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Experimental and Molecular Oncology

Ph.D. thesis: *"Seroprevalence of HHV8 in Mozambique in relation with HIV/AIDS. Clinical evidence of Kaposi Sarcoma in a HIV+ cohort. Correlation with immune and viral state and Sarcoma regression in patients under HAART and treated or not treated with cytostatics*."

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INTRODUCTION

Kaposi 's Sarcoma (KS) was first described in 1872 by the Hungarian dermatologist Moritz Kaposi.

There are four different forms of KS:

Classic KS causes multiple skin lesions on the lower limbs. It is mainly seen in elderly men in Mediterranean or Eastern European regions.

Endemic (African) KS is found in children and young men in equatorial Africa. It is more virulent than the classic form.

Acquired (Posttransplant) KS occurs in people treated with immunosuppressive drugs, especially those who have received organs transplants. It goes away when the drugs are stopped.

Epidemic (AIDS related) KS is the form associated with HIV infection. It tends to follow a more variable but potentially more aggressive course than other forms of KS.

KS is an angioproliferative illness with multifactor origin and several types differing in their clinical and epidemiological patterns. It generally arises like an inflammatory hyperplasia, and the angiogenesis process plays a primary role in the development of the disease. It evolves into disseminated nodular lesions which are very much alike a true sarcoma.

KS most commonly presents as skin lesions which often appear when the immune system is still relatively intact. As long as it is confined to the skin, KS is not fatal and it is unlikely to be serious.

While it is most commonly found on the skin, KS can occur anywhere in the body.

A major advance in our comprehension of KS epidemiology came from studying the distribution of 13.616 cases among 90.990 persons with AIDS reported to the Centres for Disease Control, Atlanta, until March 31, 1989. Detection of KS in 21% of males who acquired HIV1 through homosexual or bisexual contacts in comparison with <7% in all other HIV transmission group such as heterosexuals of Caribbean, African and other origin, intravenous drug users, transfusion recipients and persons with haemophilia suggested that AIDS KS might be a sexually transmitted infection (11)

Symptoms.

When KS lesions first appear on the skin, they are often flat patches with a pink or blood-bruise colour in white people. In black people as hyper pigmented black patches. They develop into nodules: hard, raised, round or oval lumps. Their colours is violet, bluish or reddish in light-skinned people. When the nodules are present, they often appear in

roughly symmetrical patters on each side of the body and may follow the skin fold lines of the body. In some cases, the nodules can ulcerate, bleed and become infected.
Lesions can appear also in lymph nodes, lungs or intestine. In intestine, KS is usually harmless but can sometimes cause a blockage, resulting in nausea, vomiting, abdominal pain and occasionally bleeding.
In the lymph nodes, blocked fluid drainage may cause swelling, especially in the feet, lower legs or genitals. Occasionally swelling can occurs around the eyes.
Pulmonary KS is the most serious form, and can be fatal. It can lead to recurrent chest infections or accumulation of fluid on the lung (pleural effusion). There may be blood in split, cough and breathlessness.
KS lesions sometimes occur in addition to a condition to a condition called Multicentric Castleman's Disease (MCD). This is a disorder of resulting from over-activity of lymph tissue. MCD is characterised by swollen lymph nodes, fever, fatigue, weight loss, night sweats, blood disorders and sometimes liver and spleen irregularities.

Aetiology: the Human herpes virus 8

The manifestation of the disease is strongly related with the infection by HHV8 Human Herpesvirus (16), and the seroconversion to HHV8 preceded the onset of KS in every case. (97). This is an herpes virus, first named Kaposi's sarcoma associated herpesvirus (KSHV).
In 1994 the group of Yuan Chang (30), using the method for searching DNA sequences differently expressed in KS and normal tissues, put into evidence, in the Kaposi sarcoma lesions, a herpesvirus-like DNA sequence, named KSHV or HHV-8. In March 1996 researchers in San Francisco successfully grew the virus in culture.
The new sequences showed relevant levels of homologies with oncogenic gamma-herpesvirus sequences (Herpesvirus saimiri and Epstein-Barr virus). Using RT-PCR, the HHV-8 sequences were found in all epidemiological forms of Kaposi:
HHV-8 was also found in a rare B lymphoma (BLCB) and in the Castleman syndrome, a rare disease with poor prognosis.
KS is the result of various stimuli promoting the micro vascular inflammation.
Sequence analysis placed HHV8 (named also Kaposi Sarcoma Herpes Virus, KSHV) in the gamma2 (Rhadinovirus) lineage of the gammaherpesvirinae along with Herpesvirus Saimiri (HVS). The closest human relative is the gamma1 (Lymphoxryptovirus) herpesvirus Epstein Barr Virus (EBV). This finding is intriguing for the relationship that

gammaherpesviruses have with lymphoproliferative disorders and cancers: HVS can induce malignant lymphomas in owl monkeys and EBV infection is linked to a fatal B cell proliferation in young males with lymphoproliferative disease, posttranplant lymphomas, immunoblastic lymphomas in AIDS patients, Burkitt's lymphoma, Nasopharyngeal Carcinoma and Hodgkin's Disease.

The genome of HHV8 has a 140,5 kb-long unique region (LUR) which is flanked by multiple 801 bp terminal repeat sequences. Within the LUR, 81 potential Open Reading Frames (ORFs) with more than 100 amino acids have been identified, and several additional spliced genes have since been added to this list. The numbering of HHV8 ORFs is based on positional homologies with HVS due to substantial co-linearity between these genomes whereas ORFs without positional homologues are numbered consecutively with a K prefix. The presence in the viral genome of open reading frames with significant homology to mammalian genes involved in cellular growth control, suggests that "molecular mimicry of cell cycle regulatory and signalling proteins is a prominent feature of this virus" (123)

Comparison of protein sequences predicted from the genomes of Varicella Zooster virus (VZV) and EBV suggests a common mammalian herpes viral ancestor. (35)

Analysis of genome suggests that 42 genes are common to a set of genes that the mammalian herpes viral ancestor may have possessed.

The most variable region to date in the HHV8 genome appears to be OFRK1. Phylogenetic analyses using ORFK1 sequences obtained from different geographic regions, including Classic and AIDS KS, enabled the definition of 4 major subtypes, A-D. The subtype B is predominated in Africa, while subtypes A and C were found more frequently in Europe. No correlation was noted between subtype and more aggressive HHV8 related disease, or geographic regions.(33)

<u>Transmission</u>

HHV8 is spread sexually, through mother to child contact, and via organ transplant. A group of studies suggests that the relatively high levels of HHV8 in saliva may also contribute to HHV8 transmission. (115, 24)

Sexual transmission

Formal evidence has been acquired using seroepidemiological results from several cross-sectional and prospective cohort studies on homosexual men. Sexual transmission and

particularly homosexual transmission were reported from the Sydney HIV cohort (62) the San Francisco Young Men Study (14) and from an important cohort in London (130).

Childhood transimssion

Transmission before puberty appears to be rare in the United States (15) but does occur in countries where HHV8 is more widespread. Evidence for intrafamilial clustering has been seen in Italy (5) and it is measured also in Uganda ((93) Cameroon (56) Egypt (4) South Africa. Prevalence of HHV8 is high in this last setting and transmission appears to be occurring in childhood as well as among adults, increased in later adulthood (< 18 months 37.5%, 19-120 months 38.5%, 15-34 years 32.1%, 35-69 years 62.8%). (143). Most of the paediatric cases were aged 6-10 years. A significant increase in the occurrence of total childhood cancers was found. This is mostly due to a highly significant increase in the incidence of paediatric Kaposi's sarcoma ($p = 0.000016$), which is causally related to HIV infection, and a significant increase in the incidence of retinoblastoma ($p = 0.02$), which has an unknown relation to HIV infection (113).

Infection in children less than 10, are probably the results of mother to child transmission, although whether this occured pre- peri- and post-partum is not yet known (19). A sequent study showed that the probability of mother to child transmission increased with increasing maternal antibody titer (123) In children less than 2 years, HHV8 infection is however rare, even in endemic countries, arguing against transmission through breast milk (56, 60, 86, 93.)

Parenteral transmission

The presence of HHV8 antibodies prior to transplantation in transplanted patients suggest that Posttransplant KS is mainly due to reactivation of HHV8 (112 50*)* KS remission seems to coincide with reduction or cessation of immunosuppression (102) 1998) Evidence is mixed on transmissibility of HHV8 by blood transfusion.(14)*,* (91) (94) reported an association with blood transfusion in Ugandian children.

<u>Sarcoma development: Possible co factors.</u>

It is evident that the incidence of HHV-8 infection is far higher than the prevalence of KS, suggesting that viral infection per se is not sufficient for the development of aggressive phenotype and that one or more additional cofactors are required. Some studies found an association between high anti HHV8 antibody titre and an old age, but other associations that may be factors in the development of high anti HHV8 titers include exposure to poverty or a low socioeconomic status environment and consumption of traditional maize

beer. (144) One hypothesis suggests that KS develops because the immune system is over-activated, with elevated cytochine levels stimulating the growth of early KS cells, which can eventually become cancerous. Once these cells have arisen in one part of the body they may spread in the bloodstream until they lodge in other tissues to cause additional lesions. Several cofactors have been supposed for the development of the Kaposi sarcoma, for example oncostatine M (98). Oncostatin M is a growth factor that is secreted by activated T cells that stimulates angiogenesis. An other proposed factor was the human papilloma virus, (HPV) (69). HPV 16 related DNA sequences in Kaposi's Sarcoma. Lancet 339:515-518, 1992) associated with cervical and anal cancer. Environmental cofactors are proposed (142) and iron exposure (126). Ttransmission of HHV8 infection it seems facilitated by contact with blood sucking arthropods (31).

HHV8 and HIV

Also the presence of HIV itself play a role in KS. Infections such as HIV that activate T cells, may lead to increase in levels of Oncostatin M. Studies have shown that the HIV tat gene seems cause the development of KS-like lesions in mice, and the Tat protein produced by this gene stimulates the growth of human cells in test tubes and the progression of KS appears to be due to the deregulated expression of oncogenes and oncosuppressor genes, to the long-lasting expression of the HHV8 latency genes promoted by the proliferative and angiogenic effects of the HIV-1 Tat protein (23, 49).
In a study (29) plasmas of 18 Tanzanians with sarcoma were analysed. Of them, 14 were 14 HIV+. Among these patients, the HHV8 viral load shows a median of 2075 copies/ml; in the negative group it is 450 copies/ml. Despite the exiguity of the sample, this datum seems to suggest a cross-signalling pathways between the tat protein and HHV8 DNA in the pathogenesis of KS.

HHV8 bears a gene (K1) encoding a transmembrane protein with an immunoreceptor tyrosine-based activation motif. This motif is present in receptors that mediate inflammation, K1 may activate cells in which it is expressed, as well as other cells in a paracrine manner. K1 cooperates in signaling with HIV-1 Tat, suggesting that both of the proteins from these viruses converge to reach an enhanced level of inflammation that may underlie progressive KS.(124) The level of nucleotide sequence of K1 gene was studied in a Zimbabwean group of 500 subjects. Data provide evidence of the relationship between HHV8 lytic replication and untreated HIV1 infection (21).

Morini (103) had proved that HIV tat can activate sarcoma cells derived from sporadic or iatrogenic lesions, suggesting that in patients with AIDS tat can cooperate with VEGF, thus contributing to the aggressiveness of the KS/AIDS lesions, and levels of VEGF-A are higher in HIV+ patients, with or without KS, compared with individuals without HIV infection (7). However, the serum concentration of VEGF does not seem to be useful for monitoring the disease progression, since it is not directly linked with the HIV1 or HHV8 infection, or with conditions associated with Kaposi sarcoma in AIDS. (120).
In the case of coinfection HHV8/HIV1 it is interesting to notice the correlation between the viral loads of HHV8 and HIV1. In a study (134) on 54 consecutive plasma samples from HIV1+ patients with Kaposi, an association between the two viremias was found, even if both positive and negative trends have been observed. An association with the value of CD4+ lymphocytes has also been found, while high HHV8 viral loads correlate also with HHV8 antibody titres measured with immunofluorescence. Other data, instead, (12) suggest that the HHV8 viral load in patients with KS is relatively low, and does not differ in HIV1- or HIV+ patients.

Also an higher HHV8 antibody titre is associated with the risk to develop KS (121) It is apparent that, as the HIV epidemic advances in regions of the world with endemic KS, the clinical presentation and natural history of the endemic KS are blending with those of the epidemic or AIDS-associated disease, leading to a reduction in the mean age of the cases and a nearly identical incidence in men and women. In regions of the world where patients have ready access to such chemotherapy, the impact of treatment with highly active antiretroviral drugs on the incidence and natural history of KS has been dramatic (105)
Some studies showed a high HHV8 seroprevalence in HIV1+ groups, and evidenced the association between HHV8 antibody levels and immunologic state, or antibody titre and risk of developing Ks (76).
In effect human immunodeficiency virus and KS herpesvirus/human herpesvirus-8 (KSHV/HHV8) co infection, leads to the development of an angiogenic-inflammatory state that is critical in the pathogenesis of KS. KS is driven by KSHV/HHV8-specific pathways, which include viral G protein-coupled receptor (vGPCR), viral interleukin-6 (vIL-6), and viral chemokine homologues. In addition, cellular growth/angiogenic pathways, such as vascular endothelial growth factor (VEGF), insulin-like growth factor, platelet-derived growth factor (PDGF), angiopoietin and matrix metalloproteinases (MMPs) are "pirated" by KSHV/HHV8 (39, 40)

Therefore in Western countries, the KS diffusion is epidemiological related with HIV-1 infection, where historically KS is diagnostic of AIDS. During the initial phase of the AIDS epidemic, KS was only observed in men who had homosexual relations. Some studies show that one third of patients with HIV1 HHV8 co infection can develop sarcoma. KS used to be the most commonly diagnosed HIV related malignancy. With the development of better treatments of KS and the use of antiretroviral therapy, the incidence of KS declined significantly at the end of the 1990s in Western Countries and it is rarely a cause of death. Nerveless, KS is associated with an elevated risk of death and there is some evidence that it can accelerate HIV disease.
The reason why some people infected with both HIV and HHV8 develop KS while others do not, appears to be linked to HHV8 rather than CD4 cell count. A study found that the presence of HHV8 genetic material in both blood cells and saliva was associated with KS, while people with HHV8 only in saliva were much less likely to have KS lesions (22)
Many studies focused the relationship between the two viruses in Western Countries (1, 34, 44, 71, 116) like in the EuroSIDA study, or in the study of Rezza et al. (121) in Italy to evaluate temporal trends of Kaposi's sarcoma (KS) and of the KS-related human herpesvirus (HHV-8) among homosexual men who seroconverted for HIV between 1984 and 1997 (122).

The introduction of highly active antiretroviral therapy (HAART) has radically changed the clinical course of human immunodeficiency virus (HIV) infection. The EuroSIDA Study study assessed the change in the incidence of Kaposi sarcoma (KS) among European patients with HIV since the introduction of HAART and to identify the factors associated with the development of KS among patients receiving HAART. The current incidence of KS among patients with HIV is less than 10% of the incidence reported in 1994 and most individuals who developed KS while receiving HAART began treatment with low CD4 cell counts and developed KS within 6 months of the initiation of HAART (101)
Atkinson (8) identified AIDS related KS cases in linked United States, AIDS and cancer registries for 164.000 people. KS incidence was highest in gay men, lower for heterosexual men and lowest for women (5,7 vs 0,4 per 100 person years). Relative risk adjusted for age, race, location and year of AIDS onset for injecting drug use were o,9 (95% CI 0,8, 0,9) for gay men, 1,1 (95% CI 0,7, 1,6) for hetesexual men and 1,3 (95% CI 0,9, 1,8) for women.

Analysing the epidemiology of KS in Scotland, Brewster (20) noted that the AIDS associated KS was associated with an increased morbidity and shorter survival, compared with the traditional, non AIDS KS seen in older men.
Gao reported that in the Multicenter AIDS Cohort Study the median time from seroconversion to HHV8 and development of KS was 33 months (range 6-75 months) (53)
Studies of antibody kinetics for IgG against HHV8 put into evidence that more than half the patients with KS/AIDS had seroconversion before developing sarcoma, but it does not seem that the risk of developing the tumour is strictly associated with the duration of the infection.
The immune effects of HHV8 in healthy adults consist of the chronic inhibition of the type 1-cytotoxic response of T cells, not depending from the HIV infection (37).

HHV8 and KS in Sub Saharan Africa

Data on HV negative patients HHV8 prevalence and KS incidence in Africa are scarce. A little more works are founded about HIV epidemic and KS disease.
In Sub-Saharan Africa the HIV epidemic has a high prevalence, but in these areas the KS can be found also in HIV negative patients, and according to some studies, the HHV8 virus diffusion is endemic. A study brought on in Malawi (37) in a cohort made of 272 patients and 24 healthy individuals, Seroprevalence is between 67% and 54% respectively; it grows with age, but it is not linked with HIV positivity, nor with the percentage of CD4 lymphocytes, nor the diagnosis of Kaposi (10 cases in total). Women show lower titres than men. In Zambia a study determined virus prevalence estimates and the risk factors associated with HHV-8 infection. Cross-sectional, enrolment visit data were analyzed from a prospective cohort study of perinatal transmission of HHV-8. Among 3,160 antenatal women serologically screened for HHV-8 40.2% were seropositive. (79)
Nevertheless, the Kaposi sarcoma associated with AIDS (AKS) is particularly aggressive, and is one of the main neoplasias in the Africans areas affected by both viruses.
Kaposi is endemic in South Africa well before AIDS epidemic. It is now growing, (127) and it seems that the risk of developing sarcoma is associated with antibody levels, but at a given titre the risk is higher for HIV+ subjects compared with HIV- subjects. In hospitalised coloured patients seroprevalence is about 30% adjusted by age and sex. It grows with age and it is similar in men and women. Seroprevalence lowers with levels of education, and it is lower in the whites than in the blacks. (129) In an other study (144) in a sample of 2191 HlV-1 negative patients black in-patients in Johannesburg and Soweto, South Africa,

39.0% were positive for antibodies against HHV-8. Patients with high anti-HHV-8 antibody titers are characterized by older age. In Uganda, where the KS in a common cancer, the seroprevalence is 47% in a blood donor group. (66).
In Zambia (63) prevalence results to be 48.4% in pregnant women, with a prevalence of 51.1% among HIV-positive women and 47.3% among HIV-negative women.
A study by Phiri (117*)* in Zambia shows a prevalence of 25% in 36-months-old children, while seropositive children or children born to seropositive mothers are more easily infected by HHV8.
In other African coutries, the situation seems slightly different, as shown by a study conducted on 407 pregnant women in Senegal, where the prevalence of sarcoma is low also in people with HIV. 14.3 % of the studied population resulted positive for HHV8 antibodies and did not show coinfection with HIV. Prevalence was higher among miscarrying women (17.2% vs. 4.9%). Moreover, an association has been revealed between mother infection and low weight of children at birth (54).
Just to mention some data to have an idea, in Japanese healthy adults seroprevalence is 0.2%. (51) In Northern Europe it is 3-7%, while it grows in Southern Europe. In Italy and Greece it is 35% (119).
The detection of HHV8 in PBMCs of AIDS –KS patients and HIV infected controls, has demonstrated a strong association between HHV8 infection and KS, even in a region of high HHV8 seroprevalence as The Gambia (6).

KS in the HAART age

In Countries with limited resources, in the absence of highly active antiretroviral therapy (HAART), KS was treated for palliative reasons with chemotherapy or not treated at all. Less than 1% of AIDS patients are receiving HAART in sub Saharian Africa yet, *(*95) but with the increase of the use of HAART also in developing countries the way to treat KS could dramatically change. In United States for example before the era of HAART 15% of HIV patients presented KS as the primary AIDS defining event.(65). Over the past two decades, in several African Countries with high HIV prevalence, the KS incidence have seen a 10- 20 fold increase. In Western Countries HAART has caused a sharp decline in the incidence of the disease, due to the immune restoration following the antiretroviral treatment and there are indications that this coincides with immune control of HHV8.
In effect, AIDS correlated KS could be considerate as an Opportunistic Infection (OI). In absence of HAART there is no cure for AIDS KS. No local or systemic therapy has proven

to increase survival. Chemotherapy is often used when patients have important lesions in visible area of the body, extensive painful skin lesions and oedema, oral lesions that cause obstruction or evidence of a rapid tumour progression. The natural history of the KS is changed with HAART. Several studies have shown a response rate up to 90% after two years of HAART treatment. *(* (46). Bourboulia at al. report that there is an apparent restoration after a relatively long (> 24 months) period of treatment, bur they think these immune responses could contribute to the decreased incidence of KS during HAART, but it is unlikely to be a complete explanation for the often rapid resolution of KS when HAART is started (18) Once patients are responding to therapy (partial or complete response) the chemotherapy can be interrupted while immune restoration does the work, even in pulmonary KS (87)

Some studies compared different regimens based on non nucleoside reverse transcriptase inhibitors or protease inhibitors (118).
The effectiveness of the antiretroviral therapy seems to be demonstrated, particularly using protease inhibitors, to achieve the regression of AIDS-related sarcoma. The clinical response correlates with the decrement of plasmatic levels of RNA and the increment of CD4+ T-lymphocytes. (26).
Some authors analyzed data from a group of HIV positive patients presenting with KS and under HAART, demonstrating that there were no differences between patients in remission and progression in age, CD4 cell count, and HIV VL at KS diagnosis. In a study of Thirlwell, was reported that, among 70 HIV infected patients with KS, 9 had visceral organ involvement, CD4 cell count average at 212, and 11 with undetectable viral load. 20 of them were o HAART. Of the 50 not on HAART at diagnosis, 35 were treated only with HAART and at one year, only 5 of these patients required chemotherapy. Tirelli reported that the HAART may be a useful alternative to immune response modifiers (es interferon) during the less aggressive stages of KS. In a study of was reported that HAART was associated with an improved survival (p=0,0001) and 81% reduced risk of death. Also in the Swiss HIV Cohort Study AIDS defining illnesses including KS declined by about 80%. A retrospective study in New York demonstrated the significantly longer survival in HIV infected patients with KS treated with HAART. Complete or good KS remission was reported in Dupin studies. (45, 83, 135).

KS treatment

There is a range of treatments available for KS. These include local or general chemotherapy and pathogenesis-based treatments.

Local therapy consists in localised radiation therapy (radiotherapy) it can be used to treat KS lesions in the mouth or throat, painful skin lesions or lesions that are causing blockages in the lymph nodes of the face, arms and legs. Radiotherapy kills the overactive tumour cells with a series of low doses of radiation, leaving the rest of the body untouched. Side-effects can include short-term reddening of the skin, hair loss and, in the mouth, inflammation of the mucous membranes. The lesions usually leave a scar, like a mile, where pigmentation remains in the skin.

Other approaches to treating skin lesions include removing them surgically or freezing them with liquid nitrogen (cryotherapy).

Interferon alpha has been reported as a helpful treatment. The best results were reported when it was used to treat early KS.

Cytotoxic chemotherapy is the most used treatment in limited resource Countries, but could cause different side-effects, of course. Usually it is used a combination of three different drugs, doxorubicin, bleomycin and vincristine (ABV).

In Mozambique, particularly, the protocol provide, based on corporal surface calculation, the following drugs:

Doxorrubicina 50mg, maximum 500 mg/month (monthly treatment cycle). The first day doxorrubicina half dose and the second day half doxorrubicina dose+vincristina + bleomicina.

The Vincristina dose is 1,4mg and maximum 2 mg/month.

The Bleomicina dose is 10-20 units monthly, diluted in physiologic, maximum 400 units monthly

This combination increase the median survival in patients with aggressive cutaneous or visceral KS, as reported in several papers, first in Gill (57).

In Europe and United States, researchers have developed new, less toxic formulations of chemotherapy drugs. Two drugs, liposomal doxorubicin and liposomal daunorubicin are now considered to be the standard of care for KS. Other therapies, such as angiogenesis inhibitors, are under investigation in clinical trials. (2).

Nerveless, ABV seems to be the most reasonable treatment option for AIDS-KS patients in resource-limited countries at the moment, comparing the cost-effectiveness of other kind

of treatment (pegylated liposomal doxorubicin (PLD) and liposomal daunorubicin (DNX)). (139).

Recently, researchers have started to look for drugs that inhibit HHV8. as drugs used to treat other herpes virus infections, such as ganciclovir, foscarnet, cidofovir and aciclovir. Even if the effects of the cidofovir is controversial, some authors reported effects used in association with liposomal daunorubicin. (140).
The choice of treatment for Kaposi Sarcoma in AIDS patients in Western Countries depends on several factors.
It includes of course an attentive evaluation of disease extension, but also of the progression rapidity, symptoms, and of the objective to join.
The maximum HIV suppression associated to Oppurtunistic Infection (IO) prophylaxis and treatment are crucial instruments also in Kaposi Sarcoma therapy.
In patients with spread and proliferative muco cutaneous disease, the systemic therapy could just consist in HAART administration. The HAART therapy, as primary therapy, gives an objective remission rate of 66-68%, with a complete remission rate of about 35%. The needed average time to obtain a complete response is normally between 2 and 4 months and the anti neoplastic response is generally correlated with the patient's immunological reconstitution (106). The chemotherapy treatment is reserved to patients with quickly progressive disease, visceral symptomatic disease, pulmonary localization and/or important oedema. The chemotherapy is indicated also in patients with neoplastic progression on HAART treatment.

Several chemotherapy drugs, when used in a single combination, show an activity in treating Kaposi Sarcoma in HIV/AIDS patients. They are adriamicine (ADM), the vinca's alkaloids (V) (vinblastine, vincristine, vindesine, vinorelbine), bleomicine (B) and taxole (TAX), and allow to obtain some objective responses with a response rate between 30 and 70 %, while in a large amount the responses consist of partial remissions.
In Kaposi Sarcoma treatment, it was approved also the use of two liposomal antracyclines, the ADM and the DNM-L, which have an objective response rate between 25 and 79%. Comparing the DNM-L with AB, the first drug shows the same objective response rate of 15%, also with a severe leucopoenia, consistently higher if compared with polichemotherapy.

In patients with pulmonary Kaposi Sarcoma, the DNM-L, 60 mg/m, shows an objective response rate of 32% with a quick symptom regression in about 70% of patients.
The most frequent toxicity remains the neutropoenia, documented among 85% patients.
Based on these data, the liposomal antracyclines and especially the ADN-L are, at the moment, the golden standard in first line chemotherapy for treating Kaposi Sarcoma.
The vinorelbine, 30 mg/m^2 , administered twice a week induces 43% objective responses with a rate of complete remissions of 9%, but it also causes severe leucopoenia in 45% of the patients (138).
Therefore, the HAART is an essential therapeutic strategy in Kaposi Sarcoma /HIV AIDS patients. It could be used as single anti neoplastic therapy in first stage disease, or with a slowly proliferative disease, with a little tumour load and/or when the growth is compatible with the long latency of the HAART anti neoplastic activity. Out of the antiretroviral drugs advisable for Kaposi Sarcoma patients we can find Protease Inhibitors and especially the Indinavir.

In patients with rapid proliferative disease, the golden standard is the chemotherapy alone or in association with the antiretroviral therapy (based on the patient tolerability) with HAART in the follow up. The liposomal antracyclines are the golden standard among the antiblastic drugs, as first line chemoterapy, while the BV/ABV regimens could be considered a good choice for patients with reduced marrow reserve.
A salvage chemotherapy must consider the use of Taxole and vinorelbine, this one for patients with poor general conditions. Finally, it is always useful to perform the prophylaxis of Pneumocystis Carinii pneumonia in patients with CD4+ count < 200.

The DREAM Program

DREAM (Drug Resource Enhancement against AIDS and Malnutrition) is the Community of Sant' Egidio Program to fight AIDS in Sub Saharian Africa, a total-approach program for treating the disease. It has been active in the field since March 2002, after two years of preparation work. The Program has an innovative approach, including anti-retroviral therapy, in developing Countries, as institutional partner. The Community of Sant'Egidio has a long history in Mozambique, from the humanitarian aid sent in the early 1980s to the official mediation between the guerrillas and the government leading to the General peace agreement signed in Rome on 4 October 1992 after 27 months of negotiations. Today, Mozambique is a major example of democratic rebirth in sub-Saharan Africa. This special link with Mozambique has led the Community of Sant' Egidio to choose this country as the first where to pilot the DREAM Program.

The HIV/AIDS pandemic

Starting in the early 1980s, AIDS had a development without comparison in the history of human pathologies. The tens of millions of people stricken in every country in the world bear witness to a pandemic that can truly lay claim to being the first pathology of the era of globalization. A radically new situation such as this requires radically new responses. Models must be identified that take into account both the features of the illness and the available possibilities for treatment. More than two thirds of those with HIV/AIDS – tens of millions of people – live in Africa. And only a very small number of these people, mostly those with some economic means, have access to antiretroviral treatment. With such a serious emergency, while intervention must be quick, what is needed immediately is to start laying the foundations for building a medium- and long-term response.

The antiretroviral therapy

The advent of HAART (Highly Active Anti-Retroviral Therapy) in the mid-1990s radically changed the natural history of the illness, transforming AIDS into a chronic pathology. But at the same time, antiretroviral therapy created new needs. To yield optimum results, this therapy requires technologically advanced monitoring and diagnostic methods, and must reach the population extensively, where it lives. The availability of highly specialized centers must be combined with the greatest possible spread and accessibility. Here is the first contradiction – one apparently difficult to solve, because the impact of the therapeutic intervention can only be limited. In brief, here lies the challenge: treatment with

antiretroviral drugs must be based on advanced diagnostic methods, by necessity localized in a large city; at the same time, to be effective, treatment has to rely on extensive distribution. Over time, it became clear that AIDS could not be fought with prevention alone; treatment was needed as well. Ongoing prevention is as necessary as ever, but on its own, it is clearly not enough. In fact, the lack of hope for access to and availability of therapy risks drastically to reduce the effectiveness of prevention, thereby lowering interest in knowing ones own condition vis-à-vis the infection: awareness with-out an available therapy would risk creating the dramatic and unbearable knowledge of a premature demise, often preceded by isolation and social condemnation.
These needs imply wide-ranging economic and political interventions, as well as highly articulated and innovative healthcare planning and organization of services. The millions of deaths, infected persons, and orphans require immediate intervention, without getting bogged down in non-essential preparatory phases. Likewise, however, the emergency intervention should be designed from the beginning with a view to developing stable facilities capable of waging the battle over the long term. To put it briefly, intervention in the struggle against AIDS must be based on immediacy and farsightedness.
In the DREAM Program, according to WHO guidelines for treatment in resource-limited settings, the first-line treatment consists of nucleoside reverse transcirptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). Protease Inhibitors (PI) are not considered as part of the first-line regimen, though they may be used in the event of treatment failure or intolerance of the first-line combinations. There are two basic fixed-dose combination regimens available:

AZT (zidovudine) + 3TC (lamivudine) + NVP (nevirapine)
or
d4T (stavudine) + 3TC (lamivudine) + NVP (nevirapine)
if the patient is anaemic

The zidovudine-containing regimen is preferred both because of its higher genetic barrier to mutations inducing viral resistance and- particularly in Mother to Child Prevention and Care, the DREAM scheme in pregnancy to prevent the mother-child transmission, because there is more experience with the use of this drug. In the event of anaemia (Hb<8 mg/dl) preference is given to the stavudine-containing regimen.
The second-line treatment includes the following drugs:

Indinavir (IDV)/ritonavir (RTV)
Nelfinavir (NFV)
Didanosine (DDI)
Abacavir (ABC)

HAART is offered to all patients who fulfill the following criteria:
WHO clinical stage III, IV or
CD4 cell count < 200 cell/ul3
CD4 cell count < 350 cell/ul3 and viral load >55.000 copies/ml

To prevent mother to child transmission, HAART is also offered to all HIV positive women starting from their 32nd week of pregnancy, irrespective of their clinical, cirological or immunological status. Women who do not meet the general criteria to start HAART stop the treatment withi n six months of delivery.

In addition to HAART cotrimoxazole prophylaxis is provided to all patients with CD4 cells count < 200 cell/ul3.

In case of Tuberculosis disease, the therapeutic indication is absolute for patients with CD4 < 50 cell/ul3, but is conditioned upon the clinical situation for those with CD4 count is greater than 50 but less then 200. When antiretroviral treatment starts being administrated to a patient with HIV infection in intensive treatment for TB, there may be paradoxical reactions between anti-TB and antiretroviral drugs (immuno-reconstruction syndrome). For this reason it is often preferred to wait for the first two months of anti TB therapy before starting HAART.

<u>A public health intervention</u>
The HIV pandemic threatens the African population, and tens of millions of Africans are currently estimated to be HIV-positive. According to UNAIDS recently published data, in 2004 about 25.4 million adults and children were infected with HIV in sub-Saharan Africa. Among them, 13.3 million are women, and the incidence rate is still very high (137).

With such figures, a strategy based only on prevention seems not effective enough, and the urgent need for an extraordinary effort to couple prevention with a more widespread use of highly active anti-retroviral therapy (HAART) is widely felt.
During the last few years, this treatment has been increasingly available in Africa (36), thanks to financing by international institutions and especially after the introduction of generic drugs, which have cut down the costs per patient/year while maintaining clinical effectiveness. (47).

It was starting from these assumptions that the Community of Sant'Egidio developed its methodology in the fight against AIDS, the DREAM Program. This horizontal public health intervention aims to take action in the various areas of the country. To be quickly operative and effective in order to respond to emergencies, DREAM has created a bureaucratically and administratively streamlined intervention model. This particularly agile system makes it possible to maintain administrative costs – including those for non-local personnel, who work exclusively free of charge – at a minimum percentage of the overall budget at all times. In other words, the whole budget is used exclusively to fund activities in the country.

Day hospital and home assistance

Antiretroviral therapy is administered to the population through day hospital and home assistance activities. In this way, lacking specialized facilities spread throughout the territory, the program is allowed to be in direct contact with the population, thereby guaranteeing the necessary control over the therapy. When the people to be treated cannot come closer to the possibility for therapy, it is DREAM that brings them closer.
However, AIDS cannot be combated exclusively with anti-retroviral drugs. It requires a more wide-ranging intervention taking into account the person's overall needs. The package of services ordinarily offered to the patient on a stable basis is comprised of voluntary counseling and testing, lab tests (biochemistry, haemochrome, CD4+ cells count, and viral load), antiretroviral therapy, treatment of opportunistic infections and sexually transmitted diseases, nutritional support, basic health education, and social support.
DREAM is also used for the purposes of research in public healthcare, epidemiology of services and their impact, in clinics, and in therapy in developing countries. The acquisition of new knowledge in research dedicated to development interventions in impoverished countries is a sound contribution in the fight against AIDS. In this sense, the program has

strong connections with the scientific world and is oriented towards gathering data for epidemiological and clinical studies, both as routine and for ad-hoc investigations.

The laboratory

To be effective, antiretroviral treatment requires strong lab support. This is why the DREAM program has included the development of highly specialized molecular biology labs, where the CD4+ count and the viral load measurement (in accordance with the indications of the diagnostic and therapeutic protocols), in addition to the basic biochemistry and haemochrome, are regularly performed, acting as the technological core of the whole program. Labs of this kind are needed in order to respond quickly and effectively to the development of drug-resistant viral strains. This is an investment for the country's future, both in training highly specialized human resources and in equipment.

Computerization.

All the DREAM program's activities are monitored through a computer network linking the various centers to one an-other and to the reference labs. This system guarantees efficient monitoring of the treated patients – including adherence to the therapy – and becomes an important source of data that can be used to continue improving the quality of the interventions, through applied research too. And it is applied research that makes it possible to perfect the forms of intervention and helps guarantee that the finest, most effective help is offered, even in sub-Saharan Africa countries.

DREAM laboratory's implementation

The Laboratories have been developed to monitor the efficacy of the HAART supplied to all the patients positive to HIV test and enrolled in the DREAM Program. In the labs there are also instruments and technologies needed to perform all the tests and assess the health status of HIV/AIDS suffering patients.

Two are the main problems to implementing a laboratory of this kind. One of this is certainly related to the diagnostic costs. Regarding drugs, in fact, an important discussion was raised in the past years, which leave to open some possibilities: today generic drugs are available, of cleared efficacy, permitting to decrease the costs sensibly.

About diagnostics, it is no possible to assert the same and this is a real limitation in use some indicators as CD4 cells count or the viral load determination in the scaling up, even if they represent significant instruments to monitoring therapy. An other important problem, it

was the necessity to implement excellent diagnosis facilities in Central Hospitals in big cities, designed with Western standard.
These two challenges, an economic one and a technical one, we faced and partially won opening the two laboratories in Maputo, Beira and Nampula, the Capital, the 2nd and 3rd cities in Mozambique.
The lab has been set up according to the safety rules in force in the developed countries. All the technicians working in the lab have been accurately trained on the instruments functions as well as on the procedure necessary to work safely.
The people working in the labs are supplied with white coats, gloves, disposable plastic ware as well as specific reagents for cleaning and decontaminating the workbenches and themselves.
A laminar flow hood is set up for viral particle treatment avoiding the dispersion of infectious film.
An autoclave supplied with disposable plastic bags sterilizes all the outgoing material (no facilities are provided in the Central Hospital to inactivate the lab waste).
The arrangement of the lab has been realized in order to separate the different analytical sections of the lab (haematology/cytometry, serology/biochemistry, molecular biology/bDNA). The structure of the building has been exploited as to have an optimized working line. It starts when the blood samples arrive and ends with the results output. A complete set of cooler/freezers has been set up to preserve the reagents and the plasma samples at the right temperature.
A proprietary software has been developed to archive and manage all the data produced in the lab. A network has been built to connect all the labs equipment and to perform remote control and validation of the labs working flow.

Cost-reduced analytical methods

About cost-reduced technologies, some methods were developed to reduce the analysis costs, using methods employing smaller volumes (micro-methods). Also about the lymphocyte sub-populations count it was evaluated and adopted a new flow cytometry protocol.
In fact, it is well known that laboratory tests are mandatory for the correct management of HAART, and that international guidelines impose specific protocols for CD4 + lymphocyte count and measurement of viral load as parameters to guide therapy in HIV-positive patients (32). Attempts to use simpler indicators, like the total lymphocyte count instead of

CD4 (9, 131) could lead to gross measurement errors in the recruitment and control of patients (85).

The need to speed up the spread of health care and diagnostic facilities to fight AIDS (89) conflicts with the exorbitant cost of routine tests in the resource-poor settings. Laboratory tests recommended by international guidelines are prohibitively expensive most of the weak economies in sub-Saharan Africa (100*).*
Research has recently been developed to identify less complex yet reliable equipment and protocols to be used in resource-limited settings, using micro-volume fluorimetry, immunomagnetic systems, cytospheres, simplified flow cytometers or modifying classic flow cytometry protocols to obtain reliable CD4 + T lymphocyte counts with simpler equipment (10,13, 25, 41, 42, 55, 58, 61, 104)
However, such alternative strategies did not always meet the major requirement of a drastic cost cutting of consumables. Flow cytometric immunophenotyping has been evolving with time into unsurpassedly precise and reliable, but increasingly complicated and expensive technology (64, 88).
Fighting AIDS effectively in resource-poor regions is therefore in urgent need of simplifying techniques to reduce the costs of reagents and minimize the specialised manpower (72, 73, 74).

Our effort was to reduce the amount of antibodies needed for a correct determination of lymphocyte subsets to monitor HIV + patients and to improve the accuracy of a gating technique still based on physical parameters. Recently, the “panleucogating” (PLG) protocol has been developed and validated in a number of settings (59).
Our effort was to corroborate these studies, particularly the one by Pattanapanyasat (114) to demonstrate that similar good results can be obtained in different settings, with different antibodies, sample volumes, lysing strategies and instruments.
This protocol is based on a simplified staining procedure using only two antibodies, CD45/CD4, and on a CD45-based gating procedure which gives a good discrimination of lymphocytes and enables the enumeration of CD4 + cells over the entire white cell population without the need for a leukocyte differential count.
An effective quality control was set up to monitoring the whole complex procedure from withdrawal to reporting, to transport and effective use in instrument methods to determining diagnostic parameters.

MATERIALS AND METHODS

Samples and sampling

To study seroprevalence of HHV8 in Mozambique in relation with HIV/AIDS, and the clinical evidence of Kaposi Sarcoma AIDS related incidence in Mozambique, it was considered the whole DREAM cohort in Mozambique. Since the start up in March 2002 till the end of 2005, when we started this part of the study, we have enrolled 14.011 HIV + patients in the Country, in 8 different health centers in the South and in the Central region, 9065 females (64,7%) and 4946 males (35,3 %). Half of them started HAART, on the base of the international protocols used by the DREAM Program. Like all patients served by DREAM, they have access to Community Care and Home Care Services (CCHC): counselling, laboratory monitoring, treatment and care (antiretroviral drugs, opportunistic infection treatment, nutritional supplement, sexually transmitted infections treatment), supply of mosquito nets and health education. At the Health Centre some parameters like weight and height were constantly monitored, to determine also the Body Mass Index. Based on this value (BMI<18), patients received Nutritional Supplement.
The diagnosis of HIV+ seropositivity, when performed in the GATV -Gabinete de Aconselhamento e Testagem Volontaria- of the Country, was reconfirmed by using rapid tests for antibody detection.

To establish the relationship between HAART, chemotherapy treatment and time of regression and/or remission we focused the study on the Machava Health Centre, close to the capital Maputo, in the South of the Country. We found 61 patients with Kaposi Sarcoma diagnosis. The data are referred to 4 years' activity (2002-2006).
All Kaposi Sarcoma diagnoses were confirmed at the Pathological Anatomy Laboratory- Central Hospital of Maputo.
Out of 61 KS patients, 31 patients died (50,8%) and 3 were transferred to other health centres. 27 patients are in follow up, being 39 the median age, respectively 15 males (56%) and 12 females (44%).

To study HHV8 seroprevalence in HIV positive patients we randomly derived a subcohort from the DREAM programme of the Community of Sant'Egidio, particularly those followed in the centre of Machava (Maputo), and Beira Mangachingussura, where a home care and community care programme, together with HAART treatment for AIDS, is being

implemented.
The samples were randomly collected from the two centres among the patients included in the Programme and the subcohort was made up of adults on antiretroviral therapy. It consisted of 161 patients, 93 (57.8%) women and 68 (42.2%) men. The average age of the sample was 31.54, with a median equal to 31.81. In the HIV positive subcohort, HIV1 viral load was measured and the immune status (CD4+ and CD8+ T lymphocytes count) was studied.

To study the general HHV8 seroprevalence in Mozambique we needed HIV negative patients. So, the HIV-negative subcohort (n=166) was made up of people admitted into the "Hospital Central de Maputo", chosen for having similar personal data to the HIV-positive cohort. The average age of the sample was 30.44, with a median equal to 31.0 with a minimum of 0 and a maximum of 57. The sex distribution was 69 males (41.6%) and 97 females (58.4%). The few children included in the studied population were tested positive because of the presence of maternal antibodies (two children under the age of 18 months in a HIV-negative population, included and tested because their data were noted but not associable to either cohort), while the results of children older than 18 months were interesting. Unfortunately, it was not possible to find out if they were children of women testing positive for HHV8. This information would have been interesting because children of women testing positive for HHV8 appear to be more frequently positive to the virus (117), and there is even speculation of a mother-to-child transmission.
The HIV screening was performed with rapid tests for HIV 1 and 2 antibody detection.
Finally, to study HHV8 prevalence, the cohort included 327 Mozambican patients, 137 males (42%) and 190 females (58%), median age 31. Among them, 161 were HIV-positive and 166 HIV-negative.

Testing

HIV screening was performed in the DREAM Center. Only for the HIV negative subcohort the sera were collected from hospitalized patients in the Hospital Microbiology Laboratory and tested in the DREAM Laboratory.
Immunophenotyping, viral load and HHV8 screening were performed in the Central Hospital DREAM Center. PCR researches were performed in Tor Vergata University (Roma, Italy) Public Health Department Laboratories, after DNA extraction.

It is noticeable to consider that almost all the experimental work of the project was done in the Laboratory of Molecular Biology of Maputo Central Hospital (Mozambique).
Once collected by venipuncture at the DREAM Center in a red cap tube for biochemistry or in a violet cap tube containing EDTA as anticoagulant, blood was stored at room temperature (but in a conditioned 25°C room) and quickly transferred to the Central Hospital DREAM Laboratory. Blood is normally transported on the same morning to the laboratory in slightly refrigerated insulated containers. In lab, plasma aliquots were separated and stored in a – 80°C freezer for viral load quantification and HHV8 screening. Fresh whole blood was used to determine haemograms, since haemoglobin levels are particularly significant for the immediate monitoring of the health state of the patient, and CD4 count was performed within few hours.
Specimens were always forwarded with a form containing at least the case clinical history record number, name of the patient, provenance, signature of the director of the Health Center, number of vials collected from each patient.
The Reception office checked that the number of vials corresponded to what declared and that they were all labeled.
Using the appropriate laboratory software, a progressive number was assigned to each specimen and this number was attached to the specimen and written on the accompanying form. Thus the specimen would be unequivocally identified throughout its life in the laboratory and the patient's privacy guaranteed.

Antibody detection for HIV

The diagnosis was performed according to the Mozambican national protocol, using a pair of rapid tests, the Abbott Determine and the Trinity Biotech Unigold. These are tests in lateral flow paper chromatography, easy to perform, rapid and simple to read. Therefore, they allow the highest effectiveness in this delicate phase of approaching the patient, giving a response within 15' and avoiding the need for recalling the patient who could have objective difficulties to come back for the response (difficult, absent or excessively expensive transportation for the majority of people etc). For this reason the test was performed at the Health Centre by especially trained nurses. Test sensibility and specificity are 99,75% and 100% for Determine and both 100% for UniGold.

Quantification of HIV1 viral RNA.

The determination of HIV1 viral load was performed with Bayer bDNA method. It is a well-known, standardized method, adopted in the country for its simplicity of execution and management.

This method has the advantage of being based on amplifying the signal and not the target as with the methods using PCR (polymerase chain reaction), thus being less susceptible to the problem of contamination. Currently, the bDNA system appears to be the one that offers the greatest coverage for the various extant HIV subtypes. Furthermore, bDNA is not subject to the errors that are typical of the PCR procedures (amplification of contaminant RNA, mutations induced by the polymerase). This results in a reduced level of sterility necessary for the analyses to be performed.

Direct research of the HHV8 virus by qualitative PCR .

The direct research of HHV8 virus is the oldest way to detect it, as shown in the first studies, i.e. Withby in 1995.

La Duca (80) reports the isolation of HHV8 with PCR from plasma with a gain equal to 7%, while a higher gain is obtained from lymphocytes (46%).

Some authors used PCR, specifically researching the ORF 21 region, which is characteristic of the lytic phase (tertiary lytic gene, as defined by Richard G Jenner).

In a first step of the work, direct extraction and amplification of the HHV8 virus from 15 plasma samples was tried. Samples were collected from HHV8- HIV positive patients, with no clinical evidence of Kaposi Sarcoma. None of them was under antiretroviral treatment. The DNA was extracted in Tor Vergata University Laboratories from frozen samples with Qiagen-QIAamp MinElute Virus kit - final volume 30 µl. Qualitative PCR was then performed in search of the ORF21 region (lytic phase) as a characteristic of HHV8, using two positive and one negative controls. it was not possible to isolate the HHV8 virus over 15 frozen plasma samples. Several difficulties have been faced. First of all, the probably least suitable matrix, the plasma used for the need of exploiting the existent serum bank. In addition, sending frozen samples from Sub Saharan Africa is very expensive and not so easy. For this reason, it was chosen to change the method to screen the cohort, because PCR was not available in Mozambique at the moment, and collecting materials from lesions in Kaposi Sarcoma patients was not authorized.

HHV8 antibody detection

Among the methods reported in literature for determining the presence of HHV8, there is the detection of latent antigens (LANA), which can be seen with Western Blot or immunofluorescence (IFA) and the detection of antigens of the lytic phase like, for example, EIA methods to determine the seroprevalence and the antibody titre of the antigenic peptide deriving from the ORF 65 region.

In this study, detection of anti-HHV8 serum antibodies was performed by means of a Biotrin immunohistochemical method (ELISA) for IgG titration. The kit is based upon a mix of synthetic peptides capable of detecting the antibodies against HHV8 viral lytic proteins. It is an immunoenzymic titration. The specifically bound antibodies are detected through an anti-human IgG-based conjugate labelled with peroxydase, and through a substrate reaction. The use of lytic peptide epitopes derived from different viral proteins guarantees both high sensitivity and specificity. There is no detectable cross-reactivity with HIV. Serum was used in the assay.

Immunophenotyping for the determination of CD4+ and CD8+ T lymphocytes

To study the immune status and to perform CD4 and CD8 T lymphocytes count, we used the Beckman Coulter EPICS XL-MCL flow cytometer, in double platform, with lyse no wash method and Beckman monoclonal antibodies. A big effort was made in order to optimize the diagnostic protocols for CD4 and CD8. The idea was to use the most possibly efficient protocol. As a matter of fact, the major obstacle to the diffusion of a Western-type anti-HIV strategy is presently the exaggerate cost of routine diagnostics. The type and number of specialized tests suggested by the international guidelines has actually unaffordable costs for most economic systems of the Sub-Saharan countries (100) Therefore, the research of application protocols allowing to get the needed information with a reduction of the number of tubes and antibodies needed for a correct determination of lymphocyte sub-populations is an answer to such an issue. Recently the 'PanLeucogating' protocol has been developed and validated; it is based on a strategy of simplified labelling with only two antibodies, CD45 / CD4, and on a gating procedure which allows a good resolution of the CD4+ lymphocytes without the necessity of leukocyte formula.

The PanLeucogating (PLG) has been compared with the protocol commonly used in several countries, based on the use of three antibodies CD3, CD4 and CD8 (99), and definitively adopted.

The comparison study was performed on 189 samples. All the HIV-positive patients were included in the DREAM program and were on antiretroviral therapy. Among them, 30 subjects (15.8%) had less than 200 CD4/µL.

The comparison study was carried out using a random selection of HIV + clinical samples from various peripheral health centers which sent materials for routine analyses to the hospital facilities. The HIV-positive status of patients had been assessed. Three mL of peripheral venous blood were taken from each patient in BD Vacutainer K 3 EDTA tubes. The samples were processed within 24 hours from collection.

The antibodies used for routine analysis were Beckman Immunotech Trio-Opticlone premixed anti-CD4(FITC) / anti-CD8(PE) / anti-CD3(PCy5). For the PLG technique a volume/volume mixture of anti-CD45(FITC) and anti-CD4(PE) Beckman Immunotech was used. When requested by clinicians, anti-CD8(PCy5) Beckman Immunotech was added to PLG. Both techniques were carried out with a lyse-no wash method with an ammonium chloride buffer.

The sample preparation procedure was the following: 50 µL of whole blood were added to 5 µL of the Trio-Opticlone mixture in one tube and to 5 µL of the PLG mixture in the other one. The test tubes were incubated in the dark at room temperature. After 30 minutes, 1 mL of lysing solution (generic lysing buffer) was added. The test tubes were incubated for another 20 minutes in the dark. The samples were then directly processed in the flow cytometer within one hour. Visual confirmation of a complete Red Blood Cells (RBC) lysing was requested to technicians. A Beckman Coulter EPICS XL MCL (with Multi Carousel Loader) flow cytometer equipped with an Argon ion laser (488 nm wavelength) was used, allowing the simultaneous measurement of four fluorescence channels. The routine protocol was based on the identification of lymphocytes by physical parameters, through a homogeneous lymphocyte gate on a Forward Scatter (FS) vs Side Scatter (SS) dot plot. Within the lymphocyte gate, the percentage of double positive elements CD3-PCy5 / CD4-FITC and CD3-PCy5 / CD8-PE, respectively, was calculated. The percentage of CD3 + CD4 + cells and CD3 + CD8 + cells was related to the total lymphocyte count obtained by the hematology analyzer, in order to give the absolute number of CD4 + and CD8 + per µL. With PLG the percentage of CD4 + (and of CD8 + cells if requested) over all leucocytes identified by CD45 was calculated, and CD3 expression was not taken into account.

Briefly, in a CD45 vs SS display, total leucocytes and lymphocytes are identified by two different gates. The cytometric percentage of lymphocytes over the total White Blood Cells

(WBC) can be calculated. In another display, the percentage of CD4 (or CD8) positive elements is extracted from total leucocytes. Using the total WBC count per µL from the hematology analyzer, the absolute CD4 + (or CD8 +) cell count is then calculated. The CD4/CD8 ratio is also available if the CD8 value is requested by the clinician. In the PLG protocol, however, CD8 + cells must be identified as CD8 +high CD45 high SS low cells only, to avoid the inclusion of CD8 +dim lymphocytes that are mostly CD3 - . The lymphocyte subsets count was carried out in dual-platform mode using a hematology analyzer Sysmex K21N, which gave the absolute lymphocyte count to be used in routine analysis, or the total white blood cell (WBC) count, to be used in the PLG.

Statistical analysis

All the clinical and haematic informations were registered using the DREAM Prog. Software, a proprietary software developed to manage the clinical process of each patient. All the visits were managed using the DREAM Prog. The software registers the following information:

folder ID, personal data, social information, visits (anamnesis, symptomatology, BMI, drug prescriptions), blood exams, appointments (examination, drugs, blood collection, medication, food integration, check, HIV test, “verify”), aids stage (international classification), kind of service, ARV therapy, date of record registration.

In order to describe the HHV8 prevalence and to determine the significance of HHV8 infection *versus* CD4 and viral load levels, and *versus* the BMI and Hb levels, SPSS software for statistical calculus was used.

To evaluate the PLG Protocol, statistical analysis was carried out by linear regression and Bland-Altman analyses. Statistical analysis was carried out using Kaleidagraph 3.5, Synergy Software. Percent and absolute values obtained from the two methods for CD4 and CD8 respectively were plotted. Linear regression analysis was used to calculate squared R. To compare the two different gating strategies, the differences of the pairs CD4%(PLG)-CD4%(Trio), CD4(PLG)-CD4(Trio) and CD8%(PLG)-CD8%(Trio) respectively, CD8(PLG)-CD8(Trio) were plotted against their average as already reported.

RESULTS

First of all, we'd like to show the results in defining the cost-effective protocol to monitoring CD4 and CD8 count in the studied population. The following tables illustrate the PLG protocol *versus* the Trio one.

The table 1 shows the regression parameters of CD4+ and CD8+ values, in percentage and in absolute count (abs), obtained with the PLG and the Trio protocols, respectively. Absolute counts were obtained using total WBC count (PLG) or lymphocyte count (Trio), respectively. In column B a cut off of clinical interest has been applied to %CD4+ and CD4+ absolute count.

Tab. 1

A	B	slope	intercept	R^2	R
%CD4(PLG) *vs* %CD4(Trio)		0,954	1,52	0,964	0,982
	%CD4(PLG) vs %CD4(Trio) <15%	*0,812*	*3,046*	*0,689*	*0,830*
	%CD4(PLG) vs %CD4(Trio) >15%	*0,947*	*0,920*	*0,947*	*0,973*
abs CD4(PLG) *vs* CD4(Trio)		0,965	27,6	0,976	0,988
	abs CD4(PLG) vs CD4(Trio) <200/µL	*1,075*	*0,038*	*0,900*	*0,948*
	abs CD4(PLG) vs CD4(Trio) >200/µL	*0,966*	*27,196*	*0,972*	*0,986*
%CD8(PLG) *vs* %CD8(Trio)		0,972	1,8	0,970	0,985
abs CD8(PLG) *vs* CD8(Trio)		0,968	58,4	0,988	0,994

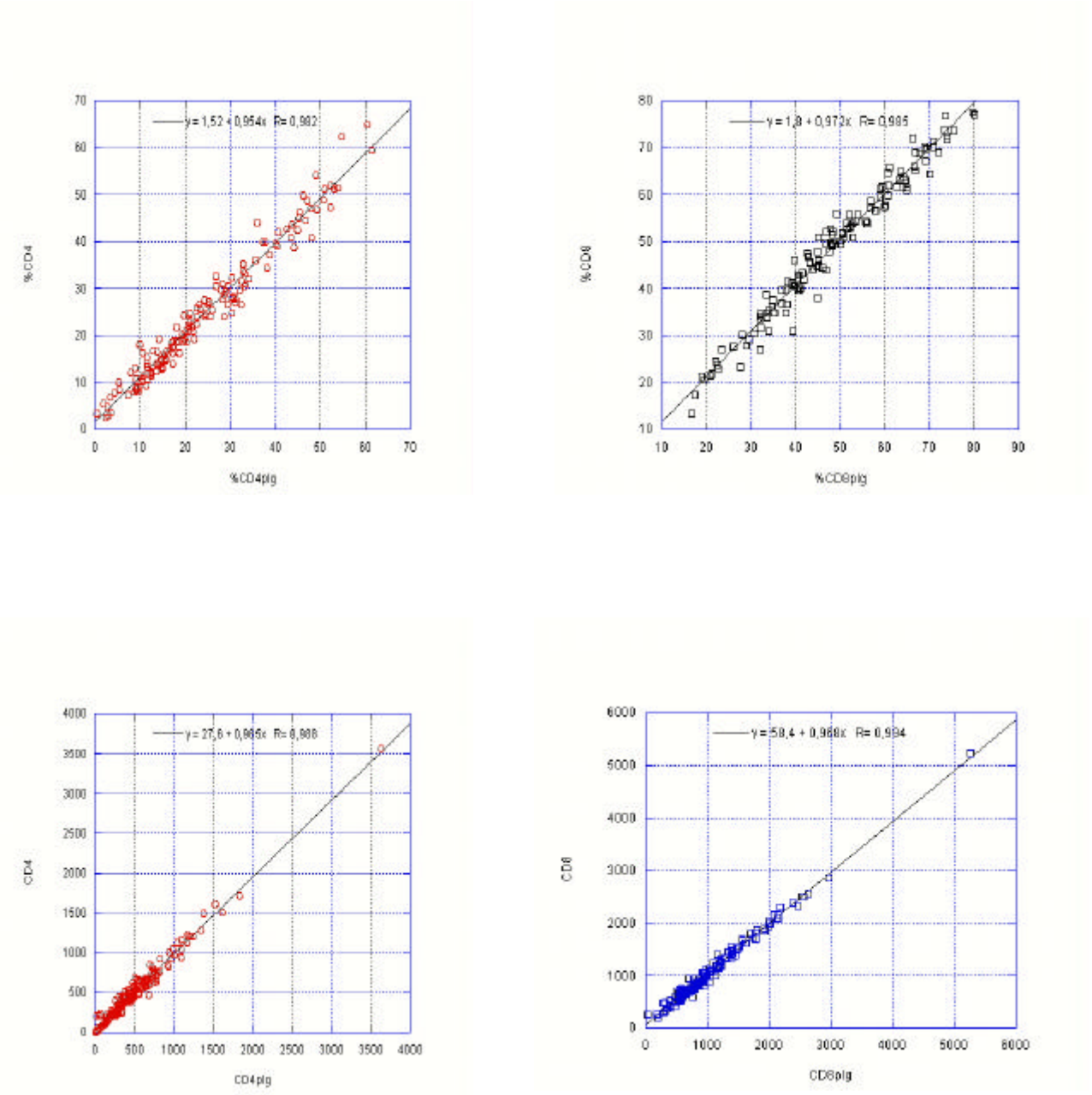
In table 2 statistical parameters obtained with Bland-Altman analysis of CD4+ and CD8+ values, are shown in percentage and in total count. They are obtained with the PLG and the Trio protocols, respectively. In column B a cut off of clinical interest has been applied to %CD4+ and CD4+ total count.

Tab.2

		mean diff	median	dev st	lo.lim.	up.lim.
%CD4(PLG)-%CD4(Trio)		-0,39	-0,07	2,69	-5,65	4,89
	%CD4(PLG)-%CD4(Trio) <15%	*-1,21*	*-0,20*	*2,39*	*-5,89*	*3,57*
	%CD4(PLG)-%CD4(Trio) >15%	*-0,02*	*0,0*	*2,74*	*-5,39*	*5,36*
CD4(PLG)-CD4(Trio)		-7,50	-2,24	61,22	-127,50	112,50
	CD4(PLG)-CD4(Trio) <200	*-9,14*	*-2,49*	*21,12*	*-50,53*	*32,25*
	CD4(PLG)-CD4(Trio) >200	*-7,17*	*-2,00*	*66,45*	*-137,42*	*123,07*
%CD8(PLG)-%CD8(Trio)		-0,45	-0,60	2,54	-5,43	4,54
CD8(PLG)-CD8(Trio)		-23,44	-17,70	71,67	-164,42	116,54

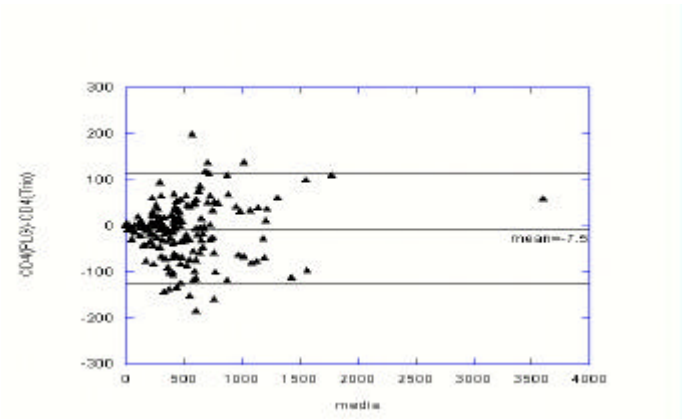
In the fig.1 it is possible to see the regression lines of CD4+ and CD8+ values, in percentage and in absolute count (abs), obtained with the PLG and the Trio protocols, respectively. Absolute counts were obtained using total WBC count (PLG) or lymphocyte count (Trio), respectively. The R value are reported on the top of each panel with the regression equation.

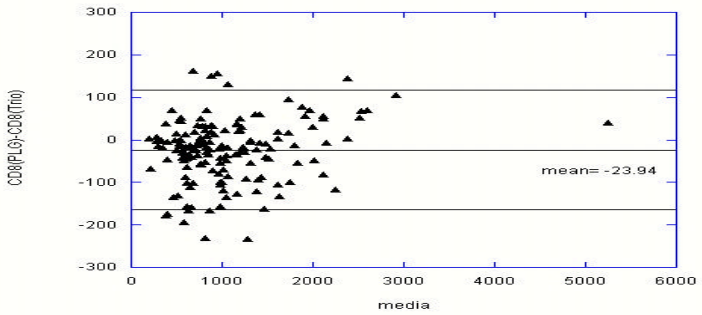
FIG.1

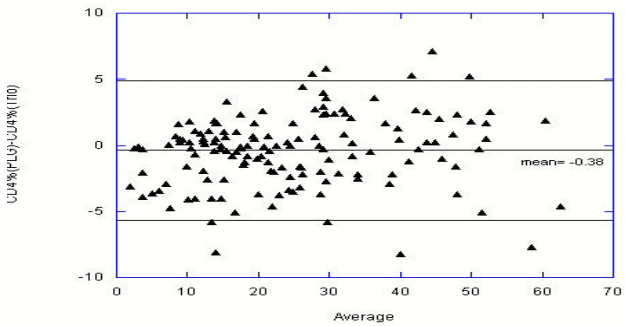


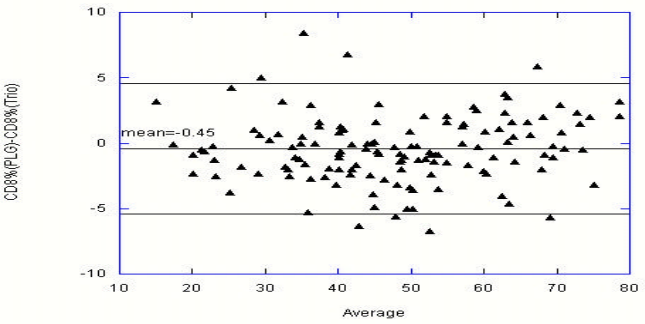
In the fig. 2 we can observe the Bland-Altman analysis of CD4 + and CD8 + . The difference of the values, in percentage and in total count, obtained with the PLG and the Trio protocols, respectively, have been plotted against the average of the same values. The mean difference of each panel is reported

FIG. 2


CD4%(PLG)-CD4%(Trio)
media
mean=-7.5


CD8(PLG)-CD8(Trio)
media
mean= -23.94


CD4%(PLG)-CD4%(Trio)
Average
mean= -0.38


CD8%(PLG)-CD8%(Trio)
Average
mean=-0.45

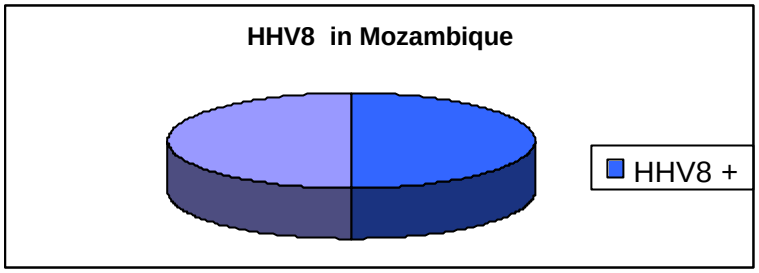
The following tables illustrate our results about seroprevalence of HHV8 in Mozambique in the population and in relation with HIV/AIDS.

In this figure 3 we can compare the HHV8 prevalence registered in our study and the HHV8 prevalence in some other Countries in Sub Saharan Africa. Mozambique is well placed in the area context, with a seroprevalence among the total population equal to 51.07%, overall higher than that registered in Zambia in a similar cohort and close to that registered in Malawi, two countries bordering Mozambique to the north and with similar populations and socio-economic situations

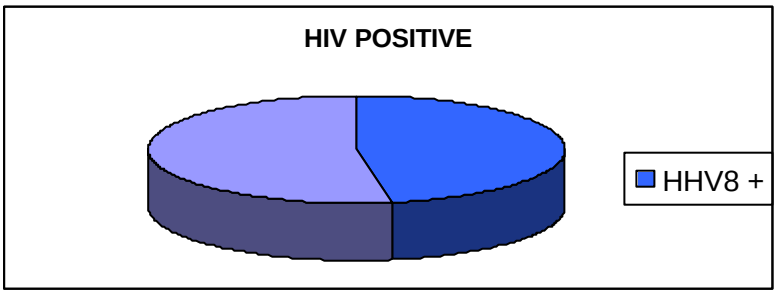
In Tab.3 are listed our data about prevalence of HHV8 in Mozambique, in comparison with the Malawian, Zambian and South African cohorts. In Mozambique the HHV8 prevalence is about 51 %

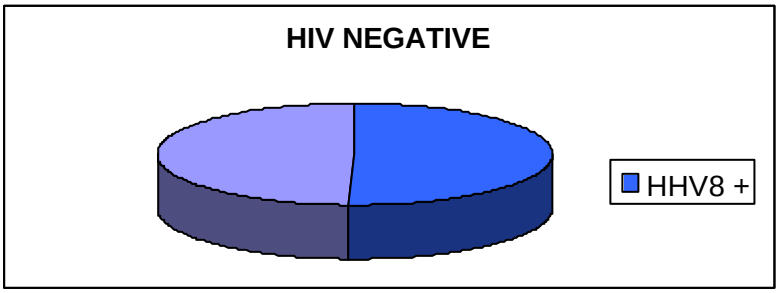
COUNTRY	SEROPREVALENCE
MALAWI (cohort of hospitalised people)	67%
MALAWI (cohort of healthy volunteers)	54%
ZAMBIA (cohort of HIV+/- pregnant women)	51,1%
ZAMBIA (cohort of HIV - pregnant women)	48,8 %
SOUTH AFRICA (hospitalised, adjusted by age and sex)	30%
MOZAMBIQUE (cohort of HIV+)	52,7%
MOZAMBIQUE (cohort of HIV -)	49,4%
MOZAMBIQUE	51,07%

In Mozambique: 51% of HHV8 positive persons.



Analysing the cohort and considering the HIV positivity, we can divide the population in two subcohort, the HIV positive, with a HHV8 prevalence of 52,7%, and the HIV negative with HHV8 prevalence of 49,4%.





In this table 4 we have the results obtained in our two sub population, HIV positive and HIV negative patients: around half (52.7%) of the HIV -positive population turned out to be positive to HHV8 too, and around 49,4 % of the HIV -negative population tested positive for HHV8, a very similar percentage

Results	Frequency	percentage
HIV positive		
HHV8 negative	76	47,2
HHV8 positive	85	52,7
TOTAL HIV positive	161	100,0
HIV negative		
HHV8 negative	84	50,6
HHV8 positive	82	49,4

TOTAL	327	

Tab 5 shows the HHV8 prevalence in the studied population described by sex ratio and median age. The median age is about 31 years in the both groups. Female ratio in about 58%.

Results	Female %	Age median	Frequency
HHV8 pos	57,8	31,8	161
HHV8 neg	58,4	31,0	166
TOTAL	58	31,0	327

In following tables we show some information about the HIV positive cohort, which is a part of the DREAM population.

In Tab.6 HIV positive cohort is studied per age median and sex ratio statistical significance, in HHV8 negative and HHV8 positive group.

HIV +	Age Median	Sig.	Female %	Sig.
HHV8 Neg	31,8	0,779	58,4	,549
HHV8 Pos	31,0	0,779	57,8	,549

There is not significance of HHV8 infection per age (p=0,779) or per sex (p=0,549) in HIV patients.

Tab 7 shows the HIV positive cohort, describing by CD4 and CD8 count levels and viral load. Significance of HHV8 infection in CD4, CD8 and viral load was studied.

	HHV8	Mean	Std. Deviation	Sig.
CD4	neg	452,81	354,992	,044
	pos	580,32	432,849	,044
	Total	516,12	387,968	
CD8	neg	790,68	447,818	,001
	pos	1237,11	1031,836	,001
	Total	1012,90	808,787	
VL	Neg	44755,54	103097,625	,766
	Pos	39692,31	83870,535	,766
	Total	40944,16	90070,027	

In the HIV positive cohort we registered a statistical significance in CD8 absolute value, comparing the HHV8 positive and HHV8 negative group (p=0.001). Not significance is observed for CD4 absolute count value, and viral load log_{10} value.

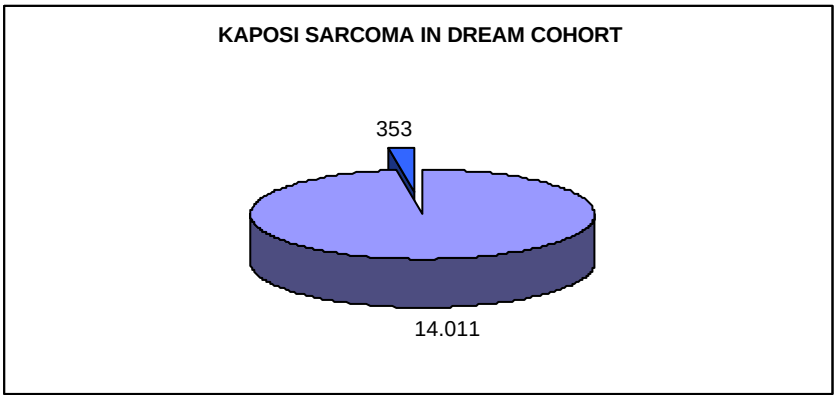
The Tab 8 illustrates in the HIV positive cohort the Body Max Index values and Haemoglobin levels. Significance of HHV8 infection in BMI and Haemoglobin levels was studied.

HHV8		MEAN	Std. Deviation	Sig.
BMI	Neg	21,8063	3,6173	,951
	Pos	20,6069	3,8605	
Hb	neg	10,937	2,3863	,035
	pos	10,460	1,9249	

In the HIV positive cohort we observe no statistical significance regarding Body Max Index values, but a significance in Haemoglobin levels between HHV8 negative and HHV8 positive group. The mean value is 10,9 g/dl for HHV8 negative patients and 10,5 g/dl for HHV8 positive patients, with a difference of 0,4 g/dl.

After measuring the HHV8 prevalence, we studied the Kaposi Sarcoma incidence in Mozambique, considering the DREAM cohort and then working only with the Machava Centre data, for a period of 4 years.

The total incidence is 25,2 °/₀₀,



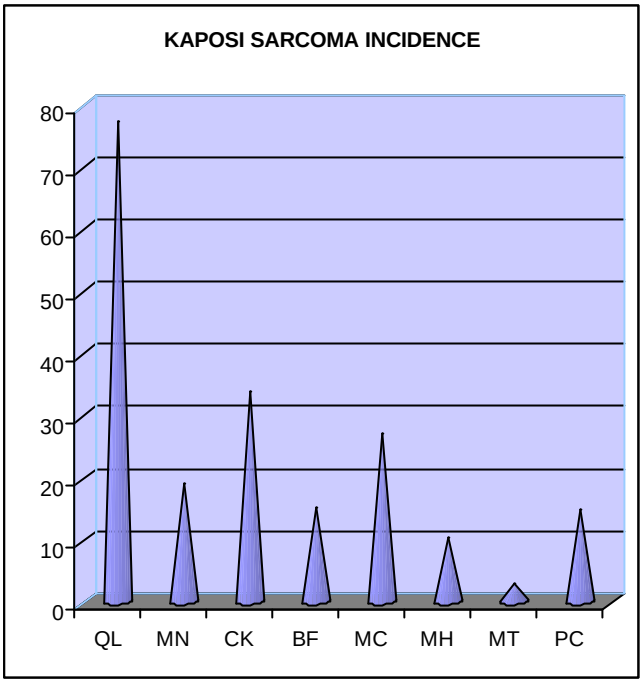
TAB 9

In the next table (9) we can observe the Kaposi Sarcoma incidence in the DREAM population since the start up in March 2002, until the end of the study, December 2006. The total incidence is 25,2 °/₀₀, but important differences are registered among the different Health Centres.

HEALTH CENTRE	KS cases number	All patients	incidence °/₀₀
Quelimane (Zambesia)	70	906	77,3
Mangachingussura (Sofala)	20	1060	18,9
Chokwe (Gaza)	131	3890	33,7
Benfica (Maputo)	14	920	15,2
Machava (Maputo)	61	2246	27,1
Mahotas ** (Maputo)	2	194	10,3
Matola * (Maputo)	4	1374	02,9
Polana Canico (Maputo)	51	3421	14,9
TOTAL	353	14011	25,2

** only MCPC*

*** Health Centre with Paediatric Assistance*



In this table 10, we can observe the sex and age distribution in the DREAM Cohort and in the group of patients with Kaposi Sarcoma diagnosis. About the 353 patients with KS, all classified as 4th WHO level, 279 of them are females (79,1%) and 74 males (20,8).

Tab.10

	Patients number	Female %	Female median age	Male median age	Sig.
DREAM Cohort	14.011	64,7 (C.I.1,004)	32	35,44	P<0,001
Confirmed Kaposi Cohort	353	79,1 (C.I.3,23)	38	40	P<0,001

In table 11 we compare the immunological and virological state of HIV positive patients with Kaposi ‘s Sarcoma and the HIV positive whole cohort (DREAM cohort). Of course, also the patients with sarcoma are part of the DREAM cohort but only for this study we analyse the data separately. Patients with Sarcoma have CD4 count and viral load levels baseline worse than the whole cohort. This is normal, because the Sarcoma is one of the

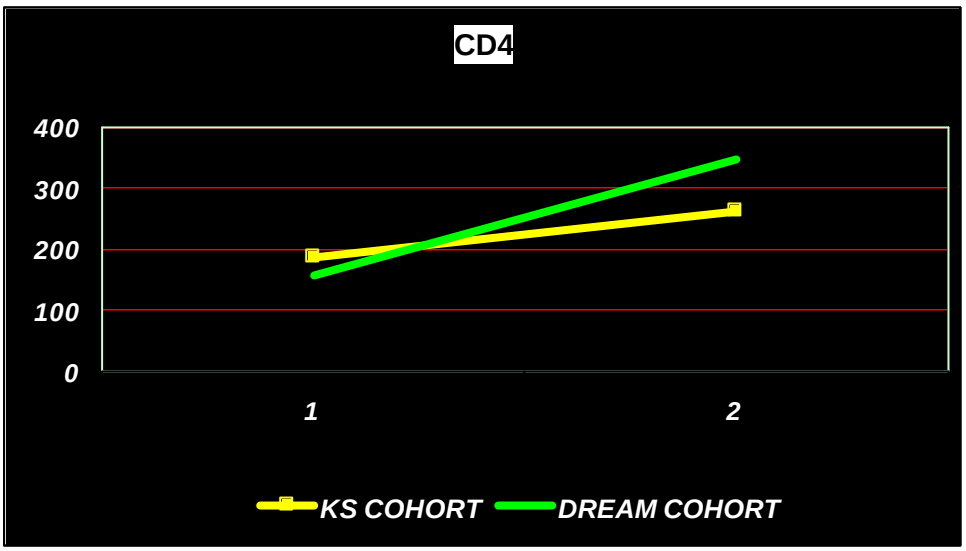
diseases that classify patient in the 4th stage. It is a signal of important compromising of the patient's state.

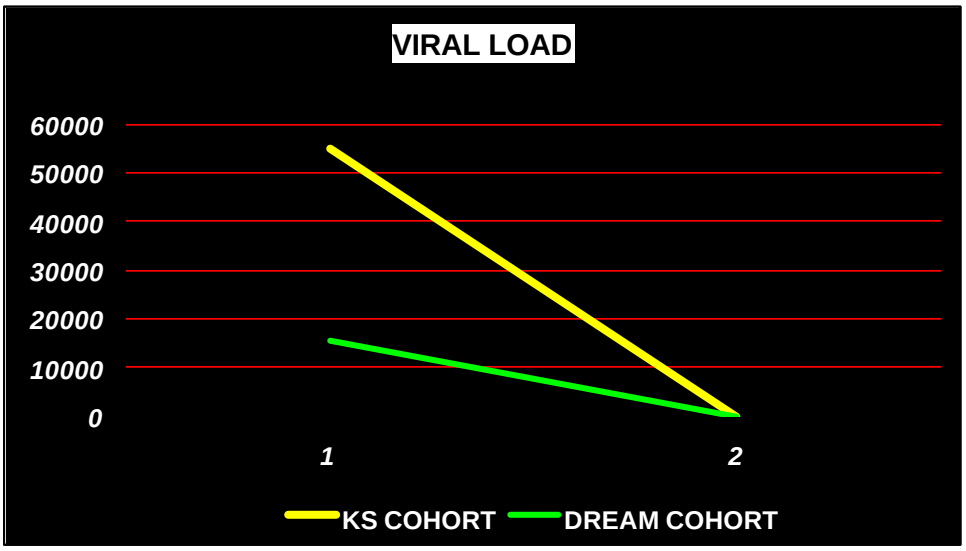
All the patients considered here are under antiretroviral treatment. After 6 months we observe in both the group a good viral response. In fact, viral load is undetectable in the Kaposi Sarcoma cohort and also in the DREAM cohort. About immunological state, we observe that both groups show an improvement, higher in the DREAM cohort, with CD4 increase of +124,5%, than the Kaposi Sarcoma group, whose improvement in CD4 count is only of 39,8 %.

Tab 11

	KS cohort baseline	DREAM cohort baseline
CD4	188 cell/ul3	155 cell/ul^3
viral load	55.000 copies/ml	15.600 copies/ml

	KS cohort on treatment after 6 months	DREAM cohort on treatment after 6 months
CD4	263 cell/ul3	348 cell/ul^3
viral load	<50 copies/ml	<50 copies/ml

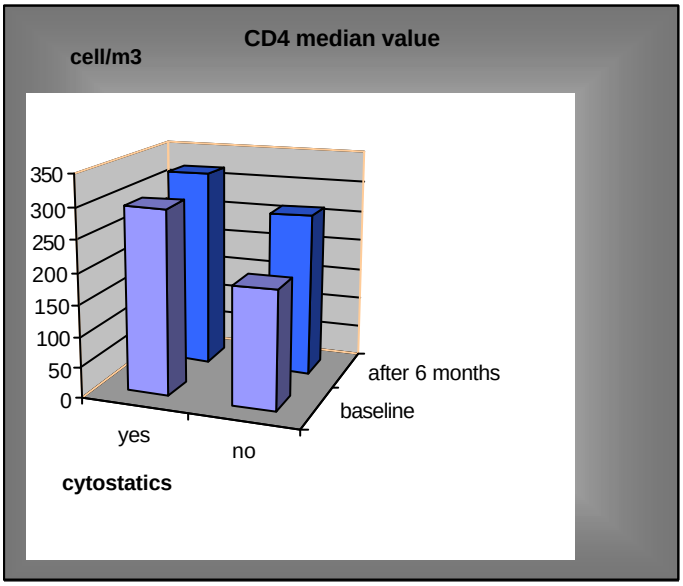




Among patients with Sarcoma, we could distinguish two different subgroups. (tab 12) One was treated also with cytostatics while the other wasn't. Regarding immunological improvement, the first group, A, show only a little gain (7,8%); the group B gain is better, 39,3%.

Tab 12

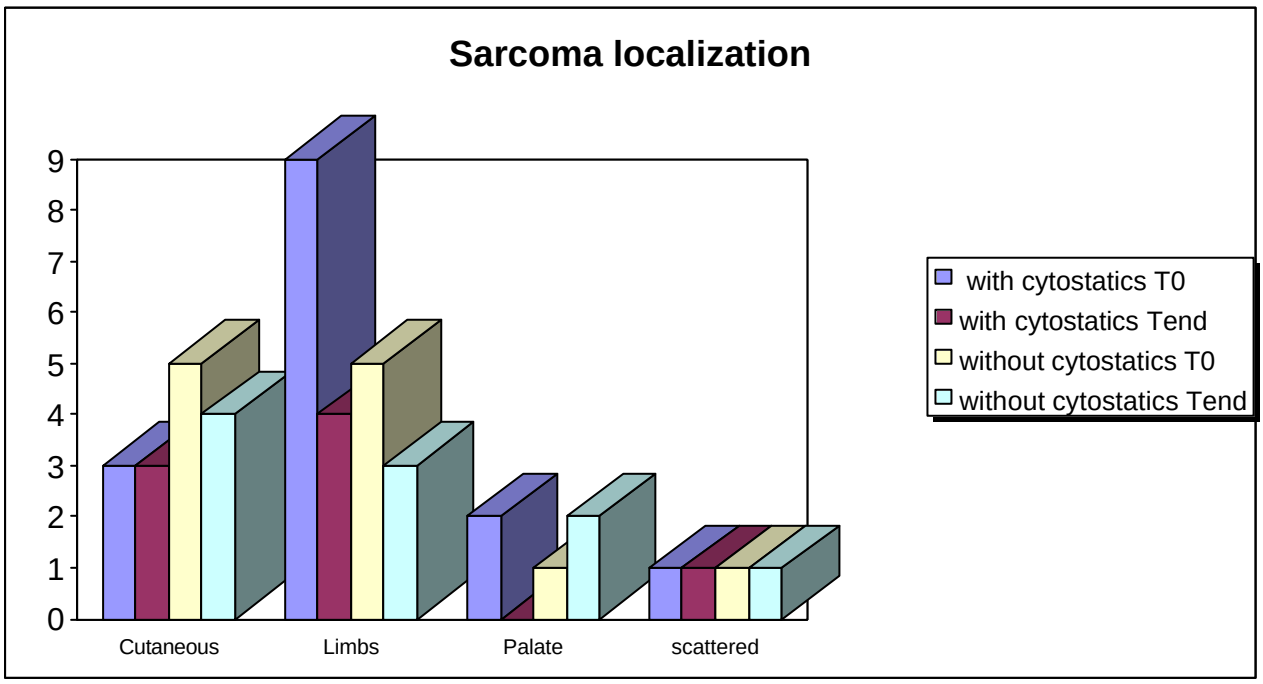
CD4 median value	with cytostatics (A) 7,8%		without cytostatics (B) 39,3%	
baseline	296 cell/ul3		188 cell/ul3	
after 6 months		319 cell/ul3		262 cell/ul3



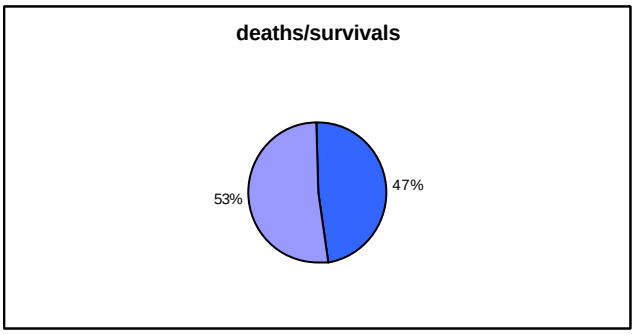
In the next table 13, we considered surviving patients with Kaposi Sarcoma coming to the Machava Center. We listed the initial diagnosis of Sarcoma, compared with time of disease remission, in group treated also with cytostatics (A, 15 patients) and the untreated group (B, 12 patients). It was not easy to determine the real point in which the sarcoma disappeared. We considered the point being the date when in the data-base reported the physician's note "asymptomatic" after several visits where they appoint the diagnosis of Sarcoma. In the treated group A 8 patients became asymptomatics (53%) and in the untreated group B 10 patients (83%).

Tab 13

SARCOMA LOCALIZATION	INITIAL DIAGNOSIS IN SURVIVAL PATIENTS WITH KS	INITIAL DIAGNOSIS IN A GROUP PATIENTS	A GROUP PATIENTS BEEN ASYMPTOMATICS	INITIAL DIAGNOSIS IN B GROUP PATIENTS	B GROUP PATIENTS BEEN ASYMTOMATICS
CUTANEOUS	8	3	3	5	4
LIMBS	14	9	4	5	3
PALATE	3	2	-	1	2
SCATTERED	2	1	1	1	1
TOTAL	27	15	8	12	10



Now we compare the surviving patients and died.



In this table 14, we compared the immunological and virological state of died patients versus surviving patients. There is a statistical significance in the immunological state difference. No statistical difference is observed in virological state.

TAB 14 CD4 BASELINE LEVELS

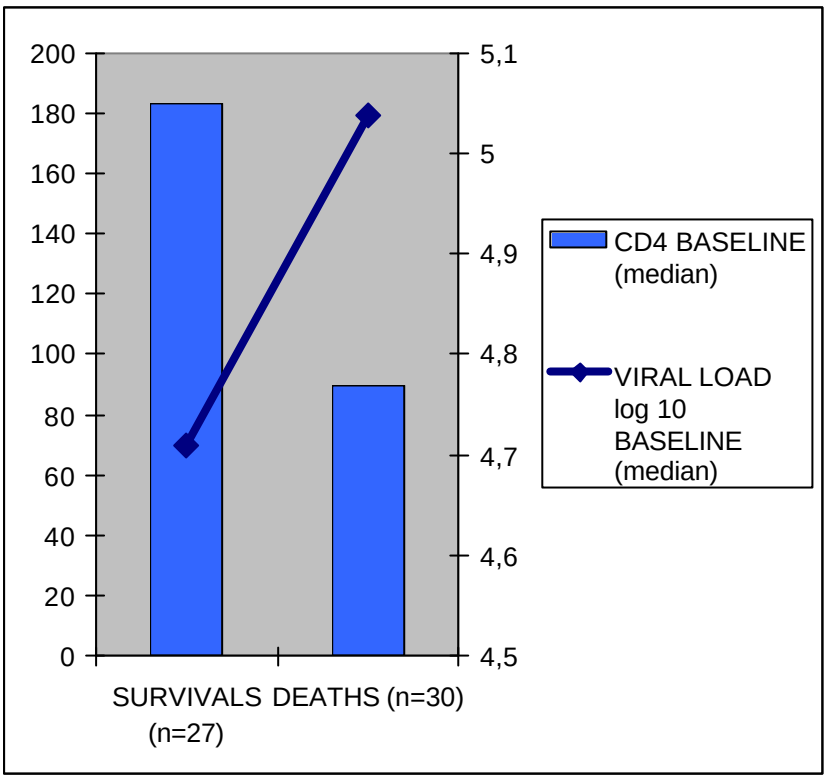
PATIENTS	MEDIAN	MIN	MAX
SURVIVALS (N=27)	183	1	528
DEATHS (N=30)	90	1	982

P<0,0005

VIRAL LOAD BASELINE LEVELS (LOG10)

PATIENTS	MEDIAN	MIN	MAX
SURVIVALS (N=27)	4,7099	0,0	5,6990
DEATHS (N=30)	5,0374	1	5,6990

p>0,005



Patients surviving have CD4 count higher than the patients died, and lower viral load baseline.

DISCUSSION

First of all, it is important to illustrate the optimisation in setting the Laboratory to obtain all the needed parameters, especially immunophenotyping, because this is a very important contribution from Pisa University and the Department of Oncology, Division of Surgical, Molecular and Ultrastructural Pathology.

The limitation of the strategy based on lymphocyte FS vs SS gate lies in the inaccurate identification of lymphocytes, especially in the presence of small monocytes and cell debris, which causes a variable degree of lymphocyte gate purity. On the contrary, in the PLG technique the panleucocyte marker CD45 displays a different quantitative expression among the various white blood cell types, which is used to accurately define leucocyte population along with their respective SS features. Lymphocytes are thus defined by a bright CD45 expression and a low side scatter (CD45 high SS low). The PLG gating strategy including CD8 causes a slight underestimation of the CD8 + cell subset and a higher CD4/CD8 ratio compared to the most credited high-technology methods that include CD3 staining.

We can observe table I summarizing the parameters of the regression lines obtained by comparing the percentage and absolute values of CD4 + and CD8 + , respectively, calculated with both protocols. In column A, the comparison between CD4% values obtained by TRIO and PLG shows an excellent correlation, with R 2 =0.964. Also the comparison between CD4 absolute value with the two methods is excellent with R 2 =0.976. As for CD8, percent comparison between TRIO and PLG shows R 2 =0.970. Absolute value comparison shows R 2 =0.988.

In the same table, in column B, the parameters of CD4 + regression, both on percentage and absolute data, have been analysed using cut off points of 15% and 200 CD4 2 /µL respectively, which clinicians consider as critical boundaries for HIV + patient classification.

The same parameters obtained in the CD4 <15% group were R 2 =0.689 and in the CD4 >15% group R 2 =0.947, respectively.

Figure 1 (a, b, c, d) depicts the correlations obtained by comparing CD4%, CD8%, CD4/µL and CD8/µL values obtained with the two protocols. The overall correlation between the

results obtained by the two protocols was satisfactory, with R always > 0.96. The correlation parameters in the subgroup with CD4 percentage <15% were worse than in the subgroup with CD4 + > 15% (R = 0.83).
Bland-Altman analyses are set out in Figure 2 (a, b, c, d), and the respective statistical parameters are summarized in Table II.

Both in the CD8 + % and CD8 + /µL comparisons the mean differences (bias) are negative, indicating a systematic underestimation of CD8 + cells with PLG. Column A summarizes statistical parameters for CD4 and CD8 percent and absolute value. The mean range difference is between -0.39 and -23.44 and the dev. standard range between 2.54 and 71. Column B illustrate the CD4% and the CD4 absolute value group stratified into the two clinical cut off values, 15% and 200 cell/µL respectively. The mean difference range is -0.02 and -9.14, dev. st. range is between 2.39 and 66.45.

The Bland-Altman stratified comparison of the two methods showed that when CD4 + cells were <15% a sizable negative bias was present, whereas when CD4 were >15% the two methods tended to overlap. This is a further confirmation of the counting inaccuracy introduced by the morphologic gating approach. CD8 absolute values also had a negative bias, and an high intercept, even if in presence of good R 2 values.
Moreover, in the CD4 <15% group the unsatisfactory agreement between the two analytical methods was also associated with a poor regression analysis (R 2 =0.689), thus confirming the bias introduced by the morphological gate. Indeed, the usage of CD8 without CD3 in the PLG protocol can be considered as an acceptable compromise between the availability of clinically relevant information and the need to contain costs, also considering the new trend by physicians that avoid to request CD8 determination. In the PLG protocol CD8 + cells must necessarily be identified as CD8 +bright only, to avoid the inclusion of CD3-neg CD8 +dim elements. This causes a systematic underestimation of percent and absolute CD8 + cells of variable magnitude, which explains the persistently negative bias when PLG-derived CD8 + cell counts are compared to the CD3-based routine method.

HHV8 prevalence in Mozambique

About the first goal of the study, HHV8 prevalence in Mozambique, the obtained results in the whole studied cohort (HIV+/- patients) showed that the HHV8 prevalence is very close to the one measured by other Authors in the area. Our data measured a prevalence of 51,07%, like in Zambia (51,1%) in a similar cohort of HIV+/- pregnant women, and in Malawi (54%) in a cohort of healthy volunteers (HIV status unknown) A lower percentage is registered in South Africa (30%). (Tab.3)

As can be seen, (Fig3), in Mozambique the HHV8 distribution is the same as in Zambia and Malawi, two countries bordering Mozambique to the north and with similar populations and socio-economic situations.

The results obtained in the two population groups are listed in table n.4: around half (52.7%) of the HIV-positive population turned out to be positive to HHV8 too, and around 49,4% of the HIV-negative population tested positive for HHV8, a very similar percentage.

In table n.5 the prevalence of HHV8 in the total studied Mozambican population described by sex ratio and median age. The median age is a little lower than the measured one in the whole DREAM cohort, sex distribution is very similar.

There isn't any influence of sex and age in the HHV8 positivity, the two groups result homogeneous by age and sex. . (tab 6)

Let us now consider some variables measured in the HIV positive sub-cohort.

As already said, for each sample the values of HIV1 viral load and the immunological parameters referred to the CD4 and CD8 sublymphocyte populations have also been determined. Depending on the reactivity to the ELISA test for HHV8, calculations have been made of the mean, with interval of confidence of 95%, standard deviation, standard error, minimum and maximum. The table n.7 summarizes the observed data and shows the significance referred to the considered variables. It can be observed that statistically significant correlation is represented only by the mean value of CD8+ lymphocytes. ($p>0,05$). In our experience it is important to monitor also Haemoglobin and BMI levels to have a quick and affordable information about the health state of the patient. When patients have BMI<18 or Haemoglobin level <8 g/dl their risk of death is increased. For this reason, for example, in the DREAM software a warning is active when these parameters are low. In the HIV sub cohort all the tested patients have BMI > 18 and no relationship exists between BMI and HHV8 positivity. The difference in Haemoglobin levels (0,5 g/dl)

seems to have a statistical (p=.035), but not clinical significance. In fact, all the tested patients have Hb > 8 g/dl. (tab 8)

The immune status seems in some way to be associated with HHV8 seropositivity, in relation either with a worse CD4 count, or with an increase of the mean of CD8 in the positive group. As known, we note the CD8+ T cell activation (43). Even if it is unclear how the immune response controls human herpesvirus 8 it seems that specific cytotoxic T lymphocytes (CTLs) may provide protection from persistent HHV8 infection. These results support the crucial role of cellular immune responses in controlling HHV8 replication, in preventing malignancies in latently infected subjects, and in conferring genuine resistance to persistent infection.(72) HHV8 interacts with the immune system through still unknown mechanisms. For example, the role of K3 and K5 in the deregulation of the Class I Histocompatibility Major Complex is being investigated (63)

Kaposi Sarcoma in HIV+ patients under ARV and treated or not treated with chemotherapy

Secondarily, we studied the clinical Kaposi Sarcoma evidence in HIV+ patients under ARV and treated or not treated with chemotherapy.

In the studied DREAM cohort, (14.011 HIV + patients in the Country) , we found 455 folder IDs with diagnosis of Kaposi's Sarcoma. Analysing the data, out of the 455 KS cases, only 353 were confirmed (78%). At this level, confirmation is made by the physician, by re-evaluating the patient situation and identifying the suspected lesions under an other disease and not KS. In Mozambique, in fact, the Kaposi Sarcoma diagnosis is usually only a clinical diagnosis, because anatomo- pathology laboratories are available only in Maputo and Beira. Among the 353 patients, only 150 (42,5%) were addressed to the Laboratory facilities, where the clinical diagnosis was always confirmed. Based on this results, we assumed that all the confirmed clinical diagnoses were correct. This way we calculated the disease incidence, (Kaposi Sarcoma cases number/whole DREAM cohort x 1000), for the considered period, about 4 years. Our results show that the Sarcoma incidence °/◦◦ in Mozambique is 25,2. The only information we have found about Sarcoma incidence in the region before the AIDS pandemic in the area is about males 0-64 years old, being the rate between 1,5-3,5 % (In the North of Mozambique the incidence was under 3,5 % and in the South of the country under 1,5%).(128)

The first evidence is that the incidence increases in the HIV+ cohort in all the Country especially in the Central area of the Country. The total incidence is tenfold the value registered in the past, before the AIDS era. If we consider Tab 9 we can observe the incidence related to each Health Centre. The highest values are registered in Quelimane (Central area) and Chokwe. In effect, we must remember that these Health Centres are inside Provincial Hospitals, and probably received patients coming from a wider area than the other ones. Mahotas has a lower incidence, but almost all the patients are children. Nevertheless, the only 2 cases observed are adults. Matola has the lowest incidence, but this is a Centre in a "Unidade de Saude Materno Infantil", (Mother-Child Health Division), so patients here are exclusively healthy women coming for the prenatal care, to whom also HIV testing is offered. Antiretroviral treatment is provided only for Mother to Child Prevention to the seropositive ones.

Kaposi Sarcoma doesn't increase in male group, like in Kaposi Sarcoma classic form, but it seems that females are more represented in the Kaposi group than in the HIV+ cohort (DREAM 64,7% C.I. 1,004, KS cohort 79,1% C.I. 3,23).

Patients with Kaposi Sarcoma are older than others. In fact, the disease is characteristic of old infection and this is the reason why the Kaposi Sarcoma cohort is more aged. tab 10

Patients with KS are identified at first contact, during the clinical visit. They represent an elevated risk group. CD4 are very low (baseline median 188 cells/ul^3) and viral load moderately high (baseline 55.000 copies/ml - 4,7404 $\log_{10}$).

All these patients started therapy immediately with the first-line regimen.

When possible, patients were transferred to the Maputo Central Hospital for specific treatment based on a combination of three different drugs, doxorubicin, bleomycin and vincristine (ABV).

Observing haematic parameters (CD4 and viral load) in the KS group, during the antiretroviral treatment, we noted a CD4 increase up to 263 cells/ul^3 median value, 40% more than the baseline value, and viral load is undetectable (<50 copies/ml) after 6 months. The same haematic parameters in the DREAM cohort (receiving HAART) are: CD4 155 (+- 54) cells/ul3 baseline, 348 (+-72) after 6 months of HAART, increasing of 124,5%, and viral load 15.600 baseline undetectable (<50 copies/ml) after 6 months decreasing of 97,9%. Tab 11

We can observe a very good response in the whole DREAM cohort on treatment, due to the efficacy of therapeutic line and the good patients' adherence. Both groups show an evident virological decrease. The immunological response is good also in the KS group,

even if among these patients a part (12%) is treated with cytostatics and the immunological improvement is lower than the whole cohort, due to the chemotherapy immunosuppression.

Analysing the KS group, and comparing CD4 values (tab 12) in patients receiving HAART and cytostatics (A), and in patients receiving only HAART (B), we can observe that in the group A the baseline CD4 median value is 296 cells/ul^3 and after 6 months 319 cells/um^3; The group B has the baseline CD4 median value 188 cells/ul^3, and after 6 months 262 cells/ul^3. It is evident that the immunological increase is difficult in group receiving cytostatics.

The problem seems to endure the indispensable time to obtain the drug's effects. If in the whole DREAM cohort, mortality is about the 7%, and patients need about 45 days' treatment , (the higher mortality is in this first period), in the KS sub cohort mortality is high (71 patients, the 13,5%) but concentrated in the first 80 days of treatment. Probably in this group of patients therapy needs more time to deploy its efficacy.

<u>Machava Centre: Patients with Kaposi Sarcoma</u>

Then, we analysed in details the first Health Centre activity, the Machava Centre, closed to the capital, Maputo, in the South of the Country. The data are referred to 4 years' activities. First of all, from our general data about HHV8 prevalence in the Country, we extrapolated the HHV8 prevalence in the local HIV+ cohort. In Machava Centre it was about 51,8% (on 133 screened patients) *vs* 51,07% being the prevalence of the Country, a very similar value. The Sarcoma incidence is 27,1 (2246 enrolled patients) *vs* 25,2 of the Country, very close to the general one. All Kaposi Sarcoma diagnoses were confirmed at the Pathological Anatomy Laboratory-Central Hospital of Maputo.

Among 61 KS patients, 31 patients died (50,8%) and 3 were transferred to other health centres.

So, we have in follow up 27 patients, 39 median age, 15 males (56%) and 12 females (44%). Here we cannot find gender difference.

Studying the 27 cases, it is evidenced that the Sarcoma diagnosis was done at the first visit, when the CD4 count was less than 200 cells/ul3 in 70% of cases, with median value of 183 cellss/ul3. As usual, all of them were in the 4th WHO Clinical Stage and they started the antiretroviral treatment.

About the diagnosis of Kaposi Sarcoma, we found 8 cutaneous, 14 lower or upper limbs localized, 3 mouth and tongue and 2 scattered Sarcomas.

At the end of the study, 4 patients had been on treatment for more then three years, 11 patients for more then two years, 9 patients for more than one year, 2 for more than six months and only 1 patient had been on treatment for just two months.
Only two patients have not immediately started the HAART treatment at the first visit, due to TB presence.
Two patients moved to the second line for treatment failure. A Protease inhibitor was introduced and the new treatment regimen was:

ddI +ABC +IND

Among the 27 patients, 15 (55,5%) started also a specific KS treatment, at the Maputo Central Hospital with doxorubicin, bleomycin and vincristine (ABV).14 of them with cytostatics and 1 patient started with radiotherapy and then was treated also with cytostatics.
As regards to antiretroviral regimen, 6 patients were treated with Indinavir (IDV) and not NVP, 5 of them were also under cytostatic treatment.
Among the 15 treated patients, one showed a complete remission, 7 were asymptomatic and 7 were in regression but still with some lesions.
Among the 12 untreated, 1 patient was in complete remission, 9 were asymptomatic and 2 still showed lesions, with one going into developing more lesions.
In the treated patients, the Sarcoma reverted in 8 patients after 12 months of both treatments (Chemotherapy and HAART).
In the untreated patients, who didn't start chemotherapy, the Sarcoma reverted in 10 cases, in 18 – 30 months, only with antiretroviral drugs.
About the 2 patients in second line, one was under chemotherapy too, while the other wasn't, and still there was an objective tumour regression within 1 year.
Among the 10 untreated patients reaching the asymptomatic state, 83%, had initial diagnosis of sarcoma (4 cutaneous, 2 mouth and tongue, 3 lower or upper limbs localized and 1 scattered sarcoma).
Among the 8 treated patients (53%) reaching the asymptomatic state, the initial diagnoses were 3 cutaneous, 4 limbs and 1 scattered sarcoma. (tab. 13). This result seems quite strange, but we must considere that the group A patients (treated also with cytostatics) have become asymptomatyc more quickly than the B group, even if it is difficult to

measure the statistical significance. In fact it was difficult to determine the exact moment when the Sarcoma disappeared.

About the 30 dead, we note that 26 patients (87%) have not started HAART (13 patients) or had been under treatment for less than 1 month (13 patients). This agreed with the evidence of the whole DREAM Cohort, where we noted that patients need at least 1 month's therapy to survive (5) Only four patients had been under antiretroviral treatment for more than 1 month when they died. About chemotherapy, only 4 patients (13,3%) started the treatment, but not the HAART. Tab 14 shows the baseline immunological and virological state for the survivors and the dead. As for immunological state, the difference between the two groups is statistically significant, with $p<0,0005$. This is the real reason for death. Unfortunately, as they were admitted, they were in worse conditions and antiretroviral therapy had no enough time to operate.

CONCLUSIONS

A major challenge for the Sub Saharan African health care providers is the implementation of an effective HIV therapeutic programme, supported by a minimum of laboratory monitoring that may guide patient management at locally affordable costs. Implementing laboratory facilities and cost-effective methods is possible in resource-limited settings. Conventional flow cytometry, adapted to meet locally affordable requirements still stands as the cheapest, yet most reliable and accurate technique to measure CD4 + T cells on a routine basis. Panleucogating uses only two antibodies (anti-CD45 and anti-CD4) and gives a clear-cut identification of CD4 + cells over the whole WBC and over lymphocytes as well. In particular CD4 + lymphocytes (CD45 high SS low CD4 high) are easily distinguished here from monocytes, whereas this does not occur when the gating strategy is based solely on physical parameters. In the PLG protocol it is also possible to add CD8-PCy5 in the same tube when the CD4/CD8 is requested. This represents a convenient and interesting alternative to the routine triple-color approach.

Viral load quantification, beside other tests as ELISA or biochemistry are now available also in Mozambique. Setting up the laboratory allowed us regular monitoring of antiretroviral therapy and potential toxicities. And in addition, it assisted us in monitoring HHV8 distribution, and studying immunological and virological characteristics of HIV+ patients with Kaposi Sarcoma.

This study confirms the free circulation of the HHV8 virus in the Mozambican population, with a prevalence rate (51,1%) similar to the one measured by other authors and with other analytical methods in bordering Countries, like Malawi and Zambia. Furthermore, it has emerged that in Mozambique, in the studied population, there is no association between seroprevalence for HIV and HHV8. The HHV8 prevalence, indeed, is similar between HIV positive and negative group. The higher incidence of Kaposi Sarcoma in the HIV-positive patients is probably related to other factors and different cofactors, such as human immunodeficiency virus (HIV) proteins, host-derived cytokines, chemokines, and growth factors, that seem to be required for the development of KS.

Immunosuppression as HIV-positive patients pass to the AIDS stage is not enough to explain the Kaposi Sarcoma clinical evidence. It should be noted that an

immunocompromised state is due to many other causes, not least, in the area under study, malnutrition, also in HIV -negative subjects. Nevertheless, using Haemoglobin levels and Body Mass Index as indicators, we have not found, in our population, any correlation with HHV8 positivity.

The expression of viral proteins capable of inactivating the p53 tumor suppressor protein has been implicated in KSHV oncogenesis, (*38)* and some authors like Whitby D and others have proposed environmental cofactors present in KS endemic regions to explain frequent reactivation of KSHV in infected subjects. (*118)*
As regards the HIV -positive cohort, the valuation of immunological and virological parameters in relation to positivity for HHV8 reveals a significant association as to CD8 values, suggesting the existence of one or more factors correlated to the two parameters. The function of cytotoxic T lymphocytes (CD8+T cells) in immunity to HHV8 in healthy individuals and in patients infected with HIV, and the role played by dendritic cells in the development of this immune response is under study. This research includes the characterization of immunodominant regions involved in the control of HHV8 infection by CD8+ T cells and their role in the progression to Kaposi's sarcoma in immunodeficient individuals*. (117)*

According to literature, the Kaposi Sarcoma related with AIDS is different from the Classic African Sarcoma. In Mozambique the incidence is higher, up to tenfold the incidence registered before the AIDS diffusion, but not related with the HHV8 prevalence. There isn't an increase in males, as in Classic form, but it seems that females are more represented in the group of patients with sarcoma. The tumour is responding to the antiretroviral treatment. The regression is evident even if patients are not treated with chemotherapy. Antiretroviral therapy increases the immunological response also in Kaposi Sarcoma patients, and also in patient group treated with cytostatics. The mortality is higher in the Kaposi Sarcoma group than in the whole cohort, but concentrated in the first 80 days of antiretroviral treatment. In addition, the group of dead patients showed worse immunological conditions. The presence of the tumour indicates a worse general condition in the patients, and an old HIV infection. Probably in this group of patients therapy needs more time to deploy its efficacy.
The evidence is that it is more important to treat AIDS, even if it is not possible to treat the correlate tumours like Kaposi Sarcoma.

Studying 27 patients in follow up in a DREAM Health Centre in Machava, since 2002 until the end of 2006, we noted that chemotherapy is not absolutely necessary to revert the most common kind of Kaposi Sarcoma excluding the pulmonary form. speeds up the regression and, even if it is always advisable, the most important thing is to start up the antiretroviral treatment, when chemotherapy is unavailable. About the use of a Protease Inhibitor like Indinavir, the antiretroviral drug often indicated as the best treatment in case of Kaposi Sarcoma in HIV positive patients, it was not possible to determine the real efficacy due to the small number of patients treated with the drug. More studies will be necessary in the future to monitor the cost-effective use of Indinavir *versus* a fixed dose regimen to treat patients with Kaposi Sarcoma.

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