNA/RNA can be extracted for genetic studies. The efficiency and yield of these methods depends on the quality of the material, and on factors like the heating temperature and the pH value of the retrieval solution used [148, 149]. The blocks used in this study were re-embedded in paraffin wax at the Department of Pathology, Haukeland University Hospital, Bergen, Norway and new 5-µm thick sections were made. These freshly cut tissue sections were re-stained with H&E, followed by a staining by the panel of monoclonal antibodies shown in Table 5.

Table 5 :Antibodies used for BL immunophenotyping

Antibody	Source	Antibody dilution	Incubation time (minutes)
CD45/M0701	DAKO	1:100	30
CD20/M0755	DAKO	1:1000	30
Bcl-6/M7211	DAKO	1:20	30
Bcl-2/M0887	DAKO	1:100	30
Ki-67/M7240	DAKO	1:250	30
CD10/NCL-CD10-270	NOVOCASTRA	1:50	60

TE9, containing 10 mM Tris, pH 9.0 and 1 mM EDTA, was used for 15 min for antigen retrieval

3.2 Clinicopathologic information

The clinical variables extracted from the histology request form included patient age, sex, site of biopsy, and HIV status (for the RLH) when available. In some of the cases, this information was incomplete or not available at all. Information regarding radiological, biochemical and hematological features associated with the tumors was also not specified. The lack of basic health services, imprecise demographic data and

poorly kept medical records all hinder accurate registration in hospital-based registries [150].

Population-based cancer registries provide reliable data both in Europe and Africa [151]. The population-based cancer registry in Uganda covers only one county in the urban and peri-urban areas around the capital, Kampala. Pathology data, which we used, have been found to include more accurate information, but may not have all the required details [152]. The cancer registries have been reported to have more complete demographic data for adults, and higher histological confirmation for childhood tumors [151, 153].It was not possible, however, to retrieve and include factors like tumor size and clinical follow up in our study.

3.3 Tissue microarray (TMA)

The tissue microarray (TMA) technology is now commonly used in tumor-based research. The technique has been validated for immunohistochemical (IHC) analysis in B-cell NHL using a large panel of antibodies [22, 154]. In our study, cores 1 mm in diameter, was taken from the BL donor blocks after having selected those with the typical BL immunoprofile and arranged in a new recipient paraffin block. The TMAs were used for all immunohistochemistry studies on BL having validated the staining on whole sections using the pRb2 monoclonal antibody. A major draw-back faced was non-informative spots caused by tissue loss due to cutting, transfer or antigen retrieval procedures like what has been reported by other authors [155]. The use of TMA has the advantage that all the slides of the study are incubated in one jar, ensuring that concentrations, temperature of all reagents, section thickness and exposure times for all steps are identical for all cases [156]. Other advantages include saving time and reagents during staining, the generation of tissue for multiple studies, and interpretations are based on findings within one small, highly defined area making their reading easier [156].

3.4 Immunohistochemical (IHC) methods

Morphologic features as seen by H&E staining and light microscopy are the main histologic methods used in histopathology diagnosis in Uganda. Immuno-histochemical methods, applicable in diagnosis, prognosis and monoclonal-based therapy in tumors [157] are not available in Uganda. In the diagnosis and classification of lymphomas, combining morphology and immunophenotype play a very important role giving most B-cell lymphomas a characteristic diagnostic profile [2].

Formalin fixation causes inter- and intramolecular protein crosslinkages which lead to masking of antigens, and reduced intensity of the final reaction in the IHC procedure [158]. Unmasking of tissue antigens for demonstration by immunohistochemistry can be achieved by enzymatic, non-enzymatic and heat-based techniques[159]. The effectiveness of IHC staining is affected by the heating conditions, the pH and type of antigen retrieval solution. Thus, individual staining conditions for each antibody must be determined [160]. Heat-induced antigen retrieval, which we used for the majority of antibodies, increases sensitivity and has proved useful in retrieval of antigens not detected when enzymatic digestion is used [160]. We also employed the test battery approach, recommended for use on all new antibodies [161] in order to determine the conditions that would give optimal staining results.

The commonly used detection methods for IHC are the avidin-biotin, streptavidin-biotin and EnVision methods. The streptavidin-biotin method is complex, and have the problem of significant background staining which is increased by heat-induced antigen retrieval [162]. We employed the EnVision method (Dako, Glostrup, Denmark), a two-step method that relies on a dextran backbone to which multiple enzyme molecules are attached. The EnVision method has been found to be faster, with less background staining than other methods, and can be used for detection of a spectrum of antigens [163-165]. In addition to peroxidase for blocking endogenous enzyme activity, we used protein block in some instances to reduce background staining by blocking non-specific binding of antibody. To evaluate the staining, at least five high-power

fields were evaluated and combined to reach an overall percentage of tumor cell staining.

In all cases, fields in different parts of the section were evaluated. Monoclonal antibodies against CD45 and CD20 were scored as positive or negative without any equivocal cases. Antibodies against Bcl-6, Bcl-2 and CD10 were judged positive when > 20% of tumor cells were positive, based on cut-off points suggested by other researchers [166, 167]. Scoring of staining for the other antibodies used in the study are indicated in the papers included in the thesis. There is, however, no uniformly agreed reporting format for immunohistochemistry results in lymphomas. Different cut-off points and scoring methods have been used by various authors in the study of antibody markers in lymphomas [166, 168-170]. There are now initiatives to improve the quality and accuracy of diagnostic slides reporting and to employ standard guidelines in report writing [171, 172].

3.5 Molecular methods

By detecting chromosomal translocations and clonal gene rearrangements, molecular genetic methods are now used as a supplement in the confirmatory diagnosis of lymphoma, and to diagnose occult disease [173]. Although this is a standard practice in many parts in Europe, this is not the case in the majority of African countries. Molecular methods are also useful in studying the biology and origin of lymphomas, in distinguishing different subtypes and in grading [174-176]. They have proven useful in prediction of prognosis in DLBCL [177, 178].

Usually, FFPE tissue is the only material available for molecular analysis in a pathology department. The extraction from fixed tissues of DNA for genetic analysis by the polymerase chain reaction (PCR) is affected by the handling the specimen before tissue fixation, the type of fixative, fixation time, and the storage conditions and duration [179]. There are various methods used for DNA extraction for PCR amplification from FFPE tissues [180]. The method we employed involved placing two

or three 8-µm sections in 190 µl buffer G2 and 10 µl proteinase K solution of the MagAttract DNA mini M48 kit (Qiagen, Hilden, Germany) and dissolving by shaking overnight at 56°C. DNA was then purified by using the kit in combination with a GenoM48 BioRobot system (Qiagen) in accordance with the manufacturers instructions. Screening for mutations was performed by direct sequencing of exons amplified by PCR. FFPE tissues from Uganda have been reported to give sub-optimal results when used for molecular studies [181]. This problem has been encountered by other researchers in our laboratory who observed that DNA extracted from FFPE tissue of Tanzanian melanoma cases resulted in less successful PCR amplification and mutation detection than DNA from Norwegian cases (H. Puntervoll, pers. communication) [182].

4. MAIN FINDINGS

In **Paper I,** we report a decrease in histopathologically diagnosed lymphomas at the peak of the HIV/AIDS in Uganda (1980-1989). Burkitt lymphomas continued to be the most frequent subtype found. Some subtypes which are common in the industrialized countries were not diagnosed.

In **Paper II**, we report on the association between EBV/HIV infection and reactive lymphoid hyperplasia (RLH). We found that RLH in Ugandan patients is frequently associated with EBV and HIV infection. The histologic features of the lymph nodes were not specific for any individual infection, but a high number of EBV-positive cases were associated with hyperplastic germinal centres while follicular fragmentation was characteristic of HIV infection.

In **Paper III,** we describe the expression of pRb2 expression in endemic BL cases from Uganda and report our findings whether tumor-associated, somatic *RBL2* mutations could be involved in deregulated cell cycle control in these cases. We found that the majority of endemic BL cases from Uganda expressed pRb2 but that somatic *RBL2* mutations had to be very rare.

In **Paper IV,** we report our findings that heterogeneous RB (pRb2 or p16) and p53 (p53, p14, or p21) pathway alterations occur frequently in BL. We found close similarities between BL and RLH in expression of the cell cycle regulator proteins examined, except for a much higher and frequent expression of p53 in BL.

In **Paper V**, we report the expression pattern of apoptosis-related proteins TSP-1, survivin and livin in BL, compared to the findings in reactive follicular hyperplasia in Ugandan patients. We found strong expression of these proteins, both in BL and RLH. No statistically significant association between the proteins in either BL or RLH could be proven.

5. DISCUSSION

5.1 METHODOLOGICAL CONSIDERATIONS

Patient records

In Paper I, we used data obtained from the patient records for the years 1980-1989, stored in the files of the Department of Pathology, MUCHS. The data were available in the department archives as recorded on the histology report forms. The quality of this data depends on the accuracy of information given by the patients, the clinician, the pathologist, and the person recording the information. It follows that the registration may suffer from mistakes, shortages and inconsistencies. Nevertheless, data from histopathology forms have been the basis for studies by other scholars, [183] and the ones we used are much the same as those accumulated by the Kampala Cancer Registry (KCR), a population-based cancer registry in Kampala, Uganda [153].

Histopathology laboratory records are useful in providing epidemiological information, but it is advised that such a laboratory should serve a defined hospital or geographical location [184]. Our clinical data are, however, neither from a single hospital nor from a single defined geographical location. There are, however, reports of agreement between the histopathology laboratory-based data and the incidence of cancer in the population, even when the laboratory data were not from a defined population base [184]. Hospital-based cancer registries have the pitfall of registering only those patients who are admitted to hospital, leading to underreporting [150]. Other problems we encountered included lack of a standardized lymphoma classification, miscoding, and coding of similar diagnostic entities in different ways. Despite these limitations, we concluded that the information obtained was sufficient for the purpose of our study and comparable to what is accepted for KCR-based epidemiologic studies[153]. Knowledge of cancer trends in Africa is reported to be low because of the large burden of communicable diseases and is mainly based on case series [185].

We compared the frequency of some major lymphoma subtypes in two periods. The lymphoma classification systems have evolved and been modified over time[186]. These changes have not been adopted yet by some histopathology laboratories. The procedure of analysing histological specimens is much the same today in Uganda as was the case in the 1960ies and 1980ies. Different and inconsistent terminology, however, has been used. In order to attain a relatively uniform classification, a lymphoma "translation system" that integrates different concepts and entities has been described [187]. Where necessary and possible we used this translation system.

An alternative to the employed method would have been complete microscopic review of the biopsies included in our series. This was attempted, but the slides and even many paraffin blocks were of very low quality because of the standard of fixation, preparation and storage and had undergone extensive deterioration. We, however, retrieved and re-embedded a selected number of BL and RLH cases of a quality sufficient for a reliable diagnosis. This yielded the material that was included in our immunohistochemical and molecular studies.

Clinicopathological variables

We obtained the clinicopathological variables by reviewing the information on the histology forms in the archives of the Department of Pathology, MUCHS. As stated above, the accuracy of this information depends on the information provider as well as the person recording it. In many instances, even information about age and gender was lacking. Clinical and follow-up information on site of disease and stage at diagnosis was also not readily available. It was therefore not possible for us to determine the prognostic significance of the markers we investigated. Cancer registries are reported to continue updating their registries even after initial diagnosis for completeness of the information [153], but this is not feasible at MUCHS. Biopsies are received from all over the country, and some investigations required for staging are not available at a number of hospitals or, where available, the patient has to meet the costs. This may also affect availability of detailed clinical parameters.

Use of archival tissue

We compiled typical BL and RLH cases from the files of the Department of Pathology at MUCHS where the surgical biopsy specimens as are stored as FFPE blocks. This provides a large tissue biobank. As the blocks are collected and stored at the time of surgical pathology diagnosis, they may not undergo the rigorous and careful procedures that would be applied to research material. The archival material may have patient information stored as well and in good settings may be used for follow-up studies of patients. Although surgical pathology specimen archives are valuable resources, little is usually done in Africa to optimize specimen storage conditions, quality assurance or quality control [188]. Formalin fixation does not necessarily destroy the antigens and it is possible to use a number of antigen retrieval methods to perform immunohistochemistry on FFPE tissues [159]. The variations in methods and quality of tissue fixation, processing and storage conditions may, however, reduce both antigen immunoreactivity and influence the result of molecular pathologic studies.

Tissue microarray (TMA)

The technique has been validated for immunohistochemical (IHC) analysis in B-cell NHL using a large panel of antibodies [22, 154]. The most important step in TMA construction is the collection of suitable samples. It is recommended that the diagnosis should be revised, fresh sections cut and stained by H&E, and the area of interest selected [155]. We initially revised the diagnosis using whole sections and a panel of antibodies. The blocks fulfilling the criteria for diagnosis of BL were then selected for TMA. After practicing, precision and accuracy were achieved and we used this technique to assemble the 51 BL cases. The slides were then stained with H&E and with the same panel of antibodies that had been used on the whole sections for confirmation of the diagnosis. This showed that the TMA method used was representative.

When staining the TMA with additional antibodies, whole sections were cut separately from ten of the cases included in the TMA block, and they were treated

and evaluated in the same way as the TMA slides. The use of TMA in studies of NHL is, as we also observed in the present work, advantageous because there are almost no cores without tumor cells. These arrays are also reported to be cost-effective in terms of reagents and materials, time for performing the immunohistochemistry and interpretation of the slides [189]. The TMA procedure is time-consuming as far as selection of representative donor blocks and cores is concerned. The major drawback that we faced, however, was loss of cores.

Immunohistochemical methods

Immunohistochemistry is widely used to study expression of specific proteins in human malignancies. In the lymphoid neoplasms, immunohistochemical expression of certain markers is among the criteria for classification [2]. The factors that have an impact on the validity of immunohistochemistry include tissue fixation and processing, unmasking of epitopes, sensitivity of the detection system and antibody quality [146]. Antigen retrieval is therefore important in order to achieve accurate and consistent results. A number of antigen retrieval methods including enzymatic and non-enzymatic methods have been described, but heat is reported to be the most important factor in unmasking antigens [159]. Different unmasking solutions are also available to achieve optimal results. The manufacturers of the various antibodies issue guidelines for suggested retrieval methods although the actual practice may vary from the guidelines. We also employed a test battery approach, recommended for use on all new antibodies [161], in order to determine the conditions that would give optimal staining results. These included testing of different retrieval buffers, with different enzymatic methods or boiling schemes, different antibody dilutions and different incubation periods. In the case of the p16 antibody, we did not need to use any antigen retrieval method.

Antigen retrieval of the archival tissue presented one of the biggest challenges of this study. There is a marked variation in the concentration of formalin used and in the fixation times of the specimens received at the pathology laboratory at MUCHS. Prolonged fixation seemed to be common for samples submitted from up-country

centres since a certain number of specimens have to accumulate before they are transported to the laboratory. Another issue regarding fixation is the unclear concentration of the formalin used. Whereas neutral, buffered formalin is not available, 40% formalin is sometimes used. At other times the pre-prepared 10% formalin solution is further diluted. Moreover, embedding of the tissue into paraffin blocks was until recently done using an open flame. This is expected to have an impact on the antigenicity of the fixated proteins. The tissue storage conditions are also not optimum, with the blocks being stored in warm, dusty rooms. These factors might have contributed to the difficulty that we encountered in antigen retrieval. This challenge was met by determining the appropriate antigen retrieval times for each antibody under consideration as described above, and also by re-embedding the specimens at the Department of Pathology, University of Bergen.

The choice of antibodies used for confirming the diagnosis of BL was based on the WHO classification of lymphomas [25]. The selection of antibody clones for the other markers was guided by accessible literature and relevance for the aims of the study. We then modified our protocols to adjust for differences in immunoreactivity due to the differences in quality of tissues and antigenicity. We applied a system for staining evaluation used by others in order to be able to compare results and because there is no generally accepted uniform method for reporting immunohistochemical staining that can be applied to most markers. Appropriate positive controls were selected from sections made from specimens known to express the antigen of interest, the most commonly used tissue being tonsils. The negative control technique used was omitting the primary antibody and otherwise staining as for the positive controls. This was done during all the rounds of staining.

Molecular methods

Formalin-fixed, paraffin-embedded tissue may be the only material available for molecular analysis in a pathology department. Archival FFPE human tissue collections are, however, reported to be in poor states of storage across the developing world, making nucleic acid extraction from these tissues difficult. The fixation of tissues in formaldehyde leads to cross-linking of tissue components, and fragmentation or destruction of nucleic acids [190]. As discussed above, successful PCR amplification of DNA from FFPE tissue may be difficult and is dependent on a variety of factors [191] which may lead to poor quality of the extracted DNA. It is therefore not strange that we found it challenging to perform the molecular studies, a problem previously encountered by other researches using FFPE tissues from Africa [181, 182]. This problem occurred despite using a kit that has been recommended as well-suited for isolating DNA from FFPE specimens [191]. The differences in conditions of fixation (time, temperature and fixative) as a barrier to extraction of quality DNA have been noted by others as well [190]. Also a group from Uganda has reported difficulty in recovering DNA from FFPE tissue despite using different methods. However, it was noted that detectable DNA after extraction always lead to successful amplification [192].

5.2 DISCUSSION OF MAIN FINDINGS

The lymphomas, a diverse group of neoplastic diseases arising from the lymphoid system, vary from highly proliferative and rapidly fatal neoplasms to indolent malignancies. NHL is the 10th most common cancer in the developing word, its high incidence in sub-Saharan African countries being attributed mainly to Burkitt lymphoma [193]. The incidence of cancer is expected to increase in the developing countries in the years to come [150]. Moreover, the current cancer incidence is most likely under-estimated in Africa. NHL are reported to be among the most common malignant tumors in Africa, but are more frequent in developed compared to developing countries [16, 185, 194]. NHL was reported to be on the increase in the United

States between 1973 and 1989 [1]. The risk of developing lymphomas, especially NHL has been found to be much higher among HIV patients compared to people without HIV/AIDS [195]. One aim of our study was to investigate the prevalence and patterns of lymphoma in the HIV/AIDS pandemic in Uganda **(PAPER I)** when a rise in the frequency of lymphomas could be expected. However, we found that there was a decrease in histopathologically diagnosed lymphomas at the peak of the HIV/AIDS epidemic in the Uganda.

This is in contrast to HIV-associated Kaposi's sarcoma which is reported to have increased significantly among Ugandan patients. The low prevalence of some cancers among HIV/AIDS patients has been attributed to low patient survival [29] which may show geographical differences and be related to the risk of intercurrent infectious diseases, the standards of health care and possibilities for treatment. Another explanation of our findings is underreporting due to shortage of resources available for diagnostic services and shortage of laboratory specialists in developing countries, Uganda inclusive [196]. Health care spending is also still low in sub-Saharan Africa and families rely on informal arrangements for health care. This may lead to the unfortunate situation that many patients, cancer patients inclusive, do not seek formal health care. NHL were the most frequent with BL the most common subtype in our series, similar to findings by other researchers [12, 93]. Very few HIV-infected patients in Uganda are on anti-retroviral therapy [197, 198],and immunosuppression due to HIV infection may account for the low number of HL. Our findings are in agreement with the findings of others who have reported a decrease in HL cases among Ugandan patients [29]

The classification of NHL has been an area of controversy. The use of widely different classifications has caused inconsistency and confusion. In the recent WHO classification of lymphomas, it is required that immunohistochemistry is applied, but this is not yet possible in our laboratory in Makerere. This is also the situation in other regions of Africa [23, 183] and is mainly attributed to the lack of resources. Some types of NHL, including body cavity-based lymphoma and primary brain lymphoma,

may be considered to be AIDS-defining when observed in a patient [41]. However, we did not find reports of either these types or of T-cell lymphomas in our study. This again could be attributed at least partly to limitations in the diagnostic facilities available.

Lymphadenopathy is a common clinical finding in Africa, which may occur in any age group in symptomatic or asymptomatic patients. Infection, autoimmune disorders, benign and malignant lymphoproliferative disorders among others, are the common causes of lymphadenopathy [199]. The most common infections causing lymphadenopathy are tuberculosis (TB), HIV, EBV, and toxoplasmosis [200]. EBV is associated with infectious mononucleosis and subtypes of both NHL and HL. HIV is associated with benign lymphadenopathy, including RLH, in addition to malignancies like NHL and Kaposi's sarcoma (KS). Lymphadenopathy has been reported to be a common finding in HIV-infected children in Uganda, and TB and KS are frequently contributing to the lymphadenopathy [201]. A large proportion of the biopsied enlarged lymph nodes is, however, diagnosed as reactive lymphoid hyperplasia (RLH) not indicative of a specific etiologic agent [202].

51.2% of the lymph node biopsies examined in 2005 at the Department of Pathology, MUCHS, were diagnosed as RLH, a finding comparable to what has been reported elsewhere [203]. The microscopic examination was done, however, without the application of ancillary techniques like immunohistochemistry. In agreement with previous reports [204], we found a predominance of follicular type of RLH (**PAPER II**). Similar to other studies [203, 204], a specific diagnosis could not be made in some of the lymph node biopsies. This may be attributed to our use of a limited panel of antibodies and ancillary techniques in trying to identify the causes. Some causes of lymphadenopathy are also difficult to diagnose when there is limited or no clinical information provided to the pathologist. However, even in cases where extensive investigations are done, it may still not be possible to prove a causative agent [202]. Specific common causes of lymphadenopathy like TB were not included in our

series. We only included the lymph nodes diagnosed as reactive lymphadenitis or non-specific lymphadenitis.

Similar to others [205, 206], we found EBV and HIV positivity as probable important causes of lymphadenopathy. Some of our cases had been serologically diagnosed with HIV, but a clinical diagnosis of EBV was not indicated in any of the patients. Although seropositivity for EBV is high in the Ugandan population [207], a clinical or histologic diagnosis of EBV-associated lymphadenopathy is not commonly made. Dose of the virus in addition to other factors may also play a role in the development and manifestation of clinical disease [208]. A high number of cases are most likely subclinical without obvious signs of infection and there is often lack of access to affordable reliable diagnostic tests [209]. Similar to other authors [210], we found concurrent EBV and HIV infection in 46 (52.2%) of the reactive lymph nodes. Lymphadenopathy, a common finding in HIV patients, presents with varied morphological picture but the most common finding is reported to be florid follicular hyperplasia [85], as was the case in our series (**PAPER II).**

EBV has been associated with the etiology of a number of diseases, Burkitt lymphoma inclusive. It is therefore not surprising that we found expression of EBV proteins in a majority of our BL cases **(PAPER IV).** Lymphomas are believed to arise from certain stages of normal lymphocyte development with oncogenic alterations or loss of responsiveness to external stimuli [60].

Altered expression of the proteins involved in the cell cycle pathway has been associated with the pathogenesis of BL [123]. However, contrary to findings reported by other authors, [211] we could not reveal any *RBL2* mutations in the tissues examined **(PAPER III).** *RBL2* mutations have, however, not been detected in sporadic BL by other researchers [212]. Although previous reports have suggested a reduction in expression of pRb2 with increasing grade of malignancy, [213, 214] we found a high expression level of pRb2 in both BL and RLH **(PAPER III).** Similar to others, [215] we also found that pRb2 expression was nuclear in the majority of cases. The function of pRb2 is reported to depend on correct subcellular localization [216]. In addition to other

mechanisms, viral protein binding [217] may be responsible for the high levels of pRb2 expression. The majority of our BL cases were positive for EBV **(PAPER IV).**

Altered expression of proteins regulating the retinoblastoma (RB-cyclin D1/cdk4/6-p16) and the p53 (p53-p21/WAF1-p14/ARF) pathways are implicated in malignant transformation [218]. We found a high frequency of Rb and p53 pathway alterations in BL **(PAPER IV)**. This is in agreement with others who have found these pathway alterations in lymphoid neoplasms [219-222]. These alterations have been reported to have prognostic implications [220, 221, 223]. Unfortunately, we could not investigate this in our studies because follow-up patient data were not available.

In accordance with BL being GC-derived, BL and RLH showed close similarities in the expression profile of the major cell cycle regulator proteins examined. Our findings, however, indicate that there is a major shift in the cycle from reactive to neoplastic state linked to the p53 protein. The pattern of p53 and p21 immunoreactivity is also reported to predict p53 mutation status, where p53 overexpression and p21 under expression is indicative of a p53 mutation [224]. This would indicate that the proportion of BL in our series with p53 mutations is not very different from that without mutations. This contradicts previous reports that p53 mutations are very common in BL [225]. Also contrary to previous studies [220], we found that BL and RLH are heterogeneous in the expression of the proteins belonging to the Rb and p53 pathways. We also included a higher number of BL specimens than used in the previous studies. The majority of our cases were also positive for EBV, whose role can not be neglected. Notably, there are no reported significant differences in p53 mutations in EBV-positive versus EBV-negative BL cases [226].

Deregulation of apoptosis is implicated in pathogenesis of human neoplasms where the balance of apoptosis and proliferation is shifted towards proliferation either by increased mitosis and/or reduced apoptosis [141]. Deregulated apoptosis has been reported in a number of lymphoproliferative disorders, and reported to be of prognostic significance [140]. Reduced apoptosis increases cell survival, and is achieved through the family of apoptosis inhibitor proteins. We found a high rate of

expression and close similarities in the expression profile of the inhibitor proteins examined in both BL and RLH **(PAPER V)** despite the high rate of apoptosis in both.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we have examined the diagnostic profile of lymphomas in Uganda in the HIV/AIDS era, and studied the expression profile of some cell cycle- and apoptosis-associated proteins in BL and RLH. The following conclusions may be drawn:

1. There has been a decrease in histopathologically diagnosed lymphomas in Uganda in the period 1980-1989. Burkitt lymphoma continued to be the most common subtype. Some subtypes, like follicular lymphomas, T-cell lymphomas and body cavity based lymphomas, were not reported at the peak of the HIV/AIDS era **(PAPER I).**

2. RLH in Ugandan patients was frequently associated with EBV and HIV infection. The histologic features of the lymph nodes were not specific for any individual infection. A high number of EBV-positive cases were associated with hyperplastic germinal centres. Follicular fragmentation was characteristic of HIV infection **(PAPER II).**

3. The majority of endemic BL from Uganda expressed pRb2, but somatic *RBL2* mutations affecting the protein's nuclear localization signal appeared to be very rare **(PAPER III).**

4. Heterogeneous RB (pRb2 or p16) and p53 (p53, p14, or p16) pathway alterations occurred frequently in BL. Close similarities were found between BL and RLH. A major difference, however, was found in the expression of p53, which was significantly more frequent in BL. **(PAPER IV).**

5. There was a strong expression of the apoptosis inhibitor proteins SURVIVIN and LIVIN and the pro-apoptotic protein TSP-1 in BL and RLH. **(PAPER V).**

Several forms of BL have been described. Some variants are difficult to discriminate from other subtypes of high-grade NHL [227]. Endemic BL is the most common subtype in Africa [98]. Its clinical and morphologic characteristics have made the diagnosis of this variant of BL rather straightforward and consistent.

We observed that BL is still the most common subtype of NHL diagnosed in Uganda. The tumor continues to occur in the young age group as was first described by Dennis Burkitt more than 50 years ago [86]. Surprisingly the incidence of biopsy-verified BL has not increased in Uganda during the HIV era. It would be of interest to reveal the reason why. Is it just a matter of underreporting or are there biological reasons for our findings? Similarly, there is also a need to explore further why some lymphoma subtypes, especially T-cell lymphomas, CNS lymphomas and the HIV-associated body cavity lymphomas, are not reported in the Ugandan population.

In western countries, follicular lymphoma is the most common NHL subtype. This is in sharp contrast to our findings in Uganda. The low frequency found can hardly be explained by the lack of ancillary diagnostic methods as most follicular lymphomas are easily diagnosed by conventional light microscopy if the biopsy material is adequate. One reason for the difference found may therefore be underreporting. Another possibility is the difference in life expectancy. Follicular lymphomas occur predominantly in adults after the age of 40, and are seen only occasionally in children.

We also found that a large fraction of reactive lymphadenopathy in Ugandan patients is associated with EBV and HIV infection, both of which have been implicated in the causation of BL [89]. Because HIV prevalence in Uganda is still high, and lymphadenopathy is a common finding even where HIV/AIDS is not clinically suspected, we recommend that pathologists should be particularly aware of HIV-associated lymphadenopathy in their practice.

Except for the high frequency of p53 positivity found in BL, we could not find any statistically significant difference between BL and RLH in the expression profile of

most of the cell cycle- and apoptosis-associated proteins examined. However, the expression profile of the proteins varied. Thus, our findings open up for future projects to examine whether this divergence has prognostic impact in BL similarly to what has been reported for other subtypes of NHL [221]. Extended studies are also needed to determine whether there is an association between the protein expression profile and the different variants of BL (endemic, sporadic, HIV-associated) or BL-related patient characteristics (age, gender, site of tumor, stage, survival etc.)

Progress in clinical management of lymphomas have demanded increasingly precise lymphoma classification and subtyping. In Uganda lymphoma diagnosis is still based solely on morphology. There is a strong demand for competence building in hematopathology. The procedures and methods concerning specimen handling, fixation, processing and storing have to be improved, and immunohistochemical and molecular genetic techniques need to be implemented. Moreover, the clinicians should be encouraged to provide complete patient information as this could be of vital importance for both a proper diagnosis, adequate treatment of the patient and clinicopathological studies.

The incidence of BL is high in Uganda compared to other countries. This offers great opportunities for both clinicians and pathologists to gain extraordinary high competence in this field of pathology and oncology. Further studies and competence building may lead to advances in diagnostics and therapy, improving the quality of life and prognosis for the individual patient.

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