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**KAPOSI'S SARCOMA AND MALIGNANT  
LYMPHOMAS IN TANZANIA DURING THE AIDS  
EPIDEMIC**

by

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Front cover image: photomicrograph of a double immunofluorescence staining of a nodular Kaposi's sarcoma (KS) tissue section. The punctate pink nuclear staining represents antibody bound to HHV-8 LANA and the diffuse cytoplasmic green staining is antibody to CD34 in KS spindle cells (SC), x 40.

Rear cover image: photomicrograph of a Burkitt's lymphoma section showing nuclear staining [brown] for EBV-encoded RNA (EBER) by *in-situ* hybridization (ISH). Note the "starry sky" pattern (arrows) representing negatively-staining tangible-body macrophages, x20.

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*This thesis is dedicated with love to my wife Erica,  
children Daniel, Faith and Nancy,  
and parents Rodger and Mfikile Mwakigonja.*



## **ABSTRACT**

### **Kaposi's sarcoma (KS) studies**

Kaposi's sarcoma (KS) is the most frequent AIDS tumor (AKS) and associated with HHV-8, but controversial whether a clonal tumor or mostly a reactive proliferation of endothelial spindle cells (SC). In the present studies AKS and endemic KS (EKS) biopsies were compared for expression of HHV-8-LANA, the lymphatic endothelial (LEC) markers LYVE-1 and Ki-67 (proliferation).

The study material is archival, formalin-fixed and paraffin-embedded (FFPE) surgical KS biopsies and sera from the Muhimbili National Hospital (MNH, Dar es Salaam, Tanzania) at early and late tumor stages of African AKS and EKS cases with cutaneous and oral (OKS) lesions from male, female, adult and juvenile KS patients.

KS histopathological and immunohistochemical (IHC) studies (papers I-III) by immunofluorescence (IFA) showed that: a) LANA+/CD34- cells were more frequent in early than late KS and most expressed LEC markers LYVE-1, D2-40 and VEGFR-3, suggesting that the resident LECs represent an early target of primary HHV-8 infection; b) LANA+/CD34-/LYVE-1+ cells decreased from early (25%) to late (4%) KS while LANA+/CD34+ SC increased suggesting a switch from LEC to VEC, c) LANA appeared better correlated to LYVE-1 (LEC) than to CD34 (VEC) expression suggesting heterogeneous SC permissiveness to HHV-8, d) the number of HHV-8 infected (LANA+) SC (CD34+) cells as well as LANA+ granules per SC nucleus was significantly higher in male vs. female, juvenile vs. adult and oral AKS (OAKS) vs. cutaneous KS lesions; e) similarly, tumor cell proliferation (Ki-67 immunoreactivity) in adult OAKS was significantly higher for male vs. female, juvenile vs. adult and OAKS vs. cutaneous KS lesions respectively; f) a positive correlation was apparently found between LANA+ SC and Ki-67 immunoreactivity (paper II). Together these findings suggest that tumor proliferation correlates with increasing HHV-8 LANA expression supporting the notion of viral (LANA)-driven cell proliferation during KS oncogenesis (paper II); g) a significantly higher viral load was also seen by quantitative RT-PCR in late nodular OAKS compared with cutaneous AKS lesions corroborating IHC results and indicating that the oral cavity is important for entry and/or reservoir for HHV-8 infection (papers II & III); h) the frequency of LANA+ SC in the tumor increases during the evolution of both cutaneous and oral KS lesions, suggesting a correlation between tumor growth and viral replication in the lesions, however, by RT-PCR, serum viral loads appeared to decrease in the corresponding patients (paper III), all sera from early stage (patch-plaque) KS were positive for anti-HHV-8 antibodies while all HHV-8 negative sera were from late-stage nodular KS patients (paper IV). This supports our novel notion of a stage dependent tissue-serum discrepancy in viral loads and HHV-8 antigen expression probably due to virus tissue retention, immune-segregation and/or selective clearance. Thus serum HHV-8 viral loads may not be sufficient for clinical and therapeutic prognostication (papers III & IV); i) the frequency of HHV-8 infection was high among the studied MNH cases and as expected, HHV-8 and HIV infection was more associated with KS compared to non-KS tumors and non neoplastic conditions; k) the findings by IFA and ELISA had a high HHV-8 diagnostic concordance although ELISA seemed to have higher positive predictive value (PPV), specificity as well as a high sensitivity allowing more affordable HHV-8 screening in Tanzania (paper IV). These findings also indicate the importance of routine HHV-8 screening among blood/organ donors to prevent transmission as well as in prospective transplant recipients to predict and possibly prevent iatrogenic KS (IKS) development (paper IV).

### **Malignant lymphoma (ML) studies**

MLs are second to KS as AIDS-related malignancies and have been reported to increase steadily with the HIV pandemic particularly in sub-Saharan Africa including Tanzania. The WHO classification, HIV and EBV association as well as ploidy and heterogeneity of diffuse large B-cell lymphoma (DLBCL) in Tanzania are not well documented. Our current study (paper V) has established that the WHO classification of lymphoid neoplasms can apparently be applied for the diagnosis of Tanzanian ML. Extranodal presentation of ML was frequent particularly for T-cell lymphomas (TCL). Diffuse large B-cell lymphoma (DLBCL) phenotype heterogeneity and frequency at MNH (Tanzania) was similar to that observed in Western countries suggesting applicability of similar, diagnostic, prognostic and therapeutic approaches (paper V). The attended ML at MNH had frequent aneuploidy, EBV infection as well as high DNA indices and cell proliferation (Ki-67). HIV infection was apparently associated with increased ML cell proliferation (paper V).

## LIST OF PUBLICATIONS

- I. Pawan Pyakurel, Fatemeh Pak, **Amos. R. Mwakigonja**, Ephata Kaaya, Thomas Heiden, Peter Biberfeld. Lymphatic and vascular endothelial origin of Kaposi's sarcoma spindle cells during tumor development. *Int J Cancer*. **2006 Sep 15**;119(6):1262-7.
- II. **Amos R. Mwakigonja**, Fatemeh Pak, Pawan Pyakurel, Innocent J. Mosha, Willy K Urassa, Ephata E. Kaaya, Peter Biberfeld. Oral Kaposi's sarcoma (OKS) in Tanzania: presentation, immunopathology and human herpesvirus-8 (HHV-8) association. *Oncol Rep*. **2007 Jun**;17(6):1291-9.
- III. Fatemeh Pak <sup>\*</sup>, **Amos R. Mwakigonja** <sup>\*</sup>, Parviz Kokhaei, Nahid Hosseinzadeh, Pawan Pyakurel, Ephata Kaaya, Gordana Bogdanovic, Galina Selivanova, Peter Biberfeld. Kaposi's sarcoma herpesvirus load in biopsies of cutaneous and oral Kaposi's sarcoma lesions. *Eur J Cancer*. **2007 Jul 10**; 43:1877–1882
- IV. **Amos R. Mwakigonja**, Pawan Pyakurel, Parviz Kokhaei, Fatemeh Pak, Leonard K. Lema, Ephata E. Kaaya, Peter Biberfeld. Human herpesvirus-8 (HHV-8) sero-detection and HIV association in Kaposi's sarcoma (KS), non-KS tumors and non-neoplastic conditions. *Infect Agent Cancer* **2008**, 3:10
- V. **Amos R. Mwakigonja**, Ephata E. Kaaya, Thomas Heiden, German Wannhoff, Juan Castro, Anna Porwit, Peter Biberfeld, Tanzanian malignant lymphomas: WHO classification, presentation, ploidy, proliferation, and HIV/EBV associations (Submitted).

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### **RELATED PUBLICATIONS**

- I. Kaaya EE, Castaños-Veléz E, Ekman M, **Mwakigonja A**, Carneiro P, Kitinya J, Linde A, Biberfeld P. AIDS and non AIDS-related malignant lymphoma in Tanzania. *Afr Health Sci.* **2006 Jun**;6(2):69-75.
- II. Pyakurel P, Pak F, **Mwakigonja AR**, Kaaya E, Biberfeld P. KSHV/HHV-8 and HIV infection in Kaposi's sarcoma development. *Infect Agent Cancer.* **2007 Feb** 2;2(1):4
- III. **Mwakigonja AR**, Kaaya EE, Mgaya EM. Malignant lymphomas and the HIV infection in Tanzania. *J Exp Clin Cancer Res*, **2008**, 27:9.

## LIST OF ABBREVIATIONS

AKS	AIDS associated Kaposi's sarcoma
ARL	AIDS-related lymphoma
BCBL	Body cavity based lymphoma (also called PEL)
BCL	B-cell lymphoma
bFGF	Fibroblast growth factor- $\beta$
Bim	Bcl-2-interacting mediator of cell death
BL	Burkitt's lymphoma
CDK	Cyclin dependent kinases
CGH	Comparative genomic hybridization
CHL	Classical Hodgkin's lymphoma
CHL-LD	Classical Hodgkin's lymphoma lymphocyte depletion
CHL-LR	Classical Hodgkin's lymphoma lymphocyte rich
CHL-MC	Classical Hodgkin's lymphoma mixed cellularity
CHL-NS	Classical Hodgkin's lymphoma nodular sclerosing
CKS	Classic Kaposi's sarcoma
DLBCL	Diffuse large B-cell lymphoma
E2F	DNA binding transcription factor
EBER	EBV-encoded RNA
EBV	Epstein-Barr virus
EKS	Endemic Kaposi's sarcoma
ELISA	Enzyme-linked immunosorbent assay also enzyme immunoassay (EIA)
FISH	Fluorescence <i>in situ</i> hybridization
HC	Hodgkin cell
hCG	Human chorionic gonadotropin
HDMEC	Human dermal microvascular endothelial cell
HHV-8	Human herpesvirus type 8 (also called KSHV)
HL	Hodgkin's lymphoma also Hodgkin's disease (HD)
HL-NLP	Hodgkin's lymphoma nodular lymphocyte predominant
IFA	Immunofluorescence assay
IHC	Immunohistochemistry
IKS	Iatrogenic Kaposi's sarcoma
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus (also called HHV-8)
LANA	Latency-associated nuclear antigen (also LNA-1)
LEC	Lymphatic endothelial cell
LMP	Latent membrane protein
MCD	Multicentric Castleman's disease

MHC	Major histocompatibility complex
MIR	Modulator of immune recognition
ML	Malignant lymphoma
NFkB	Nuclear factor kappa B
NHL	Non-Hodgkin's lymphoma
NK cell	Natural killer cell
ORF	Open reading frame
p53	Tumor suppressor gene
PEL	Primary effusion lymphoma (also called BCBL)
pRb	Retinoblastoma protein or gene
RSC	Reed-Sternberg cell
SC	Spindle cell
TAR	Transactivating responsive region (HIV-1 Tat binding region)
Tat	Transactivator of transcription
TCL	T-cell lymphoma
TNF	Tumor necrosis factor
VEC	Vascular endothelial cell
VEGFR	Vascular endothelial growth factor receptor
v-FLIP	Viral FLICE inhibitory protein (FLICE= FADD-like interleukin converting enzyme; FADD= Fas associated death domain)
v-GCR	Viral G-protein coupled receptor
v-IL-6	Viral interleukin 6
v-IRF	Viral interferon regulatory factor
v-MIP	Viral macrophage inflammatory protein

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## **1. BACKGROUND**

### **1.1 HIV epidemiology**

In 2005 the human immunodeficiency virus (HIV) has infected nearly 5 million and killed more than 3 million people globally and hit sub-Saharan regions the hardest, although major epidemics are now transpiring in Asia, Eastern Europe, and the Caribbean [1]. In Tanzania the national HIV prevalence has increased to a peak of 8.1% in 1995, and gradually decreased to 6.5% in 2004 which is expected to stabilize until 2010 whereas the urban HIV epidemic increased to 12.6% in 1992 and is expected to level to between 10.9% and 11.8% from 2003 to 2010 [2]. It is furthermore predicted that the number of new infections in Tanzania will increase to reach 250,000 per year in 2010 and deaths due to AIDS started in 1985 and will rise steadily to reach 120,000 deaths in 2010, with more females dying than men [2].

### **1.2 HIV pathogenesis**

HIV belongs to the lentiviruses or "slow viruses" subgroup of retroviruses which exhibits a long interval between initial infection and the onset of serious symptoms [3]. Thus after a long asymptomatic period of variable duration, HIV infection results in lowered immunity with increased susceptibility to new and/or normally latent infections and opportunistic tumors [4] characteristic for the late stage called **acquired immune deficiency syndrome (AIDS)**. HIV immunosuppression includes the lytic infection and eventual depletion of CD4<sup>+</sup> T cells which are the primary targets of HIV as well as that of other immune system cells with CD4 molecules on their surfaces [5]. HIV is also able to down-modulate the major histocompatibility complex class I (MHC-1) receptors and to compromise natural Killer (NK) cell functions [6]. The almost unique HIV-1 Vpu viral protein has two major functions, namely: degradation in the endoplasmic reticulum of the CD4 molecule and enhancement of virion release from cells [7]. The resulting progressive HIV immunosuppression as well as other associated viral, host and environmental factors, allow the development and/or reactivation of latent opportunistic infections including that of the oncogenic human herpesvirus type 8 (HHV-8) and the Epstein-Barr virus (EBV) and related opportunistic tumors such as Kaposi's sarcoma (KS) or AIDS-associated KS (AKS) and malignant lymphoma (ML), or AIDS-related lymphoma (ARL) [4, 8, 9]. The viral (HIV) factors promoting oncogenesis include cross-talk or transactivation with HHV-8 by the HIV transactivating (Tat) protein which is also angiogenic and recruiting endothelial spindle cells (SC) in KS tumorigenesis [10-12].

### **1.3 HIV oncology globally and in Africa**

Depending on the region, 25-40% of HIV-1 seropositive patients eventually develop a malignancy predominantly KS and ML as shown with the increased HIV epidemic in the USA [4]. In a study of 12,607 HIV-infected persons in Uganda, 378 (3.0%) cancers [181 (47.9%) prevalent, 197 (52.1%) incident] were identified among participants [9]. Of incident cancers, 137 (70%) were AIDS-defining cancers as KS, ML and cancer of cervix [9] and comparable results were reported in Zimbabwe [13]. Furthermore, several other tumors, referred to as non-AIDS-defining cancers, are also statistically increased in HIV-infected persons including Hodgkin's lymphoma (HL) [13].

### **1.4 Kaposi's sarcoma (KS)**

Kaposi's sarcoma (KS) first described by the Hungarian dermatologist Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin" [12, 14] is a multicentric, angioproliferative, maculo-papular eruption most often presenting primarily in the skin but also in mucosal membranes, viscerae and lymphnodes [12, 15] as a characteristic purplish lesion which blanches to pressure and progresses with woody oedema and skin hyperpigmentation to florid, ulcerative lesions. Previously a rare disease, it has become a major epidemiological concern globally and in particular sub-Saharan Africa including Tanzania mostly because of its association with the HIV infection [Acquired immunodeficiency syndrome (AIDS)-related KS or AKS] [12, 16].

Four different clinico-epidemiological KS forms with similar histopathology are now recognized [17-19] including:

1) AIDS-related KS (AKS): The most aggressive and rapidly growing KS form with early dissemination in the skin and viscera [11]. AKS is the most frequent tumor in human immunodeficiency virus type I (HIV-1) infection where it is frequent among homo-bisexual men and intra-venous (IV) drug users in Western countries but most often seen among heterosexuals in developing countries [20].

2) Classical or sporadic KS (CKS): Originally described [14] as a slow growing, indolent tumor mostly seen in the extremities of elderly Caucasian males of eastern Europe and Mediterranean origin [21].

3) Endemic African KS (EKS): Predominant in eastern and central Africa before the AIDS epidemic [22] and clinically similar to CKS, but also seen in children in a more fulminant and fatal form. The childhood EKS is mostly lymphoglandular with or without skin involvement.

4) Iatrogenic or post-transplantation KS (IKS): seen in immunosuppressed patients, e.g. transplant patients receiving immunosuppressive drugs, emphasizing the importance of immune disturbance as a co-factor in the pathogenesis of IKS and AKS, and possibly also EKS [17].

#### **1.4.1 Virology and pathogenesis of human herpesvirus type 8 (HHV-8/KSHV)**

##### ***Historical perspective***

Early studies suggested an infectious agent for KS [23, 24] and herpesvirus-like particles in tissue culture of KS specimens from different geographic regions were observed more than 30 years ago [25] and a relation to the human cytomegalovirus (HCMV) was proposed [25, 26]. However, in 1994 a new virus initially called Kaposi's sarcoma-associated herpesvirus (KSHV) was characterized in an AIDS-associated KS (AKS) lesion through isolation of DNA fragments of open reading frames (ORFs) 26 and 75 using representational difference analysis (RDA) [27, 28]. Subsequently, KSHV was demonstrated in all clinico-epidemiological forms and stages of KS [25, 29] and identified as human herpes virus type 8 (HHV-8) [30]. It was soon found to be also associated with some rare types of lymphomas in AIDS patients, namely primary effusion lymphoma (PEL) or body-cavity-based-lymphoma (BCBL) and the plasmablastic variant of multicentric Castleman's disease (MCD) [31, 32]. Currently, KSHV/HHV-8 is regarded as the primary pathogenic factor for KS development [25, 33].

##### ***HHV-8 virology and etiopathogenesis***

HHV-8 (KSHV) is a gamma-2 herpesvirus of the *Rhadinovirus* genus sharing significant sequence homology with human and non-human herpesviruses as Epstein-Barr virus (EBV), herpesvirus saimiri (HVS), rhesus rhadinovirus (RRV) and murine gammaherpesvirus 68 (MHV68) [12, 28, 34-37]. HHV-8 is a 140nm large virus, with a genome of approximately 165-170 kb double stranded linear DNA packed into an icosahedral capsid [25, 28], covered by a tegument containing proteins and enclosed during budding by the cell membrane-derived outer lipid layer envelop with various host and virus specific glycoproteins [38]. The HHV-8 genome is flanked by a varying number of highly GC-rich terminal repeats (TR) that surround the central (145kb) long unique region (LUR) that contains all of the viral open reading frames (ORF) [28]. Many of the ORF within the central LUR encode more than one protein species, owing to different splicing or alternative translational initiation sites. The LUR region encodes signalling molecules and other proteins that have homologs in the host genome [28, 39]. The HHV-8/KSHV genome has about 90 identified ORF and around 15 (K1-15) are apparently unique to the virus and over 60 show homology to other rhadinoviridae [25, 38].

Furthermore, it is now clear that viruses often resort to hijacking the cellular machinery to achieve their own purposes and recently it was shown that HHV-8 encodes various proteins with homology to host cellular genes in order to interfere with the regulation of cell signalling (molecular piracy) [12, 40]. Studies have suggested that these pirated genes may help the virus evade immune responses (including interferon-mediated anti-viral responses), disrupting cytokine regulation of cell growth, prevent cell cycle arrest and interrupt activation of apoptotic pathways [12, 40]. Such pirated genes include, complement binding protein ORF 4, interleukin-6 (IL-6)-like protein vIL-6 [ORF K2], the angiogenic interleukin-8 (IL-8) receptor vIL-8R [ORF K2], chemokines ORF K4,

ORF K4.1 and ORF K6, the anti-apoptotic factors vBCL-2 [ORF 16] and K7/vIAP (inhibitor of apoptosis protein) which is homologous to cellular survivin and also the interferon regulatory factor 1 (vIRF1) [ORF K9], D-type cyclin (vCyclin) [ORF 72], FLICE-inhibitory protein (vFLIP) [ORF K13], the adhesion-like molecule (ORF K14), the G-protein coupled receptor (vGPCR) [ORF 74] and the ubiquitin system [viral Ubiquitin E3 Ligases and deubiquitination enzyme (DUB)] [40].

Like all herpesviruses, HHV-8 exhibits a latent (non-productive) and a lytic (productive or replicative) life cycle both of which are characterized by distinct gene expression programmes [25, 33]. During latency, viral genome persists as closed circular episomal DNA [41] within the nucleus of KS tumor cells (SC) and B cells of MCD and other infected mononuclear cells [42] and is tethered to metaphase chromosomes and copied in tandem with host DNA during cell division [43]. During the lytic phase, the viral genomes replicate in a linear form expressing most of the more than 80 viral lytic genes, and produces infectious virions [25, 44]. Lytic virus expression is most frequent in MCD, moderate in KS and relatively rare in PEL cells [42]. HHV-8 latent genes which apparently, are all adjacent in the genome [45] include the latency-associated nuclear antigen type 1 (LANA-1) or ORF73/LANA-1, K13/vFLIP, ORF72/vCyclin, K12/Kaposin, K11.5/vIRF-2, K10.5/LANA-2 and K15/LAMP while lytic genes include K2/vIL-6, ORF 74/vGPCR, K9/vIRF-1, K10.5/K10.6/vIRF-3, K1,ORF16/vBCL-2, K7/vIAP, K3/MIR1, K5/MIR2, K14/viral OX2, viral macrophage inflammatory proteins (vMIPs) [ORF K4.1] [12, 25]. Although both latent and lytic genes play a role in HHV-8 pathogenesis the latent infection appears more important in oncogenesis [25]. Furthermore, only a few regions are transcriptionally active during latency, but in KS tumors, approximately 98% of the spindle cells (SC) solely express transcripts consistent with latent infection [33] thus only a small number of KS tumor cells also undergo lytic infection [44]. Recent studies indicate that these lytic cells might promote KS tumor development through several synergistic mechanisms including: viral spreading to new target cells and induction of KSHV-encoded angiogenic and inflammatory cytokines [44, 46]. Latent HHV-8 infection retains the capacity to reactivate replication in response to appropriate physiologic cues including hypoxia [25, 37, 47] and endoplasmic reticulum (ER) stress [48] as well as by the application of phorbol esters (TPA), sodium butyrate and ionomycin *in vitro* [25, 33, 48]. The regulation of tissue viral reactivation (switch) is important for understanding its transmission and pathogenesis [48] and appears to be triggered by the overexpression of ORF 50 or regulator of transcription activation (RTA) which in turn is induced by the overexpression of X box binding protein 1 (XBP-1) [25, 33, 49]. Furthermore, the TPA-induced lytic cycle appears to be regulated in part, by the mitogen-activated protein kinase (MAPK) pathways [44].

HHV-8 gains entry into host cells through endocytosis mediated by a number of viral glycoproteins (Fig 1) including K8.1A and ORF8 (gB) [50] shown to bind to cell surface heparan sulfate [51, 52] and the cell receptor integrin  $\alpha 3\beta 1$ , respectively [53, 54]. In KS patients HHV-8 is predominantly found in KS spindle cells (SC) but we and others have also demonstrated it in some lymphocytes, monocytes and keratinocytes [55, 56].

#### 1.4.2 Latent Genes in HHV-8 oncogenesis

**ORF73/LANA-1:** Latency associated nuclear antigen type 1 (LANA-1) is a 1,162 amino acids (aa) long phosphoprotein with a molecular mass of 222-234 kDa [34, 57]. It has no recognizable cellular homolog and has a multifunctional role in HHV-8 infection and pathogenesis [34]. LANA-1 is the most frequently expressed, immunogenic, latent antigen and considered thereby, to play an important role in the generation and maintenance of HHV-8 associated malignancies [57, 58].

During mitosis it is tethering extra-chromosomal (episomal) viral DNA to host chromosomes and thereby responsible for the segregation of viral episomes with the chromosomes into new daughter cells [12, 25, 34, 49, 57, 59]. LANA-1 also acts as a transcriptional regulator (activation and repression) including cell cycle regulation by binding to the tumor suppressor genes p53 (repression) and the retinoblastoma protein (pRb) [activation] [25, 57].



repression of its transcriptional activity. These different LANA-1 actions, allow latent HHV-8 infection to promote cell cycle progression and anti-apoptosis resulting in survival and multiplication of the infected cells [12, 45]. LANA-1 has also been reported to have a cell transformation activity by interaction with the cellular oncogene H-Ras and critically, the activation of telomerase [25, 57]. The LANA-1 antigen can easily be demonstrated by immunohistochemistry (IHC)/immunocytochemistry in cultured lymphoma (BCBL-1) cell lines as well as in fresh or formalin fixed, paraffin embedded biopsies (FFPE) as a speckled/punctate nuclear reactivity [12] (Fig 2). It is expressed in most SC of both early and late stage KS of all the different clinical forms (AKS, EKS, CKS and IKS) [12, 29] and can serve as a diagnostic marker in suspected HHV-8 related lesions. Several studies including our own [56, 60], have shown an increase in the number of LANA-1 positive cells during the progression of KS lesions [56, 60] including differential LANA-1 granule counts per cell nuclei in relation to clinical forms and sex allowing the detection and quantification of HHV-8 infection in KS lesions [56, 60]. Of interest, is the question as to whether this HHV-8 LANA quantitative immunoassays can also be used as prognostic/follow-up tools following conventional anti-KS and highly-active antiretroviral therapy (HAART).

**HHV-8 K13/vFLIP:** This pirated vFLIP gene, encoded by ORF K13, inhibits FADD-mediated apoptosis downstream of the Fas receptor and blocks the protease activities of caspases 3, 8 and 9 [25, 61]. vFLIP constitutively activates the NF- $\kappa$ B pathway by direct interaction with the I $\kappa$ B kinase (IKK) complex in the cytosol which also allows activation of the HIV-1 LTR [25, 61] thus activating HIV replication [62] although this pathway alone is insufficient and other factors including Toll-like receptors (TLR2 and TLR9) are also involved [63]. These two anti-apoptotic actions by viral FLIP, allow cell survival, clonal overgrowth and tumor progression [25]. Our previous reports on KS biopsies have shown a clear decrease of apoptosis associated with the concurrent increase of the anti-apoptotic vFLIP and BCL-2 during KS development from early (patch) to late (nodular) stage [64, 65]. Thus the exploitation of these anti-apoptotic pathways by HHV-8 contributes to tumor progression of KS lesions.

**HHV-8 ORF72/vCyclin:** vCyclin (vCYC, K-Cyclin, KSHV-Cyclin and vCyclin-D) is encoded by ORF 72 and has 32% identity and 54% similarity to cellular cyclin D2 with which it shares the ability to stimulate G<sub>1</sub>-to-S cell cycle transition by complexing with CDK4 and CDK6 which phosphorylate pRb to release E2F [12, 25, 65]. However, unlike cellular cyclins, vCYC-CDK6 complexes are resistant to cellular CDK inhibitory proteins (p16, p21 and p27), allowing unregulated cell cycle progression. Furthermore, vCYC/CDK6 complexes also phosphorylate the origin recognition complex 1 (ORC-1) protein thereby stimulating cellular DNA replication.

**K10.5/LANA-2:** K10.5 expresses LANA-2 (vIRF-3), which inhibits p53-mediated transactivation and apoptosis but is expressed latently only in KSHV-associated B-cell malignancies (PEL and MCD) and not in endothelial-derived KS tumors [25, 66].

**K15/LAMP:** The K15 gene encodes a latently expressed protein [the latency-associated membrane protein (LAMP)] with a cytoplasmic domain that contains SH2 motifs, interacts with TRAF (tumor necrosis factor receptor-associated factor) and antiapoptotic proteins, and represses B-cell receptor signalling [25]. One of the two K15 transcripts is expressed latently in PEL cells, is a positional homolog of transforming genes in EBV and HVS, and interacts with growth control proteins [25].

#### **K12/Kaposin**

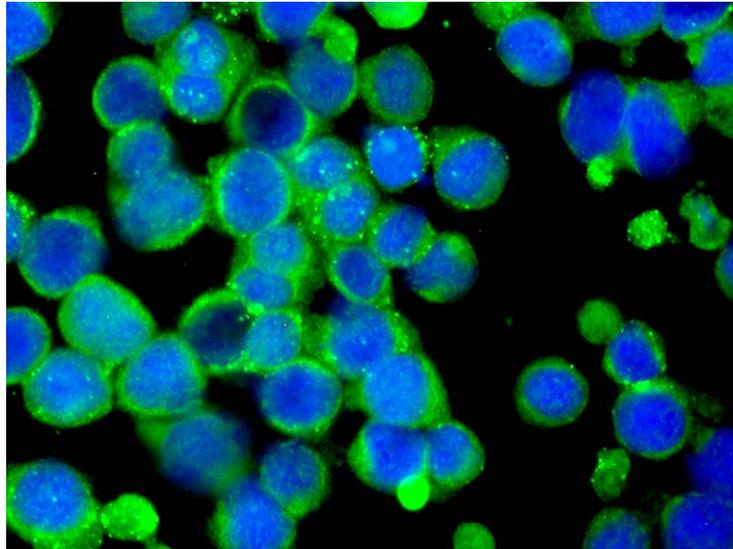
The K12 locus expresses the most abundant latent transcripts in KS and PEL tissue and is also strongly induced in lytic replication [25, 67]. Kaposin is present in three isoforms A, B and C all translated from the same region [25, 67] and Kaposin A transforms cultured cells and drives tumorigenesis when these cells are introduced into nude mice [25, 68]. Kaposin B present in all HHV-8 infected cells enhances the release of pathogenetically important proinflammatory cytokines by activating the p38 mitogen-activated protein kinase (MAPK)-MK2 kinase pathway and blocking cytokine mRNA decay [69].

**K11.5/vIRF-2:** K11.5 expresses a latent homolog of cellular IRFs that inhibits (i) NF- $\kappa$ B and cellular IRF1- and IRF3-mediated transactivation [65], (ii) apoptosis of T-cell receptor (TCR)/CD3-stimulated Jurkat cells and (iii) double-stranded RNA protein kinase [25]. By interacting with cellular transcription factors and cofactors, vIRF-2 may modulate the expression of the early inflammatory genes and potentially deregulate the immune system [70].

#### 1.4.3 Lytic Genes and HHV-8 Pathogenesis

Latent HHV-8 B-cell infection predicts risk of future KS development, and is usually established long before the onset of KS [25]. Therefore, reactivation of productive (lytic) HHV-8 infection in the latently infected B-cell reservoir is correlated with KS development and aggressiveness [25]. Furthermore, treatment of high-risk patients with the antiviral ganciclovir blocks lytic KSHV replication and reduces KS risk [25].

Many lytic genes (Fig 3) encode growth-deregulatory and immunomodulatory proteins which enable infected cells to avoid or inhibit the host immune system [25]. Collectively, these viral proteins counteract multiple levels of the immunological response to viral infection and may play dual roles in growth modulation and immune evasion; for example, antiapoptotic proteins enable proliferation of infected-cells while inhibiting host immunocytolytic activity [25].



*Fig 3: IFA on BCBL-1 cells showing diffuse cytoplasmic lytic (K8.1) staining with positive patient serum (green) and blue DAPI nuclear (DNA) staining x 40.*

**K2/vIL-6:** The viral cytokine vIL-6 retains sequence and functional homology to cellular IL-6 and stimulates multiple cellular pathways to cell proliferation [25]. vIL-6, but not human IL-6, protects PELs and heterologous cells from the antiviral, cytostatic effects of IFN- $\gamma$  and cells stably expressing vIL-6 secrete increased VEGF and induce hematopoiesis, angiogenesis and tumorigenesis when injected into nude mice [25].

**ORF 74/vGPCR:** vGPCR is a 7-transmembrane, IL-8 receptor homolog that constitutively engages pathways downstream of multiple G protein subunits [25] leading to increased transcriptional activity of their nuclear targets with stimulation of cellular proliferation, promotion of cell survival, and transformation [25]. In transient transfections, it was shown that vGPCR also activates promoters of multiple latent and lytic HHV-8 genes. Ultimately, cells expressing vGPCR secrete increased levels of autocrine and paracrine cytokines and growth factors (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8,

granulocyte-macrophage colony-stimulating factor, VEGF, bFGF, and MCP-1), as well as chemotactic factors including expression of adhesion molecules VCAM-1, ICAM-1 and E-selectin [25]. In transgenic mice, vGPCR induces multifocal, angioproliferative, KS-like lesions [25].

**K9/vIRF-1 and K10.5/K10.6/vIRF-3:** In addition to the latently expressed vIRFs discussed above, HHV-8 encodes two other homologs of these proteins [25]. vIRF-1 (K9), transforms cells in culture, is tumorigenic in nude mice, and inhibits apoptosis [25]. It also blocks programmed cell death mediated by cooperation of the cellular protein GRIM19 with IFN- $\gamma$  and retinoic acid [25]. Antisense inhibition of K9 expression in PELs demonstrated a critical role for IRF-1 in reactivation of HHV-8 from latency and lytic gene expression [25]. The other lytic viral IRF, vIRF-3, is most closely related to the cellular IRF-4 and the latent vIRF-2 and also blocks IFN signalling [25].

**K1:** K1 and K15 are positional homologs of transforming genes in EBV and HVS [25]. K1 encodes a transforming and immunomodulatory protein and it is the most highly divergent ORF in the KSHV genome. Mice transgenic for K1 develop KS-like tumors and plasmablastic lymphomas in which there is constitutive activation of NF- $\kappa$ B, Oct-2, and Lyn. K1 may also assist immune evasion of infected cells by its ability to inhibit transport of B-cell receptor complexes to the surface of B cells [25]. The K1 product is a transmembrane protein localized to the cell surface that signals constitutively through its cytoplasmic immunoreceptor tyrosine activation motif (ITAM), activating the well-characterized cellular nuclear factor of activated transcription (NFAT) growth control pathway [25]. This function of K1 is critical for HHV-8 replication, since truncation of the ITAM creates a mutant K1 that represses viral replication following reactivation from latency, in a dominant negative fashion.[25].

**ORF16/vBCL-2:** The product of HHV-8 ORF16 retains the highest (60%) sequence similarity to human bcl-2 in its BH1 and BH2 domains but little similarity in the BH3 domain and it is expressed in a lytic pattern in spindle cells and monocytes of KS lesions, and its transcript is lytically induced in PEL cells [25]. vBcl-2 is antiapoptotic; unlike its cellular counterpart, it can inhibit apoptosis induced by HHV-8 vCyc in the presence of high cdk6 and unique to vBcl-2, it cannot be converted to a proapoptotic form by caspase-mediated cleavage [25]. The antiapoptotic mechanism of vBcl-2 may be attributable to its interaction with the proapoptotic cellular protein Diva, which binds to the caspase-9 regulator Apaf-1 to prevent Bcl-XL from blocking cell death [25].

**K7/vIAP:** The 19- to 21-kDa glycoprotein product of K7 is a homolog of the cellular protein survivin-deltaEx3, a splice variant of human survivin, that protects cells from apoptosis [25]. vIAP localizes to mitochondrial membranes and inhibits apoptosis induced by the Fas and TRAIL pathways, Bax, TNF- $\alpha$  plus CHX, staurosporine, ceramide, and other chemicals [25]. It targets two critical arms of the early and late cellular apoptotic responses and acts as a protein bridge to help target Bcl-2 to activated caspase-3 and inhibit its function as an effector of cell death [25]. vIAP also binds to the cellular calcium-modulating cyclophilin ligand to enhance the cytosolic Ca<sup>2+</sup> flow and protect cells from mitochondrial damage and apoptosis [25].

**K3/MIR1, K5/MIR2:** The modulators of immune recognition (MIRs) are eponymous proteins that actively eliminate the cell surface expression of receptors recognized by the cytolytic arm of the immune system [25]. Both MIR1, encoded by K3, and MIR2, encoded by K5, specifically increase the rapid endocytosis of mature major histocompatibility complex MHC class I from the surface of infected cells and stimulate its degradation by cellular proteases; MIR2 but not MIR1 also stimulates the scavenging of B7.2 and ICAM-1 proteins from the surface [25]. Both MIR proteins are expressed in KS tissue in a lytic pattern and selectively target MHC class I but not class II; however, although K3 targets all four HLA allotypes, K5 specifically targets HLA-A and HLA-B [25].

**K14/viral OX2:** K14 encodes a homolog of the cellular OX2 protein, a glycosylated cell surface protein that is a member of the immunoglobulin superfamily and restricts cytokine production in a paracrine fashion [25]. Viral OX2 shares all of these structural cellular features but instead potently

activates inflammatory cytokine production (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) [25] important for KS pathogenesis.

**Viral macrophage inflammatory proteins (vMIPs) [K6/vMIP, K4/vMIP-II and ORF K4.1/MIP-III]:** The vMIP-I, v-MIP-II, and v-MIP-III proteins are homologs of human MIP-I. While vMIP-I is most probably a product of DE transcription, vMIP-II and vMIP-III are encoded together on an IE mRNA [25]. Furthermore, vMIP-I and vMIP-II both engage the chemokine receptor CCR-8 [25] and are highly angiogenic in chicken CAMs [25, 71]. Treatment of PELs with vMIP-I induces the secretion of VEGF-A, and dexamethasone-induced apoptosis of PELs is blocked by exogenous vMIP-I and vMIP-II (306). vMIP-III engages the CCR-4 chemokine receptor, is a selective chemoattractant for Th2 cells, and is also angiogenic in CAM assays [25].

**HHV-8 microRNAs:** Recently, MicroRNAs (miRNA), small non-coding RNA molecules which regulate expression of genes by binding to the complementary messenger RNAs have been identified in HHV-8 [72]. The HHV-8 miRNAs are confined to a region of the kaposin gene (ORF K12) [72], which is expressed by SC at all KS stages [21] and which can induce tumorigenic transformation of infected cells [73]. Removal of this miRNA may thus hinder the function of kaposin [72]. The viral and cellular genes regulated by these HHV-8 miRNAs still remain to be identified.

#### 1.4.4 HHV-8 Epidemiology

HHV-8 infection rates in various populations (Fig 4) parallels in general, the incidence of KS indicating its etiopathogenetic importance [74]. A high rate of HHV-8 infection has been documented in sub-Saharan Africa (30-70%) [Fig 4], where also the highest prevalence of KS occurs [75-77]. Relatively high or intermediate HHV-8 prevalence is noted in southern Italy and other Mediterranean areas [78]. In Italy, the prevalence varies between less than 10% in the North to more than 30% in the South [78], with the highest rates observed on the islands of Sicily and Sardinia [79]. The lowest HHV-8 prevalence has been reported in northern Europe, Asia and the United States (<10%) [80, 81], where HHV-8 infection is mostly found among homosexual men at risk of HIV infection [82] and patients with sexually transmitted diseases (STD) [83]. Based on the sequence variation in the open reading frame (ORF) K1, HHV-8 is now classified into subtypes A, B, C, D, E and N [84, 85], of which A and C are found mostly in Europe, whereas subtype B predominates in Africa [84, 85]. Subtype D is found in individuals living in the islands of Pacific ocean and Australia [86]. Subtype E has been reported in Brazil [87] and subtype N, a recently found subtype, has been identified only in South Africa [88].

**HHV-8 transmission:** Early sero-epidemiological studies of homosexual men and sex workers indicated that HHV-8 can be transmitted sexually also evident by the demonstration of the virus in semen, cervical secretions and in saliva [89, 90]. The presence of infected cells and free virus in blood of healthy individuals has also been well documented and it is not so clear when it represents a risk for blood borne transmission. Thus HHV-8 transmission has been shown after kidney transplantation [91, 92], suggesting viral reservoirs in tissues (kidney) and occasionally by blood products [93].

In endemic areas like Tanzania where KS is seen in children [16, 75, 94], oral as well as vertical route of virus transmission (transplacental, during delivery or breast feeding) has been suggested [95]. The importance of mother to child horizontal/oral transmission is indicated by the increase frequency of HHV-8 antibodies in infants by age [96] and also indicated by high HHV-8 prevalence within families in endemic areas [97].

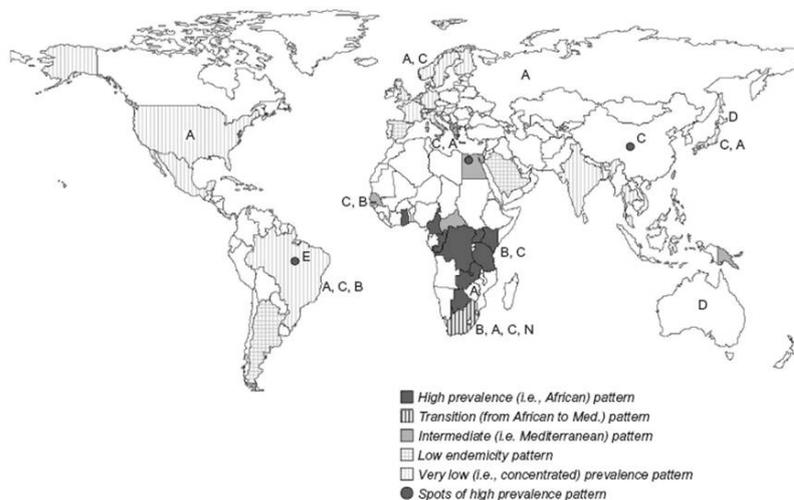


Fig 4: Global epidemiological distribution of HHV-8 infection. Adapted from Dukers & Rezza AIDS 2003, 17(12):1717-1730.

**KS, HHV-8 infection and sex:** Demographically, KS was previously related mostly to males (homosexuals and bisexuals) particularly in developed countries which suggested a possible biological gender or sexual behavior relatedness [20, 56, 98, 99]. However, the continuous narrowing of male to female ratio and high AKS frequency in sexually transmitted disease (STD) patients in sub-Saharan Africa seem to indicate the importance of a heterosexual route of KSHV/HHV-8 transmission [100, 101]. This is supported by reports of a high (1:27) male:female ratio for cutaneous non-AIDS KS (EKS) and a low (1:3) ratio for AKS in eastern Africa including Tanzania and elsewhere [20, 98-100, 102]. Interestingly, the male:female ratio is reported to be even lower (1.3:1) amongst patients with OKS in South Africa [103]. HHV-8/KSHV has been found in oral (saliva) [16, 104, 105] and genital (semen and cervical/vaginal) fluids [20, 104, 106] suggesting an important role for genital-oral and oral-oral HHV-8 transmission in KS development [16, 97, 106]. The sex differences in KS are of interest considering previous suggestions of lower female risk, possibly attributed to hormonal factors including human chorionic gonadotropin (hCG)-in pregnant women [107, 108].

**Reasons for sex differences:** These are yet to be fully clarified although various biological and socio-behavioral factors have been suggested [11]. Recently in a comparative genomic hybridization (CGH) and interphase-fluorescence *in-situ* hybridization (interphase FISH) study, we have reported cytogenetic differences between male and female KS patients including the loss of chromosome Y observed in all early and the majority of late male AKS and EKS representing a clonal genomic change [109]. Moreover, in early stage disease loss of Y chromosome was the only recurrent change found [109]. These features are apparently, mainly related to early male KS pathogenesis and may therefore indicate cytogenetic reasons for the sex differences in KS [109]. This male predominance of KS patients is as yet not fully understood and hormonal factors have been suggested to play a role [110]. Thus *in-vitro* studies on AIDS-KS derived cells indicated that glucocorticoid receptors and IL-6 may play a role in the growth of these cells [111]. Thus IL-6 production in KS-derived spindle cells was shown to be enhanced *in vitro* by glucocorticoids [110]

whereas intralesional injections of human chorionic gonadotrophin (hCG) induced regression of KS growth [111] which is compatible with the observed male KS predominance. However, the well documented male:female ratio decline for AKS [100, 101, 112] in Africa (including Tanzania) appears to reflect the ongoing HIV and AIDS epidemic which impacts more on women of the sexually active age-group. Clearly, the association of sex with KS development and HHV-8 infection has to be further elucidated.

#### **1.4.5 Summary of KS Pathogenesis**

The pathogenesis of KS is still unclear and appears complex, involving mechanisms dependent on viral and cellular factors such as, HHV-8 infection, inflammatory cytokines and angiogenesis by factors including HIV-Tat, as well as factors promoting anti-apoptosis and cell proliferation [55, 113] and microRNAs (miRNA) which have been identified in HHV-8 [72] and can induce tumorigenic transformation of infected cells [73].

**Table 1: Some KS pathogenetic factors**

<b>Pathogenic effect</b>	<b>HHV-8 factors</b>	<b>HIV factors</b>	<b>Host cell factors</b>
<b>Inflammation</b>	Kaposin B, vIRF-2, vGCPR, vOX2, vMIPs	HIV induced cytokines	Host cellular Cytokines
<b>Oncogenes</b>	LANA-1, Kaposin A, K1, miRNA		H-Ras
<b>Transcription factors</b>	LANA-1, vGCPR		
<b>Cell infection</b>	K8.1A, ORF8		heparan sulphate, $\alpha3\beta1$ integrin on ECs, SCs, MPS, B-cells, keratinocytes
<b>Cell recruitment</b>	HHV-8: VEGF, FGF		EC precursors, bone marrow stem cells
<b>Phenotype switch</b>	HHV-8		VEC & LEC phenotypes
<b>Cell proliferation &amp; cell cycle deregulation</b>	vCYC, vIL6, vGCPR	HIV Tat	Ras-ERK, CDK4, CDK6
<b>Cytogenetics</b>			Recurrent X-somal aberrations
<b>Anti-apoptosis &amp; survival</b>	vFLIP, vGCPR, vIRF-1, vBCL-2, vIAP, vMIPs, hTERT		NF- $\kappa$ B
<b>Angiogenesis</b>	vIL6, vGCPR, vMIPs	HIV Tat	VEGF
<b>Immunodeficiency</b>	AKS, IKS	AKS	AIDS, organ transplantation
<b>Immunoreactivity</b>		In AKS	
<b>Immunomodulation</b>	vIRF-2, lytic genes K1, K3, K5		
<b>Sexual behavior</b>	Sexual transmission	Sexual transmission	High risk behavior e.g. MSM, Bisexuals, IVDUs
<b>Sex (gender)</b>	Increased frequency & viral loads in males	Declining male:female ratio in AKS	Male predominance in all forms, Y-losses
<b>Age</b>	Increased viral loads in juvenile KS?	Increased frequency of AKS in the sexually active age-group	EKS & CKS in elderly, lymphoglandular EKS in juveniles, AKS in sexually active agegroup
<b>Ethnic/genetic &amp; HLA</b>	Increased frequency in Mediterranean people but decreased in West Africa		EKS & AKS in Africans, CKS in Caucasians
<b>Socio-economic status/occupation</b>	Oral (saliva) transmission in rural Africa		Low socio-economic status (SES), Life style?
<b>Geographic factors</b>			Land workers? EKS in Africa, CKS in E. Europe & Mediterranean. TPA, butyrates, ionomycin, unsafe blood, arthropods?
<b>Viral activation/replication</b>	vFLIP, K1, ORF 50 (RTA)	HIV Tat	Hypoxia, ER stress, MAPK pathway
<b>Miscellaneous</b>	Parenteral transmission	Parenteral transmission	

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*Fig 5: HHV-8 gene expression (pathogenesis) during SC development and tumor growth. Adapted from Pyakurel et al., Infect Agents Cancer 2007, 2:4.*

#### **1.4.6 Histopathology and histogenesis of KS**

The histopathology of KS is characterized by four hallmarks including an early infiltration of mononuclear (mostly lymphocytic) inflammatory cells, formation of new blood vessels (angiogenesis) appearing as small, irregular endothelial lined (vascular) slits or channels, spindle-cell (SC) development and proliferation and extravasation of erythrocytes (haemorrhage) with variable accumulation of hemosiderin pigments [15]. The SC is the characteristic neoplastic cell in KS. Furthermore, tumor formation has three chronological histopathological stages including patch, plaque (early stages of KS) as well as the advanced/late stage nodular KS. During early stages, KS thus appears as a predominantly reactive (inflammatory) cell infiltration with polyclonal proliferation of SC that can potentially regress, but usually progress to a nodular possibly clonal SC neoplasm [15, 114]. Pathognomonic for the KS evolution from early to late stage tumor lesions is the increased appearance of SC expressing CD34 [hematopoietic stem cell and vascular endothelial cell (VEC)] as well as lymphatic endothelial cell (LEC) markers [115, 116]. The early inflammatory cell infiltrate is decreased in the advanced nodular stage and remains mostly at the periphery of the dense nodular accumulation of SC. Unlike typical late metastatic cancers, KS often appears early as a multicentric tumor, with lesions arising *de novo* by a localized proliferation [117] as well as accumulation of recruited SC and

progenitors [56, 118]. These features and our previous findings indicating heterogeneity of the spindle cell compartment and lack of aneuploidy [64, 119, 120] favour a non-clonal growth of the KS lesion.

Phenotypically characteristic SCs express various (LEC and VEC) endothelial cell markers possibly representing heterogeneous cell populations of varying proportions at different tumor stages [17, 64, 119, 120]. The histogenesis of the SC in KS is elusive and controversial [121] although an endothelial phenotype has been proposed by several studies including findings of infected normal vascular endothelial lining cells in previous studies [21]. Whether SC are vascular (VEC) or lymphatic (LEC) in origin or derive from mesenchymal progenitor cells [115, 122] is still a matter of discussion, although most studies by immunohistochemistry have revealed that SC express lymphatic markers, such as D2-40 [123], LYVE-1 [115] and VEGFR-3 [124]. Furthermore, gene expression, microarray studies also show that KS neoplastic cells are closely related to lymphatic endothelial cells (LEC) coexpressing some blood vascular endothelial cell (VEC) markers and that HHV-8 can infect both LEC and VEC in vitro [125], and that infected LEC had a higher HHV-8 genome copy number than VEC. Thus in-vitro infection of CD34+ human dermal microvascular endothelial cells (HDMEC) with HHV-8 resulted in the upregulation of LEC markers such as LYVE-1 [126, 127].

Cell proliferation is relatively low in KS as shown by previous Ki-67 expression studies and by DNA flow cytometry [64]. No significant difference was observed in these studies between AKS and EKS indicating that the increased clinical aggressiveness of AKS may reflect action of various cytokines including Tat protein produced during HIV infection and by the compromised state of host immune response in HIV infection [11, 56, 64]. A low level of apoptosis in KS lesions, due to strongly expressed Bcl-2 in SC is also suggested to contribute to KS tumor growth [64].

KS spindle-like cells have been shown to occur in peripheral blood cultures of HIV infected patients with KS or at high risk for developing KS [128]. Furthermore, recent studies show that endothelial cells or their precursors residing in donor kidneys may contribute to post-renal transplant KS since KS SC in a female recipient kidney had a male (donor) karyotype and that KS SC expressed the donor HLA antigen [129]. These findings seem to indicate that KS SC and/or their progenitors can be recruited during development of the KS lesion.

#### **1.4.7 KS differential diagnosis**

Clinically KS may sometimes be difficult to differentiate from other dermal lesions as lepromatous leprosy, neurofibromatosis, dermatomycoses, papular skin rash, edematous lesions (woody edema) including elephantiasis and lymphedema [130] and skin hyperpigmentation including acanthosis nigricans [130].

On histology KS lesions may have to be differentiated from: fibroma, fibrosarcoma, leiomyosarcoma, hemangioma, hemangiosarcoma and hemangiopericytoma as well as non-neoplastic lesions including chronic, non-specific inflammation and granuloma pyogenicum/infected hemangioma.

#### **1.4.8 HIV, AIDS, HHV-8 and KS**

KS is globally the most important AIDS-related tumor (AIDS-defining malignancy) and most frequent in sub-Saharan Africa including Tanzania causing significant morbidity and mortality [25, 101, 131]. Previously a rare indolent tumor with male preponderance, AKS

has become with the HIV epidemic a common and aggressive tumor of both sexes with a decrease in male to female ratio from 19:1 to 1.7:1 particularly in East Africa [101]. Epidemiological studies indicate that the risk of developing KS increases from 5-10% in non-HIV infected individuals to 20-70% in HIV infected patients [132]. AKS progresses more aggressively, appears less sensitive to therapy and is more likely to recur post-treatment, than non-AIDS endemic KS (EKS). The oral cavity is frequently involved in AKS (oral AKS or OAKS) and used in staging of advanced HIV disease.

Some studies seem to suggest that patients infected first with HIV followed by HHV-8, progress to KS faster than those first infected with HHV-8 followed by HIV [133] suggesting that an established anti-HHV-8 immunity may have some protective effect. Furthermore, HHV-8 load is higher among HIV infected than no infected patients [11]. There seems to be a cross talk between HIV-1 and HHV-8 as recent studies have shown that HIV-1 replication stimulates HHV-8 production in PEL cell lines and peripheral blood mononuclear cells from KS patients, possibly due to the activating functions of HIV-Tat [134]. ORF50, the major transactivator of HHV-8 lytic cycle can also induce increased levels of HIV replication by interacting synergistically with HIV-1 Tat leading to increased cell susceptibility to HIV infection and transient permissiveness to HIV replication [135].

#### **1.4.9 HIV-Tat and KS**

The increased incidence of KS in patients with AIDS has been shown to be particularly related to the HIV-1 transactivating gene (Tat) protein which stimulates proliferation of spindle cells and inhibits apoptosis. The Tat protein apparently promotes AIDS KS by at least two distinct mechanisms. First, Tat competes with heparin sulfate binding sites with  $\beta$ -fibroblast growth factor ( $\beta$ -FGF) increasing the concentration this potent angiogenic factor [136, 137]. Second, HIV-1 Tat activates HHV-8 replication in BCBL-1 cells and PBMCs from patients with AIDS and PEL or KS [134] thus increasing viral load and expression of a series of viral genes including vGCR, vBCL2, and vIRF1 with oncogenic potential. Thus, evidently Tat promotes tumorigenesis of endothelial cells via stimulated synthesis of vascular endothelial growth factor and anti-apoptotic activity. Notably, the functional activity of Tat protein in the pathogenesis of AKS must clearly involve an intercellular signalling cascade since antibodies to HIV-Tat epitopes were shown to inhibit signalling mediated by Tat [138]. Furthermore, serology studies indicate a deficient anti-Tat response in AKS patients compared to HIV-positive non-AKS subjects [139, 140], indicating the importance of functional Tat to promote AKS development and aggressiveness [141]. Tat-containing supernatants specifically promote *in-vitro* growth of AIDS-KS cells which is inhibited by anti-Tat antibodies [142]. HIV Tat has the ability to activate the Ras-ERK cascade which may be relevant for endothelial cell proliferation and for Kaposi's sarcoma progression [143, 144].

#### **1.5 EBV and cancer pathogenesis**

The Epstein-Barr virus (EBV) is a human herpesvirus usually carried lifelong as an asymptomatic infection [145]. but also causative agent of infectious mononucleosis and several malignant tumors, including Burkitt's and Hodgkin's lymphoma, certain forms of T-cell lymphoma, and some epithelial tumors, such as undifferentiated nasopharyngeal carcinoma and a proportion of gastric cancers [145]. All these tumors are characterised by the presence of multiple extrachromosomal copies of the circular viral genome in the tumor cells and the expression of EBV-encoded latent genes, which appear to contribute to the malignant phenotype [146, 147]. Expression of nuclear proteins (Epstein-Barr nuclear antigens) EBNA-3A and EBNA-3C (but not EBNA-3B) are necessary to establish

lymphoblastoid cell lines (LCLs) appear to be involved in the resistance of BL cells to cytotoxic agents and cell survival [147]. EBNA-3A and EBNA-3C cooperate to downregulate the proapoptotic Bcl-2 family member (Bim) which is a critical regulator of B-cell survival and when expression is reduced a major determinant of lymphoproliferative disease in mice and humans; [147] and uniquely important in the pathogenesis of BL [147]. Thus EBV significantly increases the likelihood for B-cell lymphomagenesis in general, and BL in particular [147]. EBV infected cells show three patterns of latency (Lat I-III).

### **1.5.1 EBV and HIV**

Opportunistic DNA viruses particularly members of the herpesvirus family as EBV and HHV-8, are frequently the aetiological agents of HIV-associated lesions including Kaposi's sarcoma (KS), oral aphthous ulceration, and AIDS-associated oral lymphoma [plasmablastic lymphoma (PBL)] [105, 148]. Circulating EBV is mainly cell-associated (PBMCs) in the HIV-infected population [149]. Plasmablastic lymphoma (PBL) of the oral cavity is an aggressive neoplasm derived from B cell, considered to be the second most common among human immunodeficiency virus (HIV)-associated malignancies. [150] The presence of EBV in oral PBL suggests a direct viral role for the development of HIV-related PBL [150]. It has long been established that EBV and malaria act jointly in the pathogenesis of Burkitt's lymphoma (BL) [151]. However, it is now believed that HIV can also play a role in the pathogenesis of BL [151].

### **1.6 Malignant lymphomas (ML)**

Malignant lymphomas (ML) represent a spectrum of lymphoid neoplasms with varying prognosis including non-Hodgkin lymphoma (NHL), Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL). ML occur worldwide with an increasing incidence both in industrialized countries and Africa [4, 152-154]. Lymphomas represent today an important cause of morbidity and mortality in sub-Saharan Africa, including Tanzania, partly due to the HIV and AIDS epidemic [4, 152-154]. Recently, the World Health Organization [WHO] classification of lymphoid neoplasm which recognizes three major categories, B-cell neoplasms, T/NK-cell neoplasms and HL has been updated but not yet adopted in Tanzania thus hindering the application of comparable preventive and therapeutic measures as in the developed countries. It is now also, well documented but not in Tanzania that ML in HIV and AIDS patients also called AIDS-related lymphomas (ARL), have distinct clinical features including frequent extranodal presentation. As indicated above, the association of some ML with EBV infection is now well established [146] but this is not well documented in Tanzania. The DNA ploidy and subclassification of diffuse large B-cell lymphoma (DLBCL) is also not yet documented in Tanzania which issues are being examined in the current studies.

### **1.7 Therapeutic implications**

**1.7.1 KS:** Primary single (non-AKS) EKS,CKS & IKS lesions may benefit from low-dose localized radiotherapy (XRT) as well as intralesional chemotherapy AKS can be controlled by effective highly active anti-retroviral therapy (HAART). Severe oro-cutaneous or symptomatic visceral KS requires systemic XRT and chemotherapy with low-dose liposomal doxorubicin [155]. However, in developing countries including Tanzania, chemotherapy is rather expensive and offered to some while XRT is offered free-of-charge to all cancer patients. In Tanzania half-body irradiation is usually given to patients with local-regional KS while sequential ½-body XRT is offered to those with systemic disease.

It is also noteworthy that the only oncotherapeutic hospital in Tanzania-the Ocean Road Cancer Institute (ORCI)-is now also a care and treatment center (CTC) for HAART which AKS patients also will likely benefit from although follow-up clinical studies have not yet been documented.

#### 1.7.2 ML:

- **NHL:** treatment varies with type but anti-CD20 monoclonal antibody (rituximab) is effective in treating low-grade B-cell lymphomas (BCLs). For high-/intermediate-grade NHL the CHOP regimen (cyclophosphamide, doxorubicin, vincristine and oral prednisolone) remains the gold standard treatment and additional rituximab appears to improve the response rate and survival in BCL [155].
- **HL:** In patients with advanced disease more benefit (>50%) has been obtained from the four-drug regimen consisting of mustine, Oncovin (vincristine), procarbazine and prednisone in short courses. This has however, been displaced by new regimens including ABVD (adriamycin, bleomycin, vinblastine and dacarbazine) whose 5-year survival rate is 80% [155].
- In Tanzania, combination chemotherapy (CHOP for NHL and ABVD for HL) remain the mainstay of ML treatment and XRT is offered to those who can not afford drugs.

## 2. STUDY AIMS

### 2.1 General objectives:

1. **KS studies:** (a) the relation of KS development with HIV infection and different anatomical, sex and age settings; (b) to study histopathogenic aspects of KS spindle cells SC, their infection with HHV-8 during tumor development and (c) the role of HHV-8 in promoting spindle cell proliferation, survival and cell recruitment to KS lesions; (d) to evaluate a possible HHV-8 association with non-KS tumors and non-neoplastic conditions and (e) to study and establish a sensitive and affordable HHV-8 serological assay in Tanzania.
2. **ML studies:** to reclassify Tanzanian ML by the last WHO classification studying relation to the biological phenotype and possible viral (HIV and EBV) associations of Tanzanian ML.

Such an improved characterization of cellular and viral factors involved in the induction and development of tumors in AIDS and non-AIDS settings will help to update strategies for prevention, diagnosis, prognostication and anti-tumor therapy.

### 2.2 Specific objectives:

#### A. *KS studies (papers I-IV):*

1. To study the histopathology and presentation of KS (AKS, EKS) at MNH (II & IV)
2. To characterize spindle cells in early and late developmental tumor stages of AKS and EKS with regard to:
  - i. Expression of latency associated nuclear antigen (LANA) (I-III).
  - ii. Expression of lymphatic and vascular endothelial markers and their relationship to LANA positivity (I).
  - iii. Cell proliferation in infected (LANA+) and uninfected (LANA-) KS SC (II).
3. To evaluate and compare lesional KS HHV-8 load, of early vs. late, oral vs. cutaneous and juvenile vs. adult KS (II & III).
4. To evaluate and compare the HHV-8 DNA load and antibody immunoreactivity in patients sera and corresponding patients lesions during KS development (III & IV) and between AKS and EKS (III).
5. To study HHV-8 seroprevalence in patients with KS, non-KS tumors and non-neoplastic conditions (IV).
6. To study and establish a sensitive and affordable HHV-8 serological assay in Tanzania (IV).

#### B. *Studies (paper V) of ML at MNH (Tanzania):*

1. To characterize the presentation, histopathology and immunophenotypes according to the WHO classification.
2. To study the heterogeneity of diffuse large cell lymphomas (DLBCL).
3. To study ML DNA Ploidy.
4. To study the variability in ML cell proliferation by IHC (Ki-67) and flow-cytometry (FC).
5. To study the association of viral (HIV and EBV) infections with ML.

### 3. MATERIALS AND METHODS

**3.1 Tissue biopsies (I-V):** All tissues included in this study were routine, diagnostic surgical biopsies, fixed in (10%) buffered formalin and paraffin embedded (FFPE) at the Department of Histopathology and Morbid Anatomy, MUHAS/MNH, Dar es Salaam, Tanzania.

**3.2 Histopathology evaluation (I-V):** Biopsy sections were stained with hematoxylin and eosin (H&E) and evaluated by standard microscopy. Histopathological diagnosis was complemented by immunohistochemistry for various cellular and viral markers.

**3.3 Immunohistochemistry (IHC)/immunocytochemistry (ICC) [I-V]:** Two immunohistochemistry methods were used in these studies including the immunoperoxidase (IP) [I-V] and the immunofluorescence assay (IFA) [I-III].

**Immunoperoxidase (IP) assay [I-V]:** Immunostaining was done (Immunopathology Lab) by the avidin-biotin complex (ABC) immunoperoxidase technique as previously described [8, 156]. Briefly, tissue sections were mounted on SuperFrost® slides (Menzel GmbH & Co KG, Braunschweig, Germany) deparaffinized, rehydrated and boiled for antigen retrieval at 750W by microwave (6 min) in citrate buffer pH 6. Endogenous peroxidase activity was quenched by incubating the sections in 30% hydrogen peroxide in distilled water (30 min) at room temperature (RT) followed by washing in phosphate-buffered saline (PBS) and incubation with 1:20 normal serum from the species of the secondary antibody and washing (PBS). The sections were incubated overnight at 4°C with primary antibody as previously described [4, 152]. For bound primary antibody detection, the sections were incubated (30 min, RT) with a biotinylated anti-species (secondary) antibody, washed and followed by ABC incubation and developed (visualized) with 3,3'-diaminobenzidine (DAB) chromogen (Sigma-Aldrich, St. Louis MO, USA) as previously described [8, 156]. After PBS washing, the slides were lightly counter-stained with H & E, blued in running tap water (30 min), dehydrated in ascending grades of ethanol, cleared in two runs of xylene and mounted with coverslips using Mountex (Histolab Products AB, Göteborg, Sweden).

Negative controls included sections from tissues not expressing the respective antigens as well as substitution of the primary antibody by buffer. Positive controls included tissue sections (lymph nodes and tonsils) with known expression of the antigen under investigation. These controls were included in each experiment [4, 152].

**Tissue Immunofluorescence assay (IFA) [I-III]:** Single, double and triple antibody IFA was performed as described in Paper I-III, and previously [56]. Briefly, paraffin sections (4µ) were deparaffinized in xylene and rehydrated in descending grades of alcohol (100% - 70%) to distilled water and heated by microwave for 6 minutes at 100°C (in citrate buffer pH 6) for antigen retrieval as previously described [4, 8]. Non-specific antibody binding was blocked by incubation with normal serum (species corresponding to that of the detection system). Incubations with primary antibody were done either at 37 °C for one hour or at 4°C overnight. Detection was performed with anti-species Ig conjugated with biotin and detected with Avidin Cy5 and with anti-species Ig conjugated with FITC or Cy3 and visualised by a fluorescent microscope (Olympus BX60) equipped with digital camera (Sony DKC-5000). Appropriate filters were used for specific FITC, Cy3, Cy5, and DAPI imaging, which were edited and overlaid using Adobe Photoshop 6.0 or 7.0 (I-III).

#### **3.4 Cytology (II-V):**

1. **Cell culture (II-IV):** The HHV-8 infected body cavity-based lymphoma (BCBL-1) cells (kindly provided by G. Gaidano) and cells derived from pleural effusion lymphomas (PEL) [31] were used as positive controls for HHV-8 latent and lytic antibody immunoassays. These were cultured [Immune and Gene Therapy Lab.,

Cancercentrum Karolinska (CCK)] in RPMI 1640 medium (Gibco, BRL, UK) containing 10% heat-inactivated FCS serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C and 5% CO<sub>2</sub>. To induce lytic gene transcription, cells were cultured with 20 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [Sigma Chemical Co., St. Louis, Mo.] as previously described [20, 157], and harvested after 72 hours and washed with PBS and used for cytospin on SuperFrost® slides (Menzel GmbH & Co KG, Braunschweig, Germany). The slides were fixed for 10 minutes in 4 % paraformaldehyde (PFA) and washed before immunostaining as previously described [20].

2. **Cyto-morphometry [IV]:** Lymphoma and control tonsillar nuclei extracted by protease treatment as previously described [158, 159] (see item 3.7 on flow-cytometry below), were stained with DAPI and mounted with Vectashield [Vector Laboratories, Inc. Burlingame, CA, USA] medium for fluorescence microscopy on SuperFrost® slides. The mean size (S) and pleomorphism (P) of DAPI stained tonsillar nuclei were used as subjective unity and were termed S<sub>0</sub> and P<sub>0</sub>. Thus relative nuclear size (RNS) was the percentage size in excess of S<sub>0</sub> evaluated as S<sub>0</sub>+size increase ≤25%=S1, S<sub>0</sub>+increase of 26-50%=S2, S<sub>0</sub>+increase of 51-75%=S3 and S<sub>0</sub>+increase>75%=S4. Evaluation of nuclear pleomorphism was P1=mild, P2=moderate and P3=high. Mitotic figure counts (MFC) were evaluated on routine H & E sections. High power field (HPF) refers to x 400 microscopic magnification.

**3.5 Serum studies: (II-V):** Sera from ante-cubital fossa venous blood were collected in empty-sterile bottles from KS (II-IV), non-KS conditions and ML patients (IV & V) attending the hospital. KS patients' sera were tested routinely for HIV upon counselling by clinicians and stored at -70°C for further studies.

#### **3.5.1 Serology by enzyme-linked immunosorbent assay (ELISA)**

- **For HIV-1 (II, IV & V):** Antibodies to HIV-1 were screened using Enzygnost anti-HIV-1+2 Plus ELISA (Behring, Marburg, Germany) and reactive samples were confirmed on Wellcozyme recombinant anti-HIV-1 ELISA (Murex Diagnostic, UK) as previously described [4, 160].
- **For HHV-8 (IV):** For HHV-8 antibody evaluation an IgG enzyme immunoassay (EIA) Kit (96 Wells, HHV-317-02) from Biotrin International (Dublin, Ireland) was used. This is a direct EIA based on the binding of antibodies to lytic HHV-8 peptide antigens coupled to microtitre test strips. Bound antibodies are detected by an antihuman IgG peroxidase conjugate and a 3,3',5,5'-tetramethylbenzidine (TMB) dark blue substrate reaction (BioFX Laboratories, Inc. Owings Mills, MD). The use of lytic peptide epitopes derived from various HHV-8 viral proteins ensures both a high sensitivity and specificity. The Kit has no detectable cross-reactivity with HIV/EBV antibodies.

**3.5.2 Serology by immunofluorescent assay (IFA) [IV]:** HHV-8 serology by IFA (figure 3) was performed [Swedish Institute for Infectious Diseases Control (SMI) and Immunopathology Lab] on cytospins of BCBL-1 cells [29] using patients sera as well as control lytic (K8.1 and ORF 59) and latent (ORF 73 or LANA) antibodies [Advanced Biotechnologies Inc. (Columbia, MD)] as previously described [5,8]. Results were evaluated and documented by microphotography (Immunopathology Lab).

**3.5.3 Serology controls:** Controls included sera from known HHV-8+ KS patients and the HHV-8 infected BCBL-1 cells provided an internal positive control for IFA sensitivity. Negative controls for both IFA and ELISA included sera from known HIV and HHV-8 negative non-KS patients and PBS.

**3.6 Real-time PCR (III):** DNA was extraction from sera using the Qiamp Blood Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instruction. Real-time PCR (TaqMan) was performed using primers for the HHV-8 ORF26 region. All sera were

tested in duplicates including one inhibitor control with a positive control DNA sample added to the test specimen for control of possible inhibitory activity giving false negative results. The serum HHV-8 DNA load was determined as the mean of duplicate values. The number of HHV-8 genomes in each sample was calculated from an EBV DNA standard (Qi Amp Blood kit, Qiagen GmbH, Hilden, Germany) as previously described [90]. The ABI 7700 Sequence Detection System software created the standard curve by plotting the  $C_t$  values against each known concentration of EBV standard.

**3.7 Flow-cytometric analysis (FC) of cell nuclei [V]:** FC on suspensions of extracted ML and control tonsillar nuclei was performed as previously described [161]. Selected, non-necrotic tumour regions containing  $\geq 70\%$  neoplastic tissue in  $90\mu\text{m}$  thick sections were dissected, deparaffinized, rehydrated and digested for 1 hour at  $40^\circ\text{C}$  with  $0.1\%$  w/v Sigma protease XXIV (Sigma P8038) [Sigma-Aldrich, St. Louis, MO, USA] in Tris buffer [0.1M Tris, 0.07M NaCl (pH 7.2) [Merck, Darmstadt, Germany]. The obtained free nuclei in suspension were stained for 30' with 6-diamidino-2-phenylindole (DAPI) solution ( $10\mu\text{M}$  DAPI in 800mM disodiumhydrogenphosphate) [Sigma D9542, Sigma-Aldrich, St. Louis, MO, USA] and evaluated for DNA content by flow cytometry ( $\geq 2 \times 10^4$  nuclei per histogram). For the FC analysis, a PAS II (Particle Analysing System)-cytometer (Partec, Münster, Germany) and a LSR II Flow Cytometer (BD Biosciences, San Jose, CA) were used. The ModFit Program (Verity Software House; Topsham, ME, USA) was used for cell cycle analysis. Ploidy [DNA index (DI)] of diploid and aneuploid ML cell populations was compared to normal tonsil cells as previously described [158, 159].

**3.8 In-situ hybridization (ISH) [V]:** Detection of Epstein-Barr Virus (EBV) infection was done by automated ISH as previously described [162] (Pathology Cell analysis Lab, Cancer Center Karolinska) using a fluorescein (FITC)-conjugated oligonucleotide probe to EBV-encoded (EBER) transcripts on FFPE tissue sections optimized for use with Bond Polymer Refine Detection (DS9800) and Anti-Fluorescein Antibody (AR0833) on the Bond-max system (Leica Biosystems Nussloch GmbH, Nussloch, Germany) according to the manufacturer's instructions.

**3.9 Statistical analysis:** Data was analyzed using the EPI INFO 6 statistical software (CDC, Atlanta, Georgia, USA) [II & IV] and the Statistical Package for the Social Sciences (SPSS) [SPSS Inc., Chicago Ill] (V). The Fisher exact test was used for smaller sample sizes. P-values of  $\leq 0.05$  were considered statistically significant.

**3.10 Ethical clearance:** These studies were approved by the MUHAS Research Ethics Committee and the Ethical Committee, Karolinska University Hospital Solna (Dnr 01-096).

## **4. RESULTS AND DISCUSSIONS OF THE KS STUDIES (I-IV)**

### **4.1 General demography and frequency of the KS cohort (II)**

Approximately, 700 KS (11.1%) biopsies from 465 males and 235 females of which 488 were AKS (69.7%), 100 were EKS (16.0%) and 112 were of unknown HIV status were registered between 1990 and 2005 at the Department of Histopathology and Morbid Anatomy, MNH, Tanzania. Furthermore, a total of 78 oral KS (OKS) cases, 24 males (30.8%) and 54 females (69.2%) were collected during the period. The median age for OKS of males and females (p-value=0.03) was 38 and 31 years respectively (II).

### **4.2 Clinical presentation of the KS cohort (II-IV)**

Cutaneous KS data from Tanzania are also previously reported by us [10, 11, 15, 16, 20, 56, 64, 102, 118-120], so here the clinical picture of patients with oral KS at MNH is summarized. Most OKS patients (46/78, 59.0%) presented with primary, clinically localized disease but a greater proportion (50.0%, 12/24) of males than females (37.0%, 20/34) presented with disseminated KS at first diagnosis (II). The clinical records seem to indicate that males were 4-times more likely to develop multicentric OKS than females. Palatal KS lesions were most frequent (55.1%, 43/78) followed by the tongue (25.6%, 20/78) whereas KS in buccal mucosa (23.1%, 18/78), and other sites (tonsils, oropharynx) were rare (II).

### **4.3 General Histopathology of the KS cohort (II & IV)**

Of the studied KS cases, most were nodular stage at cutaneous (87.8%) or lymphadenopathic (12.2%) sites (IV) and out of the 78 OKS biopsies, 17 (21.8%) presented with patch KS, 13 (16.6%) with plaque, and 48 (61.5%) with nodular histopathology, respectively. Most 36/48 (75.0%) patients with nodular-stage histology had apparently non-disseminated OKS at first diagnosis (p-value=0.0013) [II] and all children with OKS (n=5) presented with nodular-stage disease (II).

### **4.4 KS spindle cell (SC) immunophenotypes (I and II)**

In both early and late stage AKS and EKS most SC expressed lymphatic markers LYVE-1, D2-40 and VEGFR-3 as well as the endothelial precursor VEGFR-2 (I) and CD34 (I & II), is the later also a marker of bone marrow derived stem-precursor cells. LYVE-1+ was the most frequent SC phenotype in early and late nodular stage KS and the majority of these cells also expressed VEGFR-3 and D2-40 (I). The expression of the lymphatic as well as vascular endothelial cell/endothelial precursor cell markers increased from early to late stage KS. These findings strongly support previous studies suggesting that individual KS SC express a lymphatic, vascular [115, 123, 125], or “mixed” phenotype [17, 64, 119, 120]. Tumor evolution from early to late stage KS thus reflects increase in SC due to both proliferation, accumulation (recruitment of SC precursors) and survival (anti-apoptosis) promoted by HHV-8 viral genes [64] (I & II).

#### **4.4.1 LANA expression and association to KS SC phenotype (I & II)**

Most CD34+ cells in early and late stage KS were immunoreactive for LANA (CD34+/LANA+) but a considerable number of CD34+ SC were LANA- at all AKS/EKS stages (I & II). However, all LANA+ cells were LYVE-1+ in early and late KS and LANA appeared better correlated to LYVE-1 than to CD34 expression [I]. This apparent heterogeneity in viral permissiveness of CD34+ SC seems less compatible with a clonal CD34+ SC proliferation and virus transfer but appears to indicate that also non-infected

CD34<sup>+</sup> SC are continuously recruited from progenitor cells and locally triggered to develop permissiveness to HHV-8 infection (I).

LANA<sup>+</sup> but CD34<sup>-</sup> (I) cells were more frequent in early than late lesions and most of these cells expressed lymphatic endothelial (LEC) markers such as LYVE-1, VEGFR-3 and D2-40 (I), suggesting that the resident LECs represent an early target of primary HHV-8 infection. This is also supported by other studies [125] showing that infected LECs have a higher HHV-8 genome copy number than VECs. Obviously a high viral copy number may result in an efficient maintenance and propagation of episomal HHV-8 DNA in dividing and migrating LECs. Also in-vitro activation of VEGFR-3 has been shown to increase endothelial cell migration and to enhance cell susceptibility to HHV-8 infection and transformation [163]. Hence, the activation of VEGFR-3 (I) in LANA<sup>+</sup>/VEGFR-3<sup>+</sup> SC observed in our study at the early stage of KS thus favours an increased endothelial cell migration (recruitment) and transformation to tumor SC including formation of pathological vascular slits during KS development.

Most likely, recruited endothelial precursor cells become infected in the lesion as evident from the increase in frequency of LANA<sup>+</sup>/CD34<sup>+</sup>/VEGFR-2<sup>+</sup> cells (I) during KS development. All these LANA<sup>+</sup>/CD34<sup>+</sup>/VEGFR-2<sup>+</sup> cells also expressed LEC markers (LYVE-1, VEGFR-3 & D2-40), indicating that after infection these cells apparently undergo a phenotype switch including upregulation of the LEC markers [115, 125, 126]. These findings also corroborate those by Wang and colleagues [125] showing that HHV-8 infects both LECs and VECs in-vitro, driving their gene expression profile closer to that of each other, as well as studies on in-vitro infection of CD34<sup>+</sup> human dermal microvascular endothelial cells (HDMEC) [127] with HHV-8 [126], showing upregulation of LEC markers such as LYVE-1 expression in the infected HDMEC. Our finding that during progression to nodular KS, the proportion of LANA<sup>+</sup>/LYVE-1<sup>+</sup>/CD34<sup>+</sup> cells increases significantly (60% to 85%) could thus indicate that HHV-8 infection promotes (a) the change of LEC towards a VEC phenotype (CD34<sup>+</sup>) (b) changes of VEC towards a LEC phenotype, (c) recruitment of CD34<sup>+</sup> endothelial progenitor cells and (d) accumulation of a mixed population according to (a), (b) and (c).

#### **4.4.2 KS SC and proliferation (II)**

A gender difference was also observed with regard to tumour cell proliferation (Ki-67 immunoreactivity) in adult OAKS with a statistically significant (p-value=0.04) difference in mean Ki-67<sup>+</sup> cell frequency for males (23.5%) and females (18.0%) [II]. This was apparent also from evaluations of proliferating HHV-8 infected SC (LANA<sup>+</sup>/CD34<sup>+</sup>/Ki-67<sup>+</sup>) or triple positive cells which were statistically significantly higher in male (median=19.3%) than in female nodular OAKS (median=9.5%, p-value=0.02) [II] and generally, showed a positive correlation (R<sup>2</sup>≈0.60%) between HHV-8 LANA<sup>+</sup> expression and Ki-67 immunoreactivity in biopsies [II]. These findings apparently indicate that LANA<sup>+</sup> cells have a proliferative advantage [164]. The proliferating LANA<sup>+</sup>/CD34<sup>-</sup> cells (II) probably represent lymphatic endothelial cells since from our study (I) all LANA<sup>+</sup>/CD34<sup>-</sup> cells were LYVE-1<sup>+</sup>. However, the LANA<sup>+</sup>/CD34<sup>-</sup>/LYVE-1<sup>+</sup> cell population decreased during the development of early (patch) to late (nodular) KS stage (I) indicating that in the initiation of the KS lesion, proliferation of resident lymphatic endothelia is necessary, but that later during KS development there is a continuous recruitment of non infected endothelial precursor cells into the KS lesion probably promoted by various inflammatory cytokines (e.g. IL-1, IL-6, TNF) [119, 165] as well as by HIV related angiogenic growth factors (FGF, VEGF, HIV-1 Tat) [32, 141, 166] Moreover, the median Ki-67<sup>+</sup> count also appeared higher in children (22.8%) than adult (17.2%) nodular OAKS [II].

#### **4.4.3 HHV-8 expression (LANA IHC) and viral load (RT-PCR) in KS lesions (II, III)**

##### ***Sex differences in LANA expression and HHV-8 load in KS lesions (II) as well as in HHV-8 antibody expression in serum of KS patients (IV)***

Nodular oral AKS (OAKS) of adult males (despite their small number) had more LANA+ cells (median=67.6%) than females (40.4%), a statistically significant difference (p-value=0.02) [II]. Similarly, LANA+ granules/nucleus (gr/n) in adult nodular OAKS showed a somewhat higher median value in male (18.5gr/n) compared to female (10.0gr/n) biopsies. Concordantly, biopsies from adult male nodular oral AKS had more (median=64.9%) LANA+/CD34+ (SC) per HPF compared to females (45.9%), a statistically significant difference (p-value=0.03) [III].

The apparently higher lesional HHV-8 content (LANA+ cell frequency and granule count/nucleus) in male than female KS lesions as well as the predilection for systemic dissemination and multi-centricity of oral lesions in males compared to females (II), agrees with the notion of KS as a predominantly male disease (II & IV). Thus at the tissue level, males may be more susceptible to HHV-8 infection than females which in part may be due to a cytogenetic predisposition in males [109] as well as other biological factors as discussed (II & IV). Furthermore, The apparent predominance of females [male:female ratio=1:2.3] in the current Tanzanian OAKS cohort (II), is obviously in contrast with the usual male predominance in KS as reported previously [20, 56, 98, 99, 103]. The reasons for this female OAKS predominance are not clear and may include a recruitment bias, but it is noteworthy that other reports from East Africa have recorded a dramatic male:female ratio decline in KS [16, 100, 101] (II).

Evidently, the finding that HHV-8 was more prevalent in male KS, is concordant to our previous reports and others [11, 16, 20, 167] on HHV-8 seroprevalence in KS and corroborates our results (II) that KS is predominantly a male disease in all its forms (paper IV). Reasons for this sex differences are yet not fully clarified although various biological and socio-behavioral factors may be considered (II & IV). Recently in a comparative genomic hybridization (CGH) and interphase-fluorescence *in-situ* hybridization (interphase FISH) study [109], we have reported cytogenetic differences between male and female KS patients including the loss of chromosome Y observed in all early and the majority of late male AKS and EKS representing a clonal genomic change [109].

##### ***Age related HHV-8 expressions in KS lesions (II)***

Evaluation of lesions in children <15 and adults ≥15 years (II) showed that childhood compared to adult OAKS had significantly higher median LANA+, LANA+/CD34+ and LANA+/CD34+/Ki-67+ cells and also higher median LANA+ granule counts (II). That OAKS in children had significantly more HHV-8 tumor cell association (LANA+ cell frequency and granule count/nucleus) than adults (II) may indicate, for unclear reasons, increased susceptibility in children and also emphasizes the importance of horizontal transmission (oral exposure) by vertical mother-to-child transmission [16, 168-170] (II).

##### ***Anatomic (oral-cutaneous) differences in LANA expression and HHV-8 load in KS lesions (II & III)***

Comparison of oral (OAKS) and cutaneous (cAKS) nodular AKS showed that LANA+ cells (II & III) were significantly (p-value =0.03) more frequent in OAKS than cAKS, both in cases matched for stage, sex and HIV-1 status and those that were unmatched (II). Also, LANA+/CD34+ and triple-positive (LANA+/CD34+/Ki-67+) SC appeared to be more frequent in nodular OAKS than cAKS (II). Furthermore, IHC findings were corroborated by viral load (in copy number/μl DNA sample) studies as determined by qRT-PCR (III). No significant difference was seen in viral load in early stage (patch/plaque) of oral (OAKS, mean=125±9) and cutaneous AKS (cAKS, mean=100±15). However, a significant

viral load difference was seen in late nodular lesions with median viral loads of 400 and 270 ( $P < 0.04$ ), for OAKS and cAKS respectively (III).

The apparent higher HHV-8 content (LANA) [II & III] and viral load (III) in oral than cutaneous AKS (cAKS) also indicates that the oral cavity is an important portal of entry and reservoir for HHV-8. In contrast, the skin although most often the primary site of KS development, seems to be less important for transmission particularly in Africa where the oral cavity and saliva reportedly are the most important routes [168, 169].

#### ***KS tumor stage differences in LANA expression and HHV-8 load (II & III)***

Stage-dependent differences in HHV-8 cell expression and viral load of cutaneous KS have been reported by us previously in studies using LANA double-triple IFA [56] as well as by a semi-quantitative PCR assay [102]. LANA IFA studies were now also conducted in oral KS tissues (II). Furthermore, our previous semi-quantitative PCR results needed to be confirmed by a quantitative real-time PCR assay (III). The increase in the number of LANA+/CD34+ (double+) and LANA+/CD34+/Ki-67+ (triple+) cells during KS development from early patch stage to the late nodular stage in cutaneous KS lesions agrees well with the stage-dependent LANA immunoreactivity (II) as well as with the significant higher viral load ( $P < 0.005$ ) found in nodular AKS (mean±sem=270±25) compared with patch/plaque AKS (mean±sem=100±15) as well as in nodular (mean±sem=185±20) vs. patch/plaque EKS (mean±sem=90±5,  $P < 0.005$ ) cases reported in our current studies (III). These results thus indicate as expected that in the tumor, HHV-8 viral load increases concordantly with the frequency of LANA+ SC during the progression of both cutaneous and oral KS lesions indicating a correlation between tumor growth and viral replication in the lesions.

#### ***HHV-8 load in AKS and EKS (III)***

Previously, we have reported no significant difference in the number of LANA+/CD34+ and LANA+/CD34+/Ki-67+ cells between AKS and EKS between biopsies of corresponding histopathological stage [56]. These results were corroborated by our quantitative real-time PCR assay study (III) showing that the comparison between patch/plaque AKS (mean±sem=100±15) and EKS (mean±sem=90±5) had approximately the same viral load, but that it appeared higher in nodular AKS (mean±sem=270±25) than EKS (mean±sem=185±20), which difference however, was not statistically significant (III). These results on AKS-EKS tissues are concordant with our previous report that in HHV-8 loads in sera of AKS and EKS patients were not statistically significantly different although the viral load appeared somewhat higher in AKS than EKS [11]. This further supports the notion of comparable pathogenic mechanisms between the two clinico-epidemiologically distinct KS forms [56]. However, the apparently higher viral content in AKS compared to EKS lesions observed in our studies is concordant with the notion of transactivation between HIV-1 and HHV-8 particularly the fact that HIV-1 Tat has been shown to activate HHV-8 replication in BCBL-1 cells and PBMCs from patients with AIDS and PEL or KS [11, 134] (III). Thus the increased clinical aggressiveness of AKS appears more mediated by HIV-1 factors as immunodeficiency (II) and immune activation than the result of different HHV-8 content in lesions and sera of AKS and EKS patients.

#### ***Differences in LANA expression and HHV-8 load in patients tissues and sera (III & IV)***

Studies comparing HHV-8 content in KS tissues/lesions and the sera of corresponding patients during the disease evolution from early patch to late nodular stage are poorly documented, apart from findings on viral load in peripheral blood mononuclear cells (PBMCs) as correlated to clinical stage [171, 172]. By RT-PCR (III), we observed that the viral load in KS biopsies during progression from patch to nodular AKS was increasing but appeared to decrease in the corresponding patients' sera which differences were statistically significant,  $P < 0.005$  (III). Furthermore, our finding that all sera from early

stage (patch-plaque) KS were positive for anti-HHV-8 antibodies while all HHV-8 negative sera were from LANA+ biopsies of late nodular stage patients (IV), supports our novel notion of stage-dependent tissue-serum discrepancy in viral loads as well as viral antigen and antibody expression probably reflecting virus tissue retention, immune-segregation with selective clearance and development of immunodeficiency during KS evolution (III & IV).

#### ***KS lesional HHV-8 expression and tumor proliferation (II)***

A sex difference was also observed in tumor cell proliferation (Ki-67 expression) in adult OAKS with a statistically significant difference in mean Ki-67+ cell frequency for males (23.5%) and females (18.0%) [p=0.04] (II). This was also apparent from evaluations of proliferating (Ki-67+), HHV-8 infected (LANA+) SC (CD34+) cells (triple positive cells) which were statistically significantly higher in male (median=19.3%) than in female nodular OAKS (median=9.5%) [p=0.02] (II). Furthermore, the frequency of triple-positive (proliferating KS SC) cells was also found to be significantly higher in male (median=17.6%, mean=20.4%) than in female OAKS (median=10.1%, mean=11.2%, p=0.03) [II]. Furthermore, the median Ki-67+ cell count also appeared to be higher in children (22.8%) than adult (17.2%) nodular OAKS. Ki-67+ expression as well as triple-positive (LANA+/CD34+/Ki-67+) spindle cells, were more frequent also, in nodular oral than in cutaneous AKS, which differences were highly statistically significant (p=0.00003 and 0.0002 respectively) [II]. These differences in HHV-8 LANA expression and cell proliferation (Ki-67+) patterns between sexes, age-groups and anatomical sites were supported by a correlation ( $R^2=0.59$  or 60%) observed between viral (HHV-8 LANA) content and cell proliferation in KS lesions (II). These findings thus support the notion of viral (LANA)-driven cell proliferation during KS oncogenesis. However, whether proliferation represents a prognostic indicator in KS as in ML [173] is yet to be clarified.

#### **4.4.4 HHV-8 and HIV-1 serology and immunology (II & IV)**

***HIV-1 serology and CD4 counts:*** The difference between HIV infection amongst KS and non-KS tumor patients was statistically significant (p-value=0.046) [IV]. ELISA studies on all (78) OKS sera showed that 74 (94.9%) were seropositive for HIV (AKS) and 4 (5.1%) cases were negative (EKS) [II]. Cell counts (FACS) of available (31) OAKS patient blood (all HIV+) showed that 25 (80.7%) had CD4 values <10 T-cells/ $\mu$ l and none >50 T-cells/ $\mu$ l. Concordantly, most patients (24/31, 77.4%) had a CD4/CD8 ratio <0.1 compatible with severe immunodeficiency (II). Furthermore, most KS cases 77.5%, (93/120) studied (IV) were HIV+ (AKS) and 22.5% (27/120) HIV- (EKS) [p<0.001] (II). However, most sera from patients with non-neoplastic conditions (70.0%, 28/40) were also HIV+ but most patients (95.8%, 23/24) with non-KS tumor were HIV negative.

***HHV-8 serology:*** All available OKS sera (33 AKS, 1 EKS) which could be screened by IFA microscopy for anti-HHV-8 antibodies were positive (II). Furthermore, most of the total cohort sera (89.0%, 164/184) were HHV-8 seropositive based on either IFA or ELISA indicating a high HHV-8 prevalence also among MNH patients whose biopsies were submitted for diagnosis (IV). HHV-8 seroprevalence was highest (93.3%, n=112/120) for KS cases, followed by non-KS tumors (91.7%, n=22/24) and lowest (75.0%, n=30/40) in non-malignant conditions, which differences were statistically highly significant (p=0.0039) [IV]. Evidently, most (70.3%, 114/162) HHV-8 seropositive patients were also, co-infected with HIV and conversely, most (80.0%, 12/20) HHV-8 seronegative were also non-reactive for anti-HIV antibodies (p=0.003). The HHV-8 seroprevalence ( $\approx$ 90%) in the studied cohort sera was higher than that in the healthy non-hospitalized population of about 50% in Tanzania [11, 20] and of 70% in sub-Saharan Africa. Obviously, this high HHV-8 frequency in patients at MNH implies a potential high risk of parenteral/iatrogenic

transmission for both patients and staff, particularly during blood transfusion and other invasive procedures, as well as for intravenous drug users [20, 74, 174]. The higher (93%) HHV-8 prevalence in KS compared to non-KS tumors and reactive lesions is consistent with a causal relationship [IV].

#### ***HHV-8 serological (IFA and ELISA) assays (IV)***

Although, HHV-8 serology is prone to observer errors, sampling bias (hospital data) and a field that is still evolving methodologically [74], IFA apparently, showed greater sensitivity than ELISA, probably because IFA methods use antibodies to both lytic and latent antigens and that lytic are reportedly more sensitive [20]. However, our finding that ELISA had apparently, higher positive predictive value as well as specificity and still had a high sensitivity was unexpected and particularly favourable as it makes HHV-8 screening more affordable in a resource-constrained country like Tanzania, mostly lacking the cell culture and fluorescence microscopy facilities needed for IFA (IV). Consequently, HHV-8 ELISA can allow larger-scale screening of HIV high-risk groups including blood/organ donors and thereby prevent KS development through early anti-HIV interventions [20, 74]. The IFA method also allows visual evaluation of both lytic and latent anti-HHV-8 antibodies titers, allowing categorization of patients as HHV-8 productive and potentially infectious and/or in a non-productive (latent) but oncogenically more risky phase [12] which can be useful for consideration of clinical or public health interventions (IV).

## **5. MALIGNANT LYMPHOMAS (ML) RESULTS & DISCUSSION [V]**

### **5.1 ML frequency, demography and presentation (V)**

During the period of study, the histopathology unit at MNH received approximately 50,000 biopsies including about 7,000 tumors out of which a total of 336 histologically diagnosed (H &E) lymphoma cases consecutively collected were evaluated. In 311 of these information on patient sex (males 63.3%, n=197/311 and females 36.7%, n=114/311) was available. Their overall age ranged from 4 to 91 years with a mean 31 and median of 30 years respectively.

Data on clinical presentation was available in 281 patients, of which 61.2%, (172) had nodal and 38.8% (109) extranodal disease at diagnosis. Extranodal presentation was found in almost half (49.5%, 54/106) of childhood ML cases and in only 8.3% (9/43) of the elderly. Anatomical sites of extranodal presentation included visceral (12.5%) bone (10.0%) and soft tissues (6.4%) while cutaneous, oral cavity, nasal and ocular in descending order were rare. Further examination of the ML anatomical distribution showed that 45.5% (n=153) had supra-diaphragmatic, 21.7% (73) sub-diaphragmatic and 21.1% (71) disseminated lymphoma.

The finding that about one-third of the ML had extranodal presentation at diagnosis is obviously of therapeutic importance and remarkably concordant with the 30-40% extranodal presentation observed among DLBCLs in Germany [175] but not previously documented in Tanzania. Nodal versus extranodal presentation is reportedly an important factor for determination of ML prognosis and pathogenicity in relation to genotypic and phenotypic differences. Corresponding to other studies [176-179] we also found that TCL were more often associated with extranodal presentation particularly cutaneous and nasal compared to other ML subtypes [176-179].

### **5.2 ML immunophenotypes and WHO histopathological subtypes (V)**

Of 174 biopsies selected and stained by H & E and immunoperoxidase assay (IHC), 158 were confirmed to be ML and 134 (84.8%) NHL, including 112 (83.6%) B-cell lymphomas (BCL) [CD20+, CD3-] and 22 (13.7%) mature T-cell lymphomas (TCL)

[CD3+, CD20-]. The BCLs included 57 DLBCL (50.9%), 23 BL (20.5%) [Figure 10 (b)] and 32 other B-cell lymphomas (28.6%). The TCL included peripheral T-cell lymphoma (unspecified) (15), extranodal NK/T-cell lymphoma (nasal type) [2 cases], angioimmunoblastic T-cell lymphoma (AIL) [2 cases] as well as an anaplastic large-cell lymphoma (1 case) and one jaw and oral cases. Furthermore, 15.2% (n=24/158) cases were HL (CD30+) mostly classical HL (CHL) of the mixed cellularity (MC) subtype but also nodular sclerosis (NS) and lymphocyte rich (LR). The CHL lymphocyte depleted (LD) as well as the non-classical nodular lymphocyte predominant Hodgkin's lymphoma (NLPHL) subtypes appeared rare.

The dominance of B-cell lymphomas, mostly DLBCL also reported in our previous study [156], and the proportions of BL, TCL and HL observed at MNH appear similar to other studies [180, 181] suggesting the applicability of the WHO classification for Tanzanian ML. Furthermore, the diagnosis of TCL and sub-phenotyping of African DLBCL by IHC using CD10, MUM1p, BCL-6 and BCL-2 cell markers as well as the WHO classification of Hodgkin's lymphoma (HL) in the current ML cohort to our knowledge is novel in Tanzania.

### **5.3 DLBCL subtypes (V)**

A total of 27 selected DLBCL biopsies were further subtyped by histopathology and immunohistology. Most (74.1%) DLBCL cases (n=20) showed completely diffuse architecture but 25.9% (n=7) had follicular remnants. Using CD10, MUM1p, and BCL-6 markers together (V), we found that slightly more DLBCL in our series showed an ABC immunophenotype (55.6%, n=15), by comparison to GCB immunophenotype 45% (12). As expected, all DLBCL with follicular remnants were GCB and most (75.0%) of those completely diffuse were ABC. In the present study DLBCL subtype was apparently, not significantly correlated to age-group, HIV serostatus, tumor proliferation (Ki-67+) or DNA index/ploidy status which however, could depend on the small sample size. The observed higher frequency of the ABC than GCB lymphomas, is concordant with several DLBCL studies from North America and Western Europe [182-184], which may support the notion of pathogenic and biological similarity between Caucasian and African DLBCL.

### **5.4 ML DNA ploidy (V)**

Overall, 40% (24/60) of the NHL cases showed DNA aneuploidy and the highest frequency (63.0%, n=17/27) was seen in the DLBCL group followed by TCL (54.5%, n=6/11) while aneuploidy was rare (14.3%, n=1) in BL cases. All three control tonsil cases were diploid. Aneuploid DNA indices (DI) ranged from 1.103 to 2.407 (mean=1.65, median=1.51, SD=0.445) of which most (50.0%) were tetraploid/multiploid ( $DI \geq 1.7$ ) [n=12/24] and 37.5% (n=9) cases were hyperdiploid ( $1 > DI < 1.3$ ). Most of the hyperdiploid ML were again DLBCL (87.5%, n=7/8) and only one TCL (12.5%) was hyperdiploid. Most of the tetraploid ML were either DLBCL (58.3%, n=7/12) or TCL (41.7%, n=5/12) but not BL or other BCL (V). Furthermore, tetraploidy appeared to be more (83.3%, 5/6) frequent among TCL cases compared to DLBCL (47.1%, 8/17).

The 40% rate of aneuploidy found among the Tanzanian ML patients indicates a relatively high prevalence of genomic instability (chromosomal aberrations) and is in general in agreement with previous reports from Western countries [185, 186] but higher than that observed among NHL in a Swedish report [187]. The high DNA indices (triploidy, tetraploidy and multiploidy) found in our current study is comparable to other reports [187, 188], and appears to correlate with biological aggressiveness and poor prognosis. The strong correlation between aneuploidy and tumor proliferation (Ki-67) found in our study was expected as previously reported by others [189] and is indicative of a biological high

tumor grade. There was no association between aneuploidy and HIV infection in our cohort but previous reports suggested higher proliferation and lower DI among HIV-associated lymphomas [190].

### **5.5 ML cell proliferation and HIV association (V)**

The mean cell Ki-67 immunoreactivity for all ML types was 40.4% (median 40.0%, SD 22.8) and ranged from 5-90%. As expected, the highest (mean=80.0%, median=82.5%, range 60-95, SD=12.25) reactivity was found among BL cases followed by DLBCL (mean 42.4%, median 40.0%, range 10-90%, SD 23.5) and closely by TCL (mean 42.3%, median 50.0%, range 10-80%, SD 24.2). Aneuploid ML cases were more frequently associated with high tumor proliferation rates (Ki-67 reactivity) compared to diploid cases (p-value 0.032). Comparison of the Ki-67 index and SPF showed a significant correlation ( $R^2=0.55$ ) between the mean SPF and Ki-67 reactivity. Furthermore, most (77.8%) of the HIV seropositive (n=7/9) ML cases had high ( $\geq 40.0\%$ ) Ki-67 reactivity (V). HIV screening (ELISA) was possible in only 10.4% (35/336) of all ML cases of which 40.0% (14/35) were seropositive. Apparently, most (85.7%, 12/14) of the positive cases were seen in the younger adults (age 18-54). Furthermore, most (77.8%) of the HIV seroreactive (n=7/9) ML showed high ( $\geq 40.0\%$ ) Ki-67 reactivity. HIV association with sex, clinical presentation (disease extent and anatomic location), EBV infection (EBER+ ISH), and DNA index (ploidy) of ML cases appeared not statistically significant which could be due to small samples. The finding that increased tumor proliferation in our current cohort appeared correlated to HIV infection, is concordant with previous reports [190, 191] and supports the notion of viral-associated/driven tumor proliferation as a biological role in the oncogenesis of HIV-related malignancies similar to that reported for HHV-8 and Kaposi's sarcoma (II).

### **5.6 EBV association of ML (V)**

EBER in-situ hybridization (ISH) (V) was evaluated on 37 ML cases of which the majority (51.4%) were positive (19/37). Interestingly, EBER+ appeared correlated to the GCB DLBCL immunophenotype (41%, 5/12) compared to a lesser (33.3%, 5/15) proportion of ABC cases (p-value<0.001). The apparent increased association of the GCB immunophenotype with EBV (EBER+ ISH) infection in our studied cohort, is of interest and is consistent with other reports [192] including that showing close association between EBV and germinal center cells during B-cell development [192] but has not been previously documented in Tanzania.

## 6. GENERAL CONCLUSIONS

**KS:** Lymphatic endothelial (LEC) markers including LYVE-1, VEGFR-3 and D2-40 were the most frequent SC phenotype in early and late nodular stage KS and HHV-8 LANA expression seemed better correlated to LYVE-1 (LEC) than to CD34 (vascular endothelial cell or VEC marker) expression in early KS suggesting that the resident LECs represent an early target of primary HHV-8 infection (I). The expression of both lymphatic (LEC) as well as vascular (VEC) markers was evident in late-stage KS suggesting a possible (LEC-VEC) phenotype switch as well as recruitment of VEC precursors during development of the KS lesion (I).

Oral Kaposi's sarcoma (OKS) is frequent (11%) among KS patients at MNH and is highly associated with HIV infection and advanced (nodular) histological stage (II).

HHV-8 lesional content (LANA immunoreactivity) is higher in nodular oral AIDS-related KS (AKS) lesions of males than females and of children than adults (II).

Males appeared to have larger tumor burden (multicentricity and systemization) although OKS frequency among females is seemingly higher (II) as well as higher HHV-8 seroprevalence (IV).

Oral AKS had higher HHV-8 content (LANA expression) than cutaneous AKS which appeared correlated to tumoral cell proliferation (Ki-67 expression) [II]. Real-time PCR studies also showed higher HHV-8 load in OKS compared to cutaneous KS (III).

HHV-8 seroprevalence was high among studied patients at MNH including AKS and HIV seronegative non-KS tumors (IV).

ELISA showed a combination of high HHV-8 sensitivity as well as higher PPV and specificity compared to IFA which however showed higher sensitivity (IV).

An apparent stage-dependent tissue serum discrepancy in HHV-8 antigen (LANA) and corresponding antibody expression (serology) [IV] was observed. Similarly, using a sensitive real-time PCR for HHV-8/KSHV, an inverted correlation of viral load in serum and corresponding KS biopsies was also found (III) which supports the notion of immune-segregation and/or selective virus clearance during KS evolution (III, IV). This finding suggests serum HHV-8 viral loads are insufficient in evaluating KS natural history, response to therapy and a possible correlation to the clinical picture.

HHV-8 screening of patients at risk of KS and of blood and organ donors particularly in high endemic areas will evidently be of help to prevent development of KS (III, IV).

**ML:** The WHO classification apparently includes the diagnosis of most Tanzanian ML types (V).

Extranodal presentation of ML was frequent particularly for T-cell lymphomas (TCL). Diffuse large B-cell lymphoma (DLBCL) phenotype heterogeneity and frequency at MNH was fully comparable to that observed in Western countries suggesting applicability of similar, diagnostic, prognostic and therapeutic approaches (V).

ML at MNH appeared to have frequent aneuploidy, EBV infection as well as high DNA indices and cell proliferation (Ki-67).

HIV appears associated with increased MNH ML cell proliferation but extended studies are needed to confirm this (V).

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## **9. APPENDIX (PAPERS I-V)**

