ASSOCIATION BETWEEN *IFN-γ, TNF-α, FcγRIIA* AND *IL-12β* GENE POLYMORPHISMS AND SUSCEPTIBILITY TO ENDEMIC BURKITT LYMPHOMA IN CHILDREN FROM WESTERN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not previously been presented to any institution or university for a degree or other awards.

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I dedicate this thesis to my son, Oluoch Kyle O'Brien for the joy and strength he bestows upon me.

ABSTRACT

Endemic Burkitt lymphoma (eBL) is an aggressive paediatric B-cell lymphoma prevalent in children from holoendemic malaria regions of sub-Saharan Africa. It is associated with early primary Epstein-Barr virus (EBV) and repetitive *Plasmodium falciparum* (Pf) malaria coinfection with high incidence in western Kenya. The lymphoma is common among children aged 2-11 years with high incidence at 5-8 years and has been associated with multiple genetic variations. Endemic BL is characterized by the overexpression of the *c-MYC* oncogene, as a consequence of the t(8:14) IGH-myc translocation. However, the translocation alone is insufficient to drive the development of eBL since normal B cells also undergo *c-myc* translocation, leading to the hypothesis that other mutational variations may contribute to tumorigenesis. Polymorphic variations in cytokine genes that affect specific cytokine transcriptional levels have been shown to influence tumoral, viral and parasitic immune responses hence increasing the risk of tumour development in several cancers. The influence of cytokine polymorphisms in the aetiology of eBL has not been exhaustibly demonstrated. In this study, a retrospective case-control study design was used to investigate the association between polymorphisms within diverse genes implicated in tumoral immune surveillance: IFN-γ (+2109C/T), *TNF-α* (-1031T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C; and the susceptibility to eBL in children from western Kenya. Specifically, the study determined the association between IFN-γ (+2109C/T), *TNF-α* (-1031T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C genotypes and *TNF-α* (-1031 T/C, -308G/A and -238G/A) haplotypes and risk of eBL development and *IFN-γ* (+2109C/T), *TNF*-α (-1031T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C gene polymorphism and EBV load among cases and controls from western Kenya. Based on the frequency of IL-12 +1188A/C low cytokine producing genotype within this population, a total of 113 eBL cases and 69 healthy age-matched control samples were used. Genomic DNA was extracted following the Qiagen™ DNAeasy protocol and used for TaqMan allelic discrimination and molecular inversion probes genotyping assays. EBV load was quantified using quantitative real-time PCR. The distribution of selected *IFN-γ, TNF-α, FcγRIIA* and *IL-12B* genotypes were determined by Fisher Exact test, while the association between the selected genotypes/TNF-α haplotypes and risk of eBL development were determined using logistic regression analysis. One-Way ANOVA was used to compare EBV load across the genotypes. The frequency of *IFN-γ* (+2109C/T), *TNF-α* (-1031T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C genotypes were not significantly different between the study groups. Furthermore, no association between *IFN-γ, FcγRIIA, IL-12B* and *TNF-α* genotypes and risk of eBL diagnosis was observed. No significant difference in the EBV viral load between IFN-γ, *TNF-α*, *FcγRIIA* and *IL-12B* genotypes were also observed. Additionally, *TNF-α* haplotypic analysis were not significantly associated with the risk of eBL development. These results demonstrate that genetic variations in selected *IFN-γ, TNF-α, FcγRIIA* and *IL-12B* genes that affect levels of their respective cytokines and affinity of FcγRIIA for IgG have no association with eBL development. This suggest that other cytokine or other genetic factors may play a role in increasing susceptibility to eBL development through other mechanisms other than *IFN-γ, TNF-α, FcγRIIA* and *IL-12B* genes. Lack of association between the selected cytokine polymorphism and development of eBL suggest that these polymorphisms are not cytokine gene risks factors for genetic screening and development of cytokine therapeutics that target prevention and management of children at risk of developing eBL within malaria endemic region of western Kenya. However, the identification of other genetic variants in cytokines and their possible association with eBL development in children from malaria endemic regions of Kenya is still critical in mapping other genetic mutations that may contribute to the pathogenesis of the lymphoma aside from the *MYC* translocation.

TABLE OF CONTENT

ACRONYMS AND ABBREVIATIONS

A:	**—**	Adenine
ADCC:	**—**	Antibody-Dependent Cellular Cytotoxicity
AID:	**—**	Activation Induced Cytidine Deaminase
BL:	**—**	Burkitt Lymphoma
C:	**—**	Cytosine
CMV:	**—**	Cytomegalovirus
EBL:	**—**	Endemic Burkitt Lymphoma
EBV :	**—**	Epstein Barr virus
Fc :	**—**	Fragment, crystallizable
G:	**—**	Guanine
GC:	**—**	Germinal Centre
HIV:	**—**	Human Immunodeficiency Virus
IFN-γ:	**—**	Interferon Gamma
IgG:	**—**	Immunoglobulin G
IGH:	**—**	Immunoglobulin Heavy Chain
IL-12:	**—**	Interleukin 12
JOOTRH:	**—**	Jaramogi Oginga Odinga Teaching and Referral Hospital
LMP-1:	**—**	Latent Membrane Protein-1
LTA :	**—**	Lymphotoxin ‘a’
LTB:	**—**	Lymphotoxin ‘b’
MIP:	**—**	Molecular Inversion Probe
MSP-1:	**—**	Merozoite surface protein-1
NF-kB:	**—**	Nuclear Factor-kappa Beta
NPC:	**—**	Nasopharyngeal Carcinoma
***Pf*:**	**—**	*Plasmodium falciparum*
SBL:	**—**	Sporadic Burkitt Lymphoma
T:	**—**	Thymidine

Th1:	**—**	T Helper-1
TNF-α:	**—**	Tumour Necrosis Factor-alpha
VCA:	**—**	Viral Capsid Antigen
3’ UTR:	**—**	3’ Untranslated Region

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CHAPTER ONE

INTRODUCTION

1.1. Background Information

Burkitt lymphoma (BL) is a highly prevalent and aggressive B cell lymphoma characterized by translocation and deregulation of *MYC* gene, a proto-oncogene involved in cell cycle progression, apoptosis and cellular transformation. The lymphoma has three distinct clinical forms recognized so far including endemic (eBL), sporadic (sBL) and immunodeficiency associated Burkitt lymphoma (Parkin *et al*., 2014). While the three clinical forms of the lymphoma are histologically identical, they present with difference in global incidence, epidemiology, clinical presentations and also genetic features (El-Mallawany *et al*., 2017). Though information on the accurate global incidence of the lymphoma is limited, BL has an incidence of 1-3 per million in the United States and Europe which is considerably lower than that of sub-Saharan Africa (Orem *et al*., 2007). In Africa, more so sub-Saharan Africa, the incidence is quite variable, ranging from a few cases to 18 per 100,000 annually (Magrath, 2012). In Kenya, the annual incidence of the lymphoma is 2.15 cases per 100,000, differentially clustered in different parts of the country based on *Plasmodium falciparum* transmission levels. Spatial clustering showed elevated levels of eBL risks in high-malaria transmission regions of western Kenya and reduced in regions where malaria is infrequent (Rainey *et al*., 2007). Therefore, eBL presents a significant public health concern in malaria endemic parts of western Kenya and additional studies to understand underlying etiological factors are needed.

Endemic Burkitt lymphoma accounts for at least 36% of childhood cancers and 70% of childhood lymphomas in sub-Saharan Africa (Orem *et al*., 2007). The lymphoma has a multifactorial aetiology, which involves genetic variations, environmental factors, infectious agents such as Epstein-Barr virus (EBV) and *Plasmodium falciparum* (*Pf)* malaria infections (Chêne *et al*., 2007). Multiple models have been proposed to explain the role of *Pf* malaria and EBV in the pathogenesis of the lymphoma. Early primary EBV infection increase risk of high EBV viral load, *MYC* translocation and transformation of B-cells and likelihood of the

lymphoma. Persistent malaria infection also impairs T-cell immune response to EBV, leads to polyclonal activation of B-cells and accumulation of EBV transformed B-cells and reactivation of activation induced cytidine deaminase (AID), an enzyme attributed to risk of *MYC* translocation and higher likelihood of eBL development (Robbiani *et al*., 2015; Rochford *et al*., 2005). In western Kenya, malaria transmission is intense and stable characterized by repetitive or chronic infection throughout the year (Piriou *et al*., 2012). Children aged 1-4 years from this region have early primary EBV infection and elevated viral loads compared to age-matched children from the neighbouring region with low and sporadic malaria transmission (Moormann *et al*., 2005; Piriou *et al*., 2012) suggesting that early primary EBV and elevated viral loads contributes to pathogenesis of eBL. As a result, children living in malaria endemic parts of western Kenya, exposed to early primary EBV infection and repetitive *Pf* malaria infection are at a higher risk of developing the lymphoma as compared to other parts of the country.

Burkitt lymphoma is characterized by the overexpression of the *c-MYC* proto-oncogene (Haluska *et al*., 1987). This is most often as a consequence of a *t(8:14)* chromosomal translocation juxtaposing the Immunoglobulin heavy chain (IGH) enhancer adjacent to *MYC* gene, while less common translocations can involve the Ig light chain loci (Bhatia *et al*., 1993; Dave *et al*., 2006). Deregulation in *MYC* alone is not sufficient to drive eBL lymphomagenesis (Richter *et al*., 2012) and other collaborating genetic mutations may be involved in initiating oncogenesis or driving tumour cell survival. Cytokine are soluble immune regulatory molecules that regulate proliferation, differentiation and normal functioning of cells (Borecký, 1992) and any variation that influences its circulating levels may affect susceptibility and progression of diseases (Feldmann, 2008). Several studies have implicated polymorphism within cytokine genes with increased risk of asymptomatic primary EBV infection and development of EBV-associated tumours (Dierksheide *et al*., 2005; Ghesquières *et al*., 2013; Lee *et al*., 2006; Steed *et al*., 2007; Wu *et al*., 2002) yet this remain uninvestigated in eBL.

Tumour necrosis factor (TNF), a potent mediator of immune regulation and inflammation can suppress tumour cell proliferation and induce regression (Green *et al*., 1982). As a pro-inflammatory cytokine, TNF-α has the ability to act as an endogenous tumour promoter that connects inflammation and carcinogenesis (Wang and Lin, 2008). Moreover, elevated serum

concentration of TNF-α was reported in chronic lymphocytic leukaemia (CLL) and shown to be correlated with known prognostic factors in the cancer (Ferrajoli *et al*., 2002). Evaluation of TNF-α concentration was also reported as a valuable parameter for reflecting the severity of staging in patients with invasive breast cancer (Sheen-Chen *et al*., 1997). Elevated plasma levels of TNF-α was associated with adverse prognostic factors and possibility of predicting a poor outcome in lymphoma patients (Salles *et al*., 1996; Warzocha *et al*., 1997). Variation in the production of TNF-α cytokine may arise from single nucleotide polymorphisms (SNPs) within the *TNF-α* promoter region (Wu *et al*., 2002). Polymorphisms within *TNF-α* (-1031T/C, -376A/G, -238A/G and -308A/G have been shown to affect transcription factor binding sites, thus modulating transcriptional regulation of the cytokine resulting into high plasma levels of the pro-inflammatory cytokine (El-Tahan *et al*., 2016). In Tanzanian children, the presence of *TNF-α* $_{-1031}$CC genotypes was associated with increased rates of malarial episodes while those with *TNF-α* $_{-308}$AA had decreased rates (Gichohi-Wainaina *et al*., 2015). Low TNF-α level as a result of loss of transcriptional regulation can impair immune responses to *Pf* malaria, leading to likelihood of EBV reactivation and therefore increasing risk of eBL development among children with the polymorphisms. While change in TNF-α level as a result of transcriptional variations in the cytokine genes may affect immune responses to *Pf* malaria and tumours and therefore increase risk of EBV reactivation, leading to higher chance of developing eBL, no study has evaluated possible association between the promoter polymorphisms and risk of developing the lymphoma.

Interferon-γ (IFN-γ) has been associated with tumour immunosurveillance through a lymphocyte-mediated response, direct actions on tumour cells and possible inhibition of tumour angiogenesis (Ikeda *et al*., 2002). *In vitro* studies have also shown that IFN-γ inhibits growth of tumour cells through the induction of apoptosis (Wall *et al*., 2003). The transcriptional levels of IFN-γ has been attributed to polymorphisms within the introns which disrupts transcription factor binding sites, leading to impaired IFN-γ production in individuals who carry the variant allele (Chevillard *et al*., 2003). Base substitution from C to T at +2109 in the third intron is functional and increase the transcriptional levels of the cytokine (Chevillard *et al*., 2003). Studies have also associated polymorphism on other *IFN-γ* intronic sequences that lead to lower cytokine levels

with the risk of developing post-transplant lymphoproliferative disorder (PTLD) and susceptibility to EBV-associated B-cell malignancies (Lee *et al*., 2006; VanBuskirk *et al*., 2001). While past studies have reported loss in IFN-γ responses and risk of eBL development and also highlighted the role of the Th1 cytokine in mediating immune responses to *Pf* malaria and EBV, the role of *IFN-γ* functional polymorphic variations in the pathogenesis of eBL development remains unexplored.

Fragment crystallizable-*γ* receptors (Fc*γ*R) are members of IG superfamily and are involved in phagocytosis and antigen presentation. The FcγRIIA has a relatively low affinity to IgG antibodies compared to other members of the Fc*γ*R family (Powell *et al*., 1999). Two allelic variations have been reported in the genetic locus of the *FcγRIIA*, 131-Arg (R131) and 131-His (H131) with arginine and histidine at position 131 of the extracellular segment of the receptor respectively. The alternative R131 allele has a lower affinity to IgG2 when compared to the reference H131 allele (Reilly *et al*., 1994). High frequency of *FcγRIIA* low affinity R131 allele was reported among EBV-positive low-grade B-cell lymphoma patients and also associated with increased Latent Membrane Protein-1 (LMP-1) expression and EBV reactivation (Diamantopoulos *et al*., 2013) Thus, children with the IgG low affinity *FcγRIIA* allele (R131) may have high EBV load which is associated with the risk of developing eBL. Despite of a possible link between this polymorphism and risk of EBV reaction or high expression of LMP-1 protein, no study has reported its possible role in the pathogenesis of the lymphoma.

Interleukin-12 (IL-12) plays a key role in the regulation of inflammation by linking innate and adaptive immune processes (Tugues *et al*., 2015). During *Pf* malaria infection, early production of the cytokine by antigen presenting cells is important in the priming of T-cells which subsequently produce IFN-γ, a Th-1 cytokine which has been associated with clearance of blood stage malaria infection (Malaguarnera *et al*., 2002). Furthermore, accessory signals from IL-12, in addition to IL-18, are also needed during innate immune responses to malaria to induce optimal production of IFN-γ (Malaguarnera *et al*., 2002). The quantity and quality of antibody responses to blood stage *Pf* malaria is also influenced by the level of IL-12 cytokine production (Su and Stevenson, 2000). Impaired production of IL-12 may facilitate tumour development among children exposed to persistent malaria and early primary EBV infection by affecting Th1

immune responses to malaria leading to loss of CD8+ T-cell responses to EBV, EBV reactivation from latency, activation of AID and transformation of B-cells, increasing susceptibility to the lymphoma. While the substitution polymorphism at *IL-12* +1188A/C 3'UTR region may influence the level of translated protein by affecting mRNA stability and transcription of the gene, thus increasing risk of eBL development among children from areas with holoendemic malaria transmission, no study has reported on its distribution among eBL cases and matched controls.

1.2. Statement of the Problem

Endemic Burkitt lymphoma is the most common paediatric lymphoma in malaria endemic region of sub-Saharan Africa. The presence of eBL in this region has been associated with holoendemic *Pf* malaria transmission and early primary EBV infection in children. The lymphoma is common among children aged 2-11 years with highest incidences reported in malaria endemic parts of western Kenya as compared to other parts of the country. Cytokines, especially Th1 cytokines such as IFN-γ, TNF-α, and IL-12 are involved in the modulation of immune responses to EBV, tumours and *Pf* malaria. The phagocytic and antigen presentation functions of FcγR have also been described in EBV and *Pf* malaria immune responses. Functional polymorphisms in *IFN-γ*, *TNF-α*, *FcγR* and *IL-12* genes that affect the transcriptional levels of the cytokine and affinity of the receptor to IgG may play a role in affecting immune responses to *Pf* malaria, EBV and tumour cells, and increasing susceptibility to eBL development among children living in malaria endemic regions of western Kenya. While polymorphisms in cytokines have been associated with susceptibility to multiple cancers, risks of malaria reinfection and poor viral immune responses and therefore high EBV load, no study has invested these specific cytokines and their genetic variants among children in western Kenya. Therefore, the current study investigated the role of these polymorphisms in the pathogenesis of the lymphoma among children from Kenya, a region with holoendemic malaria transmission and early age at primary EBV infection.

1.3. General Objective

To investigate the association between single nucleotide polymorphisms within *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C genes, *TNF-α* haplotypes and the susceptibility to endemic Burkitt lymphoma and EBV reactivation in children living in malaria endemic region of western Kenya.

1.3.1 Specific objectives

i. To determine the association between *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C polymorphism and susceptibility to eBL in children living in western Kenya.
ii. To determine the association between *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C polymorphism and EBV viral load in children from western Kenya.
iii. To determine the association between *TNF-α* (-1031 T/C, -376G/A and -238G/A) haplotypes and susceptibility to eBL in children from western Kenya.

1.3.2. Null hypotheses

i. Interferon-γ (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C polymorphisms are not associated with susceptibility to endemic Burkitt lymphoma development in children from western Kenya.
ii. Interferon-γ (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* 131His/Arg and *IL-12B* +1188A/C polymorphisms are not associated with EBV viral load in children from western Kenya.
iii. Tumour Necrosis Factor-α (-1031 T/C, -376G/A and -238G/A) haplotypes are not associated with susceptibility to eBL development in children from western Kenya.

1.4. Justification and Significance of the Study

Endemic Burkitt lymphoma is the most common childhood lymphoma in sub-Saharan Africa. Studies have shown a higher prevalence of eBL in children from the malaria endemic

regions of western Kenya and it continues to be a major public health concern within the region. A better understanding of eBL aetiology and prognosis is important in developing effective preventive and therapeutic measures and mapping those at a high risk of developing the paediatric lymphoma due to a genetic predisposition. Epstein - Barr virus and *Pf* malaria co-infection have been associated with eBL aetiology though the exact mechanism of the two factors remains unclear. Change in the transcription levels of cytokines modulates immune responses to malaria and EBV infection. Chronic presence of EBV DNA in host genome and high expression of LMP-1 has been reported in *FcγRIIA* genetic variants. Inability to inhibit tumour development, angiogenesis, and to promote regression has also been shown in individuals with low levels of IFN-γ cytokine. Identifying genetic biomarkers that regulate immune cell function and cytokine production thus affecting immune response to cancer cells, EBV and malaria parasites is important in mapping children at a greater risk of developing eBL in western Kenya. Lack of association between the selected cytokine polymorphism and development of eBL suggests that these polymorphisms are not cytokine gene risk factors for genetic screening and development of cytokine therapeutics that target prevention and management of children at the risk of developing eBL within malaria endemic region of western Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1. Endemic Burkitt Lymphoma

Burkitt lymphoma (BL) is an aggressive B-cell non-Hodgkin's lymphoma (NHL) with a high proliferative index and doubling time of 24-48 hours (Iversen *et al*., 1974). Burkitt lymphoma is classified into endemic (eBL), sporadic (sBL) and HIV- associated subtypes depending on the geographic distribution and association with Epstein-Barr virus (EBV) (Jaffe *et al*., 2001; Parkin *et al*., 2014). Sporadic BL, the subtype commonly found in western countries, accounts for 40% of lymphomas in children and 1-2% in adults (Blum, 2004) and has a lower association with EBV (5-15%) (Mbulaiteye *et al*., 2009). Endemic BL, with a 95% association with EBV (Bornkamm, 2009), is predominant in the equatorial belt of Africa and other parts of the world such as Papua New Guinea where malaria transmission is hyperendemic (Ferry, 2006).

In African Equatorial Belt, eBL accounts for 74% of childhood lymphomas and has an incidence of 5-10 per 100,000 children (Orem *et al*., 2007). Compared to sBL that frequently involves abdominal tumours, eBL is present in jaws but may also occur in the abdomen, facial bones, and other extranodal sites (Orem *et al*., 2007). Endemic BL prevalence within the malaria belt is associated with low socioeconomic status, undernourishment, malaria holoendemicity and EBV infection (Rainey *et al*., 2007). The common age of occurrence of the lymphoma within this setting is 4-7 years and the male to female ratio is 2:1 (Jaffe, 2001). EBV association with eBL was discovered in 1964 (Epstein *et al*., 1965) and subsequent studies have attempted to establish the causal role of the virus in tumorigenesis of eBL.

2.2. Co-infections in Endemic Burkitt Lymphoma

Malignant lymphomas and cancers have been associated with co-infection of pathogenic agents such as Epstein Barr Virus, Human Papilloma Virus and even *Pf* malaria. Endemic BL has been associated with early-age primary EBV infection and also geographically linked with holoendemic *P. falciparum* malaria transmission (Burkitt, 1958; Dalldorf *et al*., 1964). Epstein-Barr virus is a lymphotropic gamma herpesvirus that is spread widely within the human

population and was the first virus to be linked to human tumours (Epstein *et al*., 1965). Transmission of EBV from one person to another occurs at an early stage of life and is acquired through human saliva and breast milk contact (Daud *et al*., 2015; de-The, 1977). The virus was first isolated from an eBL tumour (Epstein *et al*., 1965) and has also been found in all cases of the lymphoma subtypes (Brady *et al*., 2007). Children who later developed eBL were also found to have high antibody titters to EBV viral capsid antigen (VCA) (de-Thé *et al*., 1978). Furthermore, primary infection of infants with EBV at an early stage in life leads to a poorly controlled infection thus postulating the increased risk of developing eBL among children in malaria endemic regions (de-The, 1977; Piriou *et al*., 2012).

In vitro, EBV has been shown to transform resting B cells leading to the formation of latently infected lymphoblastoid cells (Neitzel, 1986). In the pathogenesis of eBL, EBV may contribute to the deregulation of *MYC* gene through potentiation of gene activity and clonal expansion or direct mutagenesis (Young and Rickinson, 2004). Children living within areas of holoendemic malaria transmission of western Kenya are exposed to early age of primary EBV infection and high viral load (Piriou *et al*., 2012), thus faced with significant risk of developing eBL.

Malaria transmission is holoendemic in many parts of equatorial Africa and may occur throughout the year with primary infection experienced during the first year of life (O'Meara *et al*., 2010). The role of malaria in the pathogenesis of eBL is poorly understood, however, there is a strong geographic association between eBL and holoendemic malaria (+/-10° around equator in Africa) described as the lymphoma belt by Denis Burkitt (Burkitt, 1963). Malaria and EBV co-infection may, in concert, contribute to the development of eBL in a number of ways as reviewed by Rochford *et al*., (2005). First, malaria may disrupt EBV-specific T cell immunity leading to loss of viral control. Furthermore, malaria infection induces polyclonal B-cell expansion and subsequent reactivation of lytic EBV which may lead to expansion of latently infected B cells and thereby increases the likelihood of *MYC* translocation, the hallmark mutation in BL (Klein, 1983). *Plasmodium falciparum* malaria has also been shown to activate activation-induced cytidine deaminase (AID), an enzyme responsible for mediating class switch recombination and somatic hypermutation in immunoglobulin genes in germinal centre (GC) B cells (Torgbor *et al*.,

2014). In the presence of malaria, the number of EBV-infected B cells able to tolerate *MYC* translocation in GC increases and the probability of the translocation is also elevated due to the induction of AID (Robbiani *et al*., 2015; Torgbor *et al*., 2014; Wilmore *et al*., 2016). Therefore, persistent malaria infection in children exposed to early primary EBV infection in western Kenya may contribute to *MYC* translocation, leading to higher risk of developing eBL.

2.3. Roles of Genetic Variations in Cytokines

A diverse range of cellular stresses such as carcinogen-induced injury, infection and inflammation initiates the release of cytokines as reviewed by (Dranoff, 2004). Depending on the tumour microenvironment, cytokines are capable of modulating antitumoral responses by stimulation of effector and stromal cells at tumour sites and enhancement of tumour cell recognition by effector cells (Landskron *et al*., 2014; Lee and Margolin, 2011). However, in chronic inflammation, cytokines are capable of inducing cell transformation and malignancy, a scenario that is conditional on the balance of pro- and anti-inflammatory cytokines (Zamarron and Chen, 2011).

Different cytokines may have a role in the inhibition of human cancers (Dranoff, 2004). T-helper 1 (Th-1) cytokines have a variety of functions including regulating tumour angiogenesis, inhibiting tumour regression, and promoting apoptosis in different cancers (Braumüller *et al*., 2013). Interleukin-12 is the main orchestrator of Th-1 immune response against cancer through the initiation and maintenance of Th1 phenotype characterized by secretion of cytokines such as IL-18 and IFN-γ and may also elicit potent antitumor immune responses when exerted directly in the tumour environment (Lasek *et al*., 2014). Interleukin-12 also enhances antibody-dependent cellular cytotoxicity (ADCC) against tumour cells (Lasek *et al*., 2014). The induction of anti-angiogenic cytokines and chemokines by IL-12 such as TNF-α and IFN-γ cytokines. IFN-γ-inducible protein 10 (IP-10) and Monokine induced by gamma interferon (MIG) chemokines also reinforces the anti-angiogenic action against tumours (Angiolillo *et al*., 1996). Activated antigen presenting cells (APCs) such as the dendritic cells, monocytes, macrophages and the neutrophils are IL-12 producing cells. Though it acts on a wide variety of immune cells, the cytokine is considered important in the mediation of Th1-type

immune responses to pathogens by inducing the production of other proinflammatory cytokines such as IFN-γ and TNF-α in addition to other chemokines (Athie-Morales *et al*., 2004).

Interleukin-12 is heterodimeric and consists of two covalently linked P35 and P40 subunits (Lasek *et al*., 2014). The subunits are encoded by *IL-12A* and *IL-12B* genes which are located on chromosome 3p12q-q13.1 and 5q31-33 respectively (Sieburth *et al*., 1992). Studies have reported multiple polymorphisms on *IL-12B* gene including an *IL-12B* promoter region and a *TaqI* 3'UTR which leads to a transition from A to C at position 1188 (Seegers *et al*., 2002). The *TaqI* polymorphism (+1188A/C) is associated with an increase in the secretion of IL-12 cytokine by stimulated human monocytes. Individuals carrying homozygous wild type genotype AA are high IL-12 cytokine producers while those with a heterozygous state produce intermediate levels of the cytokine. Carrying the homozygous mutant genotype CC leads to reduced production of the cytokine, which is associated with increased risk of malaria infection (Marquet *et al*., 2008) and can potentially increase risk of eBL development.

Interferon-γ is another major Th1 cytokine that has been shown to play a role in antitumor and antiviral immune responses. Interferon-γ is produced by natural killer cells and natural killer T cells (NKT) in innate immune response and by CD4+ and CD8+ cytotoxic T cells in antigen-specific adaptive immune response (Schoenborn and Wilson, 2007). The *IFN-γ* gene contains four exons (low order polymorphisms) and three introns and is located on chromosome 12q15. A nucleotide substitution at +2109C/T has been shown to affect the expression levels of IFN-γ cytokine through a mechanism that is not yet understood (Chevillard *et al*., 2003; Henri *et al*., 2002). Two complexes have been recognized that bind to DNA containing +2109 C allele while only one of the complexes bind to +2109 T allele, which suggest possible interference with the cytokine gene transcription (Chevillard *et al*., 2003), leading to impaired tumoral, viral and parasitic immune responses and higher risks of lymphoma development.

Tumour necrosis factor alpha (TNF-α), a member of the tumour necrosis factor/tumour necrosis factor receptor family is considered as a doubled-edged sword in regard to cancer (Wang and Lin, 2008). The locus of the gene is on chromosome 6 within the human leucocyte

antigen (HLA) class III region and flanked with lymphotoxin ‘a’ (LTA) and ‘b’ (LTB) genes (Qidwai and Khan, 2011). Tumour necrosis factor alpha is involved in the modulation of immune responses and is also a pro-inflammatory cytokine involved in inflammation (Qidwai and Khan, 2011). Monocytes/macrophages and other cell types such as T are involved in the production of TNF-α. The control of production of TNF-α is tightly regulated at both transcriptional and posttranslational levels (Wilson *et al*., 1997). The transcriptional induction of TNF-α is controlled by different transcription factors including OCT1, NF-kβ and activator protein-1 (AP-1) (Qidwai and Khan, 2011).

Polymorphic variations in the promoter regions of the gene encoding the cytokine has been associated with change in cytokine levels and susceptibility to infections ((Qidwai and Khan, 2011). Tumour necrosis factor -238G/A (TNF282.2) polymorphism lies in the putative regulatory box (Y-box) of the promoter region and influences the levels of the cytokine (Qidwai and Khan, 2011). Within the Y-box, regulatory DNA binding proteins (such as NF-Y) bind, thus influencing the transcriptional regulation of the gene. The region is strongly conserved among many animal species, a further indication of its significance (D’Alfonso and Richiardi, 1994). A single -238 G to A base substitution within this region has been associated with decreased transcription of the gene (D’Alfonso and Richiardi, 1994; Morzycka-Wroblewska *et al*., 1997). A SNP at -308 (TNF308.2) leads to substitution from G to A within the promoter sequences of the gene and has been associated with a 6 to 9-fold increase in the *in vitro* transcription of the gene (Wilson *et al*., 1997). In the figure below, the different alleles within the *TNF-α* promoter region associated with interruption in cytokine expression are presented (Fig 2.1).

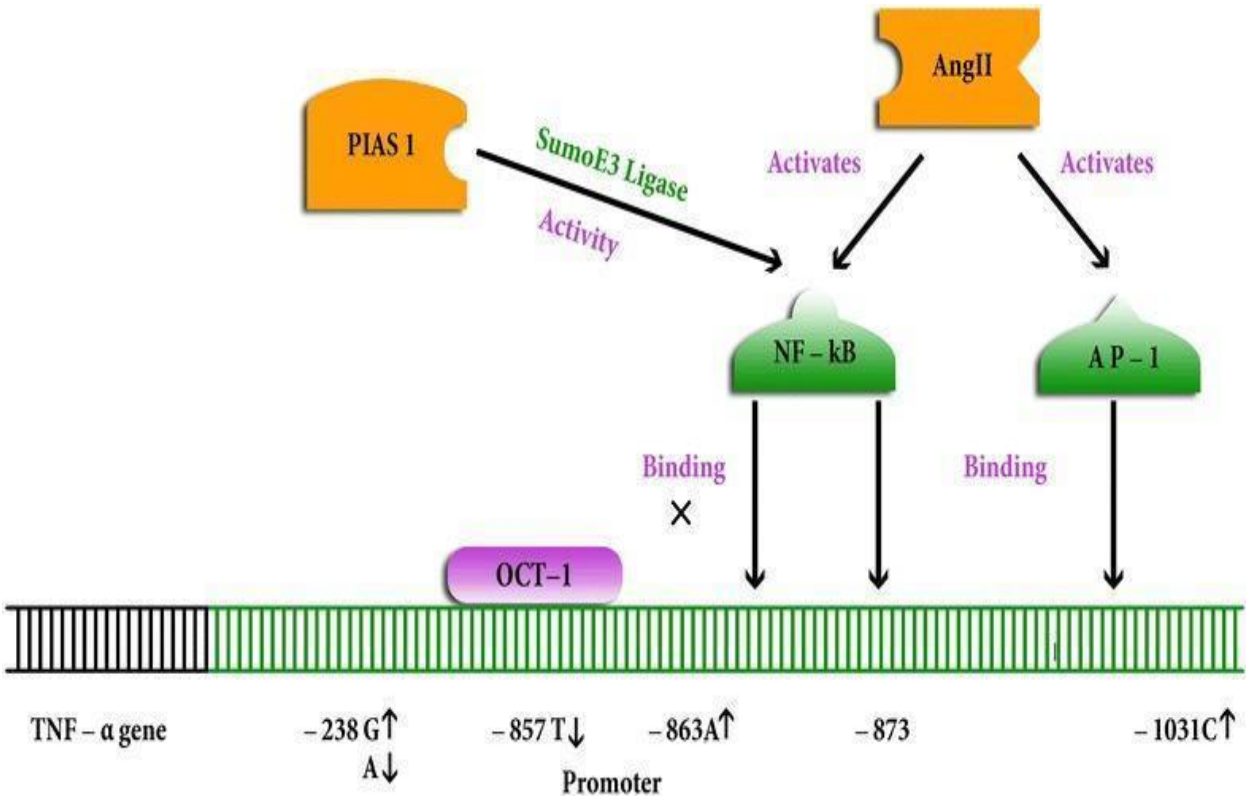


Figure 2.1**:** Molecules involved in the transcriptional induction and downregulation of TNF-α gene within the promoter region. All the alleles that lead to upregulation of TNF-α gene are represented by arrows with heads directed up while those that lead to downregulation of gene expression are represented by arrows pointing downwards (El-Tahan *et al*., 2016).

Tumour necrosis factor cytokine has been associated with the maintenance and homeostasis of immune system, inflammation and mediation of host defences. However, been reported in mediating pathological processes including chronic inflammation, autoimmunity and malignant transformations (Balkwill, 2006). Chronic inflammation attributed to this cytokine has been shown to promote tumour development and progression (Balkwill, 2006) though its role in eBL remain undefined.

Fragment, crystallizable receptors (FcRs) are transmembrane proteins that are expressed on the surface of several immune cells. Their mode of action includes activation of macrophages or cytotoxic cells, leading to enhancement of antibody-mediated phagocytosis or antibody dependent cell-mediated cytotoxicity (ADCC) in addition to the creation of a link between innate and adaptive immunity in humans (Diamantopoulos *et al*., 2013; van de Winkel and Capel,

1993). As a member of immunoglobulin superfamily, FcRs also play a role in antigen presentation (Pleass and Woof, 2001). Members of this family include Fc-γRI, Fc-γRIIA, Fc-γRIIB, Rc-γRIIIA and Fc-γRIIIB and the classification is based on differences in the affinity to immunoglobulin G (IgG) antibodies. The genes that encode all members of the FcR family are located on chromosome 1q23 (Indik *et al*., 1995). The Fc-γRII class is encoded by three genes including *Fc-γRIIA*, *Fc-γRIIB* and *Fc-γRIIC* (Brooks *et al*., 1989). The FC-γRIIA has a low affinity to IgG antibodies compared to other members of the family and is located on the surface of immune cells such as macrophages, neutrophils and eosinophils (Powell *et al*., 1999). Cells such as monocytes, macrophages, platelets and neutrophils express FC-γRIIA and are involved in innate immune responses to cancers (Pleass and Woof, 2001).

The effectiveness of Fc-γRIIA in the mediation of phagocytic function is dependent on several haplotypes (de Haas, 2001). Two allelic genes have been reported in the genetic locus of the extracellular segment of the receptor. The gene contain either G or A in codon 131, leading to 131-Arginine (C<u>G</u>T) or 131-Histidine (C<u>A</u>T) respectively within the second extracellular domain (Clark *et al*., 1989). The presence of arginine at this position is associated with a lower affinity to IgG as compared to histidine. Individuals with His-131 (H131), the A/A genotype, have a higher affinity for IgG2 as compared to those with Arg at position 131 (R131) (Reilly *et al*., 1994). While Fc-γRIIA plays a critical role in mediating antigen presentation and phagocytosis and has been associated with risks of lymphoma development, its role in eBL has not been explored.

2.4. Cytokine Genetic Variants and risk of eBL Development

The etiological factors of most B-cell malignancies are poorly understood though recent focus has shifted to the possible role of genetic variants in cytokine genes in their pathogenesis (Howell and Rose-Zerilli, 2007). Polymorphic changes in cytokine genes that influence cytokine expression levels due to interruption in the transcription factor binding sites have been studied in different B-cell lymphomas. In fact, studies have reported on the possible roles of *TNF-α* promoter variant, FcγRIIA receptor and *IFN-γ* intron polymorphism in the pathogenesis of different B-cell malignancies such as classical Hodgkin lymphoma and PTLD (Cerhan *et al*.,

2008; Ghesquières *et al*., 2013a; VanBuskirk *et al*., 2001)(Cerhan *et al*., 2008; Ghesquières *et al*., 2013b; VanBuskirk *et al*., 2001).

Tumour necrosis factor has been associated with autocrine and paracrine regulation of B cell differentiation and the production of immunoglobulin (Wu *et al*., 2002). Thus, genetic polymorphisms within the promoter region that affect the expression levels of the cytokine may contribute to the development of EBV-associated B-cell malignancies. A high prevalence of -1031C rare alleles have been found significantly among EBV positive PTLD patients as opposed to the non-PTLD transplant and health controls (McAulay *et al*., 2009). The severity of infectious diseases can be related to the TNF-α cytokine response at the genetic level (Wu *et al*., 2002). Thus, a permissive cytokine environment characterized by change in TNF-α expression levels may provide room for malignant B-cell transformation during EBV infection, leading to eBL tumorigenesis. High circulating TNF-α levels have been reported among individuals with the mutant -1031C and -308A alleles compared to the wild type -1031T and -308G respectively and has been associated with increased risks of non-Hodgkin Lymphoma (Cerhan *et al*., 2008; Sinha *et al*., 2008; Wang and Lin, 2008). In other studies, an increase in the TNF-α expression levels was associated with the emergence of tumour cells which lead to the development of malignant diseases such as chronic lymphocytic leukaemia (Ferrajoli *et al*., 2002; Szlosarek and Balkwill, 2003). Persistent inflammation triggered by high levels of TNF-α may increase chances of DNA damage, tumour angiogenesis, invasion with metastasis and local immunosuppression (Szlosarek and Balkwill, 2003). Thus, genetic variations that affect the level of TNF-α cytokine can affect the rate of progression and may contribute to the development of eBL among children.

In Th1 immune responses to tumour cells, IL-12 acts as an antitumor mediator by targeting lymphoid cells including NK and T cells, thus increasing the secretion of IFN-γ (Tugues *et al*., 2015). Thus, the actions of IL-12 cytokine which involves the direct inhibition of tumour growth by acting as an orchestrator of Th1-type immune response may play a role in the control of tumour progression. Polymorphism at +1188A/C 3'UTR of IL-12 was shown to increase the risk of developing cervical cancer, gliomas, non-Hodgkin lymphomas and lung cancer (Chen *et al*., 2009; Han *et al*., 2008). Presence of *IL-12B* +1188 C allele variant was also

associated with increased susceptibility to EBV-positive nasopharyngeal carcinoma (NPC) (Ben Chaaben *et al*., 2011). Production of IFN-γ cytokine is also known to initiate perforin-mediated inhibition of tumour development (Dranoff, 2004). Ikeda *et al*., (2002) described the possible anti-tumour action of IFN-γ in different cancer and lymphoma types. In fact, Th1 derived IFN-γ presents a dual anti-tumoral role: first, it has been shown to trigger tumoricidal activity of tumour infiltrating macrophages; second, it induces macrophages to secrete angiostatic chemokines CXCL9/MIG (monokine induced by IFN-γ) and CXCL10/IP-10 (IFN-γ inducible protein 10) (Haabeth *et al*., 2011). With the use of animal cancer models, high production of IFN-γ was described to directly inhibit tumour cell growth and induce apoptosis (Ikeda *et al*., 2002). Endogenous production of IFN-γ may also be involved in immunosurveillance of tumours through a lymphocyte mediated response, direct tumour action or possible inhibition of tumour angiogenesis (Ikeda *et al*., 2002). Studies have also shown that IFN-γ can inhibit the growth of human ovarian cancer *in vitro* (Burke *et al*., 1999; Wall *et al*., 2003) and cell proliferation of ovarian cancer cell lines (Burke *et al*., 1999). Increase in the level of IFN-γ produced has further been determined to influence the development of PTLD in renal transplant patients, an EBV co-infected condition (VanBuskirk *et al*., 2001). While multiple studies have reported potential role of IFN-γ, *TNF-α* and IL-12 polymorphisms in the development of multiple lymphomas and cancers, their possible role in eBL development remains unknown.

2.5. Cytokine Gene Variants and EBV Viral Load

Early primary EBV infection has been identified to contribute to higher risk of developing the lymphoma with studies showing that children infected early present with higher anti-VCA antibodies and increased risks of developing the lymphoma (de-Thé *et al*., 1975). Acute malaria infection also leads to sustained increase in EBV load and decrease in EBV-specific T-cell surveillance, probably contributing to the pathogenesis of eBL (Njie *et al*., 2009). Cytokines are involved in mediating immune responses to EBV and *Pf* malaria and change in transcriptional levels can contribute to high EBV viral load (Mbengue *et al*., 2016; Whittingham *et al*., 1993). Depletion in levels of IFN-γ in latently infected mice was shown to increase frequency of EBV reactivation (Steed *et al*., 2007). Normal levels of IFN-γ have also been shown to protect against liver schizogonic development of *Pf* malaria (Mellouk *et al*., 1987;

Perlaza *et al*., 2011). Individuals with low IFN-γ producing genotypes arising from polymorphisms on +2109C/T may have impaired immune response to EBV, leading to higher risks of reactivation, high viral load and likelihood of developing eBL. During malaria infection, high levels of IL-12 production protect against the parasite by enhancing erythropoiesis and reducing the chance of developing severe malaria anaemia (Mohan and Stevenson, 1998). Children with IL-12+$_{1188}$C allele and presenting with acute *falciparum* had increased susceptibility to severe malaria anaemia compared to those with wild type A allele (Ong'echa *et al*., 2011) . The levels of TNF-α have also been correlated with severity of malaria infection and considered as a prognostic marker of the parasitic infection (Singh *et al*., 2000). The presence of *TNF-α* promoter polymorphism at 238G/A was shown to influence levels of the cytokine and correlate with clinical outcome of malaria within a Nigerian cohort (Olaniyan *et al*., 2016).

In *Fcγ-RIIA*, high incidence of mutant R131 allele was reported among EBV-positive patients, an indication of the potential role of the polymorphism in maintenance of EBV latency after infection (Diamantopoulos *et al*., 2013). In fact, individuals with high EBV viral load were shown to have high prevalence of the low IgG2 affinity genotype, an indication of chronic presence of viral DNA within host genome (Diamantopoulos *et al*., 2013). The high expression of latency protein LMP1, the principle EBV oncoprotein in EBV among individuals with R131 genotype also demonstrate a possible role of Fcγ-RIIA in the expression of latency proteins (Diamantopoulos *et al*., 2013). In Classical Hodgkin Lymphoma (CHL), the R131 genotype was also found to be high among EBV positive patients as compared to EBV negative cohort (Ghesquières *et al*., 2013). The correlation of the Fcγ-RIIA R131 allele with chronic presence of EBV viral DNA in host genome and the expression of LMP1 oncoprotein may increase risk of EBV-associated malignancies such as eBL. While this polymorphism has been studied in relation to malaria in western Kenya, its association with risk to developing eBL within the same malaria endemic population remains unknown. Studies have therefore demonstrated that polymorphic variations in these cytokines and the Fcγ-RIIA that affects the affinity to IgG influences immune responses to *Pf* malaria and EBV, leading to likelihood of viral reactivation, high LMP-1 expressions and high viral load and possible risk of eBL lymphoma. However, the influence of these polymorphisms on determining the EBV viral loads among children from

malaria endemic parts of western Kenya is unknown.

2.6. Tumour Necrosis Factor Haplotypes and Risk of eBL Development

Haplotype include SNPs or other genetic markers or variants inherited together in the same chromosome with little chance of contemporary recombination (Stram, 2017). The inference of haplotypes, in addition to the individual SNPs is considered important and informative in many genetic approaches (Risch and Merikangas, 1996). The use of marker haplotype can provide additional power in the detection of association between a marker and a trait of interest (Judson *et al*., 2000). In different cancers, *TNF-α* haplotypes have been associated with increased risk of development, metastasis and also used as prognostic marker. In gastric carcinoma, the presence of *TNF-α-308*A*-associated haplotypic structure was significantly common among cases compared to controls (Canedo *et al*., 2008). Furthermore, multiple haplotype combinations, including *TNF-α* -1031/-863/-857/-308/-238 TCCGA, *TNF-α* -1031/-857/-308/-238 TCGA, *TNF-α* -1031/-857/-238 TCA and *TNF-α* -1031/-308/-238 TGA was associated with increased risk of developing HCC within a South Korean Population (Shin *et al*., 2015). In EBV associated lymphomas, the presence of *TNF-α* -1031/-863/-857/-307/-237 CACGG haplotype was associated with high risk of PTLD (McAulay *et al*., 2009). Haplotypic recombination that may affect the transcriptional levels of this cytokine may influence its role in cancer development and progression. While *TNF-α* promoter haplotypes have been studied in multiple cancers and also shown to play increase risk of EBV-associated PTLD development, its role in increasing susceptibility to eBL remains unknown.

CHAPTER THREE

MATERIALS AND METHODS

1.1. Study Area

Participants for this study were recruited from Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) located in Kisumu City (Latitude: 0°06′07″ S, Longitude: 34°45′42″ E), and Chulaimbo Health Centre located along Kisumu-Busia road (Latitude: 0°1'60" S, Longitude: 34°37'60" E). Jaramogi Oginga Odinga Teaching and Referral Hospital houses a paediatric cancer referral facility that serves the whole of western Kenya and was therefore suitable for the recruitment of eBL cases. The incidence of eBL in western region served by JOOTRH is 5-10 per 100,000 annually (Rainey *et al*., 2007), making the lymphoma one of the most prevalent in the region as shown in figure 3.2. Chulaimbo Health Centre also served as the centre for recruiting age matched non-eBL control group who live in the same region and have similar exposure to repetitive malaria and early primary EBV infection. High incidence of eBL in western Kenya has been associated with multiple factors including exposure to early primary EBV infection (Piriou *et al*., 2012) and repetitive malaria infections (Snider *et al*., 2012). Western Kenya experience holoendemic *Pf* malaria transmission with an entomological inoculation rate (EIR) of 31.1 infectious bites per person per year (Ndenga *et al*., 2006) and a malarial incidence of 1.8 to 3.6 per 100 people (Platt *et al*., 2018) annually, one of the highest in sub-Saharan Africa. The selected study site was therefore appropriate due to the high prevalence of the lymphoma, early age at EBV infection and holoendemic malaria transmission with stable infections throughout the year.

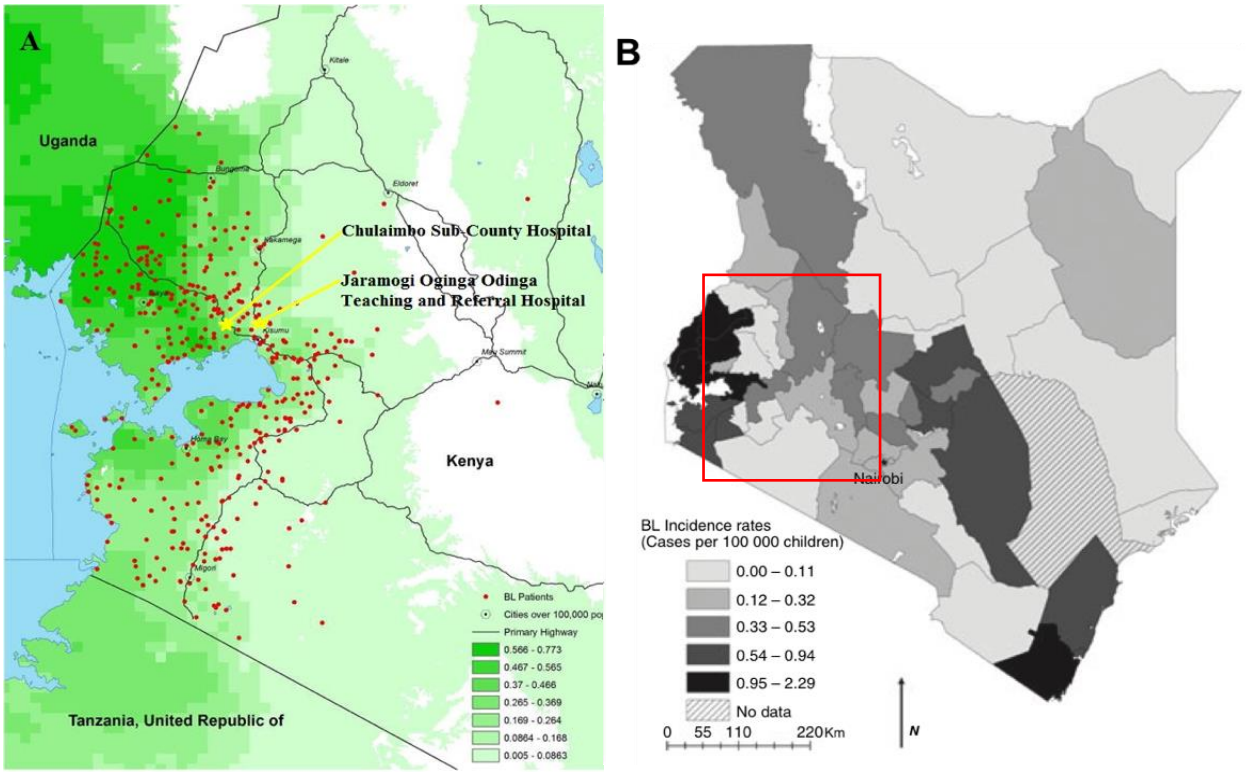


Figure 3.2: Distribution of endemic Burkitt lymphoma in western Kenya

A) Spatial distribution and catchment area in western Kenya of paediatric eBL patients admitted at JOOTRH in Kisumu, Kenya. Red dots indicate home village of 600 eBL patients enrolled at JOOTRH from 2003 to 2011. Shades of green illustrate malaria transmission intensity: light green and dark green for low and high transmission respectively (Buckle *et al*., 2016).

B) The map of Kenya showing the distribution of eBL across the country, with western Kenya and Coast leading in tumour prevalence (Rainey *et al*., 2007).

3.1 Study Population

To determine the role of cytokine gene polymorphism in development of eBL among children from western Kenya, a case-control study design was adopted in which a total of 113 histologically confirmed eBL samples, negative for tuberculosis abscess and other B-cell malignancies collected under KEMRI Scientific Ethical Review Unit SSC:1381 (Appendix 1) approval were retrospectively availed. In addition, the study also retrospectively used a total of

69 eBL-negative, age and gender matched samples of children living within the malaria endemic setting of western Kenya as the controls using a purposive sampling approach. Controls recruited from Chulaimbo Health Centre as the catchment area were matched for age, exposure to *Pf* malaria and tribe as the cases, thus limiting the number to 69 compared to 113 eBL cases who had been collected for a period of over 10 years within the project. In this study population, eBL is prevalent among children aged 2-14 with a higher risk reported in those aged 5-8 years old (Rainey *et al*., 2007).

3.2.1. Inclusion criteria

i. Only eBL patients confirmed by two independent pathologist using May-Grunewald Giemsa stain of fine needle aspirates were included in the study.
ii. All cases and controls were also within the age range of 2-14 years, tested negative for HIV and lived in western Kenya, an area with holoendemic malaria transmission.
iii. Controls were only considered for gender and age matched children negative for eBL, other lymphomas and cancers and HIV.
iv. Only those who consented to be in the study by providing a signed informed consent were included.

3.2.2. Exclusion criteria

i. Controls that had suffered from any type of lymphoma in the past
ii. Cases without confirmed diagnosis from qualified pathologists
iii. Participants who failed to sign informed consent
iv. Those who suffered from other infections such as HIV and TB.

3.2 Sample Size Determination

The study used a total of 182 participants. This sample size was calculated based on the frequency of IL-12 +1188 3'UTR genetic variants within a western Kenya population which was able to show differences in susceptibility to severe malaria anaemia among children (Ong'echa *et*

al., 2011). For a power of 80 in the determination of the proportion of alleles and distribution of *IL-12B* genetic variants within the eBL cases and non-eBL controls, the following formula was used to calculate the sample size:

$$n = \left(\frac{r+1}{r}\right)\frac{(\bar{p})(1-\bar{p})(Z_{\beta}+Z_{\alpha/2})^2}{(p_1-p_2)^2}$$

(Whitley and Ball, 2002)

Where;

n is the sample size of the case group.

r is the ratio of controls to cases (1.6).

$\bar{p}$ is a measure of variability (similar to the standard deviation)

(p_1-p_2) is the effect size (difference in proportion)

Z_{β} represents the desired power (typically 0.84 for 80% power)

Z_{α} represents the desired level of statistical significance. Typically, 1.96 is used to determine sample size (Whitley and Ball, 2002).

Odds ratio (OR) is a measure of the strength of the genetic association and is the odds of a participant having eBL given one genotype compared to the odds of not having the genotype in the presence of the lymphoma. An odds ratio of more than one was used in this case to mean that it is highly likely that a participant diagnosed with eBL had the genotype that make them susceptible to eBL. An odds ratio of 2.0 was detected based on a previous study that determined the distribution of low expressing *IL-12B* genotype within the same population (Ong'echa *et al.*, 2011).

In Ong'echa *et al*., (2011), the distribution of *IL-12B* (homozygous variant CC) which lead to reduced expression of the cytokine within the control group in western Kenya was found to be 18.6%. Therefore, the proportion of the controls living within the study population with the

genetic marker of interest was:

$$P_{cases} = \frac{ORp\ controls}{Pcontrols\,(OR-1)+1}$$

$$P_{cases} = \frac{2.0(0.186)}{(0.186)\,(2.0-1)+1} = \frac{0.372}{1.186} = 0.3137$$

From the population of cases above, the average proportion with the genetic variation of interest was:

$$\frac{0.3137 + 0.186}{2} = 0.2498$$

Therefore;

$$= 1.625 \times \frac{(0.2498)(1-0.2498)(0.84+1.96)^2}{(0.3137-0.186)^2} = 146$$

With an odds ratio of 2.0, the calculated sample size was 146. In this study, a total of 182 samples were retrospectively availed with 113 eBL cases and 69 age and gender matched controls. From a larger pool of retrospective non-eBL control samples, only 69 met the inclusion criteria for this study based on age, tribe and residence in malaria endemic parts of western Kenya.

3.3 Sample Collection and Processing

Frozen blood samples stored in EDTA microtainer tubes (Becton Dickinson, USA) were used in the extraction of DNA. The DNA extraction process was conducted according to Qiagen™ DNAeasy kit protocol (QIAGEN Sciences, Germantown, MD, USA). To the bottom of labelled 1.5ml micro-centrifuge tubes, 4µl of *RNase A* was pipetted followed by an addition of 200µl of blood cell pellet. After incubation for 2 minutes, each microtainer was briefly vortexed and pulse spanned before 20µl of *proteinase K* was added followed by brief vortexing and spinning to bring down the mixture in the micro-centrifuge. To the mixture, 200µl of *Buffer AL* (lysis buffer: guanidine hydrochloride, maleic acid) was added, briefly vortexed and spanned.

The reaction mixture was incubated at 56^0C for 10 minutes before 200µl of absolute ethanol was added to each micro-centrifuge. Vortexing was done thereafter to create homogeneity in the mixture before 630µl was pipetted to the centre of DNeasy spin column placed on top of 2ml collection tubes. Spinning followed at 6000g for a minute in an Eppendorf Centrifuge 5427 R (Eppendorf™ AG Barkhausenweg Hamburg Germany) at room temperature. The flow through was disinfected in 10% bleach and DNeasy spin column transferred to new collection tubes. To each spin column, 500µl of wash *buffer AW1* (guanidine hydrochloride) was added and incubated at room temperature for 5 minutes before centrifugation at 6000g for a minute. Again, the flow through was disinfected in 10% bleach as spin columns were transferred to new collection tubes. To each tube, 500µl of wash *buffer AW2* (guanidine hydrochloride) was added and incubated at room temperature for 5 minutes. The columns were centrifuged at 16,025g for 3 minutes, collection tubes discarded and spin column transferred to new collection tubes, dry spanned at 16,025g for 3 minutes and transferred to labelled 1.5 micro-centrifuge tubes. To each micro-centrifuge, 100µl of elution *buffer AE* was added and incubated for 5 minutes at room temperature before centrifugation at 6,000g for 1 minute for DNA elution. The elute was transferred to storage tubes and this final process repeated to increase the volume of extracted DNA to 200µl. The extracted DNA was quantified using Nanodrop 2000™ spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) for the sample set genotyped for *IL-12* +1188A/C polymorphism. For samples used for molecular inversion probe genotyping by sequencing method, Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA, USA) was done. The DNA concentration of both the cases and control samples ranged between 3.28 and 65.4ng/µl. Extracted and quantified DNA was subsequently stored at -20^0C awaiting genotyping by allele 5' discrimination assay and Molecular Inversion Probe Genotyping by sequencing method.

3.6. *IL-12B* TaqMan Genotyping

Interleukin-12B +1188A/C 3' UTR polymorphism was genotyped based on TaqMan SNP genotyping assay using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The genotyping assay ID for the SNP was *IL-12* +1188A/C (rs3212227, assay ID, C___2084293_10) (Applied Biosystems, Foster City, CA, USA). All genotyping PCR

reactions were done in a 10µl final reaction volume containing 5µl of TaqMan genotyping reaction mix, 0.5µl of SNP assay mix, 3.5µl molecular grade water, and 1µl DNA sample. The PCR cycling conditions were as indicated below; pre-PCR hold stage at 60^0C for 30 seconds, hold stage at 95^0C for 10 minutes, cycling stage at 95^0C for 15 seconds, and annealing at 62^0C for a minute. Holding stage to annealing was repeated 40 times. Genotype calling was done automatically using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) software and confirmed with the TaqMan Genotyper Software (Applied Biosystems, Foster City, CA, USA) as shown in Appendix 2. Samples that were discordant in the two genotype calling methods were repeated in subsequent runs and the whole procedure repeated. The called genotypes for the different SNPs were entered in Microsoft excel for further analysis.

3.7. Molecular Inversion Probe (MIPs) Genotyping

3.7.1. MIPs design

All MIPs for *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A) and *FcγRIIA* (HIS/ARG) were designed against the Human Genome version 19 (hg19) (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.38 and dbSNP132 https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?build_id=132) database as human reference genome. Among the Yoruba in Ibadan (YRI), the distribution of TNF-α -308G/A (rs1800629) was 79%, 19% and 1% for GG, GA and AA genotypes respectively. For IFN-γ (rs1861494), the distribution among YRI was 80% and 20% for TT and CT respectively. For FcγRIIA (rs1801274), the distribution of the genotypes among the YRI was 30%, 50% and 20% respectively for CC (Arg/Arg), CT (Arg/His) and TT (His/His) genotypes (https://www.snpedia.com/). The format of the MIPs used included a common 30bp linker flanked by an extension arm of 16-20bp and a ligation arm of 20-24bps totalling to 70 bps. The MIPs for each of the targets were chosen iteratively from the 5' to 3' end of the target region while optimizing for predicted capture efficiency. All the MIPs chosen satisfied the following criteria; a) resides on the opposite strand of the previous MIP, b) presence of <50% insert overlap with the previous MIP, c) is a predicted high performer, d) avoids single nucleotide variants from the dbSNP in target arm. All designed MIPs were column synthesized as 70-mer

oligonucleotide at 10µM concentration in 1× TE buffer, pH 8.0 (Integrated DNA Technologies, Coralville Iowa).

3.7.2. MIPs pooling and 5' phosphorylation

Before capture reactions, all MIPs were 5' phosphorylated using polynucleotide kinase (PNK). All the six MIP probes used in the study had a concentration of 10µM each and pooled together by taking 5µl of each and adding to an Eppendorf tube, forming 30µl of probes each of 10µM. Using 1µL of PNK for every nanomole probe, the total volume of PNK that was used for the phosphorylation was 0.3µl in addition to 6.5µl of water, 40µl of 10× T4 DNA ligase buffer with 10mM ATP and 30µl of MIP pool. The reaction was split into 8 tubes and the thermal cycling process performed with a lid temperature of 10°C above the incubation temperature of 47°C. The thermal cycler condition was 37°C for 45 minutes, 65°C for 20 minutes and holding stage at 4°C. After the thermal cycling, the tubes were mixed together to create a homogenous phosphorylated MIP pool which was mixed and aliquoted. The phosphorylated probes were diluted to 5µM stock from 10µM using TE buffer. Additional 500nM working solution was made from the stock by 1:10 dilution with TE buffer.

3.7.3. MIP capture

Before running MIP capture for the study samples, MIP concentration was optimized for each of the probe sets. Serial dilution was made from the stock MIP pool and test captures were done with control samples to determine the optimal working concentration. The testing was done at 8, 16 and 32 for the MIP concentration and different PCR cycles (22, 24 and 26) cycles to avoid nonspecific binding and differences compared in 2% agarose gel electrophoresis as shown in Appendix 3. The MIP capture for the samples was subsequently performed at 32 for 22 cycles with 30ng/µl of DNA due to the little primer leftover under this condition. The thermal cycler was started 4-5 minutes prior to the reaction and heated to 100°C. The cycling condition was preheated at 95°C, 95°C for 10 minutes, 60°C for 3.5 hours and 4°C holding. The dNTP was diluted by pipetting 1µl of 10nM dNTP plus 5µl 10× ampligase buffer and 44µl water making 50µl of 0.2mM dNTP. The Q5 polymerase dilution was performed by mixing 5µl of 10×

ampligase buffers and 44µl of water. Master mix of the PCR component was made by adding 0.92µl of 10× ampligase buffer, 0.2µl dNTP (0.1mM), 0.4µl MIP pool, 0.4µl ampligase at 1u/µl and 0.2µl of 0.02µM/µl Q polymerase (Qiagen, Hilden Germany). The mastermix was added into the PCR tubes followed by 30ng of sample DNA and centrifuged briefly to collect mixture at the bottom of the tube and transferred to the preheated thermal cycler at 95°C, 95°C for 10 minutes, 60°C for 3.5 hours and 4°C holding. To degrade linear DNA, exonuclease treatment was performed after removing the capture reaction from the thermal cycler and spanned down and placed on ice box for cooling. To each tube, 2µl of exonuclease (0.8µl of water, 0.2µl of 10× ampligase buffer and 0.5µl of exonuclease I) was dispensed and spanned down before incubating at 37°C (hold), 37°C for 1 hour, 95°C for 2 minutes and 4°C hold.

3.7.4. Amplification of captured DNA

Captured DNA was amplified by performing PCR in a 24µl reaction constituting 5µl of the capture reaction product, 5µl of 5× Q5 reaction buffer, 0.5µl 10mM dNTP and 0.5µl of Q5 polymerase, 1.5µl of dual forward and reverse primers, 0.75µM of barcoded primer different for each sample, and 10.75µl of PCR master mix. The program was set at 98°C for 30 seconds, 22 cycles of 98°C for 10 second, 60°C for 30 seconds, 72°C for 30 seconds and 72°C for 2 minutes before holding at 4°C.

3.7.5. MIP clean-up, pooling and Illumina sequencing

Clean-up was undertaken to remove lower weight non-specific PCR products which are 200-300bp and below self-ligation products while retaining products above 500bp. The PCR products were then pooled together and clean-up performed using 0.65 AMPure XP beads (Beckman Coulter, Brea, CA) at room temperature. Briefly, 19.5µl of XP beads (0.65 of 30µl pooled probes volume) were transferred to 1.5mL of microcentrifuge tube and the pooled PCR products dispensed while gently pipetted up and down to mix. The mixture was then incubated for 10-15 minutes and placed over a magnet until the solution was clear. With the plate still on the magnet, the supernatant was removed and 200µl of freshly prepared 80% ethanol added till beads were covered. Incubation was performed for 30 seconds and ethanol removed and the

process repeated for two complete ethanol washes before the beads were allowed to dry for five minutes. The plate was subsequently removed from the magnet and DNA eluted with 32µl of TE buffer with low EDTA (0.15mM) by pipetting thoroughly 20-30 times and incubating at room temperature for 2-5 minutes before placing on the magnet to clear. Once clear, 30µl of the elute was removed carefully without disturbing the beads and subsequently transferred to clean tubes. To improve the amount of DNA, the second PCR was undertaken with 10µl of cleaned PCR products, 4.05µl of water, 5µl of 5× Q5 reaction buffer, 5µl of 5× MMC, 0.5µl of 10mM dNTP, 0.25µl of Q5 polymerase and 0.2µl each of P2/P3 primer mix (505µM each). The program was set at 98°C for 30 seconds, X cycles of 98°C for 10 second (X is defined using the real-time PCR based on the time of reaction plateaus), 60°C for 30 seconds, 72°C for 30 seconds and 72°C for 2 minutes before holding at 4°C. The cleaned pooled PCR product was subsequently sequenced on Illumina NextSeq 500 (Illumina, San Diego, California) based on the manufacturer's instructions.

3.7.6. MIP variation calling

MIP sequences obtained from the Illumina sequencing platform were aligned to the human genome reference (hg19) using the LastZ aligner (Miller Lab, Penn State University) to the expected sequence of the target gene based on the human reference genome hg19. Mismatches were then extracted from LastZ output and a VCF file created for all the samples, including the mismatches. Annotation of the VCF file was performed using the ANNOVAR software (Open Bioinformatics) and SNP filtered based on the criteria that a position with a valid call must have at least 10 reads within the position for different samples/MIP sets.

3.8. EBV Viral Load Determination

The determination of EBV viral load for cases and control DNA samples was done according to a previously developed protocol (Hayden *et al*., 2008). For EBV QPCR, previously validated primers and TaqMan® probes (Applied Biosystem, Foster City, CA, USA) (that detect a 70bp region of the EBV DNA polymerase (BALF5) gene (Kimura *et al*., 1999) were used. The first amplicon is designed to target and amplify a 76bp conserved sequence in the EBV EBNA-1 gene

while second amplicon amplifies a spiked internal control (IC- human beta actin gene), a housekeeping gene designed and used to prevent false negative results arising from inefficient DNA extraction or inhibition of PCR amplification. The probes used were synthesized by PE Applied Biosystem (Foster City, CA, USA) and the sequences are shown in Appendix 4. Briefly, the QPCR cycle was as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 42 cycles of 15 seconds at 95°C and 1-minute hold at 60°C using CFX Connect™ Real Time Detection System (Bio-Rad, Hercules, CA, USA). For all the reactions, IQ Supermix (BioRad laboratories, Hercules, CA) was used. For the purpose of generating the standard curve, two commercially available fluorometrically quantified EBV B95-8 DNA (Advanced Biotechnologies, Inc., Columbia, MA) were used in the creation of a six point tenfold serial dilution series ranging from 2.62 copies/µl to 2.62×10^6 copies/µl. For all samples, EBV viral load were determined in duplicate and a mean value determined as the DNA copy number. The extrapolation of standard curve consisting of serially diluted EBV DNA standards was used to calculate EBV viral copy.

3.9. Data Management and Statistical Analysis

Data generated was organized in Microsoft Excel spreadsheet and analysed using R statistical packages (R Core Team, Vienna, Austria). Data collected was categorized into case number, age, affection (case/control), sex, EBV viral load and genotypes for *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* (131HIS/ARG) and *IL-12* (+1188A/C). Test for deviation from Hardy-Weinberg Equilibrium (HWE) was undertaken using Pearson goodness-of-fit χ^2 test in Genetics Package of R. Allele and genotype frequency differences between cases and controls were displayed in contingency tables. 2-by-3 genotype count and 2-by-2 allele count contingency tables were used for genotype and allele frequencies respectively (Lewis and Knight, 2012). Departure from null hypothesis that cases and controls have the same distribution of the genotypes was analysed using the Fisher's exact test in R Genetics Package. Multivariate logistic regression was used to determine the association between *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* (HIS/ARG) & *IL-12* (+1188A/C) gene variants and eBL development while controlling for possible confounders in the SNPassoc Package of R (Gonzalez *et al*., 2007). Distribution of Epstein-Barr viral load for *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA*

(HIS/ARG) & *IL-12* (+1188A/C) genotypes was described using median (Interquartile ranges). Analysis of Variance (ANOVA) was used to determine differences between *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *FcγRIIA* (HIS/ARG) & *IL-12* (+1188A/C) gene variants and EBV viral load in cases and controls. Statistical significance was set at $P \leq 0.05$.

3.10. Ethical Consideration

Permission to carry out this study was obtained from the School of Graduate Studies (SGS) of Maseno University. Ethical approval was also obtained from the Scientific and Ethical Review Committee (SERU) at the Kenya Medical Research Institute (KEMRI), Kenya and the University of Massachusetts Medical School Ethical Review Board (Appendix 1). Clinical information for the parent study was obtained under the approval of SERU of the Kenya Medical Research Institute and written informed consent obtained from study participants (Appendix 5). All participants were also identified based on a unique ID that linked the samples with the clinical and demographic information without revealing personal identifiers such as name.

CHAPTER FOUR

RESULTS

4.1. Demographic, Clinical and Laboratory Characteristics of the Study Participants at Enrolment

A total of 113 confirmed eBL patients and 69 age and gender-matched controls from malaria endemic parts of western Kenya were enrolled in this study. Table 4.1 provides a summary of the demographic, laboratory and clinical characteristics of the study participants. The age, upper and lower age percentile for cases and controls was within the bracket of risk for eBL in western Kenya. Cases and controls differed significantly with regard to EBV viral load (p<0.001). Only 16% of eBL cases in this study were parasitemic by blood smear at admission Malaria serology examination of the samples captured the history of malaria exposure of both cases and controls based on the levels of anti-merozoite surface protein 1 (MSP-1) antibodies.

Table 4.1: Demographic, clinical and laboratory characteristics of the study participants

	eBL cases	Controls	*p*-value
No. of participants (n =182)	113	69	
Gender, n, (%)			
Male	75 (66.4%)	41 (59.4%)	
Female	38 (33.6%)	28 (40.6%)	
Age, years; median (IQR)	7 (5)	5 (4)	
Malaria status, n, (%)			
Pf. Positive	16 (14.2%)	55 (79.7%)	
Pf. Negative	97 (85.8%)	14 (20.3%)	
Anti-MSP-1 antibody; Median (IQR)	25,564 (20,105)	25,480 (15,392)	0.957[a]
Median EBV copies/ng DNA (IQR)	9.113 (55.775)	0.020 (0.238)	**<0.001[a]**

[a]Statistical significance determined by the Mann-Whitney U Test. All values in bold are statistically significant at $p<0.05$. Malaria status was at time of sample collection. EBV=Epstein Barr Virus; *P.f.=Plasmodium falciparum.*

4.2. Association between IFN-γ (+2109C/T), FcγRIIA (His/Arg), IL-12 (+1188A/C), TNF-α (-1031 T/C, -308 G/A, -376 G/A and -238 G/A) Genetic Variants and eBL Development among Children from Western Kenya

The entire study population (113 cases and 69 controls) was genotyped for *IL-12* +1188 A/C 3'UTR polymorphism using 5' allele discrimination assay (Table 4.5). However, the Molecular Inversion Probe (MIP) genotyping by sequencing method adopted for *IFN-γ* (+2109C/T), *TNF-α* (-1031 T/C, -308 G/A and -238 G/A) and FC-γRIIA (His/Arg) was successful within a subset of this study population as shown in Table 4.2-4.4.

The distribution of TNF-α (-1031 T/C, -308 G/A and -238 G/A) promoter genotypes among cases and controls was comparable (p=0.710, p=0.727 and p=0.499 respectively). Furthermore, no significant deviation from Hardy-Weinberg Equilibrium (HWE) was observed in the frequency of TNF-α (-1031 T/C, -308 G/A and -238 G/A) genotypes in cases (p=0.418, p=1 and p=0.585 respectively) and controls (p=0.449, p=1 and p=0.457 respectively). Additional multivariate logistic regression controlling for age and gender was done to determine association

between TNF-α (-1031 T/C, -308 G/A and -238 G/A) promoter polymorphism and susceptibility to eBL. As shown in table 4.2, there was no significant association between the TNF-α promoter variants and susceptibility to eBL among children from western Kenya. Further stratification of genotypes into alleles followed by Fisher's exact test showed significant difference in the distribution of TNF-α -376 alleles within eBL cases and controls. However, this can be attributed to the population bias favouring the wild type G over the A allele, a scenario that also made it impossible to conduct a further multivariate logistic regression analysis on TNF-α -376 G/A role on eBL pathogenesis.

Table 4.2: Genotype and allele frequencies of the TNF-α -1031 T/C, -308 G/A, -376 G/A and -238 G/A promoter polymorphism within the study population

Cytokine Genotypes	EBL Cases	Controls	OR (95% CI)	P-Value
***TNF-α* -1031T/C**				0.710[a]
TT	40 (54.8%)	15 (65.2%)	1.000 (reference)	
TC	26 (35.6%)	7 (30.4%)	1.01 (0.27-3.69)	0.452[b]
CC	7 (9.6%)	1 (4.3%)	1.20 (0.12-12.46)	0.344[b]
TT vs. TC + CC			1.04 (0.31-3.53)	0.950[b]
CC vs. TC + TT			1.20 (0.12-11.82)	0.874[b]
TC vs. TT + CC			0.98 (0.28-3.51)	0.979
Alleles				
T	106 (72.6%)	37 (80.4%)		0.336[a]
C	40 (27.4%)	9 (19.6)		
TNF-α* -308G/*A				0.727[a]
GG	62 (84.9%)	21 (91.3%)	1.000 (reference)	
AG	11 (15.1%)	2 (8.7%)	5.84 (0.65-52.48)	0.092[b]
AA	0 (0.0)	0 (0.0)	-	
Allele				
A	135 (92.5%)	44 (95.7%)		0.737[a]
G	11 (7.5%)	2 (4.3%)		
***TNF-α* -376G/A**				0.061[a]
GG	60 (82.2%)	23 (100%)		
GA	12 (16.4%)	0 (0.0)	-	-
AA	1 (1.4%)	0 (0.0)	-	-
Allele				
G	132 (90.4%)	46 (100%)		**0.024**[a]
A	14 (9.6%)	0 (0.0)		
***TNF-α* -238G/A**				0.499[a]
GG	58 (79.5%)	21 (91.3%)	1.000 (reference)	
GA	14 (19.2)	2 (8.7%)	2.10 (0.42-10.58)	0.992[b]
AA	1 (1.4%)	0 (0.0)	-	-
Allele				
G	130 (89.0%)	44 (95.7%)		0.250[a]
A	16 (11.0%)	2 (4.3%)		

Genotype and allele frequency test were done using both Chi-square test and multivariate logistic regression. [a] Statistical significance was determined using the Fisher's exact test. [b] Multivariate logistic regression analysis was used in determining statistical significance. Multivariate logistic regression was used in determining Odds ratios (OR) at 95% confidence intervals (CI) while controlling for age and gender with the wildtype genotypes and alleles as references. Difference in the *TNF-α* -376G/A allele frequency (p=0.024) was seen, though attributed to population bias favouring the wildtype alleles and genotypes.

Difference in the frequency of Fc-γRIIA (His/Arg) genotypes in eBL cases and controls was not significant (p=0.806). No significant deviation from HWE in the genotype frequency in cases (0.701) and controls (1) was also witnessed. Further multivariate logistic regression controlling for age and gender as potential confounders showed no significant association between Fc-γRIIA (His/Arg) genotypes and susceptibility to eBL. Although heterozygous (His/Arg) individuals for FcγRIIA His/Arg polymorphism had a 2.235 odd of developing eBL relative to homozygous wildtype reference group, the results were not statistically significant (p=0.176). Additional dominant multivariate logistic regression analysis (Table 4.3) also demonstrated that carriage of the Arg allele (His/Arg + Arg/Arg) in FcγRIIA was associated with double risk for developing eBL though the findings were not statistically significant (p=0.293, OR=2.09). An overdominant analysis of His/Arg vs. Arg/Arg + His/His showed 3.25 odds of developing eBL among children with His/Arg genotype and the statistical significance was at borderline (p=0.052). Further Fisher's exact test shows no significant difference in the distribution of His and Arg alleles in eBL cases and control groups (0.874) (Table 4.3).

Table 4.3: Genotype and allele frequencies of FcγRIIA 131His/Arg polymorphism within the study population

Cytokine Genotypes	EBL Cases	Controls	OR (95% CI)	P-Value
***FcγRIIA* 131His/Arg**				0.806[a]
His/His	21 (25.3%)	8 (30.8%)	1.000 (reference)	
His/Arg	41 (50.6%	11 (42.3%)	2.24 (0.697-7.174)	0.176[b]
Arg/Arg	21 (25.3%)	7 (26.9%)	1.35 (0.372-4.928)	0.645[b]
His/His *vs.* His/Arg + Arg/Arg			2.09 (0.53-8.20)	0.293[b]
Arg/Arg *vs.* His/Arg + His/His			0.46 (0.13-1.66)	0.239[b]
His/Arg *vs.* His/His + Arg/Arg			3.25 (0.94-11.18)	0.052[b]
Allele				
His	83 (50%)	27 (52.9%)		0.874[a]
Arg	83 (50%)	25 (48.1%)		

[a] Statistical significance was determined using the Fisher's exact test. [b] Multivariate logistic regression analysis used in determining statistical significance. Multivariate logistic regression was used in determining Odds ratios (OR) at 95% confidence intervals (CI) while controlling for age and gender.

The distribution of IFN-γ +2109C/T genotypes was skewed with no IFN-γ $_{+2109}$CC genotypes recorded within the study population. No significant difference in the distribution of

IFN-γ $_{+2109}$TT and IFN-γ $_{+2109}$CT genotypes was observed (p=0.730). Additional multivariate logistic regression also showed no significant risk between IFN-γ +2109C/T genotypes and risk of eBL development after controlling for age and gender (p=0.356). Further Fisher's exact test after allele stratification also showed no significant difference in the distribution within cases and controls (Table 4.4).

Table 4.4: Genotype and allele frequencies of IFN-γ +2109C/T polymorphism in study participants

Cytokine Genotypes	eBL Cases	Controls	OR (95% CI)	P-Value
***IFN-γ* +2109C/T**				0.730[a]
TT	71 (86.6%)	24 (92.3%)	1.000 (reference)	
CT	11 (13.4)	2 (7.7%)	2.33 (0.31-17.34)	0.356[b]
CC	0 (0.0)	0 (0.0)	-	-
Allele				
T	153 (93.3%)	50 (96.2%)		0.738[a]
C	11 (6.7%)	2 (3.8%)		

[a] Statistical significance was determined using the Fisher's exact test. [b] Multivariate logistic regression analysis used in determining statistical significance. Multivariate logistic regression was used in determining Odds ratios (OR) and 95% confidence intervals (CI) while controlling for age and gender.

The distribution of IL-12 +1188A/C genotypes in eBL cases and controls was comparable (*p*=0.327) and no deviation from HWE was witnessed in cases (*p*=0.853) and controls (*p*=0.427) within this study population. Additional multivariate logistic regression controlling for age and gender as potential confounders was also insignificant. Further Fisher's exact test after allele stratification also showed no significant difference in their distribution in eBL cases and controls (Table 4.5).

Table 4.5: Genotype and allele frequencies of IL-12 +1188A/C polymorphism within study population

Cytokine Genotypes	eBL cases	Controls	OR (95% CI)	P-value
***IL-12* +1188 A/C**				0.327[a]
AA	41 (36.3%)	26 (34.7%)	1.000 (reference)	
AC	58 (51.3%)	34 (45.3%)	1.446 (0.705-2.967)	0.314[b]
CC	14 (12.4%)	15 (20%)	0.771 (0.300-1.977)	0.588[b]
AA *vs.* AC + CC			1.22 (0.63-2.39)	0.874[b]
CC *vs.* AC + AA			0.63 (0.27-1.47)	0.328[b]
AC *vs.* AA + CC			1.44 (0.76 -2.70)	0.260[b]
Allele				
A	140 (61.9%)		80 (57.9%)	0.508[a]
C	86 (38.1%)		58 (42.1%)	

[a]Statistical significance was determined using the Fisher's exact test. [b]Multivariate logistic regression analysis used in determining statistical significance. Multivariate logistic regression was used in determining Odds ratios (OR) and 95% confidence intervals (CI) while controlling for age and gender. The analysis considered the homozygous wild-type genotypes and alleles as the reference groups. The SNPassoc Package of R was used in multivariate logistic regression while Genetics Package was used in the determination of Hardy-Weinberg Equilibrium (González *et al*., 2007)(González *et al*., 2007).

4.3. Association Between TNF-α (-1031 T/C, -376G/A and -238G/A) Haplotypes and Risk of eBL Development among Children from Western Kenya

To further understand the role of *TNF-α* promoter polymorphisms and susceptibility to eBL among children from western Kenya, haplotypes for *TNF-α* (-1031 T/C, -308G/A and -238G/A) genotypes. Likelihood inference of eBL trait with haplotypes for *TNF-α* was determined through logistic regression after adjusting for the effect of age and gender. Four possible haplotypes generated from the analysis were CGA, CGG, TAG and TGG with a frequency of 9.7%, 15.3%, 6.2% and 68.5% respectively. To avoid convergence problems arising from low haplotype frequencies, the threshold was set at 0.08 thus pooling haplotypes with less than 8% frequency into a single category. In the logistic regression, TGG haplotype was used as the reference as it is a combination of the wild type alleles. From the analysis, there was no significant association between *TNF-α* haplotypes and risk of developing eBL among children from western Kenya (-1031/-376/-238 CGG, p = 0.666, CGA p = 0.120 and pooled,

p=0.064). Likelihood ratio tests for eBL trait association with haplotypes of participants determined by analysis of variance of full and reduced logistic regression models were not statistically significant (*p*=0.325) as shown in Table 4.6.

Table 4.6: TNF-α (-1031 T/C, -376G/A and -238G/A) haplotypes and risk of eBL development among children from western Kenya

Haplotype (-1031/-376/-238)	Frequency	*p*-value
TGG	68.5%	1.000 (reference)
CGG	15.3%	0.666[a]
CGA	9.7%	0.120[a]
Pooled	6.5%	0.064[a]
Likelihood ratio test		0.325[b]

Affection, age and gender were considered as covariates in the Hapassoc modelling. [a]Logistic regression controlling for age and gender was done to determine the association of different haplotypes with risk of eBL development with TGG haplotype as the reference. The global hypothesis test that *TNF-α* haplotypes increase risk of eBL development was done through likelihood ratio test. Likelihood inference of eBL trait among children from western Kenya given a specific *TNF-α* haplotype was determined by [b]ANOVA comparison of full and reduces logistic regression models from Hapassoc (Burkett *et al*., 2015).

Table 4.7: Association between IFN-γ +2109C/T (rs1861494), TNF-α -1031 T/C (rs1799964), -308G/A (rs1800629), -376G<A (rs1800750), -238G/A (rs361525), FcγRIIA His/Arg (rs1801274) and IL-12 +1188A/C polymorphisms and mean log-transformed EBV load

SNPs	Mean (EBV copies/ng DNA)	*p*-value
***TNF-α* -1031T/C**		
TT	1.595	
CT	1.698	0.147[a]
CC	4.795	
***TNF-α* -308G/A**		
GG	2.174	
AG	0.085	0.123[a]
AA	-	
***TNF-α* -376G/A**		
GG	1.771	
AG	2.254	0.307[a]
AA	-	
***TNF-α* -238G/A**		
GG	1.662	
GA	2.649	0.234[a]
AA	-	
FcγRIIA His/Arg		
His/His	2.057	
His/Arg	2.260	0.708[a]
Arg/Arg	1.381	
***IFN-γ* +2109C/T**		
CC	-	
CT	0.760	0.311[a]
TT	2.116	
***IL-12* +1188 A/C**		
AA	0.930	
AC	1.081	0.838[a]
CC	0.524	

The EBV viral loads are represented as means. [a] One-Way ANOVA test, with values being statistically significant at $p \leq 0.05$.

CHAPTER FIVE

DISCUSSION

5.1. General Introduction

Efforts to understand the pathogenesis of eBL have recently focused on the identification of other genetic factors that may augment the *MYC* translocation including transcription factor binding site mutations, and cytokine polymorphisms among other human genetic variants (Love *et al*., 2012; Mulama *et al*., 2014; Oduor *et al*., 2014; Richter *et al*., 2012). In this study, the role of genetic variants in *TNF-α* promoter region, *IFN-γ* (+2109C/T), exon H131R of FcγRIIA, *IL-12B* (+1188A/C) and susceptibility to eBL development were investigated. Through the evaluation of 113 eBL cases and 69 aged- and gender-matched controls, no significant association between *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A), *IFN-γ* (+2109C/T), exon 131 of FcγRIIA (His/Arg) and *IL-12B* (+1188A/C) was found with the susceptibility to eBL. Additional comparison of mean EBV viral load and *TNF-α* (-1031 T<C, -308G/A, -376G/A, -238G/A), third intron of *IFN-γ* (+2109C/T), exon 131 of FcγRIIA (His/Arg) and *IL-12B* (+1188A/C) genotypes was not significantly different across the groups. Furthermore, there was no significant association between *TNF-α* (-1031 T/C, -376G/A, -238G/A) haplotypes and likelihood of developing eBL among children from western Kenya.

Children aged 2-14 years and living in areas with holoendemic malaria transmission of western Kenya are at an increased risk of developing eBL with a median age bracket of 5-8 years (Rainey *et al*., 2007). In this study, the median age for controls and cases was 5 and 7 years respectively, falling within the age range of high risk of developing eBL. Only 14.2% of the cases were parasitic by blood smear at admission as compared to 79.7% of the controls, a trend that has been witnessed for eBL cases at admission. History of malaria infection for cases and controls for this study was similar as they were all recruited from an area with similar infection intensities as was confirmed by serological results for MSP-1 antibody test. The median MSP-1 antibody levels for eBL cases and non-eBL controls were not significantly different, an indication of similarity in level of *Pf* malaria seropositivity and historical exposure and infection of study population. The use of MSP-1 serology has been demonstrated to be essential in

highlighting history of *Pf* malaria infection due to its longevity in the absence of effective antimalarial treatment, frequency or persistent sub-patent infection (Drakeley *et al*., 2005). Hence the huge variation on *Pf* malaria positivity between cases and controls could be attributed to antimalarial treatment in cases when they present to the clinics when they feel unwell. Endemic BL is associated with over 95% of EBV coinfection and high viral load (Piriou *et al*., 2012) as was confirmed by the significant difference in EBV viral load in cases and controls ($p<0.001$) for this study (Table 4.1).

The current study used the distribution of IL-12B +1188A/C minor allele frequency within the study population as was reported by Ong'echa *et al*., (2011) to determine the sample size for evaluating risks of polymorphic variations within the selected cytokine genes. While the study used participants recruited from the former Siaya district which had over 90% of the samples sourced from two divisions of Karemo and Boro, the current study collected eBL cases from different parts of Luo Nyanza and are therefore more genetically diverse. Nonetheless, the lack of association reported here is consistent with past studies that have reported lack of protection against eBL among children with sickle cell trait and functional polymorphisms in IL-10 and IL-6 cytokines (Mulama *et al*., 2014; Oduor *et al*., 2014), studies that represent emerging efforts to identify other genetic variants that contribute to eBL pathogenesis in addition to MYC translocation. It also reflects the findings of (Ndede *et al*., 2017) who reported no significant difference in the circulating levels of IFN-γ and TNF-α cytokines in eBL cases and matched controls.

5.0. Polymorphism in Selected Cytokines and Risk of eBL Development

High frequency of eBL has been reported within the 'lymphoma belt', a region with holoendemic malaria exposure and early primary EBV infection in children (Rainey *et al*., 2007). However, the frequency of occurrence of eBL is still not uniform, an indication of the presence of other genetic factors that may play a role in the pathogenesis of the B-cell lymphoma. Secretion of TNF, a pro-inflammatory cytokine involved in the regulation of different biological activities, is mediated by the activation of transcription factor NF- κβ and mitogen activated protein kinases (MAPKs) (Qidwai and Khan, 2011). Factors that result into impaired levels of this pleiotropic cytokine may contribute to pathogenesis of infectious diseases and

lymphoproliferative malignancies (Qidwai and Khan, 2011). *In vitro* studies have identified TNF as an inducer of proliferation of malignant B-cells (Digel *et al*., 1989). High levels of TNF-α cytokine have been reported in lymphoproliferative disorders such as myeloma, chronic lymphocytic leukaemia and hairy cell leukaemia (Digel *et al*., 1989; Salles *et al*., 1996). Elevated levels of TNF may therefore play a role in the pathogenesis and maintenance of malignant clones in these B-cell disorders.

Polymorphisms in the promoter region of *TNF-α* have been attributed to change in the transcriptional levels of the cytokine. Gene polymorphism was identified as an independent predictor for outcome in non-Hodgkin lymphoma patients (Osman *et al*., 2012). Based on these findings, this study hypothesized that polymorphisms on the promoter region of TNF-α cytokine that affect the transcription levels may play a role in eBL development by inducing malignant B-cell clone proliferation and maintenance. Possible association between *TNF-α* (-1031 T/C, -308G/A, -376G/A, -238G/A) promoter polymorphism and susceptibility to different B-cell and EBV associated lymphomas within Caucasian and Mexican population have been reported (McAulay *et al*., 2009; Torres-Espíndola *et al*., 2014) . The *TNF-α* -1031C allele was associated with risk of developing PTLD in a study population from the United Kingdom (McAulay *et al*., 2009). The results further highlight the possibility of this SNP being used as a predictive risk factor for the development of PTLD (McAulay *et al*., 2009). In this study, there was no significant association with the presence of the CC genotype or allele. The G to A substitution at -376 in the promoter region attributed to change in the binding site for Oct-1 transcription factor, has also been associated with increased risk of developing Hodgkin lymphoma in a Mexican population(Torres-Espíndola *et al*., 2014). In this study, a population bias towards the wildtype genotype GG was noted and the low cytokine producing allele was only reported in one eBL case. The skewed distribution of the genotypes made it impossible to further model a logistic regression and determines the association with eBL development given specific genotypes. Targeting more eBL cases may help in effectively mapping out the role of this polymorphism in increasing susceptibility to eBL within this population.

Presence of high producer -308A allele in the TNF promoter region has been attributed to negative prognostic factors such as high tumour load, CNS disease and Methotrexate infusion

time in paediatric sporadic Burkitt lymphoma and B-cell acute lymphoblastic leukaemia (B-ALL) population (Seidemann *et al*., 2005). In the current study, the TNF-α 308A high cytokine producing allele was not observed in both cases and controls and the high OR and a trend (p=5.84 and 0.092, respectively) towards heterozygote genotype can be attributed to population bias preferential for the G over the A allele. The 95% CI for the heterozygote genotype also included the value of null effect, a further confirmation of no significant impact of this genotype on eBL susceptibility within this population.

Polymorphic variation on -238 characterized with substitution of G to A has also been associated with change in the transcriptional levels of the cytokine (El-Tahan *et al*., 2016). While studies on breast cancer and hepatocellular carcinoma (HCC) have shown lack of association between this polymorphism and the cancers (Hu *et al*., 2014; Zhang *et al*., 2017), this study hypothesized that the genetic variant that leads to change in transcriptional levels of the pro-inflammatory cytokine can still be functional in eBL. Just like with the two studies on breast cancer and HCC, there was no significant association between this promoter polymorphism and association with eBL development. Though past studies have indicated a possible role of TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A) promoter polymorphism in increased susceptibility to different B-cell and EBV associated lymphomas (Canedo *et al*., 2008; Judson *et al*., 2000), the current study among eBL patients found no association. This is the first study to evaluate TNF promoter polymorphisms that influence levels of the cytokine and role in the development of eBL among children exposed to high *Pf* malaria.

In this study, it was hypothesized that the IL-12 +1188A/C polymorphism that affect the transcriptional levels of the cytokine may influence Th-1 immune responses to tumour cells, thus increasing susceptibility to eBL development. The presence of CC genotype in the current study had no significant association with eBL development and the difference in distribution of the A and C allele in cases and controls was also insignificant. While those with the AC genotype had a 45% risk of having eBL, the difference was not statistically significant and the 95% CI intervals include the value of no effect. Therefore, IL-12 +1188A/C polymorphism that may affect levels of the cytokine and impair Th-1 immune responses characterized by interruption in the levels of IFN-γ and TNF- α immune responses to tumour cells does not increase risk of eBL development

among children from malaria endemic regions.

Interferon-γ is crucial in the promotion of innate and adaptive immune responses (Ikeda *et al*., 2002). In tumour immune responses, the cytokine has been associated with immunosurveillance through lymphocyte mediated response, inhibition of angiogenesis and proliferating cancer cells and direct action on tumours (Ikeda *et al*., 2002). *In vitro* study with ovarian cancer cells have also shown that IFN-γ inhibit tumour growth through the induction of apoptosis (Wall *et al*., 2003). Polymorphic variations within transcription factor binding sites of the *IFN-γ* gene have been identified and associated with impaired transcriptional levels of the cytokine (Pravica *et al*., 1999), which affects Th1 immune responses to tumour mediated by this cytokine in collaboration with other Th1 cytokines. A polymorphic substitution from C to T at position +2109 on the third intron affects the levels of the cytokine by interrupting transcription factor binding sites and may impair IFN-γ tumour immunosurveillance (Chevillard *et al*., 2003). The current study hypothesized that polymorphic variation within *IFN-γ* +2109C/T which can affect the transcriptional levels of the cytokine may impair immune response to tumour cells and increase susceptibility to eBL among children from areas with endemic malaria transmission. However, no high cytokine producing CC genotype was found within this study population and there was no significant difference in the distribution of C and T alleles in cases and controls. While the heterozygote CT genotype had an OR of 2.33, it had no significant impact on risk of eBL development and the 95% CI included the value of null effect. Thus, *IFN-γ* +2109C/T polymorphism previously associated with impaired levels of *IFN-γ* cytokine does not increase susceptibility to eBL development among children.

The mediation of phagocytic function of Fcγ-RIIA has been associated with several haplotypes (de Haas, 2001) and this has been reported in human cancers. Polymorphic change from G to A at codon 131 of the gene leads to change in amino acid from 131-Histidine to 131-Arginine. Change in genotype at this codon has been associated with low affinity for IgG2 and therefore impaired phagocytic roles of monocytes and macrophages. The presence of 131-Arg was reported in EBV positive B-cell and non-Hodgkin lymphoma patients (Diamantopoulos *et al*., 2013a; Ghesquières *et al*., 2013). The presence of the low IgG2 affinity genotype was also associated with high EBV load possibly because of poor phagocytic activity of antigen

presenting cells. Based on the reported functional association between this polymorphism and EBV-positive B-cell lymphoma, this study hypothesized that polymorphic variations within Fcγ-RIIA gene could affect immune response to EBV, leading to chronic presence of the virus in children exposed to persistent malaria infection and subsequently increasing chances of eBL development. Individuals with His/Arg genotype had a two-fold odd of having eBL within this study population (OR=2.24) though the difference was not significant (*P*=0.176). However, the 95% CI (0.597-7.174) included the value of no effect and was widely spread, an indication of low power of association and affecting its precision in defining potential risk of this genotype within this population. The current study found that polymorphism on Fcγ-RIIA gene that affects affinity for IgG2 does not significantly increase risk for developing eBL among children exposed to persistent malaria infection and early primary EBV exposure.

5.1. Tumour Necrosis Factor Promoter Haplotype and Risk of eBL Development

The incorporation of SNP-based haplotypes provides a more powerful test of genetic association as compared to individual SNPs especially when the causal genetic variants have not been typed (Lin *et al*., 2005). In tumour microenvironment, TNF-α cytokine mediates inflammations and host defences against tumour cells. High levels of the cytokine have also been associated with chronic inflammation, autoimmunity and malignant transformation, leading to cancer progression (Balkwill, 2006). Therefore, high TNF-α cytokine haplotypes can mediate tumoral immune responses, leading to susceptibility to eBL. The TNF-$\alpha_{-1031/-308/-238}$ TGA haplotype was associated with increased risk of developing hepatocellular carcinoma (HCC) in an Asian study. In the current study, no TGA haplotypic combination was found within the study population. The only haplotype combination with the -238A allele was CGA with a frequency of 9.7%. Thus, TNF-α -1031/-376/-238 haplotype combinations do not increase risk of developing eBL among children from western Kenya.

5.2. Selected Cytokine Polymorphism and EBV Viral Load

Pro-inflammatory cytokines play a critical role in human immune responses to *Pf* malaria infection but has also been attributed to immunopathology and severity of the condition

(Boström *et al*., 2012). An early production of pro-inflammatory cytokines, especially IFN-γ is essential in mounting resistance against *Pf* malaria infection and protection against clinical symptoms (Torres-Espíndola *et al*., 2014). Furthermore, TNF and IL-12 cytokines have also been shown to inhibit parasite growth and enhance phagocytic processes necessary for clearance of parasitized erythrocyte (Artavanis-Tsakonas and Riley, 2002; Torre *et al*., 2002). In this study, it was hypothesized that individual variation in the levels of IL-12, IFN-γ and TNF-α because of polymorphic variations can affect immune response to *Pf* malaria, leading to dysregulated expression of AID and high EBV viral load, further potentiating risk of eBL among children from western Kenya.

Past studies have associated TNF promoter polymorphism with increased rate of *Pf* malaria infection. In a pre-school children study in Tanzania, individuals homozygous for $TNF_{-1031}C$ allele were associated with high malaria rates while $TNF_{-308}A$ allele had decreased rates of infection (Gichohi-Wainaina *et al*., 2015). While impaired pro-inflammatory cytokine production because of genetic variants may affect immune response to *Pf* malaria and increase likelihood of AID activation and high EBV viral load, functional TNF-α (-1031T/C and -308G/A) polymorphisms associated with higher risk to malaria which can subsequently lead to EBV reactivation and polyclonal expansion of EBV infected B cells showed no differences in mean viral load in the current study. The TNF_{-308A} genotype, previously associated with decreased risk of malaria infection was not present within the study population. While the mean EBV viral load for the $TNF_{-1031}C$ was high compared to other genotypes, the difference was not statistically significant.

In response to *Pf* malaria infection, activated macrophages are also able to produce IL-12 in addition to other pro-inflammatory cytokines such as IL-18 and TNF-α (Artavanis-Tsakonas and Riley, 2002). Early production of IL-12 is crucial for the activation of T cells by maintaining the Th1 phenotype and priming NK cells, CD4+ and CD8+ T-cells towards IFN-γ producing cells, leading to production of IFN-γ which has been associated with clearance of blood stage malaria infection (Malaguarnera *et al*., 2002). During innate immune responses to malaria, NK-cells also require accessory signals from IL-12 and IL-18 for the optimal production of IFN-γ (Malaguarnera *et al*., 2002). Production of IL-12 cytokine influences the quantity and

quality of antibody responses to blood stage malaria infection (Su and Stevenson, 2000). Low levels of IL-12 cytokine can influence immune responses to viral and parasitic infections, affecting EBV reactivation status and increasing risk of related lymphoma development. Polymorphic variation in the 3'UTR region of IL-12B gene has been associated with change in the transcriptional levels of the cytokine (Seegers *et al*., 2002). High IL-12 cytokine producer genotypes have also been associated with increased risk of severe malaria anaemia in a Kenyan population exposed to repetitive malaria infection (Ong'echa *et al*., 2011). Thus, this study hypothesized that polymorphic variations in the 3'UTR region of *IL-12B* gene that affect the levels of the cytokine can impair T-cells activation and NK cell signalling leading to lower production of IFN-γ. Subsequently, this impairs immune response against blood stage *Pf* malaria, thereby influencing age at primary EBV infection, viral load and risk of eBL development among children from areas with holoendemic malaria transmission. The mean viral load for IL-12B genotypes in both cases and controls were comparable, an indication that polymorphic variations within the cytokine that affect levels may not directly affect immune responses to EBV within this study population.

Age at primary EBV infection has also been attributed to poor viral control by host immune system, thus increasing risk of eBL development (de-Thé *et al*., 1978). Children living in malaria holoendemic regions of western Kenya experience primary EBV infection earlier in life compared to those from areas with low malaria transmission (Piriou *et al*., 2012). Furthermore, younger age at time of primary infection has been associated with frequent detection of EBV and higher viral load over time (Piriou *et al*., 2012). High antibody titters against EBV VCA in children infected early with EBV was also correlated with a higher risk of developing eBL (de-Thé *et al*., 1975). Cell-mediated immune response, characterized by elevated levels of activated CD8+ cytotoxic T cells, natural killer (NK) cells and CD4+ T-cells has been observed during primary EBV infection (Snider *et al*., 2012). In this study, a polymorphic variation +2109C/T in the third intron of *INF-γ* gene previously associated with change in cytokine levels was evaluated for impaired immune response to EBV. The mean viral load in the different IFN-γ genotypes was not significantly different. The high producing IFNγ+$_{2109}$CC genotype was also absent in the individuals genotyped in the current study. Hence,

low IFN-γ responses to EBV among children from areas with holoendemic malaria transmission as has been reported in past immunological studies are not because of *IFN-γ* +2109A/G polymorphism.

The infection of B-cells by EBV and the latency of the infection have also been associated with polymorphisms within FcγRIIA gene. Fragment, crystallizable receptors, transmembrane proteins expressed on the surface of immune cells, activate macrophages and cytotoxic cells, thus enhancing antibody mediated phagocytosis and ADCC (Diamantopoulos *et al*., 2013). Polymorphism in FcγRIIA has been attributed to impaired immune responses due to loss of phagocytic function of granulocytes. Polymorphism at position 131 of the exon has been attributed to substitution of G (CGT) to A (CAT) at this position which are codons for arginine and histidine respectively. Arginine at position 131 is associated with lower affinity for IgG2 as compared to histidine-131 (Reilly *et al*., 1994). In low grade B-cell lymphomas, individuals with R-131 allele presented with high viral load and chronic presence of viral DNA in the host genome (Diamantopoulos *et al*., 2013). Moreover, R131 was also correlated with the expression of LMP1, the main oncoprotein expressed during latency of EBV (Diamantopoulos *et al*., 2013). Thus, a polymorphism that affects the affinity of the receptor for IgG may affect phagocytic properties and production of viral antigens, allowing EBV to manifest its latency programs. Based on the observations, this study hypothesized that genetic variations on FcγRIIA that affects affinity to IgG and phagocytic properties of the granulocytes may increase LMP1 expression and chronic presence of EBV, thus increasing risk of eBL among children exposed to repetitive malaria infection. The mean EBV viral load was not significantly different within the FcγRIIA genotypes in this study, an indication that H131R polymorphism may not influence immune response to EBV in children from western Kenya.

5.3. Study Limitations

The strength of genetic association studies is based on the ability to match cases and controls based on exposure to risk factors attributed to the condition. However, the study was unable to use similar number of cases and controls and the sample size was also not achieved to reflect the general distribution of the genotypes within this population. The sample size could not therefore,

acquire the expected power to conclusively demonstrate lack or presence of association between the polymorphism and eBL development. Matching of the cases and controls based on age, gender, exposure to malaria, age at primary EBV or even tribe was also not perfectly possible, creating potential confounders not included in the regression analysis of association.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary

In this study, TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A) promoter genotypes and haplotypes, third intron of IFN-γ (+2109C/T), exon 131 of FcγRIIA (His/Arg) and 3'UTR region on IL-12B (+1188A/C) genotypes were evaluated for evidence of possible role in the development of eBL among children living in malaria endemic region of western Kenya. While no significant association between the selected polymorphic variations and risk of eBL development or high EBV viral load was reported, OR greater than 1 was seen in some instances. However, the wide 95% CI in all these instances which also included the value of null effect may imply the low precision of the OR.

6.0. Conclusions

i. Polymorphisms in IFN-γ (+2109C/T), TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A), FcγRIIA H/R131 and IL-12B +1188A/C genes which affect the transcriptional levels of the cytokines and affinity of FcγRIIA to IgG are not directly associated with risk of developing eBL in children from western Kenya.

ii. Tumour necrosis factor-α TNF-α -1031/-376/-238 haplotypes have no association with risk to eBL development in children from western Kenya.

iii. Polymorphic variations in IFN-γ (+2109C/T), TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A), FcγRIIA H/R131 and IL-12B +1188A/C genes have no association with EBV viral load in children from western Kenya.

6.1. Recommendations from this Study

i. Studies designed to understand the pathogenic mechanisms of eBL and susceptibility should focus on other genetic variants aside from IFN-γ (+2109C/T), TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A), FcγRIIA H/R131 and IL-12B +1188A/C polymorphisms as they do not provide significant genetic leads to follow especially within the malaria

endemic parts of western Kenya.

ii. Efforts to understand the differences in EBV load among children in malaria endemic parts of western Kenya should focus on other polymorphic variations aside from IFN-γ (+2109C/T), TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A), FcγRIIA H/R131 and IL-12B +1188A/C genes as these not explain differences in EBV load among children living malaria endemic parts of western Kenya.

iii. Modelling of TNF-α promoter haplotype in determining possible role in increasing susceptibility to eBL should consider other loci as the three combinations do not increase risk of eBL development among children from malaria endemic parts of western Kenya and additional combinations should be considered.

6.2. Recommendations for Future Studies

i. Determination of the impact of IFN-γ (+2109C/T), TNF-α (-1031 T/C, -308G/A, -376G/A, -238G/A), and IL-12B +1188A/C polymorphisms and the systemic and local expression of the cytokines is important in deciphering their role in modulating immune response to EBV, Pf malaria and reducing risk of eBL development.

ii. Determination of the impact of FcγRIIA H/R131 polymorphism on the expression of LMP-1 protein in eBL cases and matched EBV-positive controls will also be critical in understanding the role of the genetic variant in modulating immune response to EBV and shaping risk to eBL development.

iii. Additional studies should focus on identifying EBV genetic variations that may contribute to differences in risk of eBL development by impairing host immune response to the virus through whole genome sequencing analysis.

REFERENCES

Angiolillo, A.L., Sgadari, C., Tosato, G., 1996. A Role for the Interferon-Inducible Protein 10 in Inhibition of Angiogenesis by Interleukin-12. Ann. N. Y. Acad. Sci. 795, 158–167.

Artavanis-Tsakonas, K., Riley, E.M., 2002. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes. J. Immunol. Baltim. Md 1950 169, 2956–2963.

Athie-Morales, V., Smits, H.H., Cantrell, D.A., Hilkens, C.M.U., 2004. Sustained IL-12 Signaling Is Required for Th1 Development. J. Immunol. 172, 61–69.

Balkwill, F., 2006. TNF-α in promotion and progression of cancer. Cancer Metastasis Rev. 25, 409–416.

Ben Chaaben, A., Busson, M., Douik, H., Boukouaci, W., Mamoghli, T., Chaouch, L., Harzallah, L., Dorra, S., Fortier, C., Ghanem, A., Charron, D., Krishnamoorthy, R., Guemira, F., Tamouza, R., 2011. Association of IL-12p40 +1188 A/C polymorphism with nasopharyngeal cancer risk and tumor extension. Tissue Antigens 78, 148–151.

Bhatia, K., Huppi, K., Spangler, G., Siwarski, D., Iyer, R., Magrath, I., 1993. Point mutations in the c–Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. Nat. Genet. 5, 56–61.

Blum, K.A., 2004. Adult Burkitt leukemia and lymphoma. Blood 104, 3009–3020.

Borecký, L., 1992. Cytokines in health and disease. World J. Microbiol. Biotechnol. 8, 54–57.

Bornkamm, G.W., 2009. Epstein-Barr virus and the pathogenesis of Burkitt's lymphoma: more questions than answers. Int. J. Cancer J. Int. Cancer 124, 1745–1755.

Boström, S., Giusti, P., Arama, C., Persson, J.-O., Dara, V., Traore, B., Dolo, A., Doumbo, O., Troye-Blomberg, M., 2012. Changes in the levels of cytokines, chemokines and malaria-specific antibodies in response to Plasmodium falciparum infection in children living in sympatry in Mali. Malar. J. 11, 109.

Brady, G., MacArthur, G.J., Farrell, P.J., 2007. Epstein–Barr virus and Burkitt lymphoma. J. Clin. Pathol. 60, 1397–1402.

Braumüller, H., Wieder, T., Brenner, E., Aßmann, S., Hahn, M., Alkhaled, M., Schilbach, K., Essmann, F., Kneilling, M., Griessinger, C., Ranta, F., Ullrich, S., Mocikat, R.,

Braungart, K., Mehra, T., Fehrenbacher, B., Berdel, J., Niessner, H., Meier, F., van den Broek, M., Häring, H.-U., Handgretinger, R., Quintanilla-Martinez, L., Fend, F., Pesic, M., Bauer, J., Zender, L., Schaller, M., Schulze-Osthoff, K., Röcken, M., 2013. T-helper-1-cell cytokines drive cancer into senescence. Nature 494, 361–365.

Brooks, D.G., Qiu, W.Q., Luster, A.D., Ravetch, J.V., 1989. Structure and expression of human IgG FcRII(CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. J. Exp. Med. 170, 1369–1385.

Buckle, G., Maranda, L., Skiles, J., Ong'echa, J.M., Foley, J., Epstein, M., Vik, T.A., Schroeder, A., Lemberger, J., Rosmarin, A., Remick, S.C., Bailey, J.A., Vulule, J., Otieno, J.A., Moormann, A.M., 2016. Factors influencing survival among Kenyan children diagnosed with endemic Burkitt lymphoma between 2003 and 2011: A historical cohort study. Int. J. Cancer 139, 1231–1240.

Burke, F., Smith, P.D., Crompton, M.R., Upton, C., Balkwill, F.R., 1999. Cytotoxic response of ovarian cancer cell lines to IFN-gamma is associated with sustained induction of IRF-1 and p21 mRNA. Br. J. Cancer 80, 1236–1244.

Burkitt, D., 1963. A Children's Cancer With Geographical Limitations. Cancer Prog. 92, 102–113.

Burkitt, D., 1958. A sarcoma involving the jaws in African children. Br. J. Surg. 46, 218–223.

Canedo, Paulo, Durães, C., Pereira, F., Regalo, G., Lunet, N., Barros, H., Carneiro, F., Seruca, R., Rocha, J., Machado, J.C., 2008. Tumor necrosis factor alpha extended haplotypes and risk of gastric carcinoma. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 17, 2416–2420.

Canedo, P., Duraes, C., Pereira, F., Regalo, G., Lunet, N., Barros, H., Carneiro, F., Seruca, R., Rocha, J., Machado, J.C., 2008. Tumor Necrosis Factor Alpha Extended Haplotypes and Risk of Gastric Carcinoma. Cancer Epidemiol. Biomarkers Prev. 17, 2416–2420.

Cerhan, J.R., Liu-Mares, W., Fredericksen, Z.S., Novak, A.J., Cunningham, J.M., Kay, N.E., Dogan, A., Liebow, M., Wang, A.H., Call, T.G., Habermann, T.M., Ansell, S.M., Slager, S.L., 2008. Genetic variation in tumor necrosis factor and the nuclear factor-kappaB canonical pathway and risk of non-Hodgkin's lymphoma. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 17, 3161–3169.

Chen, X., Han, S., Wang, S., Zhou, X., Zhang, M., Dong, J., Shi, X., Qian, N., Wang, X., Wei, Q., Shen, H., Hu, Z., 2009. Interactions of IL-12A and IL-12B polymorphisms on the risk of cervical cancer in Chinese women. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 15, 400–405.

Chêne, A., Donati, D., Guerreiro-Cacais, A.O., Levitsky, V., Chen, Q., Falk, K.I., Orem, J., Kironde, F., Wahlgren, M., Bejarano, M.T., 2007. A molecular link between malaria and Epstein-Barr virus reactivation. PLoS Pathog. 3, e80.

Chevillard, C., Moukoko, C.E., Elwali, N.-E.M.A., Bream, J.H., Kouriba, B., Argiro, L., Rahoud, S., Mergani, A., Henri, S., Gaudart, J., Mohamed-Ali, Q., Young, H.A., Dessein, A.J., 2003. IFN-gamma polymorphisms (IFN-gamma +2109 and IFN-gamma +3810) are associated with severe hepatic fibrosis in human hepatic schistosomiasis (Schistosoma mansoni). J. Immunol. Baltim. Md 1950 171, 5596–5601.

Clark, M.R., Clarkson, S.B., Ory, P.A., Stollman, N., Goldstein, I.M., 1989. Molecular basis for a polymorphism involving Fc receptor II on human monocytes. J. Immunol. Baltim. Md 1950 143, 1731–1734.

D'Alfonso, S., Richiardi, P.M., 1994. A polymorphic variation in a putative regulation box of the TNFA promoter region. Immunogenetics 39, 150–154.

Dalldorf, G., Linsell, C.A., Barnhart, F.E., Martyn, R., 1964. An Epidemiologic Approach to the Lymphomas of African Children and Burkitt's Sarcoma of the Jaws. Perspect. Biol. Med. 7, 435–449.

Daud, I.I., Coleman, C.B., Smith, N.A., Ogolla, S., Simbiri, K., Bukusi, E.A., Ng'ang'a, Z.W., Sumba, P.O., Vulule, J., Ploutz-Snyder, R., Dent, A.E., Rochford, R., 2015. Breast Milk as a Potential Source of Epstein-Barr Virus Transmission Among Infants Living in a Malaria-Endemic Region of Kenya. J. Infect. Dis. 212, 1735–1742.

Dave, S.S., Fu, K., Wright, G.W., Lam, L.T., Kluin, P., Boerma, E.-J., Greiner, T.C., Weisenburger, D.D., Rosenwald, A., Ott, G., Müller-Hermelink, H.-K., Gascoyne, R.D., Delabie, J., Rimsza, L.M., Braziel, R.M., Grogan, T.M., Campo, E., Jaffe, E.S., Dave, B.J., Sanger, W., Bast, M., Vose, J.M., Armitage, J.O., Connors, J.M., Smeland, E.B., Kvaloy, S., Holte, H., Fisher, R.I., Miller, T.P., Montserrat, E., Wilson, W.H., Bahl, M., Zhao, H., Yang, L., Powell, J., Simon, R., Chan, W.C., Staudt, L.M., 2006. Molecular

Diagnosis of Burkitt's Lymphoma. N. Engl. J. Med. 354, 2431–2442.

de Haas, M., 2001. IgG-Fc receptors and the clinical relevance of their polymorphisms. Wien. Klin. Wochenschr. 113, 825–831.

de-The, G., 1977. Is Burkitt's lymphoma related to perinatal infection by Epstein-Barr virus? Lancet Lond. Engl. 1, 335–338.

de-Thé, G., Day, N.E., Geser, A., Lavoué, M.F., Ho, J.H., Simons, M.J., Sohier, R., Tukei, P., Vonka, V., Zavadova, H., 1975. Sero-epidemiology of the Epstein-Barr virus: preliminary analysis of an international study - a review. IARC Sci. Publ. 3–16.

de-Thé, G., Geser, A., Day, N.E., Tukei, P.M., Williams, E.H., Beri, D.P., Smith, P.G., Dean, A.G., Bronkamm, G.W., Feorino, P., Henle, W., 1978a. Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature 274, 756–761.

de-Thé, G., Geser, A., Day, N.E., Tukei, P.M., Williams, E.H., Beri, D.P., Smith, P.G., Dean, A.G., Bronkamm, G.W., Feorino, P., Henle, W., 1978b. Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature 274, 756–761.

Diamantopoulos, P.T., Kalotychou, V., Polonyfi, K., Sofotasiou, M., Anastasopoulou, A., Galanopoulos, A., Spanakis, N., Vassilakopoulos, T., Angelopoulou, M., Siakantaris, M., Variami, E., Poziopoulos, C., Terpos, E., Kollia, P., Viniou, N.-A., 2013a. Correlation of Fc-? RIIA polymorphisms with latent Epstein?Barr virus infection and latent membrane protein 1 expression in patients with low grade B-cell lymphomas. Leuk. Lymphoma 54, 2030–2034.

Diamantopoulos, P.T., Kalotychou, V., Polonyfi, K., Sofotasiou, M., Anastasopoulou, A., Galanopoulos, A., Spanakis, N., Vassilakopoulos, T., Angelopoulou, M., Siakantaris, M., Variami, E., Poziopoulos, C., Terpos, E., Kollia, P., Viniou, N.-A., 2013b. Correlation of Fc-γ RIIA polymorphisms with latent Epstein–Barr virus infection and latent membrane protein 1 expression in patients with low grade B-cell lymphomas. Leuk. Lymphoma 54, 2030–2034.

Dierksheide, J.E., Baiocchi, R.A., Ferketich, A.K., Roychowdhury, S., Pelletier, R.P., Eisenbeis, C.F., Caligiuri, M.A., VanBuskirk, A.M., 2005. IFN-gamma gene polymorphisms

associate with development of EBV+ lymphoproliferative disease in hu PBL-SCID mice. Blood 105, 1558–1565.

Digel, W., Stefanic, M., Schöniger, W., Buck, C., Raghavachar, A., Frickhofen, N., Heimpel, H., Porzsolt, F., 1989. Tumor necrosis factor induces proliferation of neoplastic B cells from chronic lymphocytic leukemia. Blood 73, 1242–1246.

Drakeley, C.J., Corran, P.H., Coleman, P.G., Tongren, J.E., McDonald, S.L.R., Carneiro, I., Malima, R., Lusingu, J., Manjurano, A., Nkya, W.M.M., Lemnge, M.M., Cox, J., Reyburn, H., Riley, E.M., 2005. Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proc. Natl. Acad. Sci. U. S. A. 102, 5108–5113.

Dranoff, G., 2004. Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer 4, 11–22.

El-Mallawany, N.K., Mutai, M., Mtete, I., Gopal, S., Stanley, C.C., Wasswa, P., Mtunda, M., Chasela, M., Kamiyango, W., Villiera, J., Fedoriw, Y., Montgomery, N.D., Liomba, G.N., Kampani, C., Krysiak, R., Westmoreland, K.D., Kim, M.H., Slone, J.S., Scheurer, M.E., Allen, C.E., Mehta, P.S., Kazembe, P.N., 2017. Beyond Endemic Burkitt Lymphoma: Navigating Challenges of Differentiating Childhood Lymphoma Diagnoses Amid Limitations in Pathology Resources in Lilongwe, Malawi. Glob. Pediatr. Health 4, 2333794X1771583.

El-Tahan, R.R., Ghoneim, A.M., El-Mashad, N., 2016. TNF-α gene polymorphisms and expression. SpringerPlus 5.

Epstein, M.A., Henle, G., Achong, B.G., Barr, Y.M., 1965. Morphological and biological studies on a virus in cultured lymphoblasts from Burkitt's Lymphoma. J. Exp. Med. 121, 761–770.

Feldmann, M., 2008. Many cytokines are very useful therapeutic targets in disease. J. Clin. Invest. 118, 3533–3536.

Ferrajoli, A., Keating, M.J., Manshouri, T., Giles, F.J., Dey, A., Estrov, Z., Koller, C.A., Kurzrock, R., Thomas, D.A., Faderl, S., Lerner, S., O'Brien, S., Albitar, M., 2002. The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia. Blood 100, 1215–1219.

Ferry, J.A., 2006. Burkitt's lymphoma: clinicopathologic features and differential diagnosis. The Oncologist 11, 375–383.

Ghesquières, H., Maurer, M.J., Casasnovas, O., Ansell, S.M., Larrabee, B.R., Lech-Maranda, E., Novak, A.J., Borrel, A.-L., Slager, S.L., Brice, P., Allmer, C., Brion, A., Ziesmer, S.C., Morschhauser, F., Habermann, T.M., Gaillard, I., Link, B.K., Stamatoullas, A., Fermé, C., Dogan, A., Macon, W.R., Audouin, J., Cerhan, J.R., Salles, G., 2013a. Cytokine gene polymorphisms and progression-free survival in classical Hodgkin lymphoma by EBV status: results from two independent cohorts. Cytokine 64, 523–531.

Ghesquières, H., Maurer, M.J., Casasnovas, O., Ansell, S.M., Larrabee, B.R., Lech-Maranda, E., Novak, A.J., Borrel, A.-L., Slager, S.L., Brice, P., Allmer, C., Brion, A., Ziesmer, S.C., Morschhauser, F., Habermann, T.M., Gaillard, I., Link, B.K., Stamatoullas, A., Fermé, C., Dogan, A., Macon, W.R., Audouin, J., Cerhan, J.R., Salles, G., 2013b. Cytokine gene polymorphisms and progression-free survival in classical Hodgkin lymphoma by EBV status: Results from two independent cohorts. Cytokine 64, 523–531.

Gichohi-Wainaina, Wanjiku N, Melse-Boonstra, A., Feskens, E.J., Demir, A.Y., Veenemans, J., Verhoef, H., 2015. Tumour necrosis factor allele variants and their association with the occurrence and severity of malaria in African children: a longitudinal study. Malar. J. 14.

Gichohi-Wainaina, Wanjiku N., Melse-Boonstra, A., Feskens, E.J., Demir, A.Y., Veenemans, J., Verhoef, H., 2015. Tumour necrosis factor allele variants and their association with the occurrence and severity of malaria in African children: a longitudinal study. Malar. J. 14, 249.

Gonzalez, J.R., Armengol, L., Sole, X., Guino, E., Mercader, J.M., Estivill, X., Moreno, V., 2007. SNPassoc: an R package to perform whole genome association studies. Bioinformatics 23, 654–655.

González, J.R., Armengol, L., Solé, X., Guinó, E., Mercader, J.M., Estivill, X., Moreno, V., 2007. SNPassoc: an R package to perform whole genome association studies. Bioinformatics 23, 654–655.

Green, S., Dobrjansky, A., Chiasson, M.A., 1982. Murine tumor necrosis-inducing factor: purification and effects on myelomonocytic leukemia cells. J. Natl. Cancer Inst. 68, 997–1003.

Haabeth, O.A.W., Lorvik, K.B., Hammarström, C., Donaldson, I.M., Haraldsen, G., Bogen, B., Corthay, A., 2011. Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer. Nat. Commun. 2, 240.

Haluska, F.G., Tsujimoto, Y., Croce, C.M., 1987. The t(8;14) chromosome translocation of the Burkitt lymphoma cell line Daudi occurred during immunoglobulin gene rearrangement and involved the heavy chain diversity region. Proc. Natl. Acad. Sci. U. S. A. 84, 6835–6839.

Han, S.-S., Cho, E.-Y., Lee, T.S., Kim, J.W., Park, N.-H., Song, Y.-S., Kim, J.G., Lee, H.-P., Kang, S.-B., 2008. Interleukin-12 p40 gene (IL12B) polymorphisms and the risk of cervical cancer in Korean women. Eur. J. Obstet. Gynecol. Reprod. Biol. 140, 71–75.

Hayden, R.T., Hokanson, K.M., Pounds, S.B., Bankowski, M.J., Belzer, S.W., Carr, J., Diorio, D., Forman, M.S., Joshi, Y., Hillyard, D., Hodinka, R.L., Nikiforova, M.N., Romain, C.A., Stevenson, J., Valsamakis, A., Balfour, H.H., U.S. EBV Working Group, 2008. Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein-Barr virus. J. Clin. Microbiol. 46, 157–163.

Henri, S., Stefani, F., Parzy, D., Eboumbou, C., Dessein, A., Chevillard, C., 2002. Description of three new polymorphisms in the intronic and 3'UTR regions of the human interferon gamma gene. Genes Immun. 3, 1–4.

Howell, W.M., Rose-Zerilli, M.J., 2007. Cytokine gene polymorphisms, cancer susceptibility, and prognosis. J. Nutr. 137, 194S-199S.

Hu, Q., Lou, G.-G., Liu, Y.-C., Qian, L., Lv, B.-D., 2014. The Tumor Necrosis Factor-α-308 and -238 Polymorphisms and Risk of Hepatocellular Carcinoma for Asian Populations: A Meta-Analysis. Curr. Ther. Res. 76, 70–75.

Ikeda, H., Old, L.J., Schreiber, R.D., 2002. The roles of IFN gamma in protection against tumor development and cancer immunoediting. Cytokine Growth Factor Rev. 13, 95–109.

Indik, Z.K., Park, J.G., Hunter, S., Schreiber, A.D., 1995. The molecular dissection of Fc gamma receptor mediated phagocytosis. Blood 86, 4389–4399.

Iversen, O.H., Iversen, U., Ziegler, J.L., Bluming, A.Z., 1974. Cell kinetics in Burkitt lymphoma. Eur. J. Cancer 10, 155–163.

Jaffe, E.S., Weltgesundheitsorganisation, International Agency for Research on Cancer (Eds.),

2001. Pathology and genetics of tumours of haematopoietic and lymphoid tissues, World Health Organization classification of tumours. IARC Press, Lyon.

Judson, R., Stephens, J.C., Windemuth, A., 2000. The predictive power of haplotypes in clinical response. Pharmacogenomics 1, 15–26.

Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., Morishima, T., 1999. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J. Clin. Microbiol. 37, 132–136.

Klein, G., 1983. Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. Cell 32, 311–315.

Landskron, G., De la Fuente, M., Thuwajit, P., Thuwajit, C., Hermoso, M.A., 2014. Chronic Inflammation and Cytokines in the Tumor Microenvironment. J. Immunol. Res. 2014, 1–19.

Lasek, W., Zagożdżon, R., Jakobisiak, M., 2014a. Interleukin 12: still a promising candidate for tumor immunotherapy? Cancer Immunol. Immunother. 63, 419–435.

Lasek, W., Zagożdżon, R., Jakobisiak, M., 2014b. Interleukin 12: still a promising candidate for tumor immunotherapy? Cancer Immunol. Immunother. CII 63, 419–435.

Lee, S., Margolin, K., 2011. Cytokines in Cancer Immunotherapy. Cancers 3, 3856–3893.

Lee, T.C., Savoldo, B., Barshes, N.R., Rooney, C.M., Heslop, H.E., Gee, A.P., Caldwell, Y., Scott, J.D., Goss, J.A., 2006. Use of cytokine polymorphisms and Epstein-Barr virus viral load to predict development of post-transplant lymphoproliferative disorder in paediatric liver transplant recipients. Clin. Transplant. 20, 389–393.

Lewis, C.M., Knight, J., 2012. Introduction to Genetic Association Studies. Cold Spring Harb. Protoc. 2012, pdb.top068163.

Lin, D.Y., Zeng, D., Millikan, R., 2005. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. Genet. Epidemiol. 29, 299–312.

Love, C., Sun, Z., Jima, D., Li, G., Zhang, J., Miles, R., Richards, K.L., Dunphy, C.H., Choi, W.W.L., Srivastava, G., Lugar, P.L., Rizzieri, D.A., Lagoo, A.S., Bernal-Mizrachi, L., Mann, K.P., Flowers, C.R., Naresh, K.N., Evens, A.M., Chadburn, A., Gordon, L.I., Czader, M.B., Gill, J.I., Hsi, E.D., Greenough, A., Moffitt, A.B., McKinney, M.,

Banerjee, A., Grubor, V., Levy, S., Dunson, D.B., Dave, S.S., 2012. The genetic landscape of mutations in Burkitt lymphoma. Nat. Genet. 44, 1321–1325.

Magrath, I., 2012. Epidemiology: clues to the pathogenesis of Burkitt lymphoma: Review. Br. J. Haematol. 156, 744–756.

Malaguarnera, L., Pignatelli, S., Musumeci, M., Simporè, J., Musumeci, S., 2002. Plasma levels of interleukin-18 and interleukin-12 in Plasmodium falciparum malaria. Parasite Immunol. 24, 489–492.

Marquet, S., Doumbo, O., Cabantous, S., Poudiougou, B., Argiro, L., Safeukui, I., Konate, S., Sissoko, S., Chevereau, E., Traore, A., Keita, M.M., Chevillard, C., Abel, L., Dessein, A.J., 2008. A functional promoter variant in IL12B predisposes to cerebral malaria. Hum. Mol. Genet. 17, 2190–2195.

Mbengue, B., Niang, B., Niang, M.S., Varela, M.L., Fall, B., Fall, M.M., Diallo, R.N., Diatta, B., Gowda, D.C., Dieye, A., Perraut, R., 2016. Inflammatory cytokine and humoral responses to Plasmodium falciparum glycosylphosphatidylinositols correlates with malaria immunity and pathogenesis. Immun. Inflamm. Dis. 4, 24–34.

Mbulaiteye, S.M., Biggar, R.J., Bhatia, K., Linet, M.S., Devesa, S.S., 2009. Sporadic childhood Burkitt lymphoma incidence in the United States during 1992-2005. Pediatr. Blood Cancer 53, 366–370.

McAulay, K.A., Haque, T., Crawford, D.H., 2009a. Tumour necrosis factor gene polymorphism: a predictive factor for the development of post-transplant lymphoproliferative disease. Br. J. Cancer 101, 1019–1027.

McAulay, K.A., Haque, T., Crawford, D.H., 2009b. Tumour necrosis factor gene polymorphism: a predictive factor for the development of post-transplant lymphoproliferative disease. Br. J. Cancer 101, 1019–1027.

McAulay, K.A., Haque, T., Crawford, D.H., 2009c. Tumour necrosis factor gene polymorphism: a predictive factor for the development of post-transplant lymphoproliferative disease. Br. J. Cancer 101, 1019–1027.

Mellouk, S., Maheshwari, R.K., Rhodes-Feuillette, A., Beaudoin, R.L., Berbiguier, N., Matile, H., Miltgen, F., Landau, I., Pied, S., Chigot, J.P., 1987. Inhibitory activity of interferons and interleukin 1 on the development of Plasmodium falciparum in human hepatocyte

cultures. J. Immunol. Baltim. Md 1950 139, 4192–4195.

Mohan, K., Stevenson, M.M., 1998. Dyserythropoiesis and severe anaemia associated with malaria correlate with deficient interleukin-12 production. Br. J. Haematol. 103, 942–949.

Moormann, A.M., Chelimo, K., Sumba, O.P., Lutzke, M.L., Ploutz-Snyder, R., Newton, D., Kazura, J., Rochford, R., 2005. Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children. J. Infect. Dis. 191, 1233–1238.

Morzycka-Wroblewska, E., Munshi, A., Ostermayer, M., Harwood, J.I., Kagnoff, M.F., 1997. Differential expression of HLA-DQA1 alleles associated with promoter polymorphism. Immunogenetics 45, 163–170.

Mulama, D.H., Bailey, J.A., Foley, J., Chelimo, K., Ouma, C., Jura, W.G.Z.O., Otieno, J., Vulule, J., Moormann, A.M., 2014. 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. Int. J. Cancer J. Int. Cancer 134, 645–653.

Ndede, I., Mining, S.K., Patel, K., Wanjala, F.M., Chumba, D., Tenge, C., 2017. Cytokines associated with Burkitt's lymphoma in western Kenya. BMC Res. Notes 10.

Ndenga, B., Githeko, A., Omukunda, E., Munyekenye, G., Atieli, H., Wamai, P., Mbogo, C., Minakawa, N., Zhou, G., Yan, G., 2006. Population Dynamics of Malaria Vectors in Western Kenya Highlands. J. Med. Entomol. 43, 200–206.

Neitzel, H., 1986. A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum. Genet. 73, 320–326.

Njie, R., Bell, A.I., Jia, H., Croom-Carter, D., Chaganti, S., Hislop, A.D., Whittle, H., Rickinson, A.B., 2009. The Effects of Acute Malaria on Epstein-Barr Virus (EBV) Load and EBV-Specific T Cell Immunity in Gambian Children. J. Infect. Dis. 199, 31–38.

Oduor, C.I., Chelimo, K., Ouma, C., Mulama, D.H., Foley, J., Vulule, J., Bailey, J.A., Moormann, A.M., 2014. Interleukin-6 and interleukin-10 gene promoter polymorphisms and risk of endemic Burkitt lymphoma. Am. J. Trop. Med. Hyg. 91, 649–654.

Olaniyan, S.A., Amodu, O.K., Bakare, A.A., Troye-Blomberg, M., Omotade, O.O., Rockett, K.A., MalariaGEN Consortium, 2016. Tumour necrosis factor alpha promoter

polymorphism, TNF-238 is associated with severe clinical outcome of falciparum malaria in Ibadan southwest Nigeria. Acta Trop. 161, 62–67.

O'Meara, W.P., Mangeni, J.N., Steketee, R., Greenwood, B., 2010. Changes in the burden of malaria in sub-Saharan Africa. Lancet Infect. Dis. 10, 545–555.

Ong'echa, J.M., Raballah, E.O., Kempaiah, P.M., Anyona, S.B., Were, T., Davenport, G.C., Konah, S., Vulule, J.M., Ouma, C., Hittner, J.B., Perkins, D.J., 2011. Polymorphic variability in the 3' untranslated region (UTR) of IL12B is associated with susceptibility to severe anaemia in Kenyan children with acute Plasmodium falciparum malaria. BMC Genet. 12, 69.

Orem, J., Mbidde, E.K., Lambert, B., de Sanjose, S., Weiderpass, E., 2007. Burkitt's lymphoma in Africa, a review of the epidemiology and etiology. Afr. Health Sci. 7, 166–175.

Osman, N.F., Holyoake, T.L., Zeineldin, A., Elkordy, A.A., McQuaker, I.G., 2012. TNF Polymorphisms Independently Predict Outcome in Patients with B-Cell Non-Hodgkin's Lymphoma. Iran. J. Cancer Prev. 2, 19–27.

Parkin, D.M., Bray, F., Ferlay, J., Jemal, A., 2014. Cancer in Africa 2012. Cancer Epidemiol. Biomarkers Prev. 23, 953–966.

Perlaza, B.-L., Sauzet, J.-P., Brahimi, K., BenMohamed, L., Druilhe, P., 2011. Interferon-γ, a valuable surrogate marker of Plasmodium falciparum pre-erythrocytic stages protective immunity. Malar. J. 10, 27.

Piriou, E., Asito, A.S., Sumba, P.O., Fiore, N., Middeldorp, J.M., Moormann, A.M., Ploutz-Snyder, R., Rochford, R., 2012. Early age at time of primary Epstein-Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: clues to the etiology of endemic Burkitt lymphoma. J. Infect. Dis. 205, 906–913.

Platt, A., Obala, A.A., MacIntyre, C., Otsyula, B., Meara, W.P.O., 2018. Dynamic malaria hotspots in an open cohort in western Kenya. Sci. Rep. 8.

Pleass, R.J., Woof, J.M., 2001. Fc receptors and immunity to parasites. Trends Parasitol. 17, 545–551.

Powell, M.S., Barton, P.A., Emmanouilidis, D., Wines, B.D., Neumann, G.M., Peitersz, G.A., Maxwell, K.F., Garrett, T.P., Hogarth, P.M., 1999. Biochemical analysis and crystallisation of Fc gamma RIIa, the low affinity receptor for IgG. Immunol. Lett. 68,

17–23.

Pravica, V., Asderakis, A., Perrey, C., Hajeer, A., Sinnott, P.J., Hutchinson, I.V., 1999. In vitro production of IFN-gamma correlates with CA repeat polymorphism in the human IFN-gamma gene. Eur. J. Immunogenetics Off. J. Br. Soc. Histocompat. Immunogenetics 26, 1–3.

Qidwai, T., Khan, F., 2011a. Tumour necrosis factor gene polymorphism and disease prevalence. Scand. J. Immunol. 74, 522–547.

Qidwai, T., Khan, F., 2011b. Tumour Necrosis Factor Gene Polymorphism and Disease Prevalence. Scand. J. Immunol. 74, 522–547.

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. Trop. Med. Int. Health TM IH 12, 936–943.

Reilly, A. F., Norris, C.F., Surrey, S., Bruchak, F.J., Rappaport, E.F., Schwartz, E., McKenzie, S.E., 1994. Genetic diversity in human Fc receptor II for immunoglobulin G: Fc gamma receptor IIA ligand-binding polymorphism. Clin. Diagn. Lab. Immunol. 1, 640–644.

Reilly, A F, Norris, C.F., Surrey, S., Bruchak, F.J., Rappaport, E.F., Schwartz, E., McKenzie, S.E., 1994. Genetic diversity in human Fc receptor II for immunoglobulin G: Fc gamma receptor IIA ligand-binding polymorphism. Clin. Diagn. Lab. Immunol. 1, 640–644.

Richter, J., Schlesner, M., Hoffmann, S., Kreuz, M., Leich, E., Burkhardt, B., Rosolowski, M., Ammerpohl, O., Wagener, R., Bernhart, S.H., Lenze, D., Szczepanowski, M., Paulsen, M., Lipinski, S., Russell, R.B., Adam-Klages, S., Apic, G., Claviez, A., Hasenclever, D., Hovestadt, V., Hornig, N., Korbel, J.O., Kube, D., Langenberger, D., Lawerenz, C., Lisfeld, J., Meyer, K., Picelli, S., Pischimarov, J., Radlwimmer, B., Rausch, T., Rohde, M., Schilhabel, M., Scholtysik, R., Spang, R., Trautmann, H., Zenz, T., Borkhardt, A., Drexler, H.G., Möller, P., MacLeod, R.A.F., Pott, C., Schreiber, S., Trümper, L., Loeffler, M., Stadler, P.F., Lichter, P., Eils, R., Küppers, R., Hummel, M., Klapper, W., Rosenstiel, P., Rosenwald, A., Brors, B., Siebert, R., ICGC MMML-Seq Project, 2012. Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. Nat. Genet. 44, 1316–1320.

Risch, N., Merikangas, K., 1996. The future of genetic studies of complex human diseases.

Science 273, 1516–1517.

Robbiani, D.F., Deroubaix, S., Feldhahn, N., Oliveira, T.Y., Callen, E., Wang, Q., Jankovic, M., Silva, I.T., Rommel, P.C., Bosque, D., Eisenreich, T., Nussenzweig, A., Nussenzweig, M.C., 2015. Plasmodium Infection Promotes Genomic Instability and AID-Dependent B Cell Lymphoma. Cell 162, 727–737.

Rochford, R., Cannon, M.J., Moormann, A.M., 2005. Endemic Burkitt's lymphoma: a polymicrobial disease? Nat. Rev. Microbiol. 3, 182–187.

Salles, G., Bienvenu, J., Bastion, Y., Barbier, Y., Doche, C., Warzocha, K., Gutowski, M.C., Rieux, C., Coiffier, B., 1996a. Elevated circulating levels of TNFalpha and its p55 soluble receptor are associated with an adverse prognosis in lymphoma patients. Br. J. Haematol. 93, 352–359.

Salles, G., Bienvenu, J., Bastion, Y., Barbier, Y., Doche, C., Warzocha, K., Gutowski, M.C., Rieux, C., Coiffier, B., 1996b. Elevated circulating levels of TNFalpha and its p55 soluble receptor are associated with an adverse prognosis in lymphoma patients. Br. J. Haematol. 93, 352–359.

Schoenborn, J.R., Wilson, C.B., 2007. Regulation of interferon-gamma during innate and adaptive immune responses. Adv. Immunol. 96, 41–101.

Seegers, D., Zwiers, A., Strober, W., Peña, A.S., Bouma, G., 2002. A TaqI polymorphism in the 3′UTR of the IL-12 p40 gene correlates with increased IL-12 secretion. Genes Immun. 3, 419–423.

Seidemann, K., Zimmermann, M., Book, M., Meyer, U., Burkhardt, B., Welte, K., Reiter, A., Stanulla, M., 2005. Tumor necrosis factor and lymphotoxin alfa genetic polymorphisms and outcome in pediatric patients with non-Hodgkin's lymphoma: results from Berlin-Frankfurt-Münster Trial NHL-BFM 95. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 23, 8414–8421.

Sheen-Chen, S.M., Chen, W.J., Eng, H.L., Chou, F.F., 1997. Serum concentration of tumor necrosis factor in patients with breast cancer. Breast Cancer Res. Treat. 43, 211–215.

Shin, S.P., Kim, N.K., Kim, J.H., Lee, J.H., Kim, J.O., Cho, S.H., Park, H., Kim, M.N., Rim, K.S., Hwang, S.G., 2015. Association between hepatocellular carcinoma and tumor necrosis factor alpha polymorphisms in South Korea. World J. Gastroenterol. 21, 13064–

13072.

Sieburth, D., Jabs, E.W., Warrington, J.A., Li, X., Lasota, J., LaForgia, S., Kelleher, K., Huebner, K., Wasmuth, J.J., Wolf, S.F., 1992. Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. Genomics 14, 59–62.

Singh, S., Singh, N., Handa, R., 2000. Tumor necrosis factor-alpha in patients with malaria. Indian J. Malariol. 37, 27–33.

Sinha, S., Mishra, Shrawan K, Sharma, S., Patibandla, P.K., Mallick, P.K., Sharma, S.K., Mohanty, S., Pati, S.S., Mishra, Saroj K, Ramteke, B.K., Bhatt, R.M., Joshi, H., Dash, A.P., Ahuja, R.C., Awasthi, S., Consortium, I.G.V., Venkatesh, V., Habib, S., 2008. Polymorphisms of TNF-enhancer and gene for FcgammaRIIa correlate with the severity of falciparum malaria in the ethnically diverse Indian population. Malar. J. 7, 13.

Snider, C.J., Cole, S.R., Chelimo, K., Sumba, P.O., MacDonald, P.D.M., John, C.C., Meshnick, S.R., 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, e31753.

Steed, A., Buch, T., Waisman, A., Virgin, H.W., 2007. Gamma Interferon Blocks Gammaherpesvirus Reactivation from Latency in a Cell Type-Specific Manner. J. Virol. 81, 6134–6140.

Stram, D.O., 2017. Multi-SNP Haplotype Analysis Methods for Association Analysis, in: Elston, R.C. (Ed.), Statistical Human Genetics. Springer New York, New York, NY, pp. 485–504.

Su, Z., Stevenson, M.M., 2000. Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection. Infect. Immun. 68, 4399–4406.

Szlosarek, P.W., Balkwill, F.R., 2003. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. Lancet Oncol. 4, 565–573.

Torgbor, C., Awuah, P., Deitsch, K., Kalantari, P., Duca, K.A., Thorley-Lawson, D.A., 2014. A multifactorial role for P. falciparum malaria in endemic Burkitt's lymphoma pathogenesis. PLoS Pathog. 10, e1004170.

Torre, D., Speranza, F., Giola, M., Matteelli, A., Tambini, R., Biondi, G., 2002. Role of Th1 and Th2 cytokines in immune response to uncomplicated Plasmodium falciparum malaria. Clin. Diagn. Lab. Immunol. 9, 348–351.

Torres-Espíndola, L.M., Velázquez-Cruz, R., Falfán-Valencia, R., Chavez-Pacheco, J.L., Salcedo-Vargas, M., de Jesús Nambo-Lucio, M., Salinas-Lara, C., Alemón-Medina, R., Granados-Montiel, J., Reyes-Servín, M.A., Granados, J., de Jesús Castillejos-López, M., 2014a. Genetic polymorphism of tumor necrosis factor promoter region and susceptibility to develop Hodgkin lymphoma in a Mexican population. Leuk. Lymphoma 55, 1295–1299.

Torres-Espíndola, L.M., Velázquez-Cruz, R., Falfán-Valencia, R., Chavez-Pacheco, J.L., Salcedo-Vargas, M., de Jesús Nambo-Lucio, M., Salinas-Lara, C., Alemón-Medina, R., Granados-Montiel, J., Reyes-Servín, M.A., Granados, J., de Jesús Castillejos-López, M., 2014b. Genetic polymorphism of tumor necrosis factor promoter region and susceptibility to develop Hodgkin lymphoma in a Mexican population. Leuk. Lymphoma 55, 1295–1299.

Tugues, S., Burkhard, S.H., Ohs, I., Vrohlings, M., Nussbaum, K., vom Berg, J., Kulig, P., Becher, B., 2015. New insights into IL-12-mediated tumor suppression. Cell Death Differ. 22, 237–246.

van de Winkel, J.G., Capel, P.J., 1993. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol. Today 14, 215–221.

VanBuskirk, A.M., Malik, V., Xia, D., Pelletier, R.P., 2001. A gene polymorphism associated with posttransplant lymphoproliferative disorder. Transplant. Proc. 33, 1834.

Wall, L., Burke, F., Barton, C., Smyth, J., Balkwill, F., 2003. IFN-gamma induces apoptosis in ovarian cancer cells in vivo and in vitro. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 9, 2487–2496.

Wang, X., Lin, Y., 2008. Tumor necrosis factor and cancer, buddies or foes? Acta Pharmacol. Sin. 29, 1275–1288.

Warzocha, K., Salles, G., Bienvenu, J., Bastion, Y., Dumontet, C., Renard, N., Neidhardt-Berard, E.M., Coiffier, B., 1997. Tumor necrosis factor ligand-receptor system can predict treatment outcome in lymphoma patients. J. Clin. Oncol. 15, 499–508.

Whitley, E., Ball, J., 2002. Statistics review 4: Sample size calculations. Crit. Care 6, 335–341.

Whittingham, S., Naselli, G., Harrison, L.C., Boyd, A.W., Cebon, J., Jack, I., 1993. Cytokine production in response to Epstein-Barr virus infection of peripheral blood mononuclear cells *in vitro*. Immunol. Cell Biol. 71, 259–264.

Wilmore, J.R., Maue, A.C., Rochford, R., 2016. *Plasmodium chabaudi* infection induces AID expression in transitional and marginal zone B cells: Induction of AID by *Plasmodium chabaudi*. Immun. Inflamm. Dis. 4, 497–505.

Wilson, A.G., Symons, J.A., McDowell, T.L., McDevitt, H.O., Duff, G.W., 1997. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc. Natl. Acad. Sci. U. S. A. 94, 3195–3199.

Wu, M.-S., Huang, S.-P., Chang, Y.-T., Shun, C.-T., Chang, M.-C., Lin, M.-T., Wang, H.-P., Lin, J.-T., 2002a. Tumor necrosis factor-alpha and interleukin-10 promoter polymorphisms in Epstein-Barr virus-associated gastric carcinoma. J. Infect. Dis. 185, 106–109.

Wu, M.-S., Huang, S.-P., Chang, Y.-T., Shun, C.-T., Chang, M.-C., Lin, M.-T., Wang, H.-P., Lin, J.-T., 2002b. Tumor necrosis factor-alpha and interleukin-10 promoter polymorphisms in Epstein-Barr virus-associated gastric carcinoma. J. Infect. Dis. 185, 106–109.

Wu, M.-S., Huang, S.-P., Chang, Y.-T., Shun, C.-T., Chang, M.-C., Lin, M.-T., Wang, H.-P., Lin, J.-T., 2002c. Tumor Necrosis Factor–α and Interleukin-10 Promoter Polymorphisms in Epstein-Barr Virus–Associated Gastric Carcinoma. J. Infect. Dis. 185, 106–109.

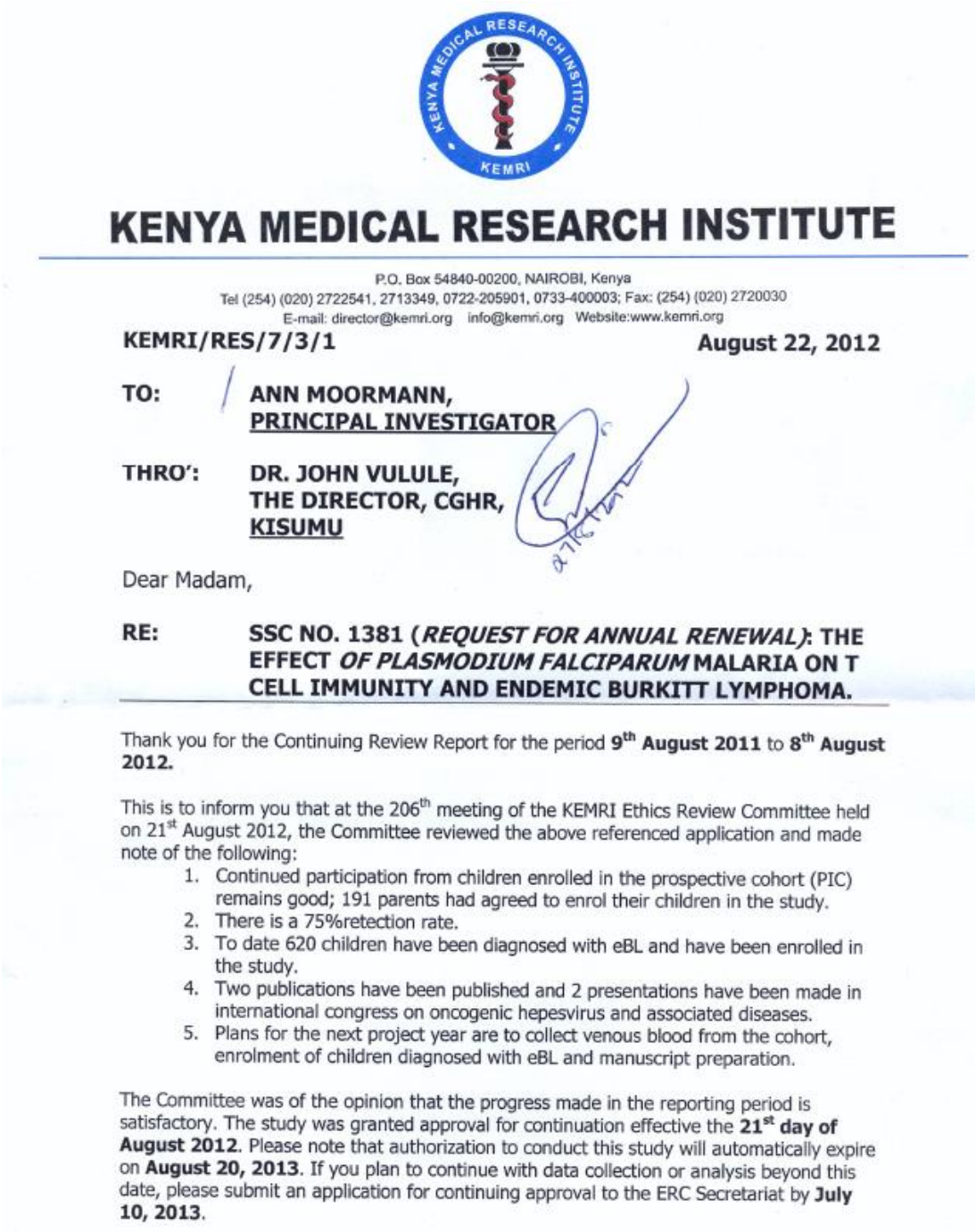
Young, L.S., Rickinson, A.B., 2004. Epstein–Barr virus: 40 years on. Nat. Rev. Cancer 4, 757–768. 2

Zamarron, B., Chen, W., 2011. Dual Roles of Immune Cells and Their Factors in Cancer Development and Progression. International Journal of Biological Sciences 7, 651–658.

Zhang, Q., Zhao, G.S., Yuan, X.L., Li, X.H., Yang, Z., Cui, Y.F., Guan, Q.L., Sun, X.Y., Shen, W., Xu, T.A., Wang, Q.S., 2017. Tumor necrosis factor alpha-238G/A polymorphism and risk of breast cancer: An update by meta-analysis. Medicine (Baltimore) 96, e7442.

APPENDICES

APPENDIX 1: SERU ETHICAL APPROVAL



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KEMRI/RES/7/3/1 **August 22, 2012**

TO: **ANN MOORMANN,**
PRINCIPAL INVESTIGATOR

THRO': **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

Dear Madam,

RE: **SSC NO. 1381 (*REQUEST FOR ANNUAL RENEWAL*): THE EFFECT *OF PLASMODIUM FALCIPARUM* MALARIA ON T CELL IMMUNITY AND ENDEMIC BURKITT LYMPHOMA.**

Thank you for the Continuing Review Report for the period **9th August 2011** to **8th August 2012.**

This is to inform you that at the 206th meeting of the KEMRI Ethics Review Committee held on 21st August 2012, the Committee reviewed the above referenced application and made note of the following:

1. Continued participation from children enrolled in the prospective cohort (PIC) remains good; 191 parents had agreed to enrol their children in the study.
2. There is a 75%retection rate.
3. To date 620 children have been diagnosed with eBL and have been enrolled in the study.
4. Two publications have been published and 2 presentations have been made in international congress on oncogenic hepesvirus and associated diseases.
5. Plans for the next project year are to collect venous blood from the cohort, enrolment of children diagnosed with eBL and manuscript preparation.

The Committee was of the opinion that the progress made in the reporting period is satisfactory. The study was granted approval for continuation effective the **21st day of August 2012**. Please note that authorization to conduct this study will automatically expire on **August 20, 2013**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **July 10, 2013**.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.

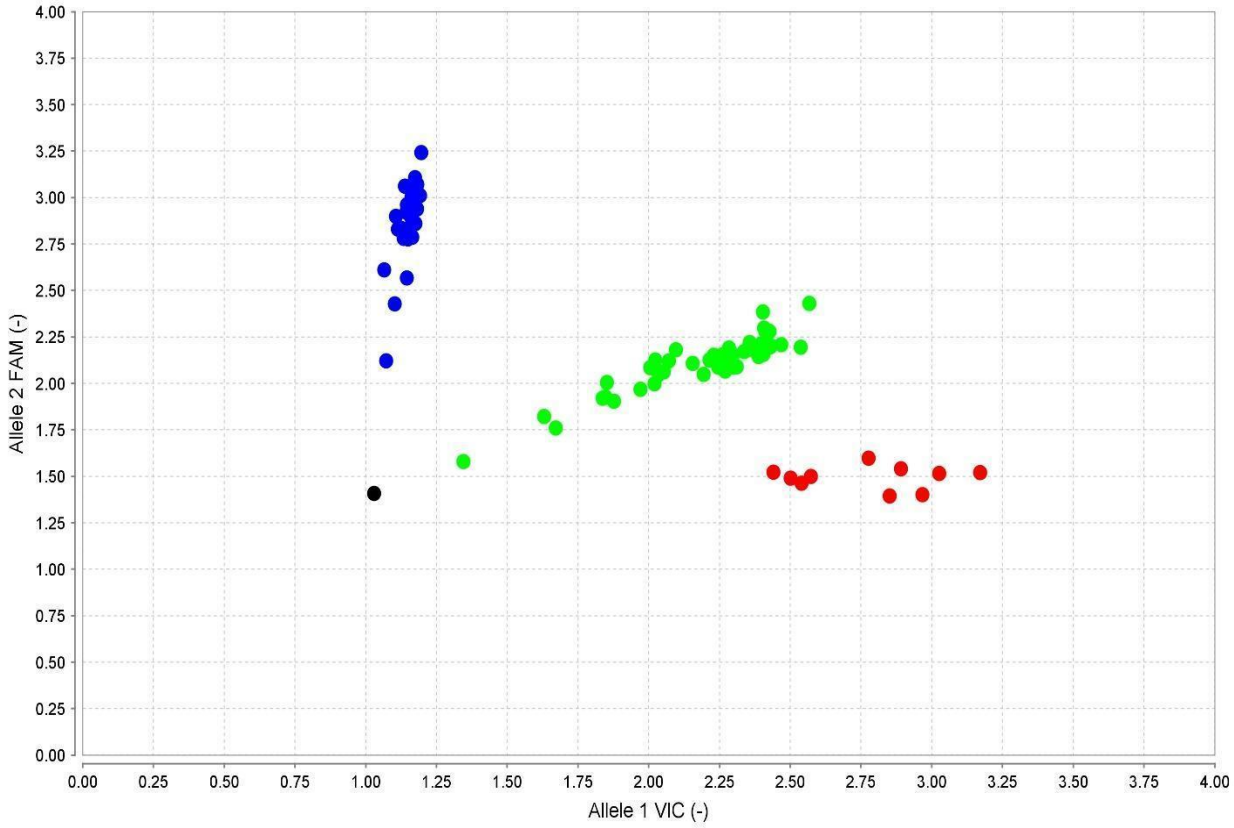
Please note that any unanticipated problems resulting from the conduct of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

You may continue with the study.

Sincerely

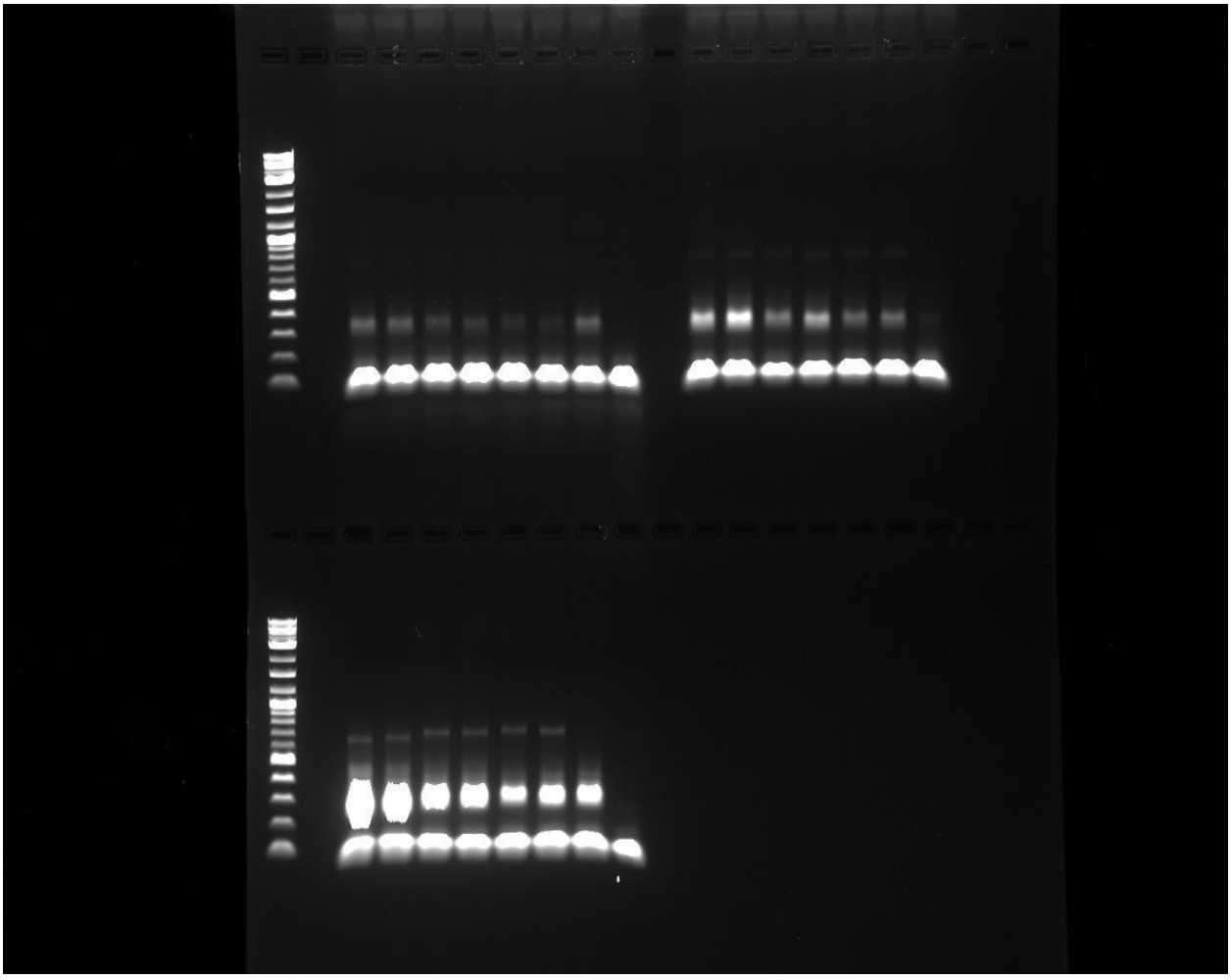
DR.BETTY NJOROGE
FOR : **DR. CHRISTINE WASUNNA,**
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX 2: IL-12 +1188A/C DISCRIMINATION PLOTS



Allelic discrimination plot of *IL-12* (+1188A/C) SNP, TaqMan® 5' Allelic Discrimination Assay technique (Applied Biosystems, Foster City, CA, USA) was used to genotype the samples. Genotype calling was done by TaqMan Genotyper Software (Applied Biosystems, Foster City, CA, USA). Blue, green and red dots represent the homozygous AA wild type, heterozygous AC and homozygous CC mutant genotypes respectively.

APPENDIX 3: OPTIMIZATION OF MIP CONCENTRATION BEFORE CAPTURE



MIP concentration optimization using controls prior to capture of study samples. The MIP concentration in the three runs represent the ladder and MIP concentration at 8X, 8X, 16X, 16X, 32X, 32X, MIP-capture_NTC and PCR_NTC. The number of cycles for the three runs from top left in a clockwise manner is 22, 24 and 26 respectively.

APPENDIX 4: PRIMER AND PROBE SEQUENCES

From: Kimura, H, *et al*. (1999). Quantitative analysis of Epstein-Barr Virus load by Using a Real-Time PCR assay. J*ournal of Clinical Microbiology.*

EBV primers

5'd CGGAAGCCCTCTGGACTTC 3' Forward Primer (FO)

5'd CCCTGTTTATCCGATGGAATG 3' Reverse Primer (RE)

EBV Probe TMP-EBV-Balf-5 with 5' FAM reporter and TAMRA BHQ-1 3' quencher

5'd FAM -TGTACACGCACGAGAAATGCGCCT-BHQ-1 3' Probe w/ FAM reporter and TAMRA quencher

Human beta-actin primers

5'd TCACCCACACTGTGCCCATCTACGA 3' Forward Primer (FO)

5'd CAGCGGAACCGCTCATTGCCAATGG 3' Reverse Primer (RE)

Human beta-actin Probe with 5'CAL Fluor Orange 560 reporter and TAMRA BHQ-1 3' quencher (read in HEX channel)

5'd CalFluorOrange -ATGCCCTCCCCCATGCCATCCTGCGT- BHQ-1 3'

APPENDIX 5: EBL PARTICIPANT CONSENT FORM

KEMRI/UMMS
CONSENT FOR INVESTIGATIONAL STUDIES
Project Title: Impact of Malaria on Shaping Immunity to EBV and Endemic Burkitt Lymphoma
Principal Investigators: Ann Moormann, Ph.D., MPH and John M. Ong'echa, Ph.D.
Consent 2: Child with eBL at enrollment

Dr. Ann Moormann from University of Massachusetts Medical School (UMMS) in the USA and her colleagues at the Kenya Medical Research Institute (KEMRI) are inviting you to enroll your child in a research study. We are doing this study because we want to understand why children living in areas with high malaria transmission are at increased risk for a form of cancer called Burkitt's lymphoma. This childhood cancer is rare in Kenya however it is more common here than in other parts of the world. Besides malaria, a virus called Epstein Barr virus (EBV) is also connected with Burkitt's lymphoma. EBV is a common virus that does not normally cause disease. This virus is transmitted by saliva. EBV infects most children by the time they are three years old. Even though this virus does not normally cause disease, it may play a role in the development of Burkitt's lymphoma. We do not know what causes Burkitt's lymphoma. The goal of this study is to understand how EBV and malaria infections may increase the risk of getting Burkitt's lymphoma. We will do this by looking at your child's defenses against EBV and malaria. We hope that the results of this study will provide some information as to how to prevent Burkitt's lymphoma.

Approximately 80 children per year diagnosed with Burkitt's lymphoma will participate in this study. This is an ongoing study over the next 5 years. If we request another blood sample from your child, you would be asked to sign a new consent form at that time.

If you agree to enter your child in this study, we will draw blood from a vein in your child's arm. If your child is less than 5 years old, we will collect approximately ½ - 1 teaspoon (2-5ml) of venous blood. If your child is older than 5 years then we will collect 1-1½ teaspoons (5-8ml) of venous blood. Only trained medical personnel will draw your child's blood. The risks for harm by having this amount of blood drawn are very low. There may be slight bleeding at the site, persistent pain, bruising or infection. Bleeding, pain and bruising should disappear after a few days. If infection occurs because of your child's participation in this study, we will cover any costs for treatment of the infection.

There are no added physical risks to your child if you consent for a blood sample to be used for research. We take steps to keep any information that identifies your child confidential. However, there is a risk that confidentiality could be lost or broken.

Since your child's medical care is provided by the hospital pediatricians and staff, the benefits of participating in the study will include provisions for transportation whenever possible to your residence after your child is discharged from hospital. Transport reimbursement will also be available for out-patient follow-up visits when your child is recovering

We will transport your child's venous blood sample to the UMMS-KEMRI laboratory in Kisumu for further testing. We may also send samples of blood to the University of Massachusetts Medical School for testing. These tests include measuring your child's blood cell defenses against EBV and malaria, testing for the presence of EBV, hemoglobin S trait and genetic factors such as human leukocyte antigen type that play a role in the body's defenses against infection and disease. Your child's blood will also be used to test for antibodies against vaccines routinely given to your child such as polio, measles, tetanus, etc. Any results from these tests will be kept confidential. To protect your child's privacy, a study number will be assigned to the blood samples. This study number will only be linked to your child's name in a database kept by the principal investigators.

Testing for HIV is routinely done as part of your child's medical care in hospital. As a part of this study, we would like to know if your child is infected with HIV. The presence of this virus can prevent the body from fighting infection and confuse our study results. If you agree to have your child tested for HIV, an HIV/AIDS counselor will talk to you. The counselor will explain how the disease is passed from one person to another and how you can prevent infection. The counselor will also explain the HIV testing and tell you if your child is infected or not. This test is strictly voluntary. The results of HIV testing will be confidential and will not be available to anyone but you. We are requesting permission to record your child's HIV test results as part of this study. You may enroll your child in this study and refuse HIV testing.

Consent to record child's HIV test result for this study yes no
(Please circle parent's response)

Storage and Use of Samples for Future Studies
Samples of your child's blood will be stored and may be used for future testing related to scientific studies not described here, including tests related to malaria, schisotsomiasis, hookworm and other diseases caused by parasites. Other viral infections may also be tested, such as cytomegalovirus or measles. Comparing immunity to different infections allows us to determine if EBV and malaria are able to cause some children to get Burkitt lymphoma.

Specimens collected from your child will be tested by laboratories working with Dr. Moormann. Some of these tests will be genetic tests. Genetic tests help us find out why some children get cancer and other children stay healthy. We all have genes but sometimes genes change. We don't always know why some genes change but sometimes this change causes the gene to act in an unhealthy way. For example, a healthy gene will tell a cell to grow and make a copy of itself. This is normal. But when gene is changed it can lose the signal that tells the cell when to stop making copies of itself. If there are too many copies of a cell, that are made too quickly and are not needed to be healthy, this process becomes what we call cancer. These gene changes are called genetic risk factors. Genes can be altered but do not always cause cancer. There are also natural defenses in the body to try to stop cancer cells. We are interested in finding out which genes lead normal cells to become cancer cells and how the body helps to stop cancer. We don't know all of the genes involved in Burkitt lymphoma. This is why we are doing this research study, so that we can find out how cancer cells start. Then we can learn how to stop it from happening or how to make a better treatment to cure it.

Because cancer is rare and other researchers study genes that may be risk factors for cancer, the results of this research, including the results of genetic testing, may be shared with other researchers. For example, the results may be entered into national databases in the United States so that researchers can ask for permission to use them for research on cancer. These results will be coded with a number and will not include your child's name or other information that could be used to identify your child. This code helps to keep your child's identity confidential. Each person's genetic information is unique to them. This means that there is a very small chance that someone could link the results back to your child. This risk of this happening is very small. However, as genetic testing becomes more common, this might become easier in the future and

there might be risks that we do not know about today. We will strive to take all precautions to prevent this from happening.

For future studies using data or specimens collected during this study, you will not be contacted for additional consent. Instead, we ask you now if we can seek permission from the ethical review committees at our institutions to use any remaining specimens for new studies. You may still participate in this study if you do not consent to us using your child's samples for future scientific studies about Burkitt lymphoma. If you check "no," then samples will be stripped of the study identification number in the database after the completion of this study, and the samples from your child will be destroyed. If you or your child changes your mind in the future, you may contact Dr. John Michael Ong'echa (0733-447920) or the KEMRI Ethical Review Committee, PO Box 54840, Nairobi 00200 at (020) 272-2541 or the Director of KEMRI, PO Box 54840, Nairobi at (020)272-2541. At your request, any existing samples linked to your child will be destroyed. However, you will not be able destroy any results that have already been used or shared. The research being conducted with these specimens and results will not directly benefit your child but the hope is that they will help children in the future.

Consent for use of child's blood samples for future studies yes no
(Please circle parent's response)

There is no immediate benefit of enrolling your child in this study. Transport home will be provided by the study if you child participates.

Summary of your rights as a participant in a research study
Your participation in this research study is voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits to which you are otherwise entitled. If you decide to join the study, you may withdraw at any time and for any reason without penalty or loss of benefits. If information generated from this study is published or presented, your identity will not be revealed. In the event new information becomes available that may affect the risks or benefits associated with this study or your willingness to participate in it, you will be notified so that you can decide whether or not to continue participating.

Contact information
One of our team members named _____ has described to you what is going to be done, the risks, hazards, and benefits involved. Further information with respect to illness or injury resulting from a research procedure as well as a research subjects' rights is available from KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi 00200 at (020) 272-2541 or the director of KEMRI, PO Box 54840, Nairobi at (020)272-2541, or the Coordinator for the Committee for the Protection of Human Subjects in Research in the United States at (508) 856-4261 or write to Committee for the Protection of Human Subjects in Research, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655 U.S.A.

Signature
Signing below indicates that you been informed about the research study in which you voluntarily agree to participate; that you have asked any questions about the study that you may have; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study. By signing this consent form, you do not waive any legal rights, and the investigator(s) or sponsor(s) are not relieved of any liability they may have. A copy of this consent form will be provided to you.

_____
Printed Name of Participant/Child

If Participant is a minor, the parent/legal guardian must sign below:

_____ Date_____
Parent or Legal Guardian signature

_____ Date_____
Cell phone contact of parent or nearest neighbor/relative

_____
Signature of Witness (if needed) Printed Name of Witness (if needed)

_____ Date_____
Signature of Investigator (Affirming subject eligibility for the study and that informed consent has been obtained).

For study coordinator:

Study assigned unique identification number: BL - _ _ _ _
(number given at time of enrollment on Form 2.1)