# Reference ranges and sources of variability of CD4 counts in HIV-seronegative women and men

M K Maini, R J C Gilson, N Chavda, S Gill, A Fakoya, E J Ross, A N Phillips, I V D Weller

**Background:** CD4 lymphocyte counts are used to monitor immune status in HIV disease. An understanding of the variability of CD4 counts which occurs in the absence of HIV infection is essential to their interpretation. The sources and degree of such variability have not been extensively studied.
**Objectives:** To establish reference ranges for CD4 counts in HIV-seronegative women and heterosexual men attending a genitourinary medicine (GUM) clinic, and to identify possible differences according to gender and cigarette smoking and, in women, any effect of the menstrual cycle, oral contraceptive use and cigarette smoking.
**Design:** Female and heterosexual male patients attending a GUM clinic and requesting an HIV-antibody test were recruited prospectively. Results from an earlier study of CD4 counts in homosexual men were available for comparison.
**Methods:** Lymphocyte subpopulation analysis on whole blood by flow cytometry.
**Results:** The absolute CD4 count and percentage of CD4 cells (CD4%) were significantly higher in women (n = 195) than heterosexual men (n = 91) [difference between the means 111 × 106/l (95% CI 41, 180) and 3·1% (1·30, 4·88)]. The absolute CD4 count and CD4% were also significantly higher in smokers (n = 143) than non-smokers (n = 140) [difference 143 (79, 207) and 2·1% (0·43, 3·81)]. Reference ranges for absolute CD4 counts (geometric mean ± 2SD) were calculated on log transformed data as follows; female smokers 490–1610, female non-smokers 430–1350, heterosexual male smokers 380–1600, heterosexual male non-smokers 330–1280. Among other variables examined, combined oral contraceptive pill use was associated with a trend towards a lower absolute CD4 count. Changes were seen in CD4% with the menstrual cycle. CD4 counts and CD4% did not differ significantly between heterosexual men and homosexual men (n = 45).
**Conclusion:** There is a significant gender and smoking effect on CD4 counts. The effects of oral contraceptive use and the menstrual cycle warrant further investigation.
(*Genitourin Med* 1996;72:27-31)

Keywords: CD4 count; reference range; HIV-seronegative

**Department of Genitourinary Medicine, Camden and Islington Community Health Services NHS Trust, The Middlesex Hospital, London W1N 8AA, UK**
M K Maini
A Fakoya

**Academic Department of Genitourinary Medicine, University College London Medical School, and Camden and Islington Community Health Services NHS Trust, London W1N 8AA, UK**
R J C Gilson
S Gill
E J Ross
A N Phillips
I V D Weller

**Department of Haematology, University College London Hospitals, London WC1E 6AU, UK**
N Chavda

Correspondence to:
Dr R J C Gilson, Academic Department of Genitourinary Medicine, University College London Medical School, The Mortimer Market Centre, Mortimer Market, Off Capper Street, London WC1E 6AU, UK.

Accepted for publication 12 September 1995

## Introduction

Cohort studies have established the prognostic value of peripheral blood CD4 counts.[1-4] They are routinely used to assess HIV-related immunosuppression, to decide when to start antimicrobial prophylaxis and antiretroviral therapy, and extensively in clinical trials. However, circulating lymphocytes represent a tiny fraction of the total body lymphoid pool. The majority are in the lymph nodes which are the main sites of HIV replication early in HIV infection.[5] A functional assessment of CD4 lymphocytes, though not currently routinely available, might provide more information.

Interpretation of the CD4 count requires an understanding of its biological variability and that due to the method of measurement.[6,7] The analysis of CD4 counts in HIV-negative populations may help to define this variability.

Heterosexuals, who constitute an increasing proportion of HIV infected patients, have not been extensively studied. Increased percentages of CD4 cells (CD4%) and absolute CD4 counts in HIV seronegative women compared with heterosexual men have been reported, but these studies have not controlled for potential confounders such as smoking and diurnal variation.[8-13]

We have measured lymphocyte subsets in HIV seronegative women and heterosexual men to identify any difference by gender and any effect of the menstrual cycle, oral contraceptive use and cigarette smoking. The study was designed to minimise the effect of procedural and diurnal variability and to collect data on potential confounders. We have compared the results with those obtained in an earlier, small study of homosexual men.

## Methods

PATIENT POPULATION

We recruited 195 non-pregnant women (median age 27·8 years, range 16·8–57·7) and 91 heterosexual men (median age 31·4 years, 18·6–66·5) between September 1992 and May 1993. All were attending the genitourinary medicine clinic at The Middlesex Hospital and were requesting an HIV-antibody test with or without other sexually transmitted disease (STD) screening. Only those testing between 9.30am and 12.30pm were included.

The study was granted ethical approval and informed consent was obtained. A piloted questionnaire was used to collect demographic and historical data, including menstrual cycle,

smoking, injecting drug misuse (IDM), and medication (last 3 months). Symptoms and STD test results were recorded.

Forty five homosexual men attending the same clinic (median age 39·5 years, range 22–59) had lymphocyte subsets measured in the same way in 1990. All were repeatedly anti-HIV-seronegative before testing and remained so for more than 6 months after.

### Laboratory methods

Blood was screened for anti-HIV-1 and anti-HIV-2 antibodies with an immunometric assay (Wellcozyme 1+2). EDTA samples, held at room temperature, were processed within 6 hours to obtain absolute lymphocyte counts (by automated white cell differential) and lymphocyte subsets. The lysed whole blood staining technique was used with Coulter monoclonals CD2, CD19, CD4 and CD8, conjugated with fluorescein isothiocyanate and phycoerythrin and a dual-colour flow cytometer (Coulter EPICS Profile II). All samples were processed in the haematology laboratory at University College Hospital which participates in a national quality assurance scheme. Correction for decreased lymphocyte purity and monocyte contamination has been evaluated in this laboratory and found to be unnecessary for fresh samples, as used here. Use of the CD4/CD8 combination of monoclonals results in the inclusion of a small number of CD8 non-T cells, but being weaker staining most are excluded by gating. Their inclusion does not affect the reported results.

Six patients were excluded; one patient with a positive anti-HIV test result, two because of insufficient blood samples and three because of delays before processing or flow cytometer faults.

### Statistical methods

The sample size was calculated to detect a difference of 150 × $10^6$/l CD4 cells between heterosexual men and women (80% power, 5% significance level). The number of women was increased to maximise the precision of the reference range in this group, and to allow subgroup analyses.

Differences between means (with 95% confidence intervals) were computed for total lymphocyte counts and lymphocyte subset counts and percent. Un-paired *t* tests and non-parametric tests were used, but gave equivalent results. Association between variables was sought by multiple linear regression analysis and a dose-response relationship with number of cigarettes smoked by calculation of Spearman's rank correlation coefficient. The

*Table 1 Association between gender, smoking, oral contraceptive use, menstrual cycle, ethnic origin, medication, injecting drug misuse, current STD and recent illness and CD4 counts and percent*

| | *CD4%* | | | *CD4 absolute count* × $10^6$/l | | |
|---|---|---|---|---|---|---|
| | *Mean* | *Difference in means* | *(95% CI) p value* | *Mean* | *Difference in means* | *(95% CI) p value* |
| *Sex* | | | | | | |
| Females n = 195 | 47·1 | 3·1 | (1·30, 4·88) p = 0·001 | 865 | 111 | (41, 180) p = 0·002 |
| Males n = 91 | 44·0 | | | 754 | | |
| *Smoking (last 3 months)* | | | | | | |
| Smoker n = 143 | 47·2 | 2·1 | (0·43, 3·81) p = 0·014 | 902 | 143 | (79, 207) p < 0·0001 |
| Non-smoker n = 140 | 45·1 | | | 759 | | |
| *Oestrogen oral contraceptive pill (last 1 month)* | | | | | | |
| User n = 54 | 45·8 | 1·3 | (−0·95, 3·56) NS | 797 | 84 | (−1, 168) p = 0·052 |
| Non-user n = 115 | 47·1 | | | 881 | | |
| *Menstrual cycle phase (non pill use for > 1 month)** | | | | | | |
| Pre-ovulatory n = 56 | 48·7 | 2·4 | (0·03, 4·79) p = 0·047 | 916 | 37 | (−68, 141) NS |
| Post-ovulatory n = 59 | 46·3 | | | 879 | | |
| Menstruating n = 11 | 51·4 | 4·3 | (0·25, 8·31) p = 0·038 | 870 | 30 | (−147, 208) NS |
| Non-menstruating n = 104 | 47·1 | | | 900 | | |
| *Ethnic Origin* | | | | | | |
| Caucasian n = 234 | 46·1 | 0·1 | (−2·99, 3·05) NS | 831 | 1 | (−117, 119) NS |
| Black n = 25 | 46·0 | | | 830 | | |
| *Medication (any medication taken in the last 3 months)*† | | | | | | |
| None n = 204 | 45·9 | 0·7 | (−1·25, 2·63) NS | 828 | 8 | (−66, 83) NS |
| Any n = 77 | 46·6 | | | 836 | | |
| *Injecting drug misuse* | | | | | | |
| Never n = 275 | 46·2 | 3·8 | (−0·62, 8·18) p = 0·09 | 832 | 60 | (−111, 231) NS |
| Ever n = 11 | 42·4 | | | 772 | | |
| *Current STD*‡ | | | | | | |
| No n = 187 | 45·9 | 0·06 | (−3·07, 3·19) NS | 810 | 70 | (−47, 194) NS |
| Yes n = 24 | 45·8 | | | 880 | | |
| *Illness (last 3 months)*§ | | | | | | |
| None n = 209 | 46·2 | 0·05 | (−2·04, 2·14) NS | 816 | 76 | (−4, 157) p = 0·063 |
| Any n = 62 | 46·3 | | | 892 | | |

*Women divided into pre/post-ovulatory, assuming ovulation occurred 14 days before the next anticipated onset of menstruation. Women who had started menstruation within 3 days of recruitment were then analysed separately.
†Treatment last 3 months: antibiotics 49; psychotropics 9; NSAIDs/other analgesics 8.
‡Current STDs: NSU 12; Chlamydia 5; HSV 2; PID 2; UTI 3 (Candida, BV, warts excluded).
§Illnesses last 3 months: viral/bacterial infection 43 (most upper respiratory tract).

*Table 2 Reference ranges for CD4 percent and absolute counts, CD8 percent and absolute counts, lymphocyte counts and CD4:CD8 ratio*

| | *CD4 geometric mean (± 2 standard deviations)* | | *CD8 geometric mean (± 2 standard deviations)* | | *Lymphocytes geometric mean (± 2 standard deviations)* | *CD4:CD8 geometric mean (± 2 standard deviations)* |
|---|---|---|---|---|---|---|
| | *%* | *Absolute count* | *%* | *Absolute count* | | |
| *Total* | | | | | | |
| Men n = 91 | 43·3 (30·4–61·6) | 710 (350–1440) | 27·0 (15·9–45·8) | 440 (200–1000) | 1·6 (0·9–2·9) | 1·6 (0·7–3·4) |
| Women n = 195 | 46·6 (34·9–62·1) | 820 (450–1510) | 26·0 (16·7–40·6) | 460 (230–910) | 1·8 (1·1–3·0) | 1·8 (1·0–3·3) |
| *Non-smokers* | | | | | | |
| Men n = 49 | 42·6 (29·5–61·6) | 650 (330–1280) | 26·7 (15·0–47·5) | 410 (170–1000) | 1·5 (0·9–2·7) | 1·6 (0·7–3·6) |
| Women n = 91 | 45·6 (34·4–60·5) | 760 (430–1350) | 26·2 (17·4–39·6) | 440 (230–830) | 1·7 (1·0–2·8) | 1·7 (1·0–3·1) |
| *Smokers* | | | | | | |
| Men n = 41 | 44·3 (31·6–62·1) | 780 (380–1600) | 27·2 (17·0–43·8) | 480 (240–960) | 1·8 (1·0–3·2) | 1·6 (0·8–3·3) |
| Women n = 102 | 47·6 (35·8–63·4) | 890 (490–1610) | 25·7 (16·0–41·5) | 480 (240–970) | 1·9 (1·1–3·0) | 1·8 (0·9–3·6) |

distribution of lymphocyte subset counts was skewed to the right and the data were therefore transformed to the log scale for the calculation of reference ranges (geometric mean ± twice the standard deviation); other analyses were not sensitive to log transformation, and therefore untransformed data was used.

## Results

Women had significantly higher CD4 counts and CD4% than heterosexual men (table 1). For CD4% the difference in the means was 3·1 (47·1 versus 44·0%) and for CD4 count the difference was 111 × $10^6$/l (865 versus 754 × $10^6$/l). No association was found with age although 85% of the patients fell within the age range of 20–40 years (data not shown). Smokers had significantly higher CD4 counts and CD4% than non-smokers. There was a positive dose-response relationship with the number of cigarettes smoked (Spearman's rank correlation coefficient 0·17, p = 0·04; data not shown).

Use of any form of hormonal contraception in the preceding 3 months had no significant effect on CD4 counts; however, among current (in the last month) users of oestrogen-containing oral contraceptive pills (OCPs) there was a trend (p = 0·052) towards a lower CD4 count but not CD4%.

An effect of the menstrual cycle was sought, after excluding women using hormonal contraception, those with erratic cycles and the post-menopausal (n = 80). Pre-ovulatory women (n = 56) had higher CD4% than post-ovulatory women (n = 59, p = 0·047). Further analysis showed that women who were menstruating (n = 11) had higher CD4% than others (n = 104, p = 0·038), but those within 2 days of ovulating did not differ significantly (data not shown). There were no significant differences in absolute CD4 counts.

Gender, smoking and recent illness showed significant associations with CD4 count after adjusting for other variables in a multiple linear regression model, suggesting that they act independently. The adjusted differences in mean CD4 count indicate the strongest association was with smoking (142/$mm^3$ higher in smokers; p = 0·0001), followed by gender (108/$mm^3$ higher in females; p = 0·002), and recent illness (80/$mm^3$ higher if recent illness, p = 0·04). When oestrogen oral contraceptive use was included in a model fitted for women only, the difference in mean CD4 count was 66/$mm^3$ (higher in non-pill users; p = 0·13). There were no statistically significant interaction effects observed. The R-square value for the multiple linear regression model was 0·12, indicating that these variables can only explain a small proportion of all inter-individual variability in CD4 counts.

Similar analyses were conducted for CD8 counts and CD8%, lymphocyte counts and CD4:CD8 ratio (data not shown). There were significant associations between total lymphocyte counts and CD8 counts and smoking, and between CD8 counts, CD8% and CD4:CD8 ratio and menstruation. There were no gender differences in CD8 counts, CD8%, CD4:CD8 ratio or total lymphocyte count.

Table 2 shows the reference ranges calculated for male and female non-smokers and smokers for CD4% and absolute counts, CD8% and absolute counts, total lymphocyte counts and CD4:CD8 ratios.

The mean CD4 count and CD4% in the group of 45 homosexual men tested in 1990 (mean CD4 count 830, CD4% 44·3) were not significantly different from the heterosexual men in this study. The reference range for CD4 counts was 420–1510 × $10^6$/l and for CD4% 32·1–59·9.

## Discussion

This study of HIV-seronegative GUM clinic attenders confirms that CD4 counts are significantly higher in women than heterosexual men after controlling for other sources of variability. Bofill *et al*[8] found that seronegative women had CD4 counts that were 28% higher, but a disproportionate number of the women were tested in the afternoon, so the gender difference might have been attributable to diurnal variation. Several studies have reported the same gender difference, but have not controlled for other factors including smoking,[8,9,12,13] pregnancy,[8,11] intercurrent infections,[8,9,11] ethnicity,[12,13] or HIV status.[9,12,13]

Procedural factors affect the reliability of lymphocyte subset counts. In this study all samples were kept at room temperature until the total lymphocyte count was measured by automated counter in a single laboratory, within 6 hours. In other studies the procedure was either not specified[9,14] or was not uniform[8] leading to only CD4% being reported.[10,11]

Flow cytometry is more reproducible than manual fluorescence as used in other studies[15 16] and whole blood staining eliminates prior lymphocyte separation, a potential source of cell loss.[9 12–14 17]

CD4-lymphocyte counts exhibit circadian variation of up to 60% with the nadir at about 11.00 am.[15 18] In this study all samples were taken between 9.30am and 12.30pm. Circannual variability in CD4% has been reported, with higher levels in April[19] and October/November,[19 20] but no such effect was seen in the present study (data not shown). CD4% has been shown to increase with age by 1% per decade,[9–11] but no such effect was detectable in this study. As reported previously[9 21] there were no racial differences in CD4 counts, although our study population was largely Caucasian.

Smoking is associated with a leucocytosis, a lymphocytosis and an increase in CD4% and CD4 absolute counts.[17 22 23] This was confirmed here and shown to be independent of gender. There was a dose-response effect of smoking, consistent with previous reports[17 23] and reports of reversibility of the effect in ex-smokers.[17 22] These findings strengthen the evidence for a causal relationship.

We included only patients who were tested HIV-antibody negative. Although 29% of the heterosexuals had had unprotected intercourse in the 3 months prior to testing, the chance of any of them being in the seroconversion window period is very small, given the overall prevalence of HIV of one in 286 recruits. None had a history of other serious illness associated with CD4 lymphopaenia.[24–27] No significant association was found between CD4 counts and the presence of an STD diagnosed at the time, although the number of patients was small. As expected, there was an association between other recent illness, most commonly upper respiratory tract infections, and an increase in CD4 count.

Treatment with cyclophosphamide or prednisolone has been associated with a decrease in CD4 counts,[28] but in this study no association with medication was observed. Only 11 patients had a history of injecting drug misuse; however, there was a trend ($p = 0{\cdot}09$) towards them having a lower CD4%. We found no difference in either CD4 counts or CD4% between heterosexual men and the group of homosexual men tested in 1990.

One possible explanation for the observed gender difference in CD4 counts is a sex hormone effect. Circulating lymphocytes have receptors for androgens and oestrogens,[29] and these hormones also exhibit diurnal and circannual rhythmicity.[16 19 30] If the adult female pattern of hormones is responsible for the difference in CD4 counts, the gender difference might be expected to appear at puberty. Tollerud *et al* found a gender difference in 17–19 year olds, but not in 12–16 year olds.[14] In adults under 40 years or age, Ohta *et al*[13] found higher CD4 counts in women than in men, but no difference in those over 40. A small study[31] found that hormone replacement therapy had no effect on CD4 counts in post-menopausal women. Changes in CD4 count and CD4% have been observed in pregnant women,[32] who were therefore excluded from our study.

A previous small study found that the menstrual cycle and OCP use had no effect on CD4% but did not control for other variables.[33] Our results suggest that OCP users may have lower CD4 counts and menstruation may be associated with higher CD4%.

Interestingly, although several factors have been shown to be associated with CD4 count variability in this study, these factors still only account for a small proportion of the variability observed (R-square 0·12). Other influences still need to be sought.

The calculated reference ranges for CD4% may be more stable than CD4 count, since the total lymphocyte count used to calculate the CD4 absolute count shows considerable variability, both within an individual and between laboratories.[34] CD4% may be a better prognostic guide than the absolute count.[35 36]

Knowledge of CD4 count reference ranges is essential in assessing the degree of immunosuppression in HIV-positive, asymptomatic individuals. A CD4 count of $500 \times 10^6$/l has been regarded as a clinically significant level, below which antiretroviral therapy should be started. The data here show that the reference range of CD4 counts extends well below 500. Awareness of the factors influencing counts and the potential magnitude of their effect should help in interpreting CD4 count changes and designing clinical trials. However, findings in HIV-seronegatives cannot be directly extrapolated to HIV-seropositives. For example, the effect of smoking on CD4 counts may be attenuated within 2 years after HIV-seroconversion[23 37] by a more rapid decline in CD4 counts in the HIV-infected smokers.[23] Diurnal variation may also be reduced in HIV seropositives.[34 38]

The gender difference observed in seronegatives may also be lost in seropositives. Women may have a faster decline in CD4 count following HIV infection.[39] Alternatively, HIV-infected women may have higher circulating CD4 counts than men but this may not have any functional significance. If this were true, they would develop opportunistic infections at higher counts; recent evidence is against this (AC Lepri, personal communication).

Whether the rate of progression to symptomatic HIV-disease and AIDS is the same in HIV-infected men and women is uncertain. If disease progression were slower in women, this would be consistent with a higher pre-infection CD4 count being associated with slower progression to AIDS.[40] Testing such hypotheses is hindered by the need to control for the other factors affecting disease progression.

We gratefully acknowledge the financial support of the AIDS Education and Research Trust (AVERT), and Camden and Islington Community Health Services NHS Trust. We thank Professor S J Machin and Mr Alan Carter, Department of Haematology, UCLH, for the help with the lymphocyte subset analyses and Professor R S Tedder, Division of Virology, UCLMS, for the HIV serology.

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