Journal of Clinical Immunology, Vol. 25, No. 2, March 2005 (© 2005)
DOI: 10.1007/s10875-005-2818-y

Determinants of CD4 Counts Among HIV-Negative Ethiopians: Role of Body Mass Index, Gender, Cigarette Smoking, Khat (*Catha Edulis*) Chewing, and Possibly Altitude?

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Accepted: November 24, 2004

To study the determinants of CD4% and CD4 counts among HIV-negative Ethiopians, and to identify factors susceptible to explain the low CD4 counts observed among Ethiopian subjects. Cohort studies among factory workers in Akaki and Wonji, Ethiopia. Clinical and laboratory examinations, including determination of HIV serological status and T-cell subsets, were performed during follow-up visits every six months. In addition, micronutrients (retinol, carotenoids, tocopherol, transferrin receptor, and selenium) plasma concentrations were determined in a subset of 38 HIV-positive and 121 HIV-negative participants. HIV-negative participants with at least one CD4 count measurement were 157 females in Akaki, 203 males in Akaki, and 712 males in Wonji. CD4 counts were independently and positively associated with body mass index (through an increase in lymphocyte counts), female gender (through an increase in CD4%), cigarette smoking (through an increase in CD4%), khat chewing (through an increase in both lymphocyte counts and CD4%), and Akaki study site (through a large increase in lymphocyte counts compensating a decrease in CD4%). Intestinal parasitic infections were not associated with CD4% or CD4 counts. Retinol, carotenoids, and α-tocopherol plasma concentrations decreased with HIV infection and advancing immunosuppression, but were not associated with CD4 counts among HIV-negative subjects. Low body mass index among Ethiopians may have contributed to their overall low CD4 counts. Other factors remain to be elucidated.

KEY WORDS: CD4 counts; micronutrients; BMI; altitude; Ethiopia.

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INTRODUCTION

Many of the immunological disturbances observed among patients infected by the human immunodeficiency virus (HIV) are related to the decline in CD4+ T-cell (CD4) counts during the course of infection, and the prognostic value of CD4 counts for the development of acquired immune deficiency syndrome (AIDS) or death is well established (1). In the search for reference values for CD4 counts, several factors were found to be associated with higher CD4 counts, among which female gender (2, 3) and smoking (3) were important. Differences in baseline CD4 counts were also observed across various ethnic groups, and Ethiopians appeared to have some of the lowest CD4 counts worldwide (4–15). This finding triggered our interest in studying factors associated with differences in CD4 counts among Ethiopians. Special emphasis was given to the role of micronutrients, considering the generally low nutritional status of Ethiopians (during the last Demographic Health Survey in 2000, 30.1% of 13,447 Ethiopian adult women had a body mass index <18.5 kg/m^2) (16), and the results of a randomized trial showing an increase in CD4 counts among HIV-infected African pregnant women taking vitamin supplements (17).

SUBJECTS AND METHODS

Subjects

Participants were factory workers enrolled in two ongoing cohort studies of HIV incidence and progression,

performed by the Ethio–Netherlands AIDS Research Project (ENARP) at the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia. Detailed description of the cohort studies has been reported elsewhere (18, 19). One site was located in Akaki (a suburb of the capital Addis Ababa, at 2300 m altitude) and the second site was in Wonji (a sugar estate, 114 km south east of Addis Ababa, at 1500 m altitude). All study participants came to the study clinic every six months for a questionnaire on health and behaviors, a clinical examination by a medical doctor, and collection of blood and stool samples for laboratory analysis. Not all questionnaire or medical data were collected at each visit: for instance, whereas weight was measured at each visit, questions on cigarette smoking and khat chewing were not repeated after the cohort intake. Baseline (intake) data were used for these two variables in this study. The cohort studies started in February 1997 in Akaki, and October 1997 in Wonji. Results as of 30 June 2001 were used in this analysis. The protocol of both cohort studies has been approved by both EHNRI and the National Ethical Clearance Committee. Informed consent was obtained from each subject. Pre- and post-test counselling for HIV testing was made available to all study participants.

Blood Collection and HIV Serology

Whole blood was collected into EDTA Vacutainer tubes between 8:30 and 11:30 am and transported to the ENARP laboratory on the same day of collection. Upon arrival, the tubes were well mixed and 500 μL of the whole blood was transferred into nunc tubes for FACScan and haematological analysis. The presence of HIV antibodies was detected on plasma using HIVSPOT rapid assay (Genelabs Diagnostics, Singapore) and an enzyme linked immunosorbent assay (ELISA) (Vironostika HIV Uni-Form II plus O, Organon Teknika, Boxtel, The Netherlands). Plasma samples tested positive by any or both tests were confirmed by Western Blot analysis (HIVBLOT 2.2, Genelabs Diagnostics, Singapore).

Leucocyte Count and T Cell Immunophenotyping

Absolute number of leucocytes per μL of whole blood was obtained using a Coulter counter T540 (Coulter Electronics, Florida, USA). Lymphocyte subsets were determined by flow cytometry using a FACScan (Becton Dickinson, San Jose, California, USA) as described in detail previously (13). Lymphocyte subsets were determined for all HIV-positive participants. For HIV-negatives, lymphocytes determination differed depending on the study site: in Akaki, it was done in 10% of all participants at each follow-up visit and in rotation; in Wonji, it was done on all participants at each visit for the first three years of the study, and then restricted to only 10% of participants.

Stool Microscopy

Stool examination for parasitic infection was performed on fresh stool at the study sites on the same date of blood sample collection and consisted of: direct examination in saline and iodine preparations; concentration in formalyn-ethyl-acetate; Baermann for *Strongyloides stercoralis* larvae; and Kato thick smear for schistosome eggs (in Wonji only). In Wonji, another stool specimen was collected and analyzed three days later, and two samples from the same stool specimen were examined separately.

Micronutrient Analysis

A total of 159 samples, 121 from HIV-negative, and 38 from HIV-positive participants, were randomly selected for the micronutrient study. All samples for the micronutrient study were from male subjects to remove the gender effect on CD4 counts. Sampling was stratified by quartile of CD4 counts among HIV-negative participants. Samples were kept at -80°C at ENARP and transported in dry ice to Europe for analysis of vitamin A, vitamin E, carotenoids, transferrin receptor and selenium. Part of the samples taken for vitamin A, E and carotenoids analysis were extracted in a room illuminated with yellow light at the Department of Human Nutrition Wageningen University (Wageningen, The Netherlands). Plasma level of vitamins and carotenoids were analyzed in the same department with reverse-phase High Performance Liquid Chromatography (HPLC) system Termo Separation Products with two detectors UV1000 UV/VIS and UV3000/FOCUS with gradient solvents as mobile phase. Within and between run coefficients of variation were 3.1 and 5.2% for vitamin A, 7.1 and 6.8% for lutein, 5.2 and 4.9% for zeaxanthine, 5.3 and 7.1% for β-cryptoxanthine, 7.4 and 9.1% for α-carotene, 6.2 and 7.8% for β-carotene, 9.9 and 8.3% for lycopene, 5.1 and 6.5% for α-tocoferol, and 15.5 and 17.2% for γ-tocoferol. Plasma transferrin receptor was analyzed in Velp hospital laboratory, Velp, the Netherlands. Within and between run coefficients of variation were 3.0 and 4.4%, respectively. Within and between run coefficients of variation were 2.1 and 2.5%, respectively.

Statistical Analysis

Difference between proportions of categorical variables or distribution of continuous variables between two groups were examined using Chi-square test for independence and Mann–Whitney U test, respectively. Test for

trends were done using a non-parametric test for trends (Culicz). Predictors of lymphocytes counts, CD4 percent (%) and CD4 counts, were identified from a dataset including repeated measurements from the same individuals participating in the cohort study (the mean number of CD4 counts measurements per individual was 2.1). Random-effects linear models were fitted to identify the predictors, while taking into account for correlation among repeated measurements from the same subjects. Dependent variables (e.g., CD4 counts) were normalized prior to introduction in the model, using the Box-Cox transformation function of Stata statistical package (Stata 6.0, Stata Statistical Software, College Station, TX, USA).

RESULTS

As of 30 June 2001, 803 and 855 participants had been recruited in Akaki and Wonji, respectively. Of these, 95 (11.8%) and 61 (7.1%) were positive for HIV antibodies. HIV-negative participants with at least one CD4 count were 157 females in Akaki, 203 males in Akaki, and 712 males in Wonji. Their general characteristics are displayed in Table I, showing that the study population consisted of middle-aged adults with low education and income, and high intestinal parasitism. Associations between these characteristics and total lymphocyte counts/CD4%/CD4 counts were examined. Factors significantly associated with lymphocyte counts, CD4% and CD4 counts were: study site, gender, body mass index, cigarette smoking, and khat consumption. Median values of lymphocyte counts, CD4%, and CD4 counts by categories of site, gender, cigarette smoking, and khat consumption at the first visit are shown in Table II. The associations were further explored in multivariate models to identify the independent contributions of each variable to the cell counts and % (Table III). CD4 counts were independently and positively associated with body mass index (through an increase in lymphocyte counts), female gender (through an increase in CD4%), cigarette smoking (through an increase in CD4%), khat chewing (through an increase in both lymphocyte counts and CD4%), and Akaki study site (through a large increase in lymphocyte counts compensating a decrease in CD4%). As suggested by the multivariate model, the increase in CD4 counts associated with khat consumption was dose-dependent, and independent of cigarette smoking (see Fig. 1). The difference in CD4 counts was significant even when comparing occasional use (once a month; $n = 109$) versus none among non-smokers ($n = 489$) (median of 714 versus 664, respectively, $p = 0.01$). It is also noteworthy that CD4 counts were 62% higher among those chewing khat daily versus those never chewing khat (1075 versus 664, respectively, $p = 0.01$). There was no association between CD4 counts and the presence of intestinal parasites, whether the latter were considered individually (i.e., strongyloidiasis, schistosomiasis, etc.) or grouped (i.e., helminthes, protozoans). Seasonal effects were also studied by adding dummy variables for each month in the multivariate model. In Akaki, lymphocyte counts were at their lowest, and CD4% at their highest, in the month of September.

The association between CD4 counts and nutritional status was further studied in a subgroup of 38 HIV-positive and 121 HIV-negative male workers. Median [range] micronutrients concentrations among HIV-negative subjects are available in the first column of Table IV. Concentrations of retinol, carotenoids (α-carotene, β-cryptoxanthin, zeaxanthin, and lycopene), and α-tocopherol were correlated with body mass index among HIV-negative subjects. While median concentrations of retinol, carotenoids, and α-tocopherol decreased with HIV infection and increasing immunosuppression (see Table IV), there were no associations between CD4 counts or CD4% and micronutrients concentrations among HIV-negative participants. CD4 counts and CD4% did not differ by micronutrients quartiles for all micronutrients tested, even after adjustment for age, study site, body mass index, cigarette smoking, and khat consumption (data not shown).

Table I. General Characteristics of HIV-negative Participants, Akaki and Wonji

	Akaki		Wonji
	Females ($n = 157$)	Males ($n = 203$)	Males ($n = 712$)
Median age (in years)	36	38	32
% illiterate	33.1	14.8	4.1
% with income <200 Eth birr[a]	31.8	10.3	31.9
% married	77.7	77.8	75.6
Number of cigarettes/day (%)			
0	96.8	80.3	63.4
<1	2.6	0.5	17.9
1–4	0.6	5.4	7.3
5–9	0.0	8.9	6.1
10–19	0.0	4.4	3.8
>20	0.0	0.5	1.6
Khat consumption (%)			
Never	97.5	81.3	67.9
<1/month	2.5	15.8	28.6
1/week	0.0	3.0	2.5
1/day	0.0	0.0	1.0
Median body mass index (kg/m^2)	20.93	20.55	20.95
Body mass index <18.5 kg/m	10.6	17.2	11.4
% with intestinal helminthes	25.7	22.1	22.3
% with intestinal protozoans	5.3	3.5	4.2

[a] 200 Eth birr ≈ US$ 24.

Table II. Median Lymphocyte Counts (per μL), CD4 %, and CD4 counts (per μL) among HIV-Negative Cohort Participants, Akaki and Wonji

	Lymphocytes			CD4%			CD4 counts		
	Akaki		Wonji	Akaki		Wonji	Akaki		Wonji
	Females	Males	Males	Females	Males	Males	Females	Males	Males
All[a]	1845	1798	1680[b]	42	39[c]	41[b]	762	684[c]	684
[IQR]	[1512–2268]	[1462–2250]	[1350–2028]	[37–45]	[33–43]	[37–46]	[604–908]	[588–832]	[541–841]
Smoking[d]									
No		1848	1672		38	40.5		686	670
Yes		1706	1707		40[e]	42[e]		668	714[e]
Khat user[d]									
No		1846	1653		39	40		671	657
Yes		1735	1755[f]		39	42[f]		700	761[f]

[a] Numbers were 157 for Akaki females, 203 for Akaki males, and 712 for Wonji males.
[b] $p < 0.05$ compared to Akaki males.
[c] $p < 0.05$ compared to Akaki females.
[d] Smokers and khat users were too rare among women (5 and 4, respectively) for subgroup analysis.
[e] $p < 0.05$ compared to non-smokers.
[f] $p < 0.05$ compared to non-khat users.

DISCUSSION

This study confirmed previous findings of low CD4 counts among healthy adult Ethiopians (11–15). Earlier studies have shown that Ethiopians have lower lymphocyte counts, and lower CD4%, compared to Dutch (13–15). To our knowledge, similarly low CD4 counts have not been found in other African countries (5–7). We were therefore interested in studying factors which would be unique to Ethiopians and would be associated with low CD4 counts.

Our first hypothesis was that low CD4 counts in Ethiopians might be related to poor nutritional status. Indeed, malnutrition depresses immune functions (reviewed in 20), and malnutrition is common in Ethiopia. In this study, retinol (vitamin A), α- and β-carotenoids, and α-tocopherol (vitamin E) plasma concentrations were lower than those observed among adult males living in European countries, Japan, and the U.S. (21). The role of micronutrients in stimulating CD4 count production was suggested by a recent randomized trial among HIV-infected African women, which showed that vitamin supplements were associated with an increase in CD4 counts (17). However, within the range of micronutrients concentrations studied, there was no association between micronutrients levels and CD4 counts in this study. Still, low body mass index, through a decrease in lymphocyte counts, was associated with low CD4 counts, maybe explaining part of the decrease in CD4 counts observed among Ethiopians and some other populations with low body mass index [e.g., Asians, 4]. Although the observed association between BMI and total lymphocyte count is very intriguing the causal relationship between the two parameters remains to be elucidated. One possible

Table III. Independent Predictors of Lymphocyte Counts, CD4%, and CD4 Counts among HIV-negative Cohort Participants in Akaki and Wonji

	Lymphocyte counts[a]		CD4%[a]		CD4 counts[a]	
	β coefficient	p-value	β coefficient	p-value	β coefficient	p-value
Age[b]	0.0118	0.39	−0.4461	0.002	−0.0226	0.33
Female gender	−0.0285	0.93	15.724	< 0.0001	1.6335	0.003
Body mass index[c]	12.684	< 0.0001	−24.294	0.34	17.567	< 0.0001
Akaki site	1.2406	< 0.0001	−9.2141	< 0.0001	0.9831	0.02
Cigarette smoking[d]	0.0583	0.48	3.5146	< 0.0001	0.4109	0.004
Khat user[d]	0.5271	0.003	4.5823	0.015	1.3251	< 0.0001

[a] The distribution of the dependent variables were "normalized" using the Box-Cox transformation function of Stata Statistical Software (see methods); a positive coefficient indicates an increase in the lymphocyte or CD4 counts (or %) per increase in one unit of the exposure variable (positive correlation); a negative coefficient indicates a negative correlation.
[b] For an increase of 1 year of age.
[c] For an increase of 1 kg/m^2.
[d] For an increase of one category of cigarette smoking or khat consumption (see Table I).

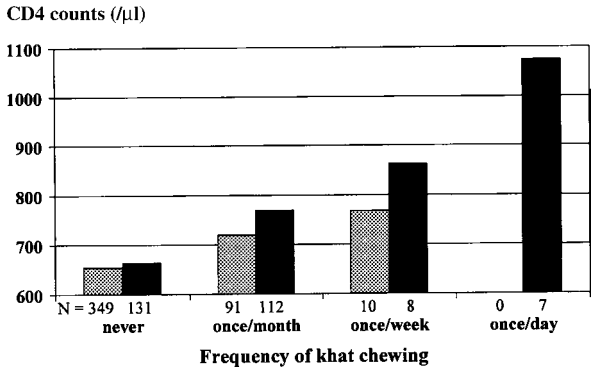

Fig. 1. Median CD4 counts (in /μL) by category of khat chewing and cigarette smoking in Wonji (light grey: non smokers and dark grey: smokers).

explanation could be that people with low BMI are more susceptible to infections that activate the immune system eventually leading to a higher turnover of lymphocytes. The findings of higher percentages of activated and dividing CD4+ and CD8+ T cells in combination with lower T cell receptor excision circles (TRECs) content in CD4+ T cells of Ethiopians compared to Dutch are in favor of this hypothesis (14, 15, 22).

A second potential explanation for low CD4 counts in Ethiopia might be the effect of ultra-violets on CD4%. Indeed, it has been shown that CD4% follow seasonal variations, with a decrease during summer time among Europeans which has been attributed to increased exposure to ultra-violets (23, 24). High exposure to ultra-violets characterizes Ethiopians from Addis Ababa, who live at high altitude (2500 m) under the heavy sunshine of sub-Saharan Africa. Of interest, subjects from Akaki, who live at higher altitude compared to those from Wonji (2300 m versus 1500 m, respectively), had significantly lower CD4%. If ultra-violets were to play a role, it would also be expected that seasonal variations would be seen in Akaki, where the months of June–September are characterized by heavy rains (the rainy season is much less pronounced at lower altitude like in Wonji). In this study, the peak CD4% was observed at the end of the rainy season in September in Akaki, giving some credit to this hypothesis.

Other factors associated with higher CD4%, such as female gender and cigarette smoking, have already been described elsewhere (2, 3). Intestinal parasitic infections did not influence absolute and relative CD4 counts in this study, not precluding nevertheless a long-term effect of repeated parasitic infections on CD4 counts. Not previously described, to our knowledge, was the association found with khat chewing. Khat (*Catha edulis*) is an evergreen tree which grows at high altitudes extending from East to Southern Africa, as well as Afghanistan, Yemen, and Madagascar (25). The chewing of khat is common in certain countries of East Africa and the Arabian peninsula. It is most valued for its stimulant effect. The alkaloid fraction, called cathinone, is the active constituent of khat, and has the basic configuration of amphetamine. Our first concern was that the association between CD4 counts and khat chewing might have been confounded by cigarette smoking, since both habits are commonly found in the same individuals. However, as shown in Fig. 1 and in the multivariate model, the effect of khat chewing on CD4 counts was independent of cigarette smoking. One must realize however that although the effect of khat was strong in the various models tried, numbers in categories of "high" khat users were small, and one may question whether the use of a product only once a month may be sufficient to induce so dramatic effects. To our knowledge, no other study has reported such associations between

Table IV. Median [Inter-Quartile Range for HIV-Negative Subjects] Nutrients Plasma Concentration Levels (in μmole/L Unless Indicated) by HIV Serological Status and CD4 Counts (per μL) Among Males in Akaki and Wonji

		HIV-positive			
Micronutrients	HIV-negative ($n = 121$)	CD4 $\geq$ 500 ($n = 6$)	CD4 between 200 and 499 ($n = 22$)	CD4 $<$ 200 ($n = 10$)	p-value[a]
Retinol	1.57 [1.16–1.96]	1.48	1.41	0.91	0.01
α-carotene	0.026 [0.015–0.036]	0.016	0.016	0.013	0.01
β-carotene	0.17 [0.09–0.30]	0.13	0.09	0.07	0.01
β-cryptoxanthine	0.48 [0.29–0.78]	0.39	0.23	0.22	0.01
Lutein	0.25 [0.17–0.35]	0.23	0.17	0.20	$<$0.01
Zeaxanthine	0.13 [0.09–0.19]	0.13	0.10	0.08	$<$0.01
Lycopene	0.016 [0.003–0.042]	0.010	0.001	0.004	$<$0.01
α-tocoferol	16.20 [12.59–21.93]	16.54	13.95	13.24	0.02
γ-tocoferol	2.24 [1.49–3.30]	1.70	2.62	2.39	$>$0.05
Transferrin receptor (in μg/L)	1.56 [1.26–2.04]	1.78	1.91	1.61	$>$0.05
Selenium	1.89 [1.60–2.11]	2.27	2.00	1.95	$>$0.05

[a]Non-parametric test for trend.

khat chewing and increase in CD4 counts. While amphetamines may have an immunosuppressive role (26), cathinone has been tested in vitro and shown to induce IL-2 production, B-cell proliferation, and CTL induction in murine cells (27). The role of IL-2 in the stimulation of proliferation and differentiation of CD4 and CD8 T lymphocytes is well known, and consistent with these results (28–30). These findings indicate that khat has indeed the potential to significantly alter the immune response.

Studies suggest so far that the level of CD4 counts prior to HIV infection does not influence prognosis for those who get infected. Males from the Multicenter AIDS Cohort Study (MACS) with high CD4 counts prior to HIV infection had, compared to others, higher viral load, and steeper slope of CD4 counts decline in the early months following infection, so that there was no evidence for a beneficial effect of higher CD4 counts before infection (31). Also, it has been shown that females have higher CD4 counts than males at the time of HIV seroconversion, AIDS development, and death, without difference in survival compared to males (32). The significance of the findings shown in this paper should not therefore be interpreted in view of their potential impact on survival after HIV infection. However, different CD4 counts may require different management guidelines for chemoprophylaxis of opportunistic infections or antiretroviral therapy. The on-going cohort studies on HIV infection progression in Ethiopia may help defining new criteria for prophylactic and therapeutic interventions among Ethiopians.

In conclusion, this study suggests a multi-factorial origin to low CD4 counts among Ethiopians. Low body mass index, by reducing the number of circulating lymphocytes, may contribute to the phenomenon. High exposure to ultra-violets, by reducing the CD4%, may also participate in it. The effect of khat on CD4 counts is intriguing and deserves further investigations.

ACKNOWLEDGMENTS

This study was carried out by the Ethiopian–Netherlands AIDS Research Project (ENARP). ENARP is a collaborative effort of the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, the Municipal Health Service, Amsterdam, the Department of Human Retrovirology of the Academic Medical Center/University of Amsterdam, and the CLB, Amsterdam, the Netherlands. ENARP is financially supported by the Dutch Ministry for Development Cooperation and the Ethiopian Ministry of Health. The authors would like to thank Dr Jean-Hervé Colle for stimulating discussions regarding the role of IL-2 in CD4 and CD8 T lymphocytes homeostasis.

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