KAPOSI'S SARCOMA: GENETIC SUBTYPES AND CLINICAL CORRELATION IN A SOUTH AFRICAN POPULATION

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Declaration

I, Thuraya Isaacs, hereby declare that the work in this dissertation/thesis is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

I would like to dedicate this thesis to my wonderful husband, Feizal, and my three boys, Imraan, Saaleh and Yaseen, for their unwavering support and encouragement throughout this journey; and for all the selfless sacrifices they have made in order for me to complete my degree. I am eternally grateful and I could not have done it without them.

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Last but not least, I would humbly like to thank the patients with Kaposi Sarcoma, without whom this research would not have been possible.

PREFACE: PERMISSION TO EXCEED THE WORD COUNT FOR LITERATURE REVIEW

Permission has been granted by the chair of the Professional Masters Committee, Prof Alan Horn, to exceed the usual word count for the literature review as recommended in the faculty guidelines, due to the complexity of the topic. In addition, the article was published in a journal with no word limit; and the referencing required by the journal and hence the thesis (modified Harvard) substantially increases the overall word count compared to other reference formats/styles such as Vancouver etc.

Abstract

Human herpes virus 8 (HHV8) is the aetiological agent of all forms of Kaposi's sarcoma (KS). Seven major subtypes (A, B, C, D, E, F, Z) based on genetic variability of open reading frame (ORF)-K1, have been identified. Numerous studies point to differing tumorigenic and pathogenic properties of the HHV8 subtypes. The study objective was to determine the prevalence of the HHV8 subtypes in a cohort of clinical and histologically confirmed KS in Cape Town, South Africa, and analyse associations between the different subtypes, clinico-epidemiological forms and clinical presentation of KS.

The clinical data was prospectively collected and recorded on a body diagram and with photographs. Demographic data was retrospectively collected from clinical records. Tissue biopsies were taken for ORF-K1 subtyping.

Out of a cohort of 103, eighty six patients were subtyped; 81 AIDS (aquired immune deficiency syndrome)-KS and 5 African endemic. Subtype A5 (42/86) and B2 (16/86) predominated. B1, B3, A1 and A4 subtypes were identified in 10/86, 9/86, 4/86 and 1/86 patients respectively. A5, B1, B2 and B3 were found in African blacks and individuals of mixed ancestry, while subtypes A1 and A4 are found only in whites and individuals of mixed ancestry.

Subtype A5 was associated with >10 KS lesions at presentation in the AIDS-cohort (32/38, p=0,050), but not in the African endemic patients (2/4, p=0,600). Subtypes A1 and A4 were less likely to be associated with poor risk tumour extension (p=0,031) and A1 was associated with lower likelihood of lower limb involvement (p=0,004).

ABBREVIATIONS	
KS	Kaposi's sarcoma
HHV8	Human herpes virus 8
PEL	Primary effusion lymphoma
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
ACTG	AIDS Clinical Trials Group
TIS	Tumour; immune status; systemic
HAART	Highly active anti-retroviral therapy
KSHV	KS-associated herpes virus
ORF-K1	Open reading frame –K1

CHAPTER 1: Literature review

Kaposi's Sarcoma: Genetic subtypes and clinical correlation in a South African population

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Total word count: 6192

INTRODUCTION

Kaposi's sarcoma (KS) is a multifocal proliferative disorder involving blood and lymphatic vessels. The aetiology of KS has been elucidated in 1994, and is due to KS-associated herpes virus also known as human herpes virus 8 (HHV8) [Chang et al., 1994]. It is also the aetiological agent of B-cell primary effusion lymphoma (PEL) and multicentric Castleman disease [Ablashi et al., 2002]. KS is an AIDS-defining disease and is the most common AIDS-related malignancy, moreover, it is one of the most common cancers in several sub-Saharan countries [Morris, 2003; Wabinga et al., 1993]. With the onset of the HIV pandemic, the prevalence of Kaposi's sarcoma (KS) in some areas of sub-Saharan Africa has risen between three and twenty fold [Parkin et al., 1999].

Six major subtypes (A, B, C, D, E and F) and at least 13 different variants or clades based on genetic variability of ORF-K1 gene sequences, have been identified [Zong et al., 2002]. The different subtypes have been shown to have variable penetrance in various population groups and are distributed along broad geographic and ethnic lines, which may have arisen through ancient human migrations [Hayward, 1999; Hayward and Zong, 2007; Zong et al., 2002; Zong et al., 1999]. Debate exists as to whether different genetic subtypes of HHV8 have different biologic and pathologic properties, which could potentially affect disease prognosis and direct future therapies. This introduction reviews the relevant literature and provides an overview of clinico-epidemiological forms of KS, the genetic subtyping of HHV8, the geographic distribution of subtypes, evolutionary advantage of HHV8, staging and an

appraisal of the literature surrounding a possible association between the different genetic subtypes of HHV8 and pathogenicity of KS.

CLINICO-EPIDEMIOLOGIC VARIANTS

Clinically KS presents as patches, plaques and nodules of varying colour from skin coloured, to red to purple to black/brown, it may present or be complicated by lymphoedema and may be complicated by ulceration (Figures 1-5). There are four principal clinico-epidemiological variants of KS, namely classic KS, African endemic KS, iatrogenic KS and AIDS-KS. As originally described by Mauritz Kaposi, classic KS is mostly seen in elderly Caucasian men, aged 50-60 years, of Mediterranean or eastern European and Jewish origin [Buonaguro et al., 2003]. It is usually chronic and locally indolent, confined to the lower extremities with a good prognosis. Iatrogenic KS has been observed following solid organ transplantation, after prolonged exposure to immunosuppression including exposure to immunosuppressive medication [Buonaguro et al., 2003; Penn, 1983]. The disease course may be chronic or progressive. Spontaneous regression is observed in the majority of patients with iatrogenic KS following discontinuation of immunosuppressive therapy. African endemic KS has four clinical variants: 1) benign nodular disease that resembles classic KS and presents with indolent, localised cutaneous tumours, but affects younger men (aged 25-40 years) 2) a locally aggressive form that mainly affects the extremities, with fungating and exophytic growth, with a tendency to invade the surrounding tissues including bone. 3) a lymphadenopathic form predominantly seen in children (aged <15 years) with a poor prognosis and 4) a florid form with widely disseminated disease and visceral involvement [Buonaguro et al., 2003; Templeton, 1981]. AIDS-related (epidemic) KS was initially described as a fulminant, disseminated form of the disease in male homosexuals, and was later found to be associated with AIDS. It presents with disseminated cutaneous and visceral disease, and affects a younger population [Beral et al., 1990; Biggar et al., 2000; Buonaguro et al., 2003; Dal Maso et al., 2009].

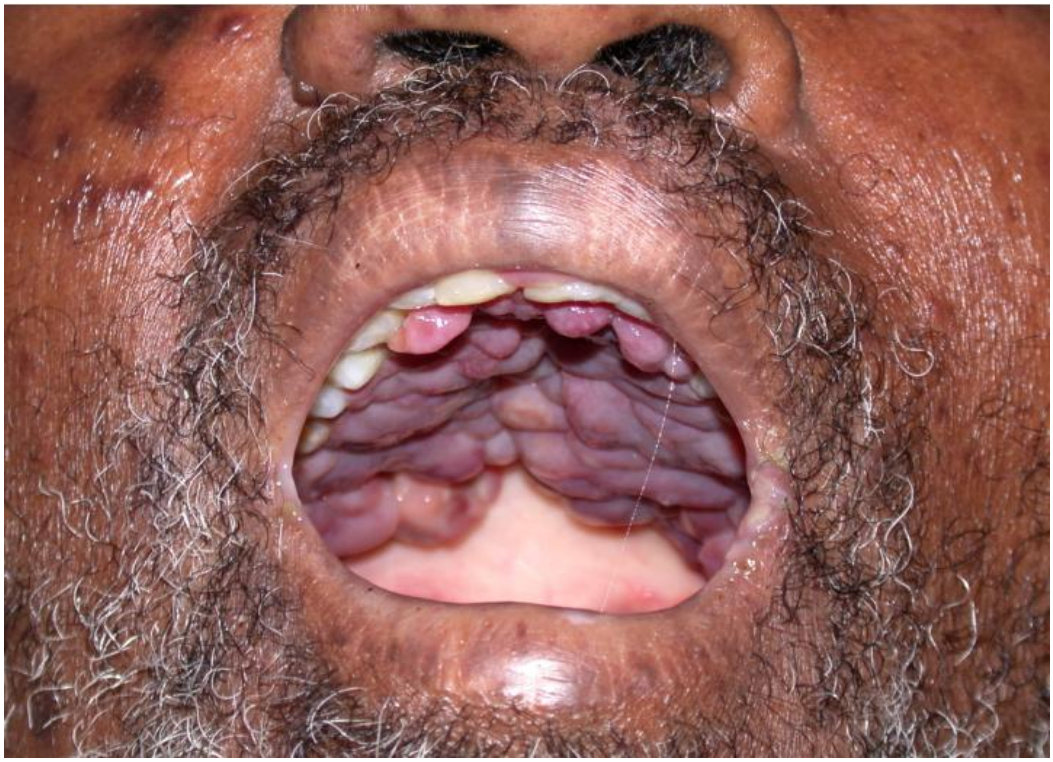

Figure 1. Nodular KS of the oral mucosa

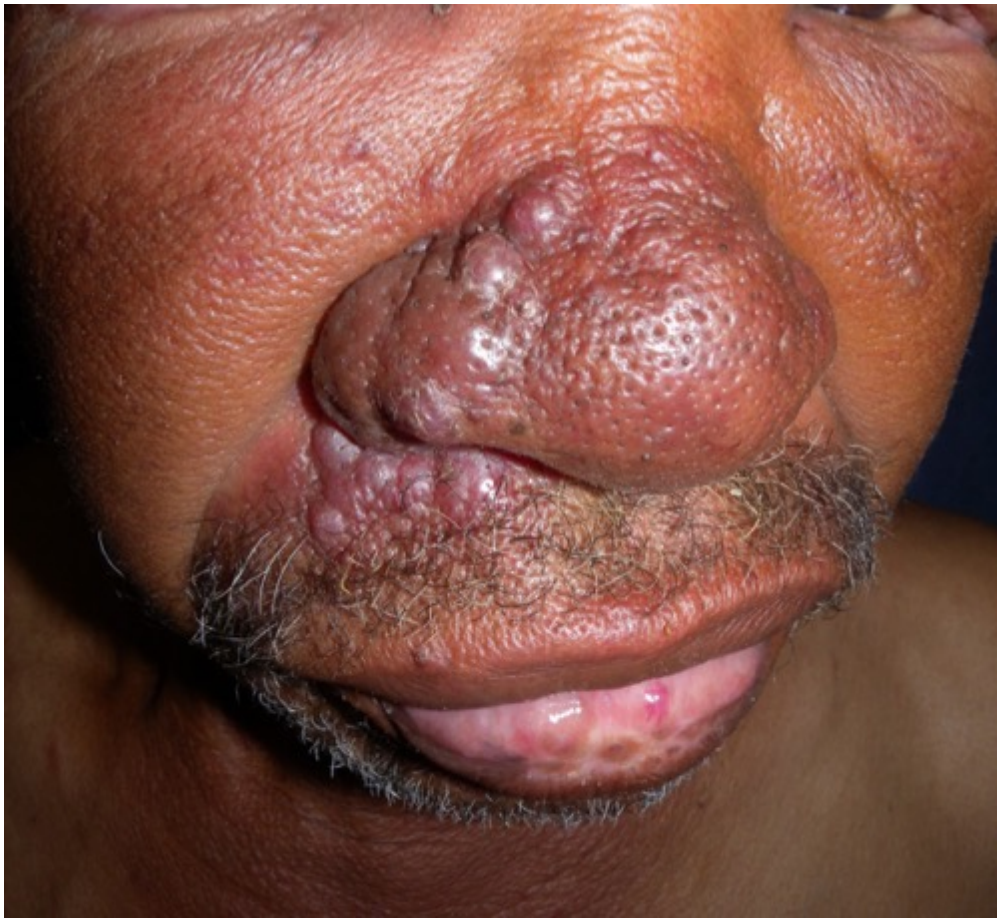

Figure 2. Nodular KS

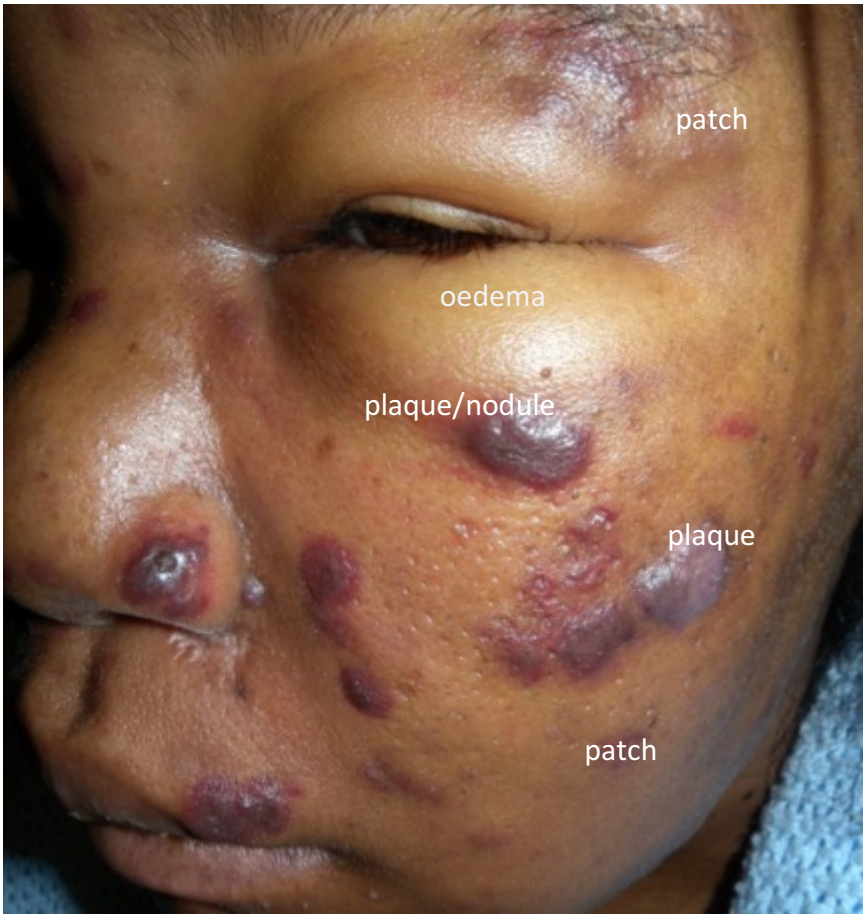

Figure 3. Patch, plaque, nodular KS with oedema

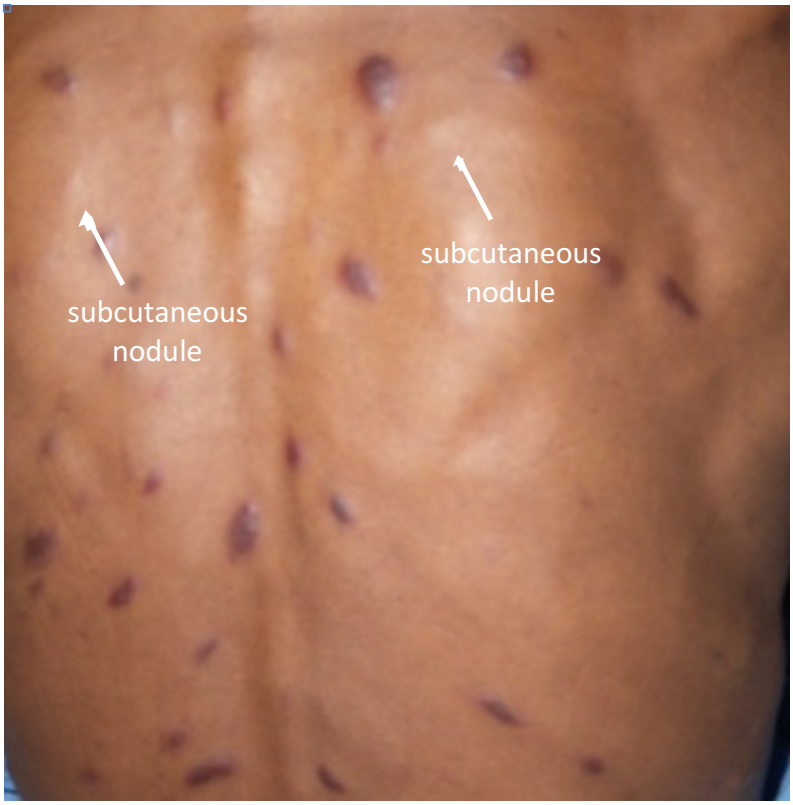

Figure 4. Patch and plaque KS showing subcutaneous nodules

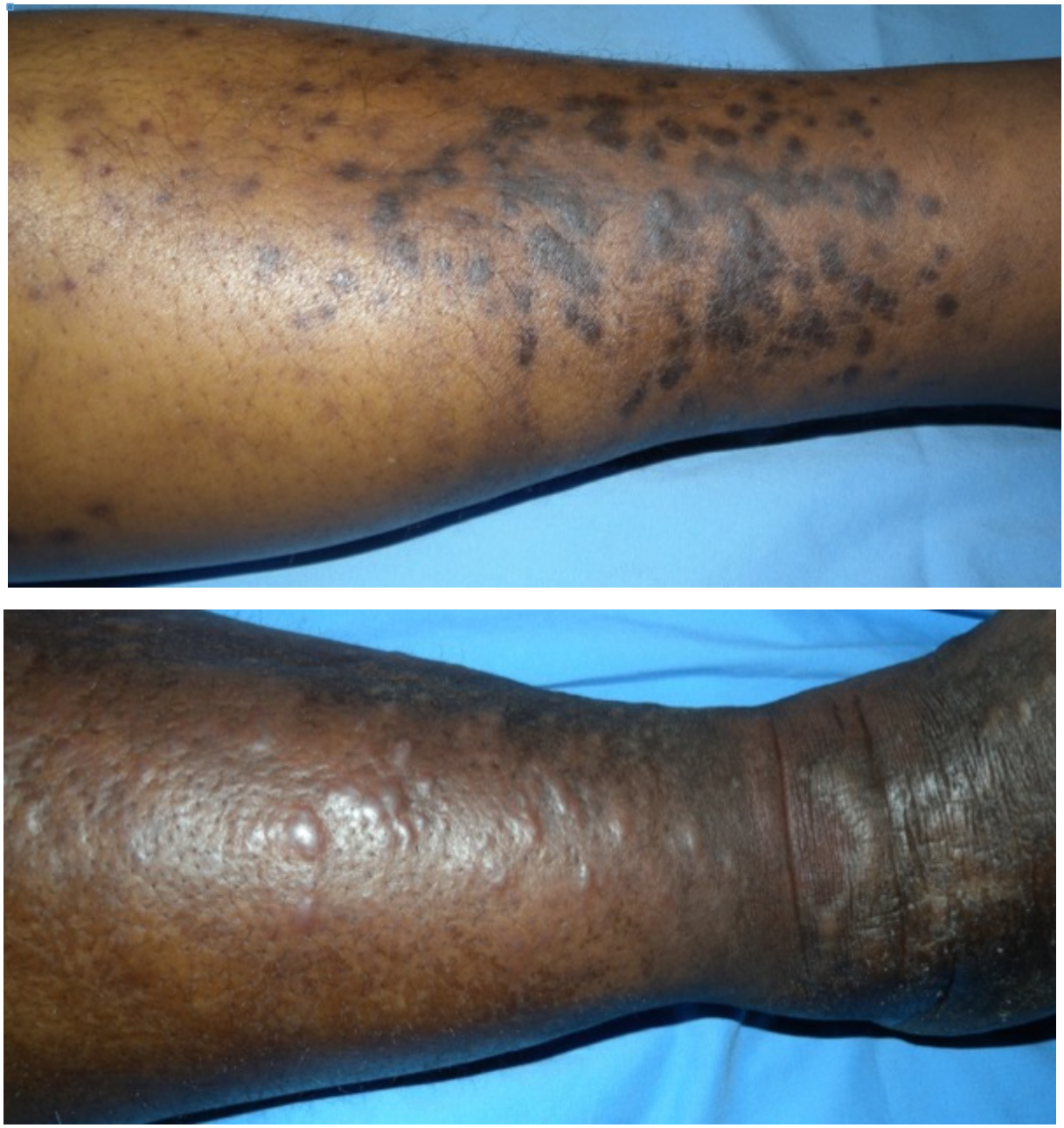

Figure 5. KS with lymphoedema

AIDS KS

With the onset of the HIV pandemic, the prevalence of Kaposi's sarcoma (KS) in some areas of sub-Saharan Africa has risen between three - and twenty fold [Parkin et al., 1999].

In sub Saharan Africa, the AIDS epidemic has led to a dramatic increase in the incidence of Kaposi's sarcoma. In countries such as Uganda and Zimbabwe, the incidence of KS has increased 20 fold to become the most common cancer in males, and the second most common in women [Wabinga et al., 1993]. A South African retrospective record review

analysed the temporal trends in the incidence of KS in blacks in KwaZulu Natal, South Africa over a period of 23 years (1983-2006). Age standardized incidence rates increased from <1 per 100,000 in 1990 to 15 per 100,000 in 2006. The age standardized incidence rates for KS increased 20-fold in men and 50-fold in women [Mosam et al., 2009]. Adult seroprevalence rates of HHV8 in South Africa amongst medical patients and patients with sexually transmitted diseases in KwaZulu Natal increased with age, ranging from 32% in age group 15-34 to 63% among adults aged 35-69 years [Wilkinson et al., 1999].

STAGING AND CLASSIFICATION

In the literature, numerous classifications have been proposed but no universally accepted classification system is available for KS. Some are classified by clinical parameters and others by histological parameters. The first classification was in 1971, when KS was first described in Africa. Taylor et al. described three main categories (1) the nodular or benign form; (2) the localized form characterized by exophytic or infiltrative growth patterns, which may involve underlying bone; and (3) the generalized form which may be either the lymphadenopathic childhood form or systemic disease with or without nodal involvement [Taylor et al., 1971] (Table 4). In 1981, Kyalwaza proposed a new classification for African KS, dividing it into indolent and aggressive disease: the indolent variety being divided into nodular and plaque disease; and aggressive disease being divided into florid, lymphadenopathic, infiltrative, visceral and bone disease. Klein in 1982 proposed yet another classification namely into nodular, generalized lymphadenopathic and locally aggressive types [Cottoni F, 1996] (Table 1).

Table 1. Kaposi's sarcoma clinical classification prior to AIDS epidemic. Adapted from Cottoni, 1996

Taylor et al (1971)	**Kyalwaza (1981)**	**Klein (1982)**
Nodular disease (Benign)	Indolent Cutaneous nodular lesions Plaques	Nodular
Locally aggressive disease Exophytic Infiltrative	Aggressive Florid lesions Lymphadenopathic Infiltrative Visceral Bone	Generalised lymphadenopathic

Generalised Lymphadenopathic Systemic with or without nodal involvement		Locally aggressive type

With onset of AIDS-KS and the presentation of more disseminated disease, a newer classification was proposed based on clinical findings that subdivided the disseminated disease seen in HIV infected homosexual males. This four-stage classification was based on the clinical appearance of tumours and on systemic involvement. It took into account parameters such as skin lesions (localized, generalized, locally aggressive) with generalized defined as more than upper or lower extremity involvement, lymph node involvement, visceral involvement, and presence or absence of systemic signs (A or B symptoms respectively) [Krigel et al., 1983]. Stage 1 is defined as cutaneous, locally indolent disease; stage 2 as cutaneous, locally aggressive with or without lymphadenopathy. Stage 3 is generalized mucocutaneous with or without lymphadenopathy; and stage 4 is visceral disease. Mitsuyaso and Groopman proposed a new four-stage classification for AIDS-KS in 1984 (Table 2). Schwartz divided KS into four stages: localized nodular, localized aggressive, generalised lymphadenopathic and disseminated visceral [Schwartz RA, 1984] (Table 2). Alessi et al divided KS into three classes. This took into account the extent of cutaneous involvement, and the presence of lymph node, mucosal, and visceral involvement as well as the presence or absence of opportunistic infections (referred to as A or B)(Table 2). Problems with these former classifications are that there was an absence of uniform characteristics; and that the meaning of clinical parameters used, such as indolent and aggressive, have not been clearly defined [Cottoni F, 1996]. Cottoni et al. proposed a new classification dividing KS into four stages which included localized, generalized, aggressive and noncutaneous disease based on lesion count, body surface area and visceral involvement [Cottoni F, 1996] (Table 2).

Table 2. Classification of AIDS-KS. Adapted from Cottoni, 1996

	Krigel et al. (1983)	**Mitsuyasu, Groopman (1984)**	**Schwartz (1984)**	**Alessi et al. (1988)**	**Cottoni (1996)**
Stage	**Tumour extension**	**Characteristics of KS**	**Characteristics of KS**	**Characteristics of KS**	**Characteristics of KS**
1	Cutaneous, locally indolent	Limited cutaneous (<10 lesions in one anatomic area)	Localized nodular (>15 lesions or restricted to one	Cutaneous (<5 lesions, or one anatomic area,	Localised (<10 lesions, or body surface

			bilateral anatomic site and few, if any, gut nodules)	+/-lymph nodes)	area <9%)
2	Cutaneous (locally aggressive +/- regional lymph node involvement)	Disseminated cutaneous (>10 lesions or more than one anatomic area)	Locally aggressive (exophytic destructive lesions and locally infiltrative cutaneous lesions)	Cutaneous with >5 lesions >1 anatomic area +/- mucosa +/- gastrointestinal	Generalised (>10 lesions, or body surface area > 9%
3	Generalised mucocutaneous +/- lymph node involvement	Visceral only (gastrointestinal; lymph nodes)	Generalized lymphadenopathic (widespread lymph node involvement, with/without skin lesions and no visceral involvement)	Mucocutaneous +/- lymph nodes and visceral or solely viscera	Aggressive (presence of one exophytic lesion with infiltrative or invasive pattern)
4	Visceral	Cutaneous and visceral	Disseminated visceral KS: widespread KS, with involvement of multiple visceral organs		Noncutaneous: absence of skin lesions
A	Asymptomatic	No systemic signs and symptoms		Opportunistic infections absent and CD4 <500	
B	Weight loss (10%) Fever >38C lasting >2 weeks- not due to identifiable infection	Weight loss >10 % body weight Fever >37,8C for >2 weeks- not due to identifiable infection		Opportunistic infections present	

The AIDS Clinical Trials Group (ACTG) oncology committee sought to develop “easily applied and unambiguous criteria for evaluation of patients with KS “ in order to evaluate efficacy of therapeutic regimes due to the lack of uniform criteria to document disease extent, tumour stage and treatment response. This was initially developed to evaluate and compare therapeutic efficacy in KS patients enrolled on ACTG therapeutic trials, but was also intended for the evaluation and comparison of efficacy of various regimens, as well as correlating features of the disease with prognosis that would facilitate the identification of optimal therapeutic strategies. It identified clinical and immunological parameters that are associated with survival (Table 3). The staging system classified patients into good risk or poor risk groups according to the TIS (tumour, immune status, systemic illness) variables; namely extent of tumour (T0: confined to the skin and/or lymph nodes and/or non nodular

oral KS vs T1: extensive oral KS including nodular oral KS, tumour associated oedema or ulceration, gastrointestinal KS, KS in other non-nodal viscera) , CD4 counts (I0:>200 cells/microliter vs I1: <200 cells/microlitre), and concomitant systemic illness (S0: absence of B symptoms, opportunistic infections vs S1: presence of B symptoms, opportunistic infections) [Krown et al., 1989].

A study of 294 consecutively enrolled patients in 8 ACTG therapeutic trials, sought to prospectively validate the ACTG staging classification for AIDS-KS. They were staged prospectively according to TIS variables and were observed for survival. Univariate and multivariate analyses were done to evaluate the association between TIS variables and survival. Survival was significantly lower in patients in poor risk categories of each of the TIS variables. Median survival for T0 and T1 were 27 and 15 months (p=0,001); for I0 and I1 were 40 and 13 months (p<0,001); and for S0 and S1 were 22 and 16 months respectively (p=0,04). Multivariate analysis showed that immune system impairment (I) was the most important predictor of survival, but also showed that in patients with a high CD4 count whose immune system was least impaired, tumour stage (T) provided significant predictive value [Krown et al., 1997].

Refined Cox models using a lower CD4 count (150 cell/microlitre) provided better discrimination between prognostic groups than the published cutoff of 200 cells/microlitre [Krown et al., 1997]. Limitations of this internal validation of the ACTG classification is that it was conducted within patients enrolled in therapeutic trials; hence may have excluded individuals at both ends of the severity spectrum with severe end organ damage or minimal disease not thought to warrant systemic therapy; and may not be generalizable.

Table 3: The AIDS Clinical Trials Group criteria

	Good Risk (0)	**Poor risk (1)**
Tumour (T)	Confined to skin and/or lymph nodes Minimal oral disease (non-nodular)	Oedema/ulceration Extensive oral KS Visceral KS
Immune status (I)	CD4 <150	CD4 >150
Systemic illness (S)	No thrush or opportunistic infections	Thrush or opportunistic infections B symptoms

	No B symptoms Karnofsky score >70	Karnofsky score <70 Other HIV related disease

Karnofsky score: See Appendix

Nasti sought to externally validate the ACTG staging system as none of the prior studies were conducted in the era of highly active anti-retroviral therapy (HAART). They concluded that CD4 level did not provide prognostic information in the post-HAART era. Only the combination of poor tumour stage (T1) and poor systemic disease (S1) risk, identified patients with unfavourable prognosis. The three year survival rates for T1S1 was 53%, which was significantly lower compared to T0S0 (88%), T1S0 (80%) and T0S1 (81%). Two different risk categories were proposed: good risk (T0S0, T1S0, T0S1) and poor risk (T1S1). Patients with T1 and pulmonary disease (T1p1) were also identified as having a poor prognosis [Nasti et al., 2003b].

A retrospective study in a resource limited setting in Uganda sought to evaluate ACTG staging criteria as predictive of overall survival among Ugandan patients with AIDS-KS. The reason for this external validation was that it had been validated in the United States and Europe, but not in resource limited settings. Multivariate analysis showed that tumour extent (HR 4,33; p <0,001) and systemic involvement (HR 1,63; p<0,01) was associated with survival. Immune status (CD4 count) was not (HR 1,25; p=0,52) [Okuku, 2012]. This confirms findings of Nasti et al [Nasti et al., 2003a; Nasti et al., 2003b].

A newer prognostic index for AIDS-KS in the era of HAART was proposed in 2006 to predict survival in KS. It was developed as a simple model to predict survival based on presenting features at the time of diagnosis of KS; and to aid in planning and evolution of trials and treatments. Variables included ACTG TIS staging; age as a categorical variable, and KS as first AIDS-defining disease. Univariate and multivariate analysis was done to identify covariates predictive of overall survival and this model was externally validated with an independent data set of KS patients. In this prospective cohort study, the authors identified and validated four prognostic factors that could be combined in a calculation to produce a prognostic score of 0 to 15 based on the clinical characteristics at the time of KS diagnosis. Having KS as the first AIDS defining illness, and an increasing CD4 count improved prognosis (in agreement with Krown et al.) while age more than 50 years old and having concomitant

AIDS associated illness conveyed a poor prognosis [Stebbing et al., 2006]. Prognostic scores of 0, 5, 10 and 15 conferred a probability of survival at 1-year of 0,993, 0,967, 0,834 and 0,378; and a 5 year-probability of survival of 0,984, 0,918, 0,631 and 0,084 respectively [Stebbing et al., 2006].

The ACTG and modified ACTG are still the most widely used prognostic indicators for KS in the published literature. Advantages of using this classification system is that it has been validated internally in the pre-HAART era; but it has also been externally validated in both Europe and in resource limited settings. Although the new prognostic index by Stebbing has been externally validated in the post-HAART era; it has not been evaluated in resource poor settings and hence may not be generalizable to our setting. It has also not been widely used even in the current literature. For this reason the modified ACTG system proposed by Nasti was used for the purposes of staging clinical presentation of KS in this study.

HHV8

HHV8 is found consistently in all KS lesions of classic, endemic, AIDS-KS, and iatrogenic KS [Zong et al., 1999]. HHV8 is a gamma 2 herpes virus (family *Herpesviridae,* subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*), that is most similar in structure and gene content to Herpesvirus saimiri (HVS) [Albrecht et al., 1992; Hayward, 1999]. The HHV8 genome harbours many genes that encode for novel proteins that are not found in other human herpesviruses. Many of these genes represent divergent viral homologs of human cellular oncogenes that were pirated by the virus, including viral interleukin-6 (vIL6), viral interleukin-8 (vIL-8) receptor, macrophage inflammatory protein (MIP), and anti-apoptotic proteins of the bcl-2 family [Hayward, 1999; McGeoch and Davison, 1999]. These are involved in activation of intracellular signaling pathways which result in a hyper-inflammatory state, angiogenesis and transformation of endothelial cells, thereby contributing to tumour formation [Wood and Feller, 2008].

HHV8 displays a latent and a lytic phase. The latent phase contributes to immune evasion and to the establishment of persistent viral infection. The major latent viral proteins include latency-associated nuclear antigen (LANA1), viral cyclin (v-cyc), and the viral Fas-associated

death domain interleukin-1B converting enzyme inhibitory protein (vFLIP). Viral G-protein coupled receptor (vGPCR) and vIL-6 are lytic-phase proteins [Wood and Feller, 2008].

LANA1 is essential to maintain latency and may have a role in immune evasion. LANA1 also inhibits the tumor suppressors p53 and retinoblastoma (Rb). This results in impaired apoptosis and activation of genes involved in angiogenesis, cell proliferation, and survival. vFLIP has been associated with cell survival, morphologic change, and inflammatory activation.

Of the lytic phase proteins, vGPCR has the capacity to transform endothelial cells by promoting their uncontrolled proliferation and by inhibition of apoptosis. Other viral lytic oncogenes, such as vIL-6, may also play a role in HHV8 mediated cell cycle dysregulation; however, their role has not yet been defined [Sunil et al., 2010].

HHV8 GENOME

HHV8 is a double-stranded (ds) DNA virus. Whereas the HHV8 genome is circular in the nucleus of latently infected cells; it is packaged into infectious viruses in a linear configuration. The HHV8 genome is mostly highly conserved, except for the two hypervariable regions on the extreme left hand side (LHS)(ORF-K1) and right hand side (RHS)(TMP/ K15) of the genome, which have been used as the basis for genetic subtyping. ORF-K1 is also called variable ITAM-containing protein (VIP) and ORF-K15 is also called latent membrane protein (LMP) or latency-associated membrane protein (LAMP).The central constant segment lies between these two hypervariable regions (Figure 6) [Zong et al., 1999].

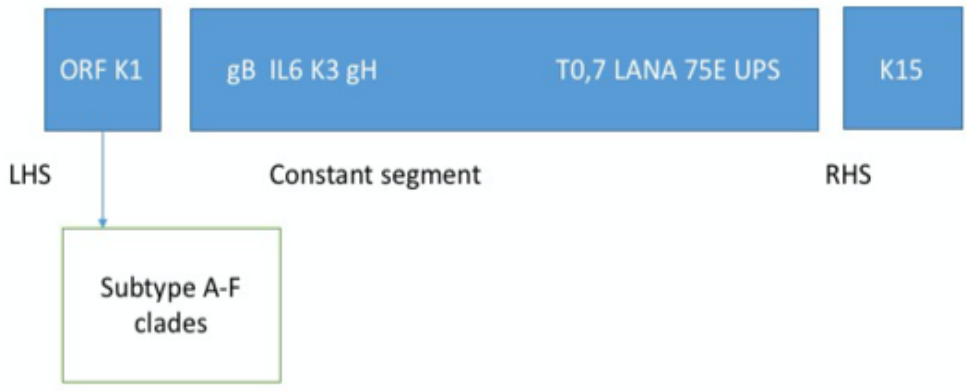

Figure 6. Diagrammatic representation of HHV8 genome and analysed loci of Hayward and Zong

ORF-K1 encodes a transmembrane glycoprotein of 46 kD on the extreme LHS of the genome that shares homology with receptors of the human immunoglobulin superfamily. ORF-K1 functions as a tyrosine kinase signaling protein; it has anti-apoptotic properties and can mimic or block activation of B-cell receptors and may play a role in establishing clinical latency [Hayward and Zong, 2007]. The ORF-K1 glycoprotein is composed of three domains: the extracellular, the transmembrane and the intracellular domain. ORF-K1 shows the highest variability within its nucleotide sequences as compared to other regions in the genome. The extracellular domain is the most variable region in the protein, consisting of two hypervariable regions, VR1 and VR2, with sequence analysis thereof demonstrating up to 85% and 60% divergence at the nucleotide and amino acid levels respectively. Analysis of these hypervariable regions of ORF-K1 has been used as the basis of HHV8 genetic subtyping [Zong et al., 1999].

The central constant region of the genome includes 10 well-characterised internal loci that have been PCR sequenced by Hayward and Zong including ORF-26, T0.7, LANA, and UPS75 [Hayward and Zong, 2007].

Apart from LANA1 the functional significance of the rest of the loci analysed have not been defined.

Unlike the hypervariability of ORF-K1 and TMP/K15 genes, the level of variability at the constant region is much smaller with only 1 to 5 % nucleotide differences, and hence they rarely have impact on primary protein structure and rarely produce amino acid changes [Hayward and Zong, 2007; Zong et al., 2002; Poole et al., 1999]]. The constant region loci can be classified into distinctive ORF-K1 linked patterns. Much greater diversity and variability is found within the constant region of samples from sub-Saharan Africa; the significance of which has not been studied [Hayward and Zong, 2007]. At the T0.7 locus, there are 9 distinct subtypes defined (A/C, F, G, J, K, B, Q, R, N). Of these, subtypes B, Q, R and N are found exclusively within sub-Saharan Africa. The level of variability in Q, R and N are more highly diverged from the Eurasian A/C subtypes. For example, subtype Q differs from Eurasian A/C by 16% nucleotide variability [Hayward and Zong, 2007; Zong et al., 2002; Poole et al., 1999].

The TMP/ K15 gene on the extreme RHS of the genome falls into three allelic subtypes referred to as P (prototype) and M (minor); and a newly recognised novel N subtype. The P allele is the predominant subtype and was found in 46/66 genomes tested and the TMP-M allele was found in 20/66 of genomes [Hayward, 1999; Poole et al., 1999]. The functional significance of these subtypes is not known.

FUNCTIONAL SIGNIFICANCE OF ORF-K1

ORF-K1 and ORF-K15 are the positional analogues (in the same position in the genome) of key latent transforming membrane proteins at the LHS and RHS of the genomes of Epstein-Barr virus (EBV), namely LMP1 and LMP2, and Herpesvirus saimiri (HVS), namely STP and TIP. These membrane proteins, LMP1 and STP, produce proliferative signals in B and T-cells; and LMP1 and TIP block B-cell and T-cell receptor signaling needed to reactivate the lytic cycle [Hayward, 1999]. ORF-K1 of HHV8 has been shown to immortalize T-cells and cause tumorigenesis to an HVS genome that has been depleted of the STP gene [Lee, 1998]. ORF-K1 contains tyrosine kinase interaction motifs that resemble the immunoreceptor tyrosine based activator motif (ITAM) found in B-cell, T-cell and FcR receptors. Hence ORF-K1 may functionally substitute for the ITAMS of B-cells and T-cells [Hayward, 1999].

ORF-K1 encodes for a protein (K1/ VIP) that is involved in cellular transformation. This transforming activity is via the phophatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt)/ mechanistic target of rapamycin (mTOR) pathway. K1 deleted viral cells showed decreased phosphorylation and activation of Akt kinase; and hence decreased viral lytic replication and decreased production of infectious virions [Zhang et al., 2016]

GENETIC SUBTYPING

Zong et al. initially carried out PCR sequencing of 3 small segments of the ORF-26 and ORF-75 genes, from 12 KS specimens [Zong et al., 2007b]. These sequences fell into 3 distinct patterns, and were used to define 3 subtypes (A, B and C). The nucleotide variability between the subtypes was low between 1,0% and 1,5%, with less than 0,1 % variability within each group. The same authors analysed the hypervariable ORF-K1 gene, which shows

the highest level of variability in the HHV8 genome, and subsequent genetic subtyping has been based on ORF-K1.

More recently, Hayward and Zong combined the analysis of three regions, the LHS side ORF-K1, the central constant segment loci and the RHS TMP allele, and have proposed a new classification of the HHV8 genome divided into 12 principal genotypes [Hayward and Zong, 2007].

ORF-K1 SUBTYPES

Different HHV8 strains have been defined by Hayward et al. and most subsequent authors as falling into distinct subgroups based on a system of PCR DNA sequence analysis of the ORF-K1 gene [Hayward et al., 1999]. Variabilities in nucleotide and amino acid sequences were calculated. Zong identified amino acid substitutions at a total of 62% of the 289 amino acid positions amongst 60 different tumour samples from the United States, Central Africa, Saudi Arabia, Taiwan and New Zealand. Initially 4 major subtypes (A, B, C, and D) and 13 distinct variants or clades were defined; of which A and B differed by 30% (varied by 85 amino acids); and A and C differed from each other by 15% (varied by 39 amino acids) [Zong et al., 1999]. B subtype predominated in KS patients from Africa or of African heritage. D subtypes were found only in classic KS patients of Pacific island heritage. C subtypes were found predominantly in classic KS, iatrogenic KS and AIDS-KS in the Middle East and Asia, while three variants A1, A4 and C3 predominated within the United States (US) AIDS-KS samples [Hayward and Zong, 2007; Zong et al., 1999; Zong et al., 2002].

To date, six major subtypes (A, B, C, D, E, and F) and numerous minor variants or clades based on genetic variability of the ORF-K1 gene sequences, have been identified with variable penetrance in different population groups, and are distributed along broad geographic and ethnic lines [Hayward, 1999; Zong et al., 2002; Zong et al., 1999] (Figure 7). In sub-Saharan Africa the B and A5 subtypes have been found to predominate in studies of tissue taken from KS patients [Cook et al., 1999; Fouchard et al., 2000; Hayward, 1999; Kajumbula et al., 2006; Lacoste et al., 2000; Meng et al., 1999; Treurnicht et al., 2002; White et al., 2008; Zong et al., 2002; Zong et al., 1999; Tornesello et al., 2010; Olp et al., 2015;

Kakoola et al., 2001]. They have also predominated in sub-Saharan Africa blood samples from KS and non-KS patients [Kajumbula et al., 2006; Meng et al., 1999; Cook et al., 1999] and buccal swabs from patients without KS [Olp et al., 2013]. A and C subtypes are found in Caucasian KS patients throughout Europe, the United States, the Mediterranean basin the Middle East and Asia [Cook et al., 1999; Zong et al., 1999], and D in the Pacific Islands and Taiwan [Zhang et al., 2008; Zong et al., 1999]. In Brazil there is a 53% prevalence of subtype E in Brazilian Indians, but the study was done on individuals without KS and therefore any association between subtype E and KS development remains to be studied [Biggar et al., 2000]. Recently subtype F has been identified in Ugandans with KS [Kajumbula et al., 2006].

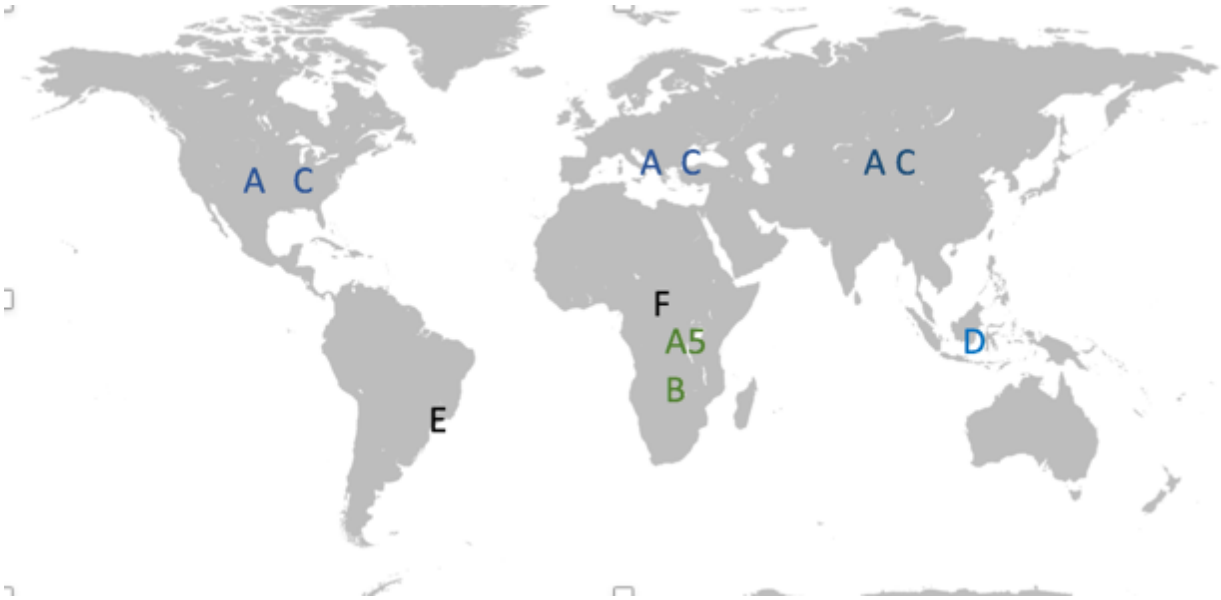

Figure 7. Map showing the geographic distribution of HHV8 ORF-K1 subtypes

ORF-K1 SUBTYPES IN SUB-SAHARAN AFRICA

Subtype A5 and B predominate in KS patients in sub-Saharan Africa (Table 4). The majority of published studies of ORF-K1 sequence analysis in Africa are of small sample size. Zong reported that among 10 endemic and AIDS-KS samples from Africa, 9 were subtype B, and 1 was subtype A5 [Zong et al., 1999]. Fouchard et al., reported that among 4 samples of African endemic and epidemic KS that were subtyped for ORF-K1, 3 out of the 4 strains were

A5, and 1 was B1 [Fouchard et al., 2000]. Among the 32 African strains from North, West and Central Africa, reported by Lacoste et al., the majority were either of the B subtype (13/32) or the A5 subtype (11/32), while the rest were subtype C. These African strains were isolated from AIDS-KS (19/32), endemic KS (5/32), multicentric Castleman disease (5/32), classic KS (1/32) and transplant KS (2/32) [Lacoste et al., 2000].

The largest analysis of ORF-K1 sequences in Africa to date is that of White et al. in Zimbabwe. Among 65 Zimbabwean AIDS-KS patients, 26/65(40%) were ORF-K1 subtype A and 39/65 (60%) were subtype B. Zimbabwean subtype A sequences grouped only with A5, while subtype B grouped with multiple clades: 26 B1 (26/39 67%), four B3 (4/39 10%) and nine B4 (9/39 39%) [White et al., 2008]. A5 and B also predominated in a Ugandan study. Among 32 samples of HHV8 DNA isolated from Ugandan KS patients, 17 grouped as A5, 9 as B1, 1 as B2, 2 as B3, 1 as subtype F and 2 as subtype C [Kajumbula et al., 2006]. These same Ugandan samples of Kajumbula were referred to in a review by Hayward and Zong, wherein data of the whole genome was presented. They did not regard the 2 B3 cases as being distinctly different from subtype B2, and included them as B2 cases [Hayward and Zong, 2007].

In South Africa with its high HIV/KS burden, there is a paucity of data regarding the epidemiology and prevalence of HHV8 ORF-K1 subtypes. In a small South African study of 21 KS patients (14 with iatrogenic KS, 6 with African endemic KS and 1 with primary effusion lymphoma) conducted in 2002, 10 patients (8 black and 2 of mixed of ancestry) were ORF-K1 subtyped. The ORF-K1 subtypes were A5 (3/10), B2 (6/10) and 1 novel B variant. These results support those of other studies in sub-Saharan Africa [Fouchard et al., 2000; Kajumbula et al., 2006; Lacoste et al., 2000; Zong et al., 1999]. Four of these 10 patients had iatrogenic KS (3 B2, 1 A5), 5 patients were African endemic KS (2 B2, 2 A5 and 1 novel B variant) and 1 had AIDS PEL (B2) [Treurnicht et al., 2002]. Hayward and Zong reported on 15 South African HHV8 KS samples (KS type not specified), which did not include those of Treurnicht. The whole genomic structure was evaluated in 14 of the samples. The ORF-K1 subtypes were A5 (7/15), B2 (3/15), B1 (3/15) and A4 (2/15) [Hayward and Zong, 2007].

Table 4. Summary of studies of ORF-K1 subtyping in sub-Saharan Africa

Study	N	Origin	*KS type and prevalence	HHV8 subtype and prevalence
Zong et al., 1999	10	Zaire Uganda Tanzania Zambia	1 African endemic 9 AIDS-KS	9 B (1 African endemic, 8 AIDS-KS) 1 A5 (AIDS-KS)
Fouchard et al., 2000	4	Central African Republic Cameroon French Guyana	1 African endemic 3 AIDS-KS	1 B1 (AIDS-KS) 3 A5 (2 AIDS-KS, 1 African endemic)
Lacoste et al., 2000	32	North, West and Central Africa	19 AIDS-KS 5 African endemic 5 Castleman 1 classic 2 transplant	14 B (8 AIDS-KS, 2 African endemic, 1 classic, 3 Castleman) 11 A5 (8 AIDS-KS, 1 African endemic, 2 iatrogenic) 2 A (1 AIDS-KS, 1 Castleman) 4 C (1 AIDS-KS, 1 African endemic, 1 iatrogenic, 1 Castleman) 1 novel subtype (AIDS-KS)
Treurnicht et al., 2002	10	South Africa	4 iatrogenic KS 5 African endemic 1 PEL	3 A5 (1 iatrogenic, 2 African endemic) 6 B2 (3 iatrogenic, 2 African endemic, 1 PEL) 1 novel B (African endemic)
Kajumbula et al., 2006	32	Uganda	KS (not specified) PBMC	17 A5 9 B1 1 B2 2 B3 1 F 2 C
Hayward and Zong, 2007	15 34	South Africa Uganda (includes those of Kajumbula et al., 2006)	KS (not specified) KS (not specified) PBMC	7 A5 3 B2 3 B1 2 A4 18 A5 10 B1 3 B2 2 C7 1 F
White et al., 2008	65	Zimbabwe	AIDS-KS	26 A5 26 B1 4 B3 9 B4

Meng et al., 1999	3 2	Uganda Zambia	AIDS-KS PBMC AIDS-KS	3 F (AIDS-KS) 2 F (AIDS-KS)
Cook et al., 1999	6 9	Uganda The Gambia	4 AIDS-KS 2 PBMC 2 no KS (HIV+) PBMC 3 AIDS-KS PBMC 6 no KS (4 HIV+; 2 HIV-) PBMC	1 A1 (AIDS-KS) 1 A5 (no KS) 4 B (3 AIDS-KS, 1 no KS) 9 B (3 AIDS-KS, 6 no KS)
Tornesello et al., 2010	12 3 6	Uganda Cameroon Kenya	African endemic African endemic AIDS-KS	5 A5 (African endemic) 2 B1 (African endemic) 4 B3 (African endemic) 1 C (African endemic) 3 A5 (African endemic) 3 A5 (AIDS-KS) 1 B1 (AIDS-KS) 1 C (AIDS-KS) 1 F (AIDS-KS)
Kakoola et al., 2001	17	Uganda	KS (not specified)	5 A5 11 B (not specified) 1 C
Olp et al., 2015	16	Uganda	12 AIDS-KS 4 African endemic	1 A5 (AIDS-KS) 8 B1 (6 AIDS-KS; 2 African endemic) 2 B3 (AIDS-KS) 5 B4 (3 AIDS-KS; 2 African endemic)
Olp et al., 2013	31	Zambia	No KS (9 HIV +; 15 HIV-; 7 HIV status unknown) Buccal swabs	17 A5 (8 HIV+; 7HIV-) 14 B (1 HIV+; 8 HIV-)

*All KS typing done on biopsy tissue from KS lesions unless otherwise specified
PBMC= peripheral blood monocyte count

PROPOSED HYPOTHESIS FOR GEOGRAPHIC DISTRIBUTION OF HHV8 SUBTYPES

Zong and Hayward proposed that HHV8 is a relatively old human virus that evolutionarily radiated along with early human populations migrating out of Africa, resulting in HHV8 genotypes that are distributed along broad geographic and ethnic lines [Hayward, 1999; Zong et al., 1999]. HHV8 like all herpes viruses is an old human virus that has co-evolved and adapted with the human host. According to the theories of Zong and Hayward, HHV8 subtypes evolved as modern humans migrated out of Africa in 3 waves: the first 100,000

years ago that failed to reach beyond the Middle East; the second 60,000 years ago which reached South Asia and Australia, and the third wave to Northern Asia and Southern Europe, 35,000 years ago. Expansions into the Americas, the Northern parts of Europe and the Pacific islands occurred 15,000 years, 10,000 years, and 4,000 years ago respectively (Figure 8). Cook and Zong proposed that subtype B is the more ancient strain, originating in Africa, and that A and C correspond to European and Northern American HHV8 genotypes [Cook et al., 1999; Zong et al., 1999]. Hayward proposed that subtype B arose 100,000 years ago as humans spread throughout Africa, subtype D was generated by a founder effect within small populations of the first human migration into South Asia 60,000 years ago; and subtype A and C arose 35,000 years ago as modern humans entered Europe and North Asia, based on evidence of human migrations and the length of the branches in the ORF-K1 phylogenetic trees [Hayward, 1999; Zong et al., 1999]. Within A and C subtypes, individual clades arose between 10,000 and 12,000 years ago. The original divergence of the B subtype into B1 and B2 clades appears to have occurred between 25 000 and 30 000 years ago [Hayward and Zong, 2007].

Studies have reported that A5 is widespread throughout Africa, and also suggests an African origin for this genotype [Lacoste et al., 2000]. According to Hayward et al., A5 may have been introduced by a single source from Europe or North Africa into a B subtype genome in ancestors of the Bantu people, which spread through sub-Saharan Africa with the Bantu expansion from West Africa about 4,000 years ago. The term Bantu people is used to denote ethnic groups in Africa who speak Bantu languages and inhabit a geographical area extending from central to southern Africa. The Bantu expansion into South Africa reached KwaZulu-Natal by 300A.D. and the Northern Province by 500A.D, and may account for the presence of A5 in South Africa. The hypothesis of Hayward et al. to explain the distribution of A5 in Africa is that A5 evolved in Africa through a rapid and recent aggressive spread through recombinant events, which was augmented by the strong selective advantage offered by the A5 allele. In support of this theory, all subtype A5 typed to date are recombinant chimeras in which a very small portion of the left hand side of the genome (ORF-K1) is A-like, hence classified as subtype A5, while the remainder is made up of the African-specific B, Q, R and N subtype constant region genomes, which as mentioned

previously, are found exclusively in sub-Saharan Africa [Hayward and Zong, 2007]. Many South African genomes with ancient N type segments of presumed Khoisan or mixed Bantu/Khoisan origin, are ORF-K1 subtype A5, suggesting that recombinant events occurred once Bantu chimeric genomes interacted with Khoisan South African genomes [Hayward, 1999; Hayward and Zong, 2007]. South Africa has a diverse population and factors such as European and Asian colonization, human migration within Africa and the resultant mixed ancestry will affect the prevalence of the subtypes in the South African population.

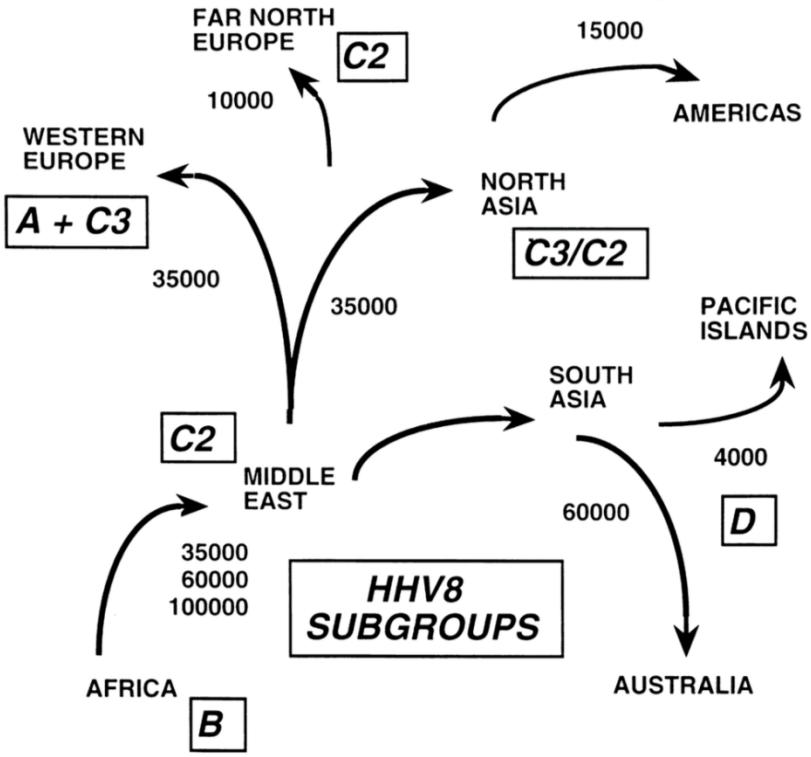

Figure 8. The global ORF-K1 subtype distribution patterns correlate with the human migration out of Africa over the past 100,000 years. Arrows indicate geographic direction and timing of migration [Hayward, 1999].

EVOLUTIONARY ADVANTAGE OF ORF-K1

Variability within the ORF-K1 gene displays very high non-synonymous rates, with up to 85% of nucleotide changes producing amino acid changes. (Nucleotide substitutions that do not lead to amino acid substitutions are called synonymous substitutions; and those that do

lead to amino acid substitutions are called non-synonymous substitutions.) This implies that the changes are not random and that a powerful biological selection process is involved [Hayward and Zong, 2007]. The advantage of the rapidity of evolution of ORF-K1 subtypes of HHV8 is yet to be elucidated, but may be due to a need of the virus to evade the immune system and establish persistent infection as described in HHV6 [Lusso et al., 1995]. Zong suggests however, that it is implausible that it is just a mechanism to evade recognition by antibodies, as the herpes-virus infected cells are more susceptible to cell-mediated rather than humoral immunity. He has proposed that the evolutionary advantage may be biological selection for recognition of cell surface markers that may allow the entry and survival of HHV8 into genetically appropriate host cells [Zong et al., 1999].

CLINICAL CORRELATION

Hayward first postulated that the ORF-K1 has a role in HHV8 biology based on the following facts. Firstly, ORF-K1 is the positional analogue of key latent transforming proteins in EBV and HVS which affect T and B-cell proliferation and tumorigenesis. Secondly, ORF-K1 contains conserved protein tyrosine kinase interaction motifs which are very rare in viral proteins. Thirdly, the observed genetic variability in ORF-K1 is not random, but appears to be due to powerful positive biological selection process. Sixty two percent of the 289 amino acids in ORF-K1 vary, and 85% of the nucleotide changes result in amino acid changes implying that the changes are not random, but due to positive selection. The level of hypervariability in ORF-K1 is not seen in any other genes of HHV8 [Hayward, 1999].

It has been proposed that different subtypes may have different pathogenic and tumorigenic properties [Hayward, 1999; Schwartz, 1996]. Conflicting data exists in the literature. Some studies have shown subtype A to be linked/associated with more rapidly progressing disease, more advanced disease and frequent mucosal and/or visceral KS [Boralevi et al., 1998; Mancuso et al., 2008; Zhang et al., 2008] (Table 5).

In an Italian study, subtype A has been linked with more rapidly progressing disease in classic KS [Mancuso et al., 2008].They classified patients with classic KS in to two groups based on the clinical evolution of the disease: of the 24/38 that were ORF-K1 subtyped, 17/24 were classified as fast progressors and 7/24 as slow progressors. HHV8 subtype A was

present in rapid progressors 12/17(71%) and in only 1/7 slow progressors, while subtype C was mainly present in slow progressors (6/7cases). Subtype A also associated with significantly higher blood HHV8 viral loads (p=0,0054). The authors concluded that careful monitoring and aggressive therapeutic protocols should be considered in patients with subtype A [Mancuso et al., 2008]. This observation confirmed previous findings of Boralevi et al., who reported that HHV8 subtype A was associated with more advanced disease, frequent mucosal and/or visceral KS (63% in A vs. 27% in B, p<0,05) amongst HHV8 isolates from 40 white French AIDS-KS cases evaluated. They concluded that subtype A displayed more aggressive pathogenicity [Boralevi et al., 1998]. Zhang et al., in Xinjang, China, showed that HHV8 subtype A was associated with more frequent mucosal lesions than subtype C [Zhang et al., 2008]. Of 27 patients included in their study (23 classic KS, 4 AIDS-KS), ORF-K1 could be amplified in 22. Subtype A was associated with significantly more frequent mucosal involvement (75% in subtype A vs 11% in subtype C; p= 0,024). Following Hayward's hypothesis that different subtypes may have different pathogenic properties, other studies addressing HHV8 epidemiology have looked for an association between subtype and clinical presentation; however no association was found [Cook et al., 1999; Lacoste et al., 2000; Meng et al., 1999; White et al., 2008; Zong et al., 1999]. Reasons for this, could include the diversity of populations studied, the heterogeneity amongst studies including methods used, different clinical parameters assessed, and population size.

Table 5. Studies showing a correlation of HHV8 subtypes with clinical presentation

Study	Sample size	Results	Conclusion
Mancuso et al., 2008	24 Italian classic KS	Subtype A present in rapid progressors (12/17) Subtype C present in slow progressors (6/7) Subtype A higher blood viral loads (p=0,005)	More careful monitoring and aggressive therapy in subtype A
Boralevi et al., 1998	40 French AIDS-KS	Subtype A more advanced, mucosal and/or visceral disease (p<0,05)	Subtype A more aggressive pathogenicity
Zhang et al., 2008	27 Chinese (23 classic KS, 4 AIDS-KS)	Subtype A more mucosal involvement than subtype C (p=0,024)	Further study is needed to establish whether HHV8 subtyping is correlated with KS clinical presentation

An association between subtypes and clinico-epidemiological forms of KS has been reported in some studies [Gazouli et al., 2004; Ramos da Silva et al., 2011] (Table 6), but not in others [Cook et al., 1999; Lacoste et al., 2000; Meng et al., 1999; White et al., 2008; Zong et al., 1999]. Gazouli reported a study of HHV8 typing in 15 iatrogenic KS, 5 AIDS-KS, 11 classic KS, and 60 healthy individuals from Greece. HHV8 ORF-K1 sequences were isolated in 30/31 KS and 10/60 healthy individuals. C3 was found in 17 cases; 12/17 were iatrogenic KS, and 5/17 healthy individuals. C1 was found in 3 cases; 2/3 were iatrogenic and 1/3 classic KS. A4 was found only in the AIDS-KS cases (5/5). A1 was found in 12 cases; 10/12 were classic KS and 2 healthy individuals. Gazouli thus suggested a possible involvement of subtype C3 in renal transplant KS, A4 in AIDS-KS and A1 in classic KS in Greece [Gazouli et al., 2004]. Distribution of ORF-K1 variants amongst classic and AIDS-KS in the United States showed predominantly C subtypes in classic KS (6/10) while AIDS-KS were predominantly subtype A (17/24; vs 6/24 subtype C), but this was not statistically evaluated [Zong, 2002]. More recently, da Silva et al. reported that out of 50 KS cases (22 AIDS-KS, 14 HIV-negative KS, 14 KS not otherwise specified) evaluated in Brazil, an elevated frequency of HHV8 subtype A was found in HIV-positive patients (15/22; 68%). Conversely, the majority of KS isolated from HIV-negative patients had subtype C (10/14; 71%). The difference in proportions between A and C subtypes within HIV-positive and HIV-negative patients was statistically significant (p=0,026, Fisher's exact test) [Ramos da Silva et al., 2011]. This data raises the question as to whether subtype A has unique biological features and suggests a possible HIV/HHV8 interaction.

Table 6. Studies showing a correlation between HHV8 subtypes and clinico-epidemiological forms of KS

Study	Sample size	Result	Conclusion
Gazouli et al., 2004 Greece	15 iatrogenic KS 5 AIDS-KS 11 classic KS 60 healthy individuals	Subtype A4 in 5/5 AIDS-KS; Subtype C in 2/3 iatrogenic KS Subtype C in 1/3 classic KS; A1 in 10/12 classic KS	Suggested a possible involvement of subtype C in iatrogenic KS; A4 in AIDS-KS; A1 in classic KS in Greece
Ramos da Silva et al., 2011 Brazil	22 AIDS-KS 14 HIV negative 14 KS not	Subtype A found in 15/22 HIV-positive Subtype C in 10/14	Distinct subtypes have a non-random distribution, possibly

	specified)	HIV-negative (p=0,026)	due to unique biological properties

Only one study has found an association between subtype B and biological properties. Kouri et al., in their study of HHV8 in 90 KS samples in Cuba, reported an association with subtype B and heterosexual behaviour (OR 3,63; CI 1,2-10,98, p=0,03), with having antecedently acquired HIV/HHV8 in Africa (p=0,0003) and with histologically nodular stage KS (OR 4,2; CI 1,1-15,7; p=0,04). They proposed that these findings may be related to a more rapid evolution and/or virulence of HHV8 subtype B [Kouri et al., 2012]. The authors concluded that this observation warrants further study as there are no consistent reports linking HHV8 subtypes with epidemiologic variants, clinical stage or with evolution of disease.

Treurnicht's study found no correlation between genetic subtype, geographical origin of the patients or the KS clinical variants (iatrogenic or African endemic KS), but the numbers in this study were very small [Treurnicht et al., 2002].

IDENTIFICATION OF GAPS OR NEEDS FOR FURTHER RESEARCH

The majority of the published studies in Africa, reported on HHV8 diversity in East [Cook et al., 1999; Hayward, 1999; Meng et al., 1999; Zong et al., 1999; Olp et al., 2015; Kakoola et al., 2001; Tornesello et al., 2010], West and Central Africa [Cook et al., 1999; Fouchard et al., 2000; Lacoste et al., 2000]. There is a paucity of data on the prevalence and epidemiology of the different HHV8 subtypes in southern Africa. A limitation of the studies conducted in Africa, are that they are all of small sample size, except for that of Kajumbula in Uganda (31 patients) and White in Zimbabwe (65 patients) [Kajumbula et al., 2006; White et al., 2008]. In the South African study of Treurnicht, only 10 cases of KS were subtyped for ORF-K1 and Hayward and Zong reported on 15 South African HHV8 KS samples [Hayward and Zong, 2007; Treurnicht et al., 2002]. There is a need for larger studies on epidemiology and prevalence of HHV8 subtypes in Africa, as well as South Africa which bear the brunt of the HIV/HHV8 KS burden.

ORF-K1 displays the most nucleotide hypervariability within the genome leading to amino acid changes, and hence may have functional significance. Despite the complexities of the

HHV8 genome including the new 12 subtypes proposed by Zong, and the limitation of only subtyping one segment of the genome; there is considerable value obtained from subtyping ORF-K1 for epidemiological reasons, as well as functional/ clinico-pathological reasons.

The subtypes of HHV8 are distributed along broad geographic and ethnic lines that have been proposed to be a result of ancient human migrations and founder effects [Hayward, 1999; Zong et al., 1999]. South Africa has a diverse population and factors such as European and Asian colonization, human migration in Africa and resultant mixed ancestry should affect the prevalence of the subtypes in the South African population. Hence it is of great importance to study the epidemiology of the genetic subtypes of the South African population.

Although, conflicting data exists, numerous studies point to differing tumorigenic and pathogenic properties of the HHV8 subtypes, in particular to increased aggressivity of subtype A [Boralevi et al., 1998; Mancuso et al., 2008; Zhang et al., 2008]. Further research is warranted to elucidate whether an association exists, which could affect disease prognosis and direct future therapies. Other than the study of Treurnicht et al. and that of Hayward and Zong, there are no recent South African studies in the literature examining the prevalence of the different subtypes and how this correlates with clinical presentation of KS [Hayward and Zong, 2007; Treurnicht et al., 2002].

A study was undertaken with the following objectives:

(1) to determine the prevalence of the HHV8 ORF-K1 subtypes; and

(2) to analyse the associations between the different HHV8 ORF-K1 subtypes, different clinico-epidemiologic forms, presentation, and stage of KS;

in a South African population with high HIV/KS burden.

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CHAPTER 2: PUBLISHED MANUSCRIPT

The results have been published in the Journal of Medical Virology 88:292-303 (2016). Attached is the published article.

Contributions of authors:

Thuraya Isaacs:

Contributed to the conception of the idea, wrote the protocol, literature review and manuscript, was responsible for data collection and analysis, corresponding author with Prof Katz

Aron Abera:

Conducted genetic subtyping, constructed the phylogenetic tree

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Genetic Diversity of HHV8 Subtypes in South Africa: A5 Subtype Is Associated With Extensive Disease in AIDS-KS

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Human herpes virus 8 (HHV8) is the etiological agent of all forms of Kaposi's sarcoma (KS). Six major subtypes (A-F), based on genetic variability of open reading frame (ORF)-K1, have been identified. Numerous studies point to differing tumorigenic and pathogenic properties of the HHV8 subtypes. The study objectives were to determine the HHV8 subtypes and their prevalence in a cohort of clinical and histologically confirmed KS in Cape Town, South Africa, and analyze associations between the different subtypes and clinical presentation of KS. Clinical records were prospectively reviewed to extract clinical presentation; demographic data were retrospectively collected and tissue biopsies were taken for ORF-K1 subtyping. Eighty six patients were subtyped; 81 AIDS (acquired immune deficiency syndrome)-KS and 5 African endemic-KS. Subtype A5 (42/86) and B2 (16/86) predominated. B1, B3, A1 and A4 subtypes were identified in 10/86, 9/86, 4/86 and 1/86 patients, respectively. A5 and B subtypes were found in African blacks and individuals of mixed ancestry, while subtypes A1 and A4 were found only in whites and individuals of mixed ancestry. Subtype A5 was associated with >10 KS lesions at presentation in the AIDS cohort (adjusted OR: 3.13; CI: 1.02–9.58). Subtypes A1 and A4 combined were less likely to be associated with poor risk tumor extension ($P = 0.031$) and A1 was associated with lower likelihood of lower limb involvement ($P = 0.019$). In conclusion, these results indicate that subtype A5 and B predominate in South Africa and A5 may be associated with more extensive disease. ***J. Med. Virol. 88:292–303, 2016***.

KEY WORDS: Kaposi's sarcoma; human herpes virus 8; HHV8; KSHV; ORF-K1

INTRODUCTION

Kaposi's sarcoma (KS) is a multifocal proliferative disorder involving blood and lymphatic vessels. The etiological agent of KS was elucidated in 1994 and is a KS-associated herpes virus (KSHV) also known as human herpes virus 8 (HHV8) (family *Herpesviridae*, subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*) [Chang et al., 1994]. This virus is also associated with the development of B-cell primary effusion lymphoma (PEL) and multicentric Castleman's disease [Ablashi

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et al., 2002]. KS is an AIDS-defining disease; it is the most common AIDS-related malignancy worldwide and is one of the most common cancers in sub-Saharan Africa [Wabinga et al., 1993; Morris, 2003].

There are four principal clinical variants of KS, namely, classic KS, African endemic KS, iatrogenic KS, and AIDS-related KS. The HIV pandemic has seen the incidence of Kaposi's sarcoma (KS) in sub-Saharan Africa rise by between three- and twenty-fold [Parkin et al., 1999]. In South Africa over a period of 23 years (1983–2006), age standardized incidence rates increased 20-fold in men and 50-fold in women [Mosam et al., 2009]. Adult seroprevalence rates of HHV8 in South Africa amongst medical patients and patients with sexually transmitted diseases in KwaZulu Natal increase with age, ranging from 32% in age group 15–34 to 63% among adults aged 35–69 years [Wilkinson et al., 1999].

HHV8 is found in KS lesions of all types. Its genome contains several genes that were pirated from the human host and play an important role in the pathogenicity of the virus. The HHV8 genome is mostly highly conserved but both ends of the genome show significant variability, which has been used as the basis for viral genetic subtyping. Six major subtypes (A, B, C, D, E, and F) and at least 13 different variants or clades based on genetic variability of ORF-K1 gene sequences, have been identified [Zong et al., 2002]. The different subtypes have been shown to have variable penetrance in various population groups and are distributed along broad geographic and ethnic lines, which may have arisen through ancient human migrations [Hayward, 1999; Zong et al., 1999; Zong et al., 2002]. Subtypes B and A5 have been found to predominate in sub-Saharan Africa [Cook et al., 1999; Hayward, 1999; Meng et al., 1999; Zong et al., 1999; Fouchard et al., 2000; Lacoste et al., 2000; Kajumbula et al., 2006]. A and C subtypes are found in Caucasian patients throughout Europe, the United States, the Mediterranean basin, the Middle East and Asia [Cook et al., 1999; Zong et al., 1999] and D in the Pacific islands and Taiwan [Zong et al., 1999; Zhang et al., 2008]. In Brazil, there is a 53% prevalence of subtype E in Brazilian Indians, but the study was done on individuals without KS and, therefore, any association between subtype E and KS development remains to be studied [Biggar et al., 2000]. In addition, recently, subtype F has been identified in Ugandans with KS [Kajumbula et al., 2006].

It has been proposed that different genotypes may have different pathogenic and tumorigenic properties [Schwartz, 1996; Hayward, 1999]. Conflicting data exist in the literature. While some studies have shown subtype A to be associated with more rapidly progressing disease, more advanced disease, frequent mucosal, and/or visceral KS [Schwartz, 1996; Mancuso et al., 2008; Zhang et al., 2008], other studies have failed to confirm this [Cook et al., 1999; Meng et al., 1999; Zong et al., 1999; Lacoste et al., 2000; White et al., 2008].

An association between subtypes and clinico-epidemiological forms of KS has also been reported in some studies [Gazouli et al., 2004; Ramos da Silva et al., 2011], but not in others [Cook et al., 1999; Meng et al., 1999; Zong et al., 1999; Lacoste et al., 2000; White et al., 2008]. An elevated frequency of HHV8 subtype A as opposed to subtype C has been reported in AIDS-KS in Brazil [Ramos da Silva et al., 2011]. Gazouli also suggested a possible involvement of subtype A4 in AIDS-KS, subtype A1 in classic KS, and subtype C3 in renal transplant KS in Greece [Gazouli et al., 2004]. These data raise the question as to whether subtype A has unique biological features. Conversely, a Cuban study of 90 KS cases, the first to report HHV8 subtype B expansion in Cuba, found an association between subtype B with heterosexual behavior (OR: 3.63, CI: 1.2–10,98; $P = 0.03$) and having acquired HHV8 in Africa ($P = 0.0003$) [Kouri et al., 2012].

There are limited data on the prevalence or epidemiology of the different HHV8 subtypes in South Africa. In a small South African study of 21 KS patients (14 iatrogenic KS, 6 African endemic KS, and 1 AIDS PEL) conducted in 2002, no correlation was reported between genetic subtype, geographical origin of patients or clinical variants [Treurnicht et al., 2002]. This lack of association may be due to the small number of patients studied and subtyped, since only 10 patients (4 iatrogenic, 5 African endemic and 1 AIDS PEL), were subtyped for ORF-K1 variants. Subtype B2 was identified in six patients (two African endemic KS, three iatrogenic KS 3, one AIDS PEL) and A5 in three patients (two African endemic KS, one iatrogenic KS). A novel B variant was found in one case of African endemic KS [Treurnicht et al., 2002]. In addition, Hayward and Zong reported on ORFK-1 subtyping in 15 South African HHV8 samples. The whole genomic structure was evaluated in 14 of the samples. The ORF-K1 subtypes were A5 (7/15), B2 (3/15), B1 (3/15), and A4 (2/15), respectively [Hayward and Zong, 2007].

Although conflicting data exist, numerous studies point to differing tumorigenic and pathogenic properties of the HHV8 subtypes. The prevalence of these subtypes seems to be population specific. On the basis of this information, a study of the prevalence of the HHV8 subtypes in a South African population, with its high KS/HIV burden was undertaken, with the aim of analyzing associations of the different HHV8 subtypes, with clinico-epidemiologic forms, presentation, and the stage of KS. These associations may impact disease prognosis and direct future therapies.

MATERIALS AND METHODS

Study Setting and Participants

The study population comprised patients presenting with an initial diagnosis of KS at Groote Schuur Hospital, a tertiary referral center in Cape Town, between January 1, 2009 and December 31, 2012.

Tissue samples consisted of 4 mm punch biopsies of KS skin lesions taken after obtaining informed

consent. The biopsies were coded with a labeling system that did not disclose the identity of the patients and frozen at −20°C. Skin lesions were photographed prior to biopsy as part of standard of care and the type and site of the lesions was recorded on body charts.

Ethics Statement

Ethics approval for this study was obtained from the University of Cape Town Human Research Ethics Committee (HREC/REF: 279/2008) and complies with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from each individual before sample collection.

Data Extraction

Patients with a clinical and histological diagnosis of KS were included in the study. Clinical records of the patients were retrospectively reviewed and the following data parameters were extracted as follows: age, sex, nationality, ethnicity (based on language as proxy), HIV status, CD4 count, whether on antiretroviral therapy (ART), chemotherapy, and presence of TB. Ethnicity was grouped as black, white, and mixed race. Mixed race in the South African context was defined as historically a mixture black and white with contributions from Malay and other smaller ethnic groups.

Prospectively collected information on clinical presentation, including the morphology of lesions and their extent and distribution based on recorded body diagrams and photographs were analyzed. Lesion morphology was classified as "patch," "plaque," "nodule," or "fungating" based on if explicitly described as such in the medical record or labeled on the body diagram, or identified from the photographs. Anatomic sites were categorized as face, oral mucosa, trunk, upper limbs, lower limbs, and genitalia. Cutaneous disease was categorized as localized or generalized, based on definitions utilized in previous literature [Nasti et al., 2003a; Mosam et al., 2008]. KS lesions confined to one anatomical area were classified as localized whether unilateral or bilateral and lesions as generalized if more than one anatomic site was involved. Cutaneous disease extent was categorized as <10 or >10, with <10 lesions indicating indolent disease [Nasti et al., 2003a].

Patients with AIDS-KS were retrospectively staged according to the modified AIDS Clinical Trials Group (ACTG) criteria proposed by Nasti in the era of highly active antiretroviral therapy (HAART). This was a refinement of the validated original ACTG criteria, which was developed as a prognostic indicator for the evaluation of AIDS-KS in the pre-HAART era [Krown et al., 1989, 1997; Nasti et al., 2003a]. This modified ACTG criteria classifies patients into risk categories for death depending on tumor extent (T1 if tumor-associated edema or ulceration, nodular oral KS, visceral KS; otherwise staged as T0) and concomitant systemic illness (S1 if opportunistic infections and/ or thrush, B symptoms present, performance status <70%, other HIV related illness; otherwise staged as S0). According to the modified staging system, the combination of poor tumor stage (T1) and poor systemic disease (S1) risk, identified patients with unfavourable prognosis especially if concomitant pulmonary involvement by KS was present [Nasti et al., 2003b]. Due to insufficient information available in the clinical records to allow accurate staging for concomitant systemic illness, patients were only staged by tumor extent (T1/T0).

PCR and Phylogenetic Analysis

Viral DNA was extracted from the biopsies using a QIAamp DNA Kit (QIAGEN) according to the manufacturer's recommendations. The full length of the ORF-K1 gene was amplified using the following primers, forward (5′-GTTCTGCCAGGCATAGTC-3′) and reverse (5′-AATAAGTATCCGACCTCAT-3′) in a reaction mix containing 1U of Taq DNA polymerase (New England Biolabs, Ipswich, USA), 2.5 mM $MgCl_2$, 200 μM each dNTPs (New England Biolabs), and 2 μl of viral DNA in 20 μl final volume. PCR reaction was performed at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min over 35 cycles [Poole et al., 1999]. The PCR product (1,065 bp) was then subjected to direct nucleotide sequencing (Macrogen, Seoul, South Korea) using the following primers: forward (5′-GGCCCTTGTGTAAACCTGTC-3′) and reverse (5′-CCTGAATGTCAGTACCAATCCA-3′) that span a region of 932 bp. Multiple sequence alignment of the HHV8 ORF-K1 region from this study and reference strains reported in the GeneBank were performed using MEGA5 [Tamura et al., 2011]. The reference sequences used are based on Zong et al. [1999], Lacoste et al. [2000], Zhang et al. [2008], and Tornesello et al. [2010]. Their accession numbers are as follows: AF133038.1 (A1), FJ884626.1 (A1), FJ884615.1 (A1), AF130305.1 (A1), AY204646.1 (A1), AY204648.1 (A2), U86667.1 (A3), AF133039.1 (A4), AF171057.1 (A5), AF178797.1 (A5), AY042955.1 (A5), AF178798.1 (A5), AF178823.1 (A5), AF178801.1 (B1), AF133040.1 (B1), AF178824.1 (B1), AF130259.1 (B2), AY042940 (B3), AF133041.1 (C1), AF133042.1 (C3), AF133043.1 (D1), AF133044.1 (D2), AF220292.1 (E), and AF178810.1 (F). Subsequently, phylogenetic trees were constructed by transforming the aligned sequence data by the neighbor-joining bootstrap values of 1,000 replicates using the maximum composite likelihood (MCL) approach.

Statistical Analysis

Data were analyzed using STATA statistical package (StataCorp, Version 13). Descriptive analysis using medians with inter-quartile ranges (IQR) were used to summarize age and CD4 count distributions. Proportions are depicted as percentages for categorical variables. A Pearson's Chi-square (χ^2) or Fisher's exact test, depending on the size of the groups compared, was used to test for strength of association between

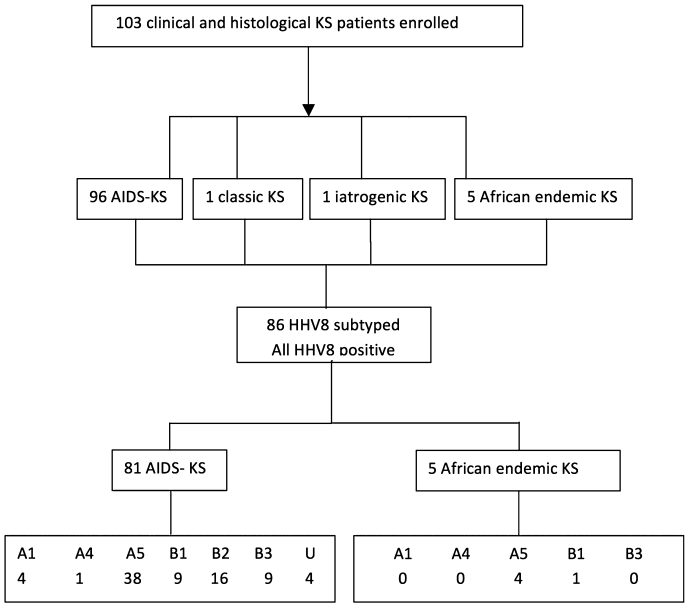

Fig. 1. Flow diagram showing the patients of the KS cohort and the determined HHV8 subtypes. U denotes an unclustered subtype.

categorical variables. Data were tested for normality and a Mann–Whitney test was used to test for strength of association for continuous variables. A significance level of $P < 0.05$ was used for all analysis.

Logistical regression was used to estimate the independent association of HHV8 subtypes with extent of KS in a multiple variable model.

RESULTS

Demographic Characteristics

Between January 1, 2009 and December 31, 2012, 103 adults were seen with a clinical and histological diagnosis of KS. AIDS-KS was diagnosed in 96 patients (93%), African endemic KS in 5 patients (5%), iatrogenic KS in 1 patient (1%), and classic KS in 1 patient (1%) (Fig. 1). Eighty-six of the 103 patients were subtyped, and 17 were excluded since subtyping was unsuccessful. Among the 86 patients subtyped, AIDS-KS was diagnosed in 81 patients (94%) and African endemic in 5 patients (6%). Fifty three of the 86 subtyped patients (62%) were male. The median age of the 86 patient cohort was 36 years (IQR 31-42). The majority of the patients, 66/86 (77%), were South African. The remaining 20 patients (17 AIDS-KS and 3 African endemic KS) were of African origin, other than South African. There was no information available from their clinical records as to their country of origin. The ethnic distribution showed 74/86 (86%) black, 10/86 (12%) mixed race, and 2/86(2%) white (Table I).

Current co-infection with tuberculosis was present in 31/86 (36%). Chemotherapy (vincristine

TABLE I. General Characteristics of Patients and Genetic Subtypes (n = 86)

Characteristic	Value
Age median in years (IQR)	36 (31–42)
Sex	
Male	53
Female	33
CD4 count median in cells/mm (IQR)	153 (83–288)
Race, number (%)	
White	2 (2)
Black	74 (86)
Mixed race	10 (12)
KS type	
AIDS-KS	81
African endemic KS	5
HHV8 subtypes	
Subtype A	47
A1	4
A4	1
A5	42
Subtype B	35
B1	10
B2	16
B3	9
Unclustered	4

and bleomycin) for KS had been received by 11/86 (13%) patients. The median number of cycles of chemotherapy received at the time seen was 1.5 (range 1–11 cycles).

Fifty six of the 81 AIDS-KS patient cohort (69%) were on ART. The median CD4 count of the 81 AIDS-KS patients was 153 cells/μl (IQR 83–288).

Sufficient clinical data were available to allow ACTG T1 staging in 71/81 AIDS-KS patients. The KS staging identified 58% (41/71) cases that were classified as poor risk tumor extension (T1).

Genetic Subtyping

Out of the 86 patients that were subtyped, 81 patients were diagnosed with AIDS-KS and 5 patients were diagnosed with African endemic KS. The ORF-K1 gene of the 86 viral isolates was sequenced and the accession numbers are indicated in Table II. A phylogenetic tree based on ORF-K1 coding region using reference sequences was constructed (Fig. 2). Subtype A5 was the most common (42/86; 49%), followed by B2 (16/86; 19%) and B1 (10/86; 12%) and B3 (9/86; 10%), which are all subtypes prevalent in Africa. In addition, four patients were infected with subtype A1 (5%) and one patient with A4 (1%) which are viral subtypes that have been shown to predominate in Europe and United States (Tables I and II). Viral sequences of four patients did not cluster with any of the classified subtypes and were termed unclustered.

Clinical Presentation

Twenty one of 86 patients (24%) presented with localized disease, and 65 patients (76%) with generalized disease (Table III).

Lesions involved the following locations on presentation: 45/86 (52%) face, 30/86 (35%) oral mucosa, 50/86 (58%) trunk, 45/86 (52%) upper limbs, 70/86 (81%) lower limbs, and 6/86 (7%) genitalia. Lesions involving two or more anatomic locations were seen in 65/86 (76%) of patients. At presentation, 62/86 (72%) presented with >10 lesions and 24/86 (28%) patients displayed less than <10 lesions (Table III).

Twenty eight of the 86 (33%) patients presented with patches, 72/86 (84%) presented with plaques, 32/86 (37%) presented with nodules, and 3/86 (4%) presented with fungating lesions. Fifty two percent (44/86) of patients presented with more than one KS morphological type, while 42/86 (49%) presented with one morphology. Dual morphology was seen in 39/86 (45%) and 5/86 (6%) presented with three morphological types (Table III). Lymphoedema was associated with KS lesions in 42/86 (49%) patients (Table III).

Clinical Correlations

Subtype A5 was strongly associated with >10 lesions at presentation in the AIDS-KS cohort. Thirty two out of 38 AIDS-KS patients who had A5 subtype had more than 10 lesions, while only 6 patients who had A5 had less than 10 lesions ($P = 0.050$). This correlation was not observed in the African endemic KS group ($P = 0.600$) (Supplementary Table S1). The strength of association of A5 with >10 lesions in AIDS-KS is increased when only the known KS subtypes are analyzed ($P = 0.026$). Multivariable analysis showed an independent association between subtype A5 and >10 lesions (adjusted OR: 3.13; CI: 1.02–9.58), (Table IV).

Subtypes A1 and A4 occurred only in AIDS-KS. Subtype A1 was less likely to be associated with lower limb involvement, which was statistically significant ($P = 0.019$). There was also a statistically significant association between subtypes A1 and A4, combined, and tumor stage. Subtypes A1 and A4 combined were less likely to be associated with poor risk tumor extension (T1) ($P = 0.031$), and hence associated with a better prognosis. Tumor stage T1 was associated with lower limb involvement ($P = 0.048$). There was a strong association between lower limb involvement and lymphoedema ($P = 0.008$).

General Correlations

There was a strong association between ethnicity and genetic subtype. Subtypes A1 ($P < 0.001$), A4 ($P = 0.023$) and A5 ($P = 0.040$) were associated with ethnicity. B1, B2, and B3 individually showed a trend toward an association with ethnicity, but this did not reach statistical significance. Subtype A1 and A4 were found only in whites and patients of mixed ancestry ($P < 0.001$ and $P = 0.023$, respectively). In contrast, subtypes A5 ($P = 0.040$) and B (B1 $P = 1.000$; B2 $P = 0.788$; B3 $P = 1.000$) were not found in white patients (Table V).

In the original cohort of 103, there was a statistically significant association between age and gender. In the age group <35 years (42), 19/42(45%) were male and 23/42(55%) were female, while in the age group >35 years (60), 43/60 (72%) were male and 17/60 (28%) were female ($P = 0.007$).

DISCUSSION

This study adds to the current knowledge of the worldwide epidemiology of HHV8 genetic subtypes, by providing the largest sample of analysis of ORF-K1 subtypes in South Africa and sub-Saharan Africa. In this study, subtypes A5 and B were found to be the predominant HHV8 subtypes. Subtype A5 was found to be associated with more extensive lesions at presentation in AIDS-KS. Subtypes A1 and A4 were not found amongst black patients. A1 was associated with a lower likelihood of having lower limb involvement. Within age group <35, there were slightly more women than men with KS, while in the age group >35 years, there were markedly more men than women ($P = 0.007$).

Subtypes A5 and B predominate in South Africa. This confirms results of previous studies that A5

TABLE II. HHV8 Subtype According to Demographic Characteristics (n = 86)

KS no.	Age	Sex	Race	KS type	ARV	CD4	Stage	HHV8 subtype	Accession number
KS3	42	M	BLACK	AIDS-KS	NO	1367	T0	A5	KP997035
KS4	39	M	BLACK	AIDS-KS	NO	66	T0	B2	KP997036
KS5	45	F	BLACK	AIDS-KS	YES	123	T1	B2	KP997037
KS6	25	M	BLACK	AIDS-KS	NO	UNKNOWN	–	B2	KP997038
KS8	30	M	BLACK	AIDS-KS	NO	239	T1	B2	KP997039
KS9	46	F	BLACK	AIDS-KS	NO	311	–	A5	KP997040
KS11	21	F	BLACK	AIDS-KS	NO	3	T0	U	KP997041
KS14	37	M	BLACK	AIDS-KS	NO	270	T1	B3	KP997043
KS17	35	F	BLACK	AIDS-KS	NO	89	T1	A5	KP997044
KS19	38	M	BLACK	AIDS-KS	NO	94	T0	B2	KP997045
KS20	31	M	BLACK	AIDS-KS	YES	110	T0	A5	KP997046
KS22	40	M	BLACK	AIDS-KS	NO	319	T1	B1	KP997047
KS25	48	F	BLACK	AFRICAN ENDEMIC	N/A	N/A	T1	A5	KP997048
KS26	31	F	BLACK	AIDS-KS	YES	99	T1	A5	KP997049
KS28	44	F	BLACK	AIDS-KS	NO	289	T1	B2	KP997050
KS29	25	M	BLACK	AIDS-KS	NO	160	T0	A5	KP997051
KS31	50	M	BLACK	AIDS-KS	NO	74	T1	A5	KP997052
KS33	42	M	WHITE	AIDS-KS	YES	150	T0	A1	KP997054
KS34	39	F	BLACK	AIDS-KS	NO	219	T0	B2	KP997055
KS35	26	M	BLACK	AIDS-KS	YES	91	T1	B2	KP997056
KS36	77	M	BLACK	AFRICAN ENDEMIC	N/A	N/A	N/A	A5	KP997057
KS37	41	F	BLACK	AIDS-KS	YES	25	–	A5	KP997058
KS38	36	M	BLACK	AIDS-KS	YES	225	T1	A5	KP997059
KS39	37	M	BLACK	AIDS-KS	YES	UNKNOWN	T0	B1	KP997060
KS40	62	M	MIXED RACE	AIDS-KS	NO	322	T0	A1	KP997061
KS41	36	F	BLACK	AIDS-KS	YES	699	T1	B2	KP997062
KS42	34	F	BLACK	AIDS-KS	YES	65	T1	A5	KP997063
KS44	33	M	BLACK	AIDS-KS	YES	132	T1	A5	KP997065
KS45	40	M	MIXED RACE	AIDS-KS	YES	89	T1	B2	KP997066
KS46	33	F	BLACK	AIDS-KS	NO	330	–	B3	KP997067
KS48	30	M	BLACK	AIDS-KS	NO	333	T1	B1	KP997069
KS49	38	F	BLACK	AIDS-KS	YES	178	T1	A5	KP997070
KS50	24	F	BLACK	AIDS-KS	YES	548	T1	B3	KP997071
KS51	.	.	BLACK	AIDS-KS	YES	UNKNOWN	T0	B1	KP997072
KS53	31	M	BLACK	AIDS-KS	YES	173	T1	B1	KP997074
KS54	49	M	BLACK	AIDS-KS	YES	92	T1	A5	KP997075
KS55	40	M	MIXED RACE	AIDS-KS	YES	UNKNOWN	T1	U	KP997076
KS56	25	F	BLACK	AIDS-KS	YES	320	T0	B3	KP997077
KS57	36	M	BLACK	AIDS-KS	YES	270	T1	A5	KP997078
KS58	58	F	BLACK	AIDS-KS	YES	231	T0	B3	KP997079
KS60	56	M	MIXED RACE	AFRICAN ENDEMIC	N/A	N/A	N/A	B1	KP997081
KS61	35	M	BLACK	AIDS-KS	YES	61	T0	B2	KP997082
KS62	32	M	BLACK	AIDS-KS	YES	133	T0	A5	KP997083
KS63	34	F	BLACK	AIDS-KS	YES	77	T1	B3	KP997084
KS64	31	F	BLACK	AIDS-KS	YES	99	T1	A5	KP997085
KS65	43	M	WHITE	AIDS-KS	YES	50	T0	A4	KP997086
KS67	34	M	BLACK	AIDS-KS	YES	547	T1	A5	KP997087
KS68	34	F	BLACK	AIDS-KS	YES	10	T0	A5	KP997088
KS69	26	F	BLACK	AIDS-KS	NO	110	T1	A5	KP997089
KS70	35	F	BLACK	AIDS-KS	YES	138	T0	A5	KP997090
KS72	30	F	MIXED RACE	AIDS-KS	YES	555	T1	A5	KP997091
KS73	24	F	BLACK	AIDS-KS	YES	288	T1	A5	KP997092
KS74	51	M	BLACK	AIDS-KS	YES	UNKNOWN	T1	A5	KP997093
KS76	31	F	BLACK	AIDS-KS	YES	220	T0	B3	KP997094
KS77	49	M	BLACK	AIDS-KS	YES	71	T1	B2	KP997095
KS78	37	M	BLACK	AIDS-KS	YES	UNKNOWN	T0	B1	KP997096
KS79	42	M	BLACK	AIDS-KS	YES	14	–	A5	KP997097
KS80	41	M	BLACK	AFRICAN ENDEMIC	N/A	N/A	N/A	A5	KP997098
KS83	39	F	MIXED	AIDS-KS	YES	163	T0	B3	KP997100

(*Continued*)

TABLE II. (*Continued*)

KS no.	Age	Sex	Race	KS type	ARV	CD4	Stage	HHV8 subtype	Accession number
			RACE						
KS84	27	M	BLACK	AIDS-KS	YES	313	T0	A5	KP997101
KS88	36	M	BLACK	AIDS-KS	YES	220	T0	B1	KP997104
KS90	30	M	BLACK	AIDS-KS	YES	130	–	A5	KP997106
KS91	38	M	BLACK	AIDS-KS	YES	129	T0	A5	KP997107
KS92	32	M	BLACK	AIDS-KS	YES	539	T1	U	KP997108
KS93	29	F	BLACK	AIDS-KS	YES	256	–	A5	KP997109
KS101	47	M	BLACK	AIDS-KS	N	396	T1	A5	KP997110
KS103	36	M	BLACK	AIDS-KS	YES	208	T0	A5	KP997111
KS104	29	M	MIXED	AIDS-KS	YES	149	T0	A1	KP997112
			RACE						
KS107	27	F	BLACK	AIDS-KS	YES	40	T1	A5	KP997113
KS108	26	F	BLACK	AIDS-KS	YES	15	–	B2	KP997114
KS109	38	M	BLACK	AIDS-KS	NO	129	T0	B1	KP997115
KS113	52	F	BLACK	AIDS-KS	YES	UNKNOWN	T1	A5	KP997117
KS115	63	M	BLACK	AIDS-KS	YES	250	T1	A5	KP997118
KS117	31	F	BLACK	AIDS-KS	NO	1	T0	B2	KP997119
KS119	53	F	BLACK	AIDS-KS	YES	69	T1	A5	KP997120
KS121	31	M	BLACK	AIDS-KS	NO	403	T1	B1	KP997122
KS123	34	M	BLACK	AIDS-KS	NO	225	T1	A5	KP997124
KS125	65	M	BLACK	AIDS-KS	NO	339	T0	A5	KP997125
KS126	36	M	BLACK	AIDS-KS	YES	83	T1	A5	KP997126
KS127	30	M	BLACK	AFRICAN ENDEMIC	N/A	N/A	T0	A5	KP997127
KS130	47	F	BLACK	AIDS-KS	YES	483	T1	B3	KP997128
KS131	35	M	BLACK	AIDS-KS	NO	251	–	B2	KP997129
KS132	26	M	BLACK	AIDS-KS	YES	153	T0	B2	KP997130
KS136	41	M	MIXED	AIDS-KS	NO	174	–	A1	KP997133
			RACE						
KS137	53	M	MIXED	AIDS-KS	YES	UNKNOWN	T1	U	KP997134
			RACE						
KS138	38	M	MIXED	AIDS-KS	YES	56	T1	A5	KP997135
			RACE						

ARV, antiretroviral therapy; T1, tumor-associated edema or ulceration, nodular oral KS, visceral KS; T0, confined to skin and or lymph nodes and/or non-nodular KS of the palate; N/A, not applicable; U, unclustered.

and B are widespread in Africa [Cook et al., 1999; Hayward, 1999; Meng et al., 1999; Zong et al., 1999; Fouchard et al., 2000; Lacoste et al., 2000; Treurnicht et al., 2002; Kajumbula et al., 2006]. The finding that A5 was the predominant subtype is consistent with that of Hayward and Zong [2007] but is different from the finding of another South African study that showed B2 as the predominant subtype [Treurnicht et al., 2002]. This difference is quite likely due to the small number of patients studied and subtyped by Treurnicht et al., only 10 patients (8 black and 2 of mixed of ancestry). Four of these 10 patients had iatrogenic KS (3 B2, 1 A5), 5 patients were African endemic KS (2 B2, 2 A5 and 1 novel B variant) and 1 had AIDS PEL (B2). In the current study, only one patient had iatrogenic KS and could not be subtyped.

Zong and Hayward proposed that HHV8 is a relatively old human virus that evolutionarily radiated along with early human populations migrating out of Africa, resulting in HHV8 genotypes that are distributed along broad geographic and ethnic lines [Hayward, 1999; Zong et al., 1999]. Cook and Zong proposed that subtype B is the more ancient strain, originating in Africa, and that A and C correspond to European and Northern American HHV8 genotypes [Cook et al., 1999; Zong et al., 1999]. The current findings that all strains of subtype B (B1, B2, and B3) are present in black patients or those of mixed ancestry supports this proposal. Other studies have reported that A5 is widespread throughout Africa, and suggest an African origin for this genotype [Lacoste et al., 2000]. According to Hayward et al., A5 may have been introduced by a single source from Europe or North Africa into a B subtype genome in ancestors of the Bantu people, which spread through sub-Saharan Africa with the Bantu expansion from West Africa about 4,000 years ago [Hayward and Zong, 2007]. The term Bantu people is used to denote ethnic groups in Africa who speak Bantu languages and inhabit a geographical area extending from Central to Southern Africa. The Bantu expansion into South Africa reached KwaZulu-Natal by 300 A.D. and the Northern Province by 500 A.D, and may account for the presence of A5 in South Africa. The hypothesis of Hayward et al. to explain the distribution of A5 in Africa is that A5 evolved in Africa through a rapid and recent aggressive spread through recombinant events, augmented by the strong selective advantage offered by the A5 allele [Hayward, 1999]. In support of this theory, all

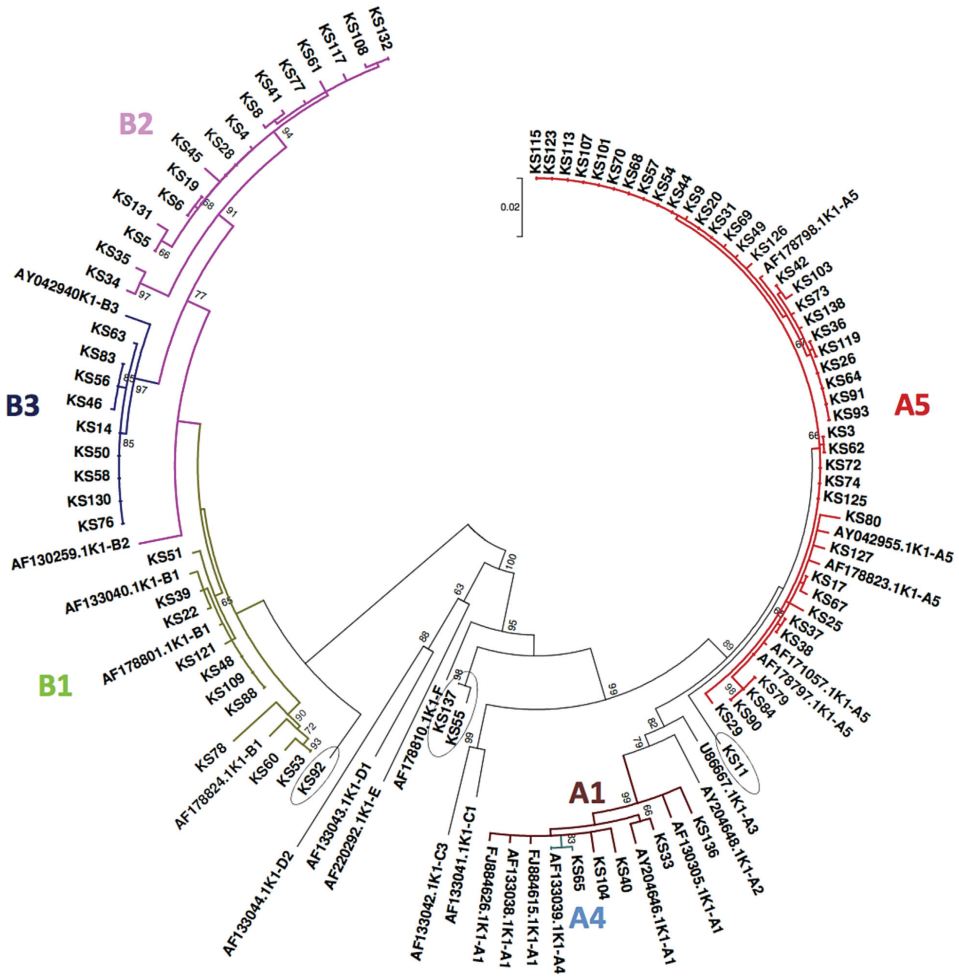

Fig. 2. Phylogenetic tree based on ORF-K1 coding region constructed by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach from 86 samples and other related reference strains obtained from GenBank database. The numbers given at each node represent the percentage bootstrap values (for 1,000 replications). Bootstrap value numbers less than 60 are not shown in the figure. Isolates that are circled are variants that did not cluster and are not subtyped.

subtype A5 typed to date are recombinant chimeras in which a very small portion of the left hand side of the genome (ORF-K1) is A-like, hence classified as subtype A5, while the remainder is made up of the African-specific B, as well as Q, R, and N subtypes constant region genomes [Hayward and Zong, 2007]. However, many South African genomes with ancient N type segments of presumed Khoisan or Xhosa origin, are ORF-K1 subtype A5, suggesting that more recent recombinant events occurred once Bantu chimeric genomes interacted with Khoisan genomes [Hayward and Zong, 2007].

According to Hayward and Zong, many South African genomes contain a much greater degree of variability in both the constant region and right hand side of the genome [Hayward and Zong, 2007]. Treurnicht et al. [2002] reported the presence of B3/C2 ORF26 constant region subtypes in A5 ORF-K1

TABLE III. Clinical Characteristics of Patients (n = 86)

Characteristic	Value (%)
Distribution	
Localized	21 (24)
Generalized	65 (76)
Morphology	
Patch	86 (33)
Plaque	72 (84)
Nodule	32 (37)
Fungating	3 (4)
Sites	
Face	45 (52)
Oral mucosa	30 (35)
Trunk	50 (58)
Upper limb	45 (52)
Lower limb	70 (81)
Genitalia	6 (7)
Lymphoedema	42 (49)
Number of lesions at presentation	
<10 lesions	24 (28)
>10 lesions	62 (72)

subtypes in South Africa. This suggests the hypothesis that the positive selective advantage of A5, may be as a result of the recombinant chimera, and not necessarily due to A5 alone. The prevalence of A5 in the current study (43/87, 49%) is consistent with that of 53% found in 31 AIDS-KS patients in Uganda [Kajumbula et al., 2006], and that of 40% found in 65 AIDS-KS patients in Zimbabwe [White et al., 2008], and 47% (7/15) found in 15 South African KS patients [Hayward and Zong, 2007]. European Subtypes A1 and A4 were found only amongst whites and patients of mixed ancestry, who were HIV positive. In our study, none of the European subtypes were present in black patients. This was also the case in Hayward and Zongs' study where the only two A4 ORF-K1 subtypes were in patients of Dutch descent [Hayward and Zong, 2007]. This supports the proposal that genetic subtypes of HHV8 are distributed along geographic and ethnic lines [Hayward, 1999; Zong et al., 1999, 2002]. South Africa has a diverse population and factors such as European and Asian colonization and human migration in Africa and the resultant mixed ancestry may account for the prevalence of the European subtypes in the South African population.

Four of the patients (5%), all AIDS-KS patients had viruses that failed to cluster with any of the known subtypes and these viruses were termed unclustered. This could be due to either recombination events between two existing subtypes or the development of new evolving strains.

Controversy exists around the pathogenicity and virulence of HHV8 subtype A. [Boralevi et al., 1998; Hayward, 1999; Meng et al., 1999; Zong et al., 1999; Lacoste et al., 2000; Mancuso et al., 2008; White et al., 2008; Zhang et al., 2008]. In an Italian study, subtype A has been linked with more rapidly progressing disease in classic KS [Mancuso et al., 2008]. Boralevi et al. reported that out of 40 HHV8 isolates from French AIDS-KS cases evaluated, subtype A was associated with more advanced disease, frequent mucosal and/or visceral KS (63% in subtype A vs. 27% in subtype B, $P < 0.05$) and concluded that subtype A displayed more aggressive pathogenicity [Boralevi et al., 1998]. This was confirmed in a study of 23 classic KS and 4 AIDS-KS cases by Zhang et al., in Xinjang, China, that showed that HHV8 subtype A was associated with more frequent mucosal lesions than subtype C [Zhang et al., 2008]. These data raise the question as to whether subtype A has unique biological features which makes it more pathogenic, and hence prognostically relevant.

In the present study, subtype A5 was found in patients who presented with more extensive disease, that is, more than 10 lesions. This suggests that A5 subtype may be associated with more extensive disease and supports the findings of Mancuso, Boralevi, and Zhang that subtype A may have more aggressive characteristics of pathogenicity [Boralevi et al., 1998; Mancuso et al., 2008; Zhang et al., 2008]. The current study describes the first reported observation of a possible association between subtype A5 and clinical presentation of KS, in particular more extensive disease in AIDS-KS. However, additional

TABLE IV. Association of Human Herpes Virus 8 Subtypes With Extent of Kaposi's Sarcoma

Subtypes		<10 lesions	>10 lesions	Unadjusted OR (95%CI)	Unadjusted *P*-value	Adjusted[a] OR (95%CI)	Adjusted[a] *P*-value
A1	Neg	21	63	1		1	
	Pos	3	1	0.11 (0.11–1.16)	0.067	0.11 (0.01–1.48)	0.096
A4	Neg	23	62	1		1	
	Pos	1	0	–	N/A	–	N/A
A5	Neg	16	28	1		1	
	Pos	8	34	2.42 (0.91–6.50)	0.078	**3.13 (1.02–9.58)**	**0.046**
B1	Neg	20	56	1		1	
	Pos	4	6	0.54 (0.14–2.10)	0.37	0.98 (0.15–6.52)	0.986
B2	Neg	18	52	1		1	
	Pos	6	10	0.58 (0.18–1.81)	0.347	0.49 (0.15–1.68)	0.258
B3	Neg	22	55	1		1	
	Pos	2	7	1.40 (0.27–7.27)	0.689	0.87 (0.13–5.63)	0.883

[a]Logistic regression model adjusted for age, sex, CD4 count, OR(95% CI) = odds ratio (95% confidence interval).

TABLE V. Correlation of Subtypes With Ethnicity

	Black (%)	Mixed race (%)	White (%)	Total	*P*-value
A1	0 (0)	3 (30)	1 (50)	4	**<0.001**
A4	0 (0)	0 (0)	1 (50)	1	**0.023**
A5	40 (56)	2 (20)	0 (0)	42	**0.040**
B1	9 (12)	1 (10)	0 (0)	10	1.000
B2	15 (94)	1 (6)	0 (0)	16	0.788
B3	8 (89)	1 (11)	0 (0)	9	1.000
Unclustered	2 (3)	2 (20)	0 (0)	4	0.092
Total	74	10	2	86	

larger studies are required in order to verify this finding and correct for cohort-related biases. This could have important implications on disease prognosis and determining therapeutic options and response to treatment. More aggressive therapy, namely ART with combined chemotherapy may be warranted in AIDS-KS patients with subtype A5 at the onset of disease.

An association between subtypes and clinico-epidemiological forms of KS has been reported in some studies [Gazouli et al., 2004; Ramos da Silva et al., 2011], but not in others [Cook et al., 1999; Meng et al., 1999; Zong et al., 1999; Lacoste et al., 2000; White et al., 2008]. Most recently, da Silva et al. reported that out of 50 KS patients evaluated in Brazil, an elevated frequency of HHV8 subtype A was found in HIV-positive patients (15/22; 68,2%). Conversely, the majority of KS isolated from HIV-negative patients had subtype C. The difference in proportions between A and C subtypes within HIV-positive and HIV-negative patients was statistically significant ($P = 0.026$, Fisher's exact test) [Ramos da Silva et al., 2011]. Distribution of ORF-K1 variants among classic and AIDS-KS in the United States showed predominantly C subtypes in classic KS, while AIDS-KS were predominantly subtype A, but this was not statistically evaluated [Zong et al., 2002]. Gazouli also suggested an association of subtype A4 in AIDS-KS and A1 in classic KS in Greece [Gazouli et al., 2004]. The present study found a strong association between subtype A5 and extent of disease in AIDS-KS, but not in African endemic KS. However, this conclusion may be because the African endemic group in the study consisted of only five patients. In the present study, subtype A1 was associated with a lower likelihood of lower limb involvement. The lower limb is the typical site for classic KS. However, in the current study A1 and A4 were all AIDS-KS patients. In addition, A1 and A4 were associated with a lower likelihood of poor risk tumor extension, hence a better prognosis.

There were slightly more women than men in the age group <35 years affected with KS, suggesting an earlier age of presentation of KS in women, while above 35 years, there were markedly more men affected. This finding that KS occurs at an earlier age in women compared to men, is consistent with a study of 152 patients conducted in KwaZulu-Natal, South Africa, as well as with smaller retrospective studies conducted in Europe and United States [Cooley et al., 1996; Nasti et al., 1999; Mosam et al., 2008]. Reasons proposed for this early age of presentation in women include that the risk for developing female KS is closely related to the epidemiology of the HIV. In KwaZulu-Natal, the age-specific distribution pattern for female KS [Mosam et al., 2008] was essentially identical to the age specific distribution pattern of HIV reported in an earlier study [Mosam et al., 2008; Rollins et al., 2002]. Health-seeking behavior and access to health care may also contribute to this finding. Women may have been exposed to HIV testing during antenatal care, hence referred to HIV treatment centers earlier resulting in earlier KS identification.

There are several limitations to the current study. A major limitation of this study is that only one segment of HHV8 genome, ORF-K1, was subtyped. It would have been more useful to evaluate the constant region as well the right hand side of the genome, especially with respect to recombinant genomes in A5, as well as the greater degree of hypervariability in the constant and RHS of South African genomes [Hayward and Zong, 2007]. Hayward and Zong address this aspect of only using one segment of the genome to make conclusions about pathological correlations [Hayward and Zong, 2007; Zong et al., 2007]. The interpretation of biological and pathological correlations from one segment has to take into account the geographical patterns, the frequency of heterogeneity in the virus, and recombinant and chimeric genomes [Zong et al., 2007]. However, if the assumption is made based on the 14 South African samples of Hayward and Zong that all of the South African genomes are non A/C in the bulk center blocks and non-M at the K15 right hand side of the genome [Hayward and Zong, 2007], then one can generalize from the ORF-K1 data alone. Any future studies looking at correlations between ORF-K1 and pathogenicity should take into account other polymorphic loci within the genome and the possibility of recombinant genomes.

The morphological description of KS relied on observer's subjective documentation on body diagrams, photographic records, and clinical records. However, all the patients were seen by dermatologists of the same department, making the morphological descriptions more uniform. Complete ACTG staging was not available for patients, and there was limited assessment of visceral involvement. This was due to the standard of care offered in the oncology unit which is based on the tenant that treatment would remain unchanged irrespective of visceral involvement. Clinical data on the duration on ART in AIDS-KS cases were not available, which may have impacted on clinical presentation. Other potential confounding variables that were not controlled for include disease duration and whether on chemotherapy. Subtyping of

ORF-K1 was only possible in 86 cases. Subtyping was unsuccessful because of failure to PCR amplify the DNA region combined with insufficient DNA. This is consistent with previous studies, such as that of Zhang, where only 22/27 could be successfully amplified, and that of White where only 65/171 could be amplified due to insufficient DNA [White et al., 2008; Zhang et al., 2008].

Language was used as proxy for ethnicity. This may not be the most accurate measure of ethnicity, but was the only means of assessing ethnicity in a retrospective study. The findings that there was a strong association between genetic subtypes and ethnicity, strengthens the use of language as proxy for ethnicity.

Variability within the ORF-K1 gene displays very high nonsynonymous rates, with up to 85% of nucleotide changes producing amino acid changes, implying that a powerful biological selection process is involved [Hayward and Zong, 2007; Zong et al., 1999]. The advantage of the rapidity of evolution of ORF-K1 subtypes of HHV8 is yet to be elucidated, but may be due to a need of the virus to evade the immune system and establish persistent infection as described in HHV6 [Lusso et al., 1995] or due to increasing the pathogenicity and virulence of HHV8. Infection with HHV8 may also potentially have an as yet unknown evolutionary advantage to the human host as has been postulated for EBV and HHV6 [Hesla et al., 2013].

In summary, subtypes A5 and B were found to predominate in South Africa, and are distributed along broad ethnic lines. More extensive disease was noted in AIDS-KS associated A5 subtype. A1 and A4 combined were associated with a lower likelihood of poor risk tumor extension, and A1 with a lower likelihood of lower limb involvement. Further research is needed to confirm the proposal that A5 may be a more pathogenic strain than subtype B in Africa.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

ADDENDUM TO MANUSCRIPT AS REQUESTED BY EXAMINER

ADDENDUM 1: DETAIL ON 4 UNCLUSTERED SAMPLES

Four samples did not cluster with any of the major subtypes and were labelled as unknown/unclustered. These four samples, although unique, did have some features suggesting a major subtype; KS92 was B-like, KS55 and KS137 were F-like while KS11 was A-like.

ADDENDUM 2: DETAIL ON B3

The B3 variant is distinguishable from other B variants by a motif at the beginning of VR2 (residues 194-198). Kajumbula et al identified two sequences that belonged to a new variant which they called B3. These sequences clustered with previously identified B sequences from Ugandan samples (Ugd1, Ugd19, Ugd7, Ugd2, Ugd21 and Ugd26) [Kajumbula et al., 2006, Kakoola et al., 2001].
In our study, we have used AY042940 (Ugd26) as a reference sequence for the B3 variant. This is comparable to White et al 2009 who used Ugd26 (AY042940) as well as the other 5 sequences (Ugd1, Ugd19, Ugd7, Ugd2 and Ugd21) as B3 references in their phylogenetic analysis [White et al., 2009]. Olp et al used AY042941 (Ugd19) as their B3 reference sequence which clustered with Ugd26 [Olp et al., 2015]. Hence the B3 sequences in this study, cluster with the B3 sequences of Kakoola, Kajumbula, White and Olp [Kajumbula et al., 2006]; Kakoola et al., 2001; White et al., 2009; Olp et al., 2015].

CHAPTER 3: APPENDICES

APPENDIX 1

KARNOFSKY SCORE [Karnofsky et al: Cancer 1:64, 1948]

Able to carry on normal activity; no special care is needed	100 Normal; no evidence of disease 90 Able to carry on activity; minor signs or symptoms of disease 80 Normal activity with effort; some signs and symptoms of disease
Unable to work; able to live at home and care for most personal needs; a varying amount of assistance is needed	70 Cares for self; unable to carry on normal activity or to do active work 60 Requires occasional assistance but is able to care for most needs 50 Requires considerable assistance and frequent medical care
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly	40 Disabled; requires special care and assistance 30 Severely disabled; hospitalization is indicated although death not imminent 20 Very sick; hospitalization necessary; active supportive treatment is necessary 10 Moribund, fatal processed progressing rapidly 0 Dead

APPENDIX 2

Supplementary Table S1: Correlation of subtypes with number of lesions

	>10 (%)	<10 (%)	Total	P value
A1	1 (2)	3 (13)	4	0,064
A4	0 (0)	1 (4)	1	0,279
A5	34 (55)	8 (33)	42	0.074
AIDS-KS	32 (52)	6 (25)	38	0,050
African endemic	2 (3)	2 (8)	4	0,600
B1	6 (10)	4 (17)	10	0,287
B2	10	6	16	0,365
B3	7	2	9	1,000
Unclustered	4 (6)	0 (0)	4	0,263
Total	62	24	86	

APPENDIX 3

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30 July 2013

HREC REF: 417/2013

Prof A Katz
N2.02 WNB
IIDMM
FHS

Dear Prof Katz

PROJECT TITLE: KAPOSI'S SARCOMA: GENETIC SUBTYPES AND CLINICAL CORRELATION IN SOUTH AFRICAN POPULATION LINK TO 279/2008 (MMed Candidate – Dr Thuraya Isaacs)

Thank you for your letter dated 22 July 2013, addressing the issues raised by the Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year till the 28 August 2014.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

We acknowledge that the following MMed Candidate, Dr T Isaacs will also be involved in this study.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research

APPENDIX 4

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PATIENT`S INFORMATION SHEET
AND CONSENT FORM

INVESTIGATING POLYMORPHISMS IN THE KAPOSI'S SARCOMA-ASSOCIATED HERPES VIRUS G-PROTEIN-COUPLED RECEPTOR GENE IN SOUTH AFRICAN KAPOSI'S SARCOMA TUMOURS

Kaposi's sarcoma is caused by a virus named Kaposi's sarcoma-associated herpes virus (KSHV) which is also named human herpesvirus-8 (HHV-8). This disease is present in Africa and in other parts of the world including USA and Europe. However, only the viruses isolated from patients in the USA and Europe have been studied. In this project we intend to study the Kaposi's sarcoma-associated herpes virus present in South African populations. This study will inform us, if there are differences between the viruses in South Africa and those studied in the USA and Europe. If we will find differences, we will examine how the differences affect the development of the tumour.

You are invited to participate in this study and we request that you allow one of the medical doctors who is treating you to take a tiny piece of your tumour.

If you agree to participate, we will take a sample during your routine visit to the clinic. However, if we ask you to return on non-clinic days in order to perform the biopsy, we will pay for your transport costs.

A medical doctor or nurse practitioner will take a small piece of skin (4mm) out of the tumour. They will first give you an injection that may sting to anaesthetise the tumour area. The injection is like the one the dentist uses. When you can no longer feel that area, a special knife will be used to remove the skin. The area may bleed and need a stitch. After stopping the bleeding a dressing will be used to cover the wound for 24 hours after which it can be taken of and the skin washed and cleaned in the usual way. You will slowly start to feel mild soreness in the area as the anaesthetic wears off. If the wound gets red and very painful please phone ……..and we will see you as soon as possible. If the biopsy causes any complication/problem that needs medical care we will treat you free of charge.

None of the team doing this study will be paid for it. Money to pay for the tests and to refund you for your travel was given by the Medical Research Council and UCT.

The results of this study will not be used in the further management of your condition. Any information that is obtained in connection with this study that can identify you will remain confidential and will be disclosed only with your permission. If you agree to participate by signing this document, your results in so far as they are reflected in the study will be reported/disclosed (in a manner that will not identify you) to the researchers, to regulatory agencies world-wide and in scientific medical literature. The researchers will undertake to protect your confidentiality. We will give you the results of the tests as soon as we receive them. We will not be able to tell what they mean until we have finished the study and this could take 1 to 2 years.

Your decision whether or not to participate will not prejudice your future relations and with the hospital/clinic or your doctor. If you decide to participate, you are free to withdraw consent at any time and such withdrawal

will not jeopardise any further treatment or your relationship with the hospital/clinic or your medical attendants.

I ……..(Patient's full name) have read the attached information leaflet about the study. I fully understand the reasons why the study is being done. I am aware that a sample of my tumour will be taken for purposes of this study.

I voluntarily agree to participate in the study without any coercion. I also know that should I change my mind about participating in the study, I may do so without compromising my future prospects of treatment or care.

…….. …../……./…...

(Patient's signature) (Date)

…….. ……..

(Investigator) (Witness)

APPENDIX 5

Journal of Medical Virology

Periodicals Inc.

Edited By: ARIE J. ZUCKERMAN

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ISI Journal Citation Reports © Ranking: 2013: 21/32 (Virology)
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Formal taxonomic nomenclature
In formal taxonomic usage, the first letters of virus order, family,subfamily, genus and species names are capitalized and the terms are printedin italics. Other words in the species name are not capitalized unless theyare proper nouns or parts of nouns, for example *West Nile virus* . Informal usage, the name of the taxon should precede the term for the taxonomicunit; for example: "the family *Paramyxoviridae*," "the genus *Morbillivirus*." The following represent examples of full formal taxonomic terminology:
1 Order *Mononegavirales*, Family *Rhabdoviridae*, genus *Lyssavirus*, Species *Rabies virus*.
2 Family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus*, species *Vaccinia virus*.
3 Family *Picornaviridae*, genus *Enterovirus*, species *Poliovirus*.
4 Family *Bunyaviridae*, genus *Tospovirus*, species *Tomato spotted wilt virus*.

Vernacular taxonomic nomenclature

In formal vernacular usage, virus order, family, subfamily, genus and species names are written in lower case Roman script; they are not capitalized, nor are they printed in italics or underlined. In informal usage,the name of the taxon should not include the formal suffix, and the name of the taxon should follow the term for the taxonomic unit; for example "the picornavirus family," "the enterovirus genus." One particular source of ambiguity in vernacular nomenclature lies in the common use of the same root terms in formal family, genus or species names. Imprecision stems from not being able to easily identify in vernacular usage which hierarchical level isbeing cited. For example, the vernacular name "paramyxovirus" might refer to the family *Paramyxoviridae*, the subfamily *Paramyxovirinae*, or one species in the genus *Respirovirus*, such as *Human parainfluenza virus 1* .The solution in vernacular usage is to avoid ‘‘jumping’’ hierarchical levels and to add taxon identification wherever needed. For example, when citing thetaxonomic placement of *Human parainfluenza virus 1*, taxon identification should always be added: " *Human parainfluenza virus 1* is a species in the genus *Respirovirus*, family *Paramyxoviridae*." In this example, as is usually the case, adding the information that this virus is also a member of the subfamily *Paramyxovirinae* and the order *Mononegavirales* isunnecessary.

It should be stressed that italics and capitals initial letters need to be used only if the species name refers to the taxonomic category. When the name refers to viral objects such as virions present in a preparation or seen in an electron micrograph, italics and capitals initial letters are not needed and the names are written in lower case Roman script. This also applies when the names are used in adjectival form, for instance tobacco mosaic virus polymerase. The use of italics when referring to the name of a species as a taxonomic entity signals that it has the status of an officially recognized species. The 7th ICTV Report (Van Regenmortel, M.H.V. et al., 1999, Academic Press) should be consulted to ascertain which names have been approved as official species names. When the taxonomic status of a new putative species is uncertain or its position within an established genus has not been clarified, it is considered a tentative species and its name is not written in italics although its initial letter is capitalized.

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Lazinski DW, Taylor JM. 1993. Structure and function of the delta virus antigens. In: Hadziyannis SJ, Taylor JM, Bonino F, editors. Hepatitis delta virus—molecular biology, pathogenesis, and clinical aspects. New York: Wiley-Liss, Inc. p 35–44.
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??? Production Questions ???

JMV Production
E-mail: jmvprod@wiley.com

APPENDIX 6

KS no:_____ Age:_____ Gender: M/F

Race:_____Ethnicity:_____

HIV status:_____ On ARV: Y/N CD4 count:_____/not documented

Coinfection TB: Y/N/not documented

SKIN EXAM:

Extent: Localised KS(Confined to one anatomical area): Y/N

Generalised/ Disseminated Cutaneous KS(2 or more sites): Y/N

Morphology: Patch Y/N

Plaque Y/N

Nodule Y/N

Fungating Y/N

Anatomic site: Face/neck Y/N

Oral Y/N

Trunk Y/N

Upper limb Y/N

Lower limb Y/N

Genitalia Y/N

Number of lesions:

<10

>10

Lymphoedema: Y/N

Histological confirmation: Y/N

HHV8 confirmation: Y/N

PHOTOS: Y/N_____

HHV 8 GENOTYPE:_____