PRESENCE OF CLASSIC KAPOSI'S SARCOMA WITH KSHV AND EBV VIREMIA, IMMUNE SUPPRESSION AND INCREASED PERIPHERALLY CIRCULATING MONOCYTES

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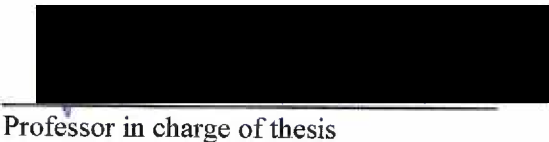

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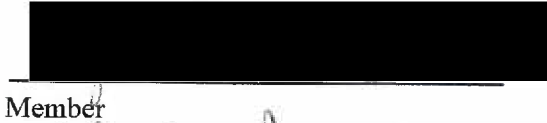

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B. ABSTRACT

Background: Classic Kaposi's sarcoma (CKS) is an inflammatory-mediated neoplasm that is endemic among elderly people of Mediterranean descent. While the etiology of CKS remains unclear, lesions develop in the presence of KS associated herpesvirus (KSHV), which is possibly reactivated by immunosuppression.

Methods: Using data and specimens from a case-control study of CKS in Sicily, Rome and Naples, we assessed peripheral blood KSHV and Epstein-Barr Virus (EBV) DNA load and hematological parameters, including proportions of CD4+ and CD8+ lymphocytes as markers of CKS risk among individuals manifesting antibodies against KSHV latent nuclear antigen (LNA)-1 by immunofluorescence assay (IFA). We used TaqMan real-time PCR to quantify KSHV-K6 and EBV-pol DNA copies/10^6 from cryopreserved peripheral blood mononuclear cells (PBMC) standardized to the human single copy ERV3 gene. CD4+ and CD8+ lymphocytes were detected by flow cytometry on fresh whole blood. Data were analyzed by logistic regression.

Results: Among 106 CKS cases and 137 KSHV LNA-1+ controls, KSHV DNA was detected in a greater proportion of cases (39.6%) than controls (16.1%; $p<0.0001$), whereas no difference in EBV DNA detection was observed by case-control status ($p=0.95$). Increasing levels of KSHV DNA load were associated with CKS compared to subjects with undetectable KSHV viremia (OR=3.34, 95% CI 1.43-7.83 with ≤52 KSHV copies/10^6 PBMC and OR=3.94, 95%CI 1.79-8.67 with >52 KSHV copies/10^6 PBMC; p-trend<0.0001). Likewise, KSHV DNA load was significantly associated with extent of disease (p-trend=0.005). A low absolute lymphocyte count (<1800 cells/μL) was associated with the presence of CKS (OR=1.76, 95% CI 1.04-2.96), although CKS risk was unrelated to levels of CD4+ lymphocytes ($p=0.70$), CD8+ lymphocytes ($p=0.23$) and CD4:CD8 ratio ($p=0.10$). With low lymphocytes, the probability of having CKS

was significantly increased both in the absence of KSHV viremia (OR=2.12, 95% CI 1.05-4.27) and in the presence of KSHV viremia (OR=7.26, 95% CI 2.83-18.66). Patients with CKS were significantly more likely to have monocytes above the median (≥408 cells/μL) and mild thrombocytopenia (<212 K/μL) compared to KSHV LNA-1 positive controls (OR=2.16, 95% CI 1.27-3.70 and 2.49 95% CI 1.19-5.20, respectively). In a multivariate model including gender, processing laboratory and an interaction between category of age and lymphopenia, CKS risk was elevated significantly with KSHV viremia (OR=4.12, 95% CI 2.02-8.44), monocytosis (OR=2.00, 95% CI 1.04-3.83) and among participants less than 70 years of age, a lymphocyte count below the median (OR=8.71, 95% CI 2.83-26.76).

Conclusion: Detectable PBMC KSHV DNA load is strongly associated with CKS. These data corroborate the specificity of KSHV for CKS pathogenesis and suggest an association with decreased platelet levels, monocytosis and a mild immune suppression, particularly among participants less than 70 years of age.

CHAPTER 1. INTRODUCTION

Classic Kaposi's sarcoma (CKS) is a rare inflammatory-mediated neoplasm that is endemic among elderly men of Eastern Mediterranean descent, particularly in southern Italy[1-4]. CKS lesions are characterized by multicentric red-purple patches on the skin that advance to plaque and nodular stages[2,5]. Histologically, KS lesions are derived from a lymphatic endothelial origin[6-10] and include proliferating spindle-shaped KS tumor cells that represent a heterogeneous population dominated by activated endothelial cells and macrophages of monocytic origin[5,11-16]. The extravasation of red blood cells, angiogenesis and infiltration of inflammatory cells including monocytes/macrophages, capable of producing pro-inflammatory cytokines[17], are hallmarks of early stage KS lesions[8,17-19]

While the etiology of CKS remains largely unclear, lesions develop in the presence of KS associated herpesvirus (KSHV)[20]. As a recently discovered member of the gammaherpesvirus subfamily, KSHV is characterized, in part, by its ability to persist in the host and alternate between a lytic and latent state. We hypothesize that when an imbalance between viral states occurs, an increase in lesional infiltrating inflammatory cells and subsequent upregulation of pro-inflammatory cytokines results in a vicious cycle of viral persistence and immune dysregulation thought to be important for the maintenance and progression of KS lesions. A change in immune competence, marked either by overall immune activation or suppression, may propagate this imbalance, thereby perpetuating the cycle. However, overall immune competence relative to KSHV lytic and latent infection and risk for disease as well as its progression in the absence of Human Immunodeficiency Virus (HIV)-1 infection, remain unclear.

Like KSHV, Epstein-Barr (EBV) is a gammaherpesvirus. Accordingly, EBV establishes persistence in the host, shares homology and cellular targets for infection and possibly similar

regulation pathways that may ultimately contribute to overall immune competence and to the formation of early CKS. The presence of EBV DNA has not been significantly correlated with CKS in the past[21,22]. However, it remains possible that the interaction between active co-infection with KSHV and EBV is related to the presence of CKS and markers of immune competence.

Thus, we examined the relationship between the presence of KSHV DNA load and surrogate cross-sectional measures of immune competence as predictors for the presence of CKS compared to controls that were seropositive for antibodies against KSHV latent nuclear antigen (LNA-1). Additionally, presence of CKS relative to EBV viremia alone and active co-infection with KSHV was evaluated to differentiate KSHV specific associations from a generalized activation of latent gammaherpesvirus due to immune dysregulation.

CHAPTER 2. METHODS

Study population

Study subjects from the Italian Kaposi's Sarcoma Case-Control study were used to assess the relationship between the presence of CKS with detectable levels of KSHV and EBV DNA in PBMCs and hematologic parameters. As described elsewhere[23], answers to a questionnaire, brief medical history, and blood were collected from participants from Eastern and Western Sicily including the provinces of Palermo, Ragusa, Siracusa and Catania, as well as sites located in Rome and Naples between April 13, 1998 and October 8, 2001.

CKS cases were enrolled from a compiled patient roster of population-based cancer registries and major referral centers. Eligible cases were defined as Italians 18 years of age or older, with a histologically confirmed diagnosis of CKS (International Classification of Disease-Oncology, third edition, M9140/3) and evidence for KSHV latent antibody by enzyme immunobsorbant assay (EIA) and immunofluorescence assay (IFA), living in the designated geographic regions at the time of study enrollment. One to two population-based comparison subjects were selected for each KS case during the same timeframe and frequency matched on age ($\pm$ 5 years or $\geq$ 80 years of age), sex and geographic region. Potential controls were screened for KSHV LNA-1 detection. Those testing seropositive by IFA were invited to provide informed consent and enrolled in the Italian Kaposi's Sarcoma Case-Control study. HIV-1 seropositive cases and controls were excluded. Approvals from appropriate Institutional Review Boards from the United States and Italy were obtained.

Laboratory methods

HHV-8 serostatus was previously determined by detection of LNA-1 in a 1:120 dilution of sera by immunofluorescence assay (IFA) using the BCBL-1 cell line with and without induction by tetradecanoyl phorbol-ester acetate (TPA)[23].

Viral loads of KSHV and EBV were examined in peripheral blood mononuclear cells (PBMC) by quantitative real time PCR. PBMC were frozen and stored in liquid nitrogen until tested. DNA was subsequently extracted from PBMC using the QIAmp DNA blood kit (Qiagen Inc. Valencia, CA), and the number of copies of KSHV and EBV DNA determined as previously described[24-27]. For KSHV, quantitative real-time amplifications were performed in triplicate against the KSHV K6 gene using the primers 5'-CGCCTAATAGCTGCTGCTACGG-3' and 5'-TGCATCAGCTGC CTAACCCAG-3' and the probe: 5'-CACCCACCGCCCGTCCAAATTC-3', on an ABI Prism 7700 (P.E. Biosystems, Foster City, CA). For EBV, amplifications were performed against the EBV pol gene region using the primers: 5'-AGTCCTTCTTGGCTAGTCTGTTGAC-3' and 5'-CTTTGGCGCGGATCCTC-3' and the labeled probe: 5'-O-CATCAAGAAGCTGCTGGCG GCC-8-3'. The number of KSHV and EBV copies detected in the triplicate samples were averaged and normalized to the number of PBMC (copies/10^6 cells) as determined by parallel quantification of the human ERV-3 gene, using the following primers 5'-CATGGGAAGCAAGGGAACTAATG-3, and 5'-CCCAGCGAGCAATACAGAATTT-3' and the labeled probe: 5'-(FAM)TCTTCCCTCGAACCTGCACCATCAAGTCA(TAMARA)-3'[28]. The lower limit of detection for KHSV and EBV were 3 and 10 copies per million PBMC, respectively.We measured hematologic parameters including leukocyte count and differential count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red-cell distribution width, platelet count, mean platelet volume, and platelet distribution width using fresh venous whole blood. Complete blood counts were performed by standard protocol using a Coulter S-Plus Jr. automated hematology analyzer

(Coulter Electronics, Hialeah, FL), which has a linear range of 0 to 999 x 10^9/L for platelets and 0 to 99 x 10^9/L for white blood cells. Peripheral blood lymphocytes were evaluated by flow cytometry on fresh whole blood. Total T-lymphocytes (helper/inducer and suppressor/cytotoxic) were estimated using monoclonal antibodies for CD4 and CD8. Absolute numbers and percentages of total lymphocytes and corresponding subpopulations were determined. All blood studies were analyzed by centralized laboratories (F. Vitale, Dipartimento di Igiene e Microbiologia "Giuseppe D'Alessandro", Universitá degli studi di Palermo and G. Rezza, Laboratorio di Epidemiologia e Biostatistica, Istituto Superiore di Sanitá, Rome) at the time of study enrollment.

Statistical analysis

We conducted all analyses using strategies appropriate for a frequency-matched case-control study. Age was divided into tertiles (<69, 69-79 and >79 years of age). Study sites were stratified by North and South, corresponding with 2 centralized processing and hematology laboratories located in Rome and Palermo, respectively. Blood cell parameters were dichotomized at median values and, KSHV and EBV viremia were dichotomized into 2 groups, present or absent, where DNA levels < 10 copies per 10^6 cells were considered absent and DNA levels ≥ 10 copies per 10^6 cells were considered present. For blood parameters with significance, $p \leq 0.10$, categories were split at the 10th percentile. Non T-lymphocytes, a surrogate for B lymphocytes, were defined as the difference between total lymphocytes and the sum of total CD4+ and CD8+ T-lymphocytes. KSHV and EBV DNA load were further evaluated by tertiles based on the distribution of detectable PBMC DNA in controls. Individuals with KSHV DNA load <10, 10-52 and >52 copies per 10^6 cells were considered to have none, low and high levels of viremia, respectively. Likewise, persons with detectable EBV viremia <10, 10-342 and >342 copies per 10^6 cells were considered to have none, low and high levels of viral load. We used Pearson χ^2 and 2-sample t-

test to assess differences in proportions and means, respectively. For log transformation, 0 values of viral load were replaced with half of the lowest value used for detectable load (5 copies per 10^6 cells). The geometric mean was used to compare continuous levels of KSHV and EBV DNA load.

The presence of CKS was estimated by the odds ratio (OR) and corresponding 95% confidence interval (CI) calculated by logistic regression[29]. Trends were assessed by modeling ordered categorical variables as continuous. All analyses with respect to the relationship between the presence of disease and viremia or hematological markers were adjusted for category of age, gender and processing laboratory site. Additional potential confounders were examined but excluded from final adjustment because they were either unrelated to CKS or PBMC KSHV DNA detection and quantification. These factors included smoking status, comorbidities, personal hygiene, sexual history, factors associated with childhood crowding and absolute platelet count. Statistical significance, based on multivariate logistic models, was calculated by the maximum conditional likelihood test and chi-squared. Significant differences between strata were determined by the Breslow-Day, χ^2 test for homogeneity. Variation in the magnitude of the effect of immune suppression and presence of CKS by category of age was assessed by including an interaction term combining age and lymphopenia into the fully adjusted model. The likelihood ratio was used to compare full and main effects models. A 2-tailed p-value less than or equal to 0.05 was considered significant. All analyses were conducted using STATA version 8.0 (College Station, TX, USA).

CHAPTER 3. RESULTS

KSHV and EBV viremia

During enrollment, 141 CKS cases and 192 healthy volunteers with evidence for KSHV latent antibody were enrolled in the parent Italian Kaposi's Sarcoma Case-Control study. For all current analyses, cases and controls reporting use of corticosteroid or cancer chemotherapy were excluded. Of the remaining 288 therapy-naïve participants, DNA from 20 incident cases (diagnosed ≤1 year from enrollment), 85 prevalent cases (diagnosed >1 year from enrollment), 1 case with unknown disease chronicity, and 137 controls were available for analysis. Approximately 21% of all CKS cases had current lesions are the time of enrollment. Hematologic parameters were available for 88.5% of the therapy-naïve population.

As shown in Table 1, a significant difference in the detection of KSHV DNA load in PBMCs was observed in cases compared to controls, both seropositive for anti-KSHV latent antigen (39.6% versus 16.1%, respectively, $p<0.00001$). No difference in the detection of EBV DNA load was observed by case-control status (55.2% versus 54.8%, respectively; $p=0.95$). DNA detection frequencies of both gammaherpesviruses were similar by category of age and processing laboratory. However, female cases had a significantly higher proportion of detectable PBMC EBV DNA (72.4%) compared to their male counterparts (48.7%; $p=0.03$).

Among persons with detectable viremia, KSHV DNA load was modestly higher in CKS cases compared to controls (mean $\log_{10}$ KSHV load per 10^6 cells = 2.06 and 1.71, respectively; p-value=0.09; data not shown). No difference was observed in either the detectability of EBV DNA or its quantification by case-control status. However, the detection of PBMC EBV DNA was higher overall than viremia by KSHV (55.0% versus 26.3% for EBV and KSHV, respectively; $p<0.00001$), suggesting greater ubiquity of EBV in this population.

KSHV DNA was detected significantly more often in PBMCs from cases compared to KSHV seropositive controls (OR=3.42, 95% CI 1.85-6.32; Table 2). By quantifying the level of detectable KSHV DNA, CKS patients were approximately 3-fold more likely to have low levels (10 – 52 copies per 10^6 cells) and 4 times more likely to have high levels (>52 copies 10^6 cells) of KSHV DNA load detected in PBMCs than controls without detectable KSHV viremia (p-trend<0.0001).

No association with gradient levels of EBV DNA load and risk for CKS were detected (p-trend=0.89). CKS cases were less likely to have dual EBV and KSHV viremia than KSHV viremia alone, although the difference was not statistically significant. Among participants with detectable KSHV DNA, EBV viremia did not significantly contribute to the probability of CKS (OR=0.63, 95% CI 0.19-2.11; data not shown).

Hematological parameters

In general, lower mean values of red blood cell (RBC) parameters were seen in CKS cases compared to controls (Table 3). While only marginal differences in mean values of hematocrit and hemoglobin were seen between the two groups, the mean platelet count was significantly lower in cases (204 K/μL) than in controls (214 K/μL; p≤0.047). As shown in Table 4, patients with CKS were significantly more likely to exhibit low platelets (<212 K/μL) compared to controls (OR=2.49, 95% CI 1.19-5.20), with a similar probability when thrombocytopenia was redefined at the 10th percentile (<150 cells/μL; OR=2.49, 95% CI 0.93-6.69; data not shown).

Overall, mean values of white blood cell (WBC) parameters were also lower in cases. Despite similar distributions of leukocytes between groups (p=0.30), mean total lymphocytes were significantly lower in CKS cases than controls (p=0.014). In participants <69 years of age, mean

absolute lymphocytes were modestly lower among cases compared to controls ($p=0.06$). However, no trend with lymphocyte levels and increasing age (Figure 1) or with disease chronicity (data not shown) was observed. Patients with CKS were significantly more likely to have low lymphocyte counts (<1800 cells/μL) overall, compared to controls (OR=1.76, 95% CI 1.04-2.96; Table 4). In support of this association, CKS risk was significantly elevated when lymphopenia was redefined using values below the 10th percentile (<1000 cells/μL; OR=5.76, 95% CI 1.52-21.75, data not shown).

CKS was weakly related to low levels of T-lymphocyte subpopulations, including absolute CD4+ cells (OR=1.11, 95% CI 0.66-1.85), CD8+ cells (OR=1.38, 95% CI 0.82-2.33) and CD4:CD8 ratio (OR=0.64, 95% CI 0.38-1.09). Among persons with low CD4+ count, 73.2% had total lymphocytes below the median, suggesting that low total CD4+ cells may be directly related to the availability of total lymphocytes for analysis. However, after controlling for absolute CD8+ T-lymphocytes and non T-lymphocytes, estimates related to CD4+ lymphopenia as well as CD4:CD8 ratio and CKS remained unchanged ($p \geq 0.89$).

Monocytes, a cellular target for productive KSHV infection, were higher in CKS cases compared to controls ($p=0.007$). An elevated total monocyte count above the median (≥408 cells/μL) was detected nearly twice as often in CKS cases than in controls. This association was not altered when monocytes were expressed as a percent of total leukocytes ($p=0.001$) or redefined at the 90th percentile (≥630 cells/μL; OR=2.30, 95% CI 1.30-4.07; data not shown).

As shown in Table 5, in the presence of KSHV viremia, patients with CKS were significantly more likely to exhibit mild thrombocytopenia (<212 K/μL; OR=9.88, 95% CI 2.83-34.44), though only modestly in the absence of viremia (OR=1.67, 95% CI 0.59-4.63). Likewise, in the presence of detectable KSHV DNA in PBMCs a high absolute monocyte count was associated

with the presence of CKS (OR=6.09, 95% CI 2.45-15.13), but significance was not achieved without KSHV viremia (OR=1.53, 95% CI 0.75-3.10). With low lymphocytes (<1800 cells/μL), the probability of disease was significantly increased both in the presence and absence of KSHV viremia (OR=7.26, 95% CI 2.83-18.66 and OR=2.12, 95% CI 1.05-4.27, respectively).

To assess differences in survivorship among patients with CKS relative to KSHV viremia and hematologic parameters, we conducted a subanalysis of incident and prevalent disease. Among persons with detectable viremia, the level of KSHV DNA load was significantly higher in incident CKS cases compared to controls (mean $\log_{10}$ KSHV load per 10^6 cells = 2.36 and 1.74, respectively; p=0.02), although levels of KHSV DNA load did not differ between incident and prevalent cases (p=0.19). The mean platelet count was significantly lower in patients with greater disease chronicity (prevalent disease >5 years) compared to controls (mean=178.89 and 214.26, respectively; p=0.009) and among cases, a low platelet count (<212K/μL) was more frequent in prevalent compared to incident CKS (72.2% and 45.5%, respectively; p=0.03). Among incident cases, low lymphocyte levels (<1800 cells/μL) were observed significantly more often than among controls (OR=3.14, 95% CI 1.06-9.28), though only modestly when controls were compared to cases with prevalent disease (OR=1.57, 95% CI 0.90-2.72). Monocytes were higher in CKS cases with both incident (p=0.048) and prevalent disease (p=0.02) compared to controls.

Overall, 21.2% of CKS cases did not have current lesions at the time of enrollment. Most risk estimates described above were not substantially different in an analysis restricted to CKS patients with at least one current CKS lesion. The exception was that KSHV DNA detected in PBMCs was significantly associated with having at least one current lesion (OR=4.40, 95% CI 2.27-8.53) and was unrelated to patients without lesions (OR=1.22, 95% CI 0.37-4.05). Likewise, compared to patients with CKS but without current lesions, KSHV viremia was found only

slightly more often among patients with one CKS lesion (OR=1.26, 95% CI 0.26-6.10) and significantly more often with multiple lesions (OR=4.66, 95% CI 1.35-16.11; p-trend=0.005, data not shown).

In a multivariate model (Figure 2) including category of age, gender, processing laboratory, presence of KSHV viremia, an absolute lymphocyte count below the median and absolute monocyte count above the median and an interaction term combining the effects of lymphopenia and category of age, the likelihood of CKS was elevated significantly with detectable KSHV viremia (OR=4.12, 95% CI 2.02-8.44), monocytosis (OR=2.00, 95% CI 1.04-3.84) and among participants less than 70 years of age, mild lymphopenia (OR=8.71, 95% CI 2.83-26.76). Absolute CD4+ T-lymphocytes, platelets and basophils were considered jointly in multivariate analysis, but did not contribute to overall fit of these data. Factors associated with CKS were not substantially altered in multivariate analyses restricted to incident or prevalent CKS cases. No additional joint effects, confounding or colinearity between variables were observed.

CHAPTER 4. DISCUSSION

The consistent presence of KSHV in tissues from patients with all forms of KS, its viral oncogenic properties[30] and presence antecedent to KS lesion formation[24] indicate that this virus plays an important role in KS pathogenesis. Compared to KSHV LNA-1 seropositive controls, our findings show that CKS cases were significantly more likely to have KSHV DNA detected in their PBMCs. Based on a similar study design in HIV-1 positive men who have sex with men (MSM) from the US, the risk associated with AIDS-KS after adjusting for HIV-1 load, was similar to our findings (OR=7.9)[31]. However, residual confounding or factors associated with HIV-1 infection may have magnified the effect of KSHV viremia on the risk of disease. We found that KSHV viremia was detected more often among CKS cases with multiple lesions compared to those with one or no current lesion, thus corroborating the role of viremia with extent of disease as previously demonstrated in patients with AIDS associated-KS[24]. However, the 3.3-fold increase in risk of CKS observed with the presence of low viremia compared to the 3.9-fold risk with high viremia suggests a predominately qualitative association in which the presence or absence, rather than the level, of viral load contributes to the likelihood of disease. Conversely, levels of EBV DNA load did not differ by case-control status, corroborating prior investigations[21,22]. Co-infection with KSHV and EBV significantly predicted presence of CKS, but was not different than the estimate achieved by KSHV infection alone. Thus, these findings support the specificity of KSHV for KS.

Immunosuppression plays a distinct and critical role in the pathogenesis of AIDS-associated KS. KS is an opportunistic AIDS-defining malignancy[11] with high incidence rates among other immunosuppressed persons including those undergoing solid organ transplantation[32,33] or receiving immunosuppressive therapy[34]. Additionally, KS is characterized, in part, by its ability to spontaneously resolve when immunity improves[35-37]. However, the role of immune competence

in Classical KS is controversial. Evidence suggests that the pathogenesis of CKS may be related to both immune stimulation, marked by upregulated levels of inflammatory markers including neopterin and β2-microglobulin[38-40] and immune suppression, marked by a reduction in T-cells[41]. Touloumi et al, reported elevated risk of CKS with low absolute lymphocytes (≤1750 cells/μL; OR=2.06) and CD4+ lymphopenia (≤1200 cells/μL; OR=2.73) compared to hospital-based controls in Greece[38]. Risk for CKS with CD8 lymphopenia (<536 cells/μL) was increased, though not significantly (OR=1.86)[38]. Similar evidence exists for mild immune suppression and risk for CKS from other investigations conducted throughout Italy and Greece, but the KSHV serostatus of the comparison groups was unknown[40,42,43].

Our estimates are based on the comparison of CKS cases to KSHV LNA-1 seropositive controls, which allow us to examine cofactors necessary for pathogenesis beyond that which could be attributed to KSHV infection. While similar distributions of CD4 and CD8 T-lymphocytes were observed between groups in our study population, mild lymphopenia was observed more often in our CKS cases compared to controls. Sub-analyses suggested that modest lymphopenia was only associated with recently diagnosed CKS and in patients less than 70 years of age. While an explanation for this is not immediately obvious, interactions between disease markers and age are not unusual. For example, prior to the availability of Highly Active Antiretroviral Therapy (HAART), severe CD4+ lymphocyte deficiency was more highly correlated with AIDS risk for adults than for children and adolescents[44]. Additional investigation is needed to validate whether the risk of CKS with lymphopenia differs by age and, if so, to probe its mechanism including the possibility of immune senescence with increasing age.

Monocytes and B cells are known cellular targets for KSHV infection. Contrary to prior reports[38], we observed an increase in the number of peripherally circulating monocytes among incident and prevalent CKS cases both before and after adjusting for the total number of lymphocytes and

presence of PBMC KSHV viremia. In addition, we found a slight non-significant increase in non-T cells, a surrogate for B cells, among cases (OR=1.37). These findings support observations of Drs. Blasig and Monini suggesting that peripherally circulating monocytes are essential for promoting CKS lesion formation and maintenance by differentiating into macrophages that harbor latent infection and that recruit KSHV to tissues by propagating reactivated virus to neighboring cells[12,13]. Given that monocytes are cellular targets for KSHV lytic infection[13], we might expect patients with CKS to have more circulating monocytes to accommodate higher viral burden and therefore, as a consequence rather than a cause of their disease. However, even CKS patients without current lesions had significantly higher absolute monocytes. Thus, the contribution of these macrophage precursors may be less directly related to tumor burden than to propagating virus.

Based on univariate analysis, we report for the first time that platelet counts were lower in CKS patients with KHSV viremia, particularly among those with chronic disease. This finding suggests that platelets might be associated with the pathogenesis of CKS either locally at the lesion site(s) or as a result of a more generalized inflammatory process precipitated by active KSHV infection, which alters platelet homeostasis.

As with any case-control study, our study has limitations. First, while TaqMan PCR, used for the detection and quantification of KSHV and EBV DNA load, is highly accurate and reproducible, we might expect a greater proportion of our CKS cases to show evidence of KSHV viremia given the endemicity of this virus particularly in Southern Italy[22]. Thus, we may be nondifferentially underestimating the prevalence of viremia in this study population and correspondingly, underestimating the relative odds of CKS given the presence of KSHV viremia. On the contrary, it is unlikely that misclassification of platelet, lymphocyte or monocyte cell counts would have transpired either by laboratory method or specimen handling, given that blood parameters did not

significantly differ by processing laboratory. Second, universally accepted normal ranges for clinically useful components of the peripheral blood vary substantially. Therefore, we used the median, in addition to the 10th percentile, to define abnormal blood parameters based on the distribution in our comparison population. Accordingly, lymphocyte, platelet and monocyte levels associated with CKS in this investigation are not considered clinically significant. Nonetheless, they may ultimately prove of value in the understanding CKS pathogenesis or in the determination of those at highest risk.

In this context, we believe that it is unlikely that lymphopenia differentially affected survivorship between cases and controls because our definition of lymphopenia was well within normal clinical limits (<1800 cells/μL). Additionally, low lymphocyte counts were detected with greater frequency in CKS patients younger than 69 years of age and among patients with incident CKS. However, patients with incident disease were not significantly younger than patients with prevalent disease or compared to controls. Third, while we restricted our study population to include participants not using immune modulating drugs, we cannot rule out the possibility that incomplete information about concomitant medication use, particularly among younger participants, did not influence hematologic parameters. Likewise, comorbidities unaccounted for and beyond those considered in the analysis could potentially confound our findings. Finally, while these cofactors are important in CKS etiology, we are limited by the extent to which the detection of KSHV DNA in PBMCs, immune suppression and hematologic parameters can be considered the cause rather than a consequence of CKS.

CHAPTER 5. SUMMARY AND CONCLUSIONS

In summary, we found that the presence of KSHV viremia was the single most important factor associated with CKS (OR=4.12), followed by a modestly elevated monocyte count (≥408 cells/µL; OR=2.00) and among the youngest participants, evidence for a reduction in the absolute number of lymphocytes (<1800 cells/µL; OR=8.71). Our findings corroborate the specificity of KSHV in KS pathogenesis and do not support a role for marked immune deficiency in non-AIDS KS. Instead, a mild and perhaps transient immune suppression occurs in the early stages of CKS and particularly among those less than 70 years of age. In addition, we report a reduction in platelets with KSHV viremia among cases with CKS, but not in the absence of viremia. Based on these findings, additional studies are warranted to determine what events related to immune competence take place prior to KSHV reactivation and whether an upregulation of events, including viral replication and increased circulating monocytes and their relationship with pro-inflammatory cytokines, are responsible for propagating CKS onset as well as more extensive and late staged disease.

CHAPTER 6. REFERENCES

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C. TABLES

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Table 1. Detectable KSHV and EBV DNA in PBMCs among Classic Kaposi's sarcoma cases compared to KSHV LNA-1 positive controls by gender, age and geographic region in Italy

	Case (n=106*)	Control (n=137)	p-value
Gender			
Male	76	96	
KSHV detected (%)	30 (39.5)	14 (14.6)	0.0002
EBV DNA detected (%)	37 (48.7)	50 (52.6)	0.61
Female	30	41	
KSHV detected (%)	12 (40.0)	8 (19.5)	0.06
EBV detected (%)	21 (72.4)	24 (60.0)	0.29
Age (years)			
≤69	41	44	
KSHV detected (%)	14 (34.1)	5 (11.4)	0.01
EBV detected (%)	23 (56.1)	21 (47.7)	0.44
70-79	38	59	
KSHV detected (%)	16 (42.1)	9 (15.3)	0.003
EBV detected (%)	23 (60.5)	34 (57.6)	0.78
>79	27	34	
KSHV detected (%)	12 (44.4)	8 (23.5)	0.08
EBV detected (%)	12 (46.2)	19 (59.4)	0.32
Geographic region in Italy			
South	69	111	
KSHV detected (%)	25 (36.2)	18 (16.2)	<0.0001
EBV detected (%)	35 (51.5)	58 (52.7)	0.87
North	37	26	
KSHV detected (%)	17 (45.9)	4 (15.4)	0.01
EBV detected (%)	23 (62.2)	16 (64.0)	0.88

*EBV load was not measured for 3 individuals with measured KSHV load. These include one 89 year old female case from the South, one 89 year old female control from the North and one 85 year old male control from the South

Table 2. Detection of KSHV and EBV DNA load in PBMCs among cases with Classic Kaposi's sarcoma and controls

	Cases (n=106)	Controls (n=137)	Adjusted OR*	95%CI*
Detected PBMC KSHV and EBV DNA				
KHSV DNA				
Absent	64	115	1.00	
Present	42	22	3.42	1.85-6.32
EBV DNA				
Absent	47	61	1.00	
Present	58	74	0.96	0.57-1.64
Quantified PBMC KSHV and EBV DNA load				
KSHV DNA copies per 10^6 cells				
None (<10)	64	116	1.00	
Low (10-52)	18	10	3.34	1.43-7.83
High (>52)	24	11	3.94	1.79-8.67
EBV DNA copies per 10^6 cells				
None (<10)	47	61	1.00	
Low (10-342)	30	37	0.99	0.53-1.87
High (>342)	28	37	0.93	0.49-1.77
Detected KSHV and EBV DNA load				
KSHV-/EBV-	31	55	1.00	
KSHV-/EBV+	33	58	0.97	0.52-1.81
KSHV+/EBV-	16	6	4.64	1.62-13.27
KSHV+/EBV+	25	16	2.62	1.18-5.80

* ORs adjusted for category of age, gender and processing laboratory
Numbers do not sum to totals due to missing data
Odds ratio (OR)
95% Confidence Interval (95% CI)

Table 3. Mean values and standard errors (SE) of red and total white blood cell counts including lymphocyte subpopulations and differential counts in Classic Kaposi's sarcoma cases and KHSV LNA-1 positive controls

	CKS cases (n=106) Mean (SE)	**Controls (n=137) Mean (SE)**	**p-value***
Hematocrit (%)	42.47 (0.44)	43.41 (0.37)	0.089
Hemoglobin (g/dL)	13.87 (0.15)	14.00 (0.15)	0.162
Platelets (K/μL)	203.79 (8.76)	214.26 (5.44)	0.033
Leukocytes (K/μL)	6461.26 (197.17)	6650.79 (144.00)	0.297
Total lymphocytes (cells/μL)	1788.91 (85.35)	1946.58 (61.09)	0.014
Total CD4+ lymphocytes (cells/μL)	778.41 (37.52)	819.05 (24.47)	0.063
Total CD8+ lymphocytes (cells/μL)	395.64 (19.67)	446.34 (21.31)	0.051
CD4:CD8 ratio	2.55 (0.15)	2.29 (0.11)	0.150
Non T-cells (cells/μL)	622.00 (80.31)	682.20 (38.49)	0.479
Neutrophils (cells/μL)	3714.8 (157.7)	3970.1 (129.0)	0.234
Monocytes (cells/μL)	504.0 (22.2)	436.8 (14.6)	0.007
Basophils (cells/μL)	36.4 (45.0)	61.8 (7.1)	0.013
Eosinophils (cells/μL)	182.7 (33.0)	163.0 (10.9)	0.721

* Adjusted for processing laboratory, category of age and gender using linear regression
Means are reported as non-log-transformed values for blood parameters

Table 4. Hematologic parameters in cases with Classic Kaposi's sarcoma and controls

	Cases (n=103)	Controls (n=152)	Adjusted OR*	95%CI*
Hematocrit (%)				
High (≥43.25)	48	76	1.00	
Low (<43.25)	55	76	1.17	0.68-2.04
Hemaglobin (g/dL)				
High (≥14.3)	43	77	1.00	
Low (<14.3)	60	75	1.62	0.91-2.86
Platelets (K/μL)				
High (≥212)	20	58	1.00	
Low (<212)	36	57	2.49	1.19-5.20
Leukocytes (K/μL)				
High (≥6400)	47	76	1.00	
Low (<6400)	56	76	1.17	0.71-1.95
Total lymphocytes (cells/μL)				
High (≥1800)	40	77	1.00	
Low (<1800)	63	74	1.76	1.04-2.96
CD4 lymphocytes (cells/μL)				
High (≥759)	48	75	1.00	
Low (<759)	52	75	1.11	0.66-1.85
CD8 lymphocytes (cells/μL)				
High (≥393)	43	75	1.00	
Low (<393)	56	75	1.38	0.82-2.33
CD4:CD8 ratio				
High (≥1.94)	60	76	1.00	
Low (<1.94)	39	75	0.64	0.38-1.09
Non T-cells (cells/μL)				
High (≥601)	43	75	1.00	
Low (<601)	57	75	1.37	0.82-2.31
Neutrophils (cells/μL)				
High (≥3,789)	22	46	1.00	
Low (<3,789)	27	46	1.22	0.59-2.51
Basophils (cells/μL)				
High (≥42)	16	53	1.00	
Low (<42)	36	45	2.18	1.02-4.64
Monocytes (cells/μL)				
Low (<408)	31	71	1.00	
High (≥408)	71	74	2.17	1.27-3.70
Eosinophils (cells/μL)				
High (≥132)	25	54	1.00	
Low (<132)	31	54	1.59	0.79-3.22

* ORs adjusted for category of age, gender and processing laboratory
Numbers do not sum to totals due to missing data
Odds ratio (OR)
95 % Confidence Interval (CI)

Table 5. Hematologic parameters with KSHV DNA in PBMCs among cases with Classic Kaposi's sarcoma and controls

	Cases (n=106)	Controls (n=137)	Adjusted OR*	95%CI*
Platelets and KSHV viremia				
Platelets high / KSHV DNA-	10	43	1.00	
Platelets low / KSHV DNA-	14	41	1.67	0.59-4.63
Platelets high / KSHV DNA+	8	3	10.12	1.84-55.73
Platelets low / KSHV DNA+	15	10	9.88	2.83-34.44
Monocytes and KHSV viremia				
Monocytes low / KSHV DNA-	18	48	1.00	
Monocytes high / KSHV DNA-	34	54	1.53	0.75-3.10
Monocytes low / KSHV DNA+	10	8	3.20	1.06-9.65
Monocytes high / KSHV DNA+	26	11	6.09	2.45-15.13
Total lymphocytes and KSHV viremia				
Lymphocytes high / KSHV DNA-	20	57	1.00	
Lymphocytes low / KSHV DNA	33	50	2.12	1.05-4.27
Lymphocytes high / KSHV DNA+	14	10	3.81	1.42-10.23
Lymphocytes low / KSHV DNA+	22	10	7.26	2.83-18.66

* ORs adjusted for category of age, gender and processing laboratory
Low total lymphocytes, low CD4+ cells and platelets were defined by the median in controls (1800 cells/μL, 759 cells/μL and 212 K/μL, respectively)
Numbers do not sum to totals due to missing data
Odds ratio (OR)
95% Confidence Interval (95% CI)

D. FIGURES

D.1 FIGURE LEGEND

D.2. FIGURE 1

Comparison of the absolute mean $\log_{10}$ lymphocyte level in cases with classic Kaposi's sarcoma and population-based controls stratified by category of age (≤69 years, 70-79 years and >79 years).

D.3. FIGURE 2

Adjusted odds ratios [and 95% confidence intervals (CI)] of classic Kaposi's sarcoma from the multivariate logistic regression model. The full model included gender (data not shown), as well as detection of KHSV DNA in peripheral blood mononuclear cells [(PBMC) present or absent], monocytosis (above or below the median, ≥408 cells/μL), processing laboratory (North or South) and the joint effect of low lymphocyte levels (above or below the median, ≥1800 cells/μL) and category of age (≤69 years, 70-79 years and >79 years).

Figure 1. Absolute mean $\log_{10}$ lymphocyte level in cases and controls by category of age

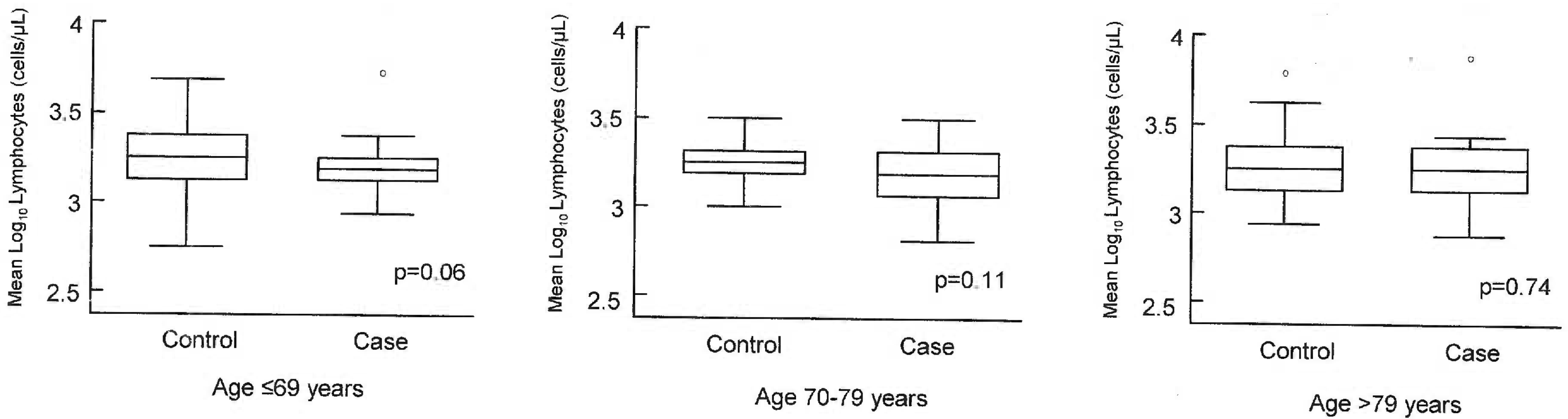

Figure 2. Multivariate model of KSHV viremia, lymphopenia, monocytosis and presence of Classic Kaposi's sarcoma