IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BURKITT'S LYMPHOMA IN CHILDREN FROM *P. FALCIPARUM*, HIV AND EBV PREVALENT SETTING AT MTRH IN WESTERN KENYA

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been submitted for any academic award in any institution; and shall not be reproduced in part or full, or in any format without prior written permission from the author and/or University of Eldoret.

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To all my family members

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ABSTRACT

Burkitt's lymphoma (BL) is a B cell non-Hodgkin's lymphoma (NHL) that affects children in equatorial belt of Africa. The region overlaps geographic areas with high prevalences of infectious diseases. Differential diagnosis of BL with tumours presenting similar clinical and morphological feature presents a challenge in the region. Many Kenyan public health institutions use clininal features and morphology by haematoxylin and eosin (H&E) to diagnose BL. This study carried out molecular characterisation of BL by immunohistochemistry (IHC), *c-myc* gene translocation, IgV_H mutation and cellular microRNA expression at Moi Teaching and Referral Hospital (MTRH) in high prevalence of EBV, HIV-1/2 and *Plasmodium falciparum* setting in Western Kenya. A prospective comparative study of 104 children with clinical and histological diagnosis of B cell NHL was undertaken. Formalin fixed paraffin embedded (FFPE) tissue sections were stained by H&E, followed by IHC for CD10, CD20, CD38, CD44, BCL-2, MYC protein and Ki-67, *c-myc* gene t(8;14) translocation by fluorescence *in-situ* hybridization (FISH), IgV_H mutations, miRNA expression, HIV-1/2 and EBV ELISA, pfHRP, HIV-1 RNA and haematogical and cytokine analyses were done at MTRH histopathology, AMPATH Reference Laboratory and the Department of Human Pathology and Oncology, University of Siena. Of the recruited NHL participants, BL accounted for 23.9%. Boys were 78.8% of cases, aged 3-16, with a mean of 8.8 ±3.7 years. Ethnic distribution of cases were; Luhya (54.6%), Kalenjin (21.2%), Luo and Kisii (9.1%) and mainly came from poorer socioeconomic backgrounds. The presentations sites were; abdomen (46%), jaw (33%) and others (21%). All BL tumours showed moderate to strong expression of CD10, CD20, CD38, high Ki-67 proliferative index (100%), MYC^+ or MYC^- expression. A MYC^+ status was associated with an unfavourable outcome. Incidence of BL was 1.4 times greater in EBER+ participants (OR: 1.39, 95% CI: 0.16–12.19) and 1.6 times greater in HIV^+ (OR: 1.58, 95% CI: 0.35–7.18), regardless of age and gender. There were variable *P.falciparum* and WBC values in various study groups. The number of IgV_H gene mutations ranged from 15-25 in BL $EBER^{+/-}$ cases and showed elevated expressions of hsa-miR-34a and hsa-miR-127 compared to control cell lines. The Th2, Th17, IL-6 ($p = 0.152$) and IL-10 ($p = 0.363$) cytokines were elevated, while Th1 cytokines IFN-γ, IL-2 and TNF-α were decreased in BL cases compared to non-BL cases. Other non lymphoid paediatric tumours occurred at MTRH. Expression of CD10, CD20, CD38, Ki-67 and $MYC^{+/-}$ can permit a more accurate BL diagnosis in addition to mutated IgV_H and upregulated IL-6, IL-10; *hsa-mR*-127 and-34a. Immunostaining of MYC protein can serve as a screening tool for which FISH test may be necessary. Pathogenesis mechanisms associated with various immune modulating infectious agents and microRNA's appeared to exist. Other molecular, immunoregulatory determinants and apparent changing anatomic site of presentation require further studies in a larger cohort over a longer period of time.

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LIST OF ABBREVIATIONS AND ACRONYMS

AICD – Activation induced cytidine deaminase

AIDS – Acquired Immunodeficiency syndrome

ARL – AIDS-related lymphomas

BCL-2 – B cell lymphoma 2 antigen

BCR- B cell receptor

bHLH – Basic helix-loop-helix protein structural motif

BL– Burkitt's lymphoma

cADPR- cylic-adenosine diphosphate ribose

CALLA-common acute lymphoblastic leukaemia antigen

CD- Cluster of differentiation

CDR- Complementarity determing region

CHOP – cyclophosphamide doxorubicin oncovin and prednisone

CIDR 1α- Cystein-rich interdormain region 1 alpha

c-myc- Myelocytomatosis oncogene that codes for a transcription factor

CMV- Cytomegalovirus

CNS – Central Nervous system

DAB – Diaminobendide tetrathydrochloride

DAPI – Diamino-2- phenyl-indole I

DLBCL- Diffuse large B cell lymphoma

DNA – Deoxyribonucleic acid

EBER – Epstein-Barr virus RNA

eBL - Endemic Burkitt's lymphoma

EBNA- Epstein-Barr virus nuclear antigen

EBV- Epstein-Barr virus

EBV-LMP 1- Epstein-Barr virus transforming protein 1

EBV-VCA – Epstein-Barr virus – viral core antigen

ELISA – Enzyme linked immunosorbant assay

EMA – Epithelial membrane antigen

ER – Endoplasmic reticulum

FFPE – Formalin-fixed paraffin embeded

FISH- Fluorescence *in-situ* hybridisation

GC – Germinal centre

HAART- Highly active antiretroviral therapy

H&E- Haematoxylin and eosin staining

HHV-8 – Human herpes virus 8

HIV – Human Immunodeficiency virus

HL –Hodgkin's lymphoma

HLA – Human leukocyte antigen

HSV1/HSV2- Herpes simplex virus 1/ Herpes simplex virus 2

ICD – International disease code

Ig - Immunoglobulin

IHC – Immunohistochemistry

IL - Interleukin

IR- Incidence rate

IREC- Institutional research ethics committee

ITN – Insecticide treated net

K Kappa

kB- Kilobase pairs

kD- kilo Dalton

KS - Karposi's Sarcoma

KSHV – Kaposi's sarcoma herpes virus

LCA – lymphocyte common antigen

LL- Lymphoblastic lymphoma

LMP- Latent membrane protein

MALT- Mucosal associated lymphoid tissue

MAX- Pprotein inhibitor

M-CSF – Monocyte colony stimulating factor

miRNA – MicroRNA

mRNA -Messenger RNA

MTRH- Moi Teaching and Referral Hospital

MYC – MYC protein

NACOSTI – Kenya National Commission for Science, Technology and Innovation

NAD- Nicotinamide adenine dinucleotide

NHL – Non Hodgkin's lymphoma

NK – Natural killer cells

PEPFAR - US President's Emergency Program for AIDS Relief

PCNL – Primary central nervous system lymphoma

PfHRP2- *Plasmodium falciparum* specific histidine rich protein 2

PI- Proliferation index

PL –Plasmablastic lymphoma

PTLD- Post transplantation lymphoproliferative disorder

q24; q32 'q' arm region 2 sub region 4 , region 3 sub region 2 cytogenetic bands

q8 -Long arm of chromosome 8

qRT-PCR- Quantitative real-time polymerase chain reaction

REAL – Revised European –America classification of lymphoid neoplasms

sBL – Sporadic Burkitt's lymphoma

SHM – Somatic hypermutation

sIgM- Surface IgM

TdT-Terminal deoxynuleotidyl transferase

TGF B – Transforming growth factor beta

Th – T- helper cells

TLR-Toll-like receptor

TRAIL- Tumour necrosis factor related apoptosis inducing ligand

UTR – Untranslating region

V_H D_H J_H -Variable, Diversity, Joining loci of genes encoding VDJ regions of heavy Ig chain

VZV – Vericella zoster virus

WHO- World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Burkitt's lymphoma (BL) was first documented by an Irish surgeon, Dennis Parson Burkitt in 1958 as a sarcoma of the jaw in Ugandan young patients, although Sir Albert Cook, a missionary doctor in Uganda, had earlier reported in 1887 seeing children with grossly distorted faces, lesions involving one or both sides of the face on upper and/or lower jaws (Ferry, 2006). Since the 1960's, endemic Burkitt's lymphoma (eBL) type has been reported in West, East and Central Africa from countries which lie in between 10^{o} N and 10^{o} S of the Equator, also known as Equatorial Belt of Africa.

Burkitt's lymphoma seems to be more common in Eastern Africa as compared to other parts of African where malaria is endemic. Incidence rates of eBL in Mali, Nigeria, Congo, The Gambia and Europe are lower than those reported in Eastern Africa (Orem, Katongole, Lambert, Sanjose, & Weiderpass, 2007). Endemic BL epidemiology is thought to overlap with the ecological zone associated with epidemic of malaria and HIV-1/2 in the region (Naresh *et al*., 2011). Burkitt's lymphoma is not only confined to the African continent but also occurs in the United States, Latin America, Brazil, Papua New Guinea and Irianjaya in Asia as well as parts of Europe, pointing to a global occurrence of this disease.

Non-Hodgkin's lymphomas (NHL) are a complex and diverse group of neoplasms. Non-Hodgkin lymphomas usually occur in children. Majority of paediatric NHL are originate from B cells and may present with clinical and pathologic features

resembling Burkitt lymphoma (BL) or other B-cell lymphomas (Lu *et al*., 2011). Burkitt's lymphoma entity further comprises a heterogeneous group of neoplasms of B cell derivation. Its characteristics can be verified by presence of molecular features of B cell, *c-myc* gene dysregulation and B cell receptor (BCR) immunoglobulin M (IgM) mutations (Hecht & Aster, 2000). In younger patients Burkitt's lymphoma tends to arise predominantly from defect in lymphocyte maturation pathway, whereas the lymphoma in older persons appears to be derived from defects on the lymphocyte activation pathway. Arrested maturation in B cell differentiation pathway expands immature B lymphocytes compartment which range from pre-B cells with intra cytoplasmic IgM to B cells with array of B cell differentiation surface markers (Dave *et al.,* 2006).

Within the Equatorial geographical boundaries, eBL is said to account for 50-75% of cancer in children with incidence rates (IR) of 5-10 cases of Burkitt's lymphoma per 100,000 children (Mbulaiteye, Bhatia, Adebamowo, & Sasco, 2011; Naresh *et al*., 2011). Burkitt's and Burkitt's-like lymphomas are the most common childhood malignant tumour with a male preponderance and peak incidence in children aged 6-7 years (Gyasi & Tettey, 2007). Burkitt's lymphoma is the most common and most frequent childhood cancer in equatorial Africa (Bellan *et al*., 2003). In Kenya, lymphomas in general, are estimated to constitute 8.5% of malignant tumours and 45% of cancers in children and young adults, majority of which are B cell non-Hodgkin's lymphoma (Cool & Bitter 1997). A 10-year average annual BL incidence rate for Kenya is estimated at 0.61 per 100 000 children by Mwanda *et al*. (2004). Incidence rate (IR) appears to vary with malaria transmission intensity: low malaria risk (BL IR = 0.39), arid/seasonal (0.25), highland region (0.66), endemic coast region

(0.68), and endemic lake region (1.23) (Rainey *et al.*, 2007; Brady, MacArthur, & Farrell, 2007; Piriou *et al.*, 2009).

Burkitt lymphoma is listed in the fourth edition of World Health Organization (WHO) 2008 classification of tumours of haematopoietic and lymphoid tissues, as a B-cell non-Hodgkin's lymphoma (NHL) with a high proliferative index. The international disease code ICD-O code of 9687/3 refers to Burkitt's lymphoma with nodal modality, while ICD-O code of 9826/3 for the leukemic modality (Jaffe *et al*., 2001). Within Burkitt's lymphoma classification, the WHO recognises three clinical variants namely; endemic (eBL), sporadic (sBL) and immunodeficiency associated BL. Endemic BL is geographically restricted to parts of Africa, Papua New Guinea and large areas of South America. The sites commonly affected by endemic Burkitt's lymphoma tumour includes; maxilla, head and the neck. Endemic Burkitt's lymphoma may also involve other facial bones, kidneys, gastrointestinal tract, ovaries, breast as well as other extranodal sites (Komatsu *et al.*, 2013). The jaw tumours appear predominantly in young children while abdominal tumours predominate with increasing child age. Terminal ileum and lymph nodes are more commonly involved in sBL. Sporadic Burkitt lymphoma (sBL) appears to have no geographical association as it occurs worldwide and mainly affects young adults and most frequently causes intra-abdominal lymphadenopathy (Magrath, 1990).

Climatic, ethnogeographic variations and infectious agents seem to affect the frequency of the different types of Burkitt's lymphoma. This may be attributed to the interplay of varied causative factors lymphomagenesis such as, exposure to ultraviolet light, chemical carcinogens, oncogenic viruses, genetic factors, and cultural practices

among populations (Makata, Toriyama, Kamidigo, Eto, & Itakura, 1996; Rainey *et al.*, 2006). A plant such as *Euphorbia tirucalli,* used in traditional remedy, a home hedge and play activities by children has been associated with Burkitt's lymphoma in previous studies. Besides geographic distribution and clinical manifestation, eBL and sBL may differ in Epstein-Barr virus (EBV) infection status. In the past, endemic Burkitt's lymphoma (eBL) has been reportedly found to be almost always associated with EBV, whereas sBL association with EBV is often low ranging from 10% to 30% positivity in different geographic areas (Bellan *et al*., 2005). Some other previous studies have specifically implied association of non Hodgkin lymphomas in general with a number of herpes viruses (Sitas *et al.,* 1997). Epstain barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), human herpes virus 8 (HHV-8) and a retrovirus human immunodeficiency virus (HIV) have been linked to increased incidences of non-Hodgkin's lymphomas previously (Thapa, Li, Jamieson, & Martínez-Maza, 2011).

Spatial-temporal non-random clustering of BL, observed in a number of studies in East Africa has been interpreted to suggest infectious agents' link in the development of Burkitt's lymphoma (Rainey *et al*., 2006). As such Burkitt's lymphoma is often thought to be a polymicrobial disease involving B lymphocytes tumour triggered by microbial-mediated immune stimulation and acquisition of spontaneous oncogenic mutations due to the ability of B cells to generate antibody diversity through VDJ_H and VJ_L genes recombinations (Rochford, Cannon, & Moormann, 2005).

Both *Plasmodium falciparum* and Epstein Barr virus (EBV) are ubiquitous human pathogens in the Equatorial Belt of Africa. A high degree of exposure to these and

possibly other pathogens are believed to play a role in the aetiology of endemic Burkitt's lymphoma (Matulima *et al.,* 2008). A number of ecological studies have implied positive association between Burkitt's lymphoma and malaria (Ogwang, Bhatia, Biggar, & Mbulatieye, 2008) and available reports show that holoendemic and/or hyperendemic malaria leads to chronic antigen immune stimulation (Ferry, 2006). Children under the age of five years are estimated to suffer an average of nine clinical episodes of malaria illness per year and many remain chronically parasitaemic all year-round (Makata *et al*., 1996). Malaria is one of the leading causes of death among youn children in Kenya and most of sub-Saharan Africa (Kenya National Bureau of Statistics (Knbs) 2010).

Infectious agents such as *P.falciparum*, EBV and HIV can stimulate the surrounding host macrophages and T cells to produce immune mediators such as monocyte colony stimulating factor (M-CSF), interleukin-8 (IL-8), IL-6, IL-10 IL-2, IL-4, tumour necrosis factor-α (TNF-α), interferon- γ (IFN-γ), IL-17, transforming growth factor-β (TGFβ) and other cytokines which may in turn drive or inhibit proliferation of B or T cells (Dranoff, 2004). Tumour cells can exploit host-derived cytokines to promote growth, increase resistance to apoptosis and foster dissemination. Interleukin-6 (IL-6) is a B cell stimulatory factor and has been shown to enhance the growth of EBV transformed lymphoblastoid cell lines in vitro and may therefore have a role in the development of BL tumour (Giadano *et al*., 1991). Decreased cytokine levels after chemotherapy in patients showing positive response to therapy. Thus, it has been postulated that characteristic clinical and histopathological features of malignant lymphomas may be due to activation of cytokine milieu (Kurzrock *et al*., 1993).

Clinical subtypes of BL and related B cell lymphomas may be histologically and morphologically indistinguishable and may or may not harbour specific signature molecular aberration such as Ig*/c-myc* (Ogwang *et al*., 2008). The *c-myc* gene is a cellular oncogene located on the long arm of chromosome 8. This Ig/*c-myc* genetic aberration is a reciprocal translocation of coding sequences of cellular *c-myc* gene on chromosome 8 (q8) and promoter sequences of heavy chain immunoglobulin genes on long arms of chromosome 14 immunoglobulin heavy (H) chain loci or light (κ) or (λ) chains immunoglobulin genes on chromosomes 2 and 22 respectively. Previous studies have indicated that translocation of *c-myc* gene and other proto-oncogene in any of these immunoglobulin (Ig) gene loci always produce cancerous B cell as in lymphomas, leukaemia, and multiple myelomas. The reciprocal translocation disrupts the structure and function of the gene, causing the *c-myc* gene to be constitutively expressed when the immunoglobulin genes are activated (Komano, Maruo, Kurozumi, Takanori, & Takada, 1999). Actively proliferating B cells have increased risk of developing genetic errors, which ultimately increases the risk of developing a cancer such as Burkitt's lymphoma (Kelly and Siebenlist 1985). The dysregulation could be triggered by infectious agents such as *P. falciparum*, Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV). Recently, some studies have shown that *c-myc* gene translocation is considered essential but may not be sufficient for BL to develop.

The precise molecular location of the breakpoints on both chromosome 8 and 14 vary. In endemic BL which is consistently associated with Epstein-Barr virus (EBV), breaks occur up to 75 kB 5' upstream of the *c-myc* oncogene, whereas in sporadic BL most of the translocation occur around exon-1 of *c-myc* gene sequence. Although the latter breakpoint alters the *c-myc* gene RNA transcripts, the breakpoint region affected is

untranslated and so the amino acid sequence of the resultant MYC protein is unchanged. The overall consequence of both rearrangements is increased expression of MYC protein (Bower, 1992).

The *c-myc* gene codes for MYC protein which has DNA binding properties The MYC protein contains two structural domains, the leucine zipper domain and the helix-loop-helix motif similar to those in other transcription factors. It is thought that at least one member of the *myc* gene family, usually *c-myc*, is expressed in all proliferating tissues. The MYC protein plays an important role in the control of proliferation and cell differentiation. Increased *c-myc* gene expression may leads to proliferation and abolishment of differentiation. The Ig/*c-myc* translocation and constitutive role of over expression of MYC protein thus contribute to the pathogenesis of BL by preventing the programmed exit of lymphocytes from the cycling compartment (Allday, 2009). Similar genetic rearrangements involving transcription factor, TCF3 and translocation of *c-myc* gene have also been described in HIV-associated BL which mostly resemble sporadic BL at molecular level (Schmitz *et al*., 2012).

Another human protooncogene is B cell leukemia-2 (*Bcl-2*) gene located on chromosome 18. It codes for BCL-2 protein, usually expressed on endoplasmic recticulum (ER), nuclear envelope and mitochondrial membrane. In B cell lymphomas with Bcl-2 gene translocation, *Bcl-2* gene on chromosome 18 locus undergo a reciprocal translocation with chromosome 14 Ig heavy (H) chain locus t(14:18), which puts *Bcl-2* gene in close proximity to immunoglobulin heavy chain gene enhancer, leading to higher levels of expression of BCL-2 protein. Values higher

than normal levels of BCL-2 inhibit apoptosis of B cells; this may equally lead to B cell tumorigenesis (Dave *et al*., 2006).

The overexpression of MYC protein in the absence of *c-myc* gene translocation suggest that a different pathogenetic mechanism other than infectious agents can cause *c-myc* gene translocation and may be involved in their malignant transformation. Gene expression at the post-transcriptional level by microRNA (miRNA) is thought to play malignant transformation role (Leucci *et al*., 2008). The miRNAs are endogenous small noncoding RNAs approximately 21–23 nucleotides, with conserved sequences expressed across diverse species of animals, plants and viruses. They mediate cleavage and/or inhibition of genomic translation by binding to 3' untranslating region (UTR) of messenger RNAs (mRNAs). For this reason, microRNAs are considered critical gene regulators and play essential role in controlling cell growth, differentiation, morphogenesis and apoptosis. Therefore, microRNAs dysregulation, often results in diseases including B cell malignancies (Esquela-Kerscher & Slack, 2006).

Patterns in microRNA expression profile and targets prediction can provide better understanding of pathogenesis of B cell malignancies including BL and possibly lead to novel strategies for improved BL therapy (Zhang *et al*., 2009). A number of studies have implicated the involvement of microRNAs in cancers in general. Therefore microRNA expression profile studies may be useful tools for diagnosis, prognosis and treatment outcome prediction in lymphomas including BL patients.

In recent years, there have been developments of several specific monoclonal antibodies that are used in identifying lymphomas. To make differential diagnosis of

Burkitt's lymphoma, immunohistochemistry (IHC) and a number of molecular diagnostic techniques are necessary. The approach requires use of amalgamation of distinct parameters namely, cell morphology, immunohistochemistry (IHC), clinical feature, molecular and cytogenetic data. The common IHC panel of antibodies that can be used in diagnosis and differentiation of lymphoid tumours from other malignant lesion includes: lymphocyte common antigen (LCA) or CD45, epithelial membrane antigen (EMA), B cell, T cells markers and secreted cytokines. There are several specific monoclonal antibodies that may be useful for lymphoma classification and differentiation. Each cluster of differentiation (CD) and markers show different pattern of positivity in Hodgkin's, non-Hodgkin's and Burkitt's lymphomas: commonly used T cell markers include: CD2, CD3, CD4, CD5, CD7, CD8, while common B cell markers include: CD 20, CD23, CD32, and CD79a; NK cell marker CD56. For non Hodgkin's lymphoma (NHL): CD15, CD30, CD20, CD45 may be a useful panel (Hedvat *et al*., 2002). Some commonly used Burkitt's lymphoma markers include: Ki-67, CD68, BCL-2, BCL-6, TdT, CD10, CD30, CD21, LMP1 (EBV), Cyclin D1, Bcl-1, MiB-1(Ki-67), PAX-5, p53, TdT and p63. In Hodgkin's lymphoma, each cluster of differentiation (CD) shows a distinct pattern of positivity in pan T cell, CD2, CD3, CD4, CD7, CD8 and pan B (Matuma 2004). Proliferative indices such as BCL-2, p53, BCL-1, BCL-6, CD20, CD23, CD79a, CD56, Ki-67 are useful lymphoma diagnostic and prognostic indicators (Matuma, 2004).

Immunophenotype of B and T cell markers can also be used to define and distinguish B-cell or T-cell lymphomas and may also have distinct prognostic value. T cell lymphomas tend to have worse prognosis than B cell lymphomas. Immunophenotype of these cells may also be useful in defining other recise entities such as small,

medium and large B cell lymphomas as well as different variants of T or NK lymphomas. Data is limited on aberrant cytometric and immunophenotype in BL to demonstrate the role of B and T cell markers in the study settings.

Generally, Immunophenotype of BL show positive reaction for monoclonal antibodies for CD3, CD45RO, CD68, CD20, CD10, BCl-2 and BCl-6. Burkitt's lymphoma is often characterized by a high degree of Ki-67, proliferation index (PI) approaching 100%, of all the malignant B cells. Most but not all cases of BL are generally considered to have dysregulation of the *c-myc* gene and carry *c-myc* 8q24 translocation with Ig heavy chain gene 14q32, t(8q24;14q32) (Queiroga *et al.,* 2008).

Several techniques are being made available to detect these lymphoma molecular characters including conventional cytogenetic analysis, multicolor fluorescene in situ hybridization (FISH), comparative genomic hybridization (CGH) array or gene expression profiling using various DNA polymerase chain reaction (PCR) techniques. In some lymphomas, genetic abnormalities are responsible for the expression of an abnormal protein such as tyrosine-kinase, transcription factor, detectable by immunohistochemistry.

Immunohistochemistry (IHC) and other molecular analyses are currently not available in many of the middle and low income countries including Kenya, despite being crucial in verifying and determining lymphoma diagnosis. These methods, when used together with IHC and fluorescent in situ hybridization (FISH), immunoglobulin (Ig) mutation and microRNA (miRNA) expression profiling can improve diagnosis of BL cases from among other B cell lymphoma cases. MicroRNA expression profiling

studies can further provide better understanting of BL tumour cell characters to help differentiate normal from cancer tissues and also to classify tumour types and grades through known or a unique miRNA expression signature of a given cancer. Burkitt's lymphoma molecular features can then be related to response to therapy and patient survival outcomes.

Accurate and reliable characterization and diagnosis of BL and other non Hodgkin's lymphomas is critical for differentiation of clinically and histologically resembling non-Hodgkin's lymphomas, disease classification, registration, clinical, epidemologic and translational research.

1.2 Statement of the Problem

Non-Hodgkin's lymphomas are the most common lymphomas in East and Central Africa (Tumwine *et al.,* 2009). Non Hodgkin's and Burkitt's lymphomas are heterogeneous in diagnostic categories and response to therapy. Previous studies of lymphoid neoplasm in East Africa and Kenya have been limited to only morphological characterization (Othieno-Abinya *et al.,* 2004). Differentiation of lymphoma subtypes based on histology alone is inadequate as similar histological features are shared by many different biological B cell lymphoma entities. A number of aggressive B cell lymphomas in children resemble BL morphologically and clinically, so cannot be reliably delineated from other subtypes by histology and clinical criteria alone. In children, BL and DLBCL lymphoma types do not differ clinically and morphologically so differential diagnosis between BL and DLBCL is often not as clear.

Distinguishing between BL and other B cell non Hodgkin's lymphomas such as BL and DLBCL is critical from clinical care and treatment outcome standpoint, as the clinical management of related B cell lymphomas may differ significantly (Dave *et al*., 2006). For instance, Burkitt's lymphoma is highly aggressive, rapidly fatal and requires a more intensive chemotherapy protocol, which typically consists of cyclophosphamide, doxorubicin, vincristine and prednisone, with monoclonal anti-B cell antibody rituximab (Bellan Stefano, Giulia, Rogena, & Lorenzo, 2010), in contrast to a closely related diagnostic entitity such as DLBCL.

The World Health Organisation classification (WHO) of tumours of the haematopoietic and lymphoid tissues, 2008 update requires that all lymphomas be classified based on immunophenotype, genetic in addition to morphology and clinical features (Campo *et al*., 2011). Unfortunately, because of lack of facilities and/or expertise for immunohistochemistry, genetic and molecular biology analysis facilities in low and middle in come countries including Kenya, the recommended WHO classification criteria is not being implemented. In the study region, non-Hodgkin's lymphomas, both T and B cell types are not differentiated into the various subtypes but diagnoses are lumped together as one entity referred to as "non Hodgkin's lymphoma". Because of relatively high incidence rate of BL in the study region, its diagnosis is often clinically presumed during care and management of patients (Ogwang, Zhao, Ayers, & Mbulaiteye, 2011). The practising clinicians in these localities have a high suspicion of BL when children present with jaw, neck, and abdominal masses even though other lymphomas such as splenic, marginal zone, DLBCL and general lymphadenopathies may be clinically indistinguishable from BL

and other non-Hodgkin's lymphomas. Lymphomas are also often difficult to distinguish from benign lymphoproliferative/inflammatory disorders and/or other non-malignant lesions in malaria, EBV and HIV-1 prevalent settings such as tuberculous adenitis. Moreover, BL in itself is heterogeneous and may overlap with gray zone-B cell lymphomas unclassifiable with features intermediate between DLBCL and BL (BL/DLBCL). Diffuse large-B-cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) have both overlapping morphologic features and even some cytogenetic features. The *c-myc* gene t(8;14) translocation largely associated with Burkitt's lymphoma is also known to occurs in 5 to 10 percent of cases of DLBCL. Because diffuse large-B-cell lymphoma is more than 20 times as common as Burkitt's lymphoma in some geographic regions, a lymphoma with a t(8;14) translocation can present a diagnostic problem. In addition, BL with different pathogenesis and aetiology such as AIDS-related BL may have different morphologic features from classical or endemic BL.

Insight and understanding of biology of normal cells of the immune system and the relationship of lymphoid neoplasm to these normal cells have the potential to help refine characterization of lymphoid neoplasm such as Burkitt's lymphoma and identification of various subtypes of non-Hodgkin's lymphomas in this study setting. This would be progress towards fulfilling the WHO criteria of immunohistochemisty (IHC) phenotyping, genetic translocation (by FISH), cell lineage, tumour location, clinical features in addition to histological characteristics, for definitive diagnosis and sub classification of lymphomas (Swerdlow *et al.*, 2008; Jaffe *et al.,* 2008).

Reliance on haematoxylin and eosin histology and light microscopy and occasionally a small panel of antibodies of immunohistochemistry staining, in middle and low

income countries in Africa including Kenya, may be insufficient for BL tumour diagnosis (Naresh *et al*., 2011). Diagnostic and clinical care decisions based on histology findings alone in these circumstances have shortcomings notably misdiagnosis of BL and other non-Hodgkin's lymphomas (Tumwine *et al.,* 2008).

It is not clearly understood whether atypical Burkitt's lymphoma is a biologically distinct entity or a morphologic variant of Burkitt's lymphoma. Immunophenotyping by immunohistochemistry and other techniques are critical in differentiating and accurately identifying various morphological forms of lymphoma entities. Accurate diagnosis of Burkitt's lymphoma is of clinical, prognostic and therapeutic importance. Accurate diagnosis of Burkitt's lymphoma should involve concurrent morphologic, immunophenotypic, molecular and cytogenetic characterization. However, in many middle and low income countries such as Kenya, this is not possible due to capacity limitations. Thus, over reliance on histologic to diagnose and classify malignant lymphomas. The reliability of haematoxylin and eosin (H&E) staining for the diagnosis of Burkitt's lymphoma has not been fully assessed in many of these countries.

The diagnostic histological hallmark for Burkitt's lymphoma is "starry-sky" pattern formed by the phagocytic histiocytes interspersed among primitive round monomorphic and deeply basophilic/lymphoblasts. This feature is also found in a number of other high grade non-Hodgkin's lymphomas and is not present in Burkitt's lymphoma with atypical morphology. Previous studies using morphology alone to identify lymphomas have shown that the diagnosis of specific types of non Hodgkin

lymphoma is low (Lukande, Wabinga, & Tumwine, 2008). This undermines patient care, cancer case registration, epidemiology and translational research.

Poor diagnostic capability and/or weak/nonexistent national cancer registries can result in limited disease descriptions and underdiagnosis of lymphomas emanating from African countries including Kenya. Immunohistochemical and molecular studies are lacking in the study region where diagnosis is largely dependent on morphology alone, hence the need for this study.

1.3 Justification of the Study

Western Kenya provides a variety of geographic features and climatic conditions, and is composed of diverse ethnic groups in Kenya including minority groups such as Asians, Somalis, Europeans, and Arabs. Moi Teaching and Referral Hospital (MTRH), is situated in Eldoret, North Rift, receives surgical biopsy specimens and patients referred from county hospitals, mission hospitals, private hospitals, clinics, and county referral hospitals across western Kenya. The population predominantly lives in agricultural or rural settlements with simple life styles with little exposure to pollution and other cancer risk factors. However, professionals, business people, office workers, and traders work and live predominantly in the urban centres.

It is difficult to precisely diagnose lymphoma and Burkitt's lymphoma in resource constrained settings. The study region covered varied geographic areas of lowland Lake Victoria and surrounding highland, associated with varied infectious diseases including malaria, EBV and HIV/AIDS among others. In this setting, infectious disease with clinical manifestations which resemble Burkitt's lymphoma clinically

such as such as parasitic infections like leishmaniasis, fungal infections, Hodgkin's and other lymphomas/leukaemia occur. The three epidemiologic variants of BL are partly defined by geographical occurrence but histologically indistinguishable and their aetiology incompletely understood (Lu *et al*., 2011).

Ancillary tests including Immunohistochemistry, molecular and immunologic techniques are therefore necessary tools in verifying diagnoses of lymphomas with equivocal morphological findings (Lukande *et al*., 2008). Analyses of the molecular features of Burkitt's lymphoma should permit a more accurate and reproducible diagnosis than can be possible with use of standard histological haematoxylin and eosin staining alone. This may be further strengthened by using cytogenetic analysis of touch preparations from fresh or frozen tissue and detection of *c-myc* gene rearrangements. The Ig/*c-myc* gene translocation may be present in some but not all BL cases. Whereas low level *c-myc* gene rearrangements have been reported in healthy Caucasians, the frequency and natural history of *c-myc* gene translocation in healthy Africans is unknown (Ogwang *et al*., 2008).

Survival rate of Burkitt's lymphoma in equatorial Africa very low compared to over 90% overall in children in Europe. Yet BL is a highly curable disorder when treated with modern intensive chemotherapy (Kelly *et al.*, 2009).

Immunohistochemical and other molecular biology techniques can greatly increase the identification of distinctive lymphoma entities as well as delineate it from condititions due to infections, lymphoproliferative disorders and non-malignant diseases. Immunophenotyping and molecular characterisation of Burkitt's lymphoma is highly likely to contribute greatly to the understanding of the biology, pathogenesis and

classification of this and other lymphomas (Patkar *et al*., 2008; Kelemen *et al*., 2010). If the findings from this and similar investigations are integrated with clinical variables and routine histopathology, the diagnoses and management would have more clinical relevance, providing information pertinent to the treatment , prognosis and epidemiological studies. The knowledge gained about molecular BL cell markers can also be used for prognostic purposes, allowing for early detection and advance prevention.

MicroRNAs profiling is a promising novel tool to explore the molecular features of BL (Lenze *et al*., 2011). Burkitt's lymphoma miRNA expression is thought to be important because of different pathogensis pathways of BL and thus a promising tool for diagnosis, prognosis and outcome prediction in lymphoma patients. Studies of miRNA expression profile are likely to provide molecular signature of BL cases whose are *c-myc* gene translocation-negative that are often wrongly treated as DLBCL. Additional molecular characteristics such as immune profiles of Burkitt's lymphoma patients can add value to diagnostic, prognostic and contribute to WHO description criteria of lymphioid neoplasms. Description of more BL molecular biomarker may help elucidate etiological mechanisms involved in BL tumorigenesis. Lymphoma molecular features can also shed more light on the underlying molecular dynamics in involved in the development of these tumours.

Findings of this study are expected to make important contribution in accurate and reproducible description and diagnosis of Burkitt's lymphomas characterization and strengthen cancer registry in Kenya. This will in turn allow monitoring of trends in

lymphoproliferative disorders in high EBV, HIV-1 and malaria prevalent settings and better diagnosis, treatment and prognosis of the various forms of Burkitt's lymphoma.

1.4 Research Questions

1. What immunophenotype and molecular features characterise Bl and treatment outcome at MTRH?

2. What is the distribution and proportion of BL in childhood Non Hodgkin's lymphomas diagnosed in MTRH?

3. What is the reliability of immunohistochemistry in diagnosing BL at MTRH?

4. What is the association of *P.falciparum*, HIV and EBV with Burkitt's lymphoma pathogenesis among children at MTRH in western Kenya?

5. What immunologic parameters characterise BL in the study setting?

1.5.0 General Objective

To characterize Burkitt's lymphoma by morphology, immunologic, immunohistochemistry, t(8; 14) *c-myc* translocation, miRNA profile and Ig mutations in high HIV-1, EBV and *P.falciparum* prevalent setting in western Kenya.

1.5.1 Specific Objectives

i. To verify molecular features of BL by immunohistochemistry, FISH, IgV_H mutations and miRNA expressions at Moi Teaching and Referral Hospital (MTRH) in western Kenya and correlate with treatment outcomes.

ii. To determine the distribution of BL at Moi Teaching and Referral Hospital (MTRH) in western Kenya

iii. To find out the reliability of immunohistochemisty, haematoxylin and eosin (H&E) in the diagnosis of Burkitt's lymphoma patients at Moi Teaching and Referral Hospital (MTRH)

iv. To determine the association of *P.faciparum,* HIV-1 and EBV and with BL pathogenesis in patients attending treatment at Moi Teaching and Referral Hospital (MTRH)

v. To evaluate the level of immune parameters, IFN-g, IL-2, and IL-6,IL-4, IL-10, TNF-a and TGF-β levels in Burkitt's lymphoma cases at Moi Teaching and Referral Hospital (MTRH)

CHAPTER TWO

LITERATURE REVIEW

2.1 Burkitt's Lymphoma

Burkitt lymphoma (BL), initially thought to be a sarcoma of the jaw, was first described in Uganda in 1958 by Denis Parson Burkitt, although, Sir Albert Cook, a missionary doctor in Uganda had reported seeing children with similar distorted facial features in 1887. Later the condition was confirmed to be a distinct subtype of B cell non Hodgkin's lymphoma (Walusansa, Okuku, & Orem, 2012). Initially, it was thought to be only confined to certain geographic areas of Africa but was subsequently reported to also occur sporadically throughout the world and in immunodeficiency conditions.

Burkitt lymphoma is a tumour of haematopoietic and lymphoid tissues. It is listed in the World Health Organization (WHO) classification of hematopoietic and lymphoid tumours as a B-cell high-grade non-Hodgkin's lymphoma, highly aggressive (with a high proliferative index), one of the fastest doubling time among lymphomas. Kinetic studies of Burkitt's lymphoma cells show a potential doubling time of about 24 hours, with a cell-loss rate of 70 percent of the cell-renewal rate (Ziegler, 1982). It is the fastest growing of human tumours. Burkitt's lymphoma is also extremely sensitive to chemotherapy. This lymphoma mostly affects individuals in the first decade of life. Since 1960's, Burkitt's lymphoma has been reported in African, United States, Latin America, Brazil and Papua New Guinea/Irianjaya in Asia, parts of Europe and other countries, implying global occurrence.

After the description of classical or endemic Burkitt's lymphoma (eBL) in East Africa, cases of sporadic BL variants were later recognised in different parts of the world, bearing close histologically and cytologically similarities to classical or endemic Burkitt's lymphoma (eBL) but quite different in clinical pattern (Cogliatti *et al*., 2007). The WHO classification recognizes three subsets of BL, namely: endemic, sporadic, and immunodeficiency associated. Morphologically and histologically, there appears to be differences between endemic BL (eBL), sporadic BL (sBL) and BL with plasmacytoid differentiation, seen among immune deficient children. Each BL subset affects different populations with different clinical presentations and forms (Swerdlow *et al.*, 2008).

Endemic Burkitt's lymphoma occurs in sub-Saharan Africa affecting mainly children, but largely confined within the lymphoma belt of equatorial regions of Africa also known as the Lymphoma Belt. The BL disease incidence varies geographically with boys seemingly more susceptible compared to girls (Gyasi & Tettey, 2007). In most cases it occurs at 4–7 years of age, with boys affected twice as frequently as girls (Mwanda *et al*., 2004) It usually involves the jaw and other facial bones, kidneys, gastrointestinal tract, ovaries, breast, and other extranodal sites (Komatsu *et al*., 2013). Sporadic BL is a worldwide phenomenon with no specific geographic or climatic association. Sporadic BL most commonly presents in the abdomen, ovaries, kidneys, omentum, and Waldeyer's tonsillar rings. Endemic BL is strongly associated with EBV infection, but the etiology of sporadic BL has yet to be defined.

The Lymphoma Belt stretches from about 10º north to 10º south of the equator (Mannucci *et al*., 2012). Within these latitudes, Burkitt's lymphoma tends to occur in

regions where the mean minimum temperature exceeds 15·5ºC and the rainfall is higher than 500 mm per year. The disease appears to be more common in damp, humid regions where the highest incidence rate of 5-15 cases/ 100,000 is found , and is absent in largely arid and semi arid regions, while the lowest incidence of 2-3 cases/1,000,000 occurs in the United States and Europe with sporadic Burkitt's lymphoma as the predominant type (Brady *et al*., 2007). Endemic Burkitt's lymphoma is usually associated with infection(s), Epstein-Barr virus (EBV) BL commonly presenting in the jaw and/or facial bones, orbit, paraspinal regions, mesentery, ovaries, breast and other extranodal sites and sensitive to chemothepy.

Burkitt's lymphoma defines a distinct syndrome of large nodal or extranodal tumours affecting the bones of jaw and abdominal viscera, mainly kidneys, ovaries, mesenteric and peritoneal structures (Hecht & Aster, 2000). Occasionally Burkitt's lymphoma patients may present with isolated tumours of the thyroid gland, distal long bone, skin, breast, testes and parotid glands. Involvement of the central nervous system is an unusual presenting feature, but it is a common manifestation of relapse after remission has been achieved with suboptimal chemotherapy (Ziegler, 1982).

In contrast, sporadic Burkitt's lymphoma occur worldwide with no geographic or climate association, infrequently involves EBV infection and commonly presents in the abdomen, often involving the distal ileum, caecum, or mesentery, ovaries, kidneys, omentum, Waldeyer's tonsillar ring and rarely involves the jaw (Ferry, 2006). Bulky and disseminated disease is present in extranodal involvement of the ovaries, Kidney, breasts, and/or central nervous system (CNS). In adults, Burkitt's lymphoma less than 1% of the disease is frequently associated with HIV infection (Newton *et al*., 2001).

Pathologically both endemic and sporadic Burkitt's lymphomas are characterized by extremely high growth fraction and spontaneous cell death. This may produce histological appearance of sheets of small to medium-sized mononiorphic lymphoid cells with prominent basophilic cytoplasm. The nuclei are usually interspersed with scattered benign macrophages containing cellular debris from apoptotic neoplastic cells, giving a "starry sky" appearance (Crawford, 2001). Cytoplasmic lipid vacuoles are usually evident on imprints and smears, suggesting defective lipid metabolism in BL tumour cells. Occasionally a predominance of larger cells is found and this may cause confusion in appearance between BL and large B-cell lymphoma such as diffuse large B cell lymphoma. This latter histologic appearance of Burkitt's lymphoma was referred to as Burkitt-like or non-Burkitt's lymphoma or atypical Burkitt's lymphoma in revised European-American lymphoma (REAL) classification (Ferry, 2006). The current WHO classification of hematopoietic and lymphoid tumours, 2008 update, instead recognizes a provisional category of aggressive B-cell lymphomas that share morphological, immunophenotypic and gene expression profile-based features intermediate between Burkitt's lymphomas and diffuse large B-cell lymphomas (BL/DLBCL), unclassifiable, with features intermediate between BL and DLBCL (Leoncini, Raphael & Stein, 2008; Swerdlow *et al*., 2008).

2.2 BL Diagnosis and Treatment

Distinguishing of BL from other forms of B cell lymphoma is critical through accurate and reproducible lymphoma diagnosis to improve management of patients with lymphoma (Adelusola, Titiloye, Rotimi, & Durosinmi, 2009).

For good outcome prediction of patients with malignant diseases like lymphomas, it is also important to know how well their immune systems recognise, respond and eliminate the pathological cell clones. T-cell immune response is one of the most considerable anti-tumour defence mechanisms (Váróczy, Gergely, Miltényi, Aleksza, & Illés, 2005). This can be done by determining the level of activated T cells/ factors participating in the anti-tumour defence in the blood of lymphoma patients both by flow cytometry and immunohistochemistry techniques. Both $CD4^+$ helper and $CD8^+$ cytotoxic T cells bear human leucocyte antigen (HLA) HLA-DR/CD38 molecules as important surface activation markers. HLA-DR/CD38 is considered as a late activation marker together with Th1, Th2, and Th17 cytokines.

A potential mechanistic link between endemic BL and HIV-1 associated BL is the polyclonal B-cell activation that occurs after malaria, EBV and HIV infections. By focusing on the interaction between malaria and EBV in areas where there is the highest incidence rate of BL and a clear epidemiological link between EBV and holoendemic malaria, is an attempt to elucidate the molecular and cellular interactions between these pathogens in the pathogenesis of BL (Rochford *et al*., 2005; Mbulaiteye *et al*., 2010). Despite the fact that HIV-1 infection is more prevalent in parts of sub-Saharan Africa than elsewhere, there are few epidemiological studies from Africa that investigated the scale of the excess risk of cancer in HIV-1 infected as compared to uninfected children (Stefan *et al*., 2011). Some reports, however, indicate that Burkitt's lymphoma that arises in the setting of both EBV and HIV-1 infections tends to appear more aggressive with immunoblastic differentiation and therefore likely to be misdiagnosed morphologically (Bishop, Rao, & Wilson, 2000). It is unclear whether BL in a geographic area, such as western Kenya with high prevalences of

viral infections like Epstein–Barr virus (EBV) and human immunodeficiency virus (HIV) is a uniform biological entity.

Most lymphoma treatment regimens are specific to certain subtype classification of lymphoma (Dave *et al.,* 2006). Compared to other high grade B cell lymphomas, Burkitt's lymphoma responds best to specific chemotherapeutic regimens that differ from other aggressive B cell lymphomas. Burkitt's lymphoma has poor outcome when treated with chemotherapeutic regimen used for diffuse large B cell lymphoma (DLBCL) and therefore continues to kill many children in resource constrained countries such as Kenya that do not have the capacity for proper disease diagnosis. Burkitt's lymphoma is rapidly fatal if untreated but it is curable with intensive CHOP chemotherapy regimen containing cycloposphamide, doxorubicin, and vincristine with monoclonal anti-B cell antibody rituximab. A relatively low-dose chemotherapy regimens such as cyclophosphamide, doxorubicin, oncovin and prednisone (CHOP) are typically used to treat diffuse large-B-cell lymphoma, which are inadequate for Burkitt's lymphoma, for which more intensive chemotherapy regimens are necessary (Dave *et al.,* 2006). Furthermore, prophylactic intrathecal chemotherapy or systemic chemotherapy that crosses the blood–brain barrier is unnecessary in most cases of diffuse large-B-cell lymphoma. However, such chemotherapy is essential for treating Burkitt's lymphoma, because of the high risk of involvement of the central nervous system associated with it. Treatment of non Hodgkin's lymphoma upfront is pegged on the histologic subtype, disease stage, phenotypic expression as well as immunologic markers (Othieno-Abinya *et al*., 2004). Thus clear distinction between Burkitt's lymphoma and diffuse large-B-cell lymphoma (DLBCL) is critical in their management.

Recent studies in developed countries show marked differences in survival rates for the different lymphoma immunophenotypes (Tumwine *et al.,* 2009). The survival rate of BL paediatric patients in equatorial Africa is still low compared to over 90% survival overall in children in Europe, due inaccurate morphologic diagnosis. Sometimes very low and not even measurable, even in major centres where it would be possible to have higher survival rates (Naresh *et al*., 2011), Unfortunately because of lack of facilities for immunohistochemistry, genetic, molecular and immunologic techniques, many facilities in Kenya are unable to adopt more accurate and reproducible techniques in the classification of lymphomas according to the criteria by WHO 2008 updated from Revised Europen-American classification of Lymphoid neoplasms (REAL).

2.3 Malaria endemicity in Kenya

In Kenya, malaria is the leading cause of death in children under 5 years of age (Kenya National Bureau of Statistics, 2010). Children under the age of five years suffer an average of nine clinical episodes of malaria illness per year and many remain chronically parasitaemic all year-round. The first infection usually occur in the first year of life, peaking at around age 5, tolerance may appear late at age 8 years, in many cases episodes of malaria level off and reduce by age 10 years (Mutalima *et al*., 2008).

Malaria may be described ecologically on the basis of transmission intensity. Kenya has widely varying rates of *P. falciparum* malaria transmission. Five malaria ecologic zones have been described, namely: low malaria risk, arid-seasonal risk, lakeside endemic malaria, highland malaria and coastal endemic malaria (Omumbo *et al.,*

1998). These are in turn defined as follows: Low risk: low parasite prevalence among children aged 0-14 years with several areas experiencing almost no malaria risk, mainly attributable to altitude or elevation and temperature restriction. Arid or seasonal risk: malaria transmission occurs in communities located near water or for a few months of the year where limited annual rainfall results in low level of malaria transmission which may be absent during the following years; low parasitaemia prevalence rates occur among children. Highland malaria: experiences an overall low disease risk on average, there are variations in low disease risk on average due to variations in rainfall and temperature between years that can lead to an epidemic. Parasite prevalence is low but varies widely over small spatial distances. Coast endemic malaria: parasite prevalence often exceeds 50% transmission and maximal disease risk, exhibit seasonality. Lakeside endemic malaria: malaria transmission and disease risk period occurs year round with parasitaemia >50% among the childhood population.

2.4 Burkitt's Lymphoma and Malaria

Denis Burkitt and his associates in 1960s, assessed the geographical distribution and the incidence of a "sarcomous lymphoma" he had described in 1958, and found the ecological distribution of Burkitt's lymphoma to be closely correlated with the same zones as malaria with temperature >15.5^{o}C and annual rainfall >500 mm, which then became known as "Lymphoma Belt" (Burkitt, 1962), suggesting that the occurrence of BL lymphoma may be linked to the distribution of *Anopheles* mosquitoes, the *Plasmodium spp* vector. Since then, both malaria and Burkitt's lymphoma have been reported 10^{o} north or south the Equator and in other areas where malaria is holoendemic such as Papua New Guinea and Brazil. The geographic coincidence of

Burkitt's lymphoma with holoendemic or hyperendemic malaria prompted Burkitt and O'Conor to postulate immune priming lymphomagenesis mechanism probably through reticuloendothelial system (Ziegler, 1982). Although *P. falciparum* is not considered oncogenic, chronic malaria could make an individual susceptible to oncogenic virus such as EBV and then go on to develop Burkitt's lymphoma (Moormann, Snider, & Chelimo, 2011). Chronic malaria infections affect the B cell compartment by inducing polyclonal activation, hyper-gammaglobulinemia leading to a dramatic increase in the levels of circulating EBV. The specific role of *P. falciparum* in the causation of BL is yet to be fully understood, but appear to follow the pattern in malaria endemic areas where the parasite infections usually occur during the first year of life, peaking around age 5 and levelling off thereafter (Orem et al., 2007). It is widely held that malaria by *P. faclciparum* play a role in Burkitt's lymphoma tumorigenesis by disrupting immune mechanisms against Epstein Barr virus (EBV) (Rochford *et al*., 2005).

In the lowlands of Kenya there is high transmission of malaria and a high incidence of Burkitt's lymphomas (Kafuko and Burkitt 1970; Mwanda *et al.,* 2004; Chene *et al.,* 2009), while the children of the same ethnic groups, from lowlands, living in urban areas or in the highlands are much less affected by both malaria and Burkitt's lymphoma (Moormann *et al.,* 2007). Other ecological studies have also pointed to a possible positive association between Burkitt's lymphoma and *P. falciparum* parasitaemia in support of previous studies (Njie *et al.,* 2009). In general, there appears to be close similarity between the world wide distributions of Burkitt's lymphoma and the distribution of holendemic and/or hyperendemic malaria.

There is delayed onset of Burkitt's lymphoma disease in children who have migrated from high risk malaria areas to low risk malaria areas and reduced incidences of BL in areas where malaria has been eradicated (Rainey *et al*., 2007). This supports the hypothesis that *P. falciparum* is an ecological co-factor of BL even though malaria biomarkers related to the onset of BL remain largely unknown. Further, BL case-control studies in Uganda and Malawi demonstrated 5-fold and 12-fold odds ratio, respectively of elevated antibodies against malaria among children with BL compared with controls (Emmanuel *et al*., 2011). Guech-Ongey *et al.* (2012) also found a lower level of malaria anti-SE36, an antigen that has been shown to elicit protective immunity to malaria, in BL children than controls in Ghana. *P. faclciparum* is postulated to induce polyclonal expansion of B cells, impair EBV-specific T-cell responses (Moormann *et al*., 2007) and preferentially stimulates the expansion of EBV- positive B cell by expression of its cystein-rich interdomain region 1-α, a pfEMP-1receptor (Emmanuel *et al.,* 2011).

The cystein-rich inter-domain region 1α (CIDR1α) of *P. falciparum* membrane protein 1 (PfEMP1) expressed on parasitised erythtocyte is a polyclonal B cell activator and leads to hypergammaglobulinaemia. Further, *P. falciparum* infection is known to have general immunosuppressive effects (Chene *et al.,* 2009). This impairs immune control of EBV leading to increased number of circulating EBV-carrying B cells which may lead to increased proliferation of EBV–carrying cells due to impaired T cell immunosurveillance and B cell polyclonal activation (Rochford *et al*., 2005). Thus chronic and intense plasmodia infection plays a crucial background role in the pathogenesis of Burkitt's lymphoma (Burkitt, 1969).

During intraerythrocytic growth of *P. falciparum*, parasite-derived proteins such as PfEMP1, RIFIN/STEVOR and SURFIN are successively expressed, exported and subsequently presented at the surface of the human red blood cell. The dominant polypeptide, *P. falciparum* (PfEMP1), is an adhesin that enables erythrocytes infected with mature stages to adhere in the micro-vasculature and sequester therein, thereby avoiding clearance by the host phagocytic system including the spleen. Latently infected B cells can proliferate and increase their number upon polyclonal activation by cystein-rich interdomain region 1 alpha (CIDR 1α) domain of PfEMP1. This domain is composed of a bundle of three α-helices that are connected by a loop to three additional helices, suggesting that PfEMP1 is a polymer of three-helix bundles (Fleire *et al*., 2006). The PfEMP1 is a large protein, 200-350 kDa encoded by a family of approximately 60 variant genes. Each individual parasite only expresses a single variant gene at a time, maintaining all other members of the family in a transcriptional silent state. Switching of expression to another variant PfEMP1 species occurs at a rate of 1% and allows the parasite to evade host immune clearance mechanisms and therefore prolongs the period of infection (Chene *et al*., 2009).

A high degree of exposure to *P. falciparum* malaria and EBV are considered prerequisites in the aetiology of endemic Burkitt's lymphoma (Piriou *et al.*, 2009). At individual level, association between BL and *P. falciparum* malaria, EBV-lytic antigen $CD8^+$ T cell interferon-γ (IFN-γ) secreting are lower in malaria holoendemic areas (Snider *et al*., 2012) probably suggesting that children repeatedly infected with *P. falciparum* malaria have a cumulative effect of loss of functional IFN- γ producing $CD8^+$ T cells in response to EBV-lytic antigens through apoptosis, deletion, exhaustion or inability to produce IFN-γ. As a result of this apparent immune

suppression, more B cells become latently infected by EBV and distorting the EBV-host balance and gradually increases the risk of BL development. The EBV-lytic antigen $CD8^+$ T cell deficiency appears to be pronounced among children 5-9 years old in malaria holoendemic areas, indicating that there may be age-dependent interaction between *P. falciparum* malaria and EBV specific T cell response (Snider *et al.*, 2012; Moormann *et al*., 2009).

Malaria caused by *P.falciparum* infection is also thought to cause numerous immunological disturbances in humans in addition to raised immunoglobulin levels such as immunoregulation of EBV-specific T cells and a tendency to develop Burkitt's lymphoma (Moorman *et al.*, 2007). The high levels of immunoglobulins found in patients with malaria is probably due to the fact that malarial parasites factors may act as a mitogen which activates B cells, prompting them to secrete immunoglobulin and autoantibodies (Katahaa, Facer, & Holborrow, 1984), this effect may be augmented by T lymphocytes (Ballet, Jaurequlberry, Dkloron, & Agrapart, 1987). The other theory of immunologic disturbance is based on the finding that malaria appears to disrupt cytotoxic T cell control of EBV-infected B cells, thus allowing the outgrowth of B cells and the production of a massive amount of immunoglobulin associated with BL development (Brady *et al*., 2007). In children with acute malaria, immunologic disturbances are linked to T helper cell deficiency. These children often have low blood levels of $CD4^+$ T lymphocytes but normal levels of cytotoxic $CD8^+$ T lymphocytes (Whittle *et al*., 1990). In addition, some experimental evidence suggests that precursor merozoite antigen of *P. falciparum* can activate EBV-infected B cells *in vitro*. Holondemic malaria leads to both hyperstimulation of the humoural immune system and secondary immuneregulation of cell mediated immunity (Bower, 1992).

Malaria infected cells are also thought to constitutively activate *c-myc* gene, a phenomenon present in BL tumours (God & Haque, 2010). The immune stimulation can also be due other infectious agents in addition to *P.falciparum*. Limited data in literature appear to support the idea that certain arboviruses transmitted by insect vectors that occur in zone as malaria could also play a role in inducing cell growth and B cell hypermutation (Orem *et al.*, 2007). Some haemoglobinopathies, such as sickle cell trait or haemoglobin traits have protective effect against malaria. The carriage of sickle cell gene, a genetic marker for reduced risk of severe malaria is reduced in children with BL**.** The geographic association with malaria and the frequent presence of EBV genome (EBER) within BL tumour cells are found in endemic BL, but not in either sporadic BL or HIV-1 associated BL is another indication of possible association.

2.5 Burkitt's Lymphoma and HIV/AIDS

Human Immunodeficiency Virus-1 (HIV-1) represents another infection that may have a role in the pathogenesis of Burkitt's lymphoma. Patients infected with the human immunodeficiency virus have an increased susceptibility to other infection and are at risk of developing malignancies (Bower, 1992). Immunodeficiency increases the risk of cancers that have specific infectious agents in their aetiology such as BL (Parkin *et al*., 2000). Burkitt and Burkitt-like lymphomas account for 30-40% of all HIV-1 associated lymphomas in developed countries. In HIV-1 patients, Burkitt's lymphoma typically occurs early in the course of HIV-1 infection, often before the development of severe immunodeficiency and infrequently in immunocompromised individuals (Bishop *et al*., 2000). But, immunodeficiency *per se* does not seem to be a prerequisite in BL development, which raises the questions whether HIV-1 has a

definite role in Burkitt lymphomagenesis, but *c-ymc* oncogene studies has been shown to be activated in HIV-1 associated Burkitt's lymphoma (Bellan *et al*., 2003).

In Western Europe and America, the increased risk of BL in persons with HIV/AIDS has focused attention to immunosuppression as a co-factor in BL development (Mbulaiteye *et al*., 2011). Where, in adults infected with HIV-1, the development of Burkitt's lymphoma is second to Kaposi's sarcoma as an AIDS case-defining condition. Unlike adults, the great majority of HIV-1 infected children in developing countries acquire the virus through vertical transmission before or during birth or in the first months of life, while the immune system is still developing and prior to exposure to many other antigens to challenge the system (Campidelli, Gazzola, Vitone, & Pileri, 2008). In Africa, there are conflicting reports on the relationship between lymphomas in general and HIV-1, some reports indicating increase in incidence whereas some others showing no increase (Kalungi, Wabinga, Molven, & Bostad, 2009). Data from a study in Uganda reported increased positive association between HIV-1 infection and Burkitt's lymphoma among children living in an area where the tumour is relatively frequent (Ogwang *et al*., 2008). The impact of infection with HIV-1 on the risk of cancer in children is less certain.

Even though HIV-1-associated Burkitt's lymphoma is relatively common in adults than children in developed countries, it may involve children in developing countries (Komatsu *et al.,* 2013). The HIV-1-associated lymphomas are listed as AIDS-related BL according to the World Health Organization (WHO) classification (Bellan *et al.,* 2005). The recent general increase incidence of NHL in sub Saharan Africa is probably due to increase in the number of HIV-1 infected patients in the region. In a

Tanzanian study, Mwakigonja, Kaaya and Mgaya, (2008) reported BL as common malignant AIDS-related lymphomas (ARL), although it is not known why BL may be common in HIV-1 and not in other forms of immunosuppressions.

Acquired Immunodeficiency syndrome (AIDS) related BL usually display an activation of *c-myc* gene translocation that is structurally similar to those found in patients with sporadic Burkitt's lymphoma (sBL) (Hecht & Aster, 2000). In addition, most AIDS related BL's in western countries are EBV negative, while in Africa they are strongly associated with EBV (Bellan *et al.*, 2003). Human immunodeficiency virus 1 (HIV-1) infection is the other known condition that is associated with the frequent occurrence of Ig/*c-myc* translocation having Burkitt or Burkitt-like lymphomas. Other studies indicate that the Tat protein from HIV-1 infected cells play a crucial role in angiogenesis through interaction with vascular endothelial growth factor (VEGF) receptors (Nyagol *et al*., 2008).

The evidence about the causal relationship between HIV-1 and Burkitt's lymphoma has been based on research studies in adult population. A number of these studies suggest approximately 2.5% of children infected with HIV-1 will develop cancer, lower than the proportion seen among infected adults (Mutalima *et al*., 2010). Stefan *et al.* (2011) also found BL to be significantly associated with HIV-1 among South African children.

Generally, HIV-1 infection induces a vigorous immune response with about 10-fold increase in polyclonal B cell activation. Polyclonal immunoglobulin production during HIV-1 infection may be a direct mitogenic effect or may be antigen specific to malaria

and HIV-1 (Allday, 2009). Parasite induced T cell immunosuppression and polyclonal B-cell activation are features of malaria, HIV-1 and other infectious agents. And this appear to suggest that HIV-1 infection may be performing an analogous role to that of holoendemic malaria in the pathogenesis of BL given that HIV-1 associated BL has been shown to carry *c-myc* translocation similar to sporadic BL (sBL) (Bower, 1992).

The predominance of HIV-1 infections in sporadic BL rather than in endemic BL reflects the stage of B-cell ontogeny at which chromosome translocation errors take place. In general, genetic errors occur during $V_HD_HJ_H$ somatic recombination in the presence of an antigen or Epstein Barr virus in case of eBL and later during isotype class switching in the absence of EBV as the case in sporadic BL (Bower, 1992). Human immunodeficiency virus-1 associated Burkitt's lymphoma is Epstein Barr Virus negative (EBV^-) in western countries and may or may not be EBV^+ in Africa (Bellan *et al*., 2003). A further potential mechanism in the pathogenesis of HIV-1 associated BL is thought to involve interleukin-6 (IL-6) and interleukin-10 (IL-10) secretions by HIV-1 infected macrophages. Both IL-6 and IL-10 are B cell stimulatory factors which enhance the growth of EBV transformed lymphoblastoid cell lines and may therefore have a role in the development of BL in HIV-1 infected individuals (Benjamin, Knobloch, & Dayton, 1992). Other molecular factors that have so far been identified as possible agents in this HIV-1 tumorigenesis trigger process include mutations of the tumours suppressor gene *p53* (Giadano *et al*., 1991) and secondary non-random chromosomal abnormalities of band 13q34 driven by this particular etiologic agent.

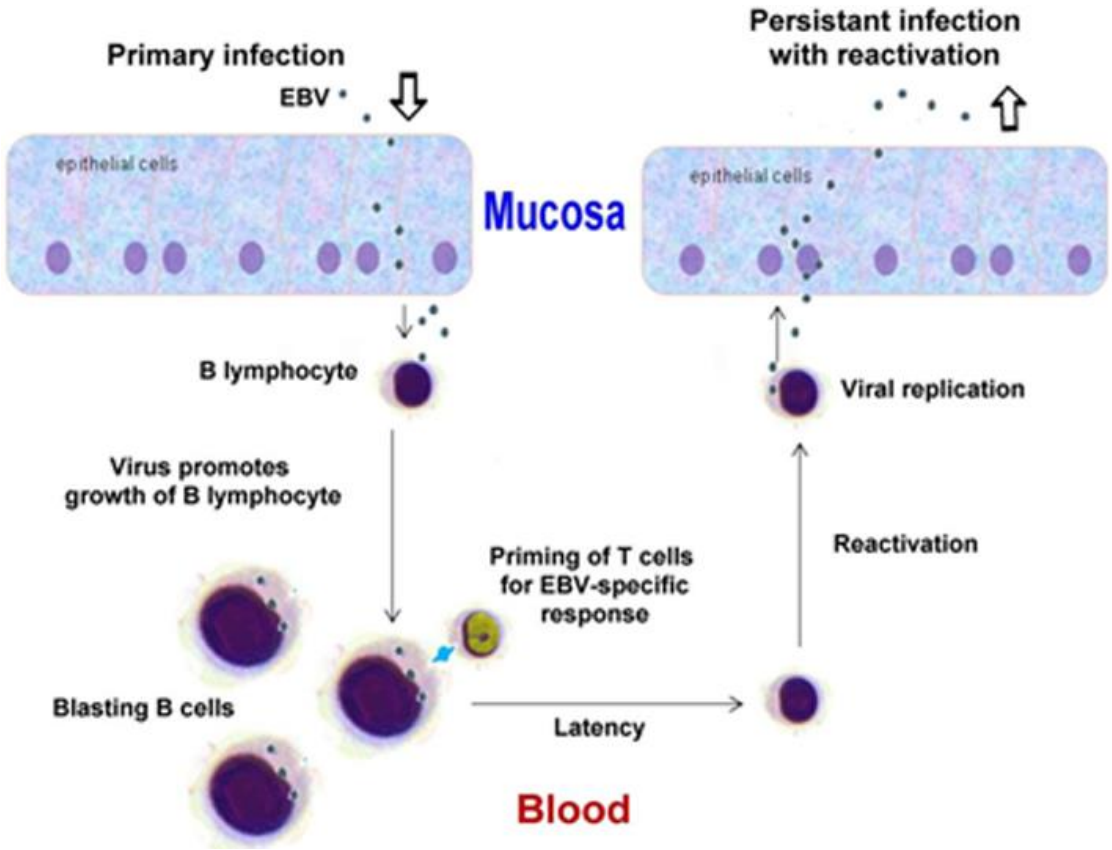
Human immunodeficiency virus-1 (HIV-1) is associated with an increased risk of malignancy, both AIDS defining and non-AIDS defining such as: Kaposi sarcoma (KS), primary central nervous system lymphoma (PCNSL) and high grade B cell non-Hodgkin lymphoma (NHL). The cancers commonly associated with HIV-1 in children include; Kaposi sarcoma, non-Hodgkin lymphoma and leiomyosarcoma (Mutalima *et al*., 2010). The pathogenesis of AIDS associated malignancies is thought to be the result of an opportunistic proliferation of cells due to oncogenic stimuli and an infection driven depressed immune system. Non Hodgkin's lymphomas are generally not considered a major cause of AIDS morbidity in Africa, a possibly of under recognition due to limited of infrastructure to accurately diagnosis these conditions (Mantina, Wiggill, Carmona, Perner, & Stevens, 2010). The fact that lymphomas may not be currently regarded as a significant cause of morbidity and/or death among HIV/AIDS patients is largely because they are not well characterized (Mantina *et al.*, 2010).

Human immunodeficiency virus associated BL (HIV-1-BL) is fairly well described in the developed world and to some extent among HIV positive adults in Africa and can be identified in many geographical area and at all ages. There is paucity of data on associatin between HIV and BL in sub-Saharan Africa, against the reality that the majority of HIV-1 infected children live (Orem *et al*., 2007).

2. 6 Burkitt's lymphoma and Epstein Barr Virus (EBV)

Herpesviridae family represents a very large but relatively clearly defined group of viruses of considerable medical importance including herpes simplex 1 (HSV1), HSV2, varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr Virus

(EBV), also known as human herpesvirus (HHV)-4, 6, 7 and 8 (Young and Rickinson, 2004). Epstein-Barr virus (EBV) is the best known and most widely studied member of the gamma (γ) herpesvirus subfamily. It is an enveloped DNA virus, surrounded by an icosahedral nucleocapsid and a tegument. The EBV genome encodes for a series of products interacting with or exhibiting homology to a wide variety of anti-apoptotic molecules, cytokines, and signal transducers, which promote EBV infection in humans. Epstein-Bar virus (EBV) infection is believed to transform and immortalise B lymphocytes, therefore the virus is potentially an oncogenic virus (Rezk & Weiss, 2007).



EBV life cyce

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Epstein Barr virus (EBV) is ubiquitous in distribution in the environment, infecting more than 90% of the human population worldwide, but the age of infection varies (Mbulaiteye *et al.*, 2006). In sub Saharan Africa, EBV infection *in vivo* generally

takes place in the first few months of life but it is usually asymptomatic with little or no evidence of clinical disease. The infection is mostly latent and asymptomatic when it occurs at an early age and probably as a result of the development to immune tolerance to the virus. Usually, proliferation of infected lymphoblastoid is controlled by inhibitory immunological mechanisms, such as EBV-specific cytotoxic $CD8^+$ T cells (Njie *et al*., 2009). Although EBV may persist in latent form in healthy carriers, the virus is associated with several human cancers (Onnis *et al.*, 2012). While an African child is thought to be infected early in life from the environment, those in developed countries are infected later during adolescent and onset of sexual debut. If the infection is acquired during adolescence or later, as is often the case in these countries, it results in infectious mononucleosis (Onnis *et al*., 2012).

Epstein-Bar virus (EBV is capable of infecting and possibly transforming B cells leading to BL pathogenesis. Epstein-Bar virus (EBV) gains access into B cells involves at least five viral glycoproteins. The EBV binding is partially mediated by the viral envelope protein gp350 which binds to complement receptor 2 (CR2) or complement receptor 3 (CR3) on B cells and tethers the virus to the B cell, allowing viral gp42 to bind to HLA class II proteins (God & Haque, 2010). Binding of EBV to the B cell, gp42 triggers membrane fusion which is carried out by the viral proteins gB, gH, and gL. Epstein-Barr virus (EBV) preferentially infects B-lymphocytes cells and memory B lymphocytes which are relatively long-lived resting cells, where the virus is able to evade recognition and attack by cytotoxic $CD8^+$ T cells and thus able to establish a persistent infection, which may be maintained throughout the host's lifetime. Infection of other cell types such as epithelial cells may also occur but less efficiently. Epstein-Barr virus (EBV) infected cells have been identified in the

oropharyngeal epithelium suggesting an epithelial cell tropism (Young & Rickinson 2004). Virall protein BMRF-2 binds cellular β1 integrins and trigger fusion of viral envelope and epithelial membrane.

The Primary Epstein Barr virus does not usually replicate in B-lymphocytes but instead establishes a latent infection, which is characterized by the limited expression of a subset of virus latent genes. On occasions, Epstein-Barr virus (EBV) may undergo lytic replication, releasing viral progeny, or instead initiate any one of the three latency programmes (Latency I, II, III), all of which involve limited gene expression. Latency I is associated with Burkitt's lymphoma and is characterized by expression of EBNA1 and small non coding EBV RNAs (EBERs). Latency II is associated with Hodgkin's lymphomas (HL) and is characterized by expression of EBNA-1, LMP-1, LMP-2, and EBERs. Latency III is frequently observed in post transplant lymphoproliferative disorders (PTLD) includes expression of all EBNAs, EBERs, and LMPs.

Majority of EBV infected B cells are recognized and targeted by T cell-mediated immune responses, but a fraction of them will instead enter the germinal centre (GC), where they express only three latent viral genes (default program or latency II) (Onnis *et al.*, 2012). In proliferating GC B cells, the process of somatic hypermutation, modifies the DNA of the variable region of immunoglobulin (Ig) genes and GC B cells finally differentiate into memory B cells or plasma cells. In this way the EBV gains access to the memory B-cell compartment of the host, its main reservoir during persistence period, when no latent viral genes are expressed. An exception occurs when the latently infected memory cells divide (during antigen re-encounter), in

which case they express the EBNA-1 protein (latency I), thereby allowing viral DNA to replicate. Epstein Barr virus (EBV) infection and persistence is usually benign and only causes disease when the host-virus balance is upset. The balance relies on equilibrium between viral latency, viral replication, and host immune responses (Donati *et al*., 2006). Persistent or life-long infections are often characterized by low level but chronic lytic reactivation and viral shedding into saliva for transmission. Lytic replication begins by the expression of the intermediate early transcription factors that control switch between lytic and latent cycles of EBV infection – BZLF-1and BRLF-1, then followed by translation of late genes encoding for structural components like the viral capsid antigen (EBV-VCA). In immunodeficiency, as opposed to Primary EBV infection which is usually followed by latency, the proliferation of infected cells can proceed unchecked, and the cells sometimes evolve from a polyclonal reactive process to a monoclonal malignant lymphoma (van den Bosch, 2004).

Epstein-Barr virus (EBV) coded genes can be grouped in; Epstein-Barr Nuclear Antigen proteins (EBNAs), Latent Membrane Proteins (LMP) and Epstein-Barr RNA (EBER-1). During persistent infection, Epstein-Barr virus (EBV) establishes different latency programmes I-III, that relate to differentiation stage, location and origin of the infected cells (Mannucci *et al*., 2012). Latently infected B lymphocytes express eight proteins–EBNA-1, EBNA-2, EBNA-3 EBNA-4 EBNA-5, EBNA-6, LMP-1, -2A and -2B and several non-translated RNA's - microRNA (miRNA). Some of these EBV-encoded RNAs (EBERs) seem to have an anti-apoptotic effect (Nanbo, Inoue, Adachi-Takasawa & Takada, 2002). Typical EBV infection produces an array of antigens including six EBNAs, early antigens, viral capsid antigen, EBV-induced membrane

antigen and latent membrane proteins (LMPs) that includes Epstein-Barr nuclear antigens (EBNA-1 EBNA-2, EBNA-3), latent membrane proteins (LMP1 and 2), and the non-coding RNAs EBER-1, EBER-2, and EBV microRNAs.

EBV latency is exclusively established in resting memory B cells; an infectious virus is produced when memory B cells switch from the latent to the lytic phase that seems to occur after proliferation and differentiation from memory to plasma cells (Chene *et al*., 2009). In vitro, latently infected B cells, the virus can be induced to enter into lytic cycle by a variety of antigenic products namely phorbol esters and anti immunoglobulins. Memory B cells seem to express none of the viral latent proteins and are invisible to the immune system. The persistence of EBV in B lymphocytes is usually maintained under host T cell control. Infection by EBV is thought to induce an early oncogenic event that results in the immortalization of B lymphocytes.

Some proteins associated with EBV infection have been shown to allow unchecked cellular proliferation by evading usual cell cycle checkpoints. The EBV virus has potent B cell growth transformation ability. This ability is thought to facilitate a *c-myc* gene translocation during normal pre-B-cell immunoglobulin rearrangement and lead to the development of a transformed B cell phenotype (Njie *et al.,* 2009). In T cell immunocompromised patients, EBV transformed cells can grow out of control as malignant lymphoproliferative lesions. For this reason, endemic BL has been considered an Epstein-Barr virus (EBV) associated malignancy among children in the HIV-1 prevalent areas and malaria endemic lymphoma belt of equatorial Africa, and also sporadically in other geographical areas where BL occurs as sporadic Burkitt's lymphoma (sBL) among adults.

Another potential contributing mechanism by which EBV infection may lead to Burkitt's lymphomagenesis is its effect on *c-myc* gene function. Chronic stimulation and expansion of B cell, by EBV and other viruses, increases the risk of *c-myc* gene translocation and other oncogenic mutations. The gene expression strategy adjusts to various cellular environment and immune responses (Klein, 2009). Epstein-Barr virus gene product BHRF-l, a Bcl-2 family homologue, has been shown to suppress apoptosis and increase cell survival, an important process in oncogenesis (Bishop *et al*., 2000). It has also been demonstrated that BHRF-l can inhibit *c-myc*-induced apoptosis without affecting *c-myc*'s capacity to promote cell growth, suggesting it may contribute to cellular immortalization and the pathogenesis of Burkitt's lymphomas (Riley *et al*., 2012).

Another supportive evidence for EBV/endemic Burkitts lymphoma association in at least 90% of cases are thought to include the presence of EBV-DNA clonally integrated into tumour tissue and seroepidemiological associations with EBV antibodies. Lymphomas that are positive for EBV express a transforming (LMP-1) (Vereide & Sugden, 2009). Viral Latent membrane proten-1 LMP-1 is thought to play a critical role in the neoplastic transformation of B lymphocytes (Tumwine, Orem, Kerchan, Byarugaba, & Pileri, 2010). Generally, the presence of latent EBV in B cell promotes genetic instability and alteration required for development in BL. EBV probably bring about transformation of B lymphocytes by activating the *c-myc* gene.

A combination of EBV and malaria infections have been linked with Burkitt's lymphoma in time and space for sometime now (Morrow, Pike, Smith, Ziegler, & Kisuule, 1971). Past studies have implied molecular links between B cells; EBV and

P. falciparum antigen pfEMP1 can directly induce EBV reactivation during malaria infections (Piriou *et al*., 2009). Other studies have further associated Burkitt's lymphoma with a reciprocal *c-myc* gene translocation and the consistent presence of EBV antigens (Chene *et al.*, 2009).

2.7.0 Molecular Features of Burkitt's lymphoma

2.7.1 Burkitt's Lymphoma and Ig/ *c-myc* Gene Translocations

A variety of primary and secondary non-random clonal cytogenetic abnormalities are found in lymphoid neoplasms, comprising translocations, inversions, insertions, duplications, amplifications, deletions, and aneusomy (Ventura *et al*., 2006). Reciprocal chromosomal translocation is another molecular feature of BL, in which the activation of the *c-myc* oncogene occurs through, juxtaposition of *c-myc* gene on chromosome 8 to the immunoglobulin (Ig) heavy chain locus on chromosome 14 or the kappa or lambda light chain locus on chromosome 2 or 22. Though some less than 10% of BL cases lack an identifiable *c-myc* gene rearrangement using fluorescene *in-situ* hybridization (FISH) analysis, both split and fusion probes for t(8;14), as well as IgH and IgL split probes (van Rijk *et al.*, 2008). The *c-myc* gene translocation associated with each BL case may also be related to the different stages of B cell maturation (Bellan *et al*., 2005).

Many of the proteins encoded by these genes play important roles in diverse cellular functions such as apoptosis inhibition, regulation of cell growth, cell cycle control, and cell differentiation. Different epidemiologic and clinical forms of BL may be histologically indistinguishable but almost always have been shown to harbour a particular molecular, Ig/*c-myc* gene translocation signature lesion (Hummel *et al*.,

2006). Many changes in lymphoid neoplasms commonly juxtapose oncogenes to the potent transcriptional enhancers associated with immunoglobulin (Ig) and T cell receptor (TCR) loci in B and T cells, respectively, often resulting in elevated levels of protein overexpression and loss of normal mechanisms of cellular control (Allday, 2009). Less commonly though, fusion genes are created that encode novel hybrid proteins, for example NPM-ALK fusion in anaplastic large-cell lymphoma (Campo *et al*., 2006).

Burkitt's lymphoma are commonly thought to be invariably associated with chromosomal translocation that dysregulate the expression of *c-myc* gene (Hecht & Aster, 2000). This gene encodes 64-kb protein belonging to basic helix-loop-helix (bHLH) transcription factors, which binds to DNA in a sequence specific manner. The product of *c-myc* gene normally plays a central role in transcription regulation of downstream genes and controls diverse cellular processes including proliferation, differentiation, metabolism, apoptosis and telomere maintenance (Lin & Flemington, 2011). Molecular cytogenetic aberration that deregulates the *c-myc* oncogene can be demonstrated by interphase and metaphase chromosome analysis by fluorescent *in situ* hybridization and other techniques.

In Burkitt's lymphoma, chromosomal translocations almost always result in the juxtaposition or reciprocal chromosomal translocations of DNA coding sequences for *c-myc* gene with sequences from immunoglobulin (Ig) genes enhancers. Immunoglobulin Ig gene enhancer elements bind to B cell specific factors capable of activating transcription from genes located up to 500 kb pairs away. Because immunoglobulin (Ig) enhancer elements are specifically active in mature B cells, their

juxtaposition to *c-myc* in BL cells drives inappropriate high levels of *c-myc* gene mRNA and MYC protein expression (Klein, 2009).

Additionally, negative regulatory sequences residing within *c-myc* gene are often removed as a direct consequence of chromosomal translocation or are mutated through other mechanisms, further contributing to increased *c-myc* activity, dysregulation of cell cycle, cellular differentiation, apoptosis, cellular adhesion, and metabolism. The consequence of activated *c-myc* gene is cell growth and proliferation that can lead to lymphomagenesis. In endemic BL the *c-myc* gene translocation is thought to involve Ig heavy chain joining (J) region while in sporadic Burkitt's lymphoma (sBL) and HIV-associated BL involve the immunoglobulin (Ig) class switch region (CSR) (Orem *et al*., 2007). Translocations of *c-myc* genes are not exclusive of BL and can also be detected in other aggressive B cell lymphoma subtypes (Snuderl *et al*., 2010).

Structurally, the *c-myc* gene is a cellular oncogene located on the long arm (q) of chromosome 8, encoding for a nuclear protein with DNA binding properties and plays a role in the control of proliferation and differentiation. Increased *c-myc* gene expression leads to proliferation and abolishment of differentiation. Reciprocal chromosomal translocation of coding sequences of cellular *c-myc* gene on the long arm of chromosome 8 band q24 and promoter sequences of heavy chain immunoglobulin genes on long arms of chromosome 14 band q32 immunoglobulin heavy chain loci, in 80% BL's or light κ or λ chains immunoglobulin genes on chromosomes 2 and 22 in 20% of BL's respectively. These translocations bring the *c-myc* oncogene into close proximity to the immunoglobulin gene inducible promoter

leading to abnormal expression of *c-myc* gene and overexpression of the functionally intact MYC protein. This reciprocal translocation disrupts the structure and function of *c-myc* gene, causing it to be constitutively expressed when immunoglobulin genes are activated.

The quantitative overexpression of *c- myc* gene has been shown to result in dysregulation of cell cycle, cell differentiation, apoptosis, cellular adhesion and metabolism, capable of blocking phenotypic maturation (Yustein & Dang, 2007). Overexpression of *c-myc* gene leads to hyper proliferation of translocation-bearing B cells, increasing their risk for developing genetic errors, which ultimately increases the risk of BL (Kelly and Siebenlist, 1985). Although *c-myc* gene translocation occurs in all cases of Burkitt's lymphoma, differences are seen in the translocation patterns in endemic and sporadic Burkitt's lymphoma. Typically, sporadic Burkitt's lymphoma has translocations involving sequences within or immediately 5' to *c-myc* gene on chromosome 8 and sequences within or near the immunoglobulin heavy chain J region on chromosome 14.

In contrast, endemic Burkitt's lymphoma tends to be characterized by a translocation involving sequences on chromosome 8 further upstream from the *c-myc* gene and sequences within or near the J_H region on chromosome. The *c-myc* gene translocation is present in approximately 90% of all BL cases. Low level *c-myc* gene rearrangements have been reported in healthy Caucasians, which perhaps the occurrence of sporadic BL and not endemic BL. The frequency and natural history of *c-myc* gene translocation in healthy Africans is unknown (Ogwang *et al*., 2008).

The unifying trait of BL variants is a deregulated and overexpression of MYC protein as a result of chromosomal translocation involving the oncogene *c-myc* gene (Klapproth & Wirth, 2010). Translocations are identical in both clinical variants but the precise molecular location of the breakpoints on both chromosome 8 and 14 vary (Bower, 1992). Endemic and sporadic forms of Burkitt's lymphoma have different breakpoints within both the *c-myc* gene locus on chromosome 8 band q24 and the Ig heavy-chain joining region on chromosome 14 while in eBL and HIV associated BL the translocation involves Ig switch region.

In endemic BL associated with Epstein-Barr virus (EBV), breaks occur up to 75 kB 5', upstream, of the *c-myc* oncogene; in sporadic BL most of the translocations occur near exon-1 of *c-myc* gene locus. This is probably reflecting defects occurring at different stages of gene rearrangement in B-cell ontology. These different breakpoints alter the *c-myc* gene RNA transcript, the region affected is untranslated (UTR) and so the amino acid sequence of the MYC protein is unchanged.

The overall consequence of rearrangements is increased expression of MYC protein rather than a qualitative change. The MYC protein is a nuclear DNA binding protein containing two structural domains, the leucine zipper domain and the basic helix-loop-helix motif previously identified in transcription factors. The MYC protein forms a heterodimer with MAX, another leucine zipper DNA binding protein, and alters the expression of a large number of cellular genes which turns the lymphoctyes cancerous (Bower, 1992)., BL. All proliferating tissues express at least one member of the *myc* gene family, usually *c-myc*. The Ig/*c-myc* translocation which results in constitutive expression of *c-myc* gene may contribute to the pathogenesis of BL by preventing the

programmed exit of lymphocytes from the cycling compartment. The *c-myc* gene rearrangements have been identified in HIV-associated BL and most resemble sporadic BL at the molecular level.

Several other proto-oncogenes in this locus produce cancerous B cell e.g. leukaemia, lymphomas multiple myelomas. The B cell leukemia-2 (*Bcl-2*) is another human proto-oncogene located on chromosome 18 its product BCL-2 is located on ER nuclear envelope and mitochondrial membrane. Translocated *Bcl-2* gene is found in B cell lymphomas. The *Bcl-2* gene on chromosome 18 locus undergo reciprocal translocation with immunoglobulin (Ig) heavy chain gene locus on chromosome 14 t(14:18), t(2;8), t(8;22) which put *Bcl-2* gene close to heavy chain gene enhancer, hence high levels of expression of BCL-2 proteins. A high level of BCL-2 is known to inhibit apoptosis of B cells (Dave *et al.*, 2006).

2.7.2 Immunoglobulin (IgH) Mutation

The survival of normal germinal centre (GC) B cells depends on the expression of an Ig receptor capable of recognizing antigen with high affinity. The ability to make high-affinity antibodies requires somatic hypermutation, in which germinal centre B cells acquire mutations at a high rate within the immunoglobulin genes in complementarily-determining regions (CDR) that encode the antigen-binding residues (Hecht & Aster, 2000). Because somatic hypermutation is apparently confined to germinal centre B cells, it marks B cells as being of germinal centre origin.

Sequence analysis of the Ig variable heavy (V_H) and light (V_L) chain genes in endemic, sporadic, and HIV-associated BL has shown that they have undergone

somatic hypermutation. Additionally, the Immunoglobulin genes of some endemic BLs show evidence of continuing somatic hypermutation, a phenomenon also seen in follicular lymphomas. This implies germinal centre B-cell origin for all forms of BL (Hetch & Aster 2000).

Neoplasm in many respects recapitulate normal stages of lymphoid cell differentiation and function, thus markers of normal counterparts are used as to classify neoplasms (Jaffe, Harris, Stein, & Isaacson, 2008). Tumours are usually categorised according to neoplastic cell origin, differentiation and maturation stage(s). But, the precise normal cell counterparts of B cells in Burkitt's lymphoma are not known.

2.7.3 Cytokines Associated with Burkitt's Lymphoma

Cytokines that are released in response to infection, inflammation and immunity can produce microenvironment that plays a role in cancer pathogenesis (Dranoff, 2004), cancer cells can also respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. Epidemiological data indicate that diverse forms of chronic inflammation markedly increase the risk of malignant transformation, meaning that unresolved host immune reactivity may promote tumour development.

Infectious agents such as, malaria, HIV and EBV stimulate immune cells and surrounding macrophages to produce activating cytokines- M-CSF, IL-8, IL-6, IFN-γ, IL-4, IL-2, IL-17A, TNF-α, IL-10, TGF-β and other cytokines that drive the proliferation of B and T cells (Ning, 2011). Interleukin-6 (IL-6) is a B cell stimulatory factor which has been shown to play a role in enhancing the growth of EBV

transformed lymphoblastoid cell lines in vitro and may therefore have a role in the development of BL (Giadano *et al.,* 1991). The frequency of non-Hodgkin lymphomas in Africa has increased since the beginning of HIV/AIDS pandemic in the early 1980's (Mantina *et al*., 2010). Benjamin *et al*. (1992) showed that B cell derived from patients with acquired immunodeficiency syndrome (AIDS) and Burkitt's lymphoma constitutively secrete large quantities of IL-10. Interleukin 10 (IL-10) exert a broad spectrum of activities such as growth and differentiation of thymocytes in presence of IL-2, and/or IL-4, co-stimulation of mast cell and IL-2 activated T- lymphocytes and probably play a role in the development of B cell abnormalities (Benjamin *et al.*, 1992).

2.7.4 Burkitts Lymphoma MicroRNA Expression

MicroRNAs (miRNAs) are small non-coding RNAs have been described as non immunogenic but seem to be crucial to the physiological regulation of gene expression at post transcriptional level by mRNA cleavage or translational inhibition, in their mature form (Bartel, 2004). Genetically, miRNA genes are located within introns of protein-coding or non-protein-coding genes or in intergenic regions as stand alone genes. They are single-stranded RNA molecules that have been shown to bind to complementary sequences in the 3'-untranslated regions of their target mRNAs leading to inhibition of translation or the degradation of the coding mRNA and consequently reduced level of the corresponding protein. In this way, MiRNAs influence important cellular processes like differentiation, proliferation and apoptosis of cells of the hematopoietic system.

Cellular miRNAs constitute approximately 1–3% of the genome and are predicted to regulate 30% of human genes. There are about 940 miRNAs identified in humans (Sandhu, Croce, & Garzon, 2011). The miRNAs are transcribed by RNA polymerase II as long primary transcripts, which are processed into approximately 70 nucleotide long precursor miRNAs by an RNAse-III-like enzyme. During transport to the cytoplasm, miRNA are made into a mature dsRNA duplex by another RNAse-III enzyme, called Dicer. The miRNA-mRNA interactions are characterized by perfect or nearly perfect Watson-Crick base pairing involving miRNA seed region typically 2–8 bases that binds the target mRNA (Sandhu *et al*., 2011). A single miRNA is predicted to target about 300 mRNAs. In addition to the canonical mechanisms of miRNA gene regulation through 3' untranslated region (UTR) interactions, other noncanonical miRNA-mediated mechanisms of mRNA expression modulation have been postulated.

Both structural and epigenetic events are thought to lead to miRNA mediated dysregulation. Many miRNA genes are frequently located near cancer susceptibility loci, which are then often subjected to genomic alterations leading to activation by translocations or ampli- fications, or loss of function due to deletions, insertions, or mutations. Modifications like miRNA promoter hypermethylation and/or histone hypoacetylation have been described in solid tumours and haematological malignancies. Aberrant miRNA expression may also result from downstream miRNA processing. For example, short hairpin-mediated silencing of RNAses involved in miRNA processing (Dicer and Drosha - Ribonuclease type III) and can lead to global repression of miRNA expression promoting cellular transformation and tumorigenesis

in vivo (Kumar *et al*., 2009). The *c-myc* gene over expression alters mRNA profiles in B-cell lymphoma via the NFκB activation pathway.

Systematic microRNA profiling in lymphoma patient samples using different miRNA expression platforms suggest that microRNAs have a role in lymphomagenesis (Onnis *et al*., 2010). Both, classic t(8;14) and variants t(8;22) or t(2;8) translocations involving *c-myc* gene are associated with *PVT1* oncogene (non-protein coding RNA gene *c-myc* gene activator), which encodes several miRNAs some of which, such as *hsa-miR-1204* have been shown to be responsible for *c-myc* gene activation. By using microarray containing oligonucleotide probes, complementary miRNA (from web-available resources), it is possible to search miRNA directed against a specific target such as *c-myc* gene. A *c-myc*-related miRNA profile can differentiate Burkitt's lymphoma from other similar B cell non Hodgkin's lymphoma such as B cell chronic lymphocytic leukaemia (CLL), mantle cell leukaemia (MCL) and follicular leukaemia (FL). Expression profile of microRNA analysis can also been used to characterize human B-cell malignancies, for instance MYC^{+} BL vs MYC^{-} BL have been shown to have upregulated-*hsa-miR-17-5p, hsa-miR-20a,* downregulated-hsa-miR-9, *hsa-miR-34b* (Zhang *et al.,* 2009). More recently attempts have been made to explain some hither to unexplained differences by evoking miRNA expression pattern, between BL subtypes, such as in *c-myc*$^{+}$ and *c-myc*$^{-}$ cases, EBV^{+} and EBV^{-} cases. This is necessary because *c-myc*$^{-}$ BL cases may represent a challenging diagnosis category to discriminate from DLBCL and from cases with intermediate features between DLBCL and BL (DLBCL/BL) (Rossi *et al*., 2012). Non-Hodgkin's lymphomas comprise a heterogeneous group of lymphoid malignancies that require comprehensive

morphologic, immunophenotyping and molecular tests for accurate description, characterization and diagnosis.

Viruses including Epstein-Barr virus and HIV-1 use miRNA to evade host immune system (Xia *et al*., 2008). Due to the non-immunogenic property of these tiny RNA molecules, viral miRNAs are good candidates to execute various viral latency associated tasks once an immune response to viral antigens has been mounted by the host (Lin & Flemington, 2011).

Dysregulation of miRNA expression has also been reported in numerous types of cancer, including lymphoma. This has made it possible to distinguish different sub groups of tumours through miRNA profiling expression patterns, for example, germinal centre B cell (GCB) and non-GCB diffuse large B cell lymphoma (DLBCL) (Lawrie *et al*., 2007).

2.7.5 Burkitt's Lymphoma Evasion of Immune Responses

The immune system is capable of generating both $CD4^+$ and $CD8^+$ T-cell responses to several latent and lytic phase EBV-associated antigens, such as LMP1, LMP2, EBNA-2, and EBNA-3. But BL infected cells generally express only the EBNA-1 protein, which is poorly antigenic and has little or no HLA class I presentation and therefore response (God & Haque, 2010). The antigen EBNA-1 escape of HLA class I presentation involves the presence of an internal glycine- alanine (Gly-Ala) repeat amino acids that has a dual role in this process. First, the Gly-Ala repeat prevents the formation of a functional complex with the proteasome, thus blocking the protein degradation necessary for HLA class I loading and presentation to $CD8^+$ T cells.

Secondly, the Gly-Ala repeat causes a decrease in the translation of the EBNA-1 mRNA, thus reducing the production of antigenic peptides. And because EBNA-1 limits its own presentation by HLA class I molecules, $CD8^+$ T cell response to EBV infected lymphoblastoid cells is largely diminished. A study by Leung, Haigh, Mackay, Rickinson and Taylor (2010) demonstrated a possible alternative method, autophagy, by which antigen presentation may allow for EBNA-1 epitopes to be displayed by HLA class II proteins. This suggests a role of $CD4^+$ T cells in recognizing the EBNA1 antigen. Another study by Chene *et al*. (2009) has shown that BL cells are deficient in their ability to functionally present antigens via the HLA class II pathway.

2.7.6 The *c-myc* Gene and Immune Evasion of BL

Cytotoxic $CD8^+$ T cells do not efficiently recognize BL cells regardless of whether EBV positive or negative. The c-myc activities appear to reduce BL immunogenicity. The c-myc expression has been shown to be inversely correlated with expression of HLA class I, in some tumors. A previous study demonstrated, an immunogenic B-cell lymphoma that is normally recognized by cytotoxic T cells was rendered non-immunogenic when *c-myc* gene was overexpressed. However, it is not known how c-myc exerts its immune inhibitory activities, but it is thought to act by altering mRNA profiles via the NFκB activation pathway. Inactivation of *c-myc* gene results in restored expression levels of accessory molecules and Ag presentation. Burkitt's lymphoma usually has little or no expression of NF-κB. Studies have shown that overexpression of *c-myc* gene leads to decreased expression of accessory molecules important in the immune response, including LFA-1, LFA-3, ICAM-1, and TAP (God & Haque, 2010). The decreased immune response may promote BL tumour growth.

2.7.7 Evasion of HLA Class I Presentation

EBNA1, the sole EBV antigen expressed in EBV positive BL, uses an internal Gly-Ala repeat to prevent its optimum presentation by HLA class I and largely escapes cytotoxic T lymphocytes (CTL) detection and also viral infection usually involve the down regulation of HLA class I protein expression.

2.7.8 Evasion of HLA Class II Presentation

There is evidence suggesting that endogenous viral antigens can also be processed and presented by HLA class II molecules for stimulation of $CD4^+$ T cells. Among the proteasome independent pathways, only macroautophagy has been observed to deliver endogenous substrates to HLA class II. Endogenous antigen can also be processed and delivered by macroautophagy to HLA class II for presentation and activation of $CD4^+$ T cells. Thus, microautophagy of EBNA-1 and association with HLA class II may provide the means by which EBV EBNA-1 antigens could be presented to $CD4^+$ T cells via the HLA class II pathway.

2.8.0 Techniques of Investigation

2.8.1 Immunohistochemistry (IHC)

The principle of immunohistochemistry (IHC) has been known from the 1930s. Since then, improvements have been made in protein conjugation, tissue fixation methods, detection labels and microscopy, making IHC an important tool in tumour diagnosis and research today (Key, 2006). Immunohistochemistry combines histological, immunological and biochemical techniques to identify tissue components using the

interaction of target antigens with specific antibodies tagged with a visible label to serve as a colour signal when visualised through a light microscope. Immunohistochemistry technique is a powerful method for localizing specific markers in formalin-fixed, paraffin-embedded (FFPE) tissues sections. It makes it possible to visualize both the distribution and localization of specific cellular components within cells and in the proper tissue context. Using specific tumour markers, the method is useful in the diagnosis of a cancer as benign or malignant, determine the stage and/or grade of a tumour, or identify the cell type and origin of a metastasis and find the site of the primary tumour.

Immunohistochemistry as a technique plays a key role in the diagnosis and classification of haemopoietic and lymphoid tissue neoplasia including BL. Morphologically BL is a malignancy of monotonous medium-sized blastic lymphoid cells that show round nuclei with clumped chromatin and multiple, centrally located nucleoli that infiltrate nodal and extra-nodal sites. The histological hallmark of BL is the presence of numerous apoptotic cells within scattered pale phagocytic macrophages giving a characteristic “starry sky” microscopic appearance of sections viewed at low power (Crawford, 2001). Burkitt’s lymphoma with atypical morphology does not show this and moreover a number of B cell lymphomas have similar morphologic appearances (Bellan *et al*., 2003).

Phenotypically, Burkitt's lymphoma cells are commonly $CD10^{+}$, $CDI9^{+}$, $CD20^{+}$, $CD22^{+}$ and $CD79a^{+}$ while Terminal deoxynuleotidyl transferase (TdT) marker is rarely expressed (Hedvat *et al*., 2002). Surface IgM and either kappa (κ) or lambda (λ) light chains are most commonly expressed and on some occasion IgG or IgA

expression is found (Cho, 2011). The Burkitt-like subtype may variably express surface immunoglobulin and occasionally expresses cytoplasmic immunoglobulin. The proliferation marker, Ki-67, is usually strongly positive in both types approaching proliferative index of 100% of tumour cells (Allday, 2009). Both Burkitt's and Burkitt-like lymphoma are believed to be derived from blastic B cells in the early germinal centre (GC). These are B cells that have rearranged their immunoglobulin genes but have yet to undergo antigen selection and have not entered the memory B-cell pool (Bishop *et al*., 2000). Generally, childhood B cell lymphomas have arrested B cell differentiation pathway compared to adults' activation pathway. In childhood neoplasms, the immature B lymphocytes are diverse and heterogenous ranging from pre-B neoplasias, in which only intraplasmic IgM is detectable to B cells neoplasias with a full array of differentiated B cell surface markers (Ziegler, 1982).

There are several specific monoclonal antibodies that are used in lymphoma classification. These antibodies were directed against cell markers designated cluster of differentiation (CD). Each cluster of differentiation (CD) shows a different pattern of positivity in non-Hodgkin's/Hodgkin's lymphoma, pan T cell, CD2, CD3, CD4, CD7, CD8 and pan B, CD 10, CD19, CD20, CD22, CD23, CD79a, sIgM, BCL-6 (Pileri *et al*., 2000; Matuma, 2004). Proliferative indices such as BCL-2, p53, BCL-1, BCL-6, CD20, CD23, CD79a, CD56, Ki-67 are useful diagnostic and prognostic indicators (Matuma, 2004). Immunohistochemistry (IHC) and molecular diagnosis are therefore required to make differential diagnosis of Burkitt's lymphoma. Both Immunohistochemistry and other molecular analyses are currently not available in most developing countries including Kenya, but are crucial in verifying the diagnosis and effective treatment of Burkitt's lymphomas (Lukande *et al*., 2008).

2.8.2 Immunohistochemistry antibodies used in lymphoma classification

BCL-2

BCL-2 is a member of a family of proteins that are involved in apoptosis. The protein BCL-2 is an integral inner mitochondrial membrane protein of 25 kD but also has a wide tissue distribution. It is considered to act as an inhibitor of apoptosis. For this reason, *bcl-2* gene expression is inhibited in germinal centres (GC) where apoptosis forms part of the B cell production pathway.

In 90% of follicular lymphoma a translocation occurs which juxtaposes the *bcl-2* gene at 18q21, to an immunoglobulin gene in chromosome 14. Thus, this t(14;18) translocation which can deregulate expression of this gene. Over expression of *bcl-2* gene can be demonstrated immunohistochemically in the vast majority of follicular lymphoma.

CD 10

Cluster of differentiation 10 (CD10), also called neprilysin, is a 100 kD cell surface metalloendopeptidase which inactivates a variety of biologically active peptides. It was initially identified as the common acute lymphoblastic leukaemia antigen (CALLA) and was thought to be tumour-specific.

Subsequent studies, however, have shown that CD10 antigen is expressed on the surface of a wide variety of normal and neoplastic cells. In other lymphoid malignancies, CD10 antigen is expressed on cells of lymphoblastic lymphoma (LL), Burkitt's lymphoma (BL) and follicular lymphoma (FL).

CD10 antigen is identifiable on the surface of normal early lymphoid progenitor cells, immature B cells within adult bone marrow and germinal centre B cells within lymphoid tissue.

It is also expressed in various non-lymphoid cells and tissues, such as breast myoepithelial cells, bile canaliculi, fibroblasts, with especially high expression on the brush border of kidney and gut epithelial cells.

CD20

The CD20 antigen is a non-glycosylated phosphoprotein of approximately 33 kD which is expressed on normal and malignant human B cells and is thought to act as a receptor during B cell activation and differentiation.

The CD20 antigen is expressed on normal B cells from peripheral blood, lymph node, spleen, tonsil, bone marrow, acute leukaemia and chronic lymphocytic leukaemia.

CD38

The CD38 molecule is a type II single transmembrane glycoprotein with a molecular weight of 46 kD. It is an ectoenzyme with the activities of ADP-ribosyl cyclase, cyclic ADP-ribose (cADPR) hydrolase, and NAD glycohydrolase and is involved in both the formation and hydrolysis of cADPR, a second messenger that regulates the mobilization of intracellular $Ca2^{+}$ ions.

Although the CD38 molecule was originally identified as a T lymphocyte differentiation antigen, it is reported to be expressed in a wide range of cells and

tissues. The CD38 antigen can deliver potent growth and differentiation signals to lymphoid and myeloid cells.

It is found on immature cells of the B and T cell lineages but not on most mature resting peripheral lymphocytes. It is also present on thymocytes, pre-B cells, germinal centre B cells, mitogen-activated T cells, Immunoglobulin-secreting plasma cells, monocytes, NK cells, erythroid and myeloid progenitors in the bone marrow and brain cells.

CD38 antigen has also been reported in neurofibrillary tangles, the pathological indicator of Alzheimer's disease that occurs in the neuronal perikarya and proximal dendrites.

CD44

The CD44, Hermes antigen (H-CAM) is an 80 to 95kD transmembrane glycoprotein with extensive O-linked glycosylation. The antigen is a cell surface receptor for hyaluronate, suggesting a role in the regulation of cell substrate interactions, as well as cell migration. It is widely distributed cell surface adhesion molecules that participate in diverse cellular interactions.

The CD44 antigen is reported to be expressed on T cells, B cells, monocytes, granulocytes, erythrocytes and weakly on platelets. Other CD44 antigen positive cell types are reported to include epithelial cells, glial cells, fibroblasts and monocytes.

Increased expression of CD44 antigen is found on some carcinomas and it has been reported that transition of tumour cell lines from non-metastatic to metastatic may be associated with changes in the expression of CD44 antigen variants.

Ki-67

The Ki67 antigen is a nuclear protein which is expressed in all active parts of the cell cycle (G1, S, G2 and mitosis) but is absent in resting cells (G0).

In contrast to many other cell cycle-associated proteins, the Ki 67 antigen is consistently absent in quiescent cells and is not detectable during DNA repair processes. Thus, the presence of Ki67 antigen is strictly associated with the cell cycle and confined to the nucleus, suggesting an important role in the maintenance and/or regulation of the cell division cycle. The monoclonal MIB-1 directed at MKI67 gene products is used to measure expression of Ki 67.

MYC Protein

The *c-myc* oncogene is the human cellular homologue of the avian myelocytomatosis viral oncogene, *v-myc* gene, found in several leukemogenic retroviruses. The *c-myc* gene is a nuclear phosphoprotein, which has DNA-binding activity and is implicated in the control of normal proliferation, differentiation, metabolism and apoptosis

Expression of *c-myc* gene in untransformed cells is as growth factor dependent and essential for progression through the cell cycle. Physiological *c-myc* gene is expressed during proliferation at all stages of embryonic development and in a wide variety of adult tissues.

The *c-myc* gene is a potent oncogene, its alteration and over expression in B cell neoplasm is usually associated with aggressive clinical behaviour (Ott, Rosenwald, & Campo, 2013). Enzyme pretreatment may enhance staining in some cases.

Terminal Deoxynucleotidyl Transferase (TdT)

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase of 58 kD located in the cell nucleus which catalyzes the polymerization of deoxynucleotides at the 3' hydroxyl ends of oligo or polydeoxynucleotide initiators and functions without a template. TdT is reported to be expressed in primitive T and B lymphocytes of the normal thymus and bone marrow.

The identification of TdT-positive cell populations in primary and secondary lymphoid organs during maturation of the immune system is one area of interest but it is the reported occurrence of high levels of enzyme activity in white blood cells and bone marrow in certain leukaemia which is of particular interest.

Early and precise differentiation of lymphoblastic lymphoma is crucial. TdT is reported to be expressed in lymphoblastic lymphoma (LL) and leukaemia.

The determination of TdT expression is reported to be most valuable when it is difficult to differentiate histologically between lymphoblastic lymphoma and Burkitt's lymphoma.

CD21

CD21 antigen is a type I integral membrane glycoprotein of molecular weight 140 kD, which functions as the receptor for the C3d fragment of the third complement component.

The CD21 molecule, present on mature B cells, is involved in transmitting growth-promoting signals to the interior of the B cell and acts as a receptor for Epstein-Barr virus. CD21 antigen is reported in B cell chronic lymphocytic leukemias and in a subset of T cell acute lymphocytic leukemias but is absent on T lymphocytes, monocytes and granulocytes. CD21 antigen is also reported to be expressed in follicular dendritic cells and in follicular and mantle cell lymphomas, mature leukemias and other lymphomas.

CD138/MUM-1

The MUM-1 (multiple myeloma oncogene 1) gene was originally identified because of its involvement in the t(6:14) translocation observed in multiple myeloma, which causes the juxtaposition of the MUM-1 gene to the Ig heavy chain locus.

MUM-1 is expressed in late plasma cell directed stages of B cell differentiation and in activated T cells, suggesting that MUM-1 may serve as a marker for lympho-hemopoietic neoplasms derived from these cells.

The morphologic spectrum of MUM-1 expressing cells has been found to range from that of a centrocyte to that of a plasmablast/plasma cell. Consequently the histogenic value of MUM-1 may be to provide a marker to aid in the identification of the

transition from BCL-6 positive (germinal center B cells) to CD138 positive (immunoblasts and plasma cells).

MUM-1 expression occurs in a wide range of lymphoid neoplasms including a proportion of diffuse B cell lymphomas but not myeloid or extra-hemopoietic neoplasms. MUM-1 is consistently expressed in myeloma cells, Reed Sternberg cells in classic Hodgkin's disease and activated and neoplastic T cells

CD99

CD99 is a 32 kDa transmembrane glycoprotein. Although its function is not fully understood, CD99 has been implicated in various cellular processes including homotypic aggregation of T cells, upregulation of TCR, and apoptosis of immature thymocytes. CD99 expression has been reported in a wide range of tumours, including lymphomas (URL: http://Leicabiosytem.com/ihc-fish-antibodies, 2014).

2.8.3 Fluorescence *in-situ* Hybridization (FISH)

The diagnosis of lymphomas can be a complex process, which needs to take into account clinical, morphological, immunophenotypic and genetic features (Diebold, 2001). Histopathological diagnosis of lymphomas is generally considered difficult and prone to mistakes. But since non-random chromosomal translocations are specifically involved in different lymphoma entities, the detection of these aberrations is increasingly becoming an important tool in the correct identification important in the diagnosis (van Rijk *et al*., 2008). Results of the EuroFISH programme, a concerted action to standardise translocation detection in pathology by a Euro-Fish protocol, showed that all probes were correctly cytogenetically located and that when

standardised, the protocol is robust and gave reliable results in approximately 90% of cases reviewed (van Rijk *et al*., 2008). Different lymphoma types are associated with non-random chromosomal aberration (s). The detection of these genetic aberrations is a fundamental step in the identification of the different lymphoma entities. Burkitt lymphoma (BL), is associated with a translocation involving *c-myc* gene t(8;14) in more than 90% of BL cases. Some cytogenetic alterations define clinically relevant subgroups and are, therefore, crucial for therapy decisions. For instance, gastric marginal zone lymphomas (gastric MALT lymphomas) lacking the t(11;18) involving the MALT1 gene respond to *Helicobacter pylori* eradication therapy in contrast to the t(11;18)-positive cases that do not respond (Tilly *et al*., 1994).

To detect a translocation in a tumour cell, probes with different colours on different chromosomes (usually two) are used in such a way that, in the case of a translocation, a fusion signal occurs. This procedure is feasible in cytospins or preparations of isolated nuclei, but more difficult in tissue sections where many nuclei are cut and thus a complete signal is present in a minority of cells making interpretation cumbersome. Split signal or break-apart probes use differently coloured probes on both sides of a known breakpoint region, resulting in a fused signal in the normal situation, but single colours when a break in the gene occurs (Ventura *et al*., 2006). This approach is advantageous in tissue sections since each single coloured signal indicates a specific chromosomal break.

2.8.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and colour change to identify antigen. Antigens from the sample are attached to a surface,

then, a specific antibody is applied over the surface bind the antigen. This antibody is linked to an enzyme. In the final step, an enzyme's substrate is added to produces a detectable visible signal by spectrophotometer, which indicates the quantity of antigen in the sample. Qualitative results usually based on detection of intensity of transmitted light. The signal is generated by enzymes which are linked to the detection reagents in fixed proportions, the signal allows accurate quantification. Epstein-Barr virus (EBV) IgG in plasma samples binding to EBV capsid antigen coated wells were quantified at absorbance of 450 nm within 30 minutes of preparation. Rapid ELISA was used to detect HIV-1/2 serostatus using Determine® and Uni-Gold® kits in parallel for HIV-1/2 positive cases.

2.8.5 Polymerase chain reaction (PCR)

To detect HIV-1 RNA, amplify IgVH DNA and quantify relative expression of microRNA-hsa-miR-127 and hsa-miR-34a, Polymerase chain reaction techniques were used.

Polymerase chain reaction is a technique that amplifies a copy or a few copies of a piece of DNA to generate thousands to millions of copies of the particular DNA sequence through cycles of repeated heating and cooling of the reaction for DNA and enzymatic replication of the DNA template. Primers containing sequences complementary to the target region and DNA polymerase are required to enable selective amplification. Amplification product is checked using agarose gel electrophoresis for size separation and by comparing with DNA ladder which contains DNA fragments of known size, run on the gel alongside the PCR products.

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample. Quantitative PCR measures the accumulation of DNA product after each round of PCR amplification.

Reverse transcription polymerase chain reaction (RT-PCR), a variant of PCR, is used to detect RNA expression. It is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. Quantitative PCR can quantify RNA in both relative and absolute terms, The combined technique, described as quantitative RT-PCR (qRT-PCR) is considered to be the most powerful, sensitive, and quantitative assay for the detection of RNA levels. Relative quantifications of RT-PCR involve the co-amplification of an internal control simultaneously with the gene of interest. The internal control is used to normalize the samples. Once normalized, a direct comparison of relative transcript abundances across multiple samples of mRNA is made.

The two-step reaction requires that the reverse transcriptase reaction and PCR amplification be performed in separate tubes. The one-step approach is thought to minimize experimental variation by containing all of the enzymatic reactions in a single environment.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Design

This study was a prospective hospital (MTRH) based design. The study period spanned three years between January 2011 and December 2013.

3.2 Study Site

Moi Teaching and Referral Hospital (MTRH) is the second referral and teaching hospital in Kenya, A significant proportion of patients are referrals from county and sub-county hospitals in the western Kenya region which hosts about 40% of the total Kenyan population, parts of eastern Uganda and southern Sudan. The hospital is a major health provider; patients with cancers are referred for treatment and management from all over the region.

3.3 Study Participants

One hundred and four (104) consecutive cases of clinically and histologically diagnosed paediatric BL and non-Hodgkin's lymphoms patients attending or admitted to the paediatric oncology wards at the hospital, females and males children and adolescents aged ≤18 years were recruited in the study. Of these, thirty three (33) were Burkitt's lymphoma and seventy (71) non-Burkitt's lymphoma patients. Biopsy and blood samples were taken from each consenting patient or guardian before the start of hospital treatment.

The study patient's records and files were assembled and scrutinized for information including clinical evaluation reports, demographic data: sex, age, tribe, race, religion,

area of residence, case notes, primary tumour site, clinicopathological details– histology, date of diagnosis, site involved, HIV-1/2, EBV and malaria status, any other intercurrent illnesses, complete blood count, chemotherapy protocol and number of courses and predicted response to treatment. The data was extracted and recorded in a proforma sheet and structured questionnaire (Appendix I). Participants and guardians or guardians were later interviewed by telephone or physically to follow up on participants.

3.4 Study Population

Included in this study were children and adolescents aged ≤ 18 years at MTRH in western Kenya with presumed diagnosis of Burkitt's lymphoma or non Hodgkin's lymphoma or on the basis of clinical information and/or cytological examination of fine needle aspirate (FNA) by a local pathology laboratory. The participants were from diverse ecological zones with etiological factor malaria endemicity and sporadic transmission in North Rift, Western and Lake Regions of Kenya. These regions stride the Equator and have semi tropical to tropical climate. Malaria transmission varies from hyperendemic in lowland areas around Lake Victoria to seasonal (April to July) in the highlands (elevation 1600 – 2,000 m).

3.5 Sample Size Calculation

The following formula, from Fisher, Laing and Strocker, (1998) was used to estimate the required sample size:

$$\frac{n = \left(Z1 - \frac{\alpha}{2} + Z1 - \beta\right) 2[P1(1 - P1) + P2(1 - P2)]}{(P1 - P2)}$$

n = the number of subjects required in each group

If :

$$Z1 - \frac{\alpha}{2} = 1.96$$

$$Power = 90\% = 1.64$$

$$P1 = 80\%$$

$$P2 = 40\%$$

Substituting

$$= \frac{(1.96 + 1.64)2[0.8(0.2) + 0.4(0.6)]}{(0.8 - 0.4)2}$$

$$= 32.4$$

$$\cong 32$$

A minimum of thirty two (32) participants were required in Butkitt's and non-Burkitt's arms of the study.

The parent or guardian of each child or adolescent were approached and invited to participate in the study and provided written informed consent for their child to be

included in the study. Each parent or guardian was also asked to consent to be interviewed on their household characteristics and mosquito net following a structured questionnaire (Appendix II).

A total of 104 study participants were consecuitively recruited out of which thirty three (33) were BL cases and seventy one (71) were catogorised as non-BL cases during the study period.

3.6 Inclusion Criteria

The following were eligible to participate in the study:

i) Children and adolescent aged ≤18 years from western Kenya

ii) Provisional Burkitt's lymphoma or non Hodgkin's lymphoma diagnosis

iii) Chemotherapy naïve at recruitment

3.7 Exclusion Criteria

Participants with the following criteria were excluded from the study:

i) Those with benign tumour

ii) Those with conditions other tumour

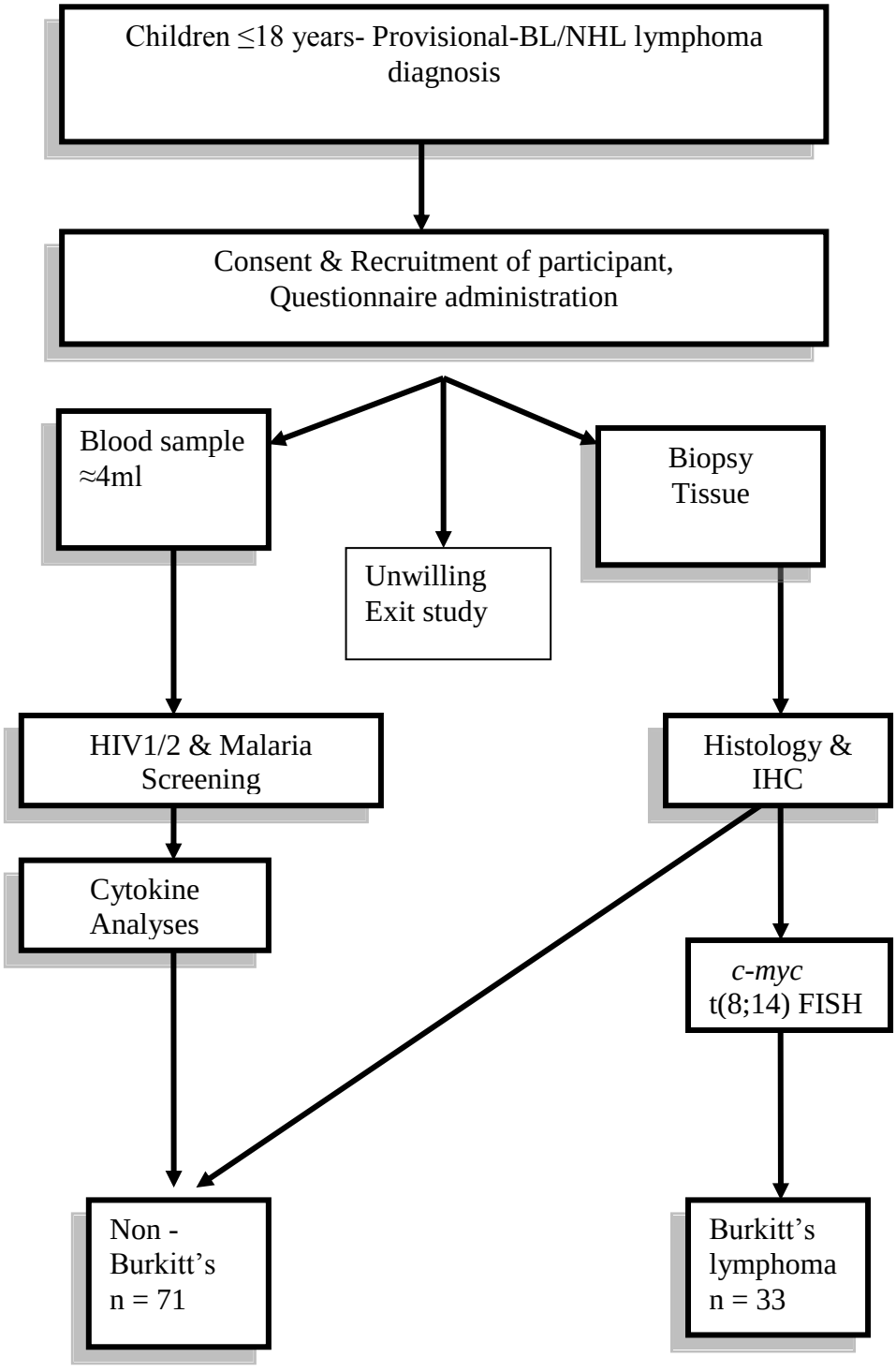
iii) Those unwilling to participate in the study

3.8 Ethical Considerations

This study was performed in conformity with the Declaration of Helsinki and permission to conduct the study and ethical clearance was obtained from Institutional Research Ethics Committee (IREC) of MTRH and Moi University before the study

begun, vide reference letter IREC/2011/04, approval number is 000654 (Appedix III). In addition authorization to conduct research was granted by the Kenya National Commission for Science, Technology and Innovation (NACOSTI) (Appendix IV). The Director of Medical Services gave authority to analyse some samples outside Kenya (Appendix III). Trained study assistants- a nurse and a laboratory technologist, were employed to recruit children and their custodians into the study and to administer questionnaires on household characteristics and mosquito net use. If considered eligible, the parent or guardian of each child were approached and invited to participate in the study. The parents or guardians of were asked to provide written consent, before enrolment of their children in the study Appendix VI). Children seen in the MTRH paediatric oncology ward with suspected cancer are routinely tested for HIV infection. Appropriately trained staff was used to provide pre- and post- HIV test counselling and obtain blood sample for laboratory testing for antibodies against HIV-1/2, EBV, malaria, cytokines as well as tissue biopsies for histology, immunohistochemisty and molecular studies.

3.9 Study workflow



3.10.0 Laboratory Methods

3.10.1 Tissue Fixation and Processing

Biopsy tissues were obtained from the the tumour site and for abdominal tumours by ultra sound guided surgery using Trucut needles (needle core biopsies). Each biopsy tissue specimen was trimmed in pieces about 1 mm diameter and fixed in freshly prepared 10% neutral buffered formalin. Optimal fixation time of 24 hours at room temperature before paraplast embedding was used.

3.10.2 Haematoxylin and Eosin Staining

Tissue slices 4µm sections were cut using a rotary microtome and then stained with haematoxylin and eosin (H&E) for histologic evaluations. The slides were interpreted by pathologists at MTRH/Moi University Histopathology Laboratory and later reviewed by Haematopathogists at a reference facility in the Department of Human Pathology and Oncology, University of Siena, Italy.

3.10.3 Immunohistochemistry (IHC) Staining

Thin tissue sections 3µm were deparaffinized in xylene and rehydrated in a graded series of ethanol. The sections were subjecte to epitope retrieval prior to sequential staining interposed with washing steps using tris- buffered saline (TBS) pH 7.6. Endogeneous peroxidise was neutrilized by peroxidise followed by protein block to reduce non-specific binding of primary antibody or polymer in Leica Bond III® stainer. A panel of monoclonal antibodies recognizing epitopes of selected antigens - CD10, CD20 CD38, CD44, CD99, CD138, Ki67, TdT, EBER, MYC and BCL-2,

were applied, in conjunction with 3,3'-diaminobenzidine tretrahydrochodie dehydrate (DAB) to visualize antibody binding after appropriate incubation at room temperature.

The staining was evaluated in at least 10 high-power fields of tumour areas, in accordance with the WHO 2008 lymphoma classification criteria. All cases were reviewed by expert haematopathologists and diagnoses were established using the set criteria of the classification criteria (Swerdlow *et al*., 2008). Agreement was reached by considering morphology on histological slides stained with Giemsa, immunophenotyping and translocation detection by FISH. BL was diagnosed as having appropriate morphology and/or distinctive immunophenotype ($CD10^+$, $CD20^+$, $CD38^+$, $CD44^-$, $BCL\text{-}2^-$, $EBER^{+/-}$ and a Ki-67 proliferative index (PI) > 90%). The percentage of Ki-67-positive cells was determined as an actual percentage of the tumour cells excluding reactive background cells. The presence of Epstein–Barr virus (EBV) in primary tumours was also assessed by IHC. The scores for Ki-67 were as percent of tumour cells staining positive.

3.10.4 MYC Protein Detection by IHC Technique

To evaluate the expression of MYC protein, commercially available monoclonal antibody (Dako®) against MYC was used on 3-µm sections, depparaffinized in xylene and rehydrated in graded alcohols. Heat-induced antigen retrieval was carried out in a microwave oven with citrate buffer at pH 6.0. Endogenous peroxidase was blocked with hydrogen peroxide at 5% and detection was performed using diaminobenzide tetrahydrochloride (DAB) system following the manufacturer's protocol.

For the presence of MYC protein, sections of each tissue blocks or representative 1-mm cores was determined by immunohistochemistry using commercially available monoclonal antibody against MYC and staining intensity was then assessed.

The reactivity for hematoxylin and eosin and immunohistochemistry were scored qualitatively for tumour cells as negative and positive.

Immunohistoschemistry staining pattern and percentage of positive neoplastic cells in each case was evaluated by at least two pathologists independently, discrepant scores were reviewed on a multi-headed Olympus® BH2 5 microscope (at x20 x40) and an agreement reached. Cases that were difficult to diagnose due to inadequate material or poor slide preparation were excluded.

3.10.5 *C-myc* Gene Translocation Detection by FISH Probes

Four-micrometre-thick formalin fixed paraffin embedded tissue sections were processed with the Histology FISH Accessory Kit (Dako®). The status of the MYC gene was evaluated by FISH using a dual-colour break-apart commercial probe (LSI MYC DC BA; Abbott Molecular, Abbott Park, IL, USA), following a standard protocol developed by the EURO-FISH programme, an initiative of European pathology laboratories to validated and standardised protocol to detect gene translocation in lymphoma entities (www.euro-fish.org) and as in Leucci *et al*. (2008) and Komatsu *et al*. (2013). Briefly, *c-myc* gene rearrangements were located using the Vysis LSI MYC dual colour break-apart rearrangement fluourescense *in-situ* hybridization (FISH) DNA Probe (# 30-191096, Abbott GmbH, Germany) according

to manufacturer's instructions. Formalin-fixed, paraffin-embedded tissue sections (4μm) were deparaffinized, air-dried, immersed in a Couplin jar filled with pre-treatment solution, and warmed at 98^{o}C for 10 minutes by means of a Whirlpool JT356 microwave oven to maximise tissue permeability and hybridization. Subsequently, the slides were cooled for 15 minutes at room temperature. After two passages of 3 minutes each in Vysis LSI/WCP hybridization buffer, excess buffer was tapped off and the slides were digested with cold pepsin for 20 minutes in a Dako Cytomation Hybridizer (Dako, Denmark). The slides were then washed twice in wash buffer for 3 minutes, dehydrated using increasing graded ethanol series, air dried, and finally 10μl of probe mixture was applied to each tissue section. The slides were then immediately covered with a cover slip and sealed with rubber cement, were then incubated at 37^{o}C in the DakoCytomation Hybridizer (Dako, Denmark) according to the manufacturer's recommendations for 13 hours. The next day, the slides were treated with stringency buffer at 73^{o}C for 10 minutes to remove the rubber cement and then rinsed twice in wash buffer for 3 minutes, dehydrated using increasing graded ethanol series, air dried, and counterstained by applying 10μl of diamino-2-phenyl-indole DAP II fluorescence to the target area of the slide and cover slip applied. At least 100 nuclei were examined for translocation involving *c-myc* gene. Hybridization signals were visualized using a Leica fluorescence microscope equipped with FITC/spectrum green, Texas red/spectrum orange, and a diamino-2-phenyl-indole I (DAP I) spectrum blue filters.

In analysis of FISH signal, a normal signal pattern of *c-myc* dual colour break apart rearrangement probe is nuclei with two yellow fusion signals, whereas in nuclei with

translocations, a yellow or red-green juxtaposed signal is obtained from one red and one green split signal (van Rijk *et al*., 2008; Bishop *et al*., 2000).

All slides were reviewed discussed with expert panel of hematopathologists according to the current criteria of the WHO for morphologic features, immunophenotype and cytogenetic findings including the presence or absence of a *c-myc* gene translocation. Tumour-biopsy specimens were classified as Burkitt's lymphoma if there was evidence of *c-myc* rearrangement, in addition to morphologic profile consistent with Burkitt's lymphoma, a Ki-67 score of more than 90 percent, and immunohistochemical evidence of CD10 or negative BCL-2, in the tumour cells.

3.10.6 Immunoglobulin Gene Mutation Analysis by RT-PCR

To determine Immunoglobulin heavy chain somatic mutation in in Burkitt's lymophoma B cells, DNA was extracted from FFPE tissue sections and then amplified. For V_H, D_H and J_H gene mutation analyses in BL cases, DNA was extracted from 20-μm–thick formalin fixed paraffin embedded (FFPE) tissue sections using a DNA extractor BioRobot EZ1 (Qiagen, Milan, Italy), in accordance with the manufacturer's protocols and using the reagents from the same supplier. The extracted DNA was then dissolved in 50-100μL distilled water, and its concentration quantified photometrically before being used as a template DNA for PCR. Control gene primer sets for quality assessment of DNA for paraffin-embedded sections were applied according to the BIOMED 2 protocol. DNA quality control PCR showed that only three of the BL cases gave amplificates of ≥300 bp or more and, thus, sufficient intact DNA for successful amplification of IgH mutation and rearrangements studies.

Amplification efficiencies ranging from 50% to 100%, Analysis of IgH rearrangement was done by sequencing of PCR amplificates with the ABI PRISM® BigDye Terminator v1.1 Ready Reaction Cycle Sequencing kit using the ABI PRISM 310 Genetic Analyzer.

3.10.7 Immunoglobulin IgV_H Amplification

To avoid cross-contamination, all procedures performed before PCR amplification such as cutting of tissue sections and proteinase K digestion were performed in a lab exclusively dedicated to this purpose and separate from other rooms in which subsequent steps were carried out, each step was carried out in separate hoods.

The DNA extracted from formalin-fixed paraffin embedded sections was amplified using a Master Cycler (Eppendorf, Hamburg, Germany) PCR with different sets of the target region primers. Polymerase chain reaction (PCR) conditions and primers have been described in detail elsewhere in Tamaru *et al*. (1995).

The amplifilication was done using 4 different sets of primers-FR2A, FR2FS, FR2BM, and FR3BM, which gave amplification efficiencies ranging from 50% to 100%, the latter obtained with FR2BM and FR3BM. The amplificates obtained with FR2A, FR2FS, and FR2BM primers were sequenced and investigated for homology with published V_H germ line sequences.

3.10.8 Immunoglobulin Heavy Chain IgV_H-IgD_H-IgJ_H Mutation Analysis

Polymerase chain reaction (PCR) amplification of IGV_H-IGD_H-IGJ_H rearrangements was performed on genomic DNA extracted from both EBV- and EBV+ BL tissue samples using consensus primers for the IGV_H FR1, along with appropriate IGJ_H genes (BIOMED-2 Protocol). The PCR products were directly sequenced with the ABI PRISM BigDye® Terminator version1.1 Ready Reaction Cycle Sequencing kit using the ABI PRISM 310 Genetic Analyzer (both from Applied Biosystems). Sequences were analyzed using the IMGT databases and the IMGT/V-QUEST tool (version 3.3.5). The following immunogenetic information were recorded for all IGV_H-IGD_H-IGJ_H rearrangements: IGV_H gene and allele usage; percentage of identity to the closest germ line IGV_H allele; V_H CDR3 length and composition, including IGD_H; and IGJ_H gene usage and IGD_H gene reading frame.

3.10.9 MicroRNA (miRNA) Expression Analysis

Extraction and Purification of Total RNA from FFPE Tissue Sections

About four (4) pieces of 5μm thick formalin fixed paraffin embedded (FFPE) sections from BL patients were used to extract total RNA, including miRNA. Total RNA including miRNA was extracted using miRNeasy® FFPE kit (Qiagen®) according to the manufacturer's instructions. Briefly, the sections were deparaffinised, digested in proteinase K, then heated on a thermoblock at 80^oC for 15 minutes followed by DNase treatment and binding to RNeasy MinElute column, from where total RNA including miRNA was then eluted in a minimum of 4μl RNase free water. The amount and quality of RNA extracted was evaluated by measuring absorbance at 260 nm, the 260/230 and the 260/280 ratios using a Thermo Scientific Nanodrop® spectrophotometer (ND-1000, Nanodrop, Thermo Scientific). The ratio of absorbance

at 260 nm and 280 nm ($A_{260/280}$) is used to assess the purity of DNA and RNA. A ratio of ~2.0 is generally accepted as pure RNA. If the ratio is appreciably lower than this, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Absorbance at 260 nm should be greater than 0.15. The ratio absorbance at 260 nm and 230 nm is used as a secondary measure of nucleic acid purity. Expected $A_{260/230}$ values range is 2.0-2.2, if the ratio is appreciably lower than the expected, it may indicate the presence of contaminants which absorb at 230 nm.

3.10.10 Expression of Human (cellular) MiRNA

The expression level of human miRNAs was evaluated using real-time quantitative reverse transcription PCR (qRT-PCR) by TaqMan® MicroRNA Assay, a two-step RT-PCR according to the manufacturer's instructions. Briefly, purified total RNA, reverse transcription was done followed by qPCR amplification, data generated was then analysed using Data Assist® software to derive relative quantitation of gene expression. To evaluate miRNAs expression, the specific c-DNA was prepared using specific TaqMan® primers for hsa-miR-34a, hsa-miR-127 and RNU-43 (Applied Biosystems, Germany) by means of reverse transcription kit (# 4366596 Applied Biosystems, Germany). The qRT-PCR was performed using TaqMan® probes, and the expression levels of miRNAs were normalized to that of RNU43, which was stably expressed among the samples. Controls were prepared from paraffin-embedded blocks made from Cag and Ramos cell lines.

3.10.11 HIV-1/2 Rapid ELISA

Human immunodeficiency virus-1/2 (HIV-1/2) serostatus of each study participants was determined using both Determine® HIV1/2 kit (Alere Medical Company, Matsudo-shi, Japan) and Uni-Gold® HIV kit (Trinity Biotech PLC, Ireland) using whole blood collected in EDTA and following manufacturer's instructions. A 50µl of sample was applied to the sample pad (marked by an arrow on the strip) and then incubated for 20 minutes. A red bar in both the patient and the control window of the strip was interpreted as positive antibodies to HIV-1/2. Positive results were confirmed with a second test using Uni-Gold® and vice versa.

3.10.12 HIV-1/2 Real Time PCR (RT-PCR) Assay

The HIV-1 RNA levels in plasma were quantitated by nucleic acid amplification of HIV-1 positive participants using Abbott RealTi*me* PCR HIV-1 kit (Germany) following the manufacturer's instructions. Briefly, RNA was extracted followed by washings. To generate amplified products, the RNA genome and related HI-1/2 sequence as internal control (IC), the target RNAs were converted to complementary DNA (cDNA) by reverse transcriptase activity, the cDNA was then amplified through repeated cycles of high and low temperatures in a thermocyler. The amount of HIV-1/2 sequence amplified was quantified using real-time HIV-1/2 oligonucleotide fluorescent probe. Fluorescence signal detected is proportional to log of HIV-1/2 RNA concentration in the sample. The assay results were reported as copies/ml.

3.10.13 EBV IgG ELISA

The detection of immunoglobulin G (IgG) subclass antibodies to Epstein Bar Virus (EBV) was done using EBV Ig ELISA kit (Human Gesellschaft, Germany) based on indirect antibody ELISA technique. Microtiter strip wells coated with EBV capsid antigen and duplicates of samples and controls were incubated and washed following manufacturer's instructions. Absorbance was then measured at 450 nm within 30 minutes of terminating the reaction using an ELISA microplate reader and results obtained compared with a cut-off value ± 15%. Patients' results equal or greater than cut-of-value plus/minus 15% were considered anti-EBV-IgG-antibody positive.

3.10.14 Malaria P. f. HRP-2 Test

Malaria test was done using malaria P.f HRP-2 whole blood test strips according to manufacturer's instructions for Burkitt's and non-Burkitt's lymphomas cases. The test is based on immunochromatographic detection of *P. falciparum* specific histidine rich protein-2 (P.f HRP-2) by coloured monoclonal antibody. Whole blood (5µl) was added to the sample pad followed by 4 drops of clearing buffer and incubation for 20 minutes at room temperature, pink bands in both the control and test regions were interpreted as positive and infection with *P. falciparum*.

3.10.15 Blood Sample Collection and Processing

About 4ml of blood samples were drawn from the median antecubital vein of the upper limb using sterile 13 x 75 mm EDTA BD Vacuitainer® Blood Collection Set. From each participant upto 4 ml was drawn and immediately labelled with the patient

identity and complete blood count done. Plasma was then separated by centrifuging at 2500 rpm for 5 minutes and stored at -80 in duplicate aliquoits of 1000 uL, until use.

3.10.16 Haematological Measurements

Automated haematology analyzer Coulter® AcT5 Diff CP (Beckman Coulter, USA) was used to determine blood count parameters including: absolute counts (cell/µL) for white blood cell, red blood cells, platelets and haemoglobin (g/dL) and percent lymphocytes in each study participants.

3. 10.17 Th1, Th2 and Th17A Cytokines Quantification by Cytometric Bead Array (CBA)

The BD® CBA Human Th1, Th2 and Th17A Cytokine Kit (#560484) was used to simultaneously detect interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor (TNF), interferon-γ (IFN-γ), and interleukin-17A (IL-17A) cytokines in plasma samples. The kit comprises seven bead populations with distinct fluorescence intensities coated with capture antibodies specific for each cytokine. The cytokine capture beads with the recombinant standards or samples were incubated and then followed by phycoerythrin (PE)-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of various cytokine. After preparation, appropriate dilutions and incubations, standards and samples were acquired on FacsCalibur® flow cytometer and data analysed using FCAP Array® software to generate results of concentrations of the various cytokine protein levels.

3.10.18 HumanTGF-β1 Cytokine Estimation by CBA Flex Kit

The BD CBA HumanTGF-β1 Flex Set kit (#560429) and BD human soluble master buffer kit (#558264), single plex assay was used to measure soluble human TGF-β1 in the samples following the manufacture's instructions, Becton-Dickison (BD®). After preparation, dilutions and incubations, standards and samples were acquired on FacsCalibur® flow cytometer and data analysed using FCAP Array® software to generate results of concentrations of TGF–β1 cytokine protein levels.

3.11 Data Management and Analysis

Data were collected and entered into a computer using software for storage and initial analysis. Descriptive analyses were performed by using frequency tables for categorical variables: sex, anatomic tumour site (face or head tumors only; abdominal for tumors involving abdominal visceral organs with or without face or head involvement using SAS version 9.1 (SAS Institute, Cary, NC), SPSS® version 20 software and Stata® version 10.0. Distributions of BL by county, ethnicity, sex, tumour anatomic site, age groups were analyzed and summarised using tables and figures. For continuous variables such as age, means and other relevant measures of central tendency were used. Statistical significance of differences in mean and median were calculated by Mann-Whitney rank-sum test. Confidence interval for median difference was also calculated. The sensitivity, specificity, negative and positive predictive values and the overall Kappa score were also calculated. Other exposure factors were examined graphically.

Odds ratios (ORs) were estimated by maximum likelihood using unconditional logistic regression using SAS software. All odds ratios relating to data on children were adjusted for child's age and sex. Analyses examining associations with EBV and malaria were restricted to HIV-1 negative cases and other controls to exclude possible confounding effects of HIV-1 infection. All p-values reported were obtained using two-sided tests of statistical significance, $p<0.05$.

3.12 Study Limitations

Neutral buffered formalin was used intermittently due to supply difficulties, this may lead to sub optimal tissue fixation.. Many tissue biopsies were vey small and some had been sectioned to near exhaustion.This limited the number of sections and amount of DNA or RNA extracts.

Other confounders such as HHV8, nutritional status, some aspects of socioeconomic status and family environment of participantsm were not captured in this study. Some aspects of participants' demographic characteristic were self-reported and therefore subjective and prone to recall bias. Follow-up loss was also a challenge.

Fluorescence in-situ hybridisation (FISH) to detect presence of *c-myc* gene re-arrangements was only done on Burkitt's lymphoma cases due to cost.

CHAPTER FOUR

RESULTS

4.1 Participants' Characteristics

Population characteristics of BL patients showed 78.8% and 21.8% males and females respectively, 9.1%, 75.8 %, 15.1% were aged >12, 5-12, and < 5 years respectively. Approximately seventy nine (79%) of BL cases were boys and their ages at diagnosis ranged from 3-16, a mean of 8.8 ±3.7 years males were more disposed, male: female ratio 4:1. Patients' demographic characteristics are as shown in Table 1.

Table 1: Demographic characteristics of participants with Burkitt's lymphoma

Characteristics	***No***	***%***
Sex (n= 33)		
Male	26	78.8
Female	7	21.2
Age at diagnosis		
>12	3	9.1
5-12	25	75.8
<5	5	15.1

Male/female ratio = 4:1

The mean age at diagnosis for plasmablastic, lymphoblastic, diffuse large B cell lymphoma and other non-Hodgkins lymphoma were: 9.0, 10.9, 14.3 and 10.3 years respectively.

4.2 Distribution of BL cases at MTRH in western Kenya

The counties of origin of cases were Busia, Kakamega, Siaya and Vihiga, all which boarder the lowlands of Lake Victoria region, had comparatively higher number of BL cases in that order, from 14 different counties in western Kenya (Figure 1).

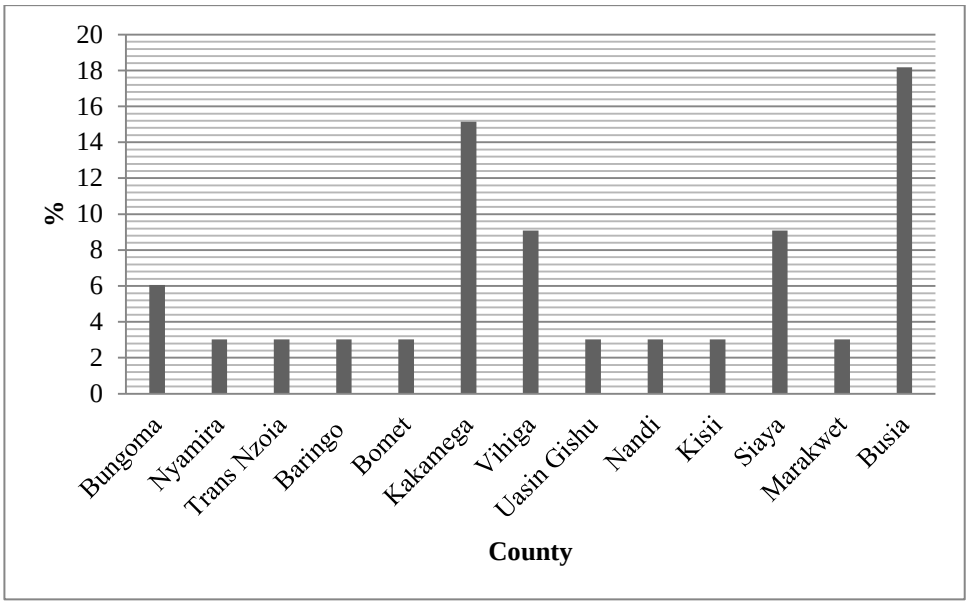


Figure 1. County distribution of Burkitt's lymphoma cases at MRTH in western Kenya

4.3 Ethnic Distribution of BL Cases

Distribution of BL cases in ethnic groups in western Kenya were as shown in Figure 2. The cases were in the order; Luhya (54.6%) > Kalenjin (21.2%) > Luo = Kisii (9.1%)>Turkana.

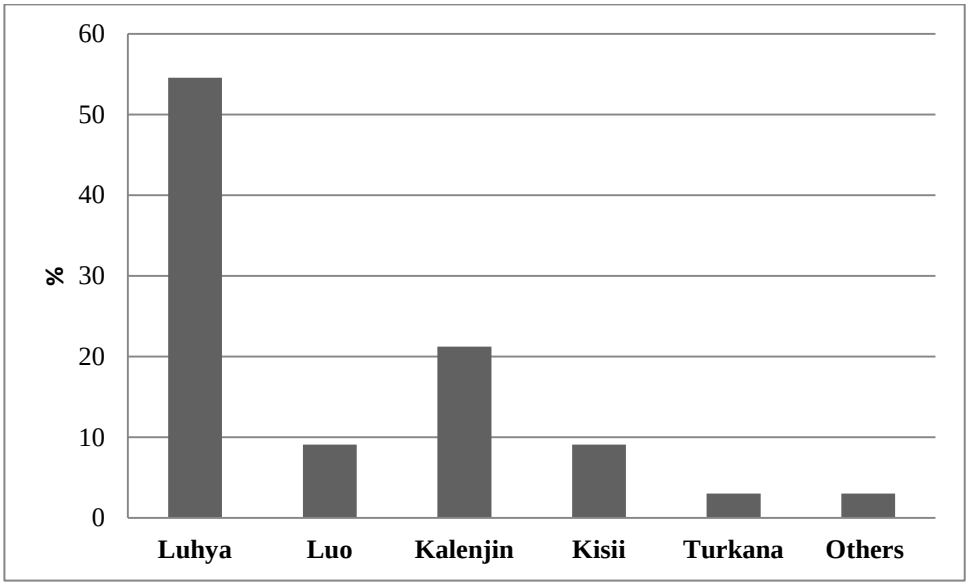


Figure 2. Ethnic distribution of BL cases among study participants

4.4 Socio-economic Characteristics

The occupations of parents/ guardians are as shown in Figure 3. Of the BL cases, 42.4%, 18.2%, 15.2%, 9.1% and 3.0% of the parents/guardians were engaged in farming, business, domestic, government/formal employment and pastoralism respectively. Farming and/or domestic work as their main preoccupation suggesting a rural life style without chemical pollutants and low level of education and therefore lower socioeconomic status compared to their urban counterparts. About 12% had no one occupation.

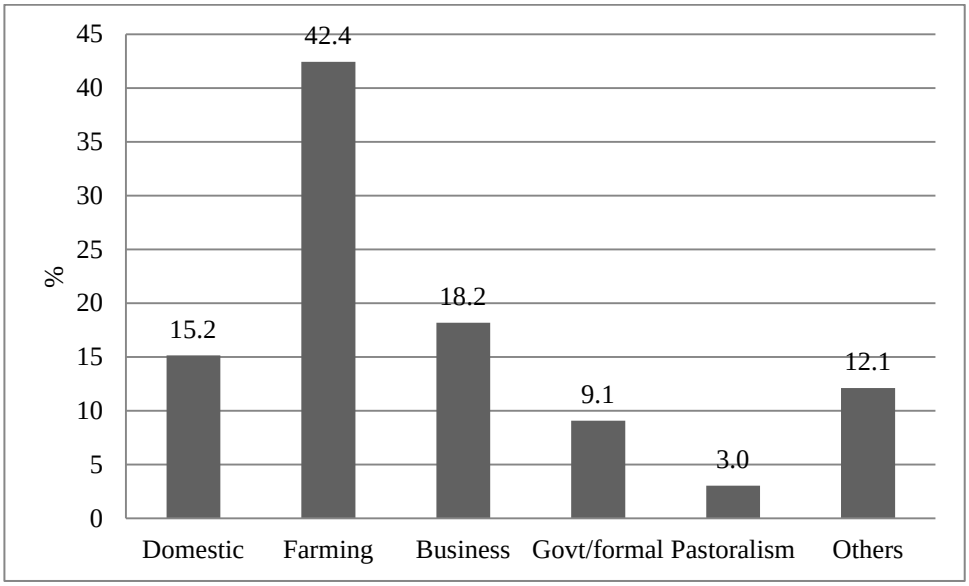


Figure 3. Occupation of BL participants' parent/guardian

4.5 Participants' Family Size

There was no familial clustering of BL since the number of siblings in families with BL ranged from 1-10 members, 39.4% of them being in the range of 1-6 members (Figure 4) with only one of them reporting a sibling tumour with physical features similar to Burkitt's lymphoma.

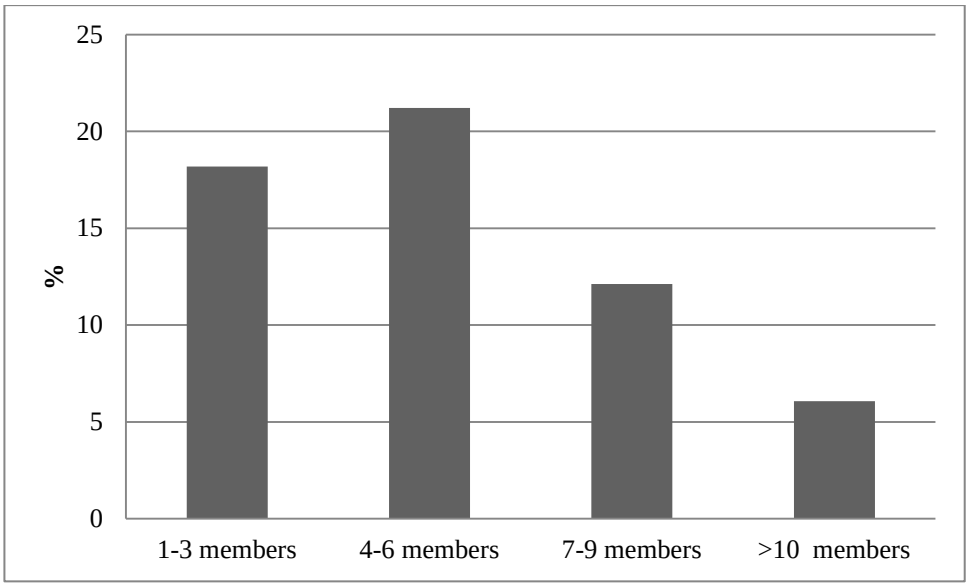


Figure 4. Participants' family size

4.6 Burkitt's Lymphoma Tumour sites

Anatomic presentations of BL showed that abdomen sites were more common than jaw, jaw and abdomen and lymph and thyroid, occurance being 46%, 33%, 12% and 3% respectively (Table 2). The EBV, HIV-1 and MYC positivity was variable at these anatomic sites-jaw, abdomen, lymph node and thyroid.

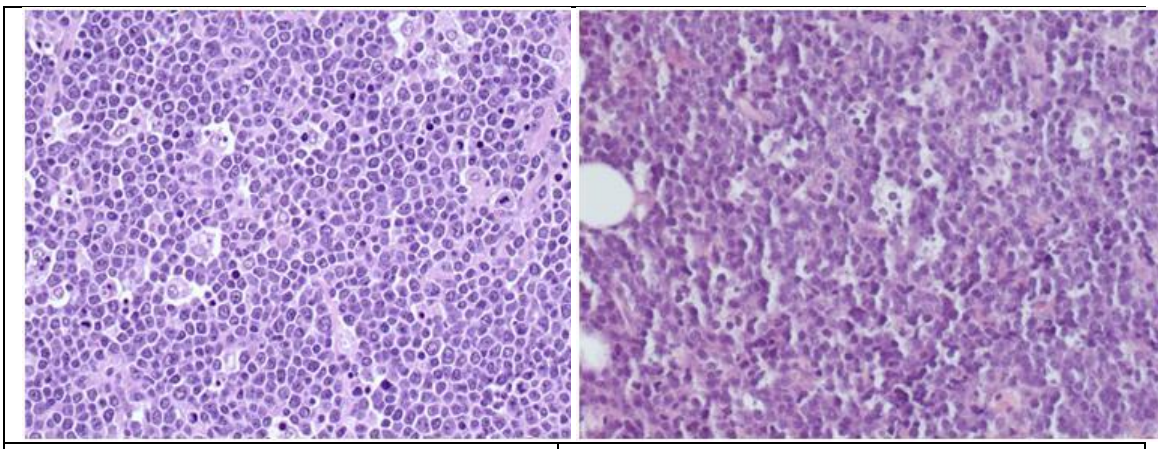
Table 2: Clinicopathological characteristics of BL patients

Cases N=33	**Site**	**Sex M/F**	**EBV+ (%)**	**HIV+**	***MYC+ve***
11	Jaw (33%)	7/4	100	1(9.1%)	10/11(90.1%)
15	Abdomen (46%)	13/2	93	3(13.3%)	15/15(100%)
4	Jaw & Abdomen (12%)	3/1	100	0.0	3/4(75.0%)
2	LN (6%)	2/0	100	0.0	2/2(100%)
1	Thyroid (3%)	1/0	100	0.0	1/1(100%)

Clinicopathologic presentation showed that abdomen>jaw>jaw and abdomen>lymph node>thyroid occurrence percent being 46%, 33%, 12%, 6% and 3% respectively. Epstein Barr virus was positive in nearly all the cases; HIV was associated with jaw and abdomen, representing 22.4% percent of all BL cases.

4.7 Burkitt's Lymphoma Diagnosis by Haematoxylin and Eosin Staining

All the 104 biopsy samples from participants were stained with haematoxylin and eosin (H&E). Of which, 33 showed overall resemblances to typical Burkitt's lymphoma morphology of cohesive monomorphic medium-sized neoplastic infiltrate, cells had round nuclei with reticulated chromatin and multiple nucleoli and a narrow rim of basophilic, vacuolated cytoplasm and higher nucleus to cytoplasmic ratio. Frequent mitotic and apoptotic figures within monotonous medium-sized blastic tumour cells presented pathognomonic view of a 'starry-sky' appearance (Figure 5). Of the 33 Butkitt's lymphoma cases, 17 (51.5%) had typical morphology, while the other 16 (48.5%) had atypical or equivocal morphology and therefore not easily distinguished by H and E staining alone.



a) BL – Typical morphology (H &E)	**b) BL –** Atypical morphology (H &E)

Deep blue stained cells = tumour cells

Figure 5. Photomicrograph of BL tumour sections stained with haematoxylin and eosin, showing typical and atypical morphologies

The two morphological forms of BL occurred in the study setting. The atypical morphology-cohesive and/or cells with greater pleomorphic nuclear sizes and shape BL was missed by H and E staining diagnosis alone. This is reflective of constrains in terms of capacity and resources at the MTRH histopathogy laboratory which may lead to misdiagnosis of BL and other diseases with similar clinical presentations.

4.8 Burkitt's Lymphoma Immunohistochesmistry (IHC) Staining Characteristics

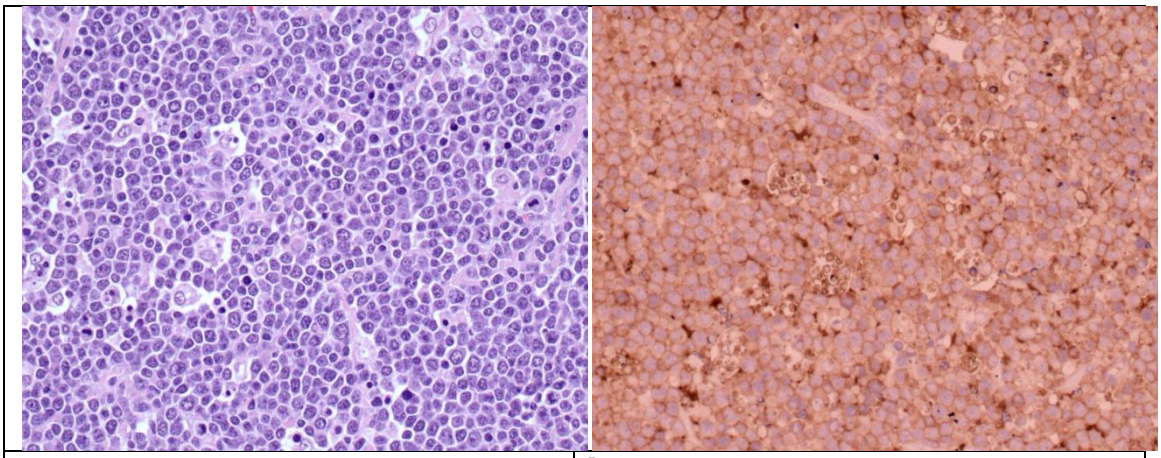
Immunohistochemical studies of 3μm biopsy tissue sections from BL participants showed characteristic expression pattern of reactivity to a series of key markers; CD38, CD44, BCL-2, CD20, CD10, Ki-67 and MYC protein as summarised in Table 3. Hundred percent of all BL tumours showed expression of CD10, CD20 and CD38 while Ki-67 expression 100%, MYC protein expression was positive in 82% of all BL tumours, while CD44 and BCL-2 markers were 100% and 89% negative in all BL tumours Table 3. The other B cell lymphomas had variable expression of these markers.

Table 3: Molecular profile of some B cell non-Hodgkin's lymphoma

		BL[1]	DLBCL[2]	LL[3]	PL[4]
		n (%)	n (%)	n (%)	n (%)
CD10	-ve	0	2 (50)	0	3 (75)
	+ve	22 (100)	2 (50)	5 (100)	1 (25)
CD20	-ve	0	0	4 (80)	4 (100)
	+ve	22 (100)	4 (100)	1 (20)	0
CD38	-ve	0	0	0	0
	+ve	17 (100)	3 (100)	5 (100)	4 (100)
CD44	-ve	22 (100)	4 (100)	5 (100)	4 (100)
	+ve	0	0	0	0
BCL-2	-ve	17 (89)	1 (25)	1 (20)	0
	+ve	2 (11)	3 (75)	4 (80)	4 (100)
Ki-67	-ve	0	0	0	0
	+ve	22 (100)	4 (100)	5 (100)	4 (100)
MYC	-ve	3 (18)	1 (33)	1 (20)	0
	+ve	14 (82)	2 (67)	4 (80)	4 (100)

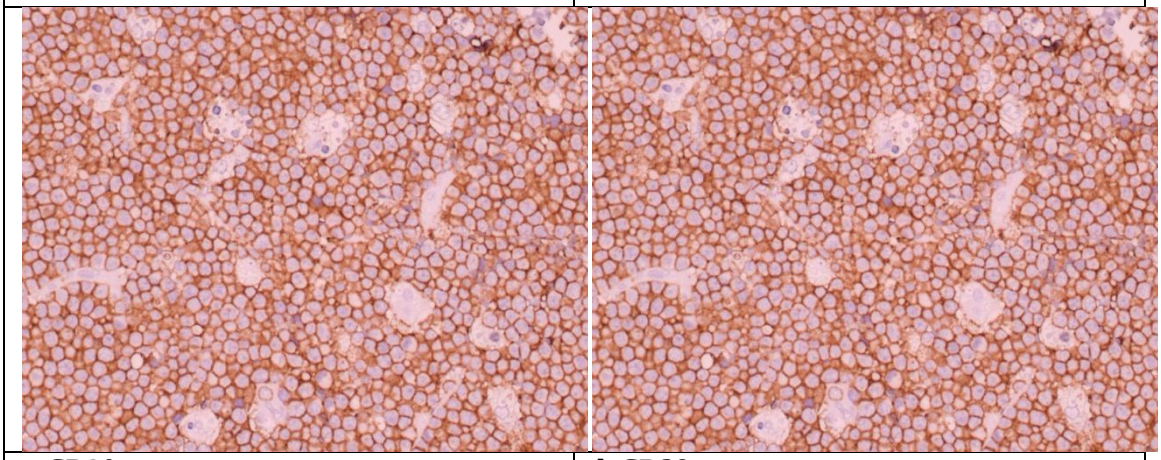
1 Burkitt's lymphoma **2** Diffuse large B cell lymphoma **3** Lymphoblastic lymphoma **4** Plasmablastic lymphoma

Both typical and atypical morphological forms of Burkitt's lymphoma in the current study showed moderate to strong expression of CD10, CD20, CD38, high Ki-67 proliferative index (PI) (100%) as shown in Figures 6 & 7. Brown stained, medium-sized with round nuclei cells, giving an appearance of monotonous field, are positive for respective cell marker. Blue or non-stained cells are negative for respective cell marker.



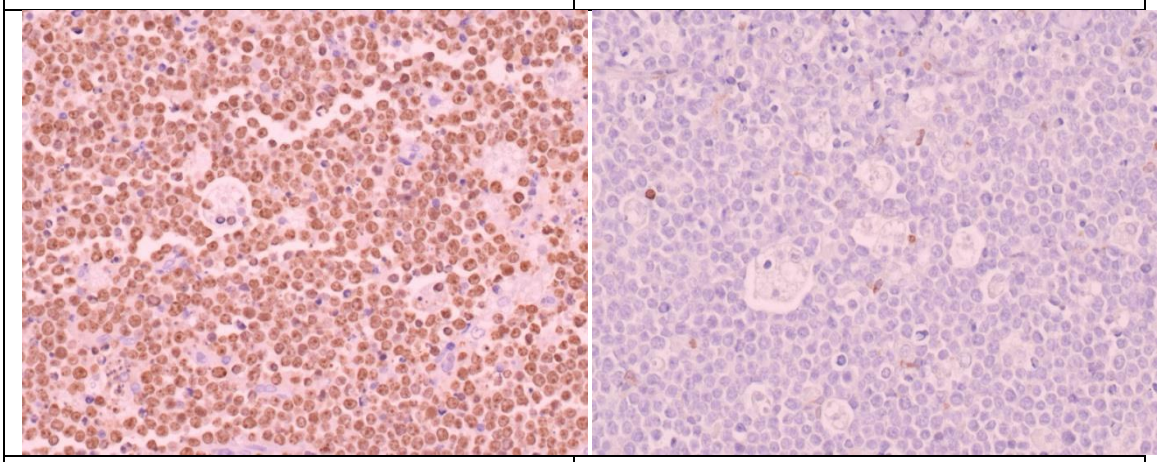
a. H&E

b. CD10



c. CD20

d. CD38

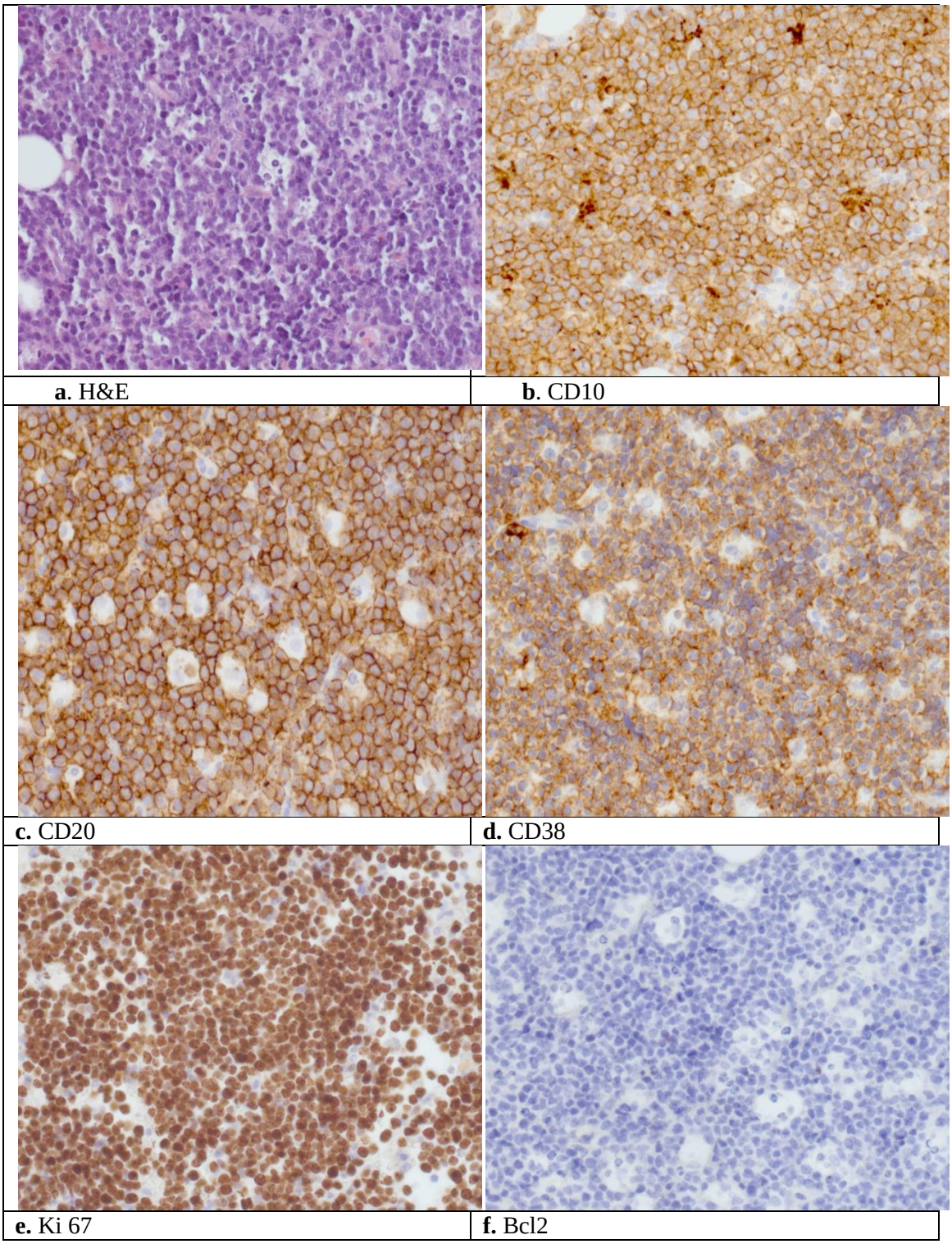


e. Ki-67

f. Bcl-2

Brown stained cells = +ve Blue /unstained cells = -ve

Figure 6. Representative immunophenotype staining of BL with typical morphology



a. H&E | **b**. CD10

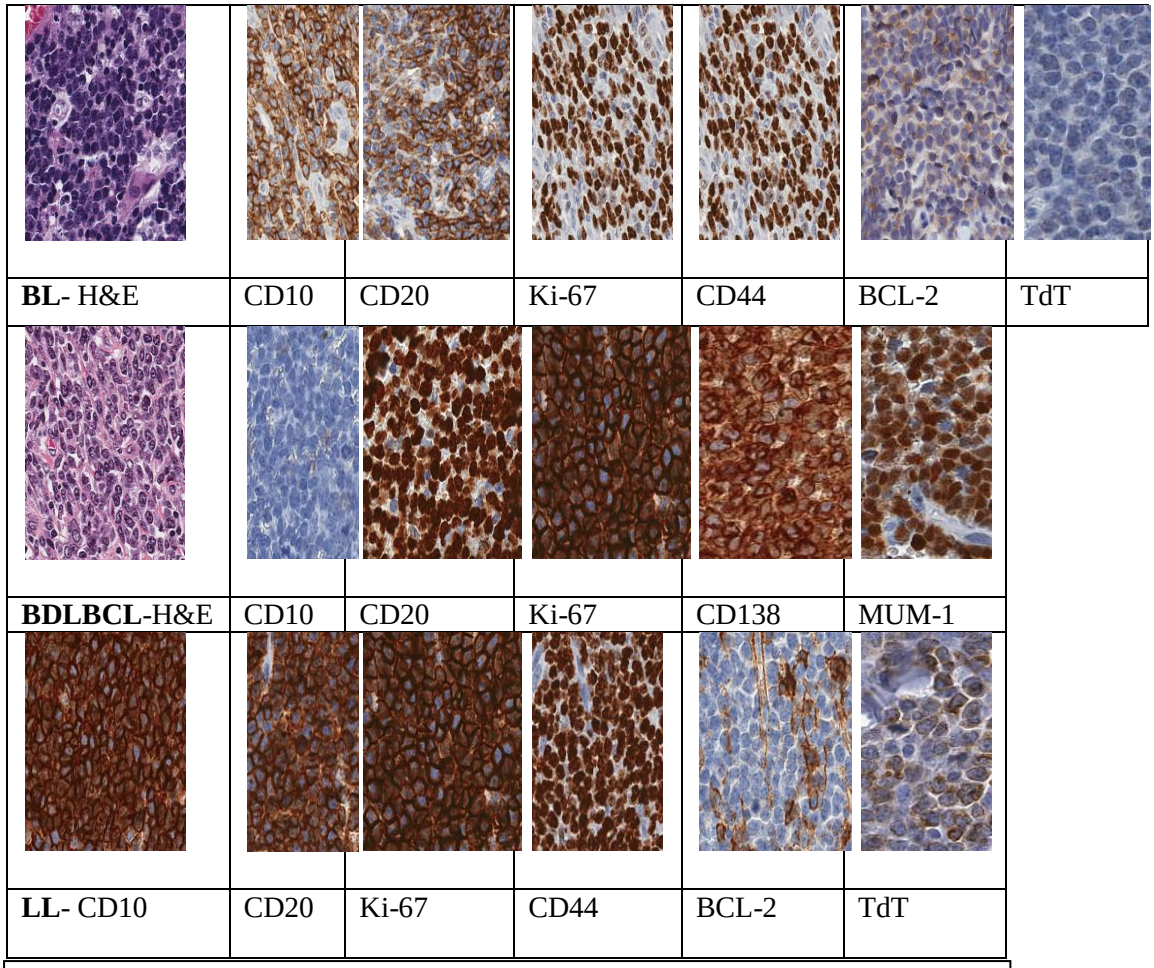
c. CD20 | **d.** CD38

e. Ki 67 | **f.** Bcl2

Brown stained cells = +ve Blue/unstained cells = -ve

Figure 7. Representative immunophenotype of BL tumour with atypical morphology

Immunohistochemical staining and histologic characteristics of BL and other aggressive B cell lymphomas are shown in Figure 8. Brown stained cells are positive while blue or unstained cells are negative for the respective cell marker.



BL- Burkitt's lymphoma, **DLBCL**-Diffuse large B cell lymphoma, **LL**-Lymphoblastic lymphoma

Figure 8. Immunohistochemical and histologic differences between BL, DLBCL and lymphoblatic lymphoma

Of the clinically presumed non-Hodgkin's lymphomas, BL was the most common (23.9%) but not other diagnostic categories namely: diffuse large B cell lymphoma (2.9%); lyphoblastic lymphoma (5.1%); plasmablastic lymphoma (2.9%) other non-Hodgkins lymphomas (16.7%) and the others being leukemias, non lymphoid

conditions as well as other lymphoproliferative disorders, on review using immunohistochemistry and other required molecular criteria (Figure 9).

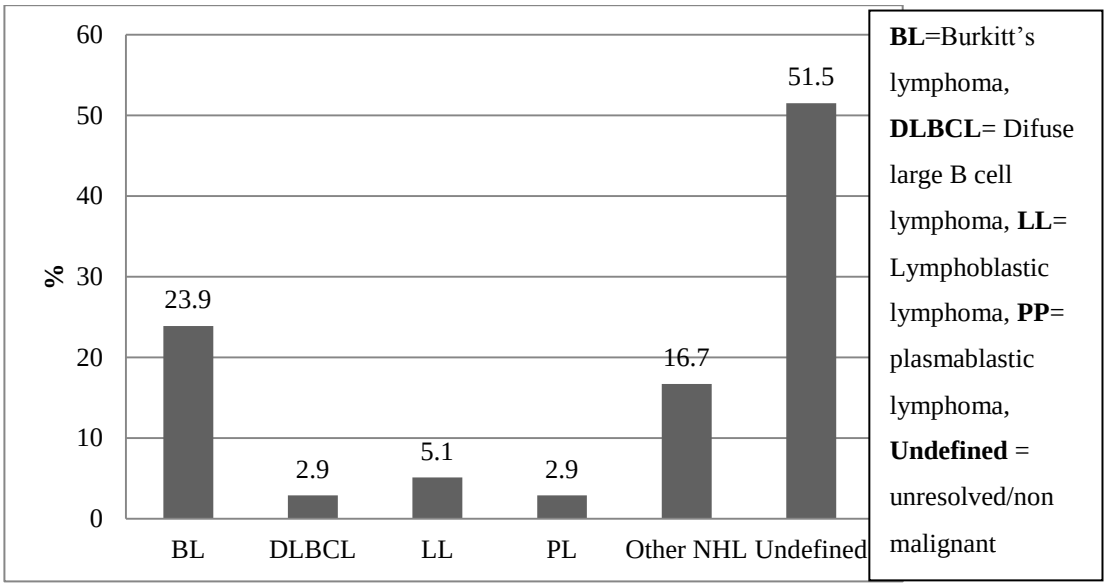


Figure 9. Lymphoma types in western Kenya

4.9 MYC Protein Detection by Immunohistochemistry (IHC)

The relationship between MYC protein expression detected by immunohistochemistry and t(8;14) *c-myc* gene translocation by fluorescence *in-situ* hybridisation (FISH) is shown in Table 4.

Table 4: Percent detection of *c-myc* translocation by IHC and FISH

		FISH c-myc	
		% -ve	**% +ve**
IHC MYC	**% +ve**	50	86.7
	% -ve	50	13.3
	Total	100	100

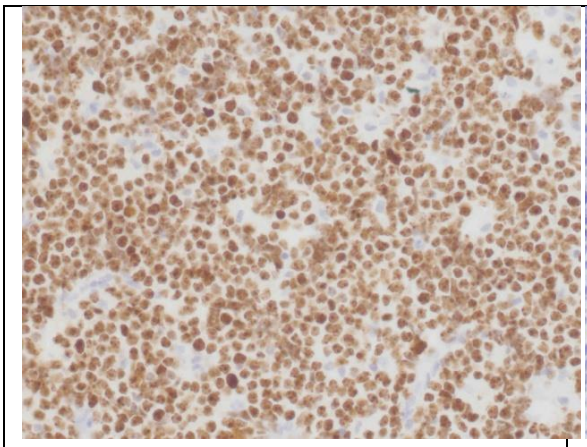
The MYC protein as detected by immunohistochemistry was 86.7% compared fluorescence *in- situ* hybridization (FISH) detection of Burkitt's lymphoma with

t(8;14) c-*myc* gene translocation. Thus, MYC protein expression detected by immunohistochemistry using commercially available antibody can equally well determine c-*myc* gene translocation found out by fluorescence *in-situ* hybridization (FISH) (Figure 10 a-e). In this study immunohistochemical detection of MYC protein expression, revealed of up to 86.7% of positive tests by fluorescence in-situ hybridization (FISH), suggesting a positive correlation between MYC protein expression and translocation by FISH (β =0.54).

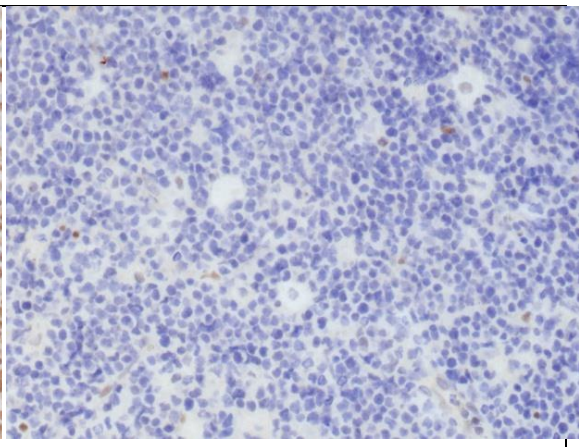
4.10 Molecular Profile and Normal B cell Counterparts of Burkitt's lymphoma

Tumour B cells in BL here showed positive staining for CD10, CD20, CD38, Ki-67 and MYC positive/negative with either typical or atypical morphologies (Figures 6 & 7). Table 7 shows percentage somatic hypermutation of some BL cases studied

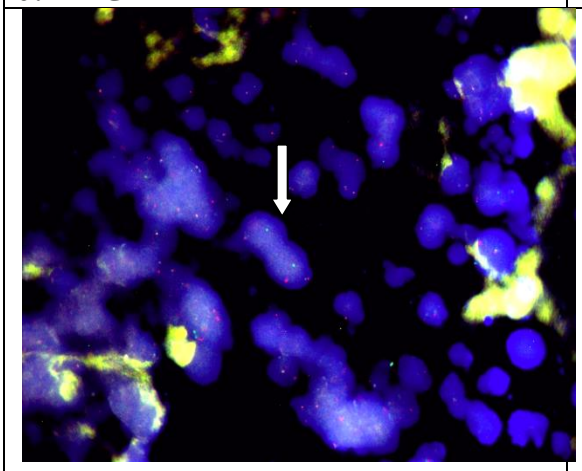
Figure 11 shows representative slides of immunohistochemistry stained and FISH *c-myc* gene translocation of BL cases. In FISH photomicrograph, green signal indicates IgH, red signal indicates *c-myc* gene and yellow signal denotes areas where green and red signals coincide. Figure 11 c & d show positive (yellow) signals for IgH/*c-myc* translocation. In this study, 13.3% *c-myc* gene translocation negative, by both fluorescence *in-situ* hybridization and immunohistochemistry BL cases were detected.



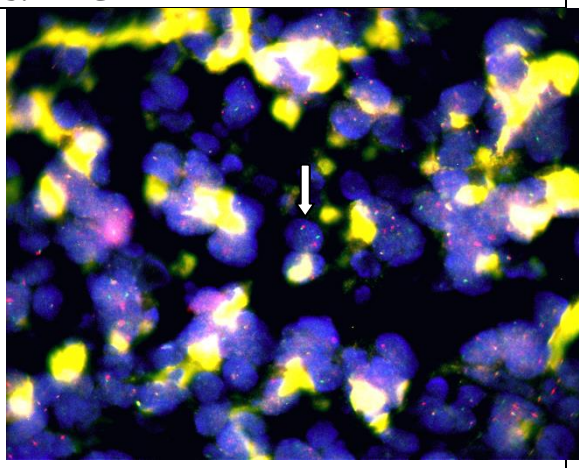
a. MYC^{+ve}



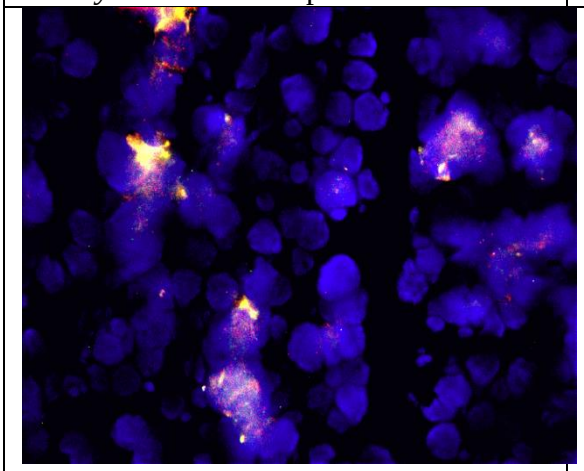
b. MYC^{-ve}



c. *c-myc* translocation positive



d. *c-myc* translocation negative



e. *c-myc* translocation negative

Figure 10. Representative slides: a & b IHC stained MYC$^{+/-}$, c & d FISH *c-myc* translocation +ve, e. FISH *c-myc* translocation –ve

⟹ Cells with green, red and yellow signals, thus positive for *c-myc* translocation

4.11 Reliability of Immunohistochemistry in BL Diagnosis

The sensitivity, specificity and predictive values of haematoxylin and eosin in the diagnosis of Burkitt's lymphoma, using immunohistochemistry (IHC) as gold standard were as shown in Table 5. Sensitivity and specificity of haematoxylin and eosin (H&E) as compared to Immunohistochemistry findings were calculated. Positive predictive value (PPV) and the negative predictive value (NPV) were also determined. Sensitivity is the probability of a positive test among patients with BL disease. Specificity is the probability of a negative test among patients without BL disease. Positive predictive value is the probability that those who test positive for BL actually have the disease while negative predictive value is the probability that those who test negative do not actually have the disease. The positive predictive value, negative predictive value, specificity and sensitivity were 53%, 89%, 94% and 40% respectively.

Table 5: Parameters for the reliability of haematoxylin and eosin in the diagnosis of Burkitt's lymphoma

Parameter	%
Sensitivity	40
Specificity	94
Accuracy	85
Positive predictive value (PPV)	53
Negative predictive value (NPV)	89

4.12 Burkitt's Lymphoma Molecular Profile and Treatment outcome

This study correlated molecular profile and treatment outcome; the results were as depicted in Figure 11. The tumour marker CD10 (β=0.40) and BCL-2 (β=0.29) appeared to be associated with favorable outcome while MYC positive (β= -0.34)

status seemed to be associated with an unfavorable outcome. The associations between the molecular markers studied and patient treatment outcomes were not significant.

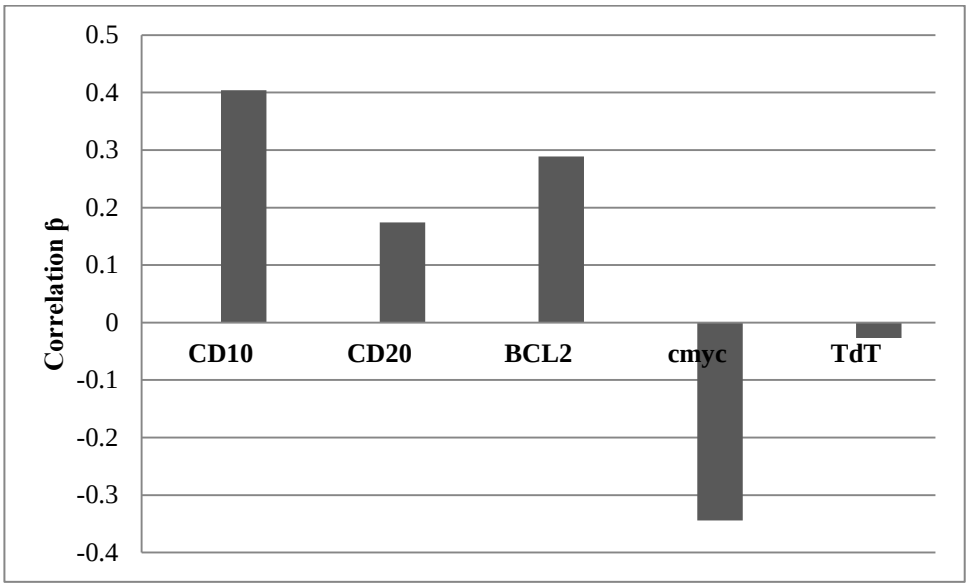


Figure 11. Correlation between molecular profiles and treatment outcome

In the correlation, the direction of treatment outcomes was from worse to good.

4.13 Association between Burkitt's lymphoma, EBV and HIV-1 Infection

Figure 12. show EBER1, EBVIgG ELISAand HIV-1 status of the study subjects.

Ninety three 93.3% of all BL participants (n=33) were positive for EBVIgG antibodies by rapid ELISA test, comparable to more than 90% of BL carrying latent EBV encoded small RNA (EBER) in the form of nuclear extra-chromosomal episomes.

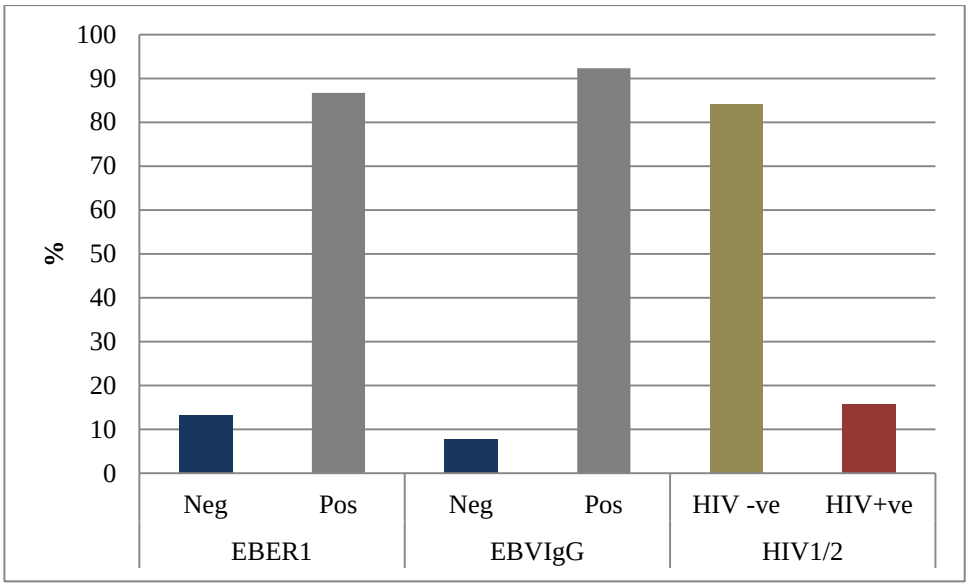


Figure 12. Levels of EBVIgG ELISA, EBER and HIV-1 status of participants

Using a log-linear model, bivariate associations between BL, EBV and HIV adjusted for age and gender, results showed that BL rates were 1.4 times greater in subjects who tested positive for EBER than those who tested negative (odds ratio, OR: 1.39, 95% CI: 0.16–12.19).

Table 6: Bivariate associations between BL, EBV$^{+/-}$ and HIV$^{+/-}$

	AOR (95% CI) EBER (+ve **vs** -ve)	AOR (95% CI) HIV (+ve **vs** -ve)
Burkitt's Lymphoma	1.39 (0.16 - 12.19)	1.58 (0.35 -7.18)

AOR = Age and gender adjusted odds ratio

In this series, the percentage of HIV-1 positive cases of Burkitt's lymphoma was (16%) with a higher viral load, Figure 13. Association between paediatric Burkitt's lymphoma and HIV-1 in the study population was 1.6 times greater in subjects who

tested positive for HIV than those who tested negative (OR: 1.58, 95% CI: 0.35–7.18), regardless of age and gender.

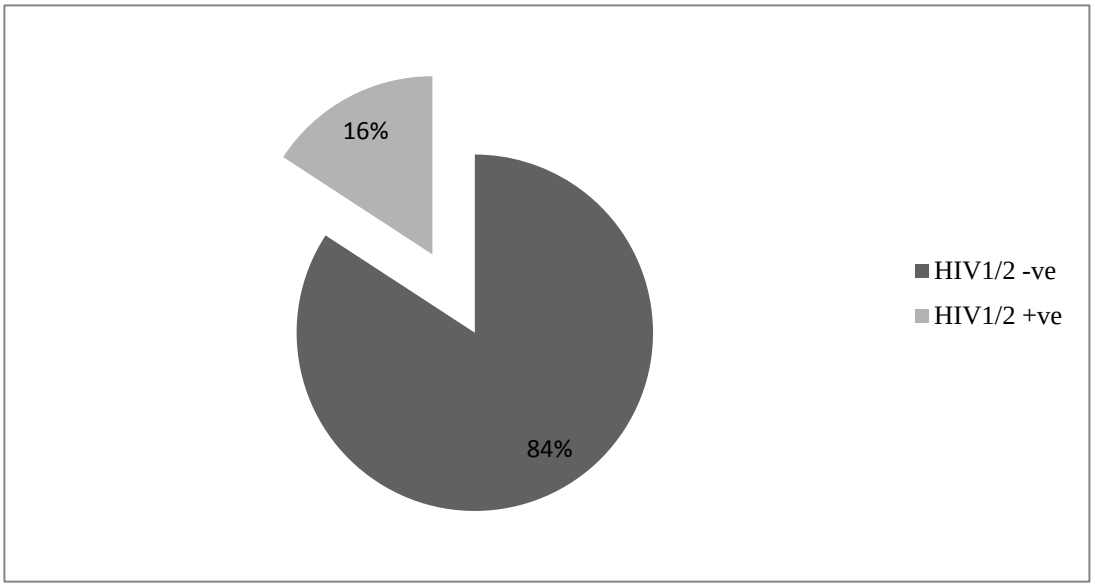


Figure 13. HIV 1/2 status BL cases

4.14 Malaria Parasite Exposure

Malaria parasite exposure, when initially evaluated using malaria immunochromatogenic *P. falciparum* antigen test, detected 5% of BL cases testing positive for the parasite antigen, possibly due to the qualitative nature of the test and ongoing anti malaria medication by the patients. Insecticide treated nets (ITN) when used as proxy to measure malaria exposure (Figure 14), showed no dramatic difference in self reported insecticide treated net (ITN) protection against malaria between BLs and non-BLs. The results show that 51.5% (n= 17) of BL cases reported household ownership and use of the mosquito nets and taking active malaria preventive measures in their family environment, compared to 57% (n= 60) in non-BL cases.

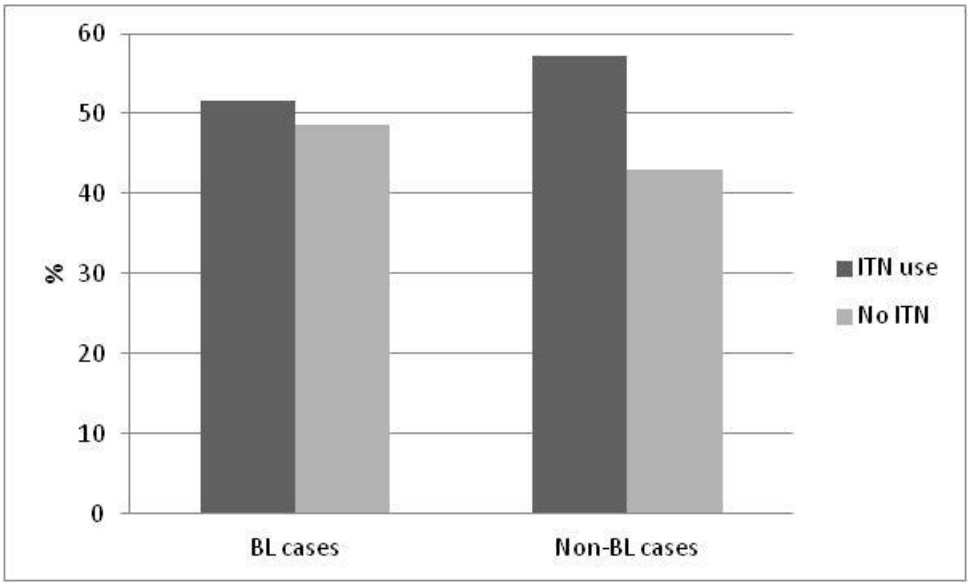


Figure 14. Insecticide-treated bed nets in BL and non-BL cases

4.15 Association beween Burkitt's lymphoma and *Plasmodium falciparum*

The study detected *P.falciparum* specific histidine rich protein 2 (p.f.HRP-2) in only 5.3% of BL paediatric cases, Figure 15.

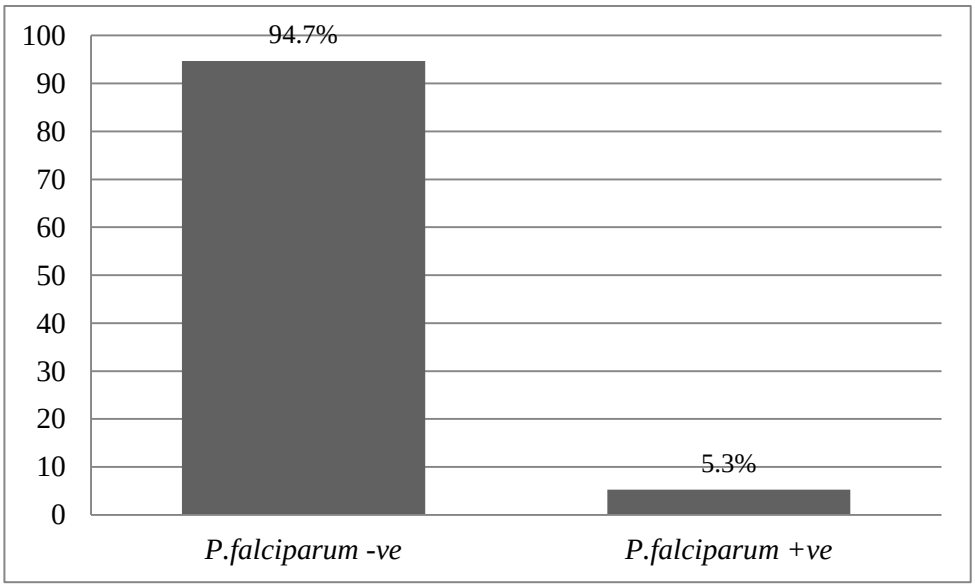


Figure 15. *P. falciparum* antigen in BL cases

This meant that there may be no marked difference in anti-*P.falciparum* antigen in blood of BL and non-BL cases.

4.16 Somatic Mutation of Immunoglobulin Heavy Chain (IgH) in BL

Table 7. shows IgV_H gene and allele alignment, percentage identity to the closest germline IgV_H allele in IMGT reference directory. Alignment score of >85% indicate the gene and allele most likely to be involved in the rearrangement.

Table 7: IgV_H gene and allele alignment and EBV status of BL cases

Case	Allele	V-gene & allele Identity (%)	V-region mutation	Amino acid Changes	*EBER*+/-
1	HomsapIgHV4-34*01 F	90.5	22	14	Positive
2	HomsapIgHV1-18*01 F	93.2	15	9	Positive
3	HomsapIgHV-13*01 F	90.4	22	13	Positive
4	HomsapIgHV3-21*01 F	89.2	25	14	Negative

The tool determines and localizes the somatic mutations of the immunoglobulin rearranged sequences (URL: http://imgt.cines.fr; Giudicelli, Chaume, & Lefranc, 2004). Cases 1-4 had V-gene and allele identity > 89% with; 22, 15, 22 and 25 IgV_H mutations respectively, cases 1-3 were positive EBER while case 4 was EBER negative.

4.17 IgV_H Gene Alignments

Figure 16. shows IgV_H gene alignment with the FR–IMGT and the CDR–IMGT delimitations. The PCR amplificate serve as input sequence which is aligned with the closest V, D and J genes and alleles in the IMGT reference directory set (www.

Imgt.cines.fr). The IMGT gene name and allele are indicated for each reference sequence. The dashes in a sequence indicate nucleotide identity while dots indicate gaps according to the IMGT numbering or nucleotides that are not taken into account for the alignment. This representation allows the comparison of the sequences and to locate somatic mutations. Cases 1-4 showed various nucleotide differences from grem line sequences corresponding to immunoglobulin somatic mutations at those particular locations.

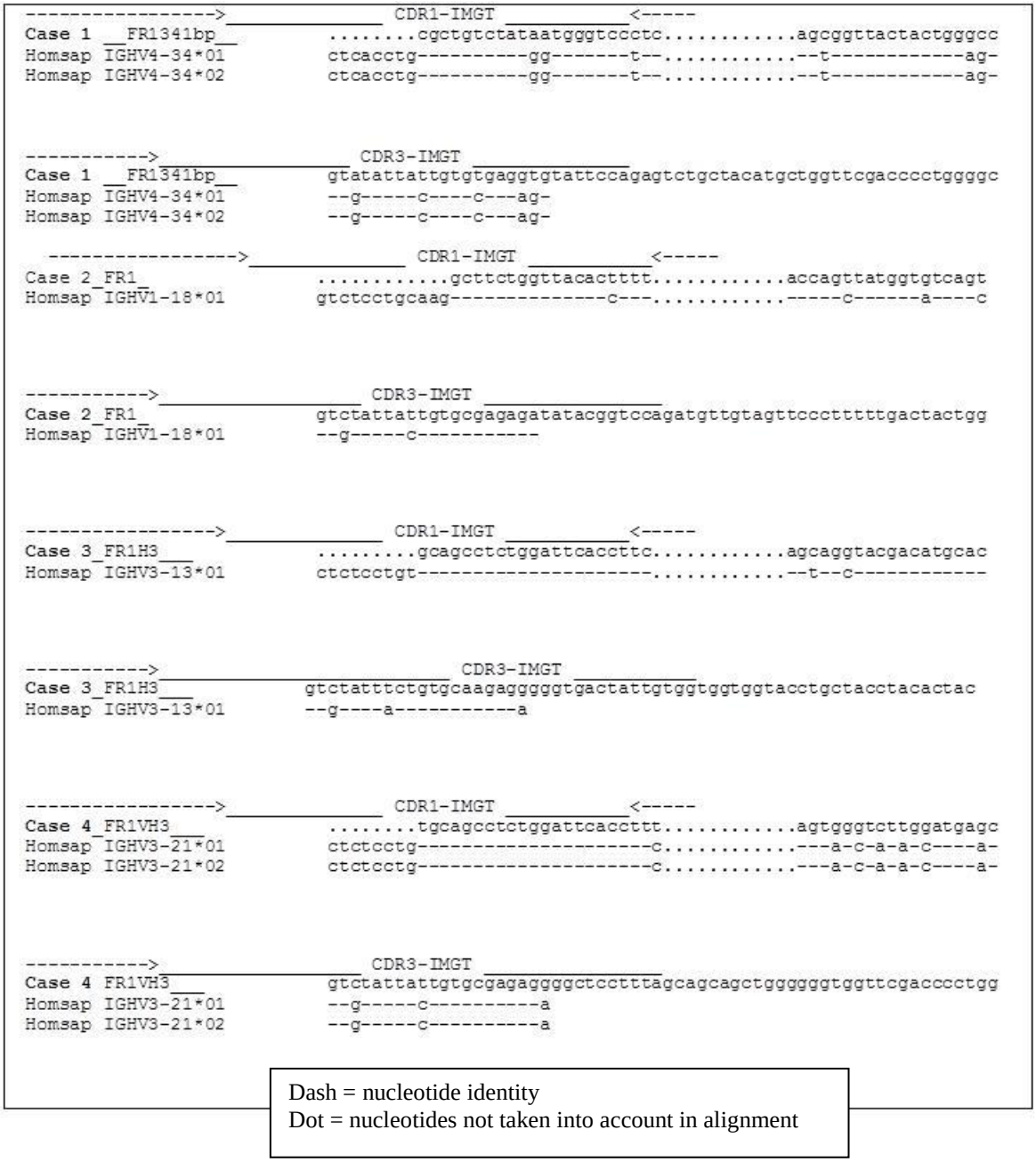


Figure 16. IgV_H gene alignment with the FR–IMGT and the CDR–IMGT delimitations

Taken together these results indicate there were differences between the input sequences and the comparison alleles in the IGMT reference directory, implying the presence of somatic mutated IgV_H gene in sequences from BL cases in this study regardless of EBER negativity or positivity of the case. The number of V_H gene mutations ranged from 15-25 (Table7).

4.18 Cellular MicroRNA Expression in Burkitt's lymphoma cases

To further asses the possibility of an alternative BL pathogenesis in addition to viral and parasite aetiology, cellular microRNA (miRNA) - *hsa-miR*-34a and *hsa-miR*-127 expression profiles in this series of Burkitt's lymphoma were investigated. Figure 17 shows the relative expression of a) *hsa-miR*-127 in BL cases, b) *hsa-miR*-127; in Ramos and Cag cell lines and c) *hsa-miR*-34a. There was a higher relative expression of 2.8 vs 2.09 of cellular *hsa-miR*-127 and has-miR-34a of MYC-positive respectively in comparison to MYC-negative BL cases, Ramos human Burkitt's lymphoma (American type) cell line (EBV-negative BL-derived cell line) and cag-multiple myeloma cell line (MYC-positive).

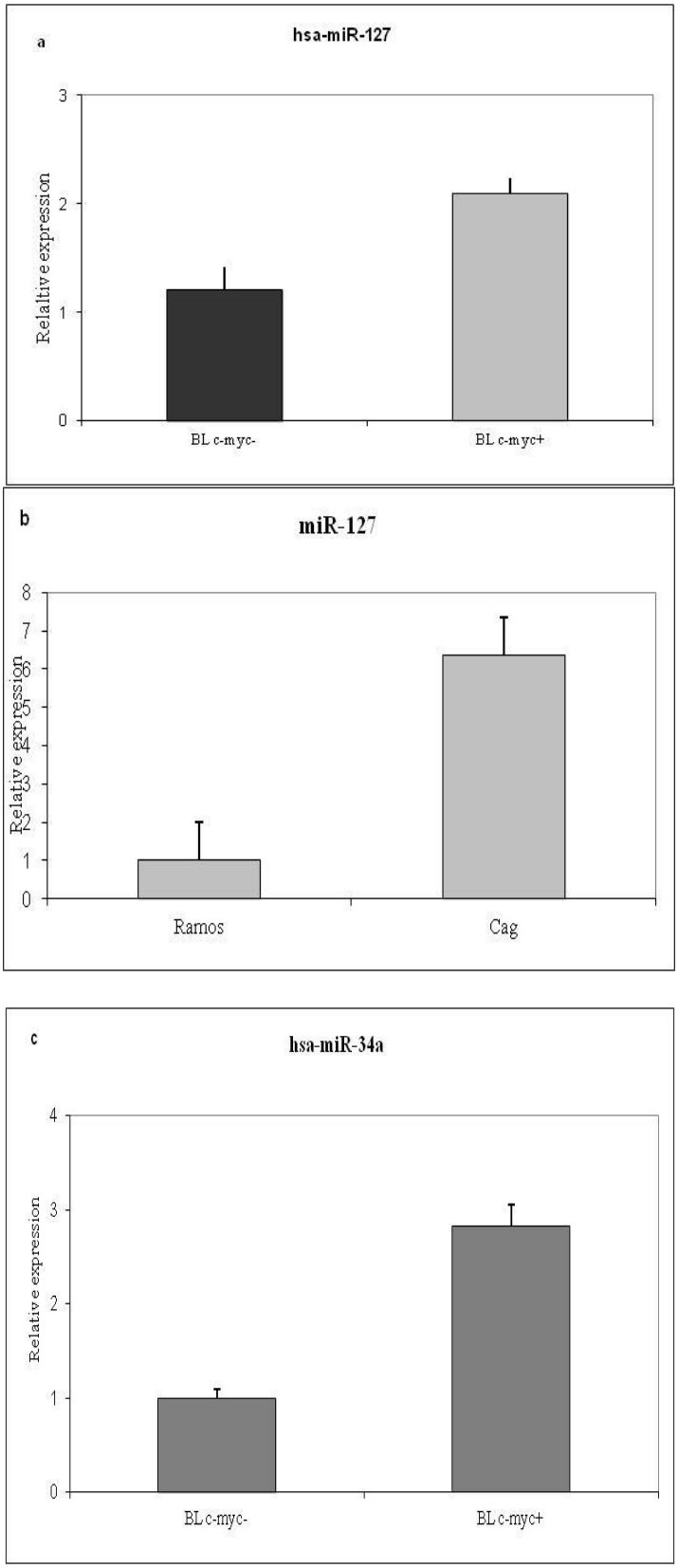


Figure 17. Relative expression of; a) *hsa-miR*-127 in BL cases, b) *hsa-miR*-127, in Ramos and Cag cell lines and c) *hsa-miR*-34a

4.19 Haematogical Parameters and Cytokines Expression in BL cases

Figure 18 and 19 show some blood count parameters in BL and non-BL participants. White blood cells, red blood cell and platelet counts were not remarkably different between BL's and non-BL's participants; the marginal median values of WBC and Median platelets levels were above normal median values in BL cases, this is suggestive of Burkitt's lymphoma in leukemic phase.

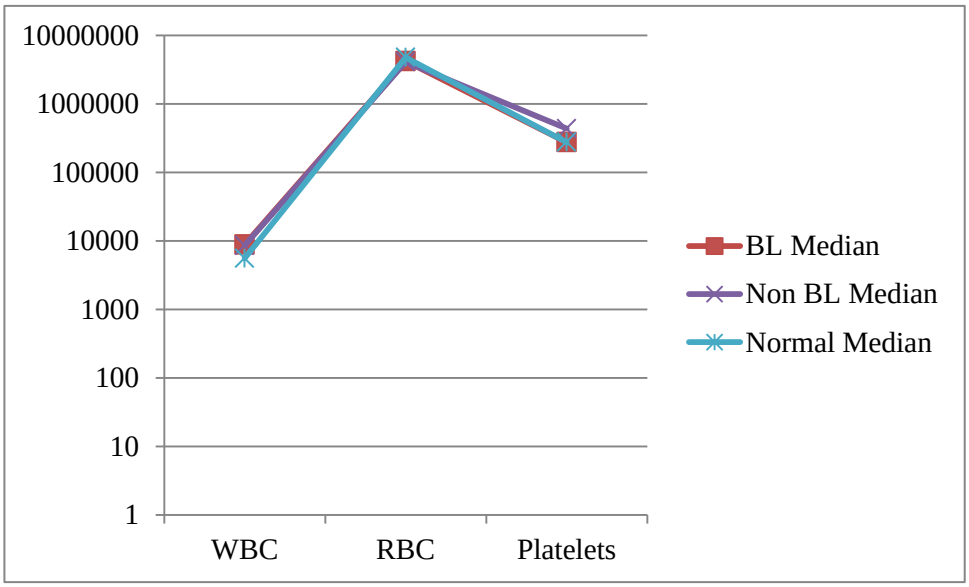


Figure 18. White, Red Blood cells and platelets in normal, BL and Non-BL cases

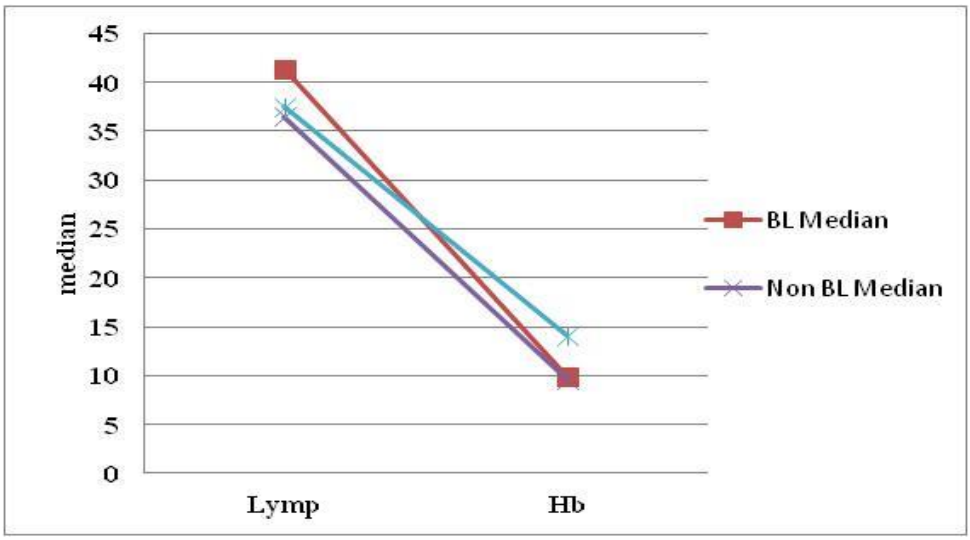


Figure 19. Median values for lymphocytes and Hb in normal, BL and Non-BL

Figure 20 shows some hematologic parameter in participants before and after treatment.

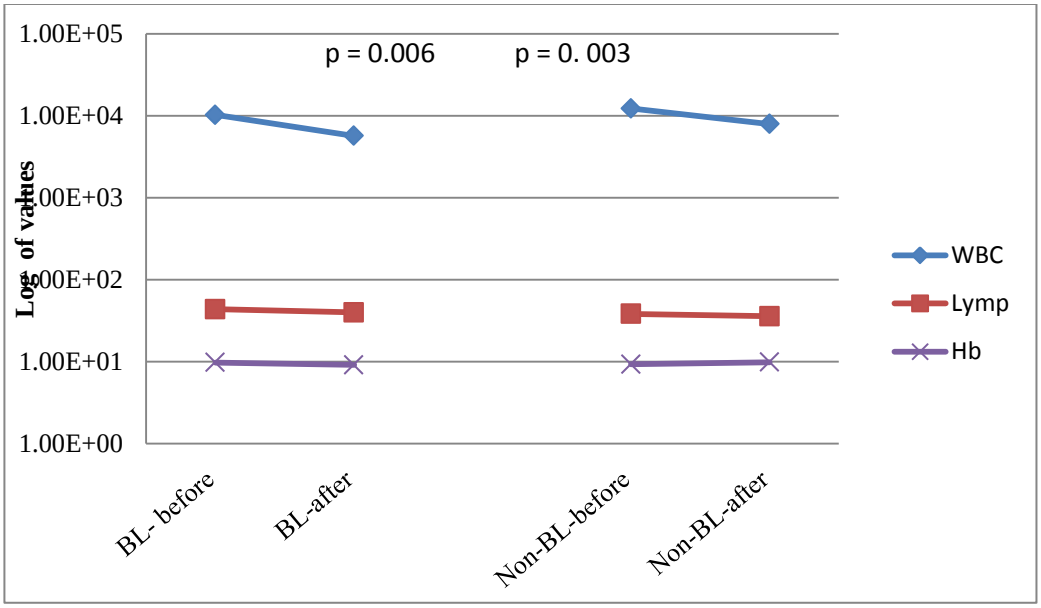


Figure 20. Log of WBC, lymphocytes and Hb before and after treatment

Figure 21 shows plasma level of some Th1, Th2, and Th17 cytokine expression in BL and non-BL participants. Figure 21 depicts differences in the expression levels of interleukin 6 (IL-6) and interleukin 10 (IL-10) in BL and non-BL participants. This study attempted evaluation of TGF-β1 however, the levels of TGF-β1, regulatory cytokine, were below the detectable limit of the BD CBA Human TGF-β1 single plex flex kit used.

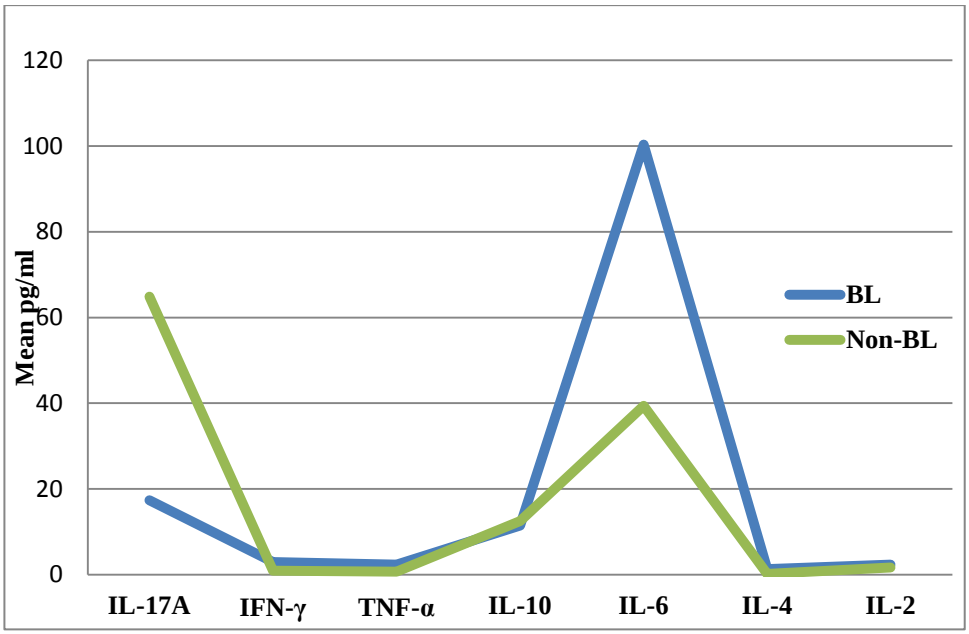


Figure 21. Th1, Th2, Th17 expression of BL and non-BL participants

The median levels of of IL-6 and IL-10 (13.83 vs 8.98 pg/ml) were higher in BL cases than (3.34 vs 1.50 pg/ml) in non-BL cases. But the mean level for IL-17A (64 pg/ml) was higher in no-BL compared to (17.38 pg/ml) for BL cases, though not statistically significant.

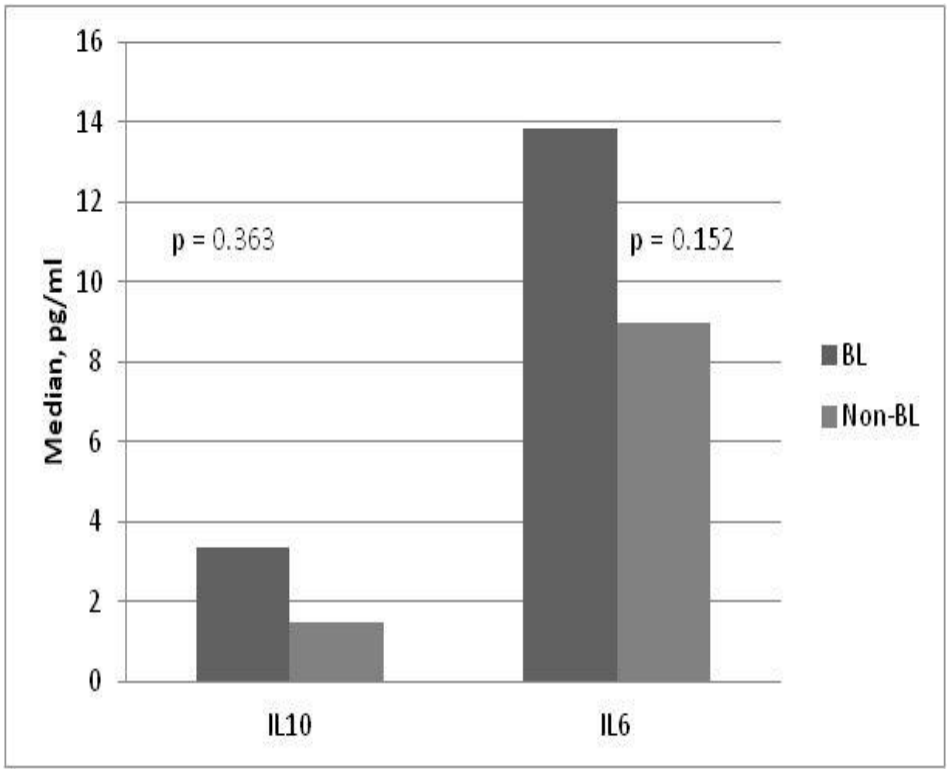


Figure 22. Comparison of IL-6/IL-10 levels in BL's and non-BL cases

CHAPTER FIVE

DISCUSSION

5.1 Study Participants' Characteristics

There was male preponderance of 78.8% compared to females (12.2%), which is in agreement with findings from several other prior studies (Kalungi *et al*., 2009).The male to female ratio of 4:1 is similar to those reported by Cool & Bitter, (1997) in Kenya but higher than those reported by Mwakigonja *et al*. (2008) in Tanzania. It is however unclear whether this male bias is due to differences in hospital seeking behaviour differences in sex between sexes, biological pathogenesis and/or yet unclear socio-environmental factors or a selection bias. For non BL participants the age at diagnosis varied widely from 2-17, a mean of 8.1 ±4.5 years probably due to different disease conditions involved in this group.

5.2 Clinicopathological Characteristics of BL cases

The mean age of 8.8 years at diagnosis for Burkitt's lymphoma cases in this study was higher than other studies by Mutalima *et al*. (2008) which reported slightly younger age of 7.1 years in children in Malawi, the difference may probably be due to the fact that many (46%) of BL cases in the present study had abdominal involvement. Children with abdominal tumour tend to be older than those who present with jaw or orbital masses (Ogwang *et al*., 2008).

The most common tumour sites were abdomen (46%), followed by jaw (33%). This is surprising and in contrast to initial reports from Uganda since the 1950's that more than 50% of BL tumours present in the jaw (Walusansa *et al*., 2012; Burkitt, 1958); Mutalima *et al*. (2008) who reported that jaw tumours involving multiple quadrants

are a characteristic feature, especially in younger children children in Malawi. Thesa findings agree with those by Ogwang *et al.* (2008), who reported abdominal presentation as the most common among children in northern Uganda. The relatively lower frequency of jaw presentation in this study may be attributable to a number of factors: jaw tumours are common in lower elevation which may have been underrepresented in this current series; improvement in technology to diagnose abdominal tumours by use of ultra sound (personal communication August 5, 2014): jaw tumours are common but treated presumptively without immunohistochemistry diagnosis. This leads to question whether the sites of BL tumour presentation might have changed over time, or it might be that a new variant of BL is present in the region. In China, Huang, Liu, Zeng, Zhang, Huang and Xu, (2015) found most extranodal and abdominal BL to be cases of sporadic Burkitt's lymphoma. A study in Ghana also observed a seemingly new trend of BL development following the pattern of westernized, rich societies, where the tumour is more likely to be presented as an abdominal tumour compared with jaw tumour (Biggar *et al*., 1981). Interestingly one unusual case presented with a thyroid mass. The tumours were not staged but were estimated to range from stage I-III according to the Ann Arbor classification scheme (Rosenwald *et al*., 2002).

5.3 Distribution of BL in Ethnic Groups

Majority of the cases were from the Luhya (54.6%), followed by Kalenjin (21.2%), Luo (9.1%) and Kisii (9.1%). The higher percentage of cases from the Luhya tribe agrees with what was reported by Rainey *et al*. (2007) in a study to evaluate spatial distribution of Burkitt's lymphoma in Kenya, but differs from Othieno-Abinya *et al*. (2004) who reported lower values for the same community. The percentage for

Kalenjin tribe is surprisingly higher than previous reports probably because this study was based in Eldoret and therefore favourable proximity and accessibility; inclusion of Mount Elgon saboat sub-tribe in Bungoma county which had higher BL incidences; the BL associated risk factors such as environmental, viral infections and malaria may have become more widespread over time from the neighbouring endemic regions due to many possible reasons. The value for Luo is lower than those reported by Makata *et al*. (1996), possibly due to distance from the study hospital Moi Teaching and Referral Hospital (MTRH) and/or other groups who study and provide BL care in the Lake Victoria region and western Kenya in general which target the disease.

5.4 Socio-economic Characteristics of BL cases

Low socio-economic status group appeared to carry a greater burden of Burkitt's lymphoma in this study. These findings are similar to those from studies by Morrow, (1974) and Biggar and Nkrumah, (1979) in which families in which Burkitt's lymphoma children tended to come from families with poorer social and economic circumstance compared to their control counterparts. Only three (3%) percent of BL cases and 9.5% non BL cases were employed in government service, probably suggesting a degree of formal education and a relatively high socioeconomic status. This means that social and economic status alone is not the only risk factors associated with Burkitt's lymphoma. Some studies have associated increased risk of BL among families with low socio- economic status to protein deficiency. Poor nutritional status is known to decrease immunosurveillance, and could increase chances for oncogenic event to occur (Rainey *et al*., 2008).

The impact of socioeconomic factors in the distribution and clinical characteristics of BL is unclear. In rural and low economic status settings in western Kenya, there exits a high likelihood of malnutrition, impaired immunity or widespread immunosuppression that may results from endemic infectious herpesviruses, *P. falciparum* and other environmental risk factors including but not limited to standing water, vegetation, and landuse practices in the population (Ogwang *et al*., 2008). Immunodeficiency following chronic infections can lead to conditions that contribute to the development of neoplasm (Vose, Chiu, Cheson, Dancey, & Wright, 2002). Reports by Orem *et al*. (2007) and Gualco, Klumb, Barber, Weiss, & Bacchi (2010) implied association of BL with lower socioeconomic status in Uganda and Brazil respectively. However, this does not appear to be the case in parts of south East Asia where BL is not endemic in similar socioeconomic environment (van den Bosch, 2004). Rainey *et al*. (2008), concluded that the non-random distribution of BL is at least, also contributed to by poor socioeconomic circumstances may be associated with early exposure to EBV, poor nutrition and/or hygiene and poor immune responses to environmental exposure to etiological agents, in a population-based case-control study in western Kenya. Thus social, economic and a range of environmental variables could thus be regarded as surrogate marker for exposure to factors that play aetiological role in BL development.

5.5 Burkitt's Lymphoma Family Clustering

This study found only one BL case in which there was reported family member with a lymphoma. similar to past epidemiologic studies of BL that have not revealed significant family clustering link to BL unlike certain childhood diseases like leukemias and other lymphomas (Rainey *et al*., 2008). This probably implies that BL

may be largely driven by somatic mutations including t(8;14) translocation events rather than inheritable traits. Some inheritable gene variant modifiers such as HbAS which provide resistance to malaria do not appear to confer protection against Burkitt's lymphoma according to a study by Mulama *et al*. (2013).

This observation on family cluster is different from earlier studies by Morrow (1974) which reported BL familial clustering tendency. Rainey *et al*. (2008) in case-control study also reported that sibship relationships, household characteristics, birth order and family environment may contribute to endemic Burkitt's lymphoma. Further studies may be necessary to elucidate the relationship.

5.6 Burkitt's Lymphoma Diagnosis by Haematoxylin and Eosin Staining

The existence of both typical and atypical Burkitt's lymphoma morphologies and similarities with related lymphoma entities as revealed by haematoxylin and eosin (H&E) staining means that morphological diagnosis using H&E alone is not sensitive enough to distinguish BL from other entities (Wilkins, 2011). The implication is that differentiating Burkitt's lymphoma from B-cell Burkitt-like lymphoma and other B-cell lymphomas with similar morphological and clinical features using H&E alone. The sensitivity of H&E was low at about 40% with accuracy of 85% which is lower than 93% reported by Lukande *et al*. (2008), but within the range of 58% to 88 as reported by Ogwang *et al*. (2011).

5.7 Burkitt's Lymphoma Immunohistochesmistry (IHC) Staining Characteristics

The immunohistochemical staining pattern and morphological spectrum of BL is broader than previously assumed, ranging from typical BL morphology to centroblast-like DLBCL morphology, expression of CD10 and Ki-67 proliferation index (PI) >90% in all cases in the absence or in combination with weak expression of BCL-2 and CD44 which is consistent with other studies by Rodig, Vergilio, Shahsafaei, and Dorfman, (2008). The Ki-67 proliferation index is in conformity with the documented high doubling time of about 24 hours of Burkitt's lymphoma from kinetic studies by Leoncini, Lazzi, Bellan, & Tosi, (2002). The percentage of cells expressing Ki-67 is an important criterion for the diagnosis decision of BL. The cut-off point for the distinction between BL and other aggressive B-cell lymphomas is generally regarded as >90%. Laboratory and/or technical inadequacies coupled with suboptimal tissue fixation could contribute to underestimation of proliferation >90%. When both CD44 and Bcl2 were negative in all BL cases, thus can serve as exclusionary biomarkers during BL diagnosis (Figure 9). The pattern of expression here shows that Burkitt's lymphoma always expressed CD10 and BCL-2 negative. Other B cell lymphoma subtypes such as DLBCL, plasmablastic, lymphoblastic lymphomas expressed TdT, CD10, and CD20 and MUM-1, similar to studies by in Uganda by Tumwine *et al*, (2008). The protein BCL-2 is a pro-apoptotic factor of the Bcl-2 gene family, and therefore promotes host cell survival. The marker Ki-67 indicates high rate of proliferation, while CD10 and CD44 signify germinal centre similarities of BL tumour cells.

Both typical and atypical morphological forms of Burkitt's lymphoma cases, showed a characteristic positive pattern of CD10, CD20, CD38, a high Ki-67 proliferative index

(PI) (>90%) and/or MYC protein expression and negative for CD44, BCL-2 and/or MYC protein expression. Expression of MYC protein and Ki-67 proliferative index (PI) of 90-100% with a negative BCL-2 was highly predictive of BL diagnosis. Both CD44 and BCL-2 negativity were exclusionary markers for BL. The observed pattern resembled that described diagnosis of Burkitt's lymphoma using an algorithmic in both resource-poor countries by Naresh *et al*, (2011). Similar immunoreactivities staining patterns have been reported and proposed for use in algorithmic diagnosis of BL by (Naresh *et al*., 2011).

These methods taken in combination, the observed immunohistochemical staining pattern, histology and clinical can help to distinguish Burkitt's lymphoma from other closely related aggressive B cell lymphoma, such as diffuse large B-cell lymphoma (DLBCL), plasmablastic lymphoma, lymphoblastic lymphoma and unclassifiable lymphoma with features intermediate between BL and DLBCL. These aggressive B cell lymphomas do not differ clinically but require different management (Bellan *et al*., 2010). Therefore, using a minimal panel of six antibodies from the studied panel, namely; CD20, CD10, BCL-2, Ki-67, CD38 and CD44, one can accurately diagnose >92% of aggressive B-cell lymphomas and identify specific subsets within it (Naresh *et al*., 2011).

5.8 Types of B cell Non-Hodgkin's Lymphoma at MTRH

Of the clinically and histologically diagnosed paediatric aggressive B cell non-Hogkin's lymphoma reviewed, this study found out that Burkitt's lymphoma accounted for 23.9% which is lower than those reported in the earlier studies in Kenya by Cool and Bitter (1997) and Parkin *et al*. (2000) but higher than those reported

between 1996-2001 from Tanzania during HIV-1 epidemic by Mwakigonja *et al*, (2008). This difference could probably be due to different accuracies of diagnostic techniques used in the different studies, the latter largely dependent on clinical and morphological methods. The other entities resolved were 5.1% lymphoblastic lymphoma, 2.9% plasmablastic lymphoma and 2.9% DLBCL. The categories of Lymphoblatic and plasmablastic lymphomas were hitherto not routinely diagnosed at MTRH histopathology laboratory, due to non existent immunohistochemistry laboratory infrastructure in the facility. The undefined category comprised of non-malignant and non-lymphoid conditions unresolved by the study criteria. Overall, these findings indicate that BL, mostly abdominal presentation, is the most common type of aggressive B-cell lymphoma in pediatric patients at MTRH in western Kenya. And that a minimal panel may be useful in diagnosing BL in limited-resource settings such as MTRH and in western Kenya. Non-lymphoid malignancies such as Wilm's tumor, neuroblastoma, nasopharyngeal carcinoma, other carcinomas and sarcomas also were reported in this study.

5.9 MYC Protein and t(8:14) Gene Translocation Detection

Close concordance in detection of MYC protein by immunohistochemistry and t(8;14) *c-myc* gene translocation by fluorescence in-situ hybridization (FISH) observed in this study, has previously demonstrated by Tapia *et al*. (2011). This suggests that in resource limited countries such as in this study setting, *c-myc* gene translocation in tumours can be evaluated in terms of MYC protein expression by immunohistochemistry staining. This can serve to select cases in which confirmatory, more expensive and technically demanding genetic test must be done. Further, there have been attempts to correlate immunonohistochemistry (IHC) and genetic

translocation. Expression patterns of TCL1, CD10, CD38, and CD44 has been found useful in identifying MYC+ tumours which harbour t(8;14) translocation. Expression of TCL1, and CD38, in absence of BCL-2 and CD44 can be useful in identifying MYC+ tumours and there is correspondence between the immunohistochemical test and FISH/cytogenetics studies (Rodig *et al*., 2008).

However, some 13.3 % of the BL cases in this study lacked identifieable c-*myc* gene translocation by Fluorescene in-situ hybridization (FISH) and MYC protein expression by immunohistochemistry (IHC). This result is slightly lower than Leucci *et al*. (2008) who found 14.3%, suggesting the possibility of an alternative BL pathogenesis for MYC negative cases, different from *c-myc* gene dysregulation (Leoncini, Leucci & Cocco, 2008). Upregulation or modulation of certain microRNAs which cause MYC protein over expression, have been proposed. MicroRNA molecule may act as tumour suppressors or oncogenes.

Lack of MYC protein expression in some BL cases in this study, supports findings by Leucci *et al*. (2008) that BL cases lacking *c-myc* gene translocation but have the morphology and the immunophenotype of BL. These findings contrasts with earlier observations that virtually all BL carry the *c-myc* oncogene translocated to an immunoglobulin (Ig) locus as a primary event (Swerdlow *et al*., 2008). It has been thought that 100% of endemic BL cases carried the t(8;14)(q21;q32) translocation (Cogliatti *et al*., 2006).

Primary karyotypic abnormalities are often closely associated with an individual lymphoma subtype, and they can be of diagnostic value. Some genetic abnormalities

are more often seen in one category of haematological malignancy, for instance the t(8;14)(q24;q32) translocation is found in Burkitt's lymphoma but, though less commonly, in diffuse large-B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and other lymphomas (Ventura *et al*., 2006). In addition to primary genetic aberration, secondary chromosomal changes may occur more commonly in some types of lymphoma than others. Some lymphomas are characterized by multiple aberrations and these are often of prognostic value. For example, t(8;14)(q24;q32) translocation is a primary aberration in endemic Burkitt's lymphoma, but it can also arise as a secondary aberration in follicular lymphoma, in which case, it is associated with a poor prognosis (Troxell, Bangs, Cherry, Natkunam, & Kong, 2005). However, *c-myc* gene translocation may also arise as a secondary event in a previously established lymphoma making such tumours behave more aggressively. In which case, *c-myc* gene translocation can be a biomarker of disease evolution and progression (Rodig *et al*., 2008). Therefore, it is important to determine and document cases and alert clinicians when *c-myc* gene translocation carrying B cell lymphomas including Burkitt's lymphomas are encountered.

The *c-myc* gene translocation to an IgH locus leads to dysregulation and constitutive expression of MYC protein, with an overall effect of uncontrolled proliferation as well as a concomitant increase in the rate of cell death (Allday *et al*., 2009). Overexpression of MYC protein contributes to proliferation through induction of activity of cyclins which then activates cyclin-dependent kinase enzymes, while repressing the activity of the cyclin inhibitor p27. The proapoptotic properties of MYC protein involve both the extrinsic factors through interactions with tumour necrosis factor related aptosis inducing ligand (TRAIL) and intrinsic factors through

interactions with p53 pathways. These properties are consistent with the observed high Ki-67 proliferative index (PI) of ≥ 90% in BL cases in this study. This means that BL cells have a high proliferative index while at the same time remain susceptible to apoptosis as in God & Haque (2010). Overexpression of MYC protein thus, drives a neoplastic B cell into cell proliferation cycle, but it also leads to apoptosis in the absence of apoptosis-inhibiting signals. Anti-apoptotic signals may be provided by other oncogenes, such as *Ras* or *Bcl-2*, or in the case of EBV-positive cells by EBNA-1, which inhibit apoptosis and are required for tumour survival (Kennedy, Komano, & Sugden, 2003), further suggesting that EBV may have an important role in BL disease process.

It appears, therefore that *c-myc* gene translocation and MYC protein overexpression is a characteristic of BL but not specific for BL (Leoncini, Leucci & Cocco, 2008). Thus, *c-myc* gene rearrangement is sensitive but not specific marker for all Burkitt's lymphoma variants. Translocations involving *c-myc* gene are occasionally seen in other B cell lymphomas (Hetch & Aster, 2000; Ventura *et al*., 2006). This study focused on t(8;14) *c-myc* gene translocation, it was not possible to determine whether different translocations apart from t(8;14) and whether other chromosomal aberrations were present. It would be worth elucidating these aspects in future studies.

It is often necessary to consider *c-myc* gene translocation by fluorescence in-situ hybridisation (FISH) results for lymphomas in formalin fixed paraffin embedded tissue biopsy together with other immunopathology reports alongside with other molecular features to get a definitive cases description. The FISH procedure would be

helpful in discriminating the most difficult lymphoma categories, but there are cost implications in resource limited settings such as in the study setting.

5.10 Normal B cell Counterpart of B cells in Burkitt's lymphoma

The precise cell counterparts of Burkitts lymphoma B cell neoplasm is not known and/or is controversial. Generally, neoplasms in many respects recapitulate normal stages of lymphoid cell differentiation and function, for instance, stages of B cell differentiation, activation and maturation. This is often used to classify the neoplasia (Jaffe *et al*., 2008). Burkitt's lymphoma tumour B cells have homing tendency to germinal centres (GC), thus BL cells morphologically tend to resemble GC cells, and express characteristic GC cell markers such as CD10, even though some authors favour the derivation of BL from GC centroblasts.

Despite positive staining for B cell antigens such as CD20 and germinal centre (GC)-associated marker like CD10 in BL tumours in this study, some B cells of these tumours phenotypically resemble centroblasts derivation by showing somatic hypermutation (Table 7). This suggestion of GC derivation contrasts the fact that some BL tumours growths are extra nodal and involve tissues that do not normally contain germinal centres under physiological condition.

5.11 Reliability of Immunohistochemistry in BL Detection

Overall, sensitivity of morphological diagnosis using haematoxylin and eosin was 40%, with immunohistochemistry (IHC) as gold standard, implying that H&E diagnosis misses to detect 60% of the people testing positive for BL by IHC. Though

specificity for the test was high at 94% meaning that 94% negative results are truly negative and only 6% may be false positive.

By using immunohistochemistry a small panel of IHC antibodies that stain B cell markers, the clinically presumed lymphomas were then classifiable into distinct lymphoma/leukaemia entities namely: Burkitt's lymphoma (BL), diffuse large B cell lymphoma (DLBCL), plasmablastic lymphoma (PL), lymphoblastic lymphoma (LL), non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma (HL) as recommended in WHO criteria for classification of haematopoietic and lymphoid tumours. This then allowed for the identification or erroneous exclusion of cases of other Hodgkin's lymphoma which are difficult to categorize using haematoxylin and eosin staining alone. Therefore commercially available IHC monoclonal antibodies and FISH molecular probes greatly improve the accurate diagnosis of Burkitt's and other lymphomas in line with the current WHO classification of haematopoietic and lymphoid malignancies criteria, similar to studies done by Naresh *et al*. (2011). Final diagnoses of lymphoma especially Burkitt's lymphoma, based on the revised World Health Organization classification should be confirmed from clinical and histopathological features using immunohistochemistry and fluorescence *in-situ* hybridization techniques. A combination of morphology, immunohistochemistry and *c-myc* gene translocation by fluorescence *in-situ* hybridization (FISH) provided improved accuracy of BL diagnosis. Naresh *et al*. (2011) demonstrated that by using a panel of six antibodies-CD20, CD10, BCL2, Ki-67, CD38 and CD44, one can accurately diagnose >92% of aggressive B-cell lymphomas and identify specific subsets within it. This thus underscores the possible impact of immunohistochemistry

and histopathology diagnoses in resource constrained medical settings such as the study hospital.

5.12 Burkitt's Lymphoma Molecular Profile and Treatment Outcome

The negative correlation between BL patients with MYC expression and treatment outcome observed in this study was in contrast to those by Leucci *et al*. (2008), who reported that BL patients without the MYC protein expression who received less aggressive therapy, usually given to DLBCL, had a poor outcome compared with patients with BL with MYC protein expressing tumours. The different is probably due to the chemotherapy regimen given in the study setting, where treatment often begins without conclusive diagnosis. Therefore, identifying a specific molecular profile in lymphomas with typical BL morphology, but negative for MYC translocation may be useful in identifying those cases that may benefit from more aggressive therapy.

5.13 Association between Burkitt's lymphoma, EBV and HIV-1 Infection

This study demonstrated EBV infection by EBV-IgG in plasma among participants and EBER1 in tissues of BL patients. Bivariate associations between BL, EBV and HIV adjusted for age and gender showed associations, however, statistically not significant. Mutalima *et al*. (2010), found no association between Burkitt's lymphoma and HIV-1, in a study of impact of HIV-1 infection on the risk of cancer among Malawian children. In developing countries, such as sub-Saharan Africa, including Kenya and other low socioeconomic settings, EBV infection is usually acquired in childhood, most often without specific symptoms. African children are infected early in life and most children in tropical Africa have acquired antibodies to EBV by their

second birthday. On the other hand EBV seroconversion tends to occur at much older ages in developed countries. When the EBV infection is delayed, it is characterised by a benign self-limited lymphoproliferation or infectious mononucleosis characterized by variable case histories (Rochford *et al*., 2005). In whatever way the disease first manifests in healthy individuals, primary infection is followed by a symptom-free carrier state.

Even though, Epstein-Barr virus (EBV) appears to infect most individuals in the study, the overwhelming majority of EBV-infected individuals do not suffer from EBV-associated malignancies, similar to observations made by De Falco *et al*. (2009). This may mean that other aetioligic factors in addition to EBV are involved in Burkitt's lymphoma pathogenesis. Epstein-Barr virus (EBV) is not generally regarded as the only driving force of BL cell proliferation; it is thought to play an important role in BL pathogenesis (Onnis *et al*., 2012).

A striking feature of EBV-positive Burkitt's lymphoma is their unique pattern of viral latent protein expression, restricted to EBV-encoded nuclear antigen 1 (EBNA-1), the same pattern of viral latency is found in latently infected memory B cells when they divide to maintain normal homeostasis in healthy carriers. Only EBNA-1 program can allow the viral genome to be transferred to daughter cells.

Previous studies, such as those those done in Uganda and Tanzania by Newton *et al*. (2001) and Mwakigonja *et al*. (2008) respectively, only very broadly associated HIV-1 infection with lymphomas in general was found. This association is well studied and documented in the developed countries among HIV-1 positive adults. The relationship

of paediatric BL and HIV-1 infection is not clearly established in the region including the study region (Orem *et al*., 2007). Thus, these HIV-1 positive paediatric Burkitt's lymphoma observed in this study are likely to be endemic Burkitt's lymphoma form than immunodeficiency or AIDS-associated BLs. This may be due to poor survival of HIV-1 perinataly infected children in the study study region and sub Saharan Africa as a whole (Parkin *et al*., 2000). Children infected by vertical transmission of HIV-1 die of AIDS during the first few months of life before other possible factors in BL pathogenesis namely: malaria, EBV, *Euphorbia tirucali*, and possibly other arboviruses play promotional role in BL tumorigensis. It is estimated that only 34% of them survive to the age of 3 years or have short survival (Campo *et al*., 2006). Human immunodeficiency 1 (HIV-1) infected individuals have increased risk of developing malignancies (Mbulaiteye *et al*., 2011). HIV-1 positive patients are reported to have a 200–1000-fold greater risk of developing BL than HIV-1 negative patients (Newton *et al*., 2001). It is widely hypothesized that immunodeficiency resulting from HIV-1 infection may be responsible for reactivation of EBV in latently infected B cells which then turn on the progresses to BL (Mutalima *et al*., 2008). In HIV-1 infected individuals, $CD4^+$ T cell count is greatly reduced, leading to diminished EBV-specific $CD8^+$ T-cell activity which then permits proliferation and reactivation of the EBV-infected B cells (Moormann *et al*., 2009). HIV-1-infected patients have a two-fold increased risk of developing malignant disease; in the head and neck the majority of cancers are Kaposi's sarcoma or oral Kaposi's sarcoma (68%), with squamous cell carcinoma and NHL accounting for 17% and 13%, respectively, and only 2% diagnosed as BL. Burkitt's lymphoma is strongly associated with HIV infection, and HIV-associated BL accounts for approximately 5–40% of cases of HIV-associated NHL.

This percentage of BL cases of HIV-1 positive BL is higher than 10% that was found by Parkin *et al*. (2000) who demonstrated that there is little or no relationship between HIV-1 infection and the development of Burkitt's lymphoma in African children who bare the largest burden of HIV/AIDS. This is probably because of recent expansion of access to life-extending anti retroviral therapy (ART) in sub-Saharan Africa and Kenya, through programs such as the Global Fund for AIDS, Malaria and Tuberculosis and the US President's Emergency Program for AIDS Relief (PEPFAR), is leading to increased survival and lengthening of life HIV-1 infected children. The HIV-1 infected participants in this study however had viral loads >100,000 copies/ml. It was not clear whether the children were on any HIV/AIDS treatment, most likely these were uncontrolled HIV-1 infection.

Human immunodeficiency virus 1 (HIV-1) infection induces a vigorous immune response with about 100-fold increase in polyclonal immunoglobulin production, polyclonal B-cell activation and secondary immunosuppression. Many parasites induce T cell immunosuppression and B-cell activation is features of both HIV and other infections like malaria infection, suggesting that each perform analogous role in the pathogenesis of BL. A study in Malawi by Mutalima *et al*. (2010) found no significant association between HIV-1 and endemic Burkitt's lymphoma. But Kalungi *et al*. (2009), found a decrease in lymphoma occurrence during HIV/AIDS era compared to 1960s before AIDS epidemic. This is also similar to what Parkin *et al*. (2000) found in Uganda that the risk of Burkitt's lymphoma is not modified by HIV infection. Morphologic and phenotypic features of plasmacytoid differentiation in some of the the BL tumours in the current study seems to suggest that these cases

might be related to HIV-1 infection similar to reports by Tumwine *et al*. (2008) and Rodig *et al*. (2008). The fact that children infected by vertical transmission die of AIDS during the first months of life due to competitive mortality from HIV-associated illnesses, before the factors involved in Burkitt's lymphoma pathogenesis such as Epstein-Barr Virus, malaria, Arboviruses, and Euphorbia tirucalli, can play their promotional role. Relative to adults, there are few published data from analytical studies on the risk of cancer in HIV-1 infected children, primarily because both cancer and HIV-1 infection are less common in children than in adults (Stefan *et al*., 2011). Studies of association between HIV-1 and Burkitt's lymphomas in adult populations have been done in the west. In the United States and Europe the increase of non-Hodgkin's lymphoma have been estimated to be 100-fold and 60-fold in populations of HIV-1 positive patients respectively during HIV/IDS pandemic (Kalungi *et al*., 2009).

The BL etiological relationships appear to be more complex and it may be that BL arises independently of evident immune impairment (Njie *et al*., 2009). Immunodeficiency-associated BL occurs in HIV-infected patients and allograft recipients. It is unclear if the HIV-1/2 positive BL cases identified here are likely to be immunodeficiency-associated BL, given the young ages of participants involved. Whereas HIV-1 associated BL can be treated with various short-term, aggressive chemotherapeutic regimens in conjunction with highly active antiretroviral therapy (HAART), toxicity and immunosupression pose a threat to the patients. The use of rituximab in immunocompromised patients is also a debated issue (God & Haque, 2010), suggesting the need for the development of less toxic and more specific immunotherapies.

On the whole, the number of HIV-1 positive children with Burkitt's lymphoma reported in the literature to date is small, as supported by this study and there remains substantial uncertainty about the role of HIV-1 (if any) in the aetiology of this common malignancy among children in parts of sub-Saharan Africa.

5.14 Malaria Parasite Exposure Prevention and BL Incidence

This study finding on malaria exposure indicated an overall increase in prevention against malaria; this supports reports by Kenya National Bureau of Statistics (2014) and studies by Emmanuel *et al*. (2011) both of which document increased protective roles of insecticide treated mosquito bed-nets (ITNs). These protective measures that guard against malaria and may be associated with decreased risk of non Hodgkin's lymphomas including Burkitt's lymphoma, this is conforms to other studies by Carpenter *et al*, (2007) in Ugandan children, but other risk factors must be playing a role as well. Past studies have demonstrated that children with *P.falciparum* malaria lose control over B-cell outgrowth and thus tumorigenesis.

One of the strongest weapons in the fight against malaria is the use of insecticide-treated nets (ITNs) while sleeping which reduces mosquito bites per night. Use of insecticide-treated nets (ITN's) is one of the main objectives for the "Roll Back Malaria" campaign in Kenya and other malaria-endemic countries to reduce the number of episodes of malaria in children less than five years. Nets reduce the human-vector contact by acting as a physical barrier and thus reducing the number of bites from infective vectors. Malaria transmission is influenced by mosquito density and by the frequency of infected mosquito bites at the household-level, which in turn is

influenced by use of pesticides, bed-nets, socio-cultural practices, and physical factors including standing water, vegetation and land use practices. These factors are often erroneously thought to be invariant over a whole region, and down play other factors that co-exist with malaria, such as intestinal helminths (Ogwang *et al.*, 2008), that can influence immune responses to malaria and in turn influence BL risk.

5.15 Association between Burkitt's lymphoma and *Plasmodium falciparum*

Past studies by Guech-Ongey *et al*. (2012) found lower levels of antibodies against *P. falciparum* in Ghana. This is similar to findings of a study by Akpogheneta *et al*. (2008) in The Gambia, endemic malaria region; children appeared to have short-lived plasma cells and experienced decline in antibody levels post infection. Given the number of reported malaria episodes among BL cases identified in the present study and evidence of previous ecological association between BL and malaria, the does appear to exist (Rainey *et al*., 2007). However, association at population level may not be true at individual level. The findings presented in this study differ from those by Carpenter *et al*. (2007) and Mutalima *et al*. (2008) who reported association between raised anti-malaria antibodies and BL cases. Even though past studies of association between Malaria and BL have been largely low resolution ecologic or geographic ones, with only a few direct population based studies to support malaria causation of BL (Orem *et al*., 2007). A Malawian study showed that children expressing high levels of antibodies for both malaria parasite and EBV had 13 times the risk of developing eBL when compared to children with control counter parts. In addition, children with malaria living in areas endemic for both EBV and malaria have been shown to have significantly higher levels of EBV antibodies than either their healthy counterparts.

The interpretation of these implied associations may be difficult, as the relationship could reflect changes that occurr after, not before onset of BL, reverse causation bias, (Baik *et al*., 2012). It may also be that an arbovirus, RNA virus transmitted by insect vectors, may play a role as final cofactor in the etiology of BL by inducing cell growth and B-cell hypermutation, but data supporting this view are scarce (Orem *et al.*, 2007).

In Kenya, malaria incidence is not only highest in the first five years of life but also in the commonest cause of morbidity and mortality below two years of age (Muga, Kizito, Mbayah, & Gakuruh, 2005). In malaria endemic parts of the country, the infection rate deceases after infancy and by five years people get infected but few clinical malaria episodes. The acquisition of immunity to malaria develops step-wise, but immunity to the parasite only develops slowly. Sterile immunity may not exist; the spleens of the exposed children become massively enlarged. In polyclonal B cell activation only a fraction of the antibodies are specific for *P. falciparum* antigens most likely as a result of polyclonal B cell activation. Prior studies indicate that malaria plays a leading role in the aetiology of Burkitt's lymphomagenesis through T cell mediated suppression either to tumour cell or Epstein-Barr virus (EBV) by precipitating a breakdown in homeostatic T cell control of EBV-infected B cells and acting as a polyclonal B cell mitogen (Schmauz, Mugerwa, & Wright, 1990). Both acute and chronic malaria infections profoundly affect the B cell compartment, inducing polyclonal activation, hyper-gammaglobulinemia and a significantly increase in the levels of circulating EBV (Chene *et al*., 2009). It is an uninterrupted presence of malaria-parasites, a never-ending activation of the immune system, splenomegaly and

concurrent infections by viruses, bacteria and parasites that endemic Burkitt's lymphoma emerges at high frequency (Chene *et al*., 2009). The cystein-rich inter-domain region1α (CIDR1-α) of the *Plasmodium falciparum* membrane protein 1 (pfEMP-1) is a polyclonal B cell activator. The CIDR1-α increases B cell survival and preferentially activates the memory compartment where EBV is known to persist. Since EBV is ubiquitous, its causative role in Burkitt's lymphoma pathogenesis may be related to differences in viral strains especially those with transformative properties. All these produce a milieu during B cell proliferation in which BL develops, including chromosomal translocation involving Ig gene loci (Futagbi, Welbeck, Tetteh, Hviid, & Akanmori, 2007).

It is generally hypothesised that hyperstimulation of B cells and suppression of T-cell activity by malaria allow for reactivation of EBV in infected B cells, which in turn increases in numbers. Suppression of T cell activity is suggested by the fact that children 5–9 years old living in areas holoendemic for malaria displayed inferior IFN-γ responses when compared to children living in regions with variable malaria transmission. This age range coincides with the peak and means age (8.8 years) of diagnosis of BL in this study. Burkitt's lymphoma B cells exhibit a deficiency in stimulation of $CD8^+$ T cells via HLA class I molecules. Amria, Cameron, Stuart, & Haque, (2008) showed that BL cells express detectable levels of HLA class II, but fail to effectively stimulate $CD4^+$ T cells.

Increasing levels of malarial antigens become trapped in secondary lymphoid organs, leading to hyperactivation of the germinal centres (GC) and increased somatic hypermutation (SHM) evidenced in this study by increased number of IgV_H

mutations. As *c-myc* gene translocations take place in the germinal centres during SHM, it is plausible to imagine an increase in the number of *c-myc* gene translocations which could ultimately lead to the over expression of MYC protein positive BL (God & Haque, 2010). Even though the role played by *c-myc* gene in the mechanisms regulating the cell cycle is not fully characterized, its increased expression is linked to cell differentiation, proliferation and apoptosis (Boxer & Dang, 2001).

The impact of malaria on the control of EBV persistence seems to be evident only before immunity to malaria is fully acquired as adults living in the same area endemic may not have detectable EBV DNA in their plasma. Endemic Burkitt's lymphoma tends to peak at ages between 5 and 8, during which malaria infection impairs the virus-host balance (Chene *et al*., 2009). Thus, age can be surrogate for cumulative exposure to deleterious infections such as malaria, HIV-1, EBV and other microbes. A non-linear risk increase in BL incidence would suggest that age may be surrogate for differences in biology of BL diagnosed at different ages, which may be independent of immunosuppression (Mbulaiteye *et al*., 2010).

In holoendemic malaria areas and where EBV-positive BL is common, *P. falciparum* infection induces the clonal expansion of EBV infected memory B cells, through interaction with Toll-like receptor 9 (TLR 9) (Onnis *et al*., 2012). When this subset of cells divides, they express EBNA1, which in turn up regulate *hsa-miR*-127 expression, and the shift to characteristic GC phenotype. The TLR9-ligand binding on its own also results in the induction of activation-induced cytidine deaminase (AICD), a critical somatic hypermutation enzyme (Peng, 2005). The active form of AICD causes DNA breaks in the heavy chain (IgH) regions, regardless of the stage of B-cell

differentiation. This is likely to predispose the cell to chromosomal aberrations such as IgH/*c-myc* translocation. This supports the view of enrichment of the BCR signalling pathway in EBV-positive Burkitt's lymphoma, suggesting a role of chronic antigenic/microbe stimulation in the pathogenesis of Burkitt's lymphoma (Piccaluga *et al*., 2011). However, it remains unclear whether *c-myc* gene translocation occurs in an EBV-infected memory B cell during clonal expansion or in a memory B cell that has re-entered the germinal centre (GC) reaction. It has recently been shown that memory B cells can be composed of antigen-dependent as well as antigen-independent subsets. The IgM memory B cells ensure the replenishment of the memory pool from antigen-experienced precursors by their rapid mobilization in germinal centres (GC). Another explanation is that Epstein-Barr virus positive and EBV negative BL may originate from different subsets of B cells (Bellan *et al*., 2005).

The normal counterpart of the neoplastic B cells in Burkitt's lymphomas (BL) is controversial. Three cellular derivation of neoplastic B cell in BL are hypothesised; precursor B cell derivation due expression of common lymphoblastic leukaemia antigen (CD10), germinal centre (GC) origin, because of homing into the GC and post GC-B cell stage. The origin of B cells in BL can be clarified by molecular biology techniques and polymerase chain reaction (PCR) procedures (Piccaluga *et al*., 2014). Pre-GC B cells, derivation include; immature SlgM+ B cells and naive mature antigen reactive SlgM+ IgD^+ B cells expressing Ig V-region genes with a germline sequence in contrast to GC B cells and post-GC B cells that contain mutated IgV_H-region genes. Given that tumorigenesis is a multistep process that occurs over long periods of time, it is virtually impossible to know how directly the final cellular or viral phenotype of BL relates to the original infected precursor cells (Onnis *et al*., 2012).

5.16 Immunoglobulin Heavy Chain (IgV_H) Somatic Mutation

Immunoglobulin gene mutations imply possible antigen selection regardless of EBV status. Studies by Bellan *et al*. (2005) demonstrated the presence of somatic mutations in all endemic BL, sporadic BL and AIDS-related BL cases. The average mutation frequency varied from 1.4% to 1.5% in sBL, from 5.0% to 5.1% in eBL, and from 5.3% to 5.4% in AIDS-related BL cases. Epstein-Barr virus (EBV) positive BL B cells carry a higher numbers of Ig VH gene heavy chain somatic mutations and signs of antigen selection. Similar to memory B cells, EBV-positive Burkitt's lymphoma B cells found mostly eBL and immunodeficiency associated BL, carry high numbers of immunoglobulin (Ig) heavy chain somatic mutations and signs of antigen selection (Bellan *et al*., 2005) (Figure 14). The fact that both EBV-positive and EBV-negative BL B cells harbour somatic mutation appear to suggest that the two entities may originate from different subsets of B cells. The expression of characteristic germinal centre (GC) cell markers such as CD10 points to a probable origin in germinal centre reaction in the pathogenesis of BL tumours.

This study shows that the derivation of neoplastic B cells in BL are not from naive, non-mutated pre-germinal centre B cells, similar to findings by Tamaru *et al*. (1995). Three out of four of these cases were both EBER and MYC positive, probably indicating post-germinal centre or memory B cell origin. The negative case also had higher number of Ig V_H gene mutation and amino acid changes- initial somatic hypermutation, thus suggesting centroblast B cell derivation and possibly implying alternative pathogenic mechanism(s) of lymphomagenesis. These findings therefore support the view that BL cells arise either from early centroblasts that are arrested

after an initial somatic hypermutation reaction, or from germinal centre B cells but not in terms of morphology and proliferation toward memory B cells because of the observed three out of four with deregulated *c-myc* gene and CD10 phenotype expression in all BL cases in this series which is similar to findings by Piccaluga *et al*. (2011). Taken together, these observations suggest that memory B cells are the normal counterpart to EBV-positive BL. However, latter findings are in contrast with the germinal centre (GC) phenotype shared by all of the BL variants. In addition, the presence of EBV in Burkitt's lymphoma probably reflects the prevailing socioeconomic status and age at seroconversion of each population of patients (van den Bosch, 2004). A study by Leucci *et al*. (2010) also concluded that both EBV-positive and EBV-negative BL may have different cells of origin. Analysis of the chromosome breakpoints involved in Ig/*c-myc* translocations appear to suggest errors in class-switch recombinations both of which are common characteristics of GC B cells (God & Haque, 2010).

5.17 Burkitt's lymphoma MicroRNA Expression pattern

MicroRNAs are a class of small RNAs that are able to regulate gene expression at the post transcriptional level. They have been shown to control cell growth, differentiation, and apoptosis, suggesting that microRNA molecules could act as tumour suppressors or oncogenes. Past studies have reported their association with fragile sites in the genome and involvement in some cancers (Leucci *et al*., 2008). MicroRNA hsa-mir-34 has been found to be down-regulated only in BL cases that were negative for *c-myc* gene translocation, probably suggesting that up regulation these and other miRNA might be responsible for *c-myc* gene dysregulation in BL and other cancers.

The up regulation of expression of hsa-miR-127 has also been shown to be related to the presence of EBV virus in BL tumour, *c-myc* gene dysregulation and alteration of memory B cell regulator in other studies by Onnis *et al*. (2012). Further, there appears to be an alternative mechanism of interaction between viral products EBNA-1 and cellular miRNAs – *hsa-miR*-127 leading to impairment of important B-cell processes such as differentiation by modulation of the master regulators of GC B cells in a B cell that is already differentiated, towards memory B cell (Leucci *et al*., 2008). It appears to be a complex interaction, among viral miRNA and host cell miRNA that may contribute to lymphomagenesis by targeting multiple host cellular pathways. The determination of expression profiles of at least some key miRNA can thus help in distinguishing BL and other cancers according to differentiation stage or cells of derivation. It has been argued that miRNA profiles could be more efficient in providing B cell derivation distinctions and better understanding of BL biology and pathogenesis by Lu *et al*. (2006).

5.18 Haematological Parameters and Cytokine Expression in BL Cases

White blood cell count levels were slightly depressed in both BL and non-Bl compared to normal ranges. This was different from Kelemen *et al*. (2010) who found elevated WBC values in atypical Burkitt's lymphoma. The levels of WBC values in BL cases and in non-BL cases reduced towards normal values significantly, $p = .006$ and $p=.003$ respectively after treatment, suggesting positive response or cytotoxic impact of chemotherapy.

Of the cytokines studied, there elevated median levels of IL-6 and IL-10 in BL cases, though not dramatic, in comparison to non-BL cases, comparable to a study by Mellgren *et al.* (2012) which reported both elevated levels of inflammatory cytokines and polymorphisms in the genes encoding interleukin (IL-10) and tumor necrosis factor (TNF-α). A study by Chopra, Chitalkar, and Jaiprakash, (2004), demonstrated that interleukin IL-6 levels are increased more than four fold in BL cases compared to healthy controls. Raised Inflammatory and regulatory cytokines have been associated with increased incidence of certain subtypes of non-Hodgkin's lymphoma and the general condition of paediatric patients is thought to be influenced by immune mediators at presentation. Overall, cytokines are believed to be involved in the pathogenesis and show enhanced expression in patients with Hodgkin's and non-Hodgkin's lymphoma.

The 81% EBER1 positivity in addition to other infectious agents including malaria and HIV-1 among in BL cases, observed in this study may be contributory to he elevated cytokine levels. Natural malarial infections is biased toward Th2-like immunity such as IL-10, comparable to Moormann *et al.* (2007) and correlates with greater parasite densities and less-effective parasite clearance of *P. falciparum*, in children. Epstein Barr virus specific $CD8^+$ T cell subset that have reduced cytotoxicity and secrete interleukin 10 (IL-10) have been reported by Chene *et al.* (2009), this constitutes an immune evasion. Furthermore, EBV latency program product, BCRF-1, has been associated with increased IL-10 (Lautscham, Rickinson, & Blake, 2003), but in this study, only human IL-10 and not the viral IL-10 homologue, BCRF1 was measured. Epstein Barr virus encoded RNA's (EBERs) have also been

reported to be responsible for increased production of the B-cell growth factor, IL-10, in EBV-positive BL lines compared to EBV-negative BL lines (Brady *et al*., 2007).

The cytokines observed in this study, TNF-α, IFN-γ, IL-6, are often associated with malaria, EBV and HIV-1 co-factors of Burkitt's lymphoma (Ning, 2011)). The levels of interleukin 6 (IL-6) in this study are higher than those reported by Aka *et al*. (2014). Interleukin 6 (IL-6) secreted by HIV infected macrophages is a B cell stimulatory factor and may enhance growth of EBV transformed lymphoblastoid cell lines in vitro and may have a role in the development of BL tumour (Bower, 1992)

The Th1 cytokines-IFN-γ, IL-2 and TNF-α were lowly expressed in BL cases probably indicating lack of immune control of transformed B cells in Burkitt's lymphoma (Ziegler, 1982). These cytokines control both lymphoid cell development and differentiation. Immune dysfunction in this compartment is thought to be the underlying basis of lymphomagenesis. Spontaneous regression and long term remissions after treatment led Burkitt and other to postulate a tumour-directed host immune response. Of interest, in EBV-infected cells, IL-10 has been shown to interact with BHRFl and promote further B-cell growth and immortalization (Benjamin *et al*., 1992). On the other hand, interleukin 10–secreting B cells (also designated B10) may inhibit immune responses to tumours.

Interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) were low, though not dramatic in BL's compared to non-BL's participants, probably due to immune T cell modulation by IL-10 and other immune modulators. The mechanisms by which IFN-γ deficiency promotes increased tumour formation are thought to be multifactorial and may include less than optimal control of tumour-cell

growth, apoptosis of T cell probably through Fas-FasL pathway, increased angiogenesis and tumour progression. The Th1 cytokines play important role(s) in immunosurveillance and control of EBV, a process that is dominated by human leukocyte antigen (HLA) class I restricted $CD8^+$ cytotoxic T lymphocyte (CTL) and IFN-γ responses to both latent and lytic viral epitopes (Moorman *et al*., 2007).

Impaired NFκB signaling and genes involved B cell receptor signaling such as STAT1 and STAT2, have been found to be involved in interferon (IFN-γ) down-regulation in BL when compared to B-lymphoblastoid cell line (Schlee *et al*., 2007) Negative regulation of STAT1 by MYC protein, expressed in 82% of BL cases in this study, is thought to occur directly, by blocking STAT1 expression or indirectly by suppressing IFN-γ induction. This means that over expression of *c-myc* gene is capable of decreasing either immune responses immunogenicity of EBV-positive or EBV-negative BL by altering genes in the NF-κB pathway. By so doing, MYC protein enhances tumour cell survival and facilitates immune evasion (God & Haque, 2010).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

In conclusion, this study demonstrated that:

1. Both typical and atypical BL morphologies occurred in the study population. Characteristic immunophenotype were $CD10^+$, $CD20^+$, $CD38^+$ & Ki-67 >90% in the absence of CD44 and BCL2 with or without MYC protein expression or *c-myc* gene translocation. Immunohistochemistry using antibody panel for these phenotypic markers, together with clinical, morphology and genetic features such as FISH can improve the accuracy of BL diagnosis in limited-resource settings such as MTRH and in Kenya. Accurate description of disease entities ensures correct entries in both MTRH and national cancer registeries.

MYC protein determination by immunohistochemistry (IHC) staining in BL tumour is a good predictor for the presence of *c-myc* gene translocation by fluorescence *in-situ* hybridization (FISH). The MYC protein as determined by immunohistochemistry can be routinely performed to delineate BL from other subtypes of aggressive B cell non-Hodgkin's lymphoma in the study setting as required by the World Health Organization (WHO) 2008 update diagnostic criteria.

Some BL molecular phenotypic characters were related to poor treatment outcome and therefore can be monitored in BL treatment and prognosis. The BLpatients with tumours expressing CD10 were to be associated with favorable outcome than those with MYC positive status.

2. Burkitt's lymphoma accounted for the largest percentage of all clinically and histological diagnosed paediatric aggressive B-cell lymphoma non Hodgkin's lymphoma followed by lymphoblastic lymphoma (LL), plasmablastic lymphoma (PL), diffuse large B cell lymphoma (DLBCL). Other non-lymphoid malignancies such as Wilm's tumor, neuroblastoma, nasopharyngeal carcinoma, other carcinomas and sarcomas also occur in the study region.

Burkitt's lymphoma occurred in patients at MTRH in western Kenya, involving children came from all ethnic communities in the region notably the Luhya, Kalenjin and Luo. Families with low socio-economic status group carry a greater burden of Burkitt's lymphoma compared to those in higher socio-economic group.

Male children and adolescents were disproportionately affected compared to their female counterparts without evidence of familial clustering. The tumour site has apparently shifting from jaw to abdominal presentation.

3. Haematoxylin and eosin (H&E) sensitivity in diagnosing BL is low in the study setting.

4. Immunoglobulin (IgV_H) studies showed rearrangement and somatic hypermutation of IgV_H, antigen experienced or GC or post GC cell of B cells origin in these BL tumours meaning antigen exposure. This implied association between Burkitt's lymphoma and microbial infections represented by EBV, HIV-1 and *Plasmodium* malaria. However, not everyone who had EBV and HIV-1 had the disease; there is likelihood of other organisms, environmental factors and/or alternative.

The BL cell spectrum in cell of derivation seen here suggested diverse origin ranging from centroblast, plasmacytoid, and memory B cell features all of which reflect the natural history of Burkitt lymphoma consisting of multiple pathogenetic events. Higher expressions of cellular microRNA- *hsa-miR* 34b and *hsa-miR* 127 both provide evidence for alternative lymphomagenesis process in EBV and MYC protein negative Burkitt's lymphoma.

5. Down regulated IL-2, TNF-α cytokines in the presence of upregulated IL-17, IL-6 and IL-10 indicated possible role of inflammatory and Th2 responses in the pathogenesis of Burkitt's lymphoma. Viral BCRF-1, a homogue for human IL-10 may be used by by Epstein-Barr virus to overvome host immune protection and allow proliferation and transformation of EBV infected B cells.

6.2 Recommendations

1. The minimal immunohistochemistry antibody panel suggested from this study should be adopted in characterising and diagnosing BL in limited-resource settings such as MTRH and in Kenya.

2. Immunochemisitry staining of MYC protein can serve as a screening tool determining *c-myc* gene translocations in tumour biopsy and only a few confirmatory genetic testing by FISH may be necessary.

3. Immunohistochemistry and molecular studies are necessary to improve accuracy of BL diagnosis.

4. Accurate disease description should improve patient management treatment outcomes for a lymphoma that is largely responds to chemotherapy and accurate cancer registry in the study region.

5. Way Forward

Immunoregulatory determinants mediated by malaria, viral infections and other environmental agents, male sex biasness require further studies. Whether the anatomical site of presentation has changed over time, EBV and *c-myc* gene translocation negative Burkitt's lymphoma found here need also to be studied as well as the role viral IL-10 homologue plays in modulating host immune response

Association of certain molecular markers and treatment outcome need further studies in a larger cohort over a longer period of time.

REFERENCES

Adelusola, K. A., Titiloye, N. A., Rotimi, O., & Durosinmi, M. (2009). Epstein Barr virus latent membrane protein-1 in Hodgkin's lymphoma in Nigerians. *African Health Sciences*, 9(3), 174–178.

Aka, P., Emmanuel, B., Vila, C. M., Jariwala, A., Nkruma, F., Periago, M. V, … Mbulaiteye, S. M. (2014). Elevated serum levels of interleukin-6 in endemic Burkitt lymphoma in Ghana. *Hematological Oncology*, 32(4), 218–220.

Akpogheneta O.J., Duah N.O., Tetteh K.K.A., Dunyo S., Lanar D.E., Pinder M. & Conway D. J. (2008) Duration of Naturally Acquired Antibody Responses to Blood-Stage *Plasmodium falciparum* Is Age Dependent and Antigen Specific. *Infection and Immunity* 76: 1748–1755

Allday, M. J. (2009). How does Epstein-Barr virus (EBV) complement the activation of Myc in the pathogenesis of Burkitt's lymphoma? *Seminars in Cancer Biology*, 19, 366–376.

Amria S., Cameron C., Stuart R., & Haque A. (2008). Defects in HLA class II antigen presentation in B-cell lymphomas. *Leukemia and Lymphoma*, 49, 2, 353–355.

Baik, S., Mbaziira, M., Williams, M., Ogwang, M. D., Kinyera, T., Emmanuel, B., … Mbulaiteye, S. M. (2012). A case-control study of Burkitt lymphoma in East Africa: are local health facilities an appropriate source of representative controls? *Infectious Agents and Cancer*, 7(1), 5. http://doi.org/10.1186/1750-9378-7-5

Ballet, J..J., Jaurequlberry, G., Dkloron. P. & Agrapart, M. (1987). Stimulation ofT lymphocyte—dependcnl diHerentiation of activated B lymphocytes in Plasmodium faciparuni supernatants. *Journal of Infectious Diseases*. 155. 1037.

Bartel D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism and function. *Cell*, 116, 281–297.

Bellan, C., Lazzi, S., Falco, G. De, Nyongo, A., Giordano, A., & Leoncini, L. (2003). Burkitt's lymphoma: new insights into molecular pathogenesis. *Journal of Clinical Pathology*, 56, 188–192.

Bellan, C., Lazzi, S., Hummel, M., Palummo, N., de Santi, M., Amato, T., … Leoncini, L. (2005). Immunoglobulin gene analysis reveals 2 distinct cells of origin for EBV-positive and EBV-negative Burkitt lymphomas. *Blood*, 106(3), 1031–6.

Bellan, C., Stefano, L., Giulia, D. F., Rogena, E. A., & Lorenzo, L. (2010). Burkitt lymphoma versus diffuse large B-cell lymphoma : a practical approach. *Haematological Oncology*, 28, 53–56.

Benjamin, D., Knobloch, T. J., & Dayton, M. A. (1992). Human B-Cell Interleukin-10: B-Cell Lines Derived From Patients With Acquired Immunodeficiency Syndrome and Burkitt's Lymphoma Constitutively Secrete Large Quantities of Interleukin-10. *Blood*, 80(5)1289-1298

Biggar, R. J., & Nkrumah, F. K. (1979). Burkitt's lymphoma in ghana: urban-rural distribution, time-space clustering. *International Journal of Cancer*, 23, 330–336.

Biggar R.J., Gardiner C, Lennette E.T., Collins W.E., Nkrumah F.K., Henle W. (1981) Malaria, sex, and place of residence as factors in antibody response to Epstein-Barr virus in Ghana, West Africa. *Lancet,* 2: 115-8

Bishop, P. C., Rao, K. V, & Wilson, W. H. (2000). Burkitt's Lymphoma: Molecular Pathogenesis. *Cancer Investigation*, 18(6), 574–583.

Bower, M. (1992). The biology of HIV-associated lymphomas. *British Journal of Cancer*, 66(3), 421–423.

Boxer, L.M. & Dang C.V. (2001). Translocations involving c-myc and c-myc function. *Oncogene*, 20, 5595–610.

Brady, G., MacArthur, G. J., & Farrell, P. J. (2007). Epstein-Barr virus and Burkitt lymphoma. *Journal of Clinical Pathology*, 60, 1397–1402.

Burkitt, D. P. (1969). Etiology of Burkitt's lymphoma-alternative hypothesis to a vectored virus. *Journal of the National Cancer Institute*, 42, 19-28

Burkitt, D. (1962). A children's cancer development dependent upon climatic factors, *Nature*, 194, 232-234.

Burkitt, D. (1958). A sarcoma involving the jaws in African children.*British Journal of Surgery*, 46, 218-223.

Campidelli, C., Gazzola, A., Vitone, F., & Pileri, S. A. (2008). HIV infection and c-MYC status in endemic Burkitt lymphoma. *Human Pathology*, 39(9), 1407–8.

Campo, E., Chott, A., Kinney, M. C., Leoncini, L., Meijer, C. J. L. M., Papadimitriou, C. S., … Swerdlow, H. (2006). Update on extranodal lymphomas . Conclusions of the Workshop held by the EAHP and the SH in Thessaloniki , Greece. *Histopathology*, 48, 481–504.

Campo, E., Swerdlow, S. H., Harris, N. L., Pileri, S., Stein, H., & Jaffe, E. S. (2011). The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*, 117(19), 5019–32.

Carpenter, L. M., Newton, R., Casabonne, D., Ziegler, J., Mbulaiteye, S., Mbidde, E., … Beral, V. (2007). Antibodies against malaria and Epstein-Barr virus in childhood Burkitt lymphoma: A case-control study in Uganda. *International Journal of Cancer*, 122(6), 1319–1323.

Chene, A., Donati, D., Orem, J., Mbidde, E. R., Kironde, F., Wahlgren, M., & Bejarano, M. T. (2009). Endemic Burkitt's lymphoma as a polymicrobial disease: new insights on the interaction between Plasmodium falciparum and Epstein-Barr virus. *Seminars in Cancer Biology*, 19(6), 411–20.

Cho, B. (2011). The next step for Burkitt lymphoma. *The Korean Journal of Hematology*, 46(2), 60–1.

Chopra, G. S., Chitalkar, P. G., & Jaiprakash, M. P. (2004). Cytokines : as useful Prognostic Markers in Lymphoma Cases. *MJAFI*, 60, 45–49.

Cogliatti, S. B., Novak, U., Henz, S., Schmid, U., Moller, P., & Barth, T. F. E. (2006). Diagnosis of Burkitt lymphoma in due time: a practical approach. *British Journal of Haematology*, 134(3), 294–301.

Cogliatti, S. B., Novak, U., Henz, S., Schmid, U., Möller, P., & Barth, T. F. E. (2007). Diagnosis of Burkitt's lymphoma in due time : a practical approach. *Diagnostic Pathology*, 2(Suppl. 1), 56.

Cool, C. D., & Bitter, M. A. (1997). The Malignant Lymphomas of Kenya : Morphology, Immunophenotype, and Frequency of Epstein-Barr Virus in 73 Cases. *Human Pathology*, 28(9), 1026–1033.

Crawford, D. H. (2001). Biology and disease associations of Epstein -Barr virus. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 356, 461–473.

Dave, S. S., Fu, K., Wright, G. W., Lam, L. T., Kluin, P., Boerma, E.-J., … Staudt, L. M. (2006). Molecular Diagnosis of Burkitt's lymphoma. *The New England Journal of Medicine*, 354(23), 2431–2442.

De Falco, G., Antonicelli, G., Onnis, A., Lazzi, S., Bellan, C., & Leoncini, L. (2009). Role of EBV in microRNA dysregulation in Burkitt lymphoma. *Seminars in Cancer Biology*, 19(6), 401–6.

Thapa, Dharma R, Xinmin Li, Beth D Jamieson, and Otoniel Martínez-Maza. 2011. "Overexpression of microRNAs from the miR-17-92 Paralog Clusters in AIDS-Related Non-Hodgkin's Lymphomas." PloS one 6(6): e20781.

Diebold, J. (2001). World health organization classification of malignant lymphomas. *Experimental Oncology*, 23, 101–103.

Donati, D., Espmark, E., Kironde, F., Mbidde, E. K., Kamya, M., Lundkvist, Å., … Falk, K. I. (2006). Clearance of Circulating Epstein-Barr Virus DNA in Children with Acute Malaria after Antimalaria Treatment. *The Journal of Infectious Diseases*, 193(7), 971–977.

Dranoff, Glenn. 2004. "Cytokines in Cancer Pathogenesis and Cancer Therapy." Nature reviews. *Cancer* 4(1): 11–22.

Emmanuel, B., Kawira, E., Ogwang, M. D., Wabinga, H., Magatti, J., Nkrumah, F., … Mbulaiteye, S. M. (2011). African Burkitt Lymphoma: Age-Specific Risk and Correlations with Malaria Biomarkers. *American Journal of Tropical Medicine and Hygiene*, 84(3), 397–401.

Esquela-Kerscher, A. & Slack, F. J. (2006). MicroRNAs with a role in cancer," *Nature Reviews Cancer*, 6(4), 259–269.

Ferry, J. A. (2006). Burkitt's Lymphoma: Clinicopathologic Features and Differential Diagnosis. *The Oncologist*, 11, 375–383.

Fleire S.J., Goldman J.P., Carrasco Y.R., Weber M., Bray D. & Batista F.D. (2006). B cell ligand discrimination through a spreading and contraction response. *Science*, 312, 738–41.

Fisher, A. A., Laing, J. E. and Strocker, J.E. (1998). Handbook for Family Planning, Operation Research Design in Sampling. *Population Council*, 40-45

Futagbi, G., Welbeck, J. E., Tetteh, J. K., Hviid, L., & Akanmori, B. D. (2007). Selective activation of TCR-gammadelta+ cells in endemic Burkitt's lymphoma. *Malaria Journal*, 6, 69. http://doi.org/10.1186/1475-2875-6-69

Giadano, G., Ballerini, P., Gong, J.Z., Mghirami, G., Neri, A., Newcombe E.R., … Dalla-Fevara R. (1991). P53 mutations in human lymphoid malignancies: association with Burkitt's lymphoma and chronic lymphocytic leukaemia. *Proceedings of the National Academy of Sciences*, 88, 5413-5417.

God, J. M., & Haque, A. (2010). Burkitt Lymphoma: Pathogenesis and Immune Evasion. *Journal of Oncology*, 2010, 2003–2005.

Gualco, G., Klumb, C. E., Barber, G. N., Weiss, L. M., & Bacchi, C. E. (2010). Pediatric lymphomas in Brazil. Clinicaal Science, 65(12), 1267–1277.

Guech-Ongey, M., Yagi, M., Palacpac, N. M. Q., Emmanuel, B., Talisuna, A. O., Bhatia, K., … Mbulaiteye, S. M. (2012). Antibodies reactive to Plasmodium falciparum serine repeat antigen in children with Burkitt lymphoma from Ghana. *International Journal of Cancer*, 130(8), 1908–14. http://doi.org/10.1002/ijc.26203

Gyasi, R. K., & Tettey, Y. (2007). Childhood deaths from malignant neoplasms in Ghana. *Ghana Medical Journal*, 41(2), 78–81.

Hecht, J. L., & Aster, J. C. (2000). Molecular Biology of Burkitt ' s Lymphoma. *Journal of Clinical Oncology*, 18(21), 3707–3721.

Hedvat, C. V., Hegde, A., Chaganti, R. S. K., Chen, B., Qin, J., Filippa, D. a., … Teruya-Feldstein, J. (2002). Application of tissue microarray technology to the study of non-Hodgkin's and Hodgkin's lymphoma. *Human Pathology*, 33(10), 968–974.

Huang H., Liu Z., Zeng H., Zhang S., Huang C. & Xu H. (2015). Clinicopathological study of sporadic BL in children. *Chinese Medical Journal*, 128(4), 510-514.

Hummel, M., Bentink, S., Berger, H., Klapper, W., Wessendorf, S., Barth, T. F. E., … Siebert, R. (2006). A Biologic Definition of Burkitt's Lymphoma from

Transcriptional and Genomic profiling. *The New England Journal of Medicine*, 354(23), 2419–2430.

Jaffe E.S., Harris N.L., Stein H., Vardiman J.W., editors. *World Health Organization Classification of tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues*. Lyon: IARC Press; 2001

Jaffe, E. S., Harris, N. L., Stein, H., & Isaacson, P. G. (2008). Classification of lymphoid neoplasms: the microscope as a tool for disease discovery. *Blood*, 112(12), 4384–99.

Kafuko, G W, Burkitt, D. P. (1970). Burkitt's lymphoma and malaria. *International Journal of Cancer*, 9, 1–9.

Kalungi, S., Wabinga, H., Molven, A., & Bostad, L. (2009). Lymphomas diagnosed in Uganda during the HIV/AIDS pandemic. *East African Medical Journal*, 86(5), 226–232.

Katahaa, P.K,. Facer, C.A. & Holborrow, E.J. (1984) Plasmoflium faleipariim products enhance human lymphocyte transformation by Epsiein Barr virus. *Clinical and Experimental Immunology*, 56, 371

Kelemen, K., Braziel, R. M., Gatter, K., Bakke, T. C., Olson, S., & Fan, G. (2010). Immunophenotypic Variations of Burkitt Lymphoma. *American Journal of Clinical Pathology*, 134, 127–138.

Kelly K. & Siebenlist U. (1985).The role of c-myc in the proliferation of normal and neoplastic cells. *Journal of Clinical Immunology*, 5, 65–77.

Kelly, J. L., Toothaker, S. R., Ciminello, L., Hoelzer, D., Lacasce, A. S., Mead, G., … Jonathan, W. (2009). Outcomes of patients with Burkitt lymphoma older than age 40 treated with intensive chemotherapeutic regimens. *Clinical Lymphoma Myeloma*, 9(4), 307–310.

Kennedy, G., Komano, J., & Sugden, B. (2003). Epstein-Barr virus provides a survival factor to Burkitt ’s lymphomas. *PNAS*, 100(24), 14269–14274.

Kenya National Bureau of Statistics (Knbs). (2014). Kenya Demographic and Health Survery. Nairobi.

Kenya National Bureau of Statistics (Knbs). (2010). Kenya Demographic and Health Survey 2008-09.

Key, M. (Ed.). (2006). Education Guide: Immunohistochemical Staining Methods (4th ed.). Dako, Carpinteria, California.

Klapproth, K., & Wirth, T. (2010). Advances in the understanding of MYC-induced lymphomagenesis. *British Journal of Haematology*, 149, 484–497.

Klein, G. (2009). Burkitt lymphoma—A stalking horse for cancer research? *Seminars in Cancer Biology*, 19(6), 347–350.

Komano, J., Maruo, S., Kurozumi, K., Takanori, O., & Takada, K. (1999). Oncogenic Role of Epstein-Barr Virus-Encoded RNAs in Burkitt’s Lymphoma Cell Line Akata. *Journal of Virology*, 73(12), 9827–9831.

Komatsu, N., Kawase-Koga, Y., Mori, Y., Kamikubo, Y., Kurokawa, M., & Takato, T. (2013). HIV-associated Burkitt lymphoma in a Japanese patient with early submandibular swelling. *BMC Research Notes*, 6(Ml), 557.

Kumar, M. S., Pester, R. E., Chen, C. Y., Lane, K., Chin, C., Lu, J., … Jacks, T. (2009). Dicer1 functions as a haploinsufficient tumor suppressor service Dicer1 functions as a haploinsufficient tumor suppressor. *Genes & Development*, 23, 2700–2704

Kurzrock R, Redman J, Cabanillas F., Jones D., Rothberg J. & Talpaz M. (1993). Serum IL-6 levels are elevated in lymphoma patients and correlate with survival in advanced Hodgkins disease and with B symptoms. *Cancer Research*, 53:2118-22.

Lautscham, G., Rickinson, A., & Blake, N. (2003). TAP-independent antigen presentation on MHC class I molecules : lessons from Epstein – Barr virus. *Microbes and Infection*, 5, 291–299.

Lawrie C.H., Soneji S., Marafioti T., Cooper C.D., Palazzo S., Paterson J.C…Hatton C.S. (2007). MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. *Int J Cancer*, 121: 1156–1161.

Lenze, D., Leoncini, L., Hummel, M., Volinia, S., Liu, C. G., Amato, T., … Stein, H. (2011). The different epidemiologic subtypes of Burkitt lymphoma share a homogenous micro RNA profile distinct from diffuse large B-cell lymphoma. *Leukemia*, 25(12), 1869–76.

Leoncini L., Leucci E. & Cocco M. (2008). c-MYC negative classical Burkitt lymphoma: alternative pathogenetic mechanism; 50th Anniversary of the Discovery of Burkitt Lymphoma, International conference on Burkitt lymphoma and related lymphoproliferative disorders, February 25-27, Kampala (Uganda).

Leoncini L., Raphael M., & Stein H. (2008). Pathology and genetics of tumors and of haematopoietic and lymphoid tissues. World Health Organisation Classification of Tumors. Lyon, IARC Press.

Leoncini, L., Lazzi, S., Bellan, C., & Tosi, P. (2002). Cell kinetics and cell cycle regulation in lymphomas. *Journal of Clinical Pathology*, 55(9) 648–655.

Leucci, E., Cocco, M., Onnis, A., Falco, G. De, Cleef, P. Van, Bellan, C., … Leoncini, L. (2008). MYC translocation-negative classical Burkitt lymphoma cases : an alternative pathogenetic mechanism involving miRNA dysregulation. *Journal of Pathology*, 216, 440–450.

Leucci, E., Onnis, A., Cocco, M., De Falco, G., Imperatore, F., Giuseppina, A., … Leoncini, L. (2010). B-cell differentiation in EBV-positive Burkitt lymphoma is impaired at posttranscriptional level by miRNA-altered expression. *International Journal of Cancer*, 126(6), 1316–26.

Leung C.S., Haigh T.A., Mackay L.K., Rickinson A. B. & Taylor G. S. (2010). Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. *Proceedings of the National Academy of Sciences*, 107 (5), 2165–2170.

Lin, Z., & Flemington, E. K. (2011). NIH Public Access. *Cancer Letters*, 305(2), 186–199.

Lu, B., Zhou, C., Yang, W., Huang, H., Gao, Z., He, Y., … Gong, L. (2011). Morphological, immunophenotypic and molecular characterization of mature aggressive B-cell lymphomas in Chinese pediatric patients. *Leukemia & Lymphoma*, 52(12), 2356–2364.

Lu, S., Bilello, J. P., Desrosiers, R. C., Edwards, R., Cai, X., Scha, A., … Cullen, B. R. (2006). Epstein – Barr Virus MicroRNAs Are Evolutionarily Conserved and Differentially Expressed. *Plos Pathogens*, 2(3), e23.

Lukande, R., Wabinga, H. R., & Tumwine, L. K. (2008). Burkitt ' s lymphoma in Uganda : the role of immunohistochemistry in diagnosis. *East African Medical Journal*, 85(5), 207–212.

Magrath I. (1990). The pathogenesis of Burkitt's lymphoma. *Advances in Cancer Research*, 55, 133-270.

Makata, A. M., Toriyama, K., Kamidigo, N. O., Eto, H., & Itakura, H. (1996). The pattern of pediatric solid tuomors in westen Kenya, East Africa, 1979-1994: an

analysis based on histopathologic study. *American Journal of Tropical Medicine and Hygiene*, 54(4), 343–347.

Mannucci, S., Luzzi, A., Carugi, A., Gozzetti, A., Lazzi, S., Malagnino, V., … De Falco, G. (2012). EBV Reactivation and Chromosomal Polysomies: Euphorbia tirucalli as a Possible Cofactor in Endemic Burkitt Lymphoma. *Advances in Hematology*, 149780.

Mantina, H., Wiggill, T. M., Carmona, S., Perner, Y., & Stevens, W. S. (2010). Characterization of Lymphomas in a High Prevalence HIV Setting Sample Population and Data Collection. *Journal of Acquired Immune Defic Syndr*, 53(5), 656–660.

Matuma, G.Z. (2004). Current advances in the diagnosis of lymphomas. *East African Medical journal*, 20,381-383.

Mbulaiteye, S. M., Anderson, W. F., Bhatia, K., Rosenberg, P. S., Martha, S., & Devesa, S. S. (2010). *International Journal of Cancer*, 126(7), 5–7.

Mbulaiteye, S. M., Bhatia, K., Adebamowo, C., & Sasco, A. J. (2011). HIV and cancer in Africa: mutual collaboration between HIV and cancer programs may provide timely research and public health data. *Infectious Agents and Cancer*. BioMed Central Ltd. http://doi.org/10.1186/1750-9378-6-16

Mbulaiteye, S. M., Walters, M., Engels, E. A., Bakaki, P. M., Ndugwa, C. M., Owor, A. M., … Biggar, R. J. (2006). High Levels of Epstein-Barr Virus DNA in Saliva and Peripheral Blood from Ugandan Mother-Child Pairs. *Journal of Infectious Diseases*, 193(3), 422–426.

Mellgren, K., Hedegaard, C. J., Schmiegelow K. & Muller K. (2012). Plasma cytokine profile at diagnosis in paediatric patients with non-Hodgkin's Lymphoma. *Journal of Pediatric Hematology/Oncology*, 34(4)271-275

Moormann, A. M., Chelimo, K., Sumba, O. P., Lutzke, M. L., Ploutz-snyder, R., Newton, D., … Rochford, R. (2005). Exposure to Holoendemic Malaria Results in Elevated Epstein-Barr Virus Loads in Children. *The Journal of Infectious Diseases*, 13210, 1233–1238.

Moormann, A. M., Chelimo, K., Sumba, P. O., Tisch, D. J., Rochford, R., & Kazura, J. W. (2007). Exposure to holoendemic malaria results in suppression of Epstein-Barr virus-specific T cell immunosurveillance in Kenyan children. *The Journal of Infectious Diseases*, 195(6), 799–808.

Moormann, A. M., Heller, K. N., Chelimo, K., Embury, P., Ploutz-, R., Otieno, J. a, … Rochford, R. (2009). Children with Endemic Burkitt Lymphoma are Deficient in EBNA1-Specific IFN-γ T Cell Responses. *International Journal of Cancer*, 124(7), 1721–1726.

Moormann, A. M., Snider, C. J., & Chelimo, K. (2011). The company malaria keeps: how co-infection with Epstein-Barr virus leads to endemic Burkitt lymphoma. *Current Opinion in Infectious Diseases*, 24(5), 435–41.

Morrow, R. H. (1974). Introduction : Burkitt's Lymphoma in Africa. *Cancer Research*, 34, 1211–1215.

Morrow, R. H., Pike, M. C., Smith, P. G., Ziegler, J. L., & Kisuule, A. (1971). Burkitt's Lymphoma : A Time-space Cluster of Cases in Bwamba County of Uganda. *British Medical Journal*, 2, 491–492.

Muga, R., Kizito, P., Mbayah, M., & Gakuruh, T. (2005). Overview of the Health System in Kenya. Kenya Service Provision Assessment Survey 2004. Nairobi.

Mulama, D. H., Bailey, J. A., Foley, J., Chelimo, K., Ouma, C., Jura, W. G. Z. O., … Moormann, A. M. (2013). Sickle cell trait is not associated with endemic Burkitt lymphoma: An ethnicity and malaria endemicity-matched case-control study

suggests factors controlling EBV may serve as a predictive biomarker for this pediatric cancer. *International Journal of Cancer*, 134(3), 645–653.

Mutalima, N., Molyneux, E. M., Johnston, W. T., Jaffe, H. W., Kamiza, S., Borgstein, E., … Newton, R. (2010). Impact of infection with human immunodeficiency virus-1 (HIV) on the risk of cancer among children in Malawi - preliminary findings. *Infectious Agents and Cancer*, 5, 5.

Mutalima, N., Molyneux, E., Jaffe, H., Kamiza, S., Borgstein, E., Mkandawire, N., … Newton, R. (2008). Associations between Burkitt lymphoma among children in Malawi and infection with HIV, EBV and malaria: results from a case-control study. *PloS One*, 3(6), e2505. http://doi.org/10.1371/

Mwakigonja, A. R., Kaaya, E. E., & Mgaya, E. M. (2008). Malignant lymphomas (ML) and HIV infection in Tanzania. *Journal of Experimental and Clinical Research*, 27(9), 1–10.

Mwanda O.W., Rochford R., Moormann A., Macneil A., Whalen C., & Wilson M.L. (2004). Burkitt's lymphoma in Kenya: geographic, age, gender ad ethnic distribution. *East African Medical Journal* 81(8)

Nanbo, A., Inoue, K., Adachi-Takasawa K. & Takada K. (2002). Epstein–Barr virus RNA confers resistance to interferon-alpha-induced apoptosis in Burkitt's lymphoma. *EMBO Journal*, 21, 954–65.

Naresh, K. N., Ibrahim, H. a H., Lazzi, S., Rince, P., Onorati, M., Ambrosio, M. R., … Leoncini, L. (2011). Diagnosis of Burkitt lymphoma using an algorithmic approach - applicable in both resource-poor and resource-rich countries. *British Journal of Haematology*, 154, 770–776.

Newton, R., Ziegler, J., Beral, V., Mbidde, E., Carpenter, L., Wabinga, H., … Jaffe, H. (2001). A case-control study of human immunodeficiency virus infection and

cancer in adults and children residing in Kampala, Uganda. *International Journal of Cancer*, 92, 622–7.

Ning, S. (2011). Innate immune modulation in EBV infection. *Herpesviridae*, 2(1), 1. http://doi.org/10.1186/2042-4280-2-1

Njie, R., Bell, A. I., Jia, H., Croom-Carter, D., Chaganti, S., Hislop, A. D., … Rickinson, A. B. (2009). The effects of acute malaria on Epstein-Barr virus (EBV) load and EBV-specific T cell immunity in Gambian children. *The Journal of Infectious Diseases*, 199(1), 31–8.

Nyagol, J., De Falco, G., Lazzi, S., Luzzi, a, Cerino, G., Shaheen, S., … Leoncini, L. (2008). HIV-1 Tat mimetic of VEGF correlates with increased microvessels density in AIDS-related diffuse large B-cell and Burkitt lymphomas. *Journal of Hematopathology*, 1(1), 3–10.

Ogwang, M. D., Bhatia, K., Biggar, R. J., & Mbulatieye, S. M. (2008). Incidence and geographic distribution of endemic Burkitt lymphoma in northern Uganda revisited. *International Journal of Cancer*, 123(11), 2658–2663.

Ogwang, M. D., Zhao, W., Ayers, L. W., & Mbulaiteye, S. M. (2011). Accuracy of Burkitt Lymphoma Diagnosis in Constrained. *Archives of Pathology and Lab Medicine*, 135, 445–450.

Omumbo J, Ouma J, Rapuoda B, Craig M.H., le Sueur D, Snow R.W., 1998. Mapping malaria transmission intensity using geo- graphical information systems (GIS): an example from Kenya. *Annals of Tropical Medicine and Parasitology*, 92: 7–21.

Onnis, A., De Falco, G., Antonicelli, G., Onorati, M., Bellan, C., Sherman, O., … Leoncini, L. (2010). Alteration of microRNAs regulated by c-Myc in Burkitt lymphoma. *PloS One*, 5(9). http://doi.org/10.1371/

Onnis, A., Navari, M., Antonicelli, G., Morettini, F., Mannucci, S., De Falco, G., … Leoncini, L. (2012). Epstein-Barr nuclear antigen 1 induces expression of the cellular microRNA hsa-miR-127 and impairing B-cell differentiation in EBV-infected memory B cells. New insights into the pathogenesis of Burkitt lymphoma. *Blood Cancer Journal*, 2(8), e84. http://doi.org/10.1038/bcj.2012.29

Orem, J., Katongole, E., Lambert, B., Sanjose, S. De, & Weiderpass, E. (2007). Burkitt ' s lymphoma in Africa , a review of the epidemiology and etiology. *African Health Sciences*, 7(3), 166–175.

Othieno-Abinya, N. ., Abwao, H. O., Maina, J. M. ., Nyabola, L. O., Opiyo, A., Njuguna, E., … Musibi, A. (2004). Non hodgkin lymphoma at kenyatta hospital. *East African Medical Journal*, 81(9), 450–458.

Ott, G., Rosenwald, A., & Campo, E. (2013). Understanding MYC driven aggressive B-cell lymphomas :pathogenesis and classification. *Blood*. http://doi.org/10.1182/blood-2013-05-498329

Parkin, D. M., Garcia-giannoli, Á., Raphael, M., Parkin, W., Martin, A., Katangole-Mbidde, E., … Ziegler, J. (2000). Non-Hodgkin lymphoma in Uganda : a case-control study. *AIDS*, 14, 2929–2936.

Patkar, N., Mehta, J., Kulkarni, B., Pande, R., Advani, S., & Borges, A. (2008). Immunoprofile of Hodgkin's lymphoma in India. *Indian Journal of Cancer*, 45(2), 59–63.

Peng S. Signaling in B cells via Toll-like receptors. *Curr Opin Immunol* 2005; 17: 230–236.

Piccaluga, P. P., Falco, G. De, Kustagi, M., Gazzola, A., Agostinelli, C., Tripodo, C., … Formica, S. (2014). Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes Gene expression analysis uncovers

similarity and differences among Burkitt lymphoma subtypes. *Blood*, 3596–3608. http://doi.org/10.1182/

Piccaluga, P. P., Falco, G. De, Kustagi, M., Gazzola, A., Agostinelli, C., Tripodo, C., … Leoncini, L. (2011). Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes subtypes. *Blood*, 117, 3596–3608.

Pileri, S. A., Ascani, S., Sabattini, E., Fraternali-Orcioni, G., Poggi, S., Piccioli, M., … Falini, B. (2000). The pathologist's view point. Part I-indolent lymphomas. *Haematologica*, 85, 1291–1307.

Piriou, E., Kimmel, R., Chelimo, K., Middeldorp, J. M., Odada, P. S., Ploutz-snyder, R., … Rochford, R. (2009). Serological Evidence for Long-Term Epstein – Barr Virus Reactivation in Children Living in a Holoendemic Malaria Region of Kenya, 1093, *Journal of Medical Virology*, 81, 1088–1093.

Queiroga, E. M., Gualco, G., Weiss, L. M., Dittmer, D. P., Araujo, I., Klumb, C. E. N., … Bacchi, C. E. (2008). Burkitt lymphoma in Brazil is characterized by geographically distinct clinicopathologic features. *American Journal of Clinical Pathology*, 130(6), 946–56.

Rainey, J. J., Mwanda, W. O., Wairiumu, P., Moormann, A. M., Wilson, M. L., & Rochford, R. (2007). Spatial distribution of Burkitt's lymphoma in Kenya and association with malaria risk. *Tropical Medicine & International Health*, 12(8), 936–43.

Rainey, J. J., Omenah, D., Sumba, P. O., Moormann, A. M., Rochford, R., & Wilson, M. L. (2006). Spatial clustering of endemic Burkitt ' s lymphoma in high-risk regions of Kenya. *International Journal of Cancer*, 127, 121–127.

Rainey, J. J., Rochford, R., Sumba, P. O., Kowuor, D., Wilson, M. L., & Moormann, A. M. (2008). Family environment is associated with endemic Burkitt lymphoma: a

population-based case-control study. *The American Journal of Tropical Medicine and Hygiene*, 78(2), 338–43.

Rezk S.A. & Weiss L.M. (2007). Epstein-Barr virus-associated lymphoproliferative disorders. *Human Pathology*, 38, 1293–304.

Riley, K. J., Rabinowitz, G. S., Yario, T. a, Luna, J. M., Darnell, R. B., & Steitz, J. a. (2012). EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. *The EMBO Journal*, 31(9), 2207–21.

Rochford, R., Cannon, M. J., & Moormann, A. M. (2005). Endemic Burkitt ' s lymphoma : a polymicrobial disease ?, Nature Reviews Immunology, 3, 182–187.

Rodig, S. J., Vergilio, J., Shahsafaei, A., & Dorfman, D. M. (2008). and CD44 Identify Aggressive Lymphomas Harboring a MYC Translocation. *Americn Journal of Surgical Pathology*, 32, 113–122.

Rosenwald, A., Wright, G., Chan, W. C., Connors, J. M., Campo, E., Fisher, R. I., … Staudt, L. M. (2002). The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *The New England Journal of Medicine*, 346(25), 1937–47.

Rossi, M., Laginestra, M. A., Gazzola, A., Sapienza, M. R., Pileri, S. a, & Piccaluga, P. P. (2012). Molecular profiling of aggressive lymphomas. *Advances in Hematology*, 464680. http://doi.org/10.1155/2012/464680

Sandhu, S. K., Croce, C. M., & Garzon, R. (2011). Micro-RNA Expression and Function in Lymphomas. *Advances in Hematology*, 2011, 347137.

Schlee, M., Holzel, M., Bernard, S., Mailhammer, R., Schuhmacher, M., Reschke, J., … Bornkamm, G.W. (2007). c-MYC activation impairs the NF-κB and the interferon response implications for for the pathogenesis of Burkitt's lymphoma. *International Journal of Cancer*, 120(7), 1387–1395.

Schmauz, R., Mugerwa, J. W., & Wright, D. (1990). The distribution of non-burkitt , non-hodgkin ’ s lymphomas in uganda in relation to malarial endemicity. *International Journal of Cancer*, 653, 650–653.

Schmitz, R., Young, R. M., Ceribelli, M., Jhavar, S., Xiao, W., Zhang, M., … Staudt, L. M. (2012). Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature*, 490(7418), 116–20.

Sitas, F., Bezwoda, W. R., Levin, V., Ruff, P., Kew, M. C., Hale, M. J., … Weaving, A. (1997). Association between human immunodeficiency virus type I infection and cancer in the black population of Johannesburg and Soweto, South Africa. *British Journal of Cancer*, 75(1997), 1991–1994.

Snider, C. J., Cole, S. R., Chelimo, K., Sumba, P. O., Macdonald, P. D. M., John, C. C., … Moormann, A. M. (2012). Recurrent Plasmodium falciparum malaria infections in Kenyan children diminish T-cell immunity to Epstein Barr virus lytic but not latent antigens. *PloS One*, 7(3), e31753. http://doi.org/10.1371/

Snuderl, M., Kolman, O. K., Chen, Y.-B., Hsu, J. J., Ackerman, A. M., Dal Cin, P., … Sohani, A. R. (2010). B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. *The American Journal of Surgical Pathology*, 34(3), 327–40.

Stefan, D. C., Wessels, G., Poole, J., Wainwright, L., Stones, D., Johnston, W. T., & Newton, R. (2011). Infection With Human Immunodeficiency Virus-1 (HIV) Among Children With Cancer in South Africa. *Pediatric Blood Cancer*, 56, 77–79.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E., Pileri, S. & Stein, H. (eds). (2008). WHO Classification of Tumours of the Haematopoietic and Lymphoid Tissue. IARC: Lyon.

Tamaru, J., Hummel, M., Marafioti, T., Kalvelage, B., Leoncini, L., Minacci, C., … Stein, H. (1995). Burkitt ' s Lymphomas Express VH Genes with a Moderate Number of Antigen-Selected Somatic Mutations. *American Journal of Pathology*, 147(5), 1398–1407.

Tapia, G., Lopez, R., Munoz-Marmol, Mate, L., Sanz, C., Tapia, G., … Marginet, R. (2011). Immunohistochemical detection of MYC protein correlates with MYC gene status in aggressive B cell lymphomas. *Histopathology*, 59, 672–678.

Tilly, H., Rossi, A., Stamatoullas, A., Lenormand, B., Bigorgne, C., Kunlin, A., … Bastard, C. (1994). Prognostic value of chromosomal abnormalities in follicular lymphoma. *Blood*, 84, 1043–1049.

Troxell, M. L., Bangs, C. D., Cherry, A. M., Natkunam, Y., & Kong, C. S. (2005). Cytologic diagnosis of Burkitt lymphoma: Role of ancillary studies. *Cancer(cancer Cytopathology)*, 105(June), 310–318. http://doi.org/10.1002/cncr.21307

Tumwine, L. K., Agostinelli, C., Campidelli, C., Othieno, E., Wabinga, H., Righi, S., … Pileri, S. a. (2009). Immunohistochemical and other prognostic factors in B cell non Hodgkin lymphoma patients, Kampala, Uganda. *BMC Clinical Pathology*, 9(1), 11. http://doi.org/10.1186/1472-6890-9-11

Tumwine, L. K., Campidelli, C., Righi, S., Neda, S., Byarugaba, W., & Pileri, S. a. (2008). B-cell non-Hodgkin lymphomas in Uganda: an immunohistochemical appraisal on tissue microarray. *Human Pathology*, 39(6), 817–823.

Tumwine, L. K., Orem, J., Kerchan, P., Byarugaba, W., & Pileri, S. a. (2010). EBV, HHV8 and HIV in B cell non Hodgkin lymphoma in Kampala, Uganda. *Infectious Agents and Cancer*, 5(1), 12.

URL: http://eurofish.org, 2014

URL:http://en.wikipedia.org/wiki/epstain, 2016

URL: http://imgt.cines.fr; Giudicelli, Chaume, & Lefranc, 2004

URL: http://Leicabiosytem.com/ihc-fish-antibodies, 2014

van den Bosch, C. A. (2004). Personal view is endemic Burkitt's lymphoma an alliance between three infections and a tumour promoter? Personal view. *The Lancet Oncology*, 5, 738–746.

van Rijk, A., Mason, D., Jones, M., Cabeçadas, J., Crespo, M., Cigudosa, J. C., … van Krieken, J. H. (2008). Translocation detection in lymphoma diagnosis by split-signal FISH : a standardised approach. *Journal of Hematopathology*, 1, 119–126.

Váróczy, L., Gergely, L., Miltényi, Z., Aleksza, M., & Illés, Á. (2005). Can CD3+/HLA-DR+ activated T cells predict the prognosis of non-Hodgkin's lymphoma patients? *Immunology Letters*, 97(1), 155–157.

Ventura, R. A., Martin-Subero, J. I., Jones, M., McParland, J., Gesk, S., Mason, D. Y., & Siebert, R. (2006). FISH Analysis for the Detection of Lymphoma- Associated Chromosomal Abnormalities in Routine. *Journal of Molecular Diagnostics*, 8(2), 141–151.

Vereide, D., & Sugden, B. (2009). Proof for EBV's sustaining role in Burkitt's lymphomas. *Seminars in Cancer Biology*, 19(6), 389–393.

Vose, J. M., Chiu, B. C., Cheson, B. D., Dancey, J., & Wright, J. (2002). Update on Epidemiology and Therapeutics for Non-Hodgkin ' s Lymphoma. *Hematology*, 241–262.

Walusansa, V., Okuku, F., & Orem, J. (2012). Burkitt lymphoma in Uganda, the legacy of Denis Burkitt and an update on the disease status. *British Journal of Haematology*, 156(6), 757–60.

Whittle, H. C., Brown, J., Marsh, K., Blackman, M., Jobe, O., & Shenton, F. (1990). The effects of Plasmodium falciparum malaria on immune control of B

lymphocytes in Gambian children. *Clinical and Experimental Immunology*, 80, 213–218.

Wilkins, B. S. (2011). Pitfalls in lymphoma pathology: avoiding errors in diagnosis of lymphoid tissues. *Journal of Clinical Pathology*, 64(6), 466–476.

Xia, T., O'Hara, A., Araujo, I., Barreto, J., Carvalho, E., Bahia, J., … Harrington Jr, William, J. (2008). *Cancer Research*, 68(5), 1436–1442.

Young, L.S. & Rickinson, A. (2004). Epstein-Barr virus: 40 years on. *Nat Rev Cancer*, 4, 757–768.

Yustein, J. T., & Dang, C. V. (2007). Biology and treatment of Burkitt's lymphoma. *Current Opinion in Hematology*, 14, 375–381.

Zhang, J., Jima, D. D., Jacobs, C., Fischer, R., Gottwein, E., Huang, G., … Dave, S. S. (2009). Patterns of microRNA expression characterize stages of human B-cell differentiation. *Blood*, 113(19), 4586–94.

Ziegler, J. L. (1982). Burkitt's Lymphoma. *Cancer Journal for Clinicians*, 32(3), 144–161.

APPENDICES

Appendix I. Data Abstraction form

BL/ / Date ……

Patient Details

Patient	Name:	ID:	Tel.:
Gender	Male ☐	Female ☐	
Age	DOB:	Years:	
Ethnicity			
Location	Residence:	Date of admission:	
	County:		
Parent/guardian	Name:	Relation:	

Diagnosis

Clinical Diagnosis		Serology tests: 1. 2. 3.
	Physician:	Others (specify):
Confirmed Diagnosis		Duration:
	By: H&E ☐ IHC ☐ FNA ☐ FCM ☐	Pathologist: Antibody panel:
Location of Tumor		Size:
		Not determined ☐
Stage at Diagnosis/ Progression		Symptom of duration:
Concurrent conditions	Malaria ☐ HIV/AIDS ☐	CMV/mononucleosis☐
	Others(specify):	

Treatment/management

Before Diagnosis

After Diagnosis

Treatment Regimen: Duration:

Treatment Outcome

Survival Yes☐ Duration after Chemotherapy
No ☐

Mortality Yes ☐ From diagnosis
No ☐

After treatment:

Quality of life

Laboratory indicators Not given ☐

Overall comments

Review visit (s) Date (s):

Comments:

Prospects of tumor free survival Good ☐ Fair☐ Bad ☐ Not predicted ☐

General comments

Data Entry by: **Sign:** **Date:**

Appendix II. Questionnaire- participant information

Name of interviewer…….

Name of interviewee……..

Patient ID……...

1. Demographic data

Sex:

male/female……...

District of birth ……..

2. Activities/occupation –farm business, domestic ……...

3. Family size

1-3 b) 4-6 c)7-9 d) 10 – above

4. Have you had malaria in the last 12 months yes/ No……

Verified with treatment /hospital records

5. Which month of the year was your most recent malaria attack? …….

How often does you/child suffer fever……

Always

ii) Always except rainy seasons

iii) Few

iv) Others specify

6. Do you have mosquito net(s) in your household? a) Yes b) No……

If yes, how many – i) one ii) two c) three d) other specify ……

……

If yes, who use it/them?

Father ii) Mother iii) children iv) All/everybody

7. Do you use any other methods of malaria vector control? Yes /No…….

If yes, which one? Home made net, smoking, spraying clearing bushes other specify……

……

8. Indoor, residual spaying in the last 6 months yes/ no……

9. Have you travelled in the last 1 month?.....

10. Which district did you visit?.....

11. Indicate the zone endemic highland arid low risk……

Name of the interviewer…….

Comments……

Verified by PI

Sign ……Date……

Comments……

Appendix III. IREC Study Approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/04
Approval Number: 000654

1st July, 2011

Isaac Ndede
Moi University
Department of Immunology
P. O. Box 4606 - 30100
ELDORET- KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
APPROVED
01 JUL 2011
P.O. Box 4606 ELDORET

Dear. Mr. Ndede

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:

"Immunohistochemical and Molecular characterization of Burkitt's Lymphoma in Children Infected with Plasmodium Falciparum, Human Immunodeficiency virus and Epstein Barr Virus in Western Kenya."

Your proposal has been granted a Formal Approval Number: **FAN: IREC 000654** on 1st July, 2011. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 2nd July, 2012. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

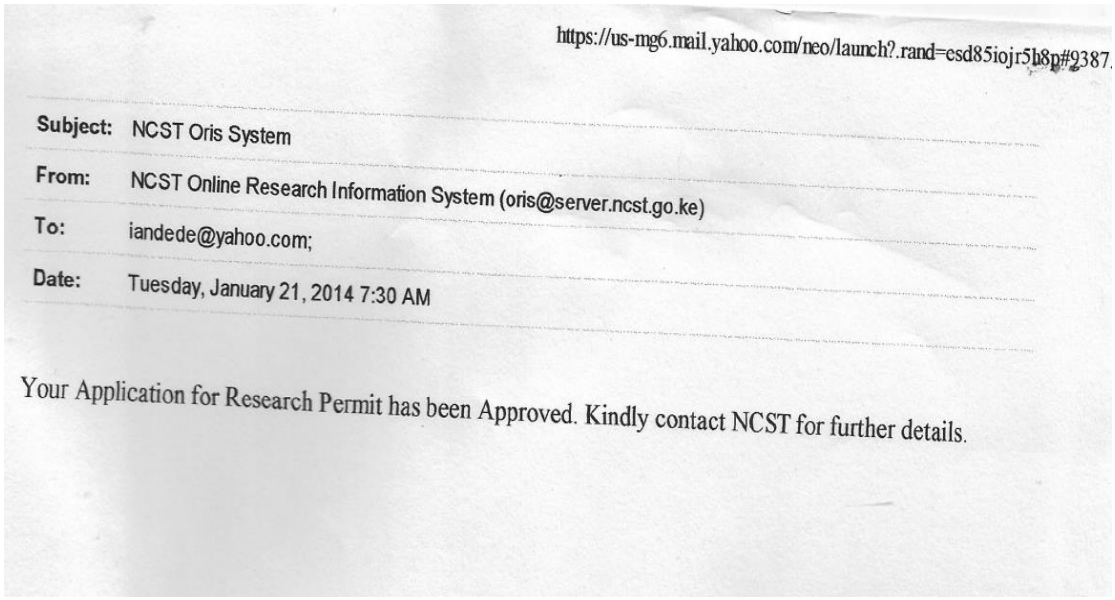
You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

01/07/2011
DR. W. ARUASA
AG. CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc: Director - MTRH
Dean - SOM
Dean - SPH
Dean - SOD

Appendix IV. NACOSTI Research Permit



https://us-mg6.mail.yahoo.com/neo/launch?.rand=esd85iojr5h8p#9387

Subject: NCST Oris System

From: NCST Online Research Information System (oris@server.ncst.go.ke)

To: iandede@yahoo.com;

Date: Tuesday, January 21, 2014 7:30 AM

Your Application for Research Permit has been Approved. Kindly contact NCST for further details.

Appendix V. Specimen shipment Authorisation

MINISTRY OF HEALTH
OFFICE OF DIRECTOR OF MEDICAL SERVICES

Telegrams:"MINHEALTH". Nairobi
Telephone; Nairobi 2717077 Fax: 2715239

OFFICE OF DIRECTOR OF
MEDICAL SERVICES
AFYA HOUSE
CATHEDRAL ROAD
P.O. BOX 30016
NAIROBI

MOH/ADM/1/1/81 VOL.1

20th December, 2013

Isaac Ndede
Moi University
Department of Immunology
P.O. Box 4606 - 30100
NAIROBI

Dear Mr. Ndede

RE: AUTHORITY TO SHIP BIOLOGICAL SAMPLES

Your request for specimen export permit dated 20th December, 2013 refers.

The title of your study is noted to be *"Immunohistochemical and Molecular characterization of Burkitt's lymphoma in Children Infected with Plasmodium Falciparum, Human Immunodeficiency virus and Epstein Barr Virus in Western Kenya".*

Authority is hereby granted for shipment of biological samples related to this research work:

- **80 Biopsy Tissue blocks**

The shipment contact details are follows:

Prof. Lorenzo Leoncini
Dept. of Medical Biotechnology
University of Siena

20/12/13

Dr. Onyancha P. K.
FOR: DIRECTOR OF MEDICAL SERVICES

Appendix VI. Consent Form

I would like to thank you for agreeing to participate in this study. The study is to characterize Burkitt's lymphomas. Please feel free to ask questions during and after the interview. Thank you.

Study No……

Title of Study:

Immunohistochemical and molecular characterization of Burkitt's lymphoma in children infected with *Plasmodium falciparum,* Human Immunodeficiency and Epstein Barr viruses in western Kenya

Investigator: Isaac Ndede

Department of Immunology

School of Medicine

P. Box 4606, Eldoret, Kenya

Informed consent

We are asking you to volunteer for a study research. This study is for Burkitt's lymphoma and non Burkitt's lymphoma patients. Before you decide to whether to take part in the study, we would like to explain the purpose of the study, the risks and benefits, and what is expected of you if you agree to be in the study.

Purpose of the Study

The purpose of this study is to characterize Burkitt's lymphoma in children aged 2-15 years in western Kenya

Procedure

Biopsy and Blood will be drawn from the study participants and sent to MTRH / AMPATH laboratories where various immune biomarkers will be determined by immunohistochemistry and flow cytometry.

Benefits

There will be no direct benefits for the participants in this study, however, the participant and others may benefit in the future from information learned from this study.

Risks

The participants may experience discomfort or pain when undergoing phlebotomy and biopsy procedures. They may also feel dizzy or faint and/or develop a bruise, swelling or infection where the needle is inserted.

Although every effort will be to protect participant's privacy and confidentiality, it is possible that others could know the participants' involvement in the study, and social harm may result.

Confidentiality

Every effort will be made to keep personal information confidential. All information obtained in this study will be treated with strict confidentiality, and divulged to any one not involved in the study. The participants' name will not be used in any publication made from this study.

Right to refuse or withdraw

Participation in this study is entirely voluntary. There is freedom to refuse to take part or withdraw at anytime, without punitive measures.

If you have any questions about this study, please contact Isaac Ndede, Department of Immunology, Box 4606, **Eldoret,** Tel. 0723247938.

Statement of consent and signatures

I have read this form or heard it read to me. I have discussed the information with study staff. My questions have been answered. I understand that my decision whether or not to take part in the study is voluntary. I understand that if I decide to join the study I may withdraw at any time. By signing this form I do not give up any rights that I have as a research participant.

…….	…….	……
Participant Name	Participant Signature	Date
…….	…….	……
Study Staff	Study Staff Signature	Date
…….	…….	……
Witness Name	Witness Signature	Date