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ROLE OF HHV-8 VIRAL PARAMETERS AND KIR/HLA  
COMPLEXES ON THE DEVELOPMENT OF CLASSIC AND  
EPIDEMIC KAPOSÍ'S SARCOMA

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## SOMMARIO

*Il sarcoma di Kaposi (KS) è una rara neoplasia di natura vascolare a diffusione multicentrica, correlata a infezione da Human Herpesvirus 8 (HHV-8). Sulla base delle caratteristiche clinico-patologiche, il sarcoma di Kaposi viene generalmente suddiviso in quattro forme: classico (cKS), epidemico (o AIDS-associato), endemico e iatrogeno. Sebbene sia stato ormai dimostrato come il virus HHV-8 sia l'agente eziologico della patologia, tuttavia è da sottolineare il fatto che l'infezione di tale virus è necessaria ma non sufficiente per lo sviluppo del sarcoma di Kaposi, e che quindi esistono altri fattori, propri del virus (modalità di trasmissione, potenzialità patogenica dei vari genotipi virali) o dell'ospite (predisposizione genetica, condizione immunologica) che possono influenzarne lo sviluppo. Le cellule natural-killer (NK) sono una componente fondamentale della risposta immune innata contro infezioni virali e come difesa per lo sviluppo di cellule tumorali: la loro attività è modulata da un complesso sistema basato sul legame di particolari recettori presenti nella loro superficie, i killer cell immunoglobulin-like receptor (KIR), a loro volta suddivisi in KIR attivatori e KIR inibitori, agli human leukocyte antigens di classe I (HLA-I), presenti sulle superficie delle cellule target. Sulla base di tutti questi dati, lo scopo di questa tesi di dottorato è di verificare se lo sviluppo del cKS e del KS epidemico sia correlato a particolari parametri virali del virus HHV-8 (presenza di anticorpi, carica virale, genotipo) o a un particolare genotipo KIR/HLA recettore/ligando dell'ospite, confrontando pazienti affetti da cKS e da KS epidemico con appropriati gruppi di controllo. Per quanto riguarda i parametri virali, gli anticorpi anti-HHV-8 sono stati trovati in tutti (100%) i pazienti cKS e nell'80% dei pazienti con KS epidemico, mentre erano presenti solo nel 50% dei soggetti di controllo HIV<sup>+</sup> e nel 10% dei soggetti sani HIV (p<0.0001). Inoltre, per quanto riguarda il diverso genotipo virale, i pazienti cKS con progressione rapida della malattia presentavano maggiormente il genotipo A del virus HHV-8 (63.6% vs 23.1%), mentre quelli con progressione lenta il genotipo C (76.9% vs 36.4%; p=0.003). Per quanto riguarda l'aspetto genetico dell'ospite, i KIR attivatori, assieme ai loro ligandi HLA, sono risultati più frequenti nei pazienti con cKS e KS epidemico rispetto ai soggetti non-KS, indipendentemente dall'infezione o meno da HHV-8 o da HIV. Prendendo in considerazione i geni KIR attivatori uno ad uno, il gene KIR2DS2 è risultato significativamente più frequente sia nei pazienti con cKS sia in quelli con KS epidemico rispetto ai soggetti sani (p=0.02 per entrambi), mentre i geni KIR2DS1 e KIR3DS1 sono stati trovati più frequentemente nei pazienti cKS (p=0.02 e p=0.04 rispettivamente). Inoltre, prendendo in considerazione il complesso KIR/HLA, il genotipo attivatore KIR2DS1/C2 è risultato positivamente associato con lo sviluppo di cKS (p=0.01). Nessuna differenza è stata invece trovata nella distribuzione dei KIR inibitori e degli HLA tra i gruppi presi in esame. In conclusione, questo studio dimostra che 1) il genotipo A del virus HHV-8 è associato ai parametri clinici peggiori della patologia, inclusa la sua progressione rapida, 2) nel cKS e KS epidemico è presente un "milieu" attivatorio KIR/HLA, e che questo milieu possa quindi essere un fattore di rischio per lo sviluppo della neoplasia.*

## ABSTRACT

*Kaposi's sarcoma (KS) is a rare vascular tumor of the skin related to Human Herpesvirus 8 (HHV-8) infection. Based on clinical and pathological characteristics, KS is divided in four different forms: classic KS (cKS), epidemic (or AIDS-associated) KS, endemic KS and iatrogenic KS. Although the link between HHV-8 and disease has been demonstrated, it is important to note that the HHV-8 infection is necessary but not sufficient to develop the disease: many important aspects remain to be elucidated, regarding both viral factors, as presence of antibody anti-HHV-8, viral transmission and pathogenic potential of different viral genotypes, and host predisposition to tumor development, different from person to person. Natural killer cells are central components of the innate immune response against viral infection and tumor growth: the modulation of their activity is a complex and multi factorial phenomenon triggered by the binding of inhibitory or activating killer cell immunoglobulin-like receptors (KIRs) to class I human leukocyte antigens (HLAs). For these reasons, the objective of this thesis is to verify whether the development of cKS and epidemic KS is related with particular HHV-8 viral parameters (viral load, genotype) or with particular host KIR/HLA receptor/ligand genotype, comparing cKS and epidemic KS patients with the appropriate control groups. Regarding the viral parameters, anti-HHV-8 antibodies were detected in all (100%) cKS patients, and in 80% of epidemic KS patients, whereas they are present in 50% of HIV<sup>+</sup> healthy individuals and in 10% of HIV<sup>-</sup> healthy donors only ( $p < 0.0001$ ). Moreover, when the HHV-8 genotypes were analyzed, A subtype was significantly more frequently isolated in cKS patients with fast progression of the disease (63.6% vs 23.1%), whereas C subtype in individuals with slow progression (76.9% vs 36.4%;  $p = 0.003$ ). Regarding the host genetic aspects, activating KIRs, together with their HLA ligands, were more frequent in cKS and epidemic KS compared to non-KS subjects, regardless of the presence or absence of HHV-8 or HIV infection. Considering the activating KIR one by one, the KIR2DS2 gene was significantly prevalent in both cKS and epidemic KS patients ( $p = 0.02$  for both), whereas KIR2DS1 and KIR3DS1 genes were more prevalent in cKS patients ( $p = 0.02$  and  $p = 0.04$  respectively). Moreover, considering the KIR/HLA complexes, the activating KIR2DS1/C2 genotype was positively associated with cKS development ( $p = 0.01$ ). Finally, the inhibitory KIR distribution, as well as the HLA ligand distribution alone, did not reveal any statistical association with cKS or epidemic KS. In conclusion, this study shows that 1) the A genotype of HHV-8 is associated with worst clinical parameters of KS, including faster progression, 2) that a KIR/HLA "activating milieu" is present in cKS and epidemic KS and that such milieu may be a risk factor for the development of the tumor.*

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## LIST OF SYMBOLS AND ABBREVIATIONS

**AIDS:** acquired immunodeficiency syndrome  
**APC:** antigen-presenting cell  
**Arg:** arginine  
**Asp:** aspartic acid  
**BCBL:** body-cavity-based lymphoma cells  
**bFGF:** basic fibroblasts growth factor  
**BK virus:** Brennan-Krohn virus  
**bp:** base pairs  
**CD:** cluster of differentiation  
**CMV:** cytomegalovirus  
**cKS:** classic Kaposi's Sarcoma  
**COX2:** cytochrome oxidase subunit II  
**cyc:** cycline  
**DC-SIGN:** dendritic cell-specific ICAM-3-grabbling nonintegrin  
**DMSO:** dimethyl sulfoxide  
**DNA:** deoxyribonucleic acid  
**dNTP:** deoxyribonucleotide triphosphate  
**DR:** death receptor  
**EBV:** Epstein Barr virus  
**EDTA:** ethylenediaminetetraacetic acid  
**FBS:** fetal bovine serum  
**FITC:** fluorescein isothiocyanate  
**FLADD:** fas-associated protein with death domain  
**FLICE:** FLADD-like IL- $\beta$ -converting enzyme  
**FLIP:** FLICE-inhibitory protein  
**Gly:** glycine  
**GPCR:** G protein-coupled receptor  
**HCV:** hepatitis C virus  
**HHV-8:** Human herpesvirus 8  
**HIV:** Human immunodeficiency virus  
**HLA:** human leukocyte antigen  
**HPV:** human papilloma virus  
**HVS:** Herpesvirus saimiri  
**ICAM:** intercellular adhesion molecule  
**Ig:** immunoglobulin  
**IL:** interleukin  
**IFA:** immunofluorescence assay  
**IFN:** interferon  
**IRF:** interferon regulatory factor  
**ITAM:** immunoreceptor tyrosine-based activation motifs  
**ITIM:** immunoreceptor tyrosine-based inhibitory motifs  
**LANA:** latency-associated nuclear antigen  
**LCL:** lymphoblastoid cell line  
**LRC:** leukocyte receptor complex

**LUR:** long unique region  
**KIR:** killer Immunoglobulin-like receptors  
**KS:** Kaposi's sarcoma  
**KSHV:** Kaposi's Sarcoma herpesvirus  
**MCD:** multicentric Castleman's disease  
**MCP:** macrophage chemotactic protein  
**MHC:** major histocompatibility complex  
**microRNA:** micro ribonucleic acid  
**MIP:** major intrinsic protein  
**mRNA:** messenger ribonucleic acid  
**NCAM:** natural cell adhesion molecule  
**NK cells:** natural killer cells  
**ORF:** open reading frames  
**PAN:** polyadenylated nuclear RNA  
**PBMC:** peripheral blood mononuclear cells  
**PBS:** phosphate buffer saline  
**PCR:** polymerase chain reaction  
**PDGF:** platelet derived growth factor  
**PEL:** primary effusion lymphoma  
**qPCR:** quantitative PCR  
**RAP:** replication associated protein  
**RBP-J:** recombination signal binding protein for immunoglobulin kappa J region  
**RDA:** representational difference analysis  
**RNA:** ribonucleic acid  
**RPM:** revolutions per minute  
**RPMI:** Roswell Park Memorial Institute  
**RTA:** replication and transcription activator  
**SEM:** standard error of mean  
**SOX:** shutoff exonuclease  
**STAT3:** signal transducer and activator of transcription 3  
**TBE:** tris borate EDTA  
**TNF:** tumor necrosis factor  
**TPA:** tetradecanoylphorbol 13-acetate  
**TR:** tandem repeated  
**TRAIL:** tumor necrosis factor-related apoptosis inducing ligand  
**TYRO:** tyrosin kinase binding protein  
**VEGF:** vascular endothelium growth factor  
**VEGFR:** vascular endothelium growth factor receptor  
**VR:** variable region  
**xCT:** exchange cysteine/glutamate transporter

# CHAPTER I

## INTRODUCTION

### **1.1 Kaposi's sarcoma**

#### **1.1.1 General characteristics**

Kaposi's sarcoma (KS) was originally described in 1872 by the Hungarian dermatologist Moritz Kaposi as “a multiple pigmented sarcoma affecting elderly Mediterranean men” [1]. To date, several epidemiological forms have been classified. Kaposi's sarcoma lesions are histologically complex and evolve over time, from a patch or plaque-like lesion at early stages to nodular lesions characteristic of disease progression. Several hypotheses regarding the pathogenesis of this vascular tumor have been suggested, including both infectious agents and cytokine-mediated processes. In 1994 a study by Chang and colleagues found a novel herpesvirus present in KS tissue [2]. This virus, called Human Herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV), is now considered the etiological factor of the disease: the infection of this virus is necessary to develop KS.

#### **1.1.2 Epidemiological forms of Kaposi's sarcoma**

Kaposi's sarcoma is a multifocal vascular neoplasm involving the skin, visceral organs and lymph nodes. The lesions are complex and contain distinctive proliferating cells with fusiform morphology, called spindle cells, activated endothelial cells and infiltrating inflammatory cells [3]. At now, based on clinical and pathological characteristics, KS is divided in four different forms: classic KS, epidemic KS, endemic KS and iatrogenic KS.

The *classic form of KS (cKS)* predominantly affects elderly male patients of Southern European heritage [4]. The tumor is also frequently described in patients from Israel, mainly in the Jewish population and in other Middle Eastern countries [5]. Disease progression in cKS patients occurs over a period of several years. The cKS can be associated with other neoplasias, like Hodgkin's lymphoma or B-cell lymphoma, due to alterations of immunological system mechanisms. The patients have plaques and red nodules, initially localized on legs, which can spread on arms and, in the 10% of cases, on internal organs [6].

The *epidemic form of KS (or AIDS-associated KS)*, that affects subjects (predominantly adult and male) with concomitant infection of HIV-1 virus, is clinically the more aggressive form of KS and it is characterized by multifocal lesions, localized in particular on head and neck, that can spread on lymph nodes, causing organ dysfunction. For this form of KS, the mortality is very high [6].

The *endemic form of KS* can be found in some equatorial countries of Africa. Here, KS is described in both young and elderly HIV-negative patients. The disease progression is more aggressive than for classical KS, in particular for children where lymph node involvement is common [7].

Finally, the incidence of this vascular tumour has increased among renal transplant recipients and other patients receiving immunosuppressive therapy. This form is known as post-transplant KS or *iatrogenic Kaposi's sarcoma* [6].

### **1.1.3 Histology of Kaposi's sarcoma**

Kaposi's sarcoma is characterized by bundles of spindle-shaped cells and endothelial-lined vascular spaces with lymphocytic infiltrates. Commonly red blood cells percolate in the spaces between the spindle cells [8]. These spindle cells produce pro-inflammatory and angiogenic factors that play an important role in the recruitment of additional cells to the sarcoma [9, 10]. Also, KS cells are highly dependent on inflammatory cytokines in culture (figure 1). This observation led to the hypothesis that KS pathogenesis may be influenced by inflammatory cytokines and angiogenic factors [11, 12]. KS is not a typical sarcoma, in that multiple lesions may appear simultaneously at several locations, but may disappear completely over time. As mentioned earlier, KS starts as a patch or plaque-like lesion at early stages and develops into the nodular lesions characteristic of later stage disease. In early stages small irregular endothelial-lined spaces, surrounded by normal blood vessels and inflammatory infiltrates of lymphocytes can be detected. As the disease progresses, the number of spindle shaped cells increases throughout the dermis and forms slit like vascular channels containing erythrocytes (patch stage). In the final, nodular stage, the lesions are characterized by sheets of spindle cells [6]. KS cells represent a heterogeneous population, as indicated by the variety of surface markers. The cells identified by histology are mainly activated endothelial cells mixed with fibroblasts, smooth muscle cells and cells of dendritic and monocytic origins [13].

The clonality of KS is frequently discussed, as the exact tumor cell type is controversial. Especially in early lesions spindle cells are a minority. Spindle cells are usually not prominent until late stage disease. It is demonstrated that spindle cells belong to an endothelium lineage that expresses the vascular endothelium growth factor receptor-3 (VEGFR-3), a marker of lymphatic vessels [14]. Other studies analyzing inactivation pattern of the X chromosome, a marker for clonality, revealed both a monoclonal and polyclonal pattern [15]. KS may be initially a non-clonal proliferation, but may sometimes develop into a clonal malignancy with metastases during advanced disease [16].

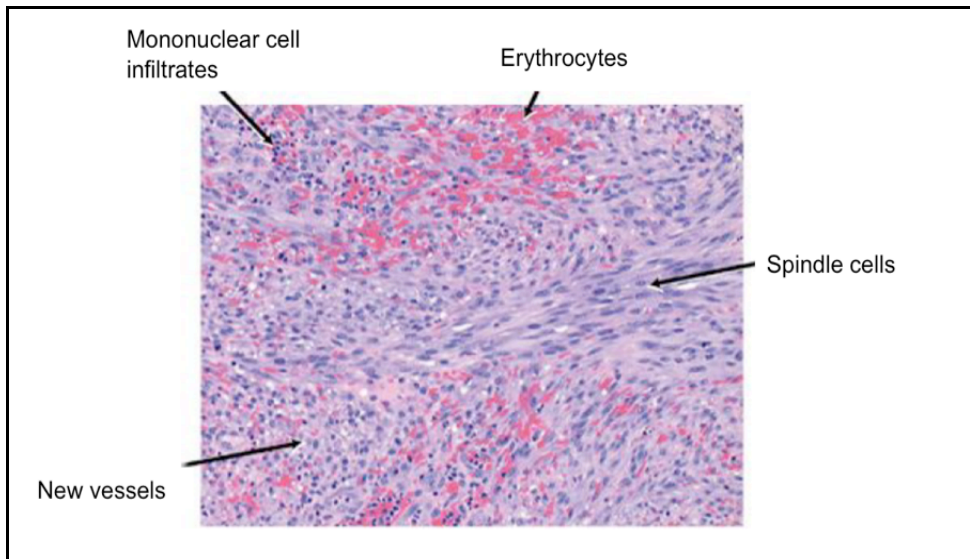


Figure 1 - Histological image of a characteristic KS lesion with hematoxylin-eosin treatment. In particular, we can see the spindle cells, with their characteristic fusiform form, and the neo-angiogenesis

#### 1.1.4 Pathogenesis of Kaposi's sarcoma

Kaposi's sarcoma lesions produce high levels of cytokines both *in vivo* and in culture. Several cytokines and growth factors have been implicated in the proliferation of KS spindle cells, including oncostatin M, platelet derived growth factor (PDGF), Interleukin-1 $\beta$  (IL-1  $\beta$ ), IL-6, IL-8, basic fibroblasts growth factor (bFGF), VEGF, macrophage chemotactic protein-1 (MCP-1), and the HIV-1 Tat protein [9,11,17]. Data from gene expression studies further support their involvement in KS pathogenesis as angiogenic properties of IL-6, bFGF and IFN- $\gamma$  (interferon gamma) could be demonstrated *in vitro* [18]. As disease progression in epidemic KS is more aggressive, it has been suggested that proteins encoded by HIV may influence tumour growth. Observations showed that the transcription activator protein, Tat, in combination with bFGF, stimulates the proliferation of KS associated spindle cells *in vitro* and can induce KS-like lesions in mice [19]. This HIV encoded protein also induces cellular genes that are pro-proliferative and pro-inflammatory (MCP-1, IL-6) in KS and possibly enhance the recruitment of leukocytes to KS lesions [20]. While *in vitro* and animal models strongly suggested that cytokines and growth factors play an important role in the development of KS [19], epidemiological data implicated a sexually transmitted cofactor [6]. AIDS patients with Kaposi's sarcoma are predominantly gay or bisexual men, rather than patients who acquired the virus through heterosexual contact, haemophiliacs or intravenous drug users [6]. These findings favour an infectious agent or co-factor in the development of KS. Infectious agents such as cytomegalovirus (CMV), HHV-6,

Human Papilloma Virus (HPV), BK virus and other viral or bacterial pathogens have all been linked to KS. However, in 1994 a research group in New York detected herpesvirus-like DNA from biopsies of epidemic KS using a subtractive DNA hybridisation technique known as representational difference analysis (RDA) [2]. The technique relies on cycles of subtractive hybridisation and polymerase chain reaction to enrich and isolate nucleotide sequences which differ between KS affected tissue and unaffected surrounding cells. Two subgenomic BamHI fragments of DNA, designated KS330<sub>Bam</sub> and KS631<sub>Bfl</sub> were isolated. Subsequent computer-assisted homology searches revealed that these novel sequences were related to the minor capsid and tegument protein genes of the lymphotropic gamma-herpesviruses Epstein Barr (EBV) and Herpesvirus saimiri (HVS) [2]. Further characterization of this newly identified the agent, called Human Herpesvirus 8 (HHV-8), and the sequencing of its whole genome [21] confirmed its classification as a member of the gamma-2 herpesvirus family [22]. Visualization of herpesvirus like particles by electron microscopy provided additional evidence for the viral origin of these sequences [23].

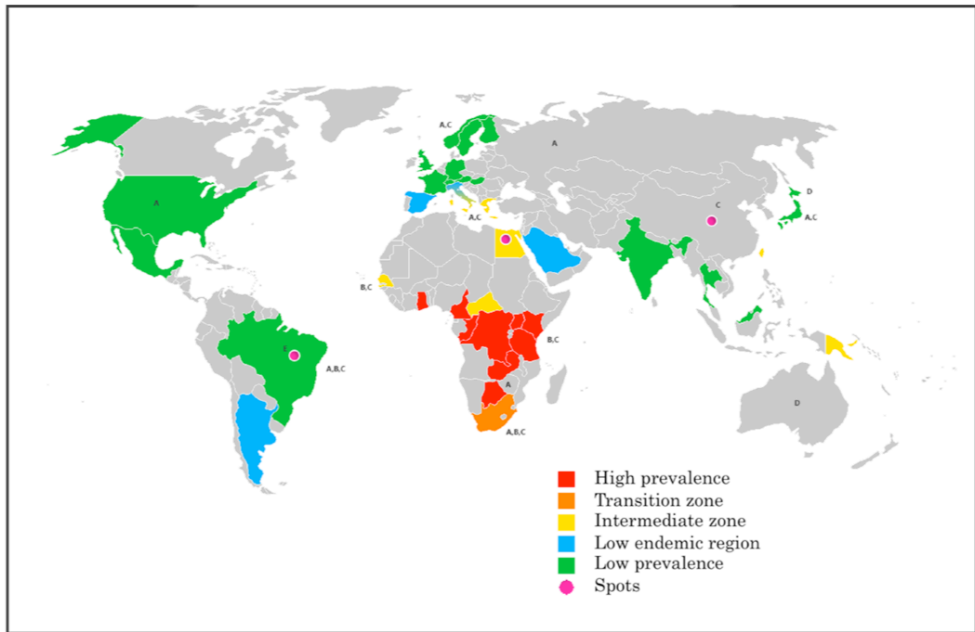
## **1.2 Human Herpesvirus 8 (HHV-8)**

### **1.2.1 General characteristics**

Human Herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated Herpesvirus (KSHV), belongs to viral Herpesviridae family, Gammaherpesvirinae subfamily, Rhadinovirus genus [22].

### **1.2.2 Epidemiology of HHV-8**

Polymerase chain reaction and serological studies have been used to define the epidemiology of HHV-8. Current knowledge suggests that HHV-8 is not ubiquitous in Northern Europe, North America and some parts of Asia, but is relatively common in Mediterranean country (Italy, Greece, Israel) and Africa [24]. In the general population of North America, Japan and Western Europe, HHV-8 seroprevalence in blood donors may be no greater than 1% (figure 2) [25]. In contrast, the seroprevalence of HHV-8 is higher in some populations at risk for sexually transmitted diseases, with the highest prevalence in homosexual male groups. Among HIV infected individuals, antibodies against the HHV-8 LANA (latency-associated nuclear antigen) protein can frequently be detected in homosexual patients (35%) as well as in 70-85% of HIV infected patients with KS. Furthermore patients who reported homosexual intercourse with other males have significantly elevated risks for KS, both in Europe and in North America [26]. In contrast, groups with low risk for the development of KS (blood donors, HIV positive women, intravenous drug users and haemophiliacs) show correspondingly low rates of seropositivity (1-3%) [27].



*Figure 2 - HHV-8 seroprevalence in the world*

Conversely, in Mediterranean Europe the virus is relatively abundant in the general population, reflecting the higher incidence of classic KS (up to 20% in south Greece and in some regions of Italy) [28]. In particular, in Italy the HHV-8 seroprevalence is relatively low in the North (5-7%), medium in the Centre (7-9%), and high in the South (23-27% with hotspot in Sicily and Sardinia). To note, in the North there are some hotspots, as along the Po river, in which the seroprevalence can reach the (figure 3) [24, 29]. Also in semen samples from Italian semen donors the prevalence of HHV-8 is high (13-23%) [30]. In Africa and the Middle East infection without disease is widespread, up to 60% seropositivity [31]. The transmission route of HHV-8 is different in Africa compared to Northern Europe and the US. While in Northern Europe HHV-8 is more likely to be acquired through sexual contact after puberty [32], in Africa, HHV-8 seropositivity is common among children from East and South Africa [33]. Since the AIDS epidemic, the incidence of KS has risen explosively. KS is now the most frequently reported tumor in several African countries, especially in Uganda, where childhood KS has risen more than 40 fold [7].



*Figure 3 - HHV-8 seroprevalence in Italy*

Studies suggest that vertical as well as horizontal transmission among children in Africa may play an important role in the route of infection [34]. Usually the mother of an HHV-8 positive child will be positive as well. How the virus is transmitted to children is unknown (possibly during child birth or breast feeding). It has been shown that saliva from PCR positive samples may contain infectious virus [35]. However, it is to note that a recent study, published by the lab in which I perform my PhD thesis, showed that in Italy HHV-8 infection is greatly enhanced within families of classic Kaposi's sarcoma patients, suggesting that close contact in general, and not only sexual contact, can contribute to the spread of the virus also in this KS form [36].



### 1.2.3 Virion structure

HHV-8 has morphological features typical of herpesviral capsids. Electron microscopy using induced Primary effusion lymphoma (PEL) cell lines revealed both intranuclear herpesvirus nucleocapsids and complete cytoplasmic virions. Capsid, 110 nm in diameter, is formed by 162 capsomeres with icosahedric symmetry and is surrounded by tegument and an by envelope, formed by a double coat of cellular lipids, associated with viral glycoprotein (figure 4). Within the nucleus, a nucleocapsid with an electro-dense core can be detected [37]. Studies on purified virions from PEL cell lines showed the presence of at least 24 virion proteins: 5 capsid proteins, 6 tegument proteins, 8 envelope glycoproteins and other 5 with not known localization. [38]. The capsid is structured by 12 pentons and 150 esons, formed respectively by 5 or 6 copies of the major capsid protein (MCP, Open Reading Frame 25, ORF-25); the capsomeres are linked by eterotrimerix structures formed by two different proteins, TRI-1 (ORF62) and TRI-2 (ORF26), mixed in 1:2 ratio.

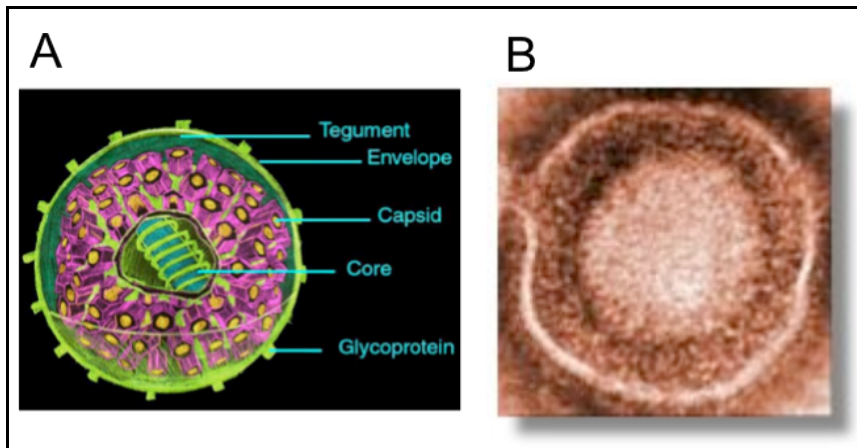


Figure 4 - HHV-8 virion showed by A: schematic cartoon; B: electronic microscopy

Little is known on real function of tegument viral proteins; however we can hypothesize that they have important regulatory function and that have a critical role on replication cycle. In particular, the protein encoded by ORF-45, early expressed in lytic viral infection, can block the innate immune response, linking the host protein IRF-7 (Interferon Regulator Factor 7), inhibiting the transcription of IFN-  $\alpha$  (interferon alpha) and IFN- $\beta$  genes [38]. Moreover, recent studies showed that this protein plays a critical role in ex novo infection and also in the release of viral progeny from infected cells [39]. The envelope is formed by herpesviridae conserved gB (ORF-8), gH (ORF-22) gL (ORF-47), gM (ORF-39) and gN (ORF-53) glycoproteins, and by ORF-K8.1, ORF-68 and ORF-28 proteins. The protein encode by ORF-K8.1 causes the viral adhesion on cell surface with a eparan-

sulfate linking, and, *in vivo*, stimulates the production of neutralizing antibody [40]. The gB protein has a site for eparan-sulfate too; moreover, in its extracellular domain, it has the aminoacidic motif Arg-Gly-Asp (RGD), that allows it to interact specifically with  $\alpha 3\beta 1$  integrin, essential for the virus for the penetration in the host cell. As other herpesviruses, gH and gL proteins formed a heterodimer: gB, gH and gL are important for the penetration in the host cell and, when they are expressed in HHV-8 infected cells, cause the polykaryocytes formation [41]. In HHV-8 virion some cell proteins are present, as  $\beta$ -actin and the myosin-II type A heavy chain, suggesting a role of the cytoskeleton on viral capsid intracellular carrying on, assembling and coming out. [38]. Finally, more than 11 viral mRNA are incorporated in the particle, probably in the tegument level. The majority is transcribed in large quantity during the lytic cycle and so they are included randomly during the phase of the viral progeny building. However, one of these mRNA, that encodes a viral protease, normally is not transcribed in high levels, suggesting that probably there are specific mechanisms for the recruitment of viral mRNA in mature virus [42].

#### 1.2.4 HHV-8 genomic organization

The genomes of gammaherpesviruses exhibit a common structure. Large collinear genomic blocks, encoding typical herpesviral proteins (DNA polymerase, thymidylate kinase, capsid proteins), are interspersed by regions containing virus type-specific Open Reading Frames (ORFs) [43]. These unique ORFs are predominantly involved in physiological processes and determine the pathogenesis of each virus. In figure 5 a schematic picture of the HHV-8 genome is shown. The complete sequence of virus has been determined from a PEL derived cell line, BC-1 [21] as well as from a KS biopsy specimen [44]. The genome is a 170 kb double stranded DNA molecule consisting of a central, low-GC content, long unique region (LUR) of 140.5 kb which is flanked each side by non coding tandemly repeated unit (TR). Each repeat is approximately 800 bp in size and high in GC content (85%) [45]. The genomic organization of HHV-8 is very similar to other Rhadinoviruses [46]. Within the LUR, 89 ORFs have been identified: 66 of them have sequence similarity to Herpes Virus Saimiri (HVS) ORFs, and for this reason the nomenclature for individual genes correspond to the HVS genome.

HHV-8 shares the genomic block organization of other herpesviruses. In HHV-8 the conserved genes are arranged in four or five blocks and include major structural proteins, DNA synthetic enzymes, glycoproteins and a viral proteinase and assembly protein. These genes are required for replication and assembly of new virus progeny. Between these blocks are non-conserved regions that show homology to other Rhadinoviruses, e.g. the v-cyclin, LANA or vGPCR (viral G Protein Couple Receptor). In addition, 15 ORFs have been identified in HHV-8 that are unique and show no similarity with other known viral genes. These unique open reading frames have been designated with a K prefix (K1-K15).

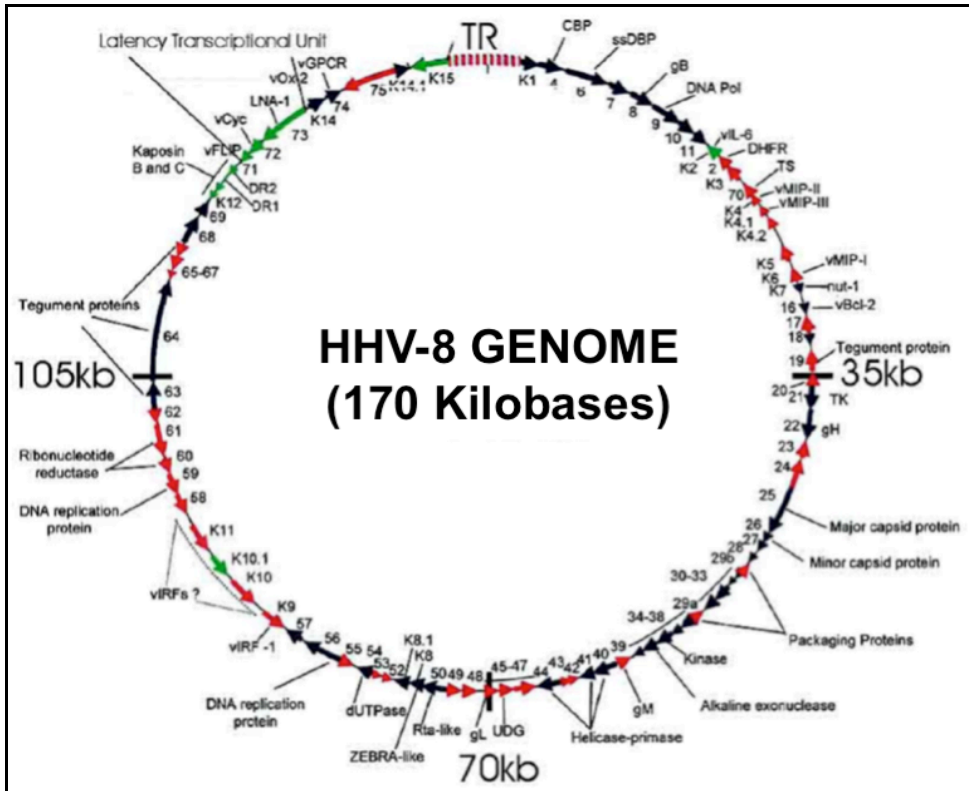


Figure 5 - HHV-8 genome in circular episome form

In the genome there are two sequences that work as origin of replication (*ori*) and they are recognized by viral DNA polymerases: the first one is between ORF-K4.2 and ORF-K5 (left *ori*) and the second one is between ORF-K12 and ORF-K71 (right *ori*). A general feature observed in DNA viruses is the presence of ORFs with significant homology to cell cycle regulatory and signaling proteins as well as proteins that can interfere with the immune system. These genes are mainly unspliced and may have been captured from the host cell during viral evolution. In HHV-8 this “piracy” of eukaryotic genes seems to be more extensive than is seen in any other herpesvirus [47]. The HHV-8 genome is equipped with several genes that encode proteins allowing the virus to escape cellular responses to viral infection. On infection, the host activates a number of anti-viral immune responses, including cell cycle arrest, apoptosis and mobilization of the immune system. HHV-8 has evolved proteins that have immunomodulatory functions such as cytokines (vMIP-I; vMIP-II) and a homolog to human IL-6 (vIL-6) [47]. Other proteins deregulate cell cycle mechanisms by interfering with receptor-mediated signal transduction pathways, including a homolog to the GPCR (vGPCR), a homolog to the Interferon Regulatory Family (vIRF), and a D-type cyclin (v-cyc) [38,47,48]. Also some viral gene products have transforming properties, e.g. ORF-K1 and

kaposin (ORF-K12) [49]. In addition, ORF-73, which encodes LANA, which has possible cellular growth-promoting activities [50]. To effectively suppress or delay apoptosis, HHV-8 carries a bcl-2 homolog (v-bcl-2, ORF-16) [51] and a protein with anti-apoptotic function (vFLIP, ORF-K13). Also viral homologous of cellular complement-binding proteins CD21/CR2 (KCP, ORF-4) and an NCAM-like adhesion protein (ORF-14) have been detected [21]. The plethora of possible interactions between these proteins makes modeling the pathogenesis of HHV-8 problematical. Apart from the proteins that may play a role in oncogenesis, several other proteins have been identified within the HHV-8 genome with importance for viral replication. Some examples include the processivity factor-9 (ORF-59) that binds the viral DNA polymerase (ORF-9) to allow synthesis of extended stretches of DNA without dissociation from the DNA template [52] and K8.1, a positional analog to HVS ORF-51, which encodes a glycoprotein expressed in lytic replication and present at the surface of infected cells and virion particles [53]. Finally, it needs to note that HHV-8 also transcribes at least 12 microRNA during the latent phase [54]. Their function is not known yet, but probably they module the host gene expression, binding themselves with cellular mRNA and interfering with their translation, or causing their degradation [55].

Otherwise, during the lytic replication, the polyadenylated nuclear RNA (PAN), 1,1 kb, is transcribed, localizing in nucleus; it represents the more abundant viral RNA within productively infected cells. Its function is not known yet: probably it works in the building of ribonucleoproteic complexes and modules processes of RNA splicing [56].

### **1.2.5 Variability of the genome**

Overall the HHV-8 genome derived from B cells or spindle cells differs only 0.4% from each other [46]. Based on sequence comparison of ORF-K1 among different HHV-8 samples, five main distinct subtypes, A, B, C, D, have been described which differ by 15 to 30% at the aminoacid level in this gene [57], showing distinctive ethnic and geographic association. Moreover, the A subtype can be divided into five distinct subgroups, referred to as A1, A2, A3, A4, and A5 variants, with the A1, A2 and A4 variants separated from the A3 and A5. Also the C subtype can be divided in four distinct subgroups, C1 and C2 (linked together), and C3 and C4 (linked together). Most epidemic KS cases in the United States are A1, A4 or C3 variants, whereas classic, iatrogenic and epidemic cases from the Middle East, Asia, and Europe are mostly classified as C1, C2 and C4 variants. Also the few cases of classic and iatrogenic Kaposi's sarcoma in United States are classified as C1, C2 and C4 variants. Examples of the B subtype were found almost exclusively in epidemic or endemic KS patients from Africa or of African heritage, whereas the rare D subtypes were found only in KS patients of Oceania or Pacific Island heritage [58]. Finally, other three minor HHV-8 subgroups had been described: E subtype, clustering in small and isolated populations of the Brazilian and Equadorian Amazon regions [59, 60], Z subtype, detected in a small cohort of Zambian children [61], and F subtype, identified in Ugandan Bantu tribe [62].

## 1.2.6 HHV-8 and oncogenesis

HHV-8 infection of endothelial cells, circulating endothelial or haematopoietic progenitors leads to changes in their morphology, glucose metabolism growth rate, lifespan and gene expression, resulting in the development of KS [63]. HHV-8 oncogenicity is reflected by the numerous pro-angiogenic molecules that are induced after infection of endothelial cells, including members of the VEGF–VEGFR family, angiopoietin family, cyclooxygenase 2 (COX2) and angiogenin [64]. However, in most experimental systems, *in vitro* infection of endothelial cells with HHV-8 leads to morphological changes and an extended lifespan and provides a survival advantage in response to apoptotic stimuli, but not full neoplastic transformation. Moreover, although HHV-8 encodes oncogenic genes that could potentially induce all KS-related malignant phenotypes, HHV-8 infection in the general population rarely leads to KS. This underscores the existence of cofactors, such as HIV or drug-induced immunosuppression, that are required for the virus to induce a tumour.

Although the vast majority of KS spindle cells are latently infected with the virus, in a small proportion of infected cells the virus undergoes lytic replication leading to the production of mature virus and cell lysis. Apart from the v-cyclin and vFLIP, the other cellular orthologues that are encoded by HHV-8 are early lytic genes that are generally expressed only in cells in which the virus is undergoing lytic replication. However, certain HHV-8 immunoregulatory and growth-promoting genes, including vIL-6, vMIR3 and vMIR5, could be activated by Notch signaling independently of the lytic transactivator RTA [65]. Moreover, limiting-dilution reverse transcription-PCR analysis shows that transcripts such as vIL-6 can be expressed in HHV-8 latency in a context-dependent manner; for example, in B cell lines [66]. This implies that the expression of HHV-8 genes might not be restricted by the classic herpesviral paradigm of latent or lytic infection, as lytic genes can be expressed without the full execution of the lytic cycle. This is an important observation when we consider the complementary role of latent and lytic gene-expressing cells in KS pathogenesis. PEL cells are latently infected with HHV-8 and were instrumental in classifying HHV-8 genes as latent or lytic, and identifying the major effectors of latent and lytic replication [67]. Many observations made from PEL-derived tumors, such as the switch to lytic replication that occurs during *in vivo* growth, are also observed in KS models, indicating that PEL is a valid model to study aspects of HHV-8 biology.

## 1.2.7 HHV-8 replication cycle

Like every herpesvirus, the starting event of HHV-8 replication cycle is the attachment of the virion with cell surface, mediated by interaction of K8.1 glycoprotein and gB with the heparan sulfate glycosaminoglycan, a molecule covalently linked to extracellular domains of a lot of membrane proteins. This interaction is not necessary for virus, but it increases the penetration efficiency, facilitating the interaction with specific receptors [68].

At now at least three different cellular proteins that could work as HHV-8 receptors are identified. The virus interacts with  $\alpha 3\beta 1$  integrin using an Arg-Gly-Asp motif present in gB protein. This integrin is expressed on many cell types susceptible to HHV-8 infection, as B-cells, endothelial cells, fibroblasts, and epithelial cells. However, although antibodies against  $\alpha 3\beta 1$  integrin interfere with virus-cell interaction, they cause just an infection reduce (about 50-70%), but not its total disappear [69]. For this reason, it is clear that  $\alpha 3\beta 1$  integrin cannot be the only host protein involved in the HHV-8 entrance. A second protein that can work as HHV-8 receptor is the exchange cystein/glutamate transporter (xCT), a protein that mediates the extracellular cystein entrance linked with glutamate exit: in fact its expression makes normally non-susceptible cells permissive to infection, whereas antibodies against xCT completely block the virus entrance [70]. In particular, this protein forms a heterodimer with CD98 on the surface cell and this complex associates itself with  $\alpha 3\beta 1$  integrin: for this reason it is hypothesized that the integrins, ubiquitary expressed in many tissues, are not real HHV-8 receptors, but they are a part of a multi-protein complex that is recognized by virus and that mediates its entrance in target cell [70]. Finally, HHV-8 uses CD209 or DC-SIGN (dendritic cell-specific ICAM-3-grabbling nonintegrin) protein, a C-type lectin, as specific receptor for the entrance in dendritic cells and in monocytes-macrophages: in fact, the link of virus to these cells and infection are blocked by antibodies against this lectin or its soluble forms [71]. In particular, the link between DC-SIGN and virus seems to be because DC-SIGN recognizes specific carbohydrate motifs in glycoprotein of viral envelope. Dendritic cells infection is associated with a reduction of DC-SIGN expression, with compromise of endocytic activity and with inhibition of antigens presentation to CD8<sup>+</sup> T cells, with consequent dysfunction of immune response [72]. So, according to all these data, it seems that HHV-8 recognizes cellular membrane proteins that are not correlated with themselves, suggesting that virus uses many receptors to infect target cells, and that they are different for each cell type.

The virus penetration, at least in some cell types, including B cells, fibroblasts and epithelial cells, happens for endocytosis of viral particle followed by fusion of envelope with the endosome membrane, due to drop in pH (ph-dependent mechanism), with the release of the capsid in cytoplasm [73]. However, according to the mechanisms of penetration of the other herpesviruses, it can not be excluded that HHV-8 can penetrate into cells through a direct fusion of viral envelope with plasmatic membrane (pH-independent mechanism). The fusogenic complex used by HHV-8 includes the gB, gH and gL glycoproteins [41]. Once released into the cytoplasm, capsid, probably in a similar way to Herpes simplex virus 1 (HSV-1), is transported through the system of microtubules to the nuclear membrane; here the viral DNA is released into the nucleus where it circularizes in episome form by action of host cell enzymes. Through interaction with cellular histonic proteins, the episome organizes itself into chromatin-like structures, becoming a substrate suitable for attachment of cellular RNA polymerase II. At this point, after a first phase of generalized gene expression, the virus normally enters in latency maintaining the expression of a limited number of viral genes (LANA, vCyc, vFLIP and Kaposin); only a small percentage of *de novo* infected cells (about 1-3%) spontaneously enters in lytic cycle, exactly as shown in cell culture

derived from PEL tumors [74]. For its functional properties, LANA protein, whose principal role is to bind stably the episome to cellular chromatin, may recruit some transcriptional repressors on the viral DNA by inhibiting the expression of the lytic genes and thus favoring the maintenance of the latency phase. However, the program of gene expression in latency does not seem to be constant: in fact, the viral gene encoding the homolog transcription factor IRF3 (LANA2 or vIRF3, encoded by ORF-K10.6/K10.5) is expressed in the PEL cells, but not in latently infected cells that are present in lesions of Kaposi's sarcoma [75]. The viral protein LANA2, together with the other viral homologs of IRF (at least three, all expressed as lytic genes), contributes to escape from innate immune response, by interfering with IFN- $\alpha$  and IFN- $\beta$  expression and with their antiviral activity [76-78]. The induction of the lytic replication is controlled by the viral protein RTA (replication and transcription activator, ORF-50) which has a N-terminal domain for dimerization and for binding to DNA, and a C-terminal domain for the transcriptional activation [65]. The RTA protein interacts with several cellular transcription factors, including STAT3 and RBP-J, activating them and thereby promoting the expression of viral lytic genes [79]. So a cascade of events starts, that leads to immediate-early and early viral genes transcription, to viral genome replication and, finally, to late genes transcription. Many chemical agents, such as phorbol esters or inhibitors of histone deacetylase, work *in vitro* to induce lytic cycle, but the specific stimuli that activate this replication in host *in vivo* are not known yet.

Most of the early genes encode enzymes (thymidine kinases, ribonucleotide reductases, DNA-polymerases and helicases), or accessory proteins, directly involved in viral genome replication. Other early proteins are involved in the escape of adaptive immune specific response, such as proteins MIR1 and MIR2 (modulators of immune recognition 1 and 2, respectively encoded by ORF-K3 and ORF-K5), inhibition of cell proteins synthesis, such as protein SOX (shutoff exonuclease, encoded by ORF-37), or in modulation of signal transduction, such as the proteins encoded by genes ORF-K1, ORF-K15 and ORF-74. In addition, among early products there are at least two proteins with regulatory functions: MTA (mRNA accumulation transcript, encoded by ORF-57), a protein that promotes viral mRNA transportation from the nucleus to the cytoplasm and that can potentiate the transcriptional activation RTA mediated, and RAP protein (replication associated protein, encoded by ORF-K8), which mediates the cell cycle blockage in G1 to promote the viral DNA synthesis instead of the cellular DNA synthesis [65]. Similar to other herpesviruses, replication of HHV-8 genome begins with a theta origin-dependent mode, that continues with a "circle rotating" origin-independent method, leading the formation of long spin concatamers, that, during the viral DNA packaging into the capsid, are cut into linear monomers. Events that are downstream of the viral genome replication have not been clarified at all.

The late genes are more abundant of the other for the availability of more molecules of DNA template at which RNA polymerase can bind: in fact, transcription of most of these genes is strongly affected by viral DNA synthesis inhibitors, such as ganciclovir, and foscarnet. The late proteins are predominantly structural proteins of capsid, tegument and envelope: the high levels of transcription correlate with the need for large amounts of structural proteins for the formation of viral progeny.

There are still many uncertainties about the final stages of the lytic replication cycle. It is assumed that the packaging of the viral genome in the new assembled capsid occurs in the nucleus and that, finally, the particle acquires tegument and envelope containing the viral glycoproteins by budding through the inner nuclear membrane. Then, the new viral particles would be transported by transcytosis in vesicles through the endoplasmic reticulum and the Golgi apparatus tanks, where the maturation of viral glycoproteins is completed. Finally, after vesicles fusion with the plasma membrane, the mature virion would be released outside the cell by exocytosis, leading the cell to death. Alternatively, the virus particle could lose the envelope during fusion with the outer nuclear membrane and acquire the final one by budding through the Golgi apparatus [65]. As for other herpesviruses, gB protein appears essential not only for binding to the target cell and the penetration, but also in the assembly phase, and in leakage of the viral progeny, since it has been shown as the deletion of the gB inhibits the release of new virions from infected cells [80]. A schematic picture of HHV-8 replicative cycle is represented by figure 6.

### **1.2.8 Detection of the HHV-8 genome in Kaposi's sarcoma**

Since its first description in 1994, HHV-8 DNA sequences have been detected by PCR in all epidemiological forms of the disease, in both fresh biopsies and in the vast majority of paraffin embedded material [81]. By using in-situ PCR, HHV-8 can be detected in all stages of the disease from early patches to nodular lesions [82]. In KS lesions, HHV-8 sequences have been localized to spindle and endothelial cells and to monocytes. Before the extensive spindle cell formation, herpesviral sequences are limited to the flat endothelial cells of the lesion [83]. The viral genome is maintained as an episome and at least one copy for cell can be detected [84]. The majority of spindle cells seem to be latently infected, with a subpopulation supporting lytic replication [85]. Several attempts to establish an *in vitro* culture system for HHV-8 using spindle cells isolated from KS have been unsuccessful. Spindle cells have a limited life in tissue culture and even well established KS cells lose the viral genome upon passage [86]. Also non-involved tissue from KS patients, including skin, lymphoid tissue, prostate, semen, saliva and PBMC, has been shown to harbor HHV-8 DNA [87].

Furthermore, in-situ hybridization to detect the minor capsid protein mRNA showed that monocytic cells are productively infected with HHV-8. However, not all monocytes stained positive and therefore transmission of virus from spindle cells to monocytes was thought to be rather unlikely [88]. It has also been demonstrated that the virus replicates in PBMCs of KS patients [84]. PCR studies of PBMCs from healthy individuals have found the virus to be uncommon [31]. In contrast in areas at high risk for KS, HHV-8 has been detected at a high frequency [89]. It has also been demonstrated that the detection of KSHV in the peripheral blood of HIV infected patients precedes KS development and is strongly correlated with increased KS risk [25].



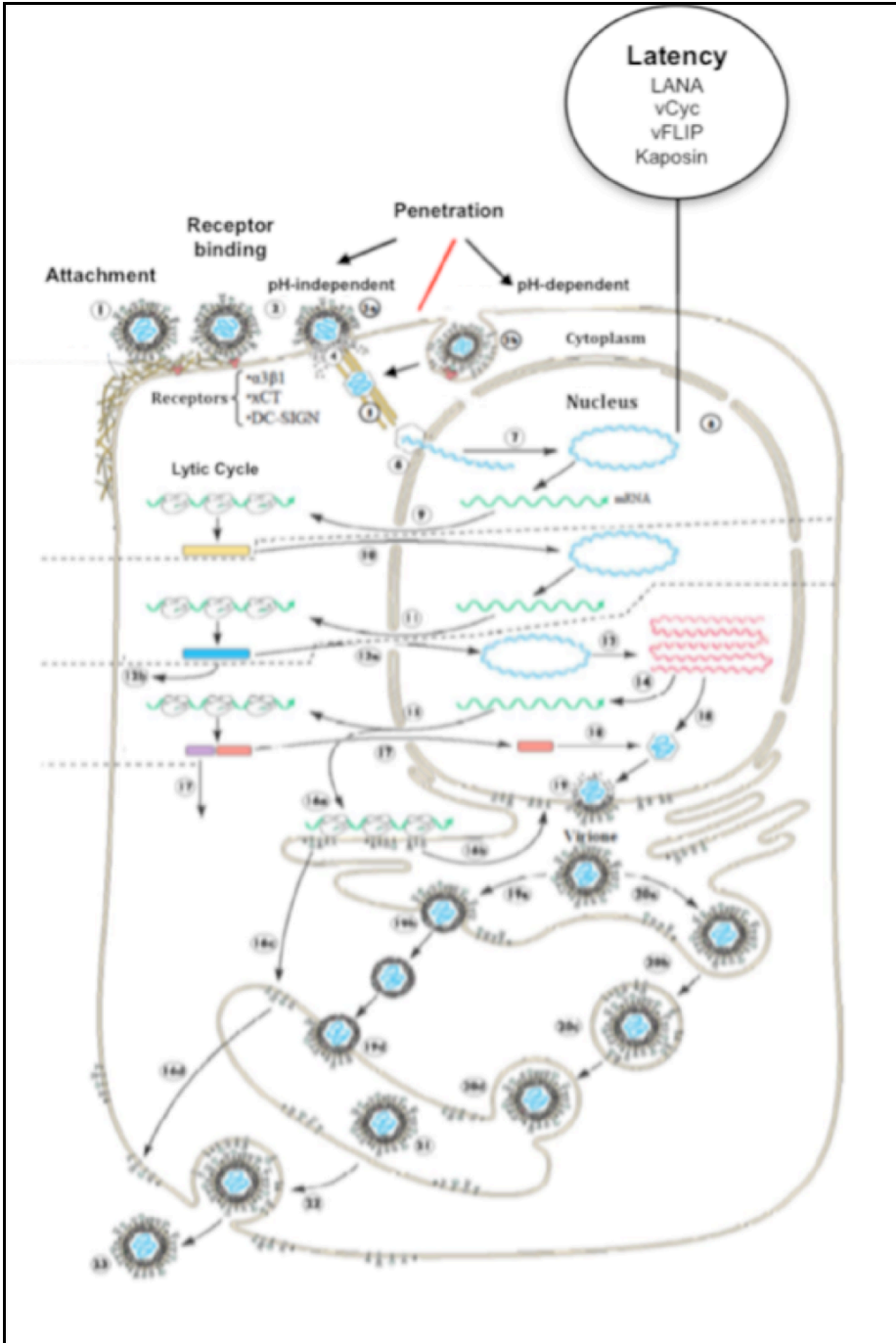


Figure 6 - HHV-8 replicative cycle (latent and lytic cycle)

### 1.2.9 HHV-8 and other lymphoproliferative diseases

Besides KS, HHV-8 sequences have also been associated with other lymphoproliferative disorders and multiple myeloma. Viral sequences have been consistently found in Primary Effusion Lymphoma (PEL) and Multicentric Castelman's Disease (MCD) [90, 91].

Primary effusion lymphoma (PEL), also called body cavity based lymphoma (BCBL), is a rare form of AIDS-associated B cell lymphoma and was initially recognized in HIV infected individuals [92]. It is seen predominantly in patients with advanced stages of immunosuppression but also cases in HIV negative patients have been reported. The lymphoma is characterized by malignant effusion in pleural and abdominal cavities, usually without significant tumor mass or lymphadenopathy [93]. Tumor cells are derived from a B cell lineage, as indicated by clonal immunoglobulin rearrangements and plasma differentiation markers. The majority of cells are co-infected with EBV and HHV-8 [94]. However, some cases have been reported where only HHV-8 is present [95]. Compared to the endothelial and spindle cells in KS lesions, HHV-8 DNA sequences have been reported to be 50 times more abundant in PEL tumor cell lines [91]. Analyses showed that PEL cells are infected with multiple episomal copies of HHV-8, 50 to 150 copies per cell were detected, whereas KS cells may contain less than one copy per cell [91]. Several B cell lines have been established from PEL patients and from the peripheral blood of PEL patients to study the pathogenesis of HHV-8 as well as to perform sero-epidemiological studies. It is likely that these B cells may be similar to EBV transformed lymphoblastoid cell lines (LCL) and produce antibodies. The majority of these B cells are latently infected with HHV-8, with approximately 2% of cells spontaneously entering the lytic replication cycle to produce infectious virions [96]. Several research groups showed that the addition of phorbol ester or sodium butyrate to HHV-8-infected PEL cell lines leads to the activation of viral gene expression [97]. As shown by Miller and colleagues in dually infected PEL cells (here the BC-I cell line), the replication of each virus can be differentially induced by chemicals such as TPA (EBV) or n-butyrate (HHV-8) [96].

Multicentric Castelman Disease (MCD), also called multicentric angiofollicular hyperplasia, is an atypical lymphoproliferative disorder described by Castelman in 1956 [98]. The disease is characterized by generalized lymphadenopathy and immunological abnormalities.

Two histological forms can be distinguished: the hyaline-vascular variant and the plasma variant. Patients with MCD tend also to develop non-Hodgkin's lymphoma or KS. The factors involved in MCD development have not yet been established. However, high levels of IL-6 have been found and suggested to play an important role in disease development [99]. HHV-8 has been detected in 50% of the plasma variant when HIV-infected, but HIV-negative cases have been reported [100]. In particular PBMCs from MCD patients have been shown to harbour the virus. Recently, large immunoblastic cells belonging to the B-cell lineage have been found to harbor the viral latent nuclear antigen (LANA) [82].

## 1.3 Natural killer cells and KIR receptors

### 1.3.1 General characteristics

Natural killer (NK) cells are recognized as a population of large granular lymphocytes in the 1970s because of their capacity to lyse spontaneously certain tumor cells (figure 7) [101]. NK cells are distinguished from B and T cells as they lack antigen-specific receptors. NK cells play an important role in early host defense against viral, bacterial, and other infections as well as tumors. NK cells exert their effector function by killing target cells without prior sensitization and producing immunoregulatory cytokines and chemokines. In human, NK cells account for ~5-15% of circulating lymphocytes and phenotypically they are characterized for the expression of CD56 and CD16 surface antigens, and for the absence of CD3 co-receptor. CD56 antigen, an isoform of the neuronal cell adhesion molecule NCAM, mediates the interaction between NK cell and target cell, whereas CD16 is a low affinity co-receptor for Fc portion of G immunoglobulins, and it is involved in antibody-mediated cell cytotoxicity [102]. Depending on CD56 and CD16 surface density, NK cells are divided into 2 sub-populations: NK cells with low CD56 density (CD56<sub>dim</sub>) and high CD16 density, that are about 90% of total NK cells, and NK with high CD56 density (CD56<sub>bright</sub>), and low or negative CD16 expression, that are about 10%.

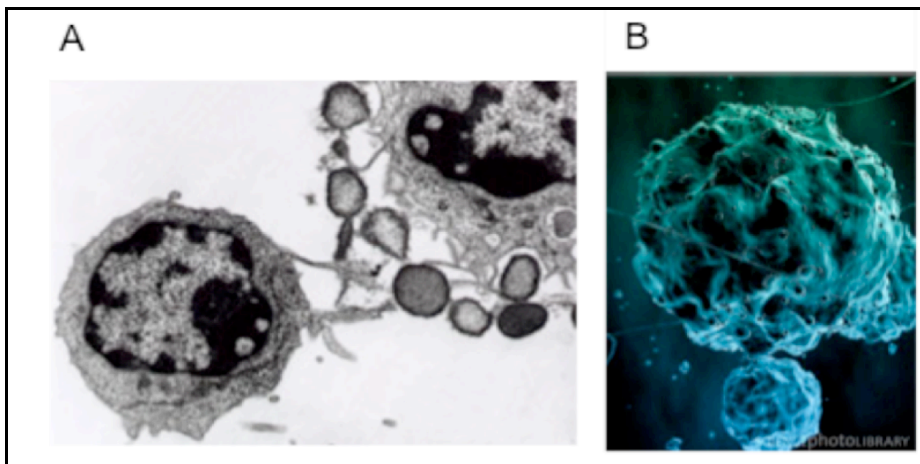


Figure 7 - (A) Natural killer cell (on the left) that attacks and destroys a tumoral cell (electronic microscopy). (B) Three-dimensional elaboration of a natural killer cell, by the scientific journal Science  
[<http://www.sciencephoto.com/media/143133/enlarge>]

The CD56<sub>dim</sub> cells mainly carry out a cytotoxic activity, whereas the CD56<sub>bright</sub> mainly secrete cytokines [102]. The NK cells constitutively express receptors with medium and high affinity for IL-2, a cytokine that induces the CD56<sub>bright</sub> proliferation and increases the CD56<sub>dim</sub> cytolytic activity [102].

Recently, NK cells have been further divided into three subsets on the basis of their cytokine production profiles: NK1 cells producing type 1 cytokine IFN- $\gamma$ ; NK2 cells producing type 2 cytokines IL-5 and IL-13 but not IL-4; and NK3 cells producing TGF- $\beta$  and IL-10 [103, 104]. In general, NK cells use three different mechanisms to lyse target cells including perforin- FasL- and TRAIL-mediated cytotoxicity. Unstimulated NK cells lyse targets primarily through the calcium-dependent release of granules containing perforin, which forms pores in target cells [105, 106]. In addition, the release of granules of granzyme A and B further enhances cell damage by DNA fragmentation [107]. Upon activation, NK cells release FasL stored within secretory lysosomes to the cell surface at functional levels and induce apoptosis in Fas<sup>+</sup> cells. TRAIL is also expressed on NK cells even without activation and can induce apoptosis in cells that express TRAIL receptors, including death receptor 4 (DR4) and death receptor 5 (DR5) [105].

### **1.3.2 Natural killer cells development**

NK cells are generated from lymphoid precursors and their complete functional and phenotypic maturation is in bone-marrow. The stroma produces the FL and FK growth factors, which cause the transformation of the original progenitor in intermediate precursor, and IL-15, cytokine that induces the differentiation of intermediate precursor in a functional CD56<sub>bright</sub> NK cells [102]. The CD56<sub>dim</sub> origin is not known yet: somebody hypothesizes the presence of an alternative unknown precursor, others suggest the same precursor stimulated by different factors, others speculate that they derive from CD56<sub>bright</sub> cells. After the release from bone-marrow, the majority of NK cells goes to peripheral blood or to spleen; just very few are in thymus and in lymph nodes [108].

### **1.3.3 Role of natural killer cells in immunity**

Increasing evidence has revealed the diverse effects and complicated role of NK cells in innate and adaptive immunity and autoimmune diseases and as NK cells can exert direct killing on target cells, produce immunoregulatory cytokines and chemokines, and interact with various immune cells [109].

#### ***1.3.3.1 Natural killer cells in innate immunity***

NK cells play a critical role in innate immune responses against infection, in particular in anti-viral immunity. NK cells contribute to anti-viral defenses by direct killing of virus-infected cells and by the production of cytokines that control viral replication [102, 110]. Many viruses develop strategies to escape recognition by

cells of the adaptive immune system, for example reducing expression of HLA molecules on the infected cell surface to block antigen presentation to T cells. Therefore, these infected cells become susceptible to NK cells lysis as they cannot engage inhibitory receptors on NK cells. However, some viruses have also evolved a number of strategies to evade NK cells, which include the expression of HLA decoy molecules that can ligate KIR, inducing NK cell inhibition and downregulation of NK cell-activating ligands [111, 112]. In addition to viral infection, NK cells also combat other types of infections including those caused by intracellular bacteria, fungi and protozoa [113]. Another important function of NK cells is to eliminate tumor cells. Many malignant cells downregulate HLA expression thus rendering them susceptible to NK cell-mediated lysis [114]. NK cells kill malignant cells through the release of perforin and granzymes and binding of the death receptors Fas and TRAIL-R on target cells [105, 106]. It is realized that although some tumor cells do not express Fas, NK cells can induce Fas expression on these targets by releasing IFN- $\gamma$  and so kill them by binding to the newly expressed Fas [106].

#### **1.3.3.2 Natural killer cells in adaptive immunity**

NK cells can shape adaptive immune responses by regulating antigen presenting cells (APC), which are responsible for taking up, processing and presenting antigens to naive T lymphocytes. Previous studies have demonstrated that NK cells can modulate dendritic cells maturation by secreting cytokines, whereas NK cells are activated by cytokines produced from mature dendritic cells [115, 116]. Reciprocal interactions between NK cells and dendritic cells may represent a key mechanism to bridge innate and adaptive responses as this affects both the magnitude and polarization of T cell responses [117]. Secondary lymphoid organs such as spleen and lymph node and inflamed tissues have been recognized as the site for NK – dendritic cells interaction for the co-localization of NK cells and dendritic cells have been observed in the sites [118]. Apart from lymphocyte priming, NK cells may also provide immunoregulatory functions on downstream adaptive responses. NK cells can directly stimulate and inhibit T cells. Some studies *in vitro* have demonstrated that NK cells can stimulate T cell proliferation and cytokine production through cell-cell interaction and cytokine secretion, while others have indicated suppressive role of NK cells on T cells mediated by direct killing and cell cycle inhibition [119, 120]. In some experimental studies, NK cell depletion revealed this dual roles of NK cells in autoimmune diseases: NK cells can either promote or suppress autoimmunity by regulating autoreactive T cells [121, 122]. In addition to regulating T cell responses, NK cells also play a role in regulating B cell responses. At first, NK cells can suppress B cell responses through direct effects on T cells [123]. Moreover, other studies indicated that NK cells, instead of direct lysis, could influence antibody production and isotype switching of B cells through cell-cell interaction and IFN- $\gamma$  [124, 125].

### 1.3.4 Natural killer cell receptors

NK cells express many types of receptors, that can be divided in activating or inhibitory receptors, according to their effect on NK cells cytotoxic activity. These receptors are usually divided in two groups:

- Killer Immunoglobulin-like Receptors (KIR)
- Heterodimeric receptors with extracellular C-type lectinic domain, like CD94/NKG2 and Ly49.

All these receptors have class I MHC (MHC-I) molecules like ligands. In humans, the HLA-A, -B and -C antigens are constitutively expressed in almost every nucleate cells, even if with different intensity [126]. In 1990, by using a tumoral model, Ljunggren and Karre [127] showed, *in vitro* and *in vivo*, that NK cells are activated by the self-antigen MHC-I loss on the target cell. This observation leads to the drafting of the “missing self” hypothesis: the loss of MHC-I on the target cell removes the inhibitory signal on the NK cells cytotoxic activity. So, recognizing MHC-I, the inhibitory receptors allow the self-tolerance and the non-self aggression by NK cells (figure 8) [128].

Recently others receptors are identified on NK cells surface, called natural cytotoxicity receptors (NKp46, NKp30, NK44) and co-receptors that recognize non-MHC molecules that are over-expressed or *de novo* expressed in cells characterized by stress, cellular activation, viral infection or tumoral transformation (for example, MICA, MICB, Rae-1 and ULBPs molecules) [129].

### 1.3.5 Killer Immunoglobulin-like Receptors (KIR)

KIR receptors are single-chain regulatory glycoproteins, belonging to immunoglobulin super-family, expressed on NK surface and can modulate their cytolytic activity. All of these receptors can recognize a particular HLA-I molecules set (see paragraph 1.3.14) [130]. Their molecular typing showed that molecules encodes by different mRNA can have the same allelic specificity, but opposite reactivity.

This phenomenon was explained by the discovery that a lot of KIR are homolog each other for extra-cellular domain, responsible for HLA recognition, but they differ for transmembrane and cytoplasmatic domains [131].

The KIR distribution in a unique NK cell population of the same subject is very heterogeneous [132]. KIR genes are not necessarily expressed in every NK cells and so every cell has a specific KIR phenotype. However, when a cell starts to express a particular combination of KIR genes, this expression pattern remains stable during the time-life as well as during the cellular division [133].

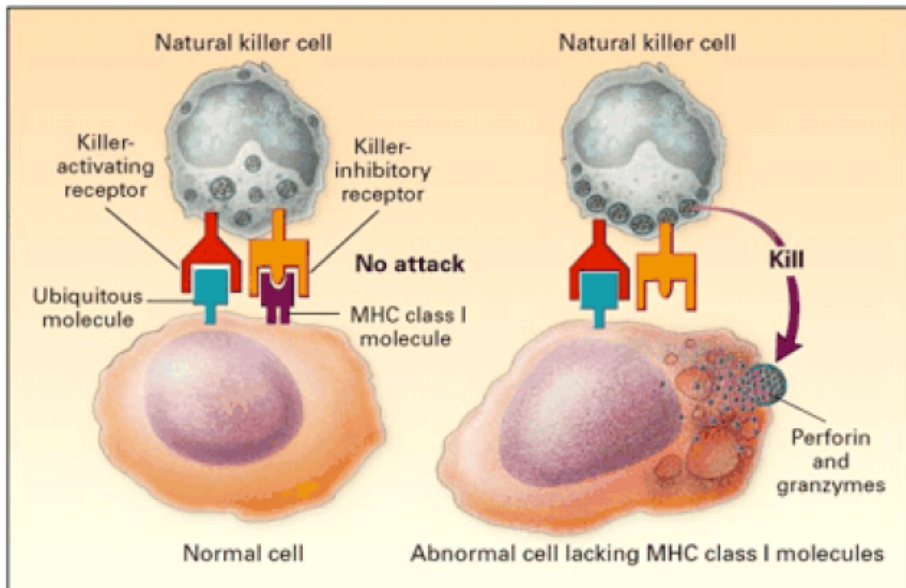


Figure 8 - NK cells activation according to “missing self” hypothesis [128]

### 1.3.6 Role of KIR on natural killer cells

KIR receptors are divided in two groups according to their function on NK cells: inhibitory KIR, if they inhibit the NK cytolytic activity, or activating KIR, if they activate it.

#### 1.3.6.1 Inhibitory KIR

The main function of this type of KIR is to protect self-cells from autologous attack by NK cells, recognizing the self-MHC-I of target cells, and blocking the cytolytic activating signaling of NK cells. In the case of downregulation or of absence of autologous HLA, due to viral infection or neoplastic transformation, these receptors are not longer able to mediate their inhibitory signaling and so the targeting cells can become susceptible to lysis by NK cells [134].

#### 1.3.6.2 Activating KIR

This receptors stimulate the NK cells cytolytic activity and their production of cytokines. Their role is not completely known yet, especially since in the same cells can be KIR couples with opposite activity. These receptors recognize their ligand

with lower affinity than the corresponding inhibitory KIR and so it is hypothesized that their stimulating activity on NK cytotoxicity and on cytokine production can happen only when, in the same time, the inhibitory signaling is down-regulated, going under a fixed critical value. Probably this mechanism is important to ensure a prevalence of inhibitory signaling to activating one, when a single NK cells express both the two types of receptors [134]. In particular, if at the same time a particular NK cell express either activating and inhibitory receptors with different HLA specificity, activating KIR works only if the target cell selectively loses every HLA allele recognized by inhibitory KIR. This mechanism is already demonstrated in some tumor and viral infection [135]. To note, the activating signal can be predominantly even if the activating KIR are over-expressed, neutralizing the inhibitory signal [135].

### 1.3.7 KIR nomenclature

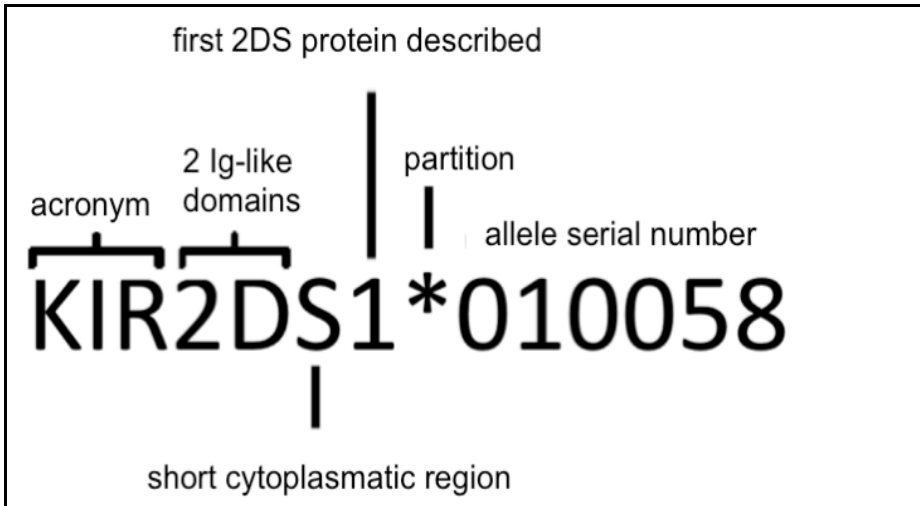
The KIR nomenclature is based on protein structure and consists of four major divisions according to two characteristics:

- the number of extra-cellular domains (2D or 3D)
- the length of cytoplasmatic domain (L or S)

In particular, if they have respectively 2 or 3 extra-cellular immunoglobulin-like domains, KIR genes are defined with 2D or 3D suffix. The L (long) or S (short) letters indicate if the receptors have a long (L) intra-cellular domain, containing two ITIM (immunoreceptor tyrosine-based inhibitory motifs) domains, that inhibits the NK cytotoxic activity, or a short (S) intra-cellular domain, containing a ITAM (immunoreceptor tyrosine-based activation motifs) domain, that stimulates the NK cytolytic activity [136]. The unique exception of this rule is KIR2DL4 that, even if it has a long intra-cellular domain, mediates an activating signaling by the interaction with the accessory protein TYRO (tyrosin kinase binding protein). Moreover, this KIR receptor seems to be the only not to have a corresponding KIR with short intra-cellular domain and, unlike the others, it doesn't have a clonal distribution, but it is expressed in almost the totally of NK cells [131, 137, 138]. Finally, in the KIR name the number that follows the L or S letter identifies each gene or molecule, whereas the numbers following the star key characterize the allele.

Two pseudogenes KIR are identified too: KIR2DP1, similar to two domain genes, and KIR3DP1: similar to KIR3DL3, it seems to represent an ancestral KIR gene [139]. The KIR nomenclature is visualized with a picture by figure 9.





*Figure 9 - Scheme of the KIR nomenclature*

### 1.3.8 KIR genomic region

The KIR locus is polymorphic and is located on the short arm of chromosome 19 (19q13.4) as part of the (LRC) Leukocyte Receptor Complex [140]. The probability of two individuals inheriting the same KIR genotype is slow, and the expression varying clonally adds another layer of complexity. Arrayed in a head-to-tail mode, KIR genes stretch over a 150 kb domain of DNA with each gene being approximately 10 to 16 kb in length [141]. Separation between all loci approximates a 2 kb stretch of DNA with the exception of a 14 kb sequence upstream from 2DS4 [142] (figure 10). Once a NK cell has committed to expressing a particular combination of KIR genes, that pattern remains stable through time and cell division [131].

### 1.3.9 KIR gene structure

KIR genes are formed by 9 exons with a very high structure nucleotidic homology among each other (more than 98%). Exons 1 and 2 encode for the recognizing domain; exons 3, 4 and 5 encode respectively for the extra-cellular domain D0, D1 and D2; exon 6 encodes for a structural extracellular portion; exon 7 encodes for transmembrane domain, and finally exons 8 and 9 encode the intracellular domain. The structure of 3D genes is formed by every exons, whereas the structure of 2D genes is formed by exons 1, 2, 4-9: a mutation localized on the third exon caused the blocking of the D0 transcription. The situation for KIR2DL4, 2DL5A and 2DL5B is different, because they are encoded by all exons with exception of exon 4, with the lacking of D1 domain. These KIR2DL genes are called type II KIR2DL,

whereas the others, activating or inhibitory, that have D0 domain, are called type I KIR2D (figure 11).  
 Finally, in KIR2DP1 pseudogene exon 3 is not encoded, causing a stop codon on exon 4, whereas in the KIR3DP1 there is a deletion on exon 2 (figure 11) [143, 144].

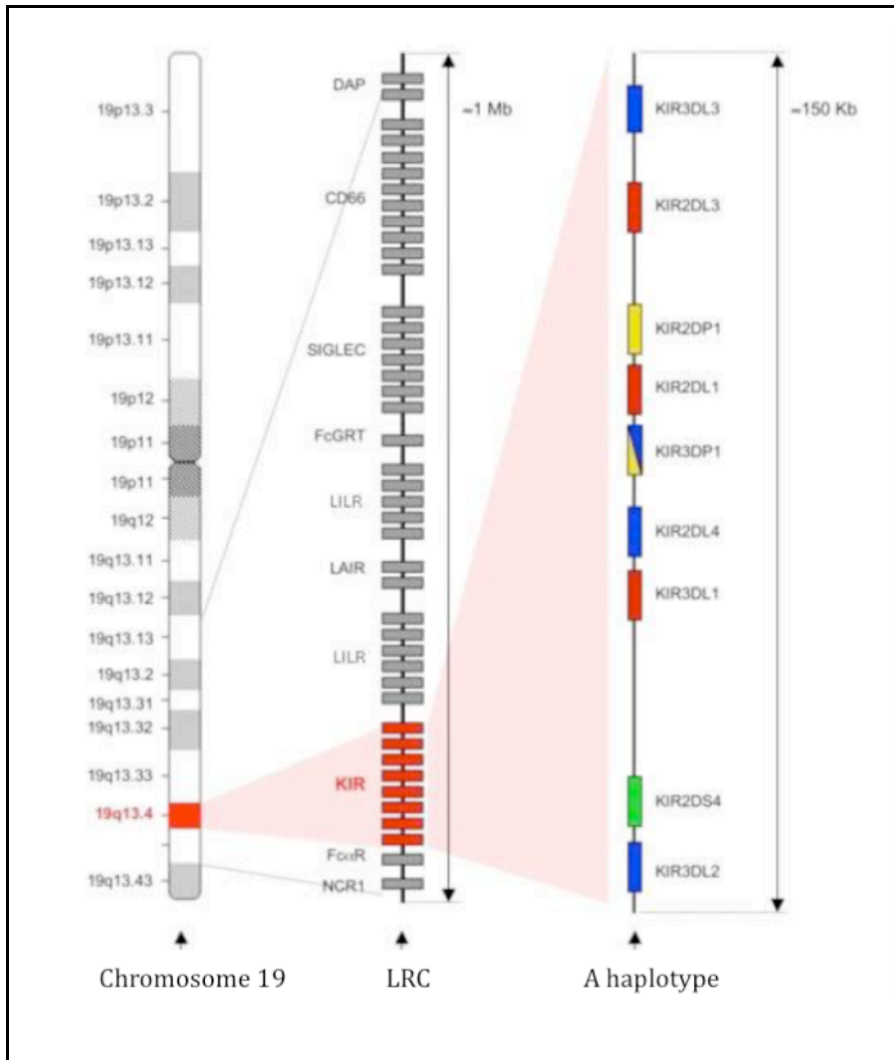


Figure 10 - Genomic organization of LRC and of a particular haplotype KIR

### 1.3.10 KIR haplotypes

Extensive variation has been shown in the number and type of KIR genes present within individuals. Both the number and the organized arrangement of inhibitory and activating KIR genes determine the particular haplotype. Over 100 different KIR haplotype profiles were described thus far and this number keeps expanding as new haplotypes are discovered [139, 143]. Dependent on the KIR genes present, two major haplotypes are formed, namely A and B (figure 12) [141].

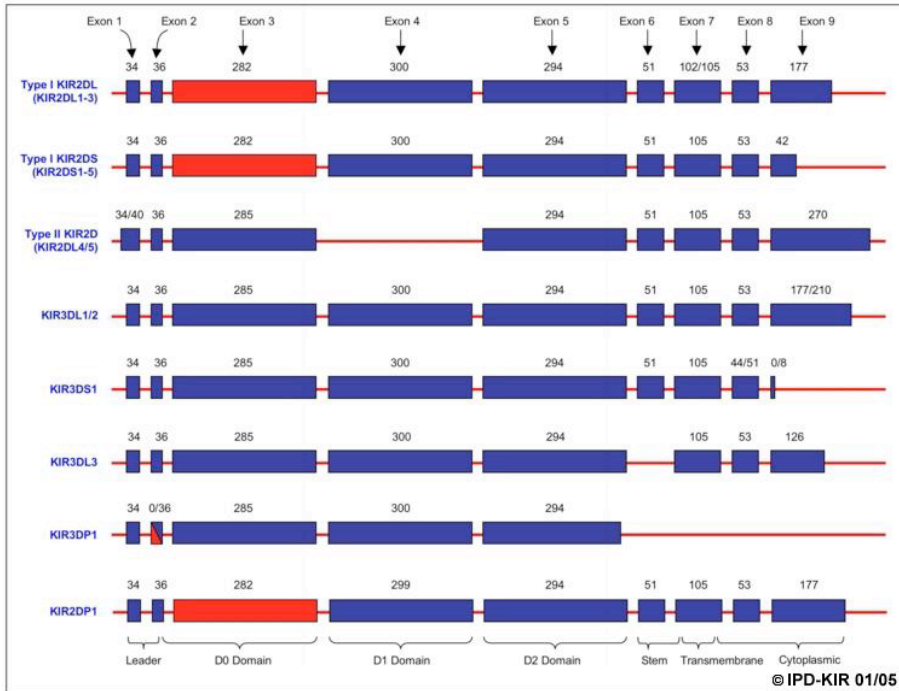


Figure 11 - KIR gene structure

#### 1.3.10.1 A haplotype

A haplotype is relatively simple, conserved and consists of a fixed number of genes (figure 12). It is composed, from centromere to telomere, by KIR3DL3, 2DL3, 2DL1, 2DL4, 3DL1, 2DS4 and 3DL2 genes. The A haplotype is more frequent in Caucasian and Japanese populations with a total frequency of 86 %, where homozygous individuals for the A haplotype make up nearly 50 % of these populations [141, 143]. Important characteristic of this haplotypes is that has only two activating KIR: KIR2DS4 and KIR2DL4. Moreover, it is demonstrated that the standard typing for KIR2DS4 gene can't discriminate the entire gene from its

common variant, equal to KIR2DS4 but with deletion of 22 bases on the sequence coding the immunoglobulin domain [139]. This deletion causes the interruption for the translation and the premature stopping on the transmembrana domain. The function of this variant of 2DS4, named KIR1D, is not known yet, but its protein structure seems not to be compatible with an activating activity. With the identification of KIR1D, the A haplotypes can be divided in 2 new haplotypes, the A-1D and the A-2DS4 haplotype: the first one is present in the 38% of Caucasian population, the second in the 11,8% [139]. The A-1D haplotype identification is very important, because it indicates that the more frequent haplotype in Caucasian population has only one activating KIR, the KIR2DL4.

### 1.3.10.2 B haplotype

B haplotype is more variable comparing to A haplotype, comprising more activating KIR genes, with gene number varying greatly from haplotype to haplotype (figure 12) [144]. Different criteria have been used for the assignment of the two haplotypes: while inclusion of 2DL1 and 2DL3 has been nominated A haplotypes, the absence of both 2DL1 and 2DL3 which has been nominated B haplotypes [139, 145]. The B haplotype dominates in Australian Aborigens with a frequency of 90 % [146], while it has similar frequency to A haplotype in Caucasian population. Among the B haplotype's gene, KIR2DL5 is relatively new, and it is divided in one expressed variant (KIR2DL5A) and one non-expressed variant (KIR2DL5B) [147]. The particularity of this gene is that these two variants don't act like allele, even if they are. In fact they are not in the same locus, and they can be found at the same time in the same haplotypes in two different positions. So, with their discovery, we can find up to 14 different loci in the same haplotypes.

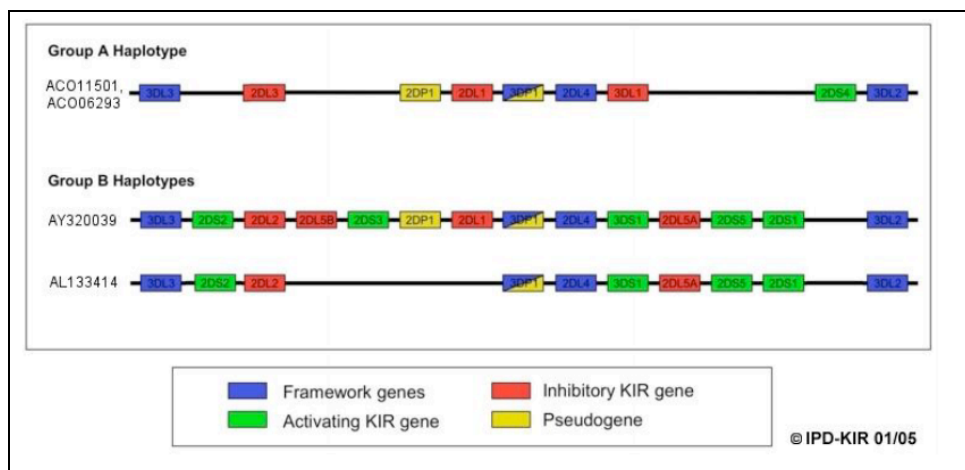


Figure 12 - A and B haplotypes

### **1.3.11 KIR gene polymorphisms**

The characteristic that mainly contributes to the variability of KIR genomic region is its genetic polymorphism, due above all to two mechanisms: point mutations and the homologous recombination [145]. KIR polymorphism is similar to MHC polymorphism, even if lower, maybe because of the minor number of KIR genes. Demonstrating this high allelic polymorphism of this region, genotyping studies distinguished, in just four loci (KIR2DL1, 2DL3, 3DL1 and 3DL2) 22 different A haplotypes, underlining that less of 0,24% of unrelated by kinship can have the identical genotype [145].

### **1.3.12 KIR receptor expression**

Human NK clones express different number and combination of KIR receptors [132]. The only requirement for each NK clone with lytic activity is that expresses at least one inhibitory receptor with specificity for a self-MHC-I molecule [132]. The inhibitory receptor can belong to either the KIR or CD94/NKG2 family. The range of different KIR receptors expressed by the repertoire of NK clones is primarily determined by the KIR genotype, but the individual HLA genotype determines which KIR can be expressed as the only inhibitory receptor on individual NK cell clones [141]. Receptor selection occurs during NK cell development to ensure only NK cells with an inhibitory receptor for a self-ligand are permitted to become armed for cytotoxicity. Thus lytic NK cells without an inhibitory receptor for self-HLA class I do not appear in peripheral circulation [132, 141].

### **1.3.13 KIR protein structure**

KIR proteins have characteristic Ig-like domains on their extracellular regions, which in some KIR proteins are involved in HLA class I ligand binding. They also have transmembrane and cytoplasmatic regions which are functionally relevant as they define the type of signal which is transduced to the NK cell. KIR proteins can have two or three Ig-like domains (hence KIR2D or KIR3D) as well as short or long cytoplasmatic tails (represented as KIR2DS or KIR2DL). Long cytoplasmatic tails contain two ITIM domains whereas short cytoplasmatic tails contain one ITAM domain. Exception to this is KIR2DL4, which contains only one N-terminus ITIM. KIR proteins vary in length from 306 to 456 amino acid residues. Although the differences in protein length are mostly the consequence of the number of Ig-like domains present, cytoplasmatic region length diversity is also an influencing factor. The leader peptide of most KIR proteins is 21 amino acid residues long. However, the presence of a different initiation codon generates a correspondingly longer leader peptide in KIR2DL4 proteins. The D0 Ig-like domain present in type II KIR2D proteins and KIR3D proteins, is approximately 96 amino acid residues in length. The D1 domain of type I KIR2D and of KIR3D proteins is 102 aminoacid residues long, while the D2 domain of all KIR proteins is 98 aminoacid residues

long. The length of the stem region varies from the 24 amino acid residues present in most KIR proteins, to only seven amino acid residues in the divergent KIR3DL3 protein. The transmembrane region is 20 amino acid residues long for most KIR proteins, but one residue shorter on KIR2DL1 and KIR2DL2 proteins as a result of a three base pair deletion in exon 7. Finally, the cytoplasmic region of KIR proteins exhibits greater length variations, ranging from 23 amino acid residues in some KIR3DS1 alleles to the 96 amino acid residues present in KIR3DL2 proteins (figure 13) [148].

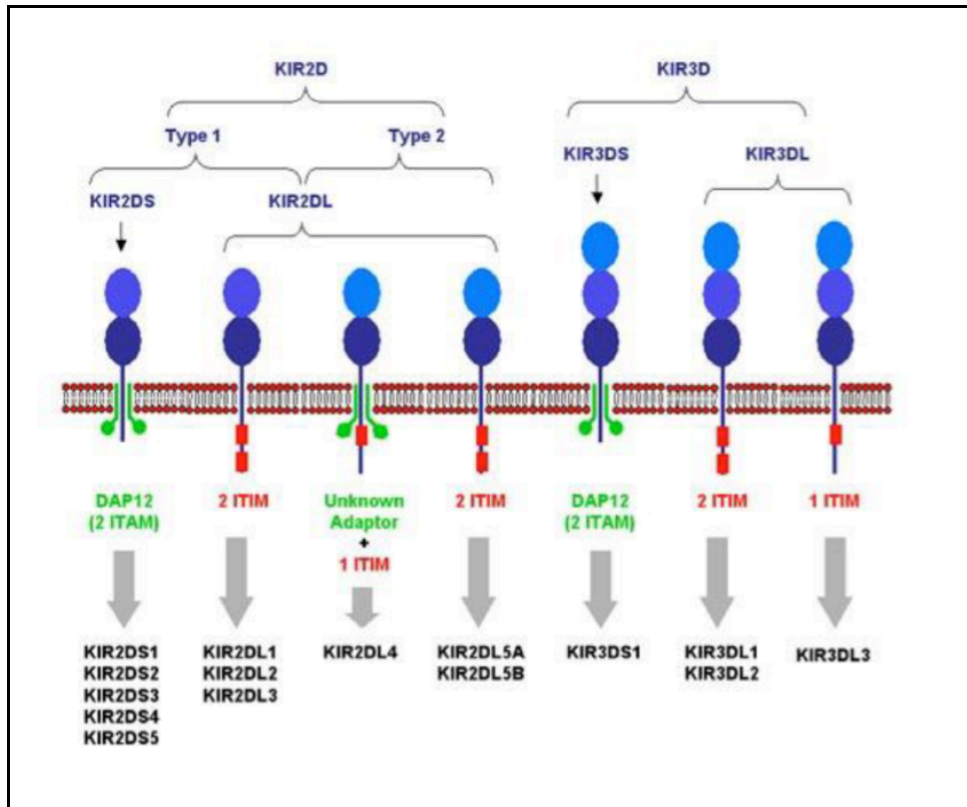


Figure 13 - KIR protein structure

### 1.3.14 KIR ligands

KIR receptors interact with the classical class I of Human Leucocyte Antigen (HLA-I) molecules. HLA is the human form of Major Histocompatibility Complex (MHC) and its gene group on chromosome 6 (6p21.3) encodes for three classes of molecules. HLA class-I has a heavy chain 44KDa polypeptide molecule consisting of three globular helical domains ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) which are expressed on the cell

surface and which inserts into the cytosol through the cell membrane. Its heavy chain is always associated with a lighter  $\beta_2$ microglobulin ( $\beta_2m$ ) chain and three different class I antigens (-A, -B and -C) are expressed; each of which displays marked allelic variation between individuals. HLA class I is virtually expressed on all cells and is responsible for the presentation of intracellular peptides associated with abnormal cellular conditions (figure 14) [149].

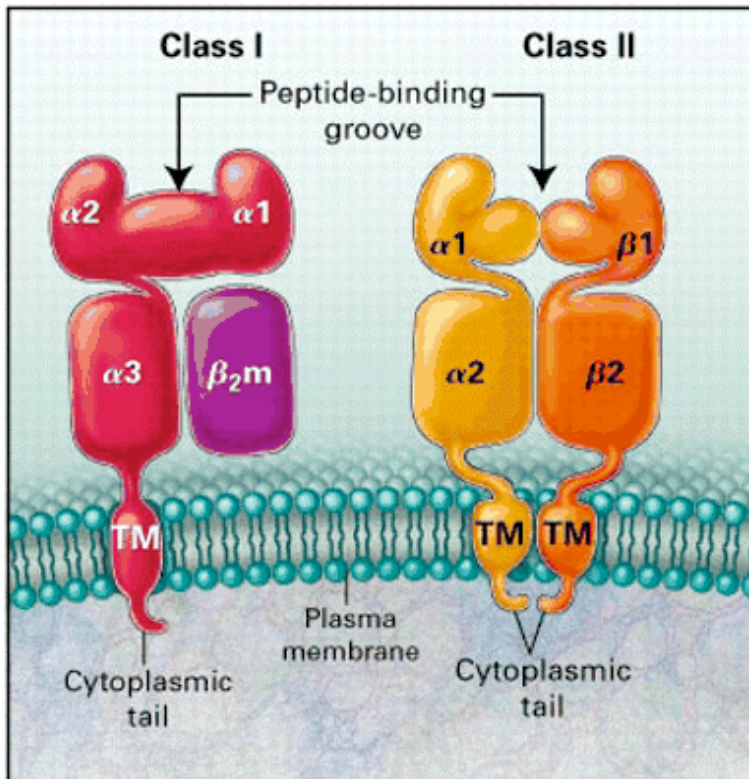


Figure 14 - Class I and class II HLA protein structure

Like HLA-I molecules, class II (HLA-II) molecules are also heterodimers, but in this case consist of two homogenous peptides, an  $\alpha$  and  $\beta$  chain, both of which are encoded in the MHC. Unlike HLA-I, the HLA-II molecules are found only on antigen-presenting cells (APC) and lymphocytes [149] (figure 14).

Peptides, generated mainly from degradation of cytosolic proteins processing by the proteasome, are transported by transporters associated with antigen processing (TAP) 1 and 2 through the endoplasmic reticulum and Golgi apparatus to the cell surface. During this process they are complexed to  $\beta_2m$ , arriving at the cell surface as an HLA class I antigen.

This peptide-HLA complex then becomes a target for cytotoxic T-cells. [150, 151]. There are many variant alleles of HLA-A, but just A\*03 and A\*11 hallotypes bind KIRs: both of them recognize and bind KIR3DL2, and A\*11 can bind KIR2DS4 too [126, 152, 153].

HLA-B alleles can be divided into two groups based on the presence of a Bw4 or Bw6 motif in the  $\alpha 1$  domain at residue 77-83 of the molecule. The unique KIR that binds with HLA-B is KIR3DL1, recognizing the HLA-Bw4 group [126, 154].

HLA-C alleles can be divided into two groups: the C1 group, having a serine (S) at residue 77 and an asparagine (N) at residue 80, and C2 group, having an asparagine (N) at residue 77 and a lysine (K) at residue 80. KIR2DL1 and 2DS1 interact with HLA-C2, while KIR2DL2, 2DL3 and 2DS2 with HLA-C1. The C specificity is due to a single substitution at 44 residue of KIR2D. KIR2DS4 interacts with both HLA-C1 and -C2, as well as with HLA-A11. [126, 152, 153]. All the known interaction between HLA and KIR, with their Ig-like domains, are summarized in table 1.

<b>KIR</b>	<b>Ig-like domains</b>	<b>Ligand</b>
2DL1	D1-D2	HLA-C2
2DL2	D1-D2	HLA-C1
2DL3	D1-D2	HLA-C1
2DL4	D0-D2	HLA-G
2DL5A	D0-D2	Unknown
2DL5B	D0-D2	Unknown
2DS1	D1-D2	HLA-C2
2DS2	D1-D2	HLA-C1
2DS3	D1-D2	Unknown
2DS4	D1-D2	HLA-C, HLA-A*11
2DS5	D1-D2	Unknown
3DL1	D0-D1-D2	HLA-Bw4
3DL2	D0-D1-D2	HLA-A*3, HLA-A*11
3DL3	D0-D1-D2	Unknown
3DS1	D0-D1-D2	HLA-Bw4

*Table 1 - Interaction between KIR receptor and HLA*

### **1.3.15 KIR and diseases**

KIR genes may predispose to human diseases: association studies have highlighted the involvement of various factors including haplotype diversity, individual gene expression and HLA ligand diversity. Although many association studies are available, at now there is only one functional study demonstrating a direct “cause and effect” relationship [155]. Cross-sectional functional studies are difficult to perform because of the inability to generate specific antibody tools against KIR.

KIR and HLA ligand interactions have been shown to be important. Clearance of



hepatitis C virus has been observed in individuals that possess homozygous 2DL3 and HLA-C1 alleles [156].

It was hypothesized that NK cells were less inhibited in this combination, as HLA-C1 alleles, were weaker at inhibiting NK cell function than HLA-C2 alleles. As a consequence there was more aggressive NK cell mediated activity against hepatitis C virus. Similarly in HIV, individuals with HLA-Bw4 and 3DS1 progressed more slowly to AIDS than those without. The mechanism for this involves ligation of activating KIR3DS1 by HLA-Bw4 ligands leading to activation and virus clearance [157]. To note, this study showed that the presence of KIR3DS1 in absence of HLA-Bw4 is associated with a more rapid progression to AIDS. Finally, a study performing on patients with nasopharyngeal cancer showed that an increasing number of activating KIR seems to correlate with the disease, and that the presence of HLA-Cw0401 is protective [158].

## CHAPTER II

### MATERIAL AND METHODS

#### **2.1 Subjects**

In total, 319 individuals were enrolled in the study; these subjects included 32 cKS patients, 20 patients with epidemic Kaposi's sarcoma, 67 HIV<sup>+</sup> healthy subjects and 200 HIV<sup>-</sup> healthy donors.

For healthy donors, subjects undergone to routinely analysis were enrolled, without known diseases at the moment of sampling, HIV negative, and with more than 60 years, so as to be comparable with patients and to minimize the possibility to have "false positivity" (i.e. subjects that may develop KS in the future).

Signed informed consent, from every subject a sample of whole blood (10 ml), and serum (5 ml) were collected. For cKS patients also saliva (about 2 ml) were collected.

#### **2.2 DNA extraction from whole blood**

Genomic and viral DNA was extracted from whole blood samples following the phenol-chloroform method.

- Thaw whole blood sample (collected in K3-EDTA tube and stored at -20°C) and transferred to a sterile 15 ml tube
- Add PBS up 15 ml, mix and centrifuge for 10 minutes at 1750 rpm
- Discard supernatant and repeat washing with PBS
- Add 1 ml of cell lysis solution (1.64 gr NH<sub>4</sub>Cl; 0.2 gr KHCO<sub>3</sub>; 0.0074 gr tetrasodium EDTA), mix and wait up 5 minutes
- Add PBS up 15 ml and centrifuge for 10 minutes at 1750 rpm
- Discard supernatant
- Put the pellet in a tube, adding about 1 ml PBS, mix and centrifuge for 10 minutes at 13000 rpm
- Discard supernatant, add 500 µl of protein lysis solution and 7.5 µl of proteinase K (20 mg/ml)
- Incubate sample at 57°C for 1 hour
- Add 800 µl of chloroform, mix and centrifuge for 10 minutes at 10000 rpm
- Transfer the upper phase (watery phase vs lower and organic phase) in a new tube with 100% ethanol
- Shack by hands till the formation of DNA flocculus
- Transfer the flocculus in a tube with 70% ethanol, washing and finally transfer it in a tube with 50 µl of H<sub>2</sub>O

## **2.3 DNA extraction from saliva**

Genomic and viral DNA extraction from saliva was performed using Oragene DNA (DNA Genotek) kits.

- Collect about 2 ml of saliva in Oragene DNA tube (the subject shouldn't have eaten, drunk, smoked or chewed gum for 30 minutes before giving their saliva sample)
- Put the tube on a flat surface
- Screw the cap onto the container, so that oragene solution can mix with saliva
- Mix gently for at least 10 seconds
- Incubate the sample at 50 °C in a water incubator overnight
- Transfer the mixed sample to a 1.5 ml to a 15 or 50 ml centrifuge tube
- Add 1 ml of cell lysis solution and 25 µl Gentra RNase A Solution (4 mg/ml)
- Vortex at high speed for 10 seconds to mix sample
- Incubate 10 minutes at room temperature
- Add 1.67 ml of protein precipitation solution to the cell lysate
- Vortex vigorously at high speed for 20 second to mix the protein precipitation solution uniformly with the cell lysate
- Incubate the samples on ice for 5 minutes
- Centrifuge at 2000 g for 5 minutes. The precipitated proteins will form a tight dark brown pellet
- Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 15 or 50 ml tube containing 5 ml of 100% isopropanol and 40 µl of Gentra Glycogen Solution (20 mg/ml)
- Mix the sample by inverting gently 50 times
- Centrifuge at 2000 g for 3 minutes; the DNA will be visible as a small white pellet
- Pour off supernatant and drain tube briefly on clean absorbent paper. Add 5 ml of 70% ethanol and invert tube several times to wash DNA pellet
- Centrifuge at 2000 g for 1 minute. Carefully pour off the ethanol
- Invert and drain the tube on clean absorbent paper and allow the sample to air dry for 5 to 10 minutes
- Add 400 µl of DNA hydration solution

## **2.4 DNA quantification**

The concentration of DNA solution was determined by spectrometer measuring DNA absorption at 260 nm wavelength, against blank, according the Lambert-Beer law:

$$A = \epsilon c l$$

with:

A = absorption

$\epsilon$  = molar absorption coefficient;  
c = concentration  
l = optical path.

The ratio A260/A280 is used to estimate the purity of nucleic acid, since protein absorb at 280nm. Generally, for DNA, a good value is between 1.8-2.0: a ratio higher than 2.0 indicates that samples may be contaminated with chloroform or phenol, whereas a ratio lower than 1.8 indicated that samples may be contaminated with proteins.

## **2.5 HHV-8 antibody detection in serum**

Antibodies against latent and lytic HHV-8 antigens were separately detected in serum samples by using an in-house indirect immunofluorescence assay (IFA), based on BCBL-1 cell lines. As written in the introduction, these cells are able to maintain the viral genome in latent episomic form at nuclear level [43], but it is also possible to induce viral replication and the consequent expression of the lytic antigens, by treatment with IL-6 and phorbol-12-myristate-13-acetate (TPA).

A cell culture system was prepared to maintain and to expand the cell line:

- Culture BCBL-1 cells in flask with cell culture medium (RPMI 1640, Sigma Aldrich, Missouri, USA, with 10% heat fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin) and glutamine (100  $\mu$ g/ml)
- Incubate at 37 °C and 5% CO<sub>2</sub>. At these growth conditions, the virus is present in cells in a latent state and only 5% of the cells are observed in active replication
- To promote also the expression of lytic antigens, add TPA (20  $\mu$ g/ml, Sigma Aldrich) and IL-6 (200 U/ml, Sigma Aldrich) to the cells and wait 72 hours: at these conditions, about 25-30% of cells show an active viral replication. In this way it is possible to study both latent and lytic phase of the virus, and consequently, to identify antibodies against latent antigens, localized at the nuclear level, and even lytic antigens, localized in the cytoplasm and on the viral cell membrane.

IFA allows a qualitative and quantitative assessment of antigen-antibody reaction that occurs at level of well-defined target structures such as the nucleus and cytoplasm. The method is called indirect because uses a secondary antibody, labeled with a fluorochrome, that can recognize the primary antibody-antigen complex. In our case, a secondary antibody directed against human IgG, labeled with FITC fluorochrome has been used.

For the fixation and permeabilization, a commercial kit (Fix and Perm, Caltag Laboratories, France) has been used.

- Wash cells and centrifuged at 1500 rpm for 10'
- Colour cells with Trypan-blue, count viable cells using a Burker chamber
- Add 800 µl of fixative to cells
- Incubate for 15' at dark at room temperature
- Add 2 ml of PBS to the suspension
- Wash cells twice by centrifugation at 1500 rpm for 5'
- Resuspend the pellet with 800 µl of permeabilization buffer
- Incubated at dark for 20' at room temperature
- Wash cells twice with 2 ml of PBS
- Put cells on the slide
- Dry the slides under a laminar flow hood
- Put the slides in cold acetone (4 °C) for 10'
- So treated, the slides can be stored at -20 °C, protected by light.

This procedure allows the entrance of fluorescein (fluorescein isothiocyanate, FITC) and antibodies within the cell (in cytoplasm and even nucleus), leaving intact the morphological characteristics. For our assays,  $6 \times 10^4$  cells have been deposited on each well of one slide. The optimal concentration has been determined by serial dilutions of the cell suspension, to have a uniform monolayer of cells in each single well.

Antibodies detection:

- Put on each spot of cells on the slide 10-15 µl of diluted (1:100 with PBS) serum sample
- Incubate in a humid chamber at 37 °C for 1 hour
- Wash the slides twice in PBS for 10' under agitation to eliminate any non-specific binding between viral and cellular antigens and serum antibodies
- Add on each wells 10 µl of FITC-conjugated (FITC-conjugated Anti-Human  $\gamma$ -Globulin with Evans Blue, Sigma Aldrich) secondary antibody, directed against human immunoglobulins
- Incubate the slides for 30' at 37 °C to allow the reaction between primary antibody and labeled secondary antibody
- Wash the slides twice in PBS
- Dry the slides on air
- Cover the slides with cover slips, helping yourself with glycerol
- Examine the slides with a fluorescence microscope.

Samples were considered positive for antibodies to latent antigens when about 80% of BCBL-1 cells in the latent stage had punctiform fluorescence in the nuclear level. By contrast, the samples were considered positive for antibodies to lytic antigens when 30% of the BCBL-1 cells in lytic stage had a uniform cytoplasmic fluorescence. As a further control of specificity, the solution of FITC-labeled secondary antibody was tested on the substrate in the absence of human serum, while as a positive control serum was used serum from a well-known Kaposi's sarcoma patient.

## 2.6 KIR and HLA genotyping

The PCR (polymerase chain reaction) is a technique that allows the enzymatic amplification *in vitro* of a specific DNA sequence, by using oligonucleotide pairs (DNA primer).

KIR genotyping was performed by SSP-PCR (single specific primer PCR) method, a technique based on a sequence-specific primer panels, using “KIR Genotyping” BAG plates for the molecular determination of loci. Every plate is formed by 24 wells with a specific primer pairs for a single allele of each locus, where PCR reaction performs. In particular, this kit allows amplifying KIR 2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS4, 3DS1, 3DL1, 3DL2, 3DL3 genes and 2DP1 and 3DP1 pseudogenes.

Similarly, also HLA-A, -B and -C typing was performed by SSP-PCR method, using “Hysto Type” BAG plate.

This is the protocol for KIR genotyping and HLA typing:

DNA (50 ng/μl)	2 μl
10 X PCR Buffer	1 μl
Taq Polymerase (5U/ μl)	0.08 μl
Sterile H <sub>2</sub> O	6.92 μl
Final volume	10 μl

These are the amplification conditions for KIR genotyping (phase 1: 10 cycles; phase 2: 20 cycles):

	Temperature	Time
Polymerase activation	94°C	2'
Denaturation 1	94°C	15''
Annealing 1	65°C	50''
Extension 1	72°C	45''
Denaturation 2	94°C	15''
Annealing 2	61°C	50''
Extension 2	72°C	45''
Final extension	72°C	10'
Blocking reaction	4°C	10'

These are the amplification condition for HLA typing (phase 1: 5 cycles; phase 2: 10 cycles; phase 3: 15 cycles):

	Temperature	Time
Polymerase activation	96°C	5'
Denaturation 1	96°C	20''
Annealing 1	68°C	30''
Extension 1	68°C	30''
Denaturation 2	96°C	20''
Annealing 2	64°C	50''
Extension 2	72°C	45''
Denaturation 3	96°C	20''
Annealing 3	61°C	50''
Extension 3	72°C	45''
Final extension	72°C	10'
Blocking reaction	4°C	10'

## **2.7 KIR haplotype and ligands**

As written in the introduction, two broad haplotypes, termed A and B, are defined based on KIR genes. A and/or B haplotypes were classified based on the criteria adopted by Middleton and colleagues (<http://www.allelefreqencies.net>) [159]. Ligands group was defined as follows:

- 1- KIRs 2DL1 and 2DS1 bind the C2 epitope (Asparagine at position 77, Lysine at position 80).
- 2- KIRs 2DL2, 2DL3, and 2DS2 bind the C1 epitope (Serine at position 77, Asparagine at position 80).
- 3- HLA-Bw4 and the HLA-Bw4\*80I (Isoleucine at position 80) subset were considered the ligand for KIR 3DL1 and 3DS1) [130, 157].

## **2.8 Agarose gel electrophoresis**

- Mix agarose powder with 1X TBE buffer to obtain a 2% final solution
- Heat in a microwave oven until gel is completely melted
- Add ethidium bromide (final concentration 1µl/ml) to allow the DNA visualization
- Pour the solution into a casting tray containing a sample comb
- Wait till the solidification of gel
- Remove the comb, using care not to rip the bottom of the wells

- Insert the gel into the electrophoretic chamber and just cover with TBE buffer
- Put samples into the wells
- Turn on the electrophoresis machine
- Wait till an adequate migration
- Visualize DNA fragments placing the gel on a ultraviolet transilluminator.

To note, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA (and RNA) and allows seeing nucleotide when lighted by ultraviolet.

## **2.9 HHV-8 viral load measurement by real-time PCR**

Real-time PCR, also called quantitative PCR (qPCR), is a technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, real time PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

HHV-8 viral load was determined by real-time PCR using an ABI PRISM 7000 SDS (Applied Biosystem, Foster City, CA). Primers and probe were designed using the Primer express software (Applied Biosystem), to obtain a 74 bp amplicon on ORF26 region of the viral genome, coding for HHV-8 minor capsid protein. PCR amplification was performed in 25  $\mu$ l reaction mixture using Taqman Universal masterMix (Applied Biosystem), 0.9 mM each primers, 0.2 mM Taqman probe and 500 ng of blood DNA or 10  $\mu$ l of DNA extracted from saliva were used as template. Each sample was analyzed in triplicate: samples that did not yield three positive reactions were repeated in triplicate and viral load results were given by the mean of the three positive reactions. Each run contained a negative control, constituted by the reaction mixture without the DNA template. Viral load was analyzed by interpolation of a standard curve obtained from serial dilution of HHV-8 quantified purified viral DNA (Advanced Biotechnologies Inc, Columbia, MD). The assay was linear from  $10^1$  to  $10^7$  copies per reaction. The interassay variability, evaluated by repeating the quantitation of the same positive specimen for 8 times, was low, with the coefficient of variation less than 20% for viral load ranging from 130 copies/ml ( $2.11 \log_{10}$ ) to  $1.3 \times 10^5$  copies/ml ( $5.11 \log_{10}$ ). The specificity was tested using as template DNA of EBV (whose genome is very similar to HHV-8 genome), which was not amplified. The lowest detection limit was 10 copies/reaction, which is equivalent to 40 copies/ $\mu$ g of DNA extracted from blood and 4000 copies/ml of saliva.



Amplification conditions (40 cycles):

	Temperature	Time
Amperase UNG activation (inside Taqman master mix)	50°C	2'
Polymerase activation	95°C	15''
Annealing	60°C	30''
Extension	60°C	30''
Blocking reaction	4°C	10'

Sequences of primers and probe, studied for the ORF26 region of the viral genome, are:

	Position	Sequence	T <sub>m</sub>
ORF26F Forward primer	47311- 47331	5'- CTC GAA TCC AAC GGA TTT GAC -3'	52.2 °C
ORF26R Reverse primer	47385- 47366	5'- TGC TGC AGA ATA GCG TGC -3'	53.1 °C
Probe	47342- 47360	6 FAM-CCA TGG TCG TGC CGC AGC A-TAMRA	57.4 °C

with:

T<sub>m</sub> = melting temperature.

The position is referred to accession number: U75698.

The lowest detection limit was 10 copies per reaction, which was equivalent to 40 copies (µg DNA extracted from blood)<sup>-1</sup> or 630 copies (ml saliva)<sup>-1</sup>.

The real time PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystem).

## **2.10 HHV-8 genotyping**

Genotype characterization of HHV-8 strains was based on sequencing of variable regions VR1 (spanning nt. 46-425), VR2 (spanning nt. 68-817) and VR3 (spanning nt. 607-942) of the hypervariable ORF-K1viral gene, coding for a transmembrane protein.

### 2.10.1 Nested PCR

Nested PCR is a variation of PCR, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first pair of PCR primers amplifies a fragment similar to a standard PCR (outer fragment). However, a second pair of primers called nested primers binds inside the first PCR product fragment to allow amplification of a second PCR (inner fragment) product, which is shorter than the first one.

#### Amplification of the outer fragment (nt. 23-987, 966 bp)

DNA (100 ng/μl)	5 μl
DMSO	2 μl
10 X PCR Buffer solution	5 μl
MgCl solution (50 mM)	2 μl
dNTPs (10 mM)	3 μl
Outer forward primer (10 μM)	3 μl
Outer reverse primer (10 μM)	3 μl
TaqGold polymerase (5U/μl)	0.3 μl
Sterile H <sub>2</sub> O	26.7 μl
Final volume	50 μl

#### Amplification conditions (30 cycles):

	Temperature	Time
Polymerase activation	94°C	10'
Denaturation	94°C	30''
Annealing	53°C	45''
Extension	72°C	2'
Final extension	72°C	7'
Blocking reaction	4°C	10'

The sequences of primers are:

	Position	Sequence	T <sub>m</sub>
OK1 forward primer	23-38	5'- GTT CTG CCA GGC ATA GTC T-3'	51.0 °C
OK1 reverse primer	987-960	5'- TCT TAC CTG AAT GTC AGT ACC A -3'	51.2 °C

T<sub>m</sub> = melting temperature.

The position is referred to accession number U75698.

Amplification of VR1 inner fragment (nt. 46-425, 377 bp)

Outer fragment (100 ng/μl)	5 μl
DMSO	2 μl
10 X PCR Buffer solution	5 μl
MgCl solution (50 mM)	1.5 μl
dNTPs (10 mM)	2 μl
Inner forward primer VR1 (10 μM)	2 μl
Inner reverse primer VR1 (10 μM)	2 μl
TaqGold polymerase (5U/μl)	0.3 μl
Sterile H <sub>2</sub> O	30.2 μl
Final volume	50 μl

Amplification conditions (phase 1: 14 cycles: phase 2: 19 cycles):

	Temperature	Time
Polymerase activation	94°C	10'
Denaturation 1	94°C	30''
Annealing 1	57°C -0.5°C each cycle	30''
Extension 1	72°C	40''
Denaturation 2	94°C	30''
Annealing 2	50°C	30''
Extension 2	72°C	50''
Final extension	72°C	5'
Blocking reaction	4°C	10'

The sequences of primers are:

	Position	Sequence	T <sub>m</sub>
VR1int forward primer	46-65	5'- CTG GGC GGC CCT TGT GTA AAC -3'	56.0 °C
VR1int reverse primer	425-405	5'- GAC TGT GTT TGA TGG CTG GC -3'	54.4 °C

T<sub>m</sub> = melting temperature.

The position is referred to accession number: U75698.

Amplification of VR2 inner fragment (nt. 68-817, 749 bp)

Outer fragment (100 ng/μl)	5 μl
DMSO	2 μl
10 X PCR Buffer solution	5 μl
MgCl solution (50 mM)	1.5 μl
dNTPs (10 mM)	3 μl
Inner forward primer VR2 (10 μM)	3 μl
Inner reverse primer VR2 (10 μM)	3 μl
TaqGold polymerase (5U/μl)	0.3 μl
Sterile H <sub>2</sub> O	27.2 μl
Final volume	50 μl

Amplification conditions (30 cycles):

	Temperature	Time
Polymerase activation	94°C	10'
Denaturation	94°C	30''
Annealing	54°C	45''
Extension	72°C	2'
Final extension	72°C	10'
Blocking reaction	4°C	10'

The sequences of primers are:

	Position	Sequence	T <sub>m</sub>
VR2 forward primer	68-88	5'- TCT TTC AGA CCT TGT TGG AC -3'	50.0 °C
VR2 reverse primer	817-801	5'- CAG AGC TAC GAG TGT CAT -3'	48.8 °C

T<sub>m</sub> = melting temperature.

The position is referred to accession number: U75698.

Amplification of VR3 inner fragment (nt. 607-942, 335 bp)

Outer fragment (100 ng/μl)	5 μl
10 X PCR Buffer solution	5 μl
MgCl solution (50 mM)	1.5 μl
dNTPs (10 mM)	2 μl
Inner forward primer VR2 (10 μM)	2 μl
Inner reverse primer VR2 (10 μM)	2 μl
TaqGold polymerase (5U/μl)	0.3 μl
Sterile H <sub>2</sub> O	32.2 μl
Final volume	50 μl

Amplification conditions (phase 1: 14 cycles; phase 2: 19 cycles):

	Temperature	Time
Polymerase activation	94°C	10'
Denaturation 1	94°C	30''
Annealing 1	63°C -0.5°C each cycle	30''
Extension 1	72°C	50''
Denaturation 2	94°C	30''
Annealing 2	56°C	30''
Extension 2	72°C	40''
Final extension	72°C	5'
Blocking reaction	4°C	10'

The sequences of primers are:

	Position	Sequence	T <sub>m</sub>
VR3 forward primer	607-628	5'- GTA TAT GTT TTT GGG CGC GTT G -3'	53.4 °C
VR3 reverse primer	942-922	5'- CCG TGC ACA AAT CGT GTA GGG -3'	55.8 °C

T<sub>m</sub> = melting temperature.

The position is referred to accession number: U75698.

## 2.10.2 DNA sequence analysis

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA.

### 2.10.2.1 Pre-labeling DNA purification

At first, the PCR product was purified to eliminate all contaminants (exceeding dNTPs or primers) that could interfere with sequencing reaction. The purification was performed by using QIAquick PCR Purification Kit Protocol Kit (Qiagen, Valencia, CA, USA).

- Add 5 volumes of Binding Buffer QG to PCR mixture
- Put the sample in purification column
- Centrifuge at 13000 rpm for 1 minute
- Add 500 µl of PE
- Centrifuge at 13000 rpm for 1 minute
- Elute the DNA from column with 50 µl of water and centrifuge at 13000 rpm for 1 minute.

Alternatively, if in the electrophoresis gel multiple lanes were present, the purification was performed by using QiAquick Gel Extraction Kit Protocol (Qiagen):

- Cut from gel the lane of the amplification of interest
- Put the cut gel in a tube
- Add 300 µl of QG buffer per 100 µg of sample
- Incubate the sample at 50 °C for 10 minutes, mixing every 2-3 minutes, to allow the gel dissolution
- Add 1 volume of isopropanol
- Put the sample in a purification column
- Centrifuge at 13000 rpm for 1 minute
- Add 500 µl of PE

- Centrifuge at 13000 rpm for 1 minute
- Elute the DNA from column with 50  $\mu\text{l}$  of water and centrifuge at 13000 rpm for 1 minute.

### 2.10.2.2 Labeling reaction

This step is necessary to incorporate fluorescent dideoxynucleotides, defined BigDye terminators into sequencing DNA. The labeling structure contains a fluorescein donor (6-FAM) molecule, linked to one of the 4 dichloroamine acceptor. The maximum excitation of every labeled is the fluorescein excitation, and the emission spectrum is the rhodamine emission spectrum. BigDye terminators are labeled with these dRhodamine molecules:

- A = dichlorine [R6G]      Green
- C = dichlorine [ROX]      Blue
- G = dichlorine [R110]      Black
- T = dichlorine [TAMRA]      Red

For this step, BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) has been used.

This is the reaction mixture:

DNA (300 ng)	x $\mu\text{l}$
2.5X Terminator Ready Reaction Mix	4 $\mu\text{l}$
Primer (pmol/ $\mu\text{l}$ )	3.2 $\mu\text{l}$
5X BigDye Sequencing buffer	2 $\mu\text{l}$
Sterile H <sub>2</sub> O (up to 20 $\mu\text{l}$ final volume)	x $\mu\text{l}$
Final volume	20 $\mu\text{l}$

Adjust the DNA (and H<sub>2</sub>O) volume to have a concentration of 300 ng.

Amplification conditions (25 cycles):

	Temperature	Time
Polymerase activation	96°C	1'
Denaturation	96°C	10''
Annealing	50°C	5''
Extension	60°C	4'
Blocking reaction	4°C	10'

### **2.10.2.3 Post-labeling DNA purification**

This procedure is necessary to eliminate the Big Dye Terminators excess that could interfere with the electrophoretic running and the detection of fluorescence. This procedure was performed by using NUCLEOSEP kit (Macherey-Nagel).

- Add to the purification column with 800  $\mu$ l of water
- Leave the column at room temperature for 2 hours
- Centrifuge the column at 3800 rpm for 2 minutes
- Add the sample (2  $\mu$ l) to the column
- Centrifuge at 3800 rpm for 2 minutes
- Collect the flow-through

### **2.10.2.4 DNA sequencing**

DNA sequencing has been performed with ABI Prism 310 Genetic Analyzer (Applied Biosystem), constituted by a capillary electrophoresis machine, a laser for to excite fluorophores, a CCD-camera to detect the signal, and a software to elaborate data. The polymer that we used for this electrophoretic experiment is POP6 polymer (Invitrogen). For each sample, 5  $\mu$ l of the purified product has been used.

### **2.10.2.5 Sequence analysis**

Sequence homology searches were determined by using BLAST. The genotype of each sample was determined by comparing its sequence with those of HHV-8 prototype [58], obtained from GenBank. Multiple sequence alignment was performed using the CLUSTAL W version 1.7 program [160]. Genotyping was confirmed with amino acidic alignment of the N-terminal VR1 region (aa 1-59), analyzing the presence of distinctive residues, as reported previously by Zong et al, 1999 [58].

## **2.11 Statistical analysis**

Group data are expressed as mean  $\pm$  SEM (standard error of mean) or as median (range). Differences in frequency were evaluated by means of a  $\chi^2$  or Fisher's exact test, and Bonferroni correction for multiple tests, as appropriate. All tests were two-sided. The association of each polymorphism with disease was measured by the Odd Ratio (OR) and a 95 % Confidence Interval (95%CI). For each independent variable, crude and adjusted odds ratios and 95 % confidence interval were calculated.

To analyze differences among groups, ANOVA and Student's t-test were



performed for parametric data, and Kruskal-Wallis test and Mann-Whitney U test for non-parametric data. For multiple tests, Bonferroni correction test was performed. Probability values of  $p < 0.05$  were accepted as significant.

## CHAPTER III

### RESULTS

#### **3.1 Clinical characteristics of study's subjects**

The clinical characteristics of the subjects examined in this study are shown in table 2.

	cKS patients	Epidemic KS patients	HIV <sup>+</sup> "non KS" subjects	HIV <sup>-</sup> healthy donors
Total number	32	20	67	200
Men/women, n	23/9	18/2	44/23	126/74
Age, yr	71.1±7.7	43.5±9.5	45±8.1	74±11.8
Slow/Fast evolution of KS	14/18	11/9	----	----

*Table 2 – Clinical characteristics of study's subjects*

Demographic analysis revealed that age was not significantly different between cKS patients and HIV<sup>-</sup> healthy donor, as well as between epidemic KS patients and HIV<sup>+</sup> healthy subjects, which are the couple to compare.

The age of cKS patients and HIV<sup>-</sup> healthy donors was significantly higher than epidemic KS patients and HIV<sup>-</sup> healthy subjects ( $p < 0.05$ ), due to the clinical characteristics of cKS, which develops particularly in old age.

About sex, no significant differences were present among the four groups, as well as about KS evolution, based on clinical parameters [161], between cKS patients and epidemic KS patients.

#### **3.2 HHV-8 seroprevalence**

HHV-8 seroprevalence was analyzed by immunofluorescence assay (IFA). As expected, all cKS patients (100%) were HHV-8 seropositive. About epidemic KS, 85.0% of cases (17/20) were seropositive. Among non-KS subjects, 47.8% (32/67) of HIV<sup>+</sup> healthy subjects, and 9.0% (18/200) of HIV<sup>-</sup> healthy donors were HHV-8 seropositive (table 3).

As expected, all these differences were significant, remarking that the HHV-8 infection is a risk factor for KS development ( $p < 0.0001$  between cKS and healthy donors;  $p = 0.003$  between epidemic KS and HIV<sup>+</sup> healthy subjects).

Table 3 describes the clinical characteristics of subjects, divided according the presence or absence of anti-HHV-8 antibodies. To note, from this point we used 33 HIV<sup>-</sup> HHV-8<sup>-</sup> healthy donors (from the 182 initially screened for HHV-8 seropositivity) for all next experiments.

	cKS patients	Epidemic KS patients	HIV <sup>+</sup> HHV8 <sup>+</sup> subjects	HIV <sup>+</sup> HHV8 <sup>-</sup> subjects	HIV <sup>-</sup> HHV8 <sup>+</sup> healthy donors	HIV <sup>-</sup> HHV8 <sup>-</sup> healthy donors
Total number	32	17/20	32	35	18	33
Men/women, n	23/9	18/2	27/5	17/18	8/10	24/9
Age, yr	71.1±7.7	43.5±9.5	46.8±9.1	44.3±8.7	83.4±11.6	70.0±12.6

*Table 3 – Subdivision of study's subjects according to HHV-8 seroprevalence*

Again, no significant differences were observed for age and sex among cKS patients, HIV<sup>-</sup> HHV-8<sup>+</sup> and HIV<sup>-</sup> HHV-8<sup>-</sup> healthy donors and among epidemic KS, HIV<sup>+</sup> HHV8<sup>+</sup> and HIV<sup>+</sup> HHV-8<sup>-</sup> subjects.

### **3.3 HHV-8 DNA in blood and saliva**

The presence of HHV-8 DNA in blood and saliva samples was analyzed in all the 170 individuals enrolled in the study. The results showed that the viral DNA could be detected in biological specimens from all the HHV-8<sup>+</sup> groups, even if not in every HHV-8<sup>+</sup> subjects. Thus, HHV-8 DNA was detected in the blood of 65.6% of cKS patients (21/32), in 45% of epidemic KS patients (9/20), in 15.6% (5/32) HIV<sup>+</sup> HHV-8<sup>+</sup> subjects and in 22.2% (4/18) of HIV<sup>-</sup> HHV-8<sup>+</sup> healthy subjects. DNA isolation was significantly more frequent in cKS patients and in epidemic KS compared to HHV-8<sup>+</sup> subjects ( $p=0.0001$  for both).

As expected, none of HHV-8 seronegative individuals had viral DNA in their biological samples.

Saliva samples were collected only in 27/32 cKS patients. In this case, HHV-8 DNA was isolated in 44.4 % of cKS patients (12/27).

The blood viral load of cKS patients was  $7.40 \times 10^3 \pm 1.64 \times 10^3$ . According to the evolution of the disease, we found that the viral load was significantly higher in cKS patients with fast progression of the disease ( $1.12 \times 10^4 \pm 2.46 \times 10^3$ ) compared to slower-progression patients ( $3.98 \times 10^3 \pm 1.51 \times 10^3$ ;  $p=0.02$ ).

For the other groups, the calculation of viral load has not been possible probably cause of the low amount of viral DNA in blood.

All these results are summarized in table 4.

### 3.4 HHV-8 genotyping

Sequence analysis of VR1, VR2 and VR3 variable region of hypervariable ORF-K1 gene has been possible in 29/32 cKS patients, in 9/20 epidemic KS, in 2/32 HIV<sup>+</sup> HHV-8<sup>+</sup> healthy subjects and in none HIV<sup>-</sup> HHV-8<sup>+</sup> healthy donors.

Nucleotide analyses of amplified HHV-8 DNA showed the subjects of 14 cases with A subtype (in particular 13 with A3 and 1 with A4), and 15 with C subtype (10 with C1 and 5 with C3) in cKS patients, 5 subjects with A subtypes (all with A1) and 4 with C subtypes (3 with C1 and 1 with C3) in epidemic KS patients, and 2 individuals with C subtypes (all with C3) in HIV<sup>+</sup> HHV-8<sup>+</sup> healthy subjects (table 4).

Taken together, the A1 HHV-8 subtype was statistically more frequent in epidemic KS patients compared to cKS patients ( $p=0.0003$ ), whereas the A3 subtype was statistically more frequent in cKS ( $p=0.01$ ).

No significant difference was found for HHV-8 viral load between cKS patients with A HHV-8 subtype ( $9.77 \times 10^3 \pm 3.22 \times 10^3$ ) and those with C subtype ( $5.78 \times 10^4 \pm 1.58 \times 10^3$ ).

Data obtained from HHV-8 phylogenetic analysis are reported in figure 15, where the clustering in A1 subtype for epidemic KS, and in A3 for cKS, is clearly shown.

	cKS patients	Epidemic KS patients	HIV <sup>+</sup> HHV8 <sup>+</sup> subjects	HIV <sup>+</sup> HHV8 <sup>-</sup> subjects	HIV <sup>-</sup> HHV8 <sup>+</sup> healthy donors	HIV <sup>-</sup> HHV8 <sup>-</sup> healthy donors
Viral DNA in blood	65.6 %	45.0 %	15.6 %	0 %	---	0 %
Viral DNA in saliva	44.0 %	---	---	---	---	---
HHV-8 genotyping	14A/15C	5A/4C	2C	---	0 %	---

Table 4 – Viral DNA and HHV-8 genotyping in study's subjects

### 3.5 HHV-8 genotyping and KS evolution

To analyze in more depth the role of HHV-8 genotyping in the disease and, in particular, in its fast or slow development, we decided to combine these results with previous data with another group of 27 cKS patients, for a total of 59 cKS subjects.

In this new sub-analysis, 33 cKS patients presented a fast cKS development, whereas 26 presented a slow evolution.

HHV-8 A subtype was observed in 21/33 (63.6%) faster-progressing patients, whereas C subtype was present only in 12/33 (36.4%) subjects. In contrast, A

subtype was observed only in 6/26 (23.1%) patients with slower disease evolution, in whom C subtype was highly prevalent (20/26, 76.9%). These differences were statistically significant ( $p=0.003$ ) (figure 16).

Due the low number of patients, we could not perform this analysis to epidemic KS patients too.

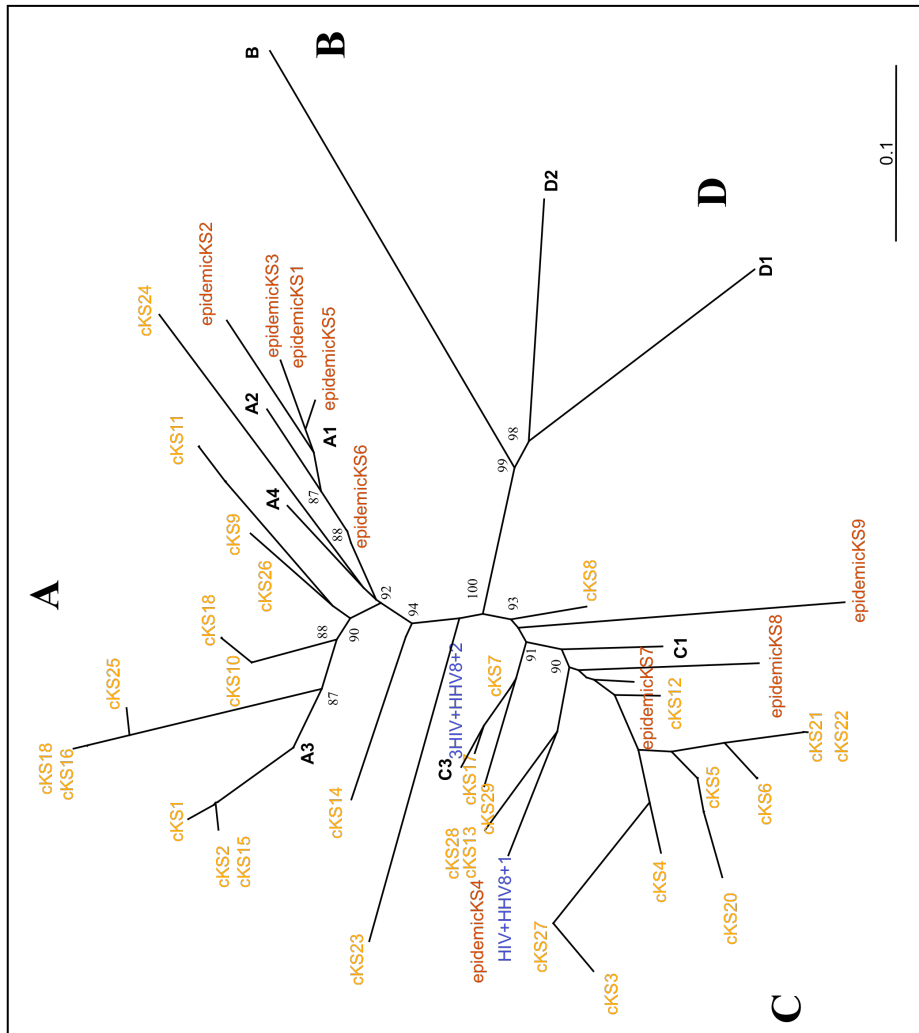
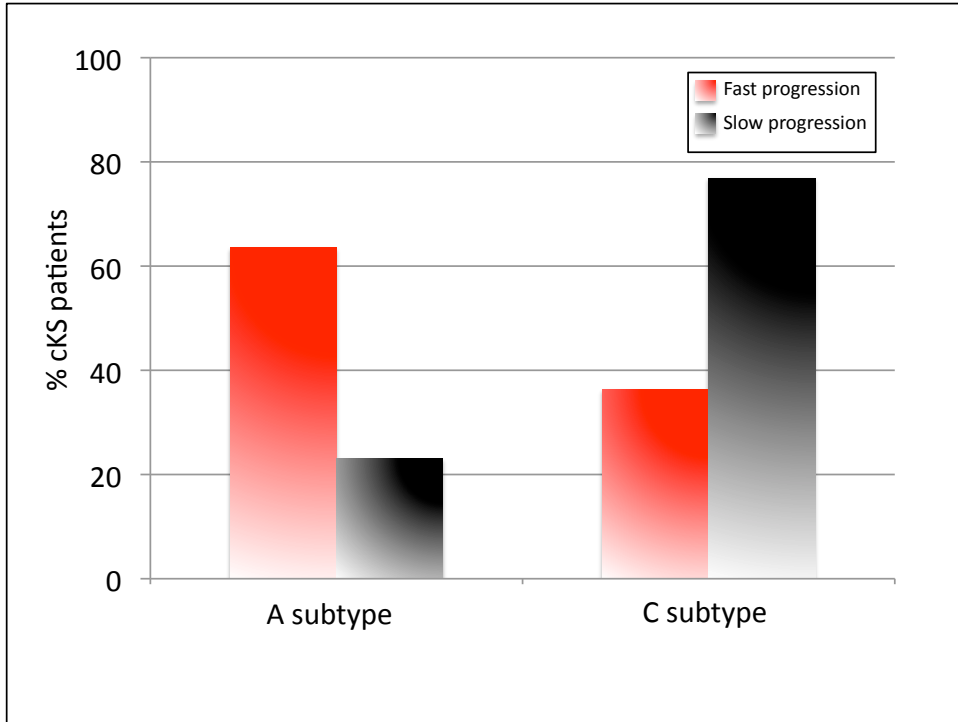


Figure 15 – Predicted phylogenetic distribution of a 942 bp segment of ORF-K1 gene amplified from cKS patients, epidemic KS patients and HIV<sup>+</sup>HHV-8<sup>+</sup> subjects. The radial phylogenetic tree was generated using CLUSTAL W. Bootstrapping for 100 replicates is noted as a percentage at major branch points. Bar, 0.1 nucleotide substitutions per site. A, B, C and D HHV-8 subtypes are also reported. Yellow: cKS patients; Orange: epidemic KS patients; Blue: HIV<sup>+</sup>HHV8<sup>+</sup> subjects.



*Figure 16 – HHV-8 A and C subtypes frequency (%) in cKS patients with fast (in red) and slow (in black) evolution.*

### **3.6 KIR and HLA genotyping**

KIR gene distribution was evaluated in all the 6 groups studied (cKS patients, epidemic KS patients, HIV<sup>+</sup> HHV-8<sup>+</sup> individuals, HIV<sup>+</sup> HHV-8<sup>-</sup> individuals, HIV<sup>-</sup> HHV-8<sup>+</sup> healthy donors and HIV<sup>-</sup> HHV-8<sup>-</sup> healthy donors), and was reported in table 5.

KIR	cKS N (%)	Epidemic KS N (%)	HIV <sup>+</sup> HHV8 <sup>+</sup> subjects N (%)	HIV <sup>+</sup> HHV8 <sup>-</sup> subjects N (%)	HIV <sup>-</sup> HHV8 <sup>+</sup> subjects N (%)	HIV <sup>-</sup> HHV8 <sup>-</sup> subjects N (%)
<i>Activating KIR</i>						
2DL4	32 (100)	20 (100)	32 (100)	35 (100)	18 (100)	32 (97.0)
2DS1	<b>21</b> <b>(65.6)</b>	7 (35.0)	10 (31.2)	10 (28.6)	<b>6</b> <b>(33.3)</b>	<b>11</b> <b>(33.3)</b>
2DS2	<b>27</b> <b>(84.4)</b>	<b>18</b> <b>(90.0)</b>	<b>17</b> <b>(53.1)</b>	<b>17</b> <b>(48.6)</b>	13 (72.2)	<b>18</b> <b>(54.5)</b>
2DS3	18 (56.3)	7 (35.0)	11 (34.4)	10 (28.6)	5 (27.8)	10 (30.3)
2DS4*001/2	7 (21.9)	1 (5.0)	11 (34.4)	12 (34.3)	3 (16.6)	2 (6.1)
2DS4*003/7	25 (78.1)	18 (90.0)	27 (84.7)	28 (80.0)	14 (78.7)	30 (90.9)
2DS5	14 (43.8)	6 (30.0)	5 (15.6)	9 (25.6)	4 (22.2)	7 (21.2)
3DS1	<b>20</b> <b>(62.5)</b>	5 (25.0)	10 (31.2)	9 (25.6)	<b>5</b> <b>(27.8)</b>	<b>10</b> <b>(30.3)</b>
<i>Inhibitory KIR</i>						
2DL1	32 (100)	19 (95.0)	30 (93.7)	33 (94.3)	18 (100)	33 (100)
2DL2	22 (68.8)	16 (80.0)	18 (56.2)	17 (48.6)	10 (55.5)	18 (54.5)
2DL3	28 (87.5)	17 (85.0)	22 (68.7)	27 (77.1)	18 (100)	30 (90.9)
2DL5A	12 (37.5)	5 (25.0)	10 (31.2)	12 (34.3)	2 (11.1)	10 (30.3)
2DL5B	7 (21.9)	7 (35.0)	11 (34.4)	14 (40.0)	2 (11.1)	5 (12.5)
3DL1	32 (100)	17 (85.0)	31 (96.8)	34 (97.1)	17 (94.4)	33 (100)
3DL2	32 (100)	20 (100)	32 (100)	34 (97.1)	18 (100)	32 (100)
3DL3	32 (100)	20 (100)	32 (100)	35 (100)	18 (100)	32 (97.0)
<i>Pseudogenes</i>						
2DP1	32 (100)	19 (95.0)	30 (93.7)	33 (94.3)	18 (100)	33 (100)
3DP1*001/2/4	0 (0)	1 (5.0)	2 (6.2)	2 (5.7)	0 (0)	0 (0)
3DP1*003	32 (100)	19 (95.0)	28 (87.5)	31 (88.6)	18 (100)	33 (100)

Table 5 – KIR gene distribution in study's subjects

In general, we found some activating KIRs significantly more frequent in KS patients compared to healthy subjects. In particular, KIR2DS2 was more frequent in both cKS and epidemic KS compared non-KS subjects, whereas KIR2DS1 and KIR3DS1 were more frequent in cKS compared healthy individuals. To note, KIR2DS4\*001/2 allele seems to be less frequent in epidemic KS patients compared to their appropriate control groups, even if the difference did not reach the level of statistical significance.

Specifically, the results that we obtained were:

### 2DS1

**cKS vs HIV<sup>-</sup> HHV-8<sup>+</sup> subjects:** p=0.01; OR: 4.20, 95%CI: 1.5-11.9

**cKS vs HIV<sup>-</sup> HHV-8<sup>-</sup> subjects:** p=0.02; OR: 3.82, 95%CI: 1.2-12.3

### 2DS2

**cKS vs HIV<sup>-</sup> HHV-8<sup>-</sup> subjects:** p=0.02; OR: 4.50, 95%CI: 1.2-17.4

**epidemic KS vs HIV<sup>+</sup> HHV-8<sup>+</sup> subjects:** p=0.007; OR: 7.94, 95%CI: 1.5-40.0

**epidemic KS vs HIV<sup>+</sup> HHV-8<sup>-</sup> subjects:** p=0.003; OR: 9.53, 95%CI: 1.9-47.4

**epidemic KS vs HIV<sup>-</sup> HHV-8<sup>-</sup> subjects:** p=0.01; OR: 7.50, 95%CI: 1.5-37.7

### 3DS1

**cKS vs HIV<sup>-</sup> HHV-8<sup>+</sup> subjects:** p=0.04; OR: 4.33, 95%CI: 1.1-18.6

**cKS vs HIV<sup>+</sup> HHV-8<sup>+</sup> subjects:** p=0.02; OR: 3.83, 95%CI: 1.2-12.4

No significant differences were observed about inhibitory KIRs, as well as the KIR genotype (AA or AB) among the six groups.

Considering overall all data of KIRs, cKS patients showed a significant higher frequency of activating KIR genes compared to subjects without the disease, regardless the infection with HHV-8 (p=0.01) (figure 17, panel A).

Similarly, epidemic KS patients had more activating KIR genes compared to their appropriate control groups (p=0.03) (figure 17, panel B).



The HLA-B and -C distribution is reported in table 6. No significant differences were observed among the groups.

<b>HLA</b>	<b>cKS</b>	<b>Epidemic KS</b>	<b>HIV<sup>+</sup> HHV8<sup>+</sup> subjects</b>	<b>HIV<sup>+</sup> HHV8<sup>-</sup> subjects</b>	<b>HIV<sup>-</sup> HHV8<sup>+</sup> subjects</b>	<b>HIV<sup>-</sup> HHV8<sup>-</sup> subjects</b>
<i>HLA-B</i>						
Bw4	18/32	15/20	16/32	25/35	13/18	19/33
Bw6	27/32	14/20	26/32	27/35	8/18	25/33
<i>HLA-C</i>						
C1 group	22/32	14/20	22/32	29/35	11/18	26/33
C2 group	26/32	16/20	24/32	22/35	15/18	24/33

*Table 6 – HLA-B and -C gene distribution in study's subjects*

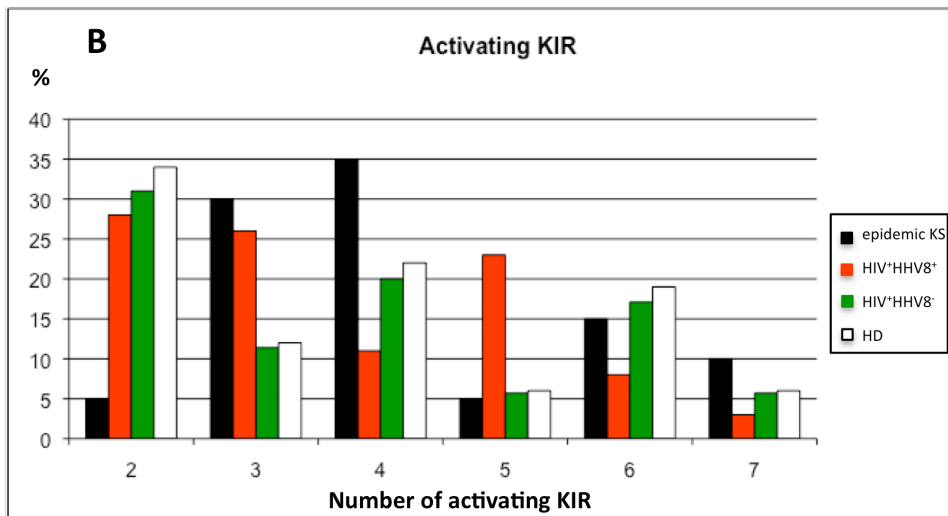
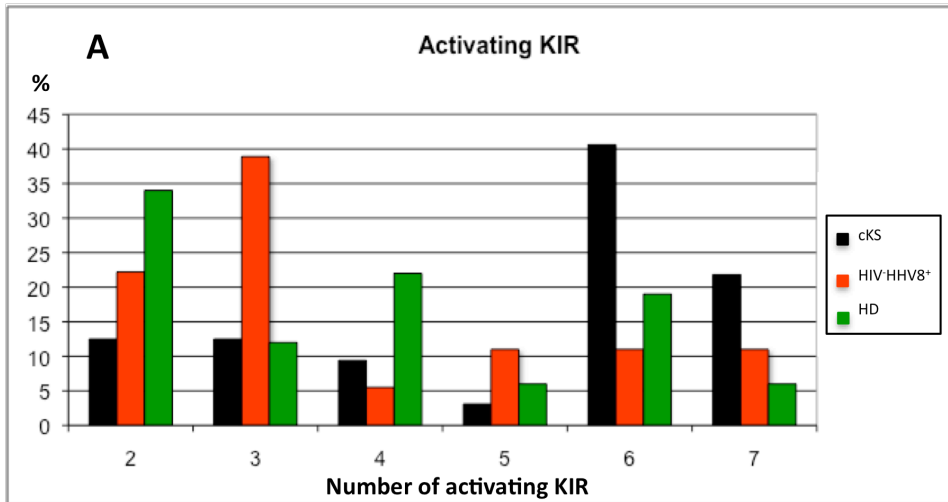


Figure 17 – panel A: activating KIR distribution in cKS patients, in HIV<sup>+</sup>HHV-8<sup>+</sup> healthy subjects and in HIV<sup>-</sup>HHV-8<sup>-</sup> healthy donors (HD).  $p=0.01$ .  
 Panel B: activating KIR distribution in epidemic KS, in HIV<sup>+</sup>HHV-8<sup>+</sup> subjects, in HIV<sup>+</sup>HHV-8<sup>-</sup> subjects and in HIV<sup>-</sup>HHV-8<sup>-</sup> healthy donors (HD).  $p=0.03$ .

### **3.7 KIR and HLA complexes**

Since KIR and HLA are strictly linked, as written in introduction, 1.3.14 paragraph, we decided to combine their data to verify if there are differences among the groups.

#### **3.7.1 KIR/HLA complexes in classic Kaposi's sarcoma**

Evaluations focusing on KIR/HLA complexes showed the presence of higher of activating KIR/HLA receptor/ligand genotyping in cKS patients compared to subjects without the disease ( $p=0.01$ ) (figure 18, panel A).

Taken KIR/HLA complex one by one, a higher frequency of KIR2DS1<sup>+</sup>/C2<sup>+</sup> genotypes was detected in cKS compared to HIV<sup>-</sup> HHV-8<sup>+</sup> individuals ( $p=0.01$ ; OR: 4.25) and to HIV<sup>-</sup> HHV-8<sup>-</sup> healthy donors ( $p=0.009$ ; OR: 5.37). The association between KIR2DS1<sup>+</sup>/C2<sup>+</sup> genotype and cKS was confirmed after binary logistic regression analysis adjusted for age and gender ( $p=0.004$ ; OR: 2.95; 95%IC: 1.42-6.15, and  $p=0.003$ ; OR: 3.02; 95%IC: 1.48-6.78 respectively).

Because HLA-Bw4 molecules are ligands of KIR3DL1 and KIR3DS1, particularly Bw4\*80I (bearing an Isoleucine at position 80) showed a stronger binding affinity with KIR3D alleles, therefore both KIR3DS1<sup>+</sup>/Bw4<sup>+</sup> and KIR3DS1<sup>+</sup>/Bw4\*80I<sup>+</sup> genotype distributions were analyzed. However, the frequency of KIR3DS1<sup>+</sup>/Bw4<sup>+</sup> as well as KIR3DS1<sup>+</sup>/Bw4\*80I<sup>+</sup> genotypes in cKS compared to healthy subjects did not reach the statistical significance. Conversely, KIR3DS1<sup>+</sup>/Bw4<sup>-</sup> frequency resulted still statistically higher in cKS than in controls ( $p=0.006$ ; OR: 6.00), confirming that the KIR3DS1 positivity, but not the KIR/HLA receptor/ligand combination 3DS1/Bw4\*80I, was associated to cKS.

Finally, no differences were found among the three groups about frequency of KIR2DS2<sup>+</sup>/C1<sup>+</sup>. All these data are summarized in figure 19, panel A.

#### **3.7.2 KIR/HLA complexes in epidemic Kaposi's sarcoma**

Similarly as cKS, also epidemic KS patients had more activating KIR genes and activating KIR/HLA receptor/ligand compared to their appropriate control groups ( $p=0.03$ ) (figure 18, panel B).

As already written (3.6 paragraph), a higher frequency of KIR2DS2<sup>+</sup> genotype was detected in epidemic KS compared to all the other appropriate control groups. However, no significant differences were found for KIR2DS2<sup>+</sup>/C1 genotypes among the groups.

The HLA ligand of KIR2DS4\*001/2 is not known yet, and so for this specific activating KIR we could not perform the KIR-HLA complex frequency analysis among the groups.

Finally, the analysis of activating/inhibitory KIR genotypes evidenced that the homozygous KIR2DS2<sup>+</sup>/2DL2<sup>-</sup> pattern was present in 3/20 epidemic KS patients, but in none of 32 HIV<sup>+</sup> HHV-8<sup>+</sup> ( $p=0.05$ ) or of 35 HIV<sup>+</sup> HHV-8<sup>-</sup> subjects ( $p=0.04$ ). All these data are summarized in figure 19, panel B.

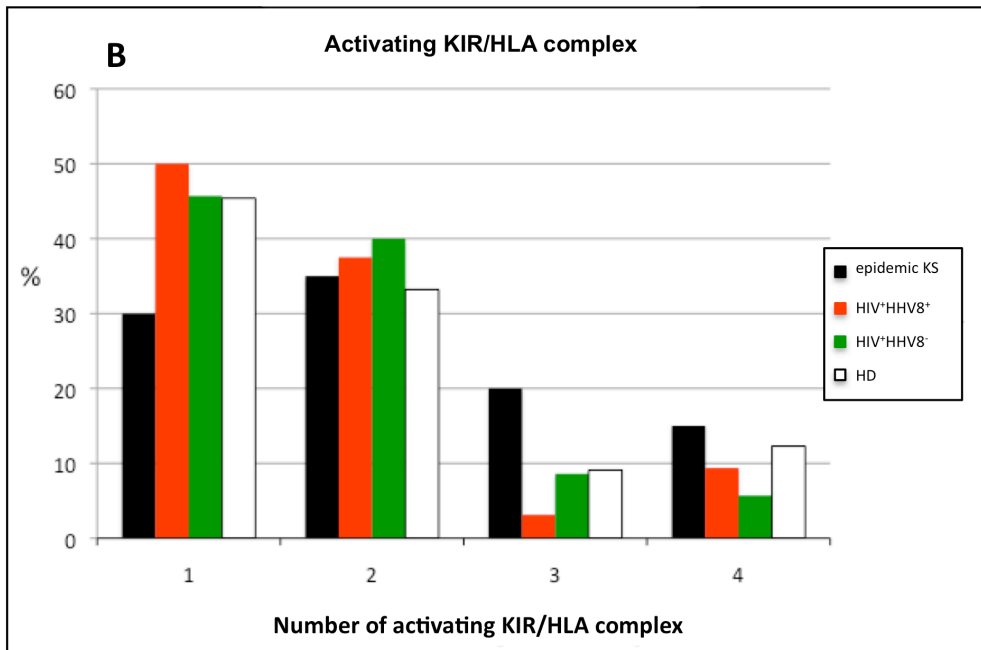
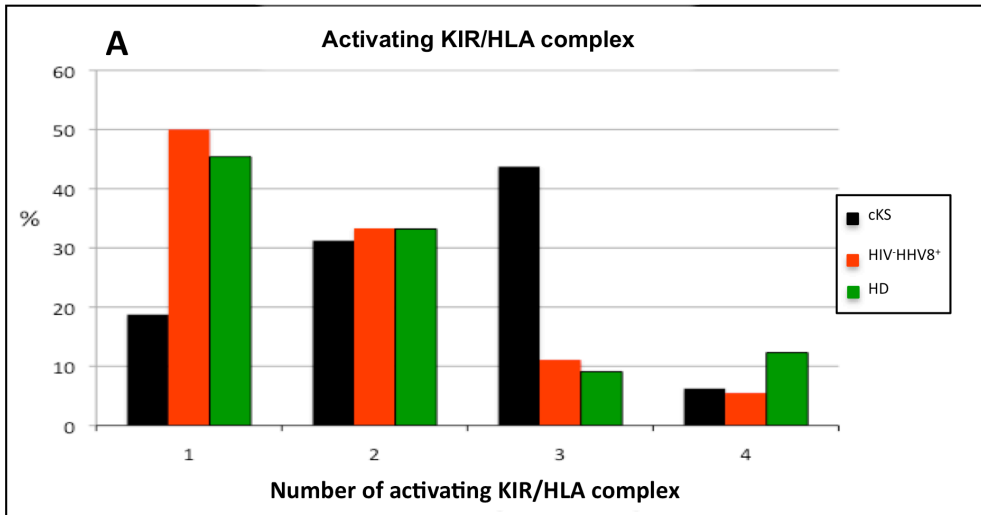
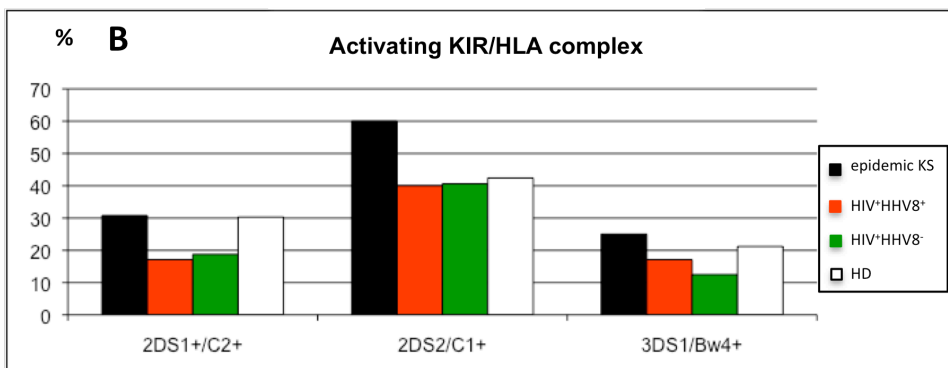
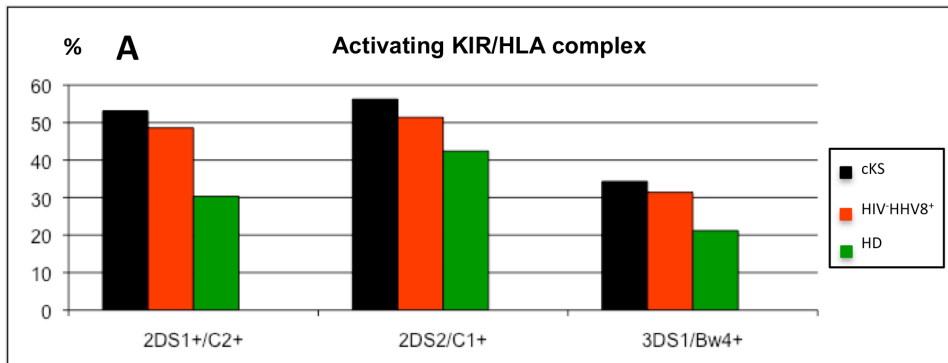


Figure 18 – panel A: activating KIR/HLA complex distribution in cKS patients, in HHV-8<sup>+</sup>HIV<sup>-</sup> healthy subjects and in HHV-8<sup>-</sup>HIV<sup>-</sup> healthy donors (HD).  $p=0.01$ . Panel B: activating KIR/HLA complex distribution in epidemic KS, in HIV<sup>+</sup>HHV-8<sup>-</sup> subjects, in HIV<sup>+</sup>HHV-8<sup>+</sup> subjects and in HIV<sup>-</sup>HHV-8<sup>-</sup> healthy donors (HD).  $p=0.03$ .



*Figure 19 – panel A: Distribution of specific activating KIR/HLA complex in cKS patients, in HHV-8<sup>+</sup> HIV healthy subjects and in HHV-8<sup>-</sup> HIV healthy donors (HD). Panel B: Distribution of specific activating KIR/HLA complex in epidemic KS, in HIV<sup>+</sup>HHV-8<sup>+</sup> subjects, in HIV<sup>+</sup>HHV-8<sup>-</sup> subjects, in HIV<sup>+</sup>HHV-8<sup>-</sup> healthy donors (HD).*

## CHAPTER IV

### DISCUSSION

Kaposi's sarcoma is a multifocal angioproliferative disorder of vascular endothelium, primarily affecting mucocutaneous tissues with the potential to involve viscera. Four clinical variants are described for the disease: classic, endemic, epidemic (or AIDS-associated), and iatrogenic KS, each with its own natural history, site of predilection, and prognosis. In absence of therapy, the clinical course of KS varies from innocuous lesions seen in the classic form to rapidly progressive and fatal lesions of epidemic KS.

Human herpesvirus 8 (HHV-8 or Kaposi's sarcoma associated herpesvirus – KSHV) is a recent member of the *Herpesviridae* family (*Gammaherpesvirinae* subfamily, Rhadinovirus genus), etiologically associated with all variants of KS.

Together with other co-factors not yet clearly defined - probably immune dysfunction, route of transmission, environment, host genetic background - HHV-8 infection is necessary for the development of KS.

Few studies have assessed the association of HHV-8 viral parameters and KS progression, and the majority of these were focalized in HIV-infected patients [162, 163]. For these reasons, in the first part of this study, immuno-virological analyses were performed in 170 KS subjects, divided in cKS patients, iatrogenic KS patients, HIV<sup>+</sup>HHV-8<sup>+</sup> or HIV<sup>+</sup>HHV-8<sup>-</sup> subjects, and HIV<sup>-</sup>HHV-8<sup>+</sup> or HIV<sup>-</sup>HHV-8<sup>-</sup> healthy individuals, to verify whether there are any association between these viral parameters and the disease.

HHV-8 antibodies were detected in all cKS patients and in 80% of epidemic KS subjects. These data are consistent with those already present in literature: although the immunofluorescence assay for detection of antibodies against HHV-8 latent nuclear antigen (LANA) is considered highly specific [164, 165], it is note that in immuno-compromised patients (as epidemic KS subjects), the LANA seroreactivity depends on CD4<sup>+</sup> lymphocytes, and that in patients with T cells lower than 100 per mm<sup>3</sup>, common in epidemic KS, the seroreactivity could not appear [166]. Also the number of HHV-8 seropositive subjects in HIV<sup>+</sup> individuals (about 50%) and in HIV<sup>-</sup> healthy donors (about 10%) is very similar to the data already present in literature regarding Italy [24, 29, 165, 167].

Sequence analysis of the HHV-8 highly variable ORF-K1 region has allowed the identification of four main HHV-8 subtypes (A, B, C, D) [168], that are differently distributed in the world: HHV-8 A and C subtypes predominate in Europe and in the United States, B subtype predominates in Africa, whereas D subtype is present almost in the Pacific islands only. It is still unclear whether different genotypes are associated with diverse rates of disease progression; similarly, the role of viral load and other possible surrogate markers on the outcome of KS is still under discussion.

In this study, HHV-8 nucleotidic sequence has been detected in blood sample of 65.6% of cKS patients and in 45.0% of epidemic KS patients, a result that confirms previous data [163, 169].

HHV-8 A and C subtypes predominate in Italy [163, 170], and also our subjects had only these kinds of HHV-8 subtypes. Interestingly, the dominant HHV-8 genotype was different in fast compared to slow progressing patients: A subtype was more frequently isolated in patients with fast progression (63.6% vs 36.4%), whereas C subtype in individuals with slow progression (76.9% vs 23.1%). HHV-8 A genotype has been associated with higher transforming potential and higher aggressiveness of KS [171-173], even if possible correlations between genotype and HHV-8-related pathologies with a different degree of aggressiveness have not confirmed by all authors [170, 174, 175]. Our results support the claim that infection with HHV-8 A subtype is indeed associated with worst clinical parameters, including faster disease.

Additional results showed that HHV-8 viral load was not detectible in non-KS HHV-8<sup>+</sup> individuals, and that it was significantly increased in cKS patients with more severe disease. This is the first time that an association between the evolution of the cKS and HHV-8 viral load has been shown: at now this link has been demonstrated for epidemic KS only [176]. However we can not exclude that this association is caused by HHV-8 subtype: a recent study showed that the A genotype is correlated with a higher viral load [177], and, in this study, we found that the majority of cKS faster evolution patients had the A HHV-8 subtype. Actually, when we analyzed the viral load in A HHV-8 subtype patients compared to C HHV-8 subtype patients, the A subtype subjects seem to have a trend for an higher viral load, even if this difference did not reach the statistical difference, probably due to the low number of subjects. A new study with more patients is needed to better understand this relation. However, if confirmed, it is a very important aspect, because in this way we would be able to prefigure a possible development of the cKS just based on HHV-8 genotyping, and it may allow to physicians to prescribe the best drug therapy already in the early stages of the disease.

About HHV-8 genotyping, we also observed that, in the A subtype, patients with the classic form of Kaposi's sarcoma were mainly grouped in A3 subtype, whereas patients with the epidemic form were mainly grouped in A1. In literature there is just a work that studied the HHV-8 genotyping in Italian patients with epidemic Kaposi's sarcoma, and it did not find any differences regarding the ORF-K1 region [178]. However, it needs to underline that in both works the ORF-K1 genotyping was performed only for a very small number of patients with epidemic Kaposi's sarcoma: in this work for just 9 patients, and in that paper for two individuals only (for one the HHV-8 genotyping was A4, for the other one C3) [178]. So, it is possible that this difference in HHV-8 genotyping between classic and epidemic Kaposi's sarcoma is just an artifact, due to the small number of subjects. A new work, focalized on ORF-K1 genotyping in epidemic form of Kaposi's sarcoma only, is necessary to verify if this relation between disease and A1 subtype of HHV-8 is true or not.

Although it is certain that HHV-8 infection plays a prominent role on the progression of KS from an angio-proliferative disorder to sarcoma, HHV-8 infection

is nevertheless not sufficient to cause such progression, as not all HHV-8-infected individuals develop the disease. The ability to kill virus-infected cells early in the immune response (the innate immune response) suggests that there may be a role for natural killer cells in determination of the course of HHV-8 infections [179]. The activity of natural killer cells is partially regulated by the balance between inhibitory and activating signals transmitted by a particular family of receptors. These receptors are located at the surface of the natural killer cell and are called killer cell immunoglobulin-like receptors (KIR). Carrying more activating KIR genes has been associated with a stronger immune response and it has been demonstrated that it is related with an increased risk for tumor development as well as for autoimmune diseases, such as psoriasis or rheumatoid arthritis [180-183]. KIR gene receptors have the capacity to recognize the human leukocyte antigen (HLA) class I molecules on target cells [184, 185]. When the expression of certain HLA molecules is downregulated by viral infections or cellular transformation, the KIR and HLA engagement will be altered. Cells that lack expression of self HLA molecules may be destroyed by cytolytic activity of natural killer cells. Moreover, when HLA molecules bind activating KIR receptors, a potent inflammatory response finalized at NK cell-mediated destruction of target cells, including transformed tumor cells and virus-infected cells, is stimulated [186]. KIR and HLA loci are highly polymorphic and map in distinct human chromosome (chromosomes 19 and 6, respectively); both KIR and the specific HLA ligands must be present in order to regulate NK cell activity, such that one without the other is functionally inert.

For all these reasons, we investigated whether specific KIR genes and their class I ligand were associated with classic Kaposi's sarcoma or epidemic Kaposi's sarcoma. Results herein, albeit stemming from analyses performed in a limited number of individuals, indicate that a statistically higher frequency of activating KIR genes together with their HLA ligands is present in HHV-8-infected patients with cKS or epidemic KS compared to non-KS patients, regardless of the presence or absence of HHV-8 or HIV infection.

Importantly, whereas activating KIR genes were more frequent in KS patients (both cKS and epidemic KS), compared to non-KS subjects, the distribution of KIR genes was similar between HHV8<sup>+</sup> or HHV8<sup>-</sup> subjects. Considering the activating KIR one by one, the KIR2DS2 gene was prevalent in both cKS and epidemic KS, whereas KIR2DS1 and KIR3DS1 were more prevalent in cKS; the similarity of KIR2DS1 and KIR3DS1 distribution may be secondary to the fact that these two genes are in linkage disequilibrium. No difference was found regarding inhibitory KIR in all the analyzed groups. Moreover, considering the KIR/HLA complex, we found that the activating KIR2DS1/C2 genotype was positively associated with cKS development, whereas the HLA ligand distribution did not reveal any statistical association with cKS. This observation, together with the fact that the OR of KIR2DS1 association increased when the KIR2DS1/C2 genotype was evaluated, may suggest that the association with the disease of KIR2DS1/C2 with cKS is mostly due to KIR-HLA interaction and not either KIR or to HLA genes separately. Data herein indicate that a KIR/HLA "activating milieu" is present in cKS and epidemic KS; such milieu may be a risk factor for the development of this tumor. Our results add KS to the list of virus-associated cancers including cervical neoplasias and nasopharyngeal carcinoma [158, 187, 188], where an increased



presence of activating KIR/HLA receptor/ligand combinations is observed and is suggested to play a role in oncogenesis.

Recent studies have shown that genotypes that increase the activation of NK cells are possibly beneficial in viral infection [189]. For example, a study performed on HIV positive subjects found that the combination of KIR3DS1 with its ligand HLA-B Bw4 80-I was associated with slower progression to AIDS, lower mean viral load, and protection against opportunistic infections [157,190]. Moreover, another work demonstrated that NK cells expressing KIR3DS1 strongly and significantly inhibited HIV-1 replication in cells expressing HLA-B Bw4 80-I as compared to NK cells that do not express KIR3DS1, providing the first functional support for the influence of KIR3DL1/S1 and Bw4 80-I on anti-HIV NK activity [191]. KIR3DS1 positive NK cells clones were preferentially activated by HIV-1 infected target cells, expressing HLA-B Bw4 80-I, resulting in lysis of these cells, indicating that NK cells identify HIV infected cells actively through KIR3DS1 and Bw4 80-I interaction.

But in cancer, especially if inflammation is a major component of tumor pathogenesis, and in which is strongly associated with progression of the disease, the condition seems opposite. For example, about virus-driven cancer, a recent work showed that strongly inhibitory KIR-HLA genotype were protective against cervical neoplasias, so that NK cells activation may contribute to a chronic inflammatory state in response to human papilloma virus (HPV), the causative agent of cervical cancer, setting the stage for carcinogenesis [154]. Moreover, an increased number of activating KIR was also found to be associated with nasopharyngeal carcinoma, a cancer that is strongly associated with EBV infection [158].

Our data are in line with this second hypothesis, in which the elicitation of inflammation and NK cells activity via KIR/HLA interactions might be directly involved in malignant transformation and tumor progression as a consequence of the production of cytokines such as TNF- $\alpha$ , IL-6 and TGF- $\beta$  that contribute to the proliferation and survival of tumor cells [192]. Non-specific inflammatory responses, such as oxidative DNA damage, may be triggered by activated NK upon KIR/HLA interaction as well, possibly facilitating the growth of oncogenic viruses [154].

## CHAPTER V

### CONCLUSIONS

Kaposi's sarcoma (KS) is a multifocal vascular neoplasm involving the skin, visceral organs and lymph nodes. The lesions are complex and contain distinctive proliferating cells with fusiform morphology, called spindle cells, activated endothelial cells and infiltrating inflammatory cells. Four clinical variants are described for the disease: classic (cKS), endemic, epidemic or AIDS-associated, and iatrogenic KS, each with its own natural history, site of predilection, and prognosis.

The link between human herpesvirus 8 (HHV-8 or KSHV) and Kaposi's sarcoma has been proven, but many important aspects including risk factors, genetic predisposition to tumor development, transmission of HHV-8, and the pathogenic potential of different genotyping remain to be elucidated.

In the first part of this thesis we decided to verify if there is association between HHV-8 viral parameters and KS type and progression, in classic and epidemic Kaposi's sarcoma.

Confirming the literature, in this study we found that the risk of Kaposi's sarcoma is related with HHV-8 seropositivity: all cKS patients and the 85% of epidemic KS patients had HHV-8 antibody in their serum, whereas about half of HIV<sup>+</sup> subjects (risk category) and 9% of healthy donors had them.

According to HHV-8 genotyping, this study showed that in cKS, A genotype was more frequently isolated in patients with fast progression (63.6%), whereas C subtype was more frequent isolated in individuals with slow progression (76.9%). The A subtype seems to have a trend for a higher viral load, although this difference did not reach a statistical difference. Moreover, among the A subtype, patients with the classic form of Kaposi's sarcoma are mainly grouped in A3 subtype, whereas patients with the epidemic form are mainly grouped in A1.

The number of KS patients in which the HHV-8 genotyping was available is not very high, so new studies focalized in particular on the genotyping of HHV-8 in a large population of cKS and epidemic KS, in every stage's disease, are needed to confirm these data, and the possible role of A subtype in a more aggressiveness of the disease.

Even if the role of HHV-8 in the pathology is demonstrated and the virus is necessary to develop the disease, HHV-8 infection is nevertheless not sufficient to cause the progression of KS from an angio-proliferative disorder to sarcoma, as not all HHV-8-infected individuals develop KS. Natural killer cells are central components of the innate immune response, and their major role is the protection against viral infection and tumor growth via direct and indirect mechanisms. The modulation of natural killer activity is a complex and multi factorial phenomenon triggered by the binding of inhibitory or activating killer cell immunoglobulin-like receptor (KIR) to class I human leukocyte antigen (HLA). For this reason, in the second part of this thesis we decided to verify if there is a different distribution of

KIR and HLA-B and HLA-C genes in patients with classic and epidemic Kaposi's sarcoma compared to the appropriate control groups.

We found that patients with cKS and epidemic KS had a statistically higher frequency of activating KIR genes compared to non-KS patients. The presence of HIV infections, as well as HHV-8 infection, did not correlate with any KIR distribution and frequency in subjects without KS. Considering the activating KIR one by one, we found that KIR2DS2 gene was more frequent in cKS, as well as in epidemic Kaposi's sarcoma compared to non-KS individuals, whereas KIR2DS1 and KIR3DS1 were more prevalent in cKS patients compared to non-KS healthy donors. Regarding the HLA distribution alone, no differences were found among the analyzed groups. About the KIR/HLA complexes, we found that the activating complexes were more frequent in cKS and epidemic KS patients compared to non-KS individuals; moreover, considering KIR/HLA complexes one by one, the study showed that the activating KIR2DS1/C2 genotype was positively associated with cKS development.

Results herein indicate that a statistically higher frequency of activating KIR genes, as well as KIR/HLA complexes is present in cKS and epidemic KS patients compared to non-KS individuals, regardless the presence or absence of HHV-8 or HIV infection.

In the future, it will be important to expand the analysis on HLA-A, another HLA ligand for KIR genes, to complete the study on KIR/HLA complex, and to include patients with the epidemic and iatrogen form of KS, to verify if also in these two different kinds of KS an "activating milieu" compared to the normal population is present.

In conclusion, although these data stem from analyses performed in a relatively small group of individuals and will need to be validated using bigger cohorts of KS patients, this study shows that KS development is related with a higher frequency of activating KIR, as well as of activating KIR/HLA complexes, and that the A genotype of HHV-8 is associated with worst clinical parameters of KS, including faster disease.

## CHAPTER VI

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## APPENDIX

### ARTICLES ON THIS ISSUE PUBLISHED IN PEER-REVIEWED JOURNALS

- Mancuso R, Brambilla L, **Agostini S**, Biffi R, Hernis A, Guerini FR, Agliardi C, Turlaki A, Bellinvia M, Clerici M. *Intrafamilial transmission of Kaposi's sarcoma-associated herpesvirus and seronegative infection in family members of classic Kaposi's sarcoma patients*. J Gen Virol 2011; 92: 744-751.
- Guerini FR, Mancuso R, **Agostini S**, Agliardi C, Zanzottera M, Hernis A, Turlaki A, Calvo MG, Bellinvia M, Brambilla L, Clerici M. *Activating KIR/HLA complexes in classic Kaposi's Sarcoma*. Infect Agent Cancer 2012; 7: 9.

### ABSTRACTS ON THIS ISSUE PRESENTED IN INTERNATIONAL CONFERENCES

- **Agostini S**, Guerini FR, Bandera A, Mancuso R, Hernis A, Agliardi C, Sabbatini F, Beretta I, Fusco C, Gori A, Clerici M. *A role for activatory KIR/HLA complexes in HIV-associated Kaposi's sarcoma development*. [Abstract] Retrovirology 2011; 8 (supplement) A209.
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