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ETIOLOGICAL RISK FACTORS AND CLINICAL CHARACTERISTICS OF CHILDHOOD NON-HODGKIN LYMPHOMA IN UGANDA

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Etiological Risk Factors and Clinical Characteristics of Childhood Non-Hodgkin Lymphoma in Uganda
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Then the Lord said "Shall I hide from Abraham what I am about to do?" Genesis 18:17 (NIV)

In honour of Mr. Stanley Orem, who firmly believed that friendship *(larem)* is the sure way to guarantee a future for the next generation.

ABSTRACT

Introduction: Incidence of non-Hodgkin lymphoma (NHL) has increased greatly over time, especially in children. Improved diagnostic methods alone cannot explain this increase, especially the increase observed in sub-Saharan Africa, where diagnostic capabilities are low.

Objectives and aims: The objectives of this study were to better understand known risk factors for NHL, such as Epstein-Barr virus (EBV), and their impact on disease characteristics. The specific aims were: I. to understand the background role of EBV, II. to elucidate the basis for and strength of the diagnosis of childhood NHL in Uganda, III. to highlight trends in characteristics of childhood NHL, and IV. to examine the impact of human immunodeficiency virus (HIV) infection.

Subject and Method: Aims I and II were studied in **Papers I and II** using samples and data from a case-control study carried out at the Mulago National Referral Hospital between 2004 and 2008. This study enrolled children with suspected tumours or masses referred to the Departments of Paediatrics, Paediatric surgery, Orthopaedics and to the Uganda Cancer Institute. In **Paper I**, EBV viral load was measured in saliva, whole blood, and white blood cells by real-time PCR, serological values for IgG-VCA, EBNA1, and EAd-IgG were measured and compared in NHL and chronic inflammatory conditions (CIC). Comparisons were also done by NHL subtypes (Burkitt lymphoma, BL and other NHL). Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated. In **Paper II** children were diagnosed with suspected NHL based on initial clinical examination; tissue samples were then taken and examined in Uganda and sent thereafter to a pathology laboratory in The Netherlands for re-examination and additional tests. Agreement between diagnoses assigned in Uganda and The Netherlands were compared using kappa statistics.

For aims III and IV a review of routine clinical records of paediatric BL patients seen at the Uganda Cancer Institute was done and reported in **Papers III and IV**. Information on demographic characteristics (age and sex), clinical features (symptoms, signs, disease site, and stage), treatment response and vital status information were obtained. In **Paper III** the frequency distribution of the clinical characteristics, treatment, and outcome of childhood BL over 20 years were summarised by means and standard deviations (SD), or proportions; differences were tested by the 2 test, t-test, z-test or analysis of variance (ANOVA) procedures. In **Paper IV** descriptive statistics of frequencies, means and SD were done using Student's t-test and Chi-square test statistic and ORs, CIs and P-values were obtained. Survival analysis was performed using the Kaplan-Meier method.

Results: In **Paper I** the most common clinical presentations were fever, night sweats and weight loss. EBV viral load in blood was elevated in BL vs other NHL (OR 6.67, 95% CI 1.32-33.69; P-value=0.04) and a significant difference in EAd-IgG was observed in NHL vs CIC (OR 0.19, 95% CI 0.07-0.51; P-value=0.001). In **Paper II**, the agreement between clinical and pathological diagnoses of NHL in Uganda was 91% (95% CI 84-95; kappa 0.84; P-value=0.001). The agreement between clinical diagnoses in Uganda and pathological diagnoses in The Netherlands was 49% (95% CI 40-59; kappa 0.04; P-value=0.612). The agreement between all pathological diagnoses assigned in Uganda and The Netherlands was

36% (95% CI 28-46; kappa 0.11; P-value=0.046). In **Paper III**, facial tumour (n=945, 77.65%) and abdominal disease (n=842, 69.19%) were the most common presentations. Significant presentation with advanced-stage disease (hepatic mass, malignant pleocytosis) was noted (P-value <0.01). Mortality was higher in older children, children with advanced-stage BL, and HIV-positive children. In **Paper IV** HIV-positive children presented significantly more often with disease in the lymph nodes (67%), liver (51%), and chest (10%). Response to chemotherapy was similar in HIV-positive and HIV-negative children although survival was poorer in HIV-positive children (median survival of 11.79 months, 95% CI 8.65-14.92; P-value<0.000).

Conclusion: This study provides additional understanding of the role of EBV in childhood NHL, shown by the significant association between virological and serological markers and common general features, suggesting a common factor. We noted a weak basis for diagnosis of childhood NHL in Uganda with a high probability of error. The presenting features of childhood NHL have not changed with time, although more children present late, especially those with HIV. Improvements in the cancer care system in Uganda should include better diagnostic and treatment services for children as a basis for better understanding of disease and high-quality research.

LIST OF SCIENTIFIC PAPERS

This thesis comprises the published papers listed below and will be referred to throughout by their Roman numbers I-IV:

I. **Orem J**, Sandin S, Mbidde E, Mangen FW, Middeldorp J, Weiderpass E. Epstein-Barr virus viral load and serology in childhood non-Hodgkin lymphoma and chronic inflammatory conditions in Uganda: implications for disease risk and characteristics. J Med Virol 2 Jun 2014. doi: 10.1002/jmv.23988.

II. **Orem J**, Sandin S, Weibull CE, Odida M, Wabinga H, Mbidde E, Wabwire-Mangen F, Meijer CJLM, Middeldorp JM, Weiderpass E. Agreement between diagnoses of childhood lymphoma assigned in Uganda and by an international reference laboratory. Clin Epidemiol 2012;4:339-347.

III. **Orem J**, Mulumba Y, Algeri S, Bellocco R, Wabwire Mangen F, Katongole Mbidde E, Weiderpass E. Clinical characteristics, treatment and outcome of childhood Burkitt's lymphoma at the Uganda Cancer Institute. Trans R Soc Trop Med Hyg 2011;105:717-726.

IV. **Orem J**, Maganda A, Katongole Mbidde E, Weiderpass E. Clinical characteristics and outcome of children with Burkitt lymphoma in Uganda according to HIV infection. Pediatr Blood Cancer 2009;52:455-458.

Additional related background publications not included in the thesis:

V. **Orem J**, Mbidde E, Weiderpass E. Current investigations and treatment of Burkitt's lymphoma in. Africa Tropical Doctor 2008;38:7–11.

VI. **Orem J**, Mbidde E, Lambert B, de Sanjose S, Weiderpass E. Burkitt's lymphoma in Africa, a review of the epidemiology and etiology African. Health Sciences 2007;7(3):166-175.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
BL	Burkitt lymphoma
CD	Clusters of differentiation
CI	Confidence interval
CIC	Chronic inflammatory conditions
CSO	Civil society organization
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
HIV	Human immunodeficiency virus
HR	Hazard ratio
LCL	Large cell lymphoma
LMPs	Latent membrane proteins
MALT	Mucosa-associated lymphoid tissue
MTS	Multi-tumour suppressor
NGO	Non-Governmental Organisation
NK	Natural killer cell
OR	Odds ratio
SD	Standard deviation
SIDA	Swedish International Development Cooperation Agency
TCR	Terminal C repeat
UNEPI	Uganda National Expanded Programme of Immunization
UNHRO	Uganda National Health Research Organisation
WHO	World Health Organisation

1 BACKGROUND

The worldwide incidence of non-Hodgkin lymphoma (NHL) has increased greatly over time (1, 2). Although a plateau has been noted in adults, there is still an upward trend in childhood NHL (2, 3). There is currently no clear explanation for this increase in childhood NHL. Improvement in diagnostic methods has been advanced as a possible explanation (1, 4), but as diagnostic capacity has not improved significantly in sub-Saharan Africa this is not a plausible explanation for the increase (Figure 1.) in this region (5, 6). Possible risk factors for childhood NHL include infectious agents, especially with viruses such as Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), or other immune disorders or environmental factors (7, 8). The study of the risk factors for and characteristics of childhood NHL are of interest in developing countries in sub-Saharan Africa such as Uganda, as a better understanding of these factors could provide opportunities for prevention and control of the disease, the benefits of which could go beyond childhood NHL to other malignancies, such as Hodgkin lymphoma and chronic inflammatory conditions (CIC), which share similar environmental, and possibly mechanistic, pathways (9).
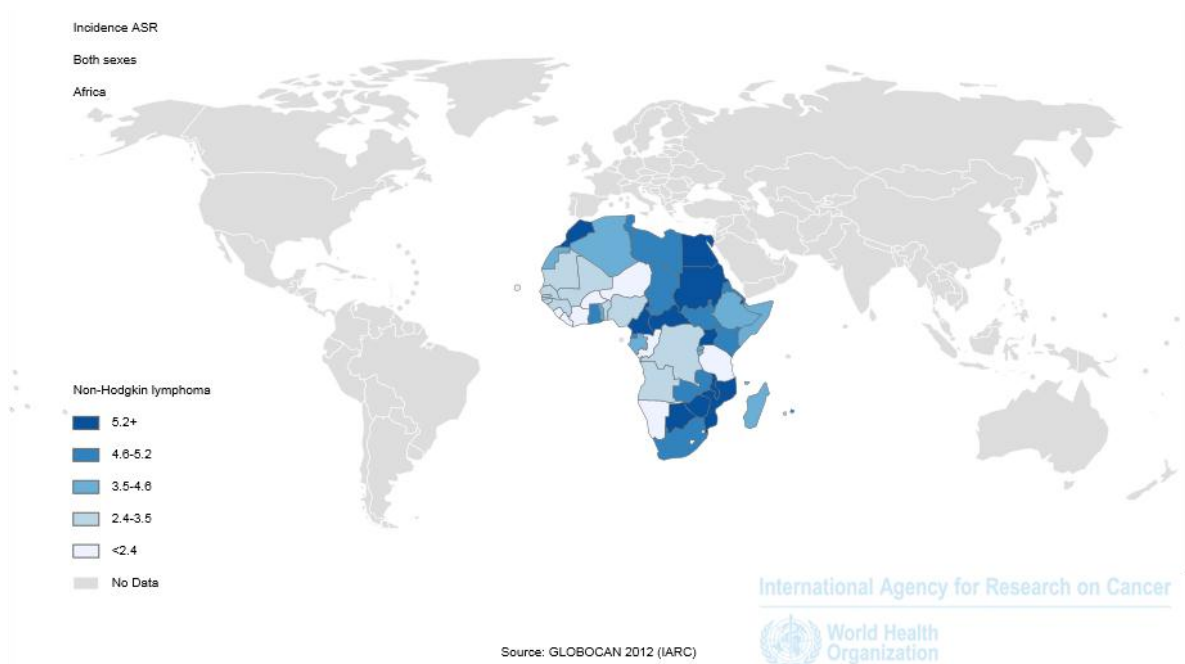
Figure 1. Incidence of non-Hodgkin Lymphoma in Africa (source:Globocan 2012, IARC)

1.1 CHILDHOOD NON-HODGKIN LYMPHOMA

NHLs are malignancies of lymphocytes and their progenitor cells, which usually develop in lymph nodes or other lymphoid tissues (10, 11). They are traditionally classified into Hodgkin lymphoma and NHL. NHL is a heterogeneous group; the World Health Organisation (WHO) classification of lymphoid malignancies specifies over 80 subtypes of NHL. NHL constitutes slightly less than 10% of cancers in children and adolescents (12), and it has one of the best outcomes of all childhood malignancies (14). Indeed, cure rates for NHL have risen to around 70%-90% (12), due in part to modern treatment based on histological diagnosis (13).

1.2 EPIDEMIOLOGY, AETIOLOGY, CELLULAR AND CLINICAL CHARACTERISTICS OF CHILDHOOD NON-HODGKIN LYMPHOMA

The worldwide incidence of NHL has greatly increased in the last few decades, especially childhood NHL (1). Lymphoma (Hodgkin lymphoma and NHL) is the third most common childhood malignancy (13), and the increasing incidence of childhood NHL has lent added importance to the investigation of its aetiology, including factors such as genetics, immune disorders, infectious agents and environmental exposures (14). Elucidating these factors may help identify new causes, as well as children with a higher risk of developing the disease. It may also enable earlier detection and treatment of NHL, and thus lead to better outcomes.

The factors responsible for the increase in NHL incidence may differ between developed and developing countries, especially when considering the acquired immune deficiency syndrome (AIDS) epidemic (15-17). In the developed world NHL accounts for approximately 7% of all childhood malignancies, but there are no precise figures available in Africa due to a lack of population-based registries (18, 19). NHL is the most frequently diagnosed malignancy in children with AIDS, and often occurs before the age of 4 years, especially following vertical transmission (19, 20). In some countries this has led to mandatory HIV screening in children who are diagnosed with NHL before the age of 3 years.

There are marked differences between childhood NHL and NHL in adults with respect to cause, subtype, staging, treatment possibilities and outcome (21). Childhood NHL is more often caused by infectious agents and more often presents as high-grade tumours with disseminated disease, whereas NHL in adults commonly presents as low- or intermediate-grade tumours (21). It has been hypothesised that early exposure to causative infectious agents could be responsible for the differences in median age at presentation. Interaction between factors associated with NHL, such as EBV, malaria, and HIV could also be contributing to the rise in childhood NHL and NHL in adults.

A study of trends of cancer incidence in Uganda from 1960-1997 reported an increase in the incidence of NHL, including Burkitt and Burkitt-like lymphoma (BL) (5). Incidence rates in adults in 1960-1994 were constant at 3.9/1000, but increased to 7.4/1000 in 1995-1997 (5). The standardised incidence rate for BL and other NHL in children in 1960-1971 was 9.6/1000, but increased to 34.3/1000 in 1991-1997 (5). This change could not be attributed to

improved coding or diagnostic capacity, and no direct causative factors could be found to explain this trend.

1.3 AETIOLOGY

The cause of NHL is mostly unknown, but several factors have been associated with lymphomagenesis. These include genetics, immune disorders, infectious agents and environmental exposures. Genetic immune disorders have been consistently associated with lymphomagenesis and include severe combined immunodeficiency, hypogammaglobulinaemia, common variable immunodeficiency, Wiskott-Aldrich syndrome, and ataxia-telangiectasia. NHLs that occur in individuals with these disorders are often associated with EBV infection (22). Along the same lines, a number of acquired immune disorders are also associated with an increased risk of NHL, notably AIDS, solid organ transplantation, and a variety of autoimmune disorders (23, 24).

1.4 CELLULAR AND CLINICAL DESCRIPTION OF CHILDHOOD NON-HODGKIN LYMPHOMA

The WHO classification of childhood NHL is based on phenotype (i.e., B-cell lineage, T-cell lineage, or natural killer [NK]-cell lineage) and differentiation (i.e., precursor vs mature) (25). Subtypes of childhood NHL are mature B-cell lymphoma (including BL and diffuse large B-cell lymphoma), lymphoblastic lymphoma (primarily precursor T-cell lymphoma and, less frequently, precursor B-cell lymphoma), and anaplastic large cell lymphoma (LCL) (mature T-cell or null-cell lymphomas) (26). Some subtypes of NHL that are usually seen in adults are also, though rarely, diagnosed in children, including peripheral T-cell lymphoma, T/NK-cell lymphomas, cutaneous lymphomas, and indolent B-cell lymphomas (e.g., follicular lymphoma) (27).

1.5 MATURE B-CELL LYMPHOMA

Mature B-cell lymphoma comprises BL and diffuse large B-cell lymphoma. The malignant cells in mature B-cell lymphoma are of B-cell phenotype (28). The malignant cells express surface immunoglobulin, especially immunoglobulin M of either kappa or lambda light chains. Other markers of B-cell phenotype such as clusters of differentiation (CD) 20 and CD22 are also present, especially in BL/leukaemia, in which the common acute lymphoblastic leukaemia antigen (CD10) is always expressed. The chromosomal translocation t(8;14) is commonly expressed in patients with BL/leukaemia, whereas t(8;22) and t(2;8) are rarely expressed. These chromosomal translocations juxtapose the cell proliferation oncogene *c-MYC* (29) and immunoglobulin locus regulatory elements, leading to inappropriate expression of *c-MYC*. morphologically, these manifest in the form of uniform, small, non-cleaved cells. The presence of *c-MYC* rearrangement is the gold standard for BL diagnosis. However in rare situations where cytogenetic information is not available, morphology can be used. A proliferation index (Ki-67^{+}) of ≥99% can complement a morphological diagnosis of BL in the absence of cytogenetic information (30).

1.5.1 Burkitt and Burkitt-like lymphoma

There are several forms of BL, which are mainly defined by geographic distribution and risk factors (31). Endemic BL is the disease originally described by Burkitt and largely found in Africa; it characteristically affects the facial skeleton of children between the ages of 2 and 9 years. Sporadic BL is the form that was described outside Africa. It is morphologically similar to endemic BL and affects mainly the abdominal viscera; it can be detected at any age and no specific cofactors have been described. The third form of BL is associated with HIV infection (HIV-associated BL). Although HIV-associated BL has been well described in the developed world and among HIV-positive adults in Africa, childhood HIV-associated BL has not been well characterised. The unifying characteristics of all patients with BL include a unique morphology and chromosomal translocations involving the proliferation of *c-MYC*, which is present in BL irrespective of geographic location or immune status (32).

1.5.2 Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma is of germinal centre phenotype. It comprises 10%-20% of childhood NHL and is mainly seen in young children (33). Its presentation is similar to BL, i.e., it is mainly localised and involves bone marrow, the central nervous system, or mediastinal disease (primary mediastinal B-cell lymphoma). However, unlike other subtypes primary mediastinal B-cell lymphoma has an inferior outcome (34).

1.6 LYMPHOBLASTIC LYMPHOMA

Lymphoblastic lymphoma comprises 20% of childhood NHL. It is usually positive for TdT; more than 75% of lymphoblastic lymphoma is of T-cell immunophenotype and 25% is of B-cell precursor phenotype (35). Lymphoblastic lymphoma does not demonstrate chromosomal translocation, but 75% of cases present with an anterior mediastinal mass, which presents clinically as an airway obstruction (dyspnea, wheezing, stridor, dysphagia), as well as possible swelling of the head and neck (36). Pleural effusions, involvement of lymph nodes, widespread dissemination to bone, skin and bone marrow, and involvement of the central nervous system are less common in lymphoblastic lymphoma than in BL (30).

1.7 ANAPLASTIC LARGE CELL LYMPHOMA

The third common subtype of NHL is anaplastic LCL which accounts for approximately 10% of childhood NHL (37, 38). While this subtype is predominantly of mature T-cell immunophenotype, null-cell lymphoma (i.e., no T-cell, B-cell, or NK-cell surface antigen expression) can also occur (40). The WHO classifies anaplastic LCL as a peripheral T-cell lymphoma characteristically expressing CD30 with chromosomal translocation of the *ALK* gene (39).

Anaplastic LCL has a broad range of presentations, including involvement of lymph nodes and a variety of extranodal sites, such as skin, bone and, less often, the abdominal viscera.

Anaplastic LCL is commonly associated with systemic symptoms (e.g., fever and weight loss) (26).

1.8   RARE CHILDHOOD NON-HODGKIN LYMPHOMA

Low- and intermediate-grade mature B-cell lymphomas, such as small lymphocytic lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, mantle cell lymphoma, myeloma, or follicular cell lymphoma are rarely seen in children (40). In the most recent WHO classification, paediatric follicular lymphoma and paediatric nodal marginal zone lymphoma were classified as specific disease subtypes (41).

Table 1. Clinical and pathological classification of childhood NHL

WHO classification	Immunophenotype	Clinical presentation	Chromosome translocation	Genes affected
Burkitt and Burkitt-like lymphoma	Mature B-cell	Intra-abdominal (sporadic), jaw (endemic) head and neck (non-jaw) (sporadic)	t(8;14)(q24;q32),t(2;8)(p11;q24), t(8;22)(q24; q11)	c-MYC, IGH, IGK, IGL, Rb2/p130
Diffuse large B-cell lymphoma	Mature B-cell	Nodal, abdomen, bone, primary central nervous system, mediastinal	Not well characterised in children	
Lymphoblastic lymphoma	T-cell or pre B-cell	Mediastinal bone marrow skin, bone	MTS1/p16ink4a deletion TAL1 t(1;14)(p34; q11), t(11;14)(p13;q11)	TAL1, HOX11
Anaplastic large-cell lymphoma	T-cell or null-cell	Variable, but systemic symptoms often prominent	t(2;5)(p23: q35)	ALK, NMP

Adapted from National Cancer Institute (http://www.cancer.gov/).

2 PATHOGENESIS OF NON-HODGKIN LYMPHOMA

2.1 GENERAL BACKGROUND ON PATHOGENESIS

The transformation of a normal cell into a neoplastic cell involves a series of genetic events that interfere with normal cell growth and differentiation. There are two groups of genes (proto-oncogenes and tumour suppressor genes), that are intimately involved in the pathogenesis of malignancies (42). Proto-oncogenes are involved in the stimulation of normal cell growth and in stopping differentiation, while tumour suppressor genes promote differentiation and limit cell proliferation. If this delicate balance is disrupted, either by activation of proto-oncogenes or loss of tumour suppressor genes, it results in unrestrained cell proliferitt, which is a common characteristic of malignant cells (42). Proto-oncogenes can be activated by genetic rearrangement (usually chromosomal translocation) or amplification, which leads to an overexpression of the oncogene protein product. The second mechanism is genetic translocation, or somatic point mutation, both of which alter the oncogene coding sequence, resulting in a novel protein with new biological properties. The mechanism of oncogene activation in childhood NHL usually involves chromosomal translocations that correlate with cell phenotype. Inactivation of tumour suppressor genes has yet to be identified as a frequent event in lymphomagenesis, although it is thought that the loss of the multi-tumour suppressor (*MTS 1*) gene may be important in the pathogenesis of certain lymphoblastic lymphomas (42).

2.2 GENETIC AND MOLECULAR BASIS OF PATHOGENESIS IN NON-HODGKIN LYMPHOMA

In both common and uncommon subtypes of NHL, alterations occur in uncommitted hematopoietic progenitors (11). There are three models of lymphomagenesis: 1) a normal lymphocyte acquires the full complement of alterations at a single stage of maturation that corresponds to the NHL phenotype; 2) one or more initiating events occur during early lymphoid maturation and the remaining alterations develop at a stage that corresponds to the NHL phenotype; 3) the initial alterations occur within hematopoietic stem cells, which then acquire further alterations at multiple stages of differentiation (11). The genetic events associated with NHL generally result in oncogene activation, and it is important to determine which factors predispose individuals to these genetic events if we are to understand lymphomagenesis in children and determine appropriate treatment and prevention strategies (11).

2.3 BURKITT LYMPHOMA AND MATURE B-CELL LYMPHOMAS

BL is characterised by the chromosomal translocation t(8;14)(q24;q32), and is less commonly associated with t(2;8)(pll;q24) and t(8;22)(q24;q11) (43). *c-MYC* is located on chromosome 8q24; the juxtaposition of *c-MYC* next to the highly active Ig genes in B precursor cells leads to the deregulation and overexpression of the c-Myc protein, a major event in the pathogenesis of BL. Molecular analysis of the chromosomal translocations associated with BL demonstrates distinct patterns of rearrangement depending on the clinical form of the

disease. The molecular breakpoints in *c-MYC* differ between the endemic and sporadic forms of BL. In endemic BL, the breakpoints are generally 5' to *c-MYC* exon 1, and the 3' border of the first exon is frequently mutated, obliterating a normal transcriptional control point (44). In sporadic BL, the *c-MYC* breakpoint is usually within exon 1 or intron 1. Therefore, the proteins coding exons 2 and 3 are disassociated from the regulatory exon 1, and cryptic promoters within intron 1 lead to unrestrained expression. In either case, the coding region remains intact while the regulatory region of exon 1 is either lost or disrupted by somatic mutation. These mechanisms lead to overproduction of the functionally active c-Myc protein. More recent data suggest that point mutations within the protein coding domains may result in a c-Myc protein with greater functional activity. *c-MYC* deregulation may also reflect the proximity to transcriptional enhancers within the translocated Ig gene. The breakpoints within the Ig heavy chain locus also differ in endemic and sporadic BL. In endemic BL, the breakpoints occur within the joining segments of IgH, reflecting a translocation that occurred during primary rearrangement of the antibody locus. In contrast, in sporadic BL most of the breaks occur in the switch region, suggesting that the chromosomal translocation occurred while the locus was undergoing an isotype switch (e.g., from IgM to IgG). Therefore, the B precursor cells where these translocations occur probably differ in endemic and sporadic BL (44).

2.4 LYMPHOBLASTIC LYMPHOMAS

The great majority of lymphoblastic lymphomas are of T-cell lineage (45). These lymphomas share a similar morphology and immunophenotype, and they show genetic alterations similar to those in T-cell leukaemia. This suggests that lymphoblastic lymphomas and T-cell leukaemia may be part of a spectrum of a single disease. Molecular studies have shown that T-cell translocations generally place a proto-oncogene, usually a transcription factor, under the control of the terminal C repeat (TCR) regulatory domains. The result is aberrant expression of the proto-oncogene which is similar to the overexpression of *c-MYC* in mature B-cell lymphoma (46).

The gene most commonly implicated in the pathogenesis of T-cell leukaemia-lymphoma is the *TALl* gene (42, 47). Alterations of the *TALl* gene occur in up to 30% of T-cell leukaemia-lymphoma, either by chromosomal translocation (3%) or by a specific 90 kb deletion of a regulatory region (26%) that is not detected by conventional cytogenetic techniques. The t(l:14) and t(l:7) juxtapose the *TALl* gene next to highly active TCR genes, and the regulatory region deletion places the *TALl* gene next to the promoter region of the T-cell active *SIL* gene (48).

2.5 LARGE CELL LYMPHOMA

In general LCL is of B- or T-cell lineage. B-cell LCL has a centroblastic or immunoblastic histology and develops in older patients (38). T-cell LCL is derived from mature or peripheral T-cells and is termed peripheral T-cell lymphoma. Most childhood peripheral T-cell

lymphomas express CD30, and are classified as anaplastic LCL. In adults however, most LCL is of B-cell phenotype and is associated with t(14;18) chromosomal translocation (26).

2.6 THE ROLE OF EPSTEIN-BARR VIRUS IN LYMPHOMAGENESIS

EBV is a lymphotropic gamma human herpes virus that is widespread among humans, and was the first virus to be associated with a human tumour (49). EBV transmission takes place early in life and in humans it is associated with contact through saliva(Figures 2 and 3). In Uganda, children with higher baseline titres of EBV antigens are at a higher risk of developing BL, and high EBV antibody levels were detected many years before BL diagnosis (50). The capacity of EBV to stimulate B-cell proliferation (Figure 2), inducing the malignant phenotype, strongly supports the suggestion that EBV plays an aetiological role in BL (51). In 1997, the International Agency for Research on Cancer concluded that there was sufficient evidence to classify EBV as carcinogenic in the causation of BL (52). EBV is known to transform resting B-cells into latently-infected lymphoblastoid cells (53). Constitutive expression of the latency state is evidenced by the presence of latent proteins consisting of six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and -LP) and three latent membrane proteins (LMPs1, 2A and 2B) (54, 55). The expression of the non-coding polyadenylated small EBV-encoded RNAs EBER1 and EBER2, called latency III, are observed in immunoblastic lymphomas. The expansion of the B-cell pool as a result of EBV infection is regulated by the immune system (56, 57).

EBV-induced cell proliferation is normally controlled by the host T-cell response. However, if this cellular immune response is blunted or altered as a consequence of a congenital or acquired immune disorder, B-cell proliferation may go unchecked (58, 59). Excessive proliferation could lead to an increased risk of recombinational events that result in chromosomal translocations associated with malignant phenotypes (60). These effects may allow further EBV-induced oligoclonal B-cell proliferation, eventually resulting in monoclonal proliferation (61). The manner in which EBV may facilitate chromosomal aberrations is unknown; indeed the direct involvement of EBV has not yet been proven. However, the mechanisms by which EBV-infected B-cells elude immune surveillance are slowly being elucidated (62). Programmed cell death (apoptosis) is part of an organism's protective response to viral infection. Many viruses, including EBV, harbour genes that encode proteins that prevent or delay apoptosis (63).
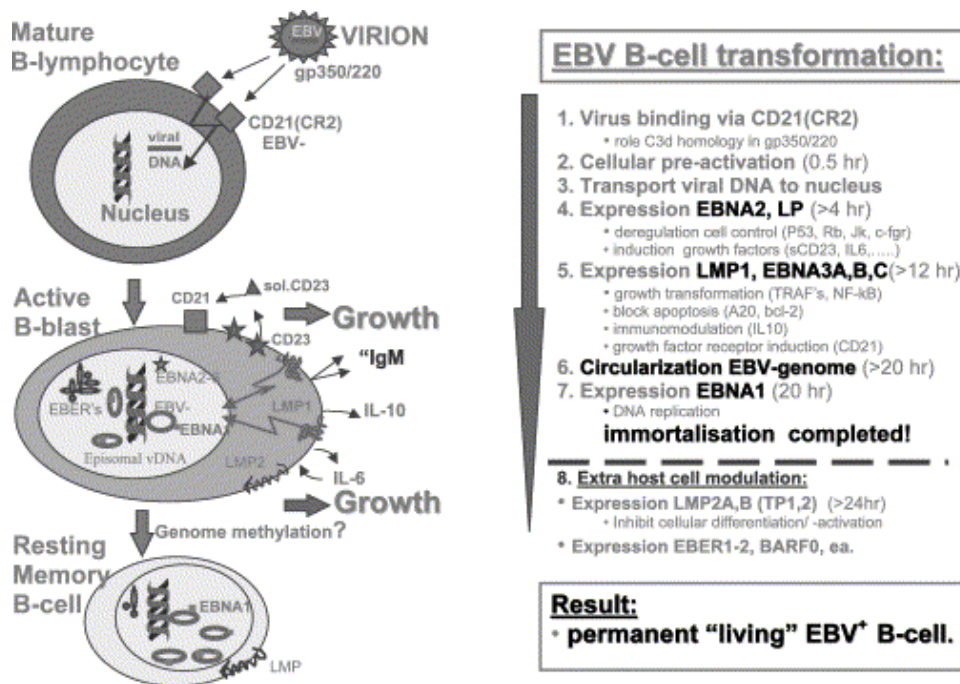
Figure 2. Role of EBV B-cell transformation and possible lymphomagenesis.

Reproduced from (51) with permission

2.6.1 The role of malaria and Epstein-Barr virus in endemic Burkitt lymphoma

The geographic differences observed in the incidence of BL may be related to the age of the individual at the time of EBV infection, or to the influence of other endemic factors, such as malaria (64). Indeed, the distribution of endemic BL coincides with the "malaria belt" in Africa (65, 66). Malaria stimulates B-cell proliferation and induces T-cell suppression (66). This makes the expanded B-cell pool prone to chromosomal translocation that brings *c-MYC* under the governance of an immunoglobulin gene t(8;14), which induces unbridled cell proliferation (55). EBV DNA is found in a high percentage of patients with endemic BL (95%), but only a minority of patients with sporadic BL (15%). EBV is a very common virus and is not limited to regions of endemic BL.

2.7 THE ROLE OF EBV IN IMMUNODEFICIENCY-RELATED LYMPHOPROLIFERATIVE DISEASE AND LYMPHOMA

The incidence of lymphoproliferative disease and NHL is 100-fold higher in immunodeficient children than in the general population (67). The cause of such immune disorders may be genetic, secondary to HIV infection or iatrogenic following transplantation (solid organ transplantation or allogeneic hematopoietic stem cell transplantation) (68, 69). NHL observed in individuals with primary immune disorders usually shows a mature B-cell phenotype and large cell histology (70), though mature T-cell leukaemia-lymphoma and anaplastic LCL have also been observed (71, 72). Children with primary immunodeficiency and NHL are more likely to present with advanced disease and with symptoms related to extranodal disease (73). HIV-associated NHL is usually aggressive, with most cases

occurring in extralymphatic sites, and can be broadly grouped into three subcategories: systemic (nodal and extranodal), primary NHL of the central nervous system, and body cavity-based NHL, also referred to as primary effusion lymphoma (74). Approximately 80% of all NHL in HIV patients is considered to be systemic. Primary effusion lymphoma, a unique lymphomatous effusion associated with human herpes virus 8 and Kaposi sarcoma herpes virus, is primarily observed in HIV-positive adults, but has also been reported in HIV-positive children. Post-transplant lymphoproliferative disorder represents a spectrum of clinically and morphologically heterogeneous lymphoid proliferations. Essentially all post-transplant lymphoproliferative disorders following hematopoietic stem cell transplantation are associated with EBV, but EBV-negative post-transplant lymphoproliferative disorder can also be seen following solid organ transplant (75).

2.8 THE ROLE OF EPSTEIN-BARR VIRUS IN CHILDHOOD CHRONIC INFLAMMATORY CONDITIONS

The persistence of infectious agents or microorganisms is the result of an inability to eliminate microbes, mainly due to ineffective host immunity (76). Disease processes often start as acute infections before assuming a persistent state (77). The role of viral persistence in the causation of CICs, which are often referred to as idiopathic conditions, is being more and more recognised and has been linked to EBV (78). Some conditions thought to be associated with EBV include rheumatoid arthritis, systemic erythematous lupus, multiple sclerosis, and chronic fatigue syndrome (79, 80). Other conditions of connective tissue vascular systems have been noted, as have disorders of the lymphoid system such as haemophagocytic lymphohistiocytosis and lymphoproliferative diseases. Cutaneous manifestations such as *hydroa vacciniforme* are also common (80). There is likely a wide spectrum of CICs associated with EBV infection. The pathogenesis of CICs starts with viral access through the epithelial barrier instigated by microbial toxins, environmental insults, or other predisposing factors that lead to a loss of epithelial integrity. Activation of resident inflammatory cells or other endogenous ligands may then follow. Macrophage activation and establishment of chronic inflammation of underlying tissues may be sequelae (81). The mechanisms that lead to the diversification of the spectrum of CICs observed have yet to be unravelled. EBV infection may fit into this mechanism through chronic evolution, viral persistence, modification of the host immune response through antibodies, and inhibition of apoptosis (82).

3 DIAGNOSIS OF CHILDHOOD NON-HODGKIN LYMPHOMA

Although the most common subtypes of childhood NHL are relatively limited to BL, lymphoblastic lymphoma and LCL, correct diagnosis and classification of these subtypes are essential (83, 84). Careful handling of pathological specimens, along with collection of proper materials for ancillary studies such as immunophenotyping, cytogenetics, or molecular studies, aids pathologists in reaching a correct diagnosis (85). The specific morphological, immunophenotypic, and genetic features of these common subtypes of childhood NHL are

well known. When NHL is a consideration within the differential diagnosis, one must consider all aspects of the clinical spectrum, such as age, comorbidities, nodal location, symptomology, and growth kinetics (86). Appropriate tissue sampling is vital for correct diagnosis and may require the examination of a sizable amount of tissue.

Although cytology by fine needle aspiration is often the first-line approach for diagnosis when NHL is suspected, the rush to employ this method should be tempered by the need for a clear histological diagnosis (87). Expert cytopathology may be available in some centres, but the addition of a core biopsy can increase diagnostic accuracy considerably (87, 88). The advantages of taking a core biopsy include the availability of rapid results, low morbidity, and low cost, in addition to providing more information than cytology. Histology minimises the potential for misdiagnosis and incorrect treatment, and this applies especially to children. Comprehensive diagnostic evaluation and classification of NHL is essential not only for treatment initiation, but also to allow for the identification of biologically distinct subtypes of NHL that require different treatment (85).

3.1 DIAGNOSTIC CAPACITY IN AFRICA

BL best exemplifies the need for accurate NHL diagnosis in Africa. This tumour has the fastest growth rate of any other currently known, with a doubling time of 24 hours (the hallmark of an aggressive disease) (89, 90). This calls for an accurate and reliable diagnostic process. Despite the typical clinical features exhibited by the disease in Africa, in order to be accurate, a diagnosis of NHL must be based on histology (91). The methods commonly employed to obtain tissue for diagnostic purposes include excisional biopsy and cytological methods such as touch preparation, fine needle aspiration, and cytocentrifuge of body cavity fluids (92, 93).

Each of these procedures has its advantages and disadvantages depending on the circumstances, which are often dictated by the clinical setting. One advantage of an excisional biopsy is that it provides a sizeable tissue sample for histology, and allows for advanced tests such as immunohistostaining (94). It also allows for the storage of pathological material, which may be useful for a later review. However, the disadvantage is that excisional biopsy requires surgery, which may take time to organise and requires skilled personnel that may not be available. Furthermore, for an excisional biopsy to be feasible patients must present early and in very good general condition. Cytological investigations, on the other hand, can render a diagnosis using body cavity fluids or aspirate samples from an accessible tumour mass.

The accuracy of fine needle aspiration in African BL has a high degree of correlation with excision biopsy, and many centres are currently using it for diagnosis and to determine appropriate treatment (95). In patients with a rapidly progressing disease, it is useful to obtain a cytological sample by fine needle aspiration for confirmatory diagnosis (96). This may be useful in HIV-associated BL, which can present as a systemic disease (97). However, in patients presenting with early-stage disease, limited disease, or with fewer complications and

a stable general condition, an excision biopsy should be advised if the medical expertise is available (98). The tissue obtained from an excision biopsy should be used for a touch preparation to guide treatment initiation pending the histological diagnosis.

The use of fine needle aspiration or other cytological methods is recommended to confirm histological findings before assigning a final diagnosis, but histological diagnosis remains the gold standard. The goal should be to improve the accuracy of NHL diagnosis while at the same time improving access to advanced tests such as immunohistochemistry, flow cytometry and molecular methods (99).

3.1.1 Staging investigations

Bone marrow aspirate and biopsy should always be performed early in the workup of patients with NHL (100). Involvement of bone marrow may lead to confusion as to whether the patient has NHL or leukaemia. Traditionally, patients with more than 25% marrow blasts are classified as having mature B-cell leukaemia, and those with fewer than 25% marrow blasts are classified as having NHL. Radiographic imaging is essential in the staging of patients with NHL (101). In developing countries, ultrasound is the preferred method for staging an abdominal mass, but computed tomography scan and, more recently, magnetic resonance imaging are often used for staging masses at other sites. Radio nucleotide bone scans should only be considered for patients with suspected bone involvement (102). The most widely used staging scheme for childhood NHL is the St. Jude Children's Research Hospital Staging System for Childhood NHL (103, 104).

Table 2. The St. Jude Staging System for Childhood NHL

Stage	Description
I	A single tumour (extranodal) or single anatomic area (nodal), excluding mediastinum or abdomen
II	A single tumour (extranodal) with regional node involvement On same side of diaphragm: Two or more nodal areas Two single (extranodal) tumours ± regional node involvement A primary gastrointestinal tract tumour (usually ileocecal) with or without associated mesenteric node involvement, grossly completely resected
III	On both sides of the diaphragm: Two single tumours (extranodal) Two or more nodal areas All primary intrathoracic tumours (mediastinal, pleural, thymic) All extensive primary intraabdominal disease; unresectable All primary paraspinal or epidural tumours regardless of other sites
IV	Any of the above with the initial central nervous system or bone marrow involvement (<25%)

Adapted from (104).

3.1.2 Treatment approach

Children with NHL should be referred to an institution with experience in the treatment of childhood tumours and that possesses a multidisciplinary team of paediatric oncologists, as this expertise represents the best way to achieve optimal survival (105, 106). Childhood NHL should generally be considered widely disseminated from the outset, even when it is apparently localised; as a result, combination chemotherapy is recommended for most patients (107). Patients with stage I and II disease have an excellent prognosis, regardless of histology (36, 108, 109).

4 PARADIGM FOR PREVENTION AND CONTROL OF INFECTION-RELATED CANCER

The high rate of infection-related cancers, such as EBV-related cancers in sub-Saharan Africa, makes prevention and control of these infections a priority (110, 111). The populations with the greatest need must be identified through risk stratification. This may encourage national cancer programmes to consider interventions for groups at high risk for cancers like BL, which is highly associated with an infectious cause (112, 113).

The early onset of EBV infection in children in developing countries warrants a better understanding of the mechanism of acquisition of EBV (Figure 3), and of the spread of EBV infection within individuals and in the community (114). This can provide opportunity for harnessing the knowledge of spread of infection in the prevention and control of related cancers within the structure of a well-planned national cancer control strategy (Figure 4).
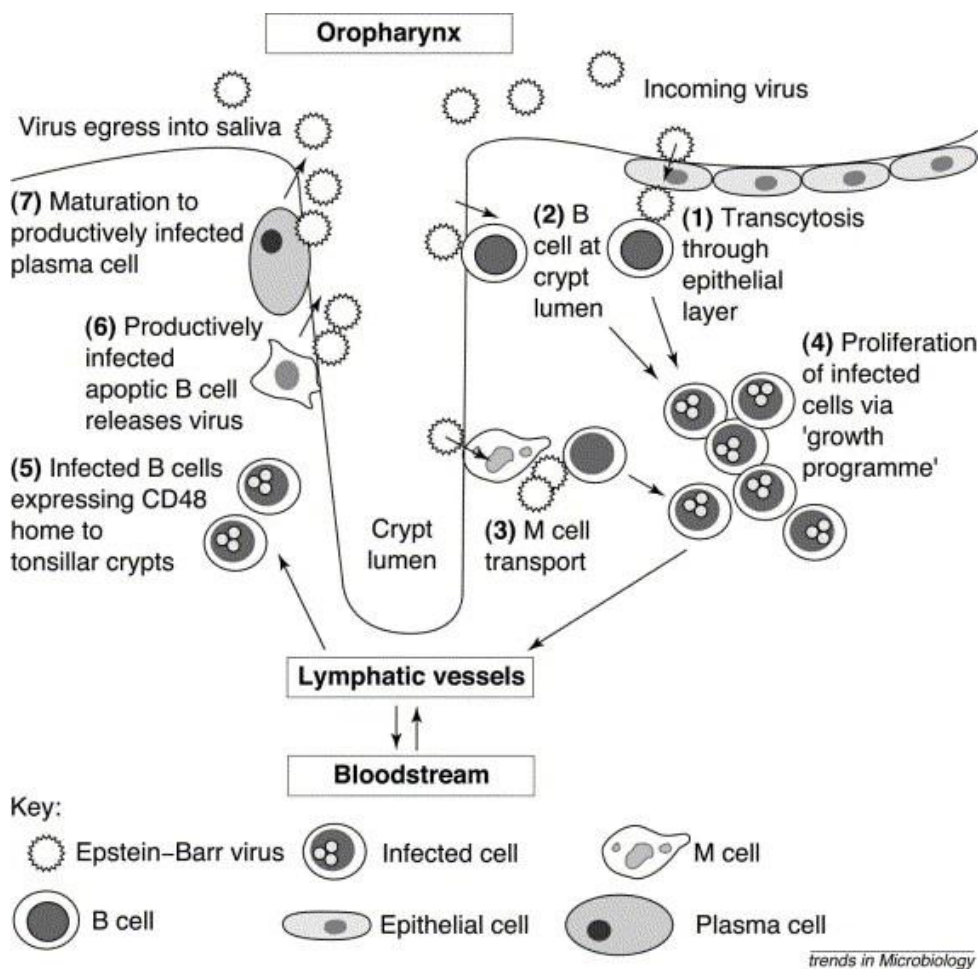
Figure 3. Mechanisms by which EBV may gain access to, and egress from, B-cells in the normal host. Reproduced from (114) with permission
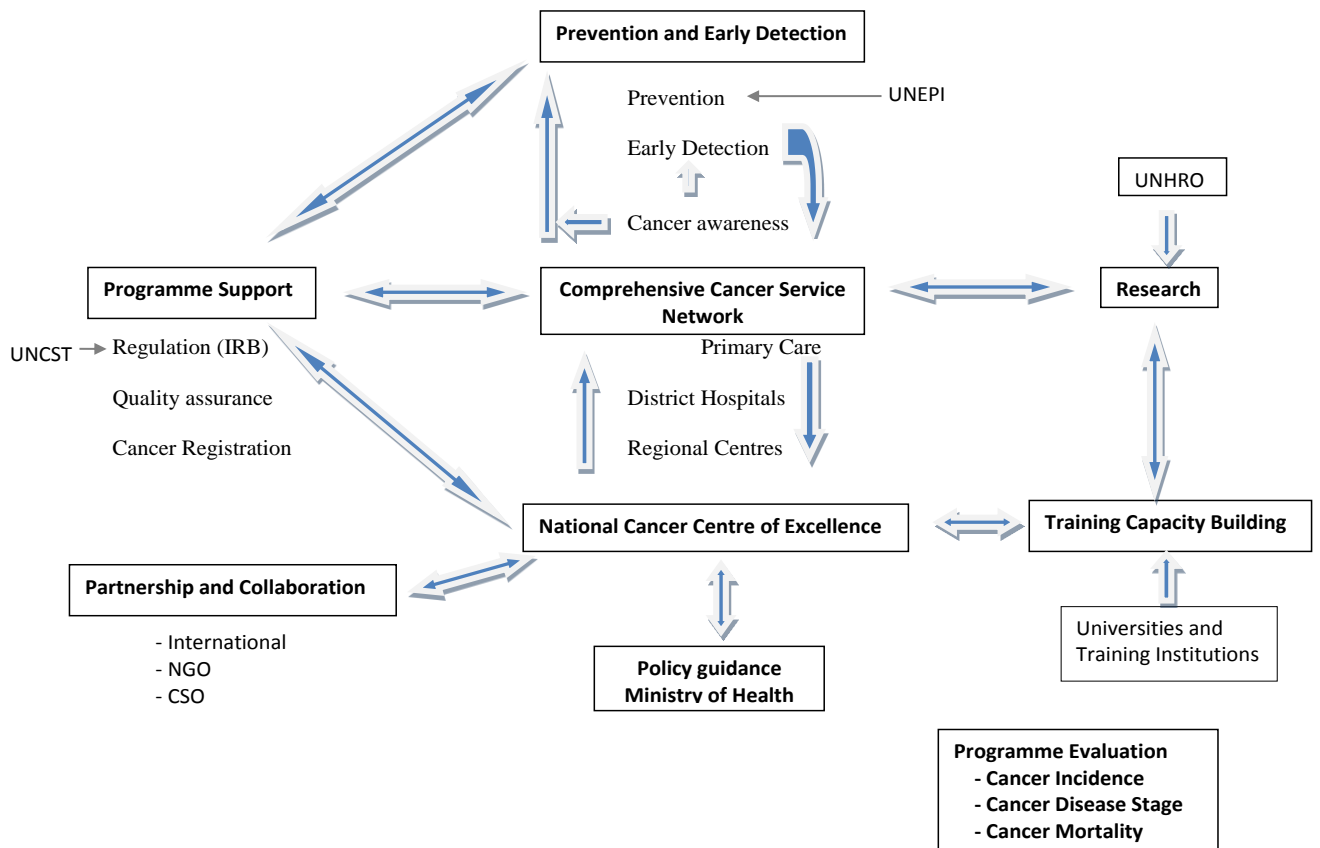
Figure 4. Outline of the comprehensive cancer prevention and control programme in Uganda

5 AIMS OF THE THESIS

The overall objective of this thesis was to further our understanding of current known risk factors for NHL, such as EBV, and their impact on disease characteristics.

The specific aims were:

I.   To better understand the background role of EBV infection in pathogenesis and characteristics of childhood NHL and CIC in a population with early exposure to EBV.

II.   To elucidate the basis for assignment of a diagnosis of childhood NHL and the strength of this diagnosis in Uganda using current practices and a justification for the introduction of advanced diagnostic methods in Uganda.

III.   To highlight trends in the clinical characteristics of the most common childhood lymphoma (BL) in an era of increasing prevalence of contributing cofactors, including malaria and HIV.

IV.   To specifically examine the impact of HIV infection on the clinical characteristics and outcome of childhood lymphoma.

6 MATERIALS AND METHODS

6.1 STUDY SITE

Papers I and II

The studies reported in **Papers I and II** were carried out at the Mulago National Referral Hospital in Kampala, Uganda, a teaching hospital of Makerere University College of Health Sciences and the Uganda Cancer Institute (Appendix 1). The Uganda Cancer Institute is the national cancer treatment centre, which operates under the authority of the Ministry of Health in Uganda and is affiliated with Makerere University Medical School. All processing of tissue samples and assignment of initial diagnoses were done at the Department of Pathology, Makerere University College of Health Sciences. Additional analysis were done at the Karolinska Institute's Department of Medical Epidemiology and Biostatistics, and confirmatory tests were done at the Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands, which is an academic centre of excellence for cancer pathology in Europe.

Papers III and IV

The studies reported in **Papers III and IV** was based on routine clinical records collected from the archives of the Uganda Cancer Institute. Paper III included information from the period 28 September 1985 to 21 February 2005, and Paper IV included information from the period between 1994 and 2004. Information from the Kyadondo Cancer Registry in Kampala was incorporated, as the Uganda Cancer Institute is situated within the catchment area of this highly-rated registry.

6.2 STUDY POPULATION

Papers I and II

The study population for Papers I and II were children with suspected tumours or masses, who were referred to the Department of Paediatrics, the Department of Paediatric Surgery, or the Department of Orthopaedics of the Mulago National Referral Hospital and the Uganda Cancer Institute (Appendix 1). Those with suspected NHL, especially those with a clinically identifiable facial tumour (who were assumed to have BL) were considered cases. The rest of the children with masses were considered controls, and they were further categorised into tumour and non-tumour controls based on the initial histological report from the Department of Pathology at Makerere University.

Papers III and IV

For **Papers III and IV** we performed a review of routine clinical records of paediatric BL cases seen at the Uganda Cancer Institute. Case notes were retrieved from the Uganda Cancer Institute archives. Information on demographic characteristics (age, sex), clinical characteristics (symptoms, disease site and stage), treatment, response, and vital status were abstracted using pre-coded forms.

6.3 DATA COLLECTION

Papers I and II

All children received a clinical diagnosis at the Mulago National Referral Hospital, based on information on presentation and physical findings (Appendix 1). Consenting children underwent clinical examination; detailed clinical and demographic information was collected by questionnaire. Excision biopsies were taken and examined morphologically in Uganda as part of standard hospital procedure. Reporting criteria for biopsy blocks was based on the presence of NHL, presence of other tumour, or presence of an inflammatory process with no tumour. Results from pathological laboratories in Uganda and The Netherlands were classified as NHL (i.e., BL or other NHL), other cancers, and CIC. NHL diagnoses and subtyping were in accordance with the WHO classification of haematological malignancies.

Papers III and IV

Data for these two papers was collected as part of the routine data collection system at the Uganda Cancer Institute. The majority of patients with suspected cancers follow the referral system from primary health care units or sub district-level health care centres to general hospitals, and onward to the Uganda Cancer Institute. Information on sex, age, symptoms, tumour presentation and disease stage at baseline, HIV status (by ELISA test, which is the most common for HIV diagnosis in Uganda), chemotherapy treatment administered, response to chemotherapy, and vital status were abstracted from clinical records.

6.4 STATISTICAL ANALYSIS

Paper I

In **Paper I** disease characteristics were explored by calculating summary statistics for all NHL and CIC separately; in a separate step BL, other NHL and CIC were compared. To analyse the association between EBV viral load by compartments (saliva, blood and white blood cells) between groups (NHL vs CIC, BL vs other NHL, and BL vs CIC) we calculated odds ratios (OR) and associated two-sided 95% confidence intervals (CI) by fitting ordinary logistic regression. We investigated the associations between level of viral load (high, intermediate, and low) and the probability of different conditions (NHL vs CIC and BL vs other NHL) within the different compartments. We also compared the association between serological markers of EBV infection by level of antibody titters (positive, normal, and elevated) and the probability of different conditions (NHL vs CIC and BL vs other NHL). We

calculated ORs and associated two-sided 95% CIs by fitting ordinary logistic regression. For all calculations we used the SAS software version 9.3.

Paper II

For **Paper II** agreement between clinical diagnoses assigned at the Mulago National Referral Hospital in Uganda, and pathological diagnoses assigned in The Netherlands was established using summary statistics and kappa statistics with two-sided 95% CIs using bootstrap techniques and a two-sided test of the hypothesis of a kappa being equal to zero. We calculated the observed agreement (percentage of samples rated equally) and two-sided 95% exact CIs. The kappa statistic measures the agreement between two raters by relating the observed proportion of equally rated samples with what is expected from chance alone, i.e., (Po - Pe)/(1 - Pe), where Po is the observed proportion of samples on which two raters agree and Pe is the proportion expected by chance alone. A value close to zero indicates no agreement, while a value close to one indicates good agreement. The statistical software R package irr version 0.83 for Debian-64 bit was used for the kappa calculations.

Paper III

For **Paper III** data on the characteristics of children with BL were summarised by means and standard deviations (SD), or with proportions; differences in proportions and means were tested by the two-test, t-test, z-test or analysis of variance (ANOVA) procedures. Time since BL diagnosis was used as the time scale in the survival analysis. Death due to BL was defined as failure, whereas all other means of leaving the study were censored. The Kaplan-Meier technique was used to calculate 5-year survival rates, and the log-rank test was used to analyse differences in survival curves. Next, the Cox proportional hazards model was fitted to estimate the hazard ratio (HR), reflecting the effect of the covariates on survival. Odds were defined as the ratio of the probability that the event of interest would occur, in this case presenting with advanced-stage disease, divided by the probability that the same event would not occur (i.e., [risk/1−risk]). Logistic regression was used to calculate ORs and 95% CIs to compare disease stages at baseline by sex and presence of ovarian mass. Both Wald and likelihood ratio tests were used to assess the statistical significance of the variables of interest. All P-values were derived from two-sided tests and a P-value <0.05 (assuming a 95% confidence level) was considered statistically significant. Cox assumptions of proportionality were evaluated both graphically and analytically, using a test based on Martingales and Schoenfeld residuals. Data analyses were performed with the Stata statistical package version 11.2 (StataCorp, LP, College Station, TX, USA).

Paper IV

For this paper a pre-coded questionnaire was used for data abstraction. Initial data entry was done using Epi-Info version 6.2 software, and exported to SPSS software. Descriptive statistics of frequencies, means and SD were calculated using the Student's t-test for analysis of continuous variables, and the Chi-square test for categorical variables. ORs, CIs, and P-

values were obtained and survival analysis was performed using the Kaplan-Meier method. Differences in the survival times of HIV-positive and HIV-negative children were obtained using the log rank test.

6.5 ETHICAL ISSUES

Papers I and II

Between 2004 and 2008, children aged 1-17 years with suspected tumours referred to the Mulago National Referral Hospital were invited to participate in a case-control study on childhood NHL (Appendix 1). In addition to parental or guardian consent, assent was requested from invited children aged 9 years or older. For children aged younger than 9 years, parental or guardian consent was considered sufficient for participation. Ethical approval for the study was obtained from the Makerere University College of Health Sciences Ethical Committee (Kampala, Uganda) and the Uganda National Council for Science and Technology.

Papers III and IV

A review of routine clinical records of paediatric BL cases seen between 1994 and 2004 at the Uganda Cancer Institute was performed. Case notes were retrieved from the Uganda Cancer Institute archives. Ethical approval for the study was obtained from the Makerere University College of Health Sciences Ethical Committee (Kampala, Uganda) and the Uganda National Council for Science and Technology.

7 SUMMARY AND DISCUSSION OF RESULTS

Paper I

Fever was the most common symptom (all NHL=83.3%, BL=88.6%, other NHL=78.3 %(Figure 5), and CIC=80.7%) followed by night sweats (BL=73.9%, other NHL=58%, and CIC=74.2%) and weight loss (BL=65.9%, other NHL=54.3%, and CIC=71%). Other common presentations were extra facial, facial, and gland swelling. HIV positivity overall was less than 10% (all NHL=10.6%, BL=11.6%, other NHL=10.2%, and CIC=6.9%) and a universal background malaria infection. There was no statistically significant difference in clinical presentation or demographic characteristics between NHL or its subtypes and CIC.

BL=46, LCL=33, DLBCL=2, Unclassified=13
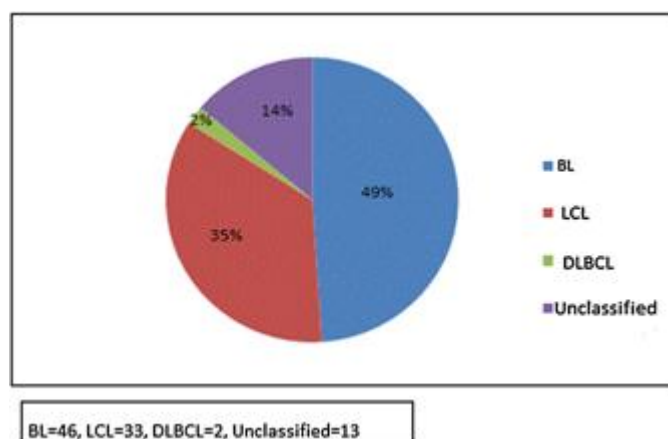
Figure 5. Subtypes of childhood non-Hodgkin Lymphoma

EBV load in saliva was not statistically significantly different in children with BL, other NHL, or CIC. EBV viral load (low, intermediate and high) in saliva was not statistically significantly different when intermediate and low levels, or high and low levels, were compared in children with NHL and CIC. This was also the case in whole blood and white blood cells. EBV IgG serology (positive, normal and elevated) was not significantly different between NHL and CIC in respect to IgG-VCA and EBNA1.

There was a significant difference in elevated levels of EAd-IgG (Table 3) in NHL vs CIC (OR 0.19, 95% CI 0.07-0.51; P-value=0.001), with NHL cases having higher EAd-IgG values. There was also a statistically significant difference between high and low EBV viral loads in blood among children with BL and those with other NHL (OR 6.67, 95% CI 1.32-33.69; P-value=0.04).

The findings of high to intermediate EBV viral load in the saliva and white blood cells of children with other NHL and CIC may point to a possible role for EBV in these diseases.

Table 3. Comparison of Epstein - Barr virus (EBV) Serology betweenNon-Hodgkin's Lymphoma (NHL) and Chronic Inflammatory Conditions (CIC)

EBV serology	All NHL N (%)	CIC N (%)	All NHL vs. CIC OR (95% CI)	P-value
VCA-p18 (normalized value)				
Positive (1)	0.001	0 (0)	—	—
Normal (1–6)	26 (19.3)	20 (21.5)	0.001–999	0.99
Elevated (>6)	108 (80.0)	73 (78.5)	0.88 (0.46, 1.69)	0.70
EBNA1-IgG				
Positive (1)	2 (1.5)	1 (1.1)	1	0.49
Normal (1–6)	40 (29.6)	34 (36.6)	0.59 (0.05, 6.77)	0.67
Elevated (>6)	29 (21.5)	5 (5.4)	0.19 (0.07, 0.51)	0.001
EAd-IgG				
Positive (1)	25 (18.5)	14 (15.0)	1	0.28
Normal (1–6)	81 (60)	74 (79.6)	0.61 (0.29, 1.27)	0.19
Elevated (>6)	29 (21.5)	5 (5.4)	0.19 (0.07, 0.51)	0.001

Paper II

Of the 314 children with a clinical diagnosis, 199 (63.4%) were boys and 115 (36.6%) were girls. The most common symptoms were fever, night sweats, and weight loss. The characteristics of the 118 children with complete diagnostic information (i.e., a clinical diagnosis and histological diagnoses from both Uganda and The Netherlands) were very similar to those of the 314 children with a clinical diagnosis only. The mean ages of these 314 and 118 children were 7.2 (SD 3.3, range 1-17) years and 7.5 (SD 3.5, range 2-17) years, respectively.

Agreement on the diagnosis (Table 4) of NHL (i.e., BL and other NHL), other cancer, or CIC between clinical diagnoses and pathological diagnoses assigned in Uganda was 91% (95% CI 84%-95%) with a kappa statistic of 0.84 (95% CI 0.75-0.92; P-value=0.001). The agreement between clinical diagnoses and pathological diagnoses assigned in The Netherlands was 49% (95% CI 40%-59%) with a kappa statistic of 0.04 (95% CI −0.10-0.17; P-value=0.612). Of the 46 children with a clinical diagnosis of CIC, 34 had a cancer diagnosis according to the laboratory in The Netherlands.

Further comparison by NHL subtype (i.e., BL or other NHL), other cancer, or CIC(Table 5), showed an agreement between clinical diagnoses and pathological diagnoses assigned in Uganda of 69% (95% CI 59%-77%) with a kappa statistic of 0.56 (95% CI 0.44-0.67; P-value=0.001). Agreement on NHL subtypes (i.e., BL or other NHL), other cancer, and CIC between clinical diagnoses and pathological diagnoses assigned in The Netherlands was 32% (95% CI 24%-41%) with a kappa statistic of 0.05 (95% CI −0.06-0.16; P-value=0.326). A direct comparison of pathological diagnoses assigned in Uganda and The Netherlands showed an agreement of 36% (95% CI 28%-46%) with a kappa value of 0.11 (95% CI −0.01-0.24; P-value=0.0459).

The agreement between pathological diagnoses (Table 6) assigned in Uganda and those assigned in The Netherlands was very low (36%). This weak agreement is probably due to the fact that pathological diagnosis in Uganda is performed at a very basic level, whereas pathologists in The Netherlands have access to additional tests for subsequent detailed characterisation by immunohistochemistry, and are thus able to make more precise diagnoses.

Table 4. Comparison of agreement between clinical diagnoses in Uganda for non- Hodgkin lymphoma (including Burkitt's lymphoma), other cancer, and noncancerous chronic conditions, and the pathology laboratories in Uganda and The Netherlands.

	Clinical diagnosis from Uganda			
	NHL, n (%)	Other cancer, n (%)	NCCC, n (%)	Total, n (%)
Pathological diagnosis **from Uganda**				
NHL	58 (94)	1 (2)	3 (5)	62 (100)
Other cancer	1 (8)	9 (69)	3 (23)	13 (100)
NCCC	1 (2)	2 (5)	40 (93)	43 (100)
Total	60 (51)	12 (10)	46 (39)	118 (100)
Kappa test statistic (95% CI)				0.84 (0.75, 0.92)
P value of the kappa statistics				<0.001
Percentage of agreement (95% CI)				91% (84%, 95%)
Pathological diagnosis **from The Netherlands**				
NHL	46 (53)	8 (9)	33 (38)	87 (100)
Other cancer	1 (50)	0 (0)	1 (50)	2 (100)
NCCC	13 (45)	4 (14)	12 (41)	29 (100)
Total	60 (51)	12 (10)	46 (39)	118 (100)
Kappa test statistic (95% CI)				0.04 (−0.10, 0.17)
P value of the kappa statistics				0.612
Percentage of agreement (95% CI)				49% (40%, 59%)

Table 5. Comparison of agreement between clinical diagnosis in Uganda for Burkitt's lymphoma, other non-Hodgkin lymphoma (excluding Burkitt's lymphoma), other cancer, and noncancerous chronic conditions, and pathology laboratories in Uganda and in The Netherlands.

	Clinical diagnosis from Uganda				Total, n (%)
	BL, n (%)	Other NHL, n (%)	Other cancer, n (%)	NCCC, n (%)	
Pathological diagnosis from Uganda					
BL	21 (62)	10 (29)	0 (0)	3 (9)	34 (100)
Other NHL	16 (57)	11 (39)	1 (4)	0 (09)	28 (100)
Other cancer	0 (0)	1 (8)	9 (69)	3 (239)	13 (100)
NCCC	1 (2)	0 (0)	2 (5)	40 (939)	43 (100)
Total	38 (32)	22 (19)	12 (10)	46 (399)	118 (100)
Kappa test statistic (95% CI)					0.56 (0.44–0.67)
P value of the kappa statistics					<0.0001
Percentage of agreement (95% CI)					69% (59%, 77%)
Pathological diagnosis from The Netherlands					
BL	19 (45)	10 (24)	0 (0)	13 (31)	42 (100)
Other NHL	10 (22)	7 (16)	8 (18)	20 (44)	45 (100)
Other cancer	1 (50)	0 (0)	0 (0)	1 (50)	2 (100)
NCCC	8 (28)	5 (17)	4 (14)	12 (41)	29 (100)
Total	38 (32)	22 (19)	12 (10)	46 (39)	118 (100)
Kappa test statistic (95% CI)					0.05 (−0.06, 0.16)
P value of kappa statistics					0.326
Percentage of agreement (95% CI)					32% (24%, 41%)

Table 6. Comparison of agreement between the pathological diagnosesassigned in Uganda and The Netherlands.

	Pathological diagnosis from Uganda				Total, n (%)
	BL, n (%)	Other NHL, n (%)	Other cancer, n (%)	NCCC, n (%)	
Pathological diagnosis from The Netherlands					
BL	22 (52)	7 (17)	1 (2)	12 (29)	42 (100)
Other NHL	8 (18)	10 (22)	8 (18)	19 (42)	45 (100)
Other cancer	0 (0)	1 (50)	0 (0)	1 (0)	2 (100)
NCCC	4 (14)	10 (34)	4 (14)	11 (38)	29 (100)
Total	34 (29)	28 (24)	13 (11)	43 (36)	118 (100)
Kappa test statistic (95% CI)					0.11 (−0.01, 0.24)
P value of kappa statistics					0.0459
Percentage of agreement (95% CI)					36% (28%, 46%)

Paper III

The mean age at diagnosis and first admission to the Uganda Cancer Institute was 6.69 years (SD 2.89), with a mean follow-up of 10.44 months (SD 23.64). Follow-up time was similar for boys and girls (P-value=0.980). While most children had a clinical diagnosis of BL, only 50.53% had a histological confirmation of the diagnosis, and 9.37% had a cytological confirmation of the disease. However, the proportion of histologically confirmed BL increased significantly in the last period of diagnosis (58.12%), which may be due to an improved capacity to perform histology for the purposes of assigning diagnoses in Uganda.

The most common symptoms at diagnosis were fever, anaemia, weight loss, night sweats, severe infection, and history of severe or recurrent malaria. Significant increases in the

proportion of children presenting with fever were observed during the study period, as well as differences over time in the proportion of children presenting with anaemia, night sweats and severe infection.

The most common tumour presentations were facial tumour and abdominal disease. One-third of girls presented with ovarian mass. Presentation with hepatic mass and malignant pleocytosis increased during the study period, whereas other tumour presentations remained similar. Most children presented with advanced-stage BL (stage C 32.12% and stage D 26.67%). Indeed, there was an increase in the overall proportion of children presenting with advanced-stage BL during the study period, although girls presented more frequently with advanced-stage disease than did boys. Girls presenting with ovarian mass were overwhelmingly diagnosed at advanced stages.

About half of the children received at least three cycles of chemotherapy. There was a small but significant increase in the proportion of children receiving zero or only one cycle of chemotherapy during the study period (P-value <0.001). Following treatment, most children achieved either complete or partial remission, yielding an objective treatment response of 75.71%, which was similar in boys and girls, and increased during the study period.

In total, 191 children died during follow-up (15.69% of 1217); among the 1206 children with information on chemotherapy, 46 died without receiving any chemotherapy, and 145 died despite having received at least one cycle of chemotherapy. Children aged 15-17 years at diagnosis had a higher mortality rate (HR 3.81, 95% CI 1.81-8.01) than children diagnosed at ages 0-4 years.

There was a significant increase in mortality rate among children with symptoms of fever, weight loss, night sweats, or severe infection at BL diagnosis, compared with children without these symptoms. Children with symptoms of anaemia and severe or recurrent malaria at BL diagnosis had a decreased mortality rate compared with children without these symptoms. Children presenting with facial tumour had a relatively decreased mortality rate (HR 0.33, 95% CI 0.25-0.45) compared with children with other tumour presentations. HIV-positive children had an increased overall mortality rate compared with HIV-negative children (HR 2.50, 95% CI 1.47-4.26). (Appendix 2c)

A decreased mortality rate was revealed with increasing cycles of chemotherapy (P-value <0.001). Children treated with at least one cycle of chemotherapy had a 90% reduction in mortality rate (HR 0.10, 95% CI 0.07-0.14). Children who achieved complete or partial remission after chemotherapy also had a lower mortality rate (HR 0.04, 95% CI 0.01-0.12; HR 0.16, 95% CI 0.05-0.53, respectively) compared with those with no response to chemotherapy. Advanced-stage disease led to poor outcome. Disease stage was associated with mortality rate (P-value <0.001) and children diagnosed at advanced stages had an increased mortality rate (HR 4.04, 95% CI 2.72-5.99) compared with those diagnosed at stage A, B and AR combined. Girls with an ovarian mass had particularly high odds to present with

advanced-stage disease compared with boys (OR 13.69, 95% CI 6.31-29.72), as well as compared with girls without an ovarian mass (OR 19.73, 95% CI 8.88-43.85).

The Kaplan-Meier survival estimates indicated that there was no overall survival difference between boys and girls (P-value=0.091) (Appendix 2d). HIV-positive children had an increased overall mortality rate compared with HIV-negative children (HR 2.50, 95% CI 1.47-4.26). HIV-negative children had higher survival than HIV-positive children (P-value <0.001)(Appendix 2c).

Paper IV

Of the 228 children (139 males, 61%; 89 females, 39%), 158 were HIV-negative and 70 were HIV-positive. Overall, the mean age was 6.9 years (HIV-positive children: 6.7 years; HIV-negative children: 7.1 years). There was no significant difference in the general characteristics of children with BL, regardless of HIV status, sex, age, and duration of illness (Table 7). Presentation with facial tumour was seen in 50 (71.4%) HIV-positive children and 121 (76.6%) HIV-negative children. The most common extrafacial presentation was abdominal, which was seen in 152 children (66.7%). HIV-positive children had significantly more liver involvement than HIV-negative children (OR 2.29, 95% CI 1.29-4.07; P-value=0.004). Another common site among HIV-positive children was the thorax (OR 5.74, 95% CI 1.44-22.91; P-value=0.01). Lymphadenopathy was seen in 108 children (47.4%), most of whom were HIV-positive. HIV-positive children were more likely to present with advanced-stage disease (OR 2.33, 95% CI 1.19-4.54; P-value=0.007).

Objective treatment response was seen in 158 (69.3%) children (Table 8). There was no significant difference in response by HIV status in all study children (HIV-positive children: OR 4.84, 95% CI 0.99-30.60; P-value=0.026) or in those with BL.

Figure 7. Demographic and clinical characteristics

	HIV+	HIV−	OR	95% CI	P-value
Demographic characteristics					
Patients (male/female)	70 (40/30)	158 (99/59)	0.80	0.45–0.41	0.43
Age (years): mean ± SD	6.6 ± 4.0	7.1 ± 3.1	—	—	0.44
Duration of illness (weeks) mean ± SD	6.3 ± 7.0	6.9 ± 7.7	—	—	0.58
Clinical characteristics					
History of fever	39	84	1.11	0.63–1.95	0.72
History of night sweats	23	33	1.85	0.99–3.48	0.05
History of weight loss	35	64	1.47	0.83– 2.59	0.18
Halitosis	20	54	0.77	0.42–1.42	0.40
Facial tumor	50	121	0.76	0.41–1.44	0.41
Abdominal tumor	52	100	1.68	0.90–3.13	0.10
Splenic mass	40	70	1.68	0.95–2.96	0.07
Hepatic mass	36	50	2.29	1.29–4.07	0.004a
Malignant ascites	12	24	1.155	0.54–2.47	0.71
Spinal cord tumor	10	17	1.382	0.60–3.19	0.45
Malignant pleocytosis	10	8	3.125	1.18–8.30	0.02
Thoracic involvement	7	3	5.741	1.44–22.91	0.011
Lymphoadenopathy	47	61	3.249	1.80–5.88	0.000
Stage					
A	11 (15.7%)	38 (24.1%)	0.59	0.26–1.30	0.16
B	13 (18.6%)	32 (20.3%)	0.90	0.41–1.94	0.77
C	20 (28.6%)	55 (34.8%)	0.75	0.39–1.44	0.36
D	26 (37.1%)	32 (20.3%)	2.33	1.19–4.54	0.007
ARb					

Table 8. Treatment and response

	HIV+	HIV−	OR	95% CI	P-value
Number of courses					
0	8 (11.4%)	12 (7.6%)	1.57	0.55–4.38	0.35
1–2	23 (32.9%)	49 (31.0%)	1.09	0.57–2.07	0.78
>2	39 (55.7%)	97 (61.4%)	0.79	0.43–1.46	0.42
Disease response					
No response	1 (1.4%)	3 (1.9%)	0.75	0.01–9.53	1.000
Complete response (CR)	25 (35.7%)	65 (41.1%)	0.79	0.43–1.48	0.44
Partial response (PR)	22 (31.4%)	46 (29.1%)	1.12	0.58–2.14	0.73
Stable disease (SD)	6 (8.6%)	3 (1.9%)	4.84	0.99–30.60	0.03a
No assessment	16 (22.9%)	41 (25.9%)	0.85	0.41–1.72	0.62

Seventy children were lost to follow-up, and 48 died. There were therefore 180 censored observations (70 lost to follow-up and 110 alive); 44 were HIV-positive children, and 136 were HIV-negative children. The rate of loss to follow-up between HIV-positive and HIV-negative children was not significantly different (OR 1.05, 95% CI 0.57-1.93; P-value=0.874). More deaths occurred in HIV-positive than HIV-negative children, (26 HIV-positive and 22 HIV-negative; OR 3.65, 95% CI 1.79-7.47; P-value=0.000).

Mean survival time for HIV-negative children was 84.95 months (95% CI 72.40-97.49 months), while for HIV-positive children it was 55 months (95% CI 28.79-82.53 months). The median survival time for HIV-negative children had yet to be reached (Figure 5) at the end of the study, while for HIV-positive children it was 11.79 months (95% CI 8.65-14.92 months). There was a significant difference in survival rates between HIV-positive and HIV-

negative children (OR 2.22, 95% CI 1.11-4.42; P-value=0.023). The log rank test showed a significant difference in survival times between HIV-positive and HIV-negative children with BL (P-value=0.0018).
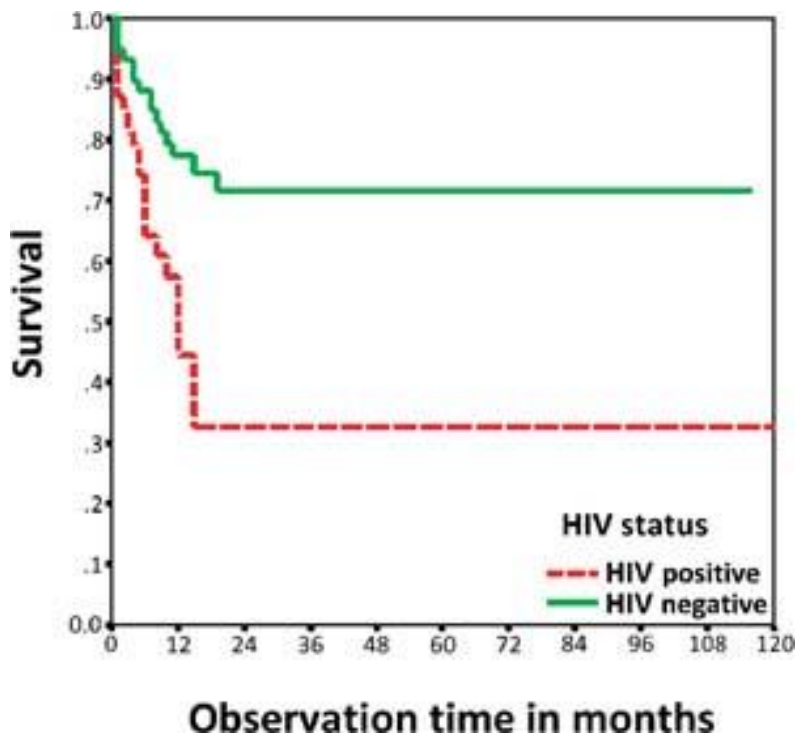

Figure 5: Survival among children with BL in Uganda according to HIV infection status

8 GENERAL DISCUSSION

8.1 METHODOLOGICAL CONSIDERATIONS

8.1.1 Study design and study population

Papers I and II are based on data from a hospital-based case-control study, and Papers III and IV are retrospective routine cohort studies based on data retrieved from the archives of the Uganda Cancer Institute. Case-control and cohort studies are common epidemiological study designs. Case-control studies can be population-based, hospital/institution-based, or nested within a cohort study. Cohort studies on the other hand, can be prospective or retrospective. Case-control studies start with a source population, which is the equivalent of a hypothetical study population in a cohort study, and from which the participants are selected.

Having a control group from the source population is vital if one is to properly understand the distribution of the exposure of interest. However, case-control studies, although highly efficient when it comes to understanding the causes of rare disease, are still subject to bias, especially selection and recall bias (115). Epidemiological studies based on data from hospitals or clinics require a high-quality reliable source (116). They are based on individual information at study entry and during the intervening period of care, and they are based to some extent on outcome information. The availability of histological confirmation of diagnoses provides a key basis for assigning participants to the correct groups, as well as categorisation and external review, all of which have an impact on the validity of epidemiological studies (117). The added benefit of cost-effectiveness puts hospital-based studies on par with population-based studies (118). All of these advantages are manifest in our hospital-based sources of information for the studies in this thesis, and the studies presented in this thesis were based on all the above mentioned principles.

Papers I and II are based on data of children with suspected NHL seen at the Mulago National Referral Hospital and the Uganda Cancer Institute(Appendix 1). Paper II mainly provided validation for the basis for NHL diagnosis and the classification of NHL subtypes from tissue samples. On the other hand, Papers III and IV employed routinely collected retrospective data from patient records at the Uganda Cancer Institute. The availability of detailed, well-maintained records with follow-up and outcome data at the Uganda Cancer Institute meant we could efficiently answer research questions regarding the clinical characteristics and outcomes of BL, one of the most common subtypes of childhood NHL in Africa. The usefulness of any study is in large part dependent on the reliability of the data and the manner in which the study is executed, which is in large part reflected in the validity of the study.

Validity is defined as the (relative) lack of systematic measurement error when comparing the actual observation with a standard, which is a reference method representing the 'truth' (119). There are two types of validity: internal and external (120).

8.1.2 Internal Validity

Internal validity is the ability of an instrument to measure what it sets out to measure. The purpose of this is to ensure that inferences made in a study are accurate. Internal validity is therefore crucial in clinical research to ensure that findings from the study sample can be extrapolated to the broader community (120). In case-control studies, internal validity guarantees a reasonable precision of the strength of the cause-effect relationship (121).

In Paper I internal validity was ensured by using clear diagnoses for group assignment, based on verified histology performed in an external cancer reference laboratory in The Netherlands, which was the main focus of the results shown in Paper II. In Paper II clinical diagnoses and pathological diagnoses from Uganda were validated in the same external reference laboratory with the sole purpose of excluding potential false diagnoses before determining which children would be classified as cases and which as controls.

In **Papers III and IV** internal validity was ensured by including only data from the archives of the Uganda Cancer Institute, hence reflecting the base population of the Uganda Cancer Institute and the Kyadondo Cancer Registry in Kampala.

There are three key factors that impact the internal validity of any study: bias, confounding and chance.

8.1.2.1 Bias

Bias refers to a mechanism that produces a lack of internal validity due to incorrect assessment or judgment of associations between an exposure and an outcome in the target population (122). There are various types of bias, but there are two main categories: information bias and selection bias (122).

Information bias may also be called observation, classification, or measurement bias. It results mainly from the incorrect determination of the exposure or the outcome, or both. To avoid information bias in cohort studies, information on outcomes should be obtained in a consistent manner for both exposed and unexposed individuals. To avoid information bias in a case-control study, data for cases and controls should be gathered in a uniform manner (123). Furthermore, the individuals collecting the data should be unaware of the status, or the assignment, of cases and controls (122, 123). This was achieved in Papers I and II, as the research assistants who collected relevant data were blinded to the assignment of cases and controls. Moreover, cases and controls were obtained from the same source population, i.e., patients from the Mulago National Referral Hospital and the Uganda Cancer Institute. The fear that this could have introduced bias was allayed by the comparability of the cases and controls with respect to clinical and demographic characteristics in both papers.

The most common type of information bias is misclassification, which arises when sensitivity and specificity of procedures to detect exposures and outcomes are not perfected (124). There are two types of misclassification: differential and non-differential. Differential misclassification results when information is gathered differently for two groups (e.g., cases

or controls) (124). This results in an increased or decreased relative risk or OR, depending on the direction of the bias. In non-differential misclassification, real differences are obscured (124). This can result from the use of an ambiguous instrument, such as a questionnaire, which leads to errors in data collection between groups such as cases and controls (125). This may shift the OR toward unity, meaning no association in a case-control study (126). In all papers included in this thesis, differential misclassification was avoided by the use of uniform standard operating procedures for obtaining data from the groups involved. Non-differential misclassification was avoided by the use of a standardised questionnaire for all the groups that were compared. Furthermore, the questionnaires were pretested to ensure reproducibility of the information obtained.

Recall bias occurs when disease status influences a participant's perception of its cause, or causes a subject to search harder for an exposure to the putative cause (124). This is common in case-control studies since participants know their disease status. It can also occur in cohort studies when subjects know their exposure status. Reporting bias occurs when participants give information they think researchers expect to hear. Ensuring similar processes for obtaining information and avoiding disclosure of the study hypothesis to participants represent some ways of avoiding reporting and recall bias. Observation bias or interviewer bias occurs when knowledge of the study hypothesis or exposure status influences data recording (127). In the papers included in the present thesis, parental recall bias was anticipated, especially in Paper I, and minimised by excluding direct and leading questions in the interviews. Furthermore, the interviewers were not aware of the study hypothesis or research questions.

Selection bias is common in retrospective studies that analyse routine data from a single population (128). The very factors that determine hospital attendance often bias the selection in hospital-based studies, and hence the data collected from the population for a retrospective study (128). In this thesis, selection bias was considered in Papers III and IV. For example, children with better family support may be more likely to go to the hospital for diagnosis and treatment. Hence children from poorer backgrounds could have been inadvertently excluded.

8.1.2.2 Confounding

Confounding is best defined as a confusing or mixing of the effect (129), i.e., the effect of an exposure is mixed with the effect of another variable, leading to bias (129). Confounding is a systematic error that can be prevented or removed using two common methods: randomisation or random assignment in experimental studies, and restriction, which involves including subjects who have the same values for variables that are suspected confounders (129). Confounding can be controlled before, or after data collection is completed.

In Paper I the inclusion and exclusion criteria applied meant confounding was largely minimised. The purpose of these approaches was to achieve homogeneity between study groups. Restriction is the simplest approach to minimising confounding; it increases the internal validity of a study at the expense of external validity. In Paper I, the restriction

applied was that only diagnoses verified and confirmed in The Netherlands were used, hence strengthening internal validity.

Another way to enhance the overall generalizability of findings to the target population is to ensure the representativity of the study sample. This includes all attributes of the study, including case selection, which should be designed to capture all cases as rapidly as possible. Although this could weaken the validity, our strategy of ensuring external pathological review for the diagnoses in Paper II went a long way in restoring internal validity. We didn't undertake any matching in our studies, nor did we undertake stratification in our studies to minimise cofounding.

8.1.2.3 *Chance*

If results cannot be explained by selection, information, or confounding bias, then chance might be another explanation (129). To ensure the validity of findings, one must ensure biases are excluded during the process, leaving only chance as an explanation. The likelihood of chance alone as an explanation for spurious findings can be minimised considerably by improving the power of the study, i.e., increasing the sample size. All our studies had reasonable power given the sample sizes, thus minimising considerably the possibility that chance could be an explanation for our findings.

8.1.3 External validity

External validity means the results from study participants can be extrapolated to the general population (155). Ideally this would be best undertaken via a census of the general population, but this is not usually possible in medical research. Therefore it is assumed that data collection via questionnaire, and sampling of tissue and body fluids are obtained in a representative manner from the underlying source population, for both cases and controls, so that results can be comparable to the base population (130).

In **Paper I** we excluded normal children as controls due to ethical concerns; hence we don't have a disease-free control group, which could have provided an ideal baseline comparison.

Paper II provided a reliable diagnosis and setting, a relatively large sample size, and pathological diagnoses that were based on the same tissue samples in Uganda and in The Netherlands. Moreover, we were able to compare clinical diagnoses in Uganda with histological diagnoses assigned in Uganda and in The Netherlands.

Paper III had a number of strengths, including the large sample size, long observation period, and the unique position of the study site, as the Uganda Cancer Institute is the only national cancer treatment centre in Uganda. Because this was a retrospective study that analysed routine data from a single cancer institute, the potential for selection bias is a major weakness, especially since children with better family support may be more likely to go to the hospital for diagnosis and treatment. Thus, our results cannot be considered truly representative of the entire country, although most suspected cancer patients, in particular

children, are indeed referred to the Uganda Cancer Institute. In the absence of routine mechanisms for tracing children who do not return for additional treatment or routine follow-up, it is not surprising that there was a substantial number of children in our study that were censored in the data analysis at last admission or visit to the Uganda Cancer Institute.

The strength of **Paper IV** was that the site is the only cancer centre in Uganda with available expertise in chemotherapy treatment in children. In addition, the Uganda Cancer Institute is in the catchments area of the Kyadondo Cancer Registry in Kampala.

8.2 ETHICAL ISSUES

8.2.1 Risk benefit to the participants

For **Papers I and II**, the consent process clearly underlined the anticipated possible risks of participation in the study, such as loss of time while involved in the study, risk of blood loss during blood collection, and discomfort due to needle prick, all of which were explained to participants. In the same way the benefits of participation were explained, these included the benefit of knowing the health status of children, prompt referral to specialists, specific knowledge of HIV status, and the national and population benefit of knowing about how NHL affects children, as well as methods of prevention and possible treatment.

For **Papers III and IV**, which were retrospective studies, anonymity of personal information was safeguard by minimising the possibility to link records to individual participants.

8.2.2 Empowerment of participants

For **Papers I and II**, during the consent process we explained to parents or guardians of participants, that participants could withdraw from the study at any time without prejudice. Contact information was given in case of questions or concerns regarding the study. Participants' privacy was respected, and access to care was guaranteed for all participants at any clinic operating within the Mulago National Referral Hospital, including care for cancer and HIV. Furthermore, we stated that care would be provided without discrimination to all invited children, whether they consented to participate or not.

8.2.3 Sources of funding for the study

Sources of funding for the studies were disclosed in all the publications as coming from the Swedish International Development Cooperation Agency (SIDA), Makerere University and the Mulago National Referral Hospital.

8.2.4 Data collection

Ethical approval for all the studies in this thesis was obtained from the Makerere University College of Health Sciences Ethical Committee and the Uganda National Council for Science and Technology. Participation was voluntary through the consent process. Informed consent was obtained from parents or legal guardians, and assent was obtained from children older than 9 years of age at enrolment. HIV counselling and testing were offered before conducting

HIV testing; results were given only to those who wanted them, followed by post-test counselling. Strict confidentiality of information was maintained for all participants. In addition children underwent clinical examination and detailed clinical and demographic information were collected by questionnaire. Data safety and disposal policy are in accordance with Institutional Review Board policies.

8.2.5 Dissemination of findings

The papers in this thesis were published in peer-reviewed journals, but no name or medical records of participants were revealed.

9 INTERPRETATION AND IMPLICATIONS

9.1 INTERPRETATION

9.1.1 Paper I

EBV viral load in whole blood was significantly higher in children with BL compared to children with other NHL. Moreover, there was a strong association of EBV viral load in blood and white blood cells with BL. The role of EBV in lymphomagenesis is intriguing, since the driver of this process is not very clear. Our study highlighted the possible association of higher viral load with NHL in general, but particularly in BL. Secondly, our study demonstrated that viral load may be linked to disease stage in BL, as those with higher viral load presented at more advanced stages, which is in line with observations from Kenya and other countries (131, 132). It has been hypothesised that the high risk of BL in sub-Saharan Africa could be linked to the level of viral activity, which is reflected in our study by viral load (31, 133).

Further to the importance of viral activity in disease aetiology and the disease process, we high EA IgG serology was significantly present in children with NHL. This is a marker of viral reactivation and of acute or chronic active EBV infection, brought about by both acute primary EBV infection and EBV reinfection. There was a highly statistically significant association between elevated EA IgG in children with NHL compared to those with CIC (134, 135).

In line with disease aetiology, we can hypothesise that there are two processes that result from EBV infection, both of which lead to lymphomagenesis. One is an active process that is marked by high level of viral load and DNA, which could be exemplified by BL. The other is a reactive process that is marked by the development of antibodies, especially EA IgG, which could be exemplified by other subtypes of NHL and possibly CIC in childhood (136). Our study has therefore responded more specifically to questions raised by earlier investigators in the field of EBV and childhood NHL, especially BL (137-139).

9.1.2 Paper II

We have confirmed that the capacity for correctly diagnosing childhood NHL is low in Uganda. Unfortunately, our findings are not unique to Uganda, nor are they limited to children with lymphoma (140). This conclusion is supported by findings in a number of African countries. Moreover, we showed that clinical criteria have no role in the diagnosis of childhood cancers in low-income settings (141, 142). Our study has by far the most objective findings that have come out of Africa on the magnitude of diagnostic challenges in paediatric cancer.

9.1.3 Paper III

Changes in BL tumour presentation have been suggested in other parts of Africa, such as Nigeria, Kenya, and Ghana (143-145). In the 1980s, changes in BL tumour presentation were reported in Ghana, where more children presented with abdominal disease rather than the usual facial tumours (145). In Nigeria, similar changes in tumour presentation were reported, and more recent observational studies in Kenya suggested that the clinical manifestations of BL vary across different regions of the country (144, 146).

We did not observe changes in the major clinical characteristics of BL during our 20-year observation period, nor did we see changes in age and sex distribution. It is our belief that the changes in tumour presentation reported elsewhere may have been due to random variations, since no clear explanations were given for the observations.

Unlike Kaposi sarcoma, there has not been a dramatic decrease in the incidence of HIV-associated NHL since the introduction of highly active antiretroviral therapy. These clinical-epidemiological observations emphasise differences in risk factors and tumorigenesis of AIDS-defining malignancies and reinforce the need to understand the aetiological mechanisms that lead to BL.

9.1.4 Paper IV

Earlier in the HIV epidemic most studies looked for an association between HIV and childhood BL, but the association could not be clearly demonstrated (19, 147). In a series of 50 consecutive patients with BL subjected to HIV testing at Uganda Cancer Institute in the early 1990s, none was found to be HIV-positive (Professor EK Mbidde, unpublished work). It was concluded that BL in Ugandan children was mainly of endemic form and not associated with HIV. Our finding is the first detailed evaluation of the characteristics of HIV-associated BL in children from Africa (148). HIV-associated BL in Uganda tends to affect extrafacial sites, mainly lymphnodes, abdominal sites, and thoracic sites, which is similar to reports from developed countries. Our study is also the first to clearly show a similarity in response to chemotherapy between HIV-positive and HIV-negative children, and finally the significant negative impact of HIV infection on the survival of HIV-positive children with BL. This is the first report of its kind and offers clinicians insight and hope for effective treatment of children with BL (148). The finding that HIV-positive children present more

commonly with lymphadenopathy supports earlier observations from Malawi and elsewhere (149).

9.2 IMPLICATIONS

9.2.1 Clinical and diagnostic implications

Paper I demonstrated the commonality of systemic clinical symptoms (fever, weight loss, night sweats, and lymphadenopathy) in children with NHL and CIC in Uganda. These features are not discriminatory and could be a source of diagnostic confusion if not verified through reliable laboratory tests. Hence clinical findings alone should not be the sole basis for NHL diagnosis or treatment initiation. Our findings suggest for the first time that there are common subtypes of NHL other than BL in Ugandan children. The other common subtypes of childhood NHL we found included LCL and diffuse large B-cell lymphoma, and these should be considered in children with NHL.

Paper II confirmed the caution mentioned in Paper I that clinical diagnosis alone should not be used as a basis for treatment initiation in children with NHL in Uganda. A high probability of error even after pathological diagnosis in Uganda emphasises the challenges of diagnosing childhood NHL in Uganda, and the weak agreement observed between pathological diagnoses from Uganda and The Netherlands supports this. The main difference between the two settings is that pathological diagnosis in Uganda is performed at a very basic level, whereas in The Netherlands the use of advanced tests for detailed characterisation, such as immunohistochemistry, is routine. There is therefore a dire need to upgrade diagnostic capabilities in Uganda.

Paper III indicated that the well-known clinical characteristics of BL (facial features, age and sex distribution) have not changed in the last 20 years. However, girls presenting with ovarian masses are likely to present with advance-stage disease. Therefore girls should be highly scrutinised for the presence of ovarian BL to facilitate early diagnosis. The increase in the frequency of BL in children presenting with malignant pleocytosis and hepatic masses implies the need for routine cerebrospinal fluid analysis and abdominal scans. Paper III also underlines a number of factors associated with a poor prognosis of BL, including some symptoms (fever, weight loss, and night sweats), severe bacterial infection, and HIV infection. Factors associated with a better prognosis included facial tumour, early-stage disease, and initiation of chemotherapy.

Paper IV showed that, although for a long time BL in Ugandan children was thought to be mainly endemic BL and thus unrelated to HIV, a sizable number of HIV-positive children do present with BL. Moreover, irrespective of HIV status, BL in Ugandan children presents commonly as a jaw mass. This implies that presentation of facial disease alone is not discriminatory for endemic and HIV-associated BL in Uganda. However, the presence of extrafacial features, particularly lymphadenopathy or at abdominal and thoracic sites, are common in HIV-positive children. The response to chemotherapy for both endemic and HIV-

associated BL is similar, although survival is poorer in HIV-positive children, possibly due to the lack of availability of antiretroviral therapy at the time of the study.

9.2.2 Health system and policy implications

Paper I showed a low enrolment rate into the study due to lack of consent. This points to the need for a deliberate policy that helps the community to see the value of research in children and encourages them to allow their children participate in research studies.

Paper II provided a strong basis for the establishment of a more reliable diagnostic system for cancer in general in Uganda, through infrastructure development and augmentation of the capacity to obtain and process good quality samples. For children in particular our findings provided a compelling case to improve the quality of diagnosis of childhood NHL in Uganda. A central national reference laboratory specialised in cancer is needed. This laboratory should be adequately equipped and staffed, have reliable access to laboratory supplies, and regular quality upgrades. It should be policy that clinical diagnosis alone not be used as the basis for initiating treatment of any type for children with NHL in Uganda, including BL.

Paper III highlighted the plight of children with BL. Indeed, despite the fact that this cancer is highly curable with chemotherapy alone, many children in Uganda still die from BL due to poor socioeconomic circumstances and inability to reach hospital to initiate or complete treatment, which was a major determinant of outcome. Improvement in the health care system in Uganda is needed, specifically for early cancer diagnosis; increasing access to histological confirmation and rapid referral to the Uganda Cancer Institute for specialised cancer care would improve the chances for children with BL to be diagnosed at earlier stages and properly treated, resulting in lower mortality.

Paper IV indicated that HIV-associated BL is common in Uganda and behaves similarly to endemic BL, hence the need to routinely test for HIV in children presenting with BL.

9.2.3 Cancer control implications

Paper I further supports the well-known role of EBV in the aetiology of BL and the possible link between EBV and other childhood NHL and CICs. **Paper I** also affirms the route of EBV transmission, as well as a potential preventive strategy, including vaccination. **Paper II** showed the need for accurate diagnosis to improve cancer surveillance through registration, whereas **Paper III** showed the importance of improving the cancer reporting and surveillance system in the country, which is in line with the national cancer policy and control programme. There is a need for mechanisms that can be used to trace children who do not return for additional treatment, as a substantial number of children in our study were lost to follow-up. **Paper IV** showed the need to control the development of cancer in children with HIV.

9.2.4 Research implications

The findings in **Paper I** will likely stimulate new research on the role of EBV viral load and serology in NHL diagnosis, and risk stratification for children with EBV-related conditions. Secondly, evaluation of the spectrum of EBV-related childhood malignancies and other conditions in Uganda is an important research area. The role of other common diseases, including HIV and malaria, in the aetiology of childhood malignancies requires further exploration. The relationship between EBV and NHL, especially the specific role of EBV in NHL subtypes and other childhood illnesses, needs to be better understood. This will help to design better clinical and preventive interventions for EBV-related illnesses in children in Uganda. Understanding the spread of EBV in the community, and the implementation of preventive strategies including vaccination, are sorely needed in developing countries. **Paper I** also supports the association of biological markers of EBV with childhood NHL, irrespective of subtype, in Uganda.

Meanwhile, **Paper II** underscores the necessity of building research capacity for tissue handling, processing, storage, and pathology, and the benefit this would present for both clinical and pathological research opportunities in Uganda.

Paper III challenges the current assumption that the clinical characteristics of endemic BL in African children have changed over the last 50 years.

Finally **Paper IV** raises the need for research that will lead to better characterisation of children with BL, in order to understand whether there is a distinct HIV-associated BL that exists alongside endemic BL in the region. The role of other infectious agents and HIV in this process and in outcomes is another important research area. Indeed, this will help to find better treatment methods for HIV-positive children with BL, including feasibility of specific chemotherapy protocols with minimal myelotoxicity. Since socioeconomic factors have a big impact on outcome, studies looking at factors that influence compliance in children with BL are important in social science research.

10 CONCLUSIONS

AIM I: To understand the background role of EBV infection in pathogenesis and characteristics of childhood NHL and CICs in a population with early exposure to endemic infections

We have shown in this study that children with NHL and CIC in Uganda present with similar features, especially fever, weight loss, and night sweats. These clinical characteristics are not discriminatory, which suggests a common background process. We also showed that EBV viral load in blood is statistically significantly elevated in children with BL, but not those with other NHL. We also observed a highly elevated expression of EA IgG in NHL, but not CIC. This study therefore suggests that EBV could be associated with childhood NHL irrespective of subtype in Uganda. Therefore further characterisation of the relationship between EBV and NHL is needed, especially the specific role of EBV in disease subtypes and other childhood illnesses. This will help in designing better clinical and preventive interventions for EBV-related illnesses in children in Uganda.

AIM II: To elucidate the strength of and basis for assignment of childhood NHL diagnosis in Uganda using current practices and to justify the introduction of advanced diagnostic methods in Uganda.

Clinical diagnosis of NHL in Uganda has a high probability of error compared with pathological diagnosis assigned in Uganda and in The Netherlands. In addition, agreement on the pathological diagnosis of NHL between Uganda and The Netherlands was very low. Clinical diagnosis alone should therefore not be the basis for initiating treatment of NHL in Uganda, including BL, because the probability of diagnostic error is high. There is a need to improve the quality of diagnosis of childhood NHL in Uganda by creating an efficient system within the public health sector for the collection, handling, processing, and storage of biological samples. A national reference laboratory specialised in cancer is therefore warranted in Uganda.

AIM III: To highlight the trends in the clinical characteristics of the most common childhood lymphoma (BL) in an era of increasing prevalence of contributing cofactors, including malaria and HIV.

Major clinical characteristics of BL have remained unchanged over the last 20-plus years of observation. However, subtle changed have been observed: more children are presenting with advance-stage disease, especially in girls with ovarian masses and children with HIV. This was confirmed by the increase in the proportion of children with malignant pleocytosis, liver masses and systemic symptoms. Mortality from childhood NHL has not changed over time; HIV-positive children still had a higher mortality rate than HIV-negative children throughout the study period. Improvements in the health care system in Uganda, including facilities for early cancer diagnosis, increased access to histological confirmation, and rapid referral to the Uganda Cancer Institute for specialised cancer care, would improve the chances that children

with BL would be diagnosed at earlier stages and properly treated, resulting in lower mortality.

AIM IV: To specifically examine the impact of HIV infection on the clinical characteristics and outcome of childhood lymphoma.

Children with BL in Uganda present frequently with facial disease, irrespective of HIV status. However HIV-associated BL tends to also present with affected extrafacial sites, mainly lymphadenopathy, abdominal sites, and thoracic sites. There is no difference in response to chemotherapy, but HIV-positive children with BL have poorer survival than HIV-negative children with BL. There is a need for further characterisation of BL to understand the role of HIV in the process and outcome of this disease. This understanding would help improve treatment approaches and possibly the prognosis of HIV-positive children with BL.

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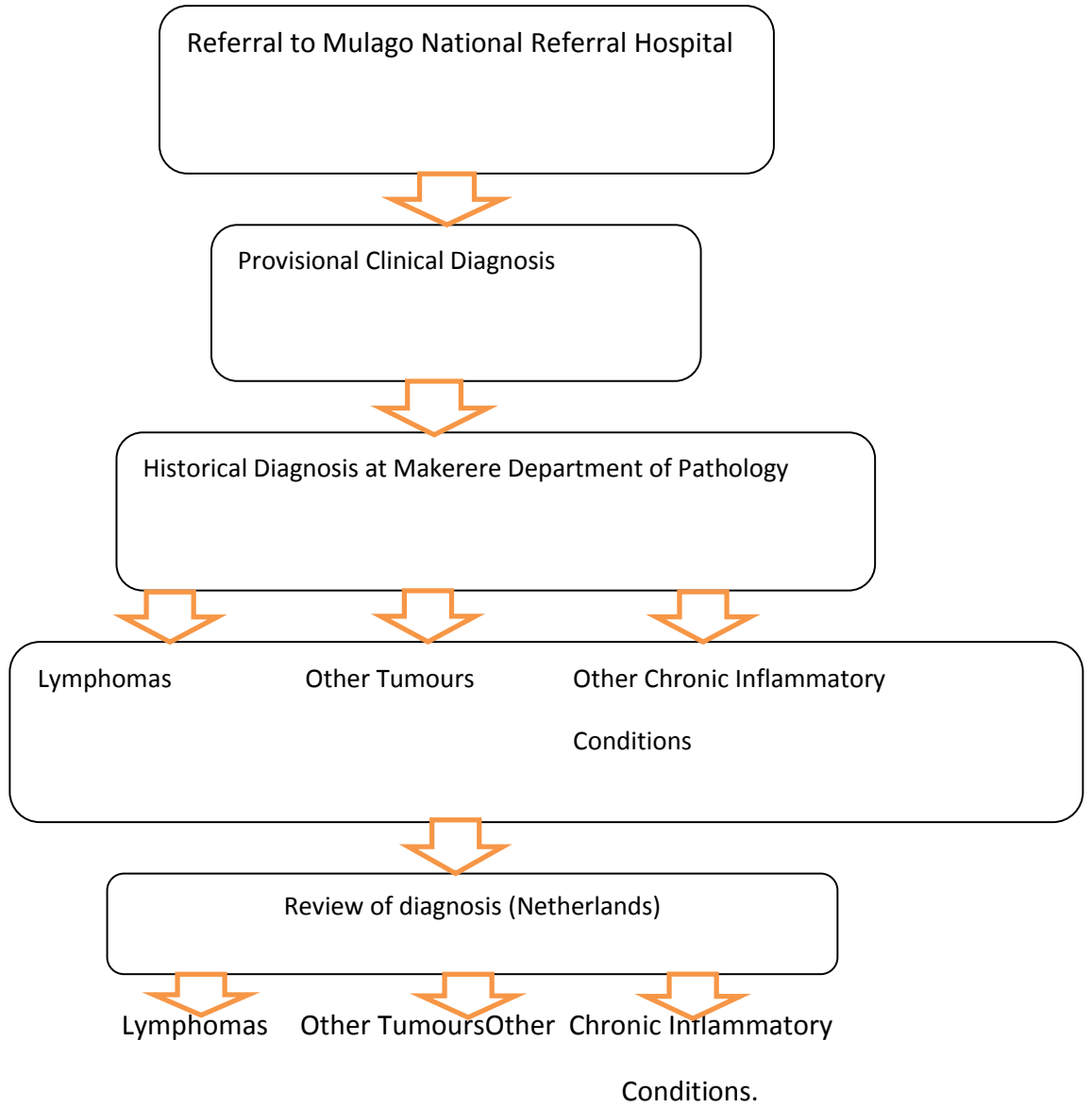
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13 APPENDICES

Appendix 1.Outline of Study Diagnostic Process for Papers I and II

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┌─────────────────────────────────────────────┐
│  Referral to Mulago National Referral Hospital │
└─────────────────────────────────────────────┘
                    ⇩
┌─────────────────────────────────────────────┐
│  Provisional Clinical Diagnosis                │
└─────────────────────────────────────────────┘
                    ⇩
┌─────────────────────────────────────────────┐
│  Historical Diagnosis at Makerere Department of Pathology │
└─────────────────────────────────────────────┘
         ⇩              ⇩              ⇩
┌─────────────────────────────────────────────┐
│  Lymphomas    Other Tumours    Other Chronic Inflammatory │
│                                Conditions      │
└─────────────────────────────────────────────┘
                    ⇩
┌─────────────────────────────────────────────┐
│  Review of diagnosis (Netherlands)             │
└─────────────────────────────────────────────┘
         ⇩              ⇩              ⇩
   Lymphomas    Other TumoursOther  Chronic Inflammatory
                                    Conditions.
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Appendix 2. Supplemental materials from Paper III on survival among 1217 children with Burkitt Lymphoma by period of diagnosis, HIV status, ART era
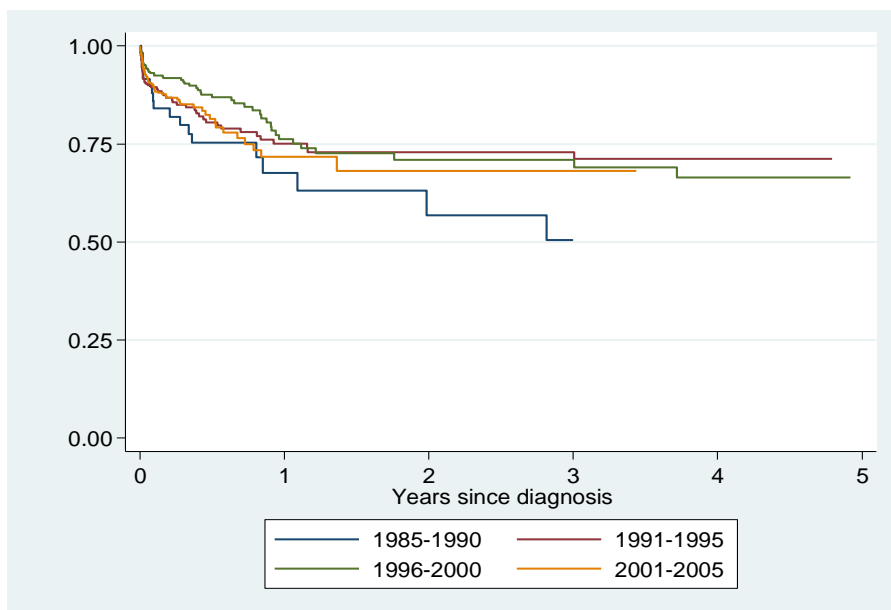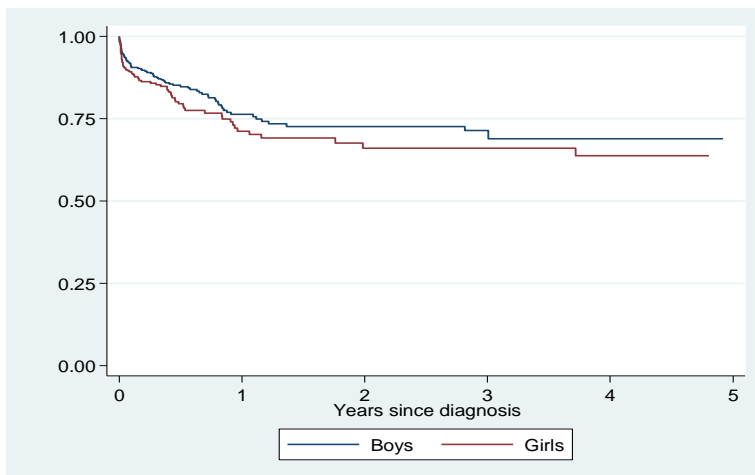

a. Survival in years among 1217 children aged <18 years diagnosed with Burkitt's lymphoma at the Uganda Cancer Institute during 1985–2005 according to period of diagnosis. Log-rank test: $\chi^2_{(1)} = 5.72$, P = 0.126.
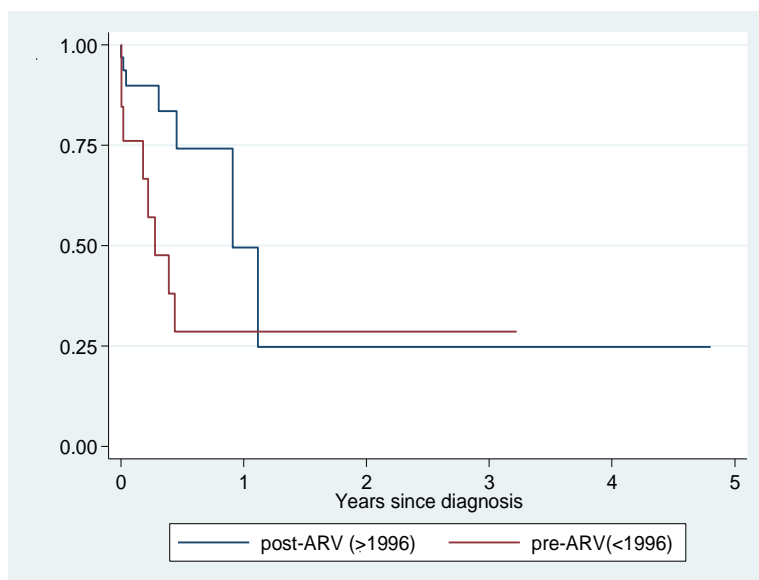
b. Survival in years among 45 HIV-positive children aged <18 years diagnosed with Burkitt's lymphoma at the Uganda Cancer Institute during 1985–2005 according to the introduction of antiretroviral (ARV) drugs in the country. Log-rank test: $\chi^2_{(1)} = 3.51$, $P = 0.061$.
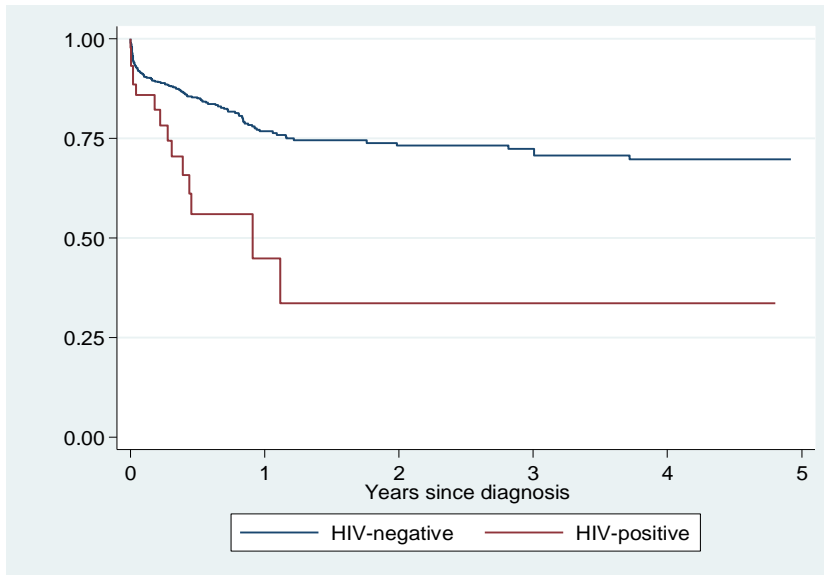
b. Survival in years among 1163 children aged <18 years diagnosed with Burkitt's lymphoma at the Uganda Cancer Institute during 1985–2005 according to HIV status. Log-rank test: $\chi^2_{(1)} = 12.62$, $P < 0.001$.

c. Survival in years among 1217 children aged <18 years diagnosed with Burkitt's lymphoma at the Uganda Cancer Institute during 1985–2005. Log-rank test: $\chi^2_{(1)} = 2.87$, $P = 0.091$.