

THE ROLE OF IMMUNOSUPPRESSION AND IMMUNE-ACTIVATION IN CLASSIC KAPOSI'S SARCOMA

Giota TOULOUMI¹, Angelos HATAKIS^{1*}, Irini POTOURIDOU², Ioanna MILONA¹, John STRARIGOS², Andreas KATSAMBAS², Gaetano GIRALDO³, Elke BETH-GIRALDO³, Robert J. BIGGAR⁴, Nancy MUELLER⁵, and Dimitrios TRICHOPOULOS^{1,5}

¹Department of Hygiene and Epidemiology, Athens Medical School, Athens, Greece

²Department of Dermatology and Venereology, "Andreas Sygros" Hospital, University Clinic, Athens, Greece

³Department of Experimental Oncology and AIDS Reference Center, Istituto Nazionale Tumori "Fond. G. Pascale", Naples, Italy

⁴Viral Epidemiology Branch, National Cancer Institute, Bethesda, MD, USA

⁵Department of Epidemiology, Harvard University, School of Public Health, Boston, MA, USA

Immunodeficiency and elevated levels of cytokines have been associated with the development of Kaposi's sarcoma (KS) lesions in patients with AIDS and iatrogenic immunodeficiency. However, their role in classic KS (CKS) is unclear. We measured peripheral blood cell levels, including T-cell subsets, as well as neopterin and β_2 -microglobulin in 91 HIV-negative Greek patients with histologically confirmed CKS and in 107 controls matched for age and sex. CKS cases had slightly lower leukocyte counts ($p = 0.08$) and lymphocyte counts ($p = 0.02$). Although the percentage of CD4 and CD8 T-lymphocytes were not significantly different from controls ($p = 0.10$ and $p = 0.45$, respectively), CD4 T-lymphocytes were lower in cases than controls (812 cells/ μ L and 1,009 cells/ μ L, respectively; $p = 0.01$); part of this difference resulted from the lower lymphocyte counts ($p = 0.07$ after adjusting for lymphocyte counts). However, neopterin and β_2 -microglobulin were both considerably elevated [geometric mean (95% CI): 8.35 (7.27–9.73) nmol/L and 2,904 (2,479–3,401) μ g/L in cases and 5.86 (5.40–6.35) nmol/L and 2,042 (1,880–2,218) μ g/L in controls, respectively]. We conclude that CKS patients are predominantly characterised by immune activation, although an element of minor immunosuppression may also be present. *Int. J. Cancer* 82:817–821, 1999.
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Prior to the AIDS epidemic, Kaposi's sarcoma (KS), a tumour of endothelial cell origin, was a rare diagnosis in Europe, occurring mainly in adult men of Mediterranean or Jewish origin (Wahman *et al.*, 1991; Beral, 1991). In Africa, a more aggressive form of KS was endemic in adults and children (Wahman *et al.*, 1991; Beral, 1991). With the AIDS epidemic, KS has become frequent in Western countries, preferentially affecting homosexual men infected with HIV-1 (Biggar *et al.*, 1984; Lifson *et al.*, 1990). Although KS has been studied for over 100 years, its pathogenesis remains unknown. With AIDS-KS seen in more than 15% of AIDS cases, interest in the epidemiology and aetiology of KS has increased greatly.

Several studies have suggested that a sexually transmitted infectious agent may be causally associated with the development of KS (Beral, 1991; Archibald *et al.*, 1992). A recently described new agent, human herpesvirus 8 (HHV-8), has been linked with KS, but its exact role in the development of KS lesions remains unclear (Chang *et al.*, 1994; Boshoff *et al.*, 1995; Gao *et al.*, 1996). The association of AIDS and other causes of immunodeficiency with KS (AIDS-KS and iatrogenic KS) suggests that immunosuppression facilitates the development of KS lesions (Beral, 1991; Schulz and Weiss, 1995). However, the role that immunosuppression plays in the development of Mediterranean (called classical KS or CKS) and African KS is not clear. The limited number of studies on these forms of KS have given contradicting results (Kestens *et al.*, 1985; Marining *et al.*, 1985).

Studies that examined the role of cytokines in KS lesions have suggested that high levels of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, tumour necrosis factor (TNF) α and β and interferon- γ (IFN- γ), in the setting of a dysregulated immune system may be essential in the development of KS (Ensoli *et al.*,

1994; Barillari *et al.*, 1992). It has been proposed that chronic and repeated infections may provide the continuous stimulus for inflammatory cytokines production that contribute to the pathogenesis of KS, and that, herpesviruses (such as HHV-8) may intensify the course of KS by production of IFN- γ and other inflammatory cytokines (Samaniego and Gallo, 1996). Increased levels of serologic markers that directly or indirectly reflect activation of the endogenous interferon system, such as levels of β_2 -microglobulin and neopterin, have been shown to be associated with poor prognosis in asymptomatic HIV-1 infected subjects and in patients with AIDS-KS (Fahey *et al.*, 1990). However, limited data are available on the levels of these markers in patients with KS not associated with AIDS (Kestens *et al.*, 1985).

The investigation of the possible interaction between non-AIDS KS and immunity could contribute to better understanding the pathogenesis of the disease. Our purpose is to assess the immune competence of the CKS patients in an ongoing case-control study in Greece, one of the Mediterranean countries where CKS is primarily found.

MATERIAL AND METHODS

Patients

The population of our study is part of an ongoing case-control study started in 1989 in Athens, Greece. In Athens, the "Andreas Sygros" Hospital is the reference hospital for serious skin diseases in Southern and Central Greece (Touloumi *et al.*, 1997). Attending physicians at this hospital were contacted weekly for suspected cases of KS (*i.e.*, patients with skin lesions) who were negative for antibodies to the human immunodeficiency virus (HIV). All cases were interviewed and biopsy material and blood specimen were obtained. After interviewing patients, contacting the attending physicians and abstracting medical records, patients were classified as having CKS or iatrogenic KS. Only patients with CKS were included in the present analysis. All cases were histologically confirmed and were further characterised as incident cases, if they were diagnosed for first time within 1 year prior to the time the blood specimen and the questionnaire were taken, and prevalent cases otherwise. An effort to collect clinical and laboratory data to assess the existence of KS lesions in internal organs was unsuccessful since the majority of the patients denied the necessary medical work-up.

Two hospital control groups were selected: patients with orthopaedic diseases and patients with basal skin cancer (BSC). Controls were excluded if they had prior or current immunosuppressive

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*Correspondence to: Department of Hygiene and Epidemiology, Athens Medical School, M Asias 75, Athens 11527, Greece. Fax: +301 7704225. E-mail: ahatazak@atlas.uoa.gr

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treatment, prior or current malignant disease and/or diabetes. For orthopaedic controls, blood specimens were obtained prior to surgery. Cases and controls were frequency-matched for age and sex.

Methods

Peripheral blood lymphocyte subpopulations were evaluated by flow cytometry in the National Retrovirus Reference Center (Athens, Greece) using the same reagents during the whole study period. Monoclonal antibodies for CD3, CD4, CD8, HLA-DR, CD16 and CD56 were used for the estimation of total T-lymphocytes helper/inducer, suppressor/cytotoxic, B-lymphocytes and natural killer (NK) cells (Beckton-Dickinson, San Jose, CA). Absolute numbers and percentages of total lymphocytes were calculated. Neopterin (Immuntest Neopterin, Henning Berlin, Germany) and β_2 -microglobulin (β_2 -microglobulin RIA; Abbott, Chicago, IL) were measured in serum by commercially available assays.

Statistical analysis

In the statistical analysis, the haematological parameters were treated either as continuous variables or ranked into quartiles. The distributions of all subjects (cases and controls) were used to assign quartiles. Comparison of mean levels of the haematological variables between cases and controls were made using Student's *t* test or linear contrasts in one-way analysis of variance or non-parametric tests, as appropriate. Since cases and controls were frequency-matched, the unconditional logistic regression (controlling for the matching factors) was used to evaluate the association between the risk of being CKS case and the haematological parameters.

RESULTS

During the study period, 109 patients with diagnosed KS were examined. Eighteen were excluded from the analysis for the following reasons: 7 were renal-transplant recipients, 1 had chronic lymphocytic leukaemia, 1 had active infection and 9 were under immunosuppressive therapy. All the remaining 91 patients were histologically confirmed. Sixty-four of them (70.3%) were incident cases and 27 (29.7%) were prevalent cases. Sixteen CKS cases had been treated for their tumour, 8 with chemotherapy and 8 with local radiotherapy. However, all of them had stopped any treatment for at least 2 months prior to the time the blood specimen was taken (median time between the date of last treatment taken and the date of blood specimen: 313 days).

One hundred eight hospital controls were recruited. Forty-two of them were patients with orthopaedic diseases, and 66 had basal skin cancer. One of the orthopaedic controls was excluded because he was under corticosteroid treatment. The mean age of the CKS subjects was 72.1 years (SD = 10.9) and 75.8% were male. Both orthopaedic and BSC controls were closely matched with the CKS subjects. Among orthopaedic and BSC subjects, mean ages (SD) were 72.4 (7.8) and 68.6 (15.6) years, and 63.4% and 77.3% were male, respectively. The differences were not significant from cases or from each other.

Table I shows the mean values and standard errors for red and white blood cell parameters, lymphocyte subpopulations, neopterin and β_2 -microglobulin for cases and controls. Since the 2 control groups did not differ significantly in any of these parameters, they were combined for the analysis. On average, haematocrit (Hct) and haemoglobin (HB) tended to be lower in CKS cases compared to controls, while there was no significant difference between the 2 groups in mean platelets number. The mean white blood cell count (WBC) was marginally significantly ($p = 0.08$) lower in CKS cases than in controls. The absolute number of total lymphocytes and the T-lymphocyte subsets, namely, CD3, CD4 and CD8 T-lympho-

TABLE I – MEAN VALUES AND STANDARD ERRORS (SE) OF RED AND WHITE BLOOD CELL PARAMETERS, LYMPHOCYTE SUBPOPULATIONS, NEOPTERIN AND β_2 -MICROGLOBULIN IN CLASSIC KAPOSI'S SARCOMA (CKS) CASES AND CONTROLS

Parameter	CKS (N = 91) Mean (S.E.)	Controls (N = 107) ¹ Mean (S.E.)	<i>p</i>
Haematocrit (%)	43.40 (0.58)	45.01 (0.52)	0.040
Haemoglobin (g/dl)	13.95 (0.17)	14.41 (0.17)	0.053
Platelets (K/ μ L)	264 (9.88)	277 (8.29)	0.322
Leukocytes (K/ μ L)	6.88 (0.17)	7.34 (0.19)	0.082
Lymphocytes			
%	32.65 (1.14)	34.07 (0.99)	0.344
count (cells/ μ L)	2188 (83.08)	2498 (98.89)	0.018
CD3 T-lymphocytes			
%	67.93 (1.11)	69.06 (0.90)	0.423
count (cells/ μ L)	1485 (61.35)	1726 (74.49)	0.015
CD4 T-lymphocytes			
%	37.89 (1.17)	40.47 (1.02)	0.098
count (cells/ μ L)	812 (38.73)	1009 (55.40)	0.005
CD8 T-lymphocytes			
%	34.31 (1.15)	35.54 (1.10)	0.447
count (cells/ μ L)	760 (41.30)	897 (50.70)	0.040
CD4/CD8 (ratio)	1.39 (0.22)	1.25 (0.07)	0.518
Neopterin (nmol/L)	11.73 (1.52)	6.50 (0.38)	<0.001
β_2 -microglobulin (μ g/L)	4398.6 (701.5)	2263.3 (123.2)	0.001

¹Pooled control group.

cytes, were significantly lower in the CKS cases compared with the controls ($p < 0.05$). However, when the lymphocyte subpopulations were expressed as a percentage of the total lymphocyte count, there were not statistically significant differences between the 2 groups, although the percentage of CD4 counts was marginally lower in CKS cases ($p = 0.10$, Table I). The mean CD4/CD8 ratios were similar between CKS cases and controls. Differences in CD4 T-lymphocytes remained marginally significant ($p = 0.07$) after adjusting for total lymphocytes, whereas differences in CD3 and CD8 T-lymphocytes became insignificant ($p = 0.20$ and 0.47 , respectively). No significant differences were seen between the two groups in the absolute numbers or percentages of polymorphonuclear lymphocytes (PMN) and monocytes or in natural killer or B cells (data not shown).

Significant differences between CKS cases and controls were observed for the levels of neopterin and β_2 -microglobulin ($p < 0.01$, Table I). The mean levels of neopterin and β_2 -microglobulin were almost doubled in the CKS cases compared to controls. The distributions of both variables were highly right skewed, but non-parametric tests gave similar results. The median levels of neopterin for CKS cases and controls were, respectively, 6.83 and 5.30 nmol/L. For β_2 -microglobulin, median levels were 2,421 and 2,050, respectively. All the extreme values [$\geq 9,000 \mu$ g/L for β_2 -microglobulin ($n = 7$) and ≥ 23 nmol/L for neopterin ($n = 10$)] were observed only in the CKS cases.

Restricting analysis to the CKS cases without any treatment did not change the results. One way analysis of variance, using each control group separately gave similar results, although the significance levels were somewhat lowered by the smaller number of observations. None of the examined red and white cell parameters or lymphocyte subpopulations differed significantly between incident and prevalent CKS cases. However, incident CKS cases tended to have higher levels of neopterin and β_2 -microglobulin than prevalent CKS cases (median neopterin levels in incident and prevalent CKS cases: 7.5 and 5.8 nmol/L, respectively, $p = 0.11$; corresponding figures for β_2 -microglobulin levels: 2,738 and 1,972 μ g/L respectively, $p = 0.03$).

Table II shows the odds ratio (OR) of being CKS case compared with controls by lymphocyte subpopulation quartiles, while Table III shows the corresponding ORs by quartiles of neopterin and β_2 -microglobulin. The presented ORs are adjusted for age and sex. ORs were inversely associated with lower lymphocyte subsets. There was a tendency for increasing risk of CKS with decreasing

TABLE II – ODDS RATIO (OR) AND 95% CONFIDENCE INTERVAL (95% CI) OF BEING CLASSIC KAPOSI'S SARCOMA (CKS) CASE COMPARED WITH CONTROLS BY LYMPHOCYTE SUBPOPULATIONS, ADJUSTED FOR AGE AND SEX

Parameter	OR	95% C.I.	p
Total lymphocytes (cells/ μ L)			
Quartile 4 (\geq 2,781)	1	—	—
Quartile 3 (2,201–2,780)	1.20	(0.49–2.93)	0.691
Quartile 2 (1,751–2,200)	2.84	(1.14–7.03)	0.025
Quartile 1 (\leq 1,750)	2.06	(0.84–5.04)	0.113
CD3 T-lymphocytes (cells/ μ L)			
Quartile 4 (\geq 1,901)	1	—	—
Quartile 3 (1,491–1,900)	2.53	(0.95–6.70)	0.063
Quartile 2 (1,201–1,490)	2.88	(1.08–7.70)	0.035
Quartile 1 (\leq 1200)	3.12	(1.15–8.52)	0.026
CD4 T-lymphocytes (cells/ μ L)			
Quartile 4 (\geq 1,051)	1	—	—
Quartile 3 (856–1,050)	2.40	(0.90–6.35)	0.079
Quartile 2 (651–855)	2.64	(1.01–6.94)	0.049
Quartile 1 (\leq 650)	2.73	(1.03–7.25)	0.043
CD8 T-lymphocytes (cells/ μ L)			
Quartile 4 (\geq 1,031)	1	—	—
Quartile 3 (761–1,030)	1.28	(0.49–3.32)	0.617
Quartile 2 (536–760)	1.64	(0.62–4.05)	0.338
Quartile 1 (\leq 535)	1.86	(0.72–4.81)	0.203

TABLE III – ODDS RATIO (OR) AND 95% CONFIDENCE INTERVAL (95% CI) OF BEING CLASSIC KAPOSI'S SARCOMA CASE COMPARED WITH CONTROLS BY LEVELS OF NEOPTERIN AND β_2 -MICROGLOBULIN, ADJUSTED FOR AGE AND SEX

Parameters	OR	95% C.I.	p
Neopterin (nmol/L)			
Quartile 1 (\leq 4.70)	1	—	—
Quartile 2 (4.71–5.90)	1.73	(0.74–4.06)	0.209
Quartile 3 (5.91–8.05)	1.66	(0.69–3.95)	0.255
Quartile 4 (\geq 8.06)	5.24	(2.11–12.97)	0.000
β_2 -microglobulin (μ g/L)			
Quartile 1 (\leq 1,615)	1	—	—
Quartile 2 (1,616–2,180)	1.62	(0.66–3.94)	0.292
Quartile 3 (2,181–3,080)	2.29	(0.91–5.77)	0.080
Quartile 4 (\geq 3,081)	4.43	(1.71–11.51)	0.002

levels of CD3, CD4 and CD8 T-lymphocytes but this was less pronounced for CD8 T-lymphocytes (test for linear trend: $p = 0.01, 0.02$ and 0.11 , respectively). The ORs increased with increased levels of both neopterin and β_2 -microglobulin. Compared to subjects in the lowest quartile, subjects in the highest quartile of neopterin or β_2 -microglobulin were, respectively, 5.2 and 4.4 times more likely to be CKS cases than controls.

Overall, the Spearman's correlation coefficient between neopterin and β_2 -microglobulin was 0.71 in cases and 0.65 in controls. However, the correlation coefficients between lymphocyte subpopulations and either neopterin or β_2 -microglobulin were small in magnitude and not significantly different from zero. This suggests that the subjects with elevated levels of neopterin or β_2 -microglobulin and the subjects with decreased numbers of lymphocyte subsets do not necessarily coincide. Figure 1 shows the scatterplot of the absolute number of CD4 T-lymphocytes versus the levels of neopterin among CKS cases and controls. While the distributions shift towards higher levels of neopterin and smaller number of CD4 T-lymphocytes for the CKS patients compared to controls, the variability in the values is large in both groups. Scatterplots of the levels of neopterin against the absolute number of CD3 or CD8 T-lymphocytes as well as the corresponding scatterplots with the levels of β_2 -microglobulin were similar to that of CD4 counts and neopterin levels.

In logistic models where the levels of neopterin or β_2 -microglobulin (quartiles) and the number of CD3 or CD4 T-lymphocytes (quartiles) were simultaneously fitted, both factors were found to be independent predictors of the risk of being CKS

case with little mutual confounding. For example, for quartiles ranging from the lowest to the highest, the adjusted for β_2 -microglobulin ORs (95% CI) for CD4 T-lymphocytes were 3.0 (1.1–8.8), 2.4 (0.9–6.8), 2.2 (0.8–6.2) and 1 respectively, while the corresponding figures for β_2 -microglobulin quartiles were 1, 1.3 (0.4–3.7), 1.7 (0.6–5.1) and 4.1 (1.3–12.9).

DISCUSSION

Evidence from several studies suggests that the aetiology of KS is multifactorial. While there is justifiable interest about the role of the newly discovered HHV-8 in its aetiology, there is also no question that immunity plays a major role in its over-expression. The excess risk of KS in persons with AIDS and those receiving immunosuppressive drugs is extraordinary (Beral, 1991; Schulz and Weiss, 1995), but the role of immunosuppression in the pathogenesis of non-AIDS KS, however, remains ill-defined. So far, it has been most studied in African KS subjects. Early studies reported findings of immune using clinical parameters (Taylor and Ziegler, 1974), but this was not confirmed by a later study which examined CD4 and CD8 levels (Kestens *et al.*, 1985).

Our results show that CKS patients have significantly lower CD3, CD4 and CD8 T-lymphocytes compared with controls, but some of these differences result from the lower lymphocyte counts in CKS cases. However, differences in CD4 T-lymphocytes remained marginally significant even after adjusting for lymphocyte counts. Although the mean values of all T-lymphocyte subsets in CKS patients were well within the normal range and possible residual confounding cannot be excluded, the data suggest that a minor immunosuppression may be present in CKS patients. Similar results were found in a small Italian study (Marining *et al.*, 1985). Recently, significant reductions in CD3 and CD4 but not in CD8 T-lymphocytes in CKS subjects in Italy have been reported (Santelli *et al.*, 1988). In an earlier Greek study (Kaloterakis *et al.*, 1984), anaemia (Hct < 30%) and lymphocytopenia (total lymphocytes < 1,500 cells/ μ L) was observed in half of the 72 CKS cases studied. In our study, CKS patients tended also to have lower haematocrit and haemoglobin than controls.

Cytokines, in particular IL-1, IL-6, TNF α and β and interferon- γ , have been suggested to be important in KS genesis (Ensoli *et al.*, 1994; Barillari *et al.*, 1992). In their review of this topic, Samniengo and Gallo (1996) argue that immune activation rather than immunosuppression is the underlying stimulus for KS and suggest that HHV-8 and perhaps other herpesviruses stimulate KS by inducing inflammatory cytokines. Detection of cytokines is technically difficult because most of them are produced only locally and tightly bound to cell receptors. However, in 2 recent reports, using PBMCs tumour infiltrating lymphocytes and spindle cell cultures from lesions of AIDS-KS and CKS, it was found that γ -interferon production correlates with the presence of HHV-8 sequences and that the formation of KS lesions is upregulated by tat protein of HIV-1 in AIDS-KS (Sirianni *et al.*, 1998; Fiorelli *et al.*, 1998). We used detection of β_2 -microglobulin and neopterin as an indirect approach. These markers are interferon-induced products and can be measured in serum by commercially available kits. In our study, the most striking findings were that CKS patients had markedly increased levels of both markers. Levels in CKS patients were almost twice those found in matched controls, and all persons who had extremely high values were CKS patients.

In HIV-1 infected individuals, levels of both markers were independent predictors of AIDS onset (Fahey *et al.*, 1990), and patients with AIDS-KS tended to have high levels, which were associated with disease progression. Few studies have been published about these markers in patients with non-AIDS KS, but the data so far reported do not agree. In African patients with endemic KS, β_2 -microglobulin levels were similar in cases and matched controls (Kestens *et al.*, 1985), but, an Italian study reported

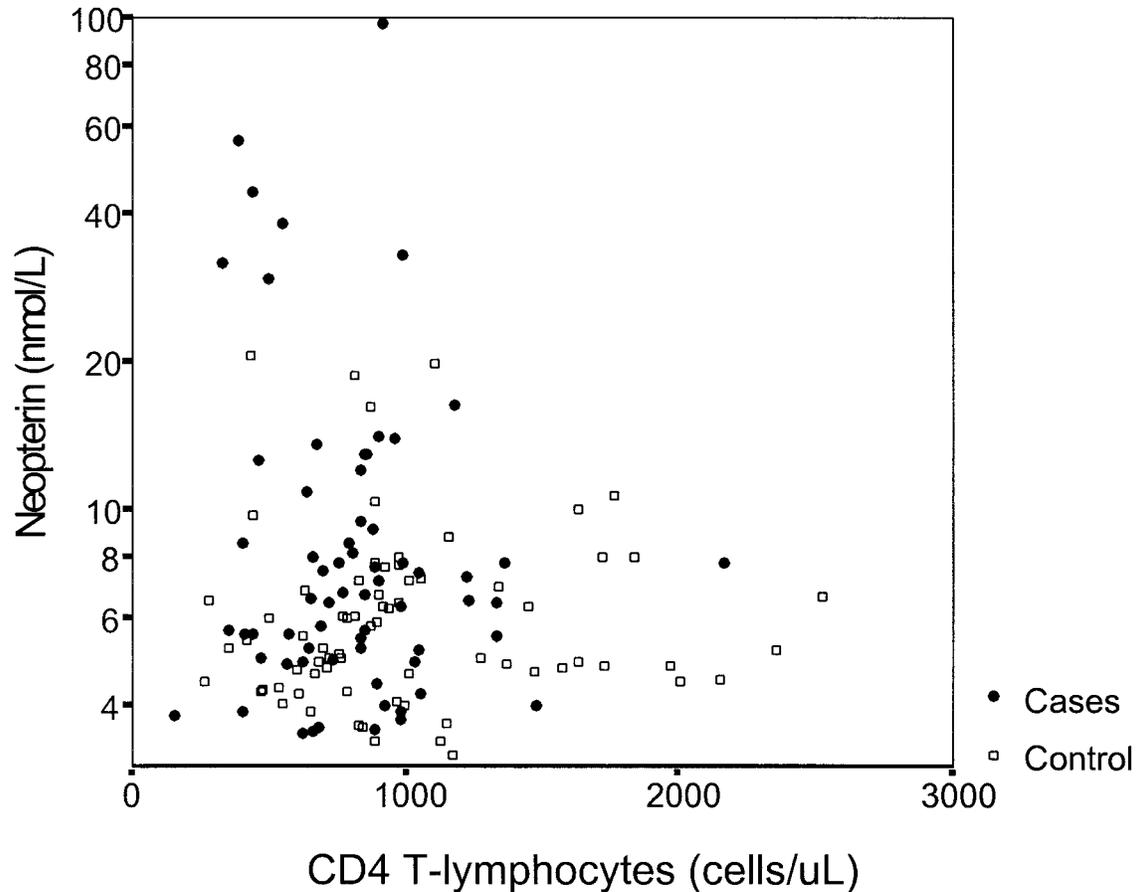


FIGURE 1 – Scatterplot of neopterin levels vs. number of CD4 T-lymphocytes in classic Kaposi's sarcoma cases and controls.

neopterin urinary excretion levels significantly higher in CKS patients than in controls (Santelli *et al.*, 1988). Our study found CKS patients to have markedly higher levels of both β_2 -microglobulin and neopterin than controls.

It has been proposed that the pathogenesis of KS may be related to both an early phase of immunostimulation and a subsequent

phase of immunosuppression (Barillari *et al.*, 1992). Our data suggest that an immune activation and maybe a minor immunodeficiency is present in CKS patients soon after KS onset and continues to be present also in patients with chronic KS. However, further investigation is required to elucidate the role of both immune activation and immunosuppression in non-AIDS KS.

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