

Effect of recent thymic emigrants on progression of HIV-1 disease

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Summary

Background The concentration of T-cell receptor-rearrangement excision DNA circles (TREC) in peripheral-blood T cells is a marker of recent thymic emigrant $\alpha\beta$ T cells. We studied the predictive ability of measurements of TREC for clinical outcome in HIV-1-infected individuals.

Methods We measured TREC in peripheral-blood mononuclear cells with a real-time PCR assay. We studied 131 Greek participants in the Multicenter Hemophilia Cohort Study who had known HIV-1 seroconversion dates. The prognostic value of baseline TREC, CD4 T-cell count, and HIV-1 RNA concentration was assessed by Kaplan-Meier and Cox's regression analysis.

Findings Four participants had progressed to AIDS by first blood sampling. Among the remaining 127 individuals, the median value of TREC per 10^6 cells was 6900 (IQR 2370–15 604). Baseline TREC values were lower in the 53 who progressed to AIDS than in those who did not (geometric mean 2843 [95% CI 1468–5504] vs 6560 [4723–9113] per 10^6 cells; $p=0.017$). The relative hazard of AIDS, adjusted for plasma viral load, CD4 T-cell count, and age at seroconversion was 1.44 (95% CI 1.04–2.01; $p=0.031$) per ten-fold increase in TREC; that for death was 1.52 (1.12–2.06; $p=0.007$). The adjusted relative hazards of death were 2.91 (1.91–4.44; $p<0.001$) per ten-fold increase in plasma HIV-1 RNA load and 1.20 (1.04–1.38; $p=0.014$) per 100-cell decrease in CD4 T-cell count.

Interpretation The concentration of TREC in the peripheral T-cell pool complements HIV-1 RNA load and CD4 T-cell count in predicting the rate of HIV-1 disease progression. Recent thymic emigrants have a role in the pathogenesis of HIV-1 disease.

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Introduction

The rate of progression of HIV-1 disease varies widely among infected individuals. Several virological and host factors influence this variability. Virological factors include the plasma HIV-1 RNA load and the biological phenotype of HIV-1 strains. Many epidemiological studies on HIV-1-infected individuals have shown a relation between continuing viral replication and the pathogenesis of HIV-1 disease, with plasma HIV-1 RNA load being a strong predictor of the rate of progression of HIV-1 disease.^{1–6} In addition, host factors have substantial impact on clinical progression. For example, variants of the genes for CC-chemokine receptors 5 and 2 (*CCR5* and *CCR2*) significantly affect the transmission of HIV-1 and the rate of disease progression.^{7–11} Acquisition of HIV-1 infection at older age is associated with faster disease progression.¹² The relation between the age at HIV-1 infection and the subsequent gradient of the fall in CD4 T-cell count has been delineated,^{13,14} but how older age leads to faster clinical progression is not yet clear. Among several proposed mechanisms is the involution of the thymus that occurs with advanced age.¹⁵

Many studies have implicated the thymus in the pathogenesis of HIV-1 infection.^{16–19} During intrathymic T-cell differentiation, progenitor cells undergo rearrangement of the $\alpha\beta$ T-cell receptor resulting in the formation of stable excisional episomal T-cell-receptor DNA circles. Since these DNA circles are not copied during mitosis, the numbers decrease during cell division or the circles are lost owing to cell death.^{20–22} The concentration of T-cell-receptor rearrangement excision DNA circles (TREC) in the peripheral T-cell pool is a marker for thymocytes that have undergone few cellular divisions after T-cell-receptor rearrangement, commonly known as recent thymic emigrants.^{23,24} Douek and colleagues²⁵ showed in peripheral-blood mononuclear cells that the predominant form of TREC declined with age and on HIV-1 infection. Subsequently, we found that in uninfected individuals TREC values remained stable and high (about 105 TREC per 10^6 peripheral-blood mononuclear cells) for the first 10–15 years of life, followed by a sigmoid drop (1.0–1.5 ten-fold decrease) between the ages of 20 and 25 years and a gradual decline (slope $-0.03 \log_{10}/\text{year}$) for older ages.²⁶ Among HIV-1-infected individuals, TREC values were significantly lower than those in age-matched normal controls, although a significant overlap of the distribution of values was observed between the two groups. In this study, we examined the effect of TREC concentration in the peripheral T cells on HIV-1 disease progression in a cohort of HIV-1-infected patients with haemophilia.

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Methods

Study participants

All clinical samples were obtained from HIV-1-infected Greek patients enrolled in the Multicenter Hemophilia Cohort Study. The 158 HIV-1-infected haemophilic white individuals have known seroconversion dates and have been prospectively followed up for more than 16 years since seroconversion.¹³ Clinical and laboratory data have been collected roughly every 6 months. For 131 study participants, at least one cryopreserved sample of peripheral-blood mononuclear cells was available during the clinical follow-up. 27 individuals had no sample of peripheral-blood mononuclear cells available and, therefore, were excluded from the study (23 died within 7 years of seroconversion and four were lost during the clinical follow-up period).

TREC concentration was measured from cryopreserved peripheral-blood mononuclear cells collected closest to the HIV-1 seroconversion date. The sample collection date at which TREC concentration was first measured was used as the day of study entry (baseline). The distribution of TREC values of HIV-1-infected patients was compared with that of 544 age-matched HIV-1-uninfected individuals described in detail previously.²⁶

In longitudinal studies, we used peripheral-blood mononuclear cells from seven participants who were long-term

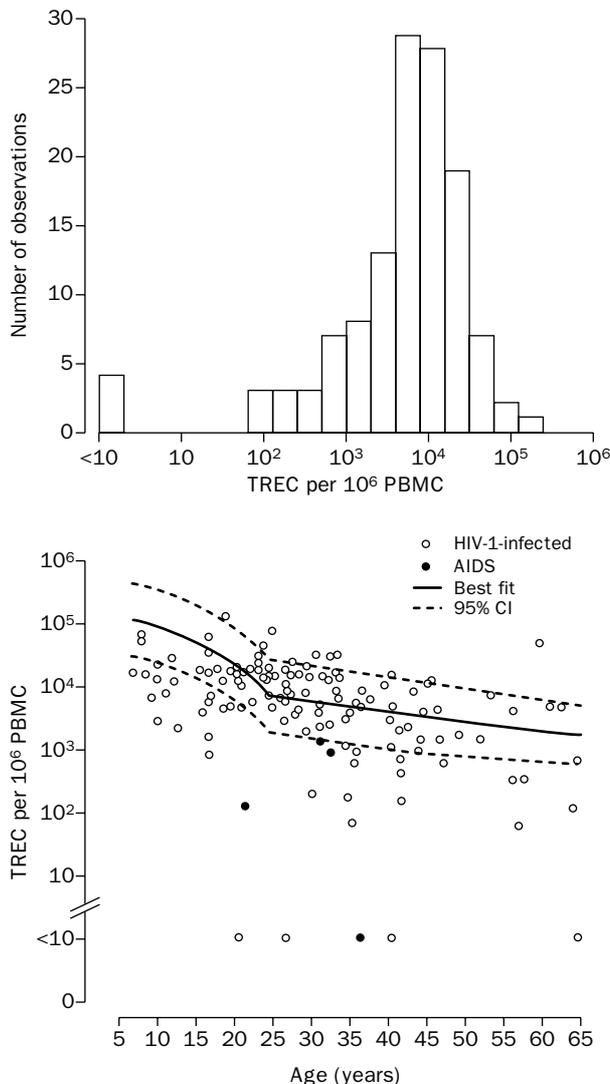


Figure 1: Distribution of TREC values at baseline and scatter plot of TREC values for each HIV-1-infected participant against age and the best-fitted curve for uninfected participants
PBMC=peripheral-blood mononuclear cells.

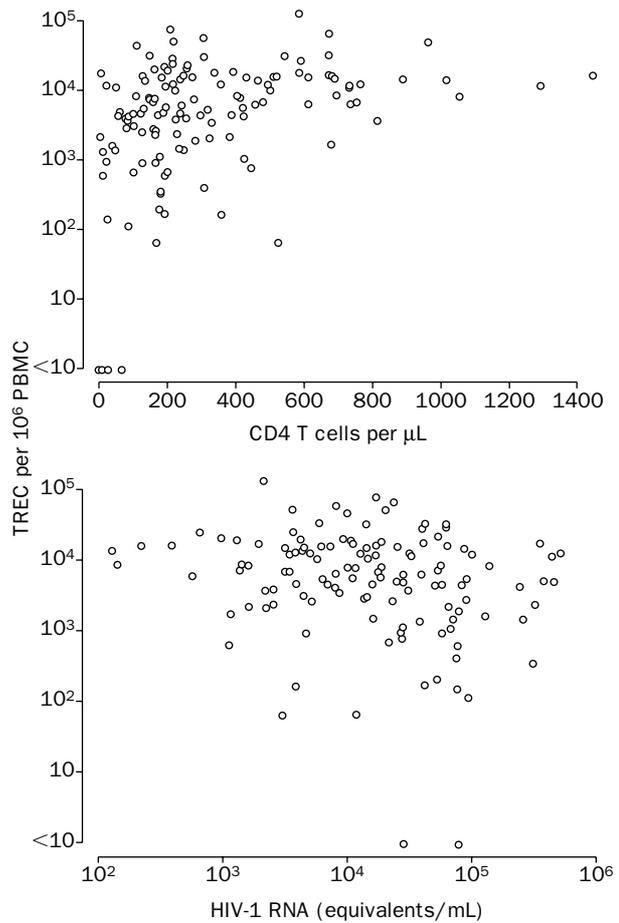


Figure 2: Relation between baseline TREC values (\log_{10} scale) and corresponding CD4 T-cell counts and HIV-1 RNA concentrations in plasma (\log_{10} scale)

non-progressors (without clinical AIDS and with CD4 T-cell counts above 500 cells/ μ L for at least 10 years since seroconversion) and from seven progressors randomly selected by a computer algorithm who developed clinical AIDS during follow-up. In these patients, TREC values were measured in all available stored samples taken before the start of highly-active antiretroviral therapy.

Procedures

Plasma HIV-1 RNA was measured with the ultrasensitive HIV-1 Amplicor Monitor assay (Roche Diagnostics, Alameda, CA, USA), which has a detection limit of 50 HIV-1 RNA copies/mL. CD4 T-cell counts were measured by flow cytometry with standard procedures.

To quantify the concentration of TREC per cell in peripheral-blood mononuclear cells, we used a molecular-beacon-based real-time PCR assay. The general method of quantifying single nucleotide sequences with nucleotide-specific molecular beacons²⁷ and real-time PCR²⁸ has been previously described. Here we used a multiplex assay to quantify simultaneously the TREC value and a *CCR5* coding sequence to measure cell equivalents in the input DNA. The sequence of the human TREC-specific molecular beacon was fluorescein-5'CGGCGTCTGCTCTTCATTACACCGTTCTCAGCCG-4-(4'-dimethylaminophenylazo)benzoic acid, and the sequences of the PCR primers were 5'-GATGGAAAACACAGTGTG-ACATGG and 5'-CTGTCAACAAAGGTGATGCCACAT. The sequence of the human *CCR5*-specific molecular beacon was tetrachrofluorescein-5'-GCGCCTATGACA-AGCAGCGGAGGAGGCGC-4-(4'-dimethylaminophenylazo)benzoic acid, and the sequences of the PCR primers were 5'-GCTGTGTTTTCGCTCTCTCCAGGA and

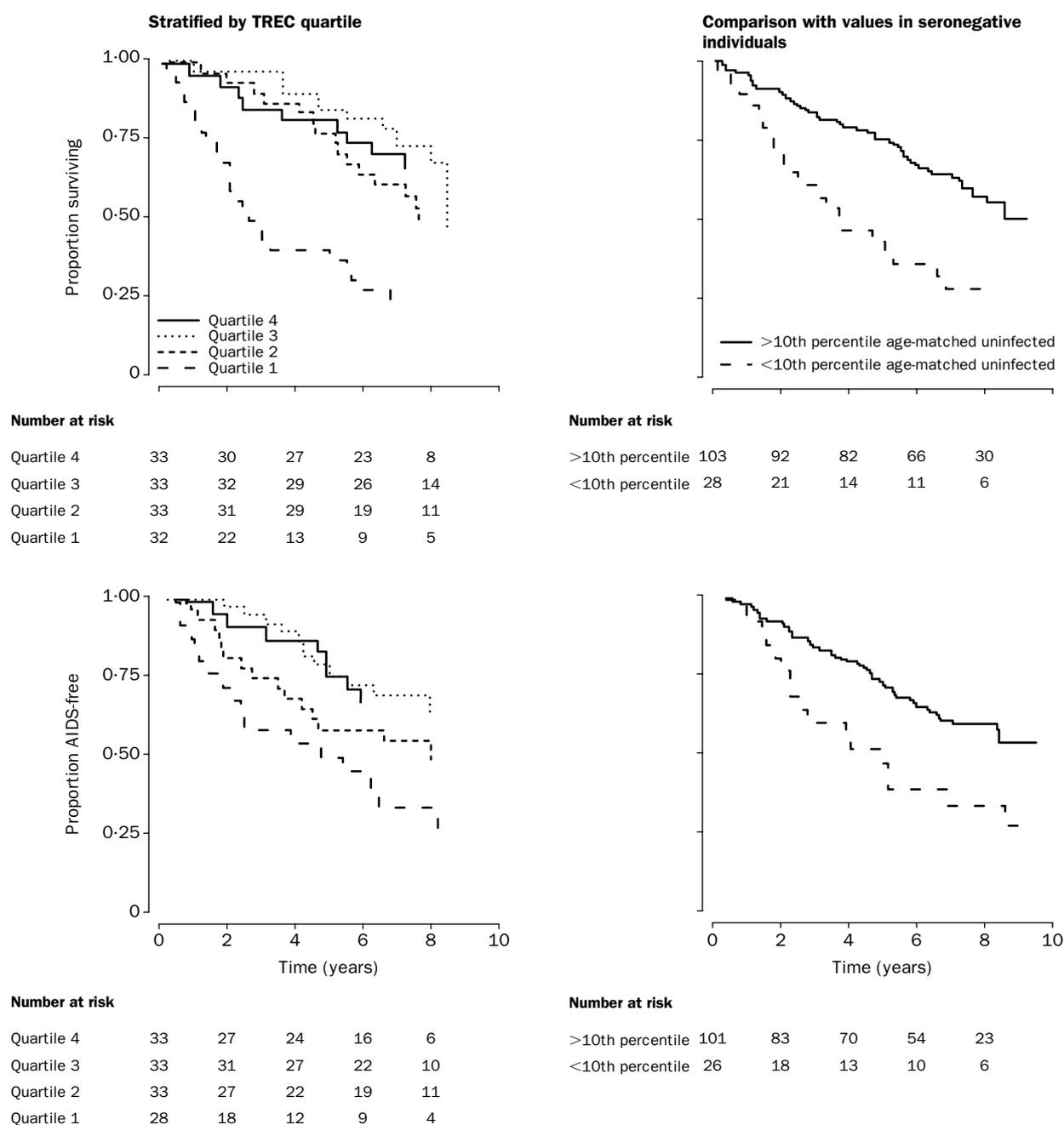


Figure 3: **Prognostic effect of TREC values on progression to AIDS and death**

For TREC distribution: quartile 1= $<1585/10^6$ PBMC; quartile 2= $1586-6310/10^6$ PBMC; quartile 3= $6311-15\ 849/10^6$ PBMC; quartile 4= $>15\ 849/10^6$ PBMC.

5'-CTCACAGCCCTGTGCCTCTTCTTC. In each molecular beacon, the first and last six nucleotide bases form the hairpin structure, and the remaining sequences are the target recognition sequences. The hairpin sequences and the length of the target recognition sequences in both molecular beacons were designed so that they would hybridise to the target amplicons at the temperature used for the corresponding PCR primer hybridisations without any cross-hybridisation to other targets.

Each 50 μ L reaction mixture contained about 1 μ g genomic DNA, 0.25 μ mol/L of each molecular beacon, 0.5 μ mol/L of each primer, 0.25 μ mol/L of each of dNTP, 2.5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA, USA), 50 mmol/L potassium chloride, 3.5 mmol/L magnesium chloride, and 10 mmol/L Tris-HCl, pH 8.3. 50 cycles of amplification (94°C denaturation for 30 s, 60°C annealing for 60 s, and 72°C polymerisation for 30 s) were run in a spectrofluorometric thermocycler (ABI PRISM 7700, Applied

Biosystems, Foster City, CA, USA). During the annealing stage of each thermal cycle, the fluorescence emission spectrum from 500 nm to 650 nm was recorded. After completion of the PCR amplification, in each sample the emission spectra for each PCR cycle were decomposed into the spectral contributions of fluorescein and tetrachlorofluorescein, and the changes in the emission spectra during the course of the amplification were analysed and compared with those from control *CCR5* and TREC samples with known concentrations of initial DNA templates. In each genomic DNA sample, peripheral-blood mononuclear cells were quantified as one cell per two *CCR5* copies (unpublished), and the TREC value was calculated as the number of TREC per 10^6 peripheral-blood mononuclear cells.

Statistical analysis

Individual gradients of log-transformed TREC concentrations and CD4 T-cell counts over time were estimated by linear

	Unadjusted		Adjusted for all other factors	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
Death				
TREC*	2.01 (1.59–2.55)	<0.0001	1.52 (1.12–2.06)	0.007
HIV-1 RNA†	3.59 (2.41–5.35)	<0.0001	2.91 (1.91–4.44)	<0.0001
CD4 T-cell count‡	1.36 (1.18–1.57)	<0.0001	1.20 (1.04–1.38)	0.014
Age at seroconversion§	1.38 (1.15–1.65)	<0.0001	1.07 (0.88–1.31)	0.498
AIDS				
TREC*	1.38 (2.41–1.39)	<0.0001	1.44 (1.04–2.01)	0.031
HIV-1 RNA†	3.05 (2.02–4.60)	<0.0001	2.80 (1.80–4.36)	<0.0001
CD4 T-cell count‡	1.28 (1.11–1.48)	0.001	1.14 (0.99–1.31)	0.079
Age at seroconversion§	1.05 (0.86–1.30)	0.620	0.86 (0.70–1.07)	0.184

Cox's proportional-hazards models were used to calculate the relative risk of death and AIDS per unit change in the covariates: *10-fold decrease in TREC per 10⁶ peripheral-blood mononuclear cells; †10-fold increase in HIV-1 RNA/mL; ‡decrease of 100 cells/ μ L; §10-year increase.

Relative risk of HIV-1-induced death and AIDS associated with changes in TREC, HIV-1 RNA, CD4 T-cell count, and age at seroconversion

regression analysis. Individual-specific gradients among different groups were compared by use of the Mann-Whitney *U* test. The relative prognostic values of baseline TREC concentrations, CD4 T-cell count, and plasma HIV-1 viral load were investigated with death and AIDS development as two critical endpoints of HIV-1 infection. Kaplan-Meier survival curves were used to estimate cumulative frequency of an endpoint, and the log-rank test was used to compare survival curves among different groups. Univariate and independent effects were assessed by Cox's proportional-hazards models. The cohort was analysed as seroprevalent (study entry was used as time 0). However, we also did sensitivity analyses, by Kaplan-Meier methods and Cox's proportional-hazards models with allowance for late entry (seroconversion date was used as time 0). Total and AIDS-free survival were calculated in study participants whose TREC values were above or below the 10th percentile of the values in the HIV-1-uninfected population, stratified in 10-year age-groups.

Results

Baseline values

TREC values were measured in the 131 of 158 haemophilic individuals whose stored peripheral-blood mononuclear cells were available. The median time between HIV-1 seroconversion and first collection of peripheral-blood mononuclear cells was 7.2 years (range 3.5–12.9 years). The mean age of the participants at study entry was 30.8 years (SD 13.7; range 7–65). Four participants had developed AIDS by the time of first collection of blood for mononuclear cells (study entry) and their TREC values ranged from less than 10 (one patient) to 1346 per 10⁶ cells. Of the 127 participants without clinical AIDS at study entry, four had fewer than 10 TREC per 10⁶ cells (figure 1). The median value of TREC per 10⁶ cells was 6900 (IQR 2370–15 604). Values were decreased in older individuals (by 0.26 log₁₀ [95% CI 0.15–0.37] or 45% [29–57] per 10 years increase in age; *p*<0.0001).

At study entry (\pm 6 months) in participants without AIDS the median CD4 T-cell count was 242 cells/ μ L (range 3–1445) and the median value for plasma HIV-1 RNA was 17 050 copies/mL (range 35–542 120). TREC values were moderately correlated with CD4 T-cell counts (Spearman's *r*=0.42, *p*<0.0001) and weakly correlated with HIV-1 RNA load (Spearman's *r*=–0.23, *p*=0.008, figure 2).

Among the 544 seronegative individuals the median value of TREC was 8327 per 10⁶ cells (IQR 2282–46 058). Overall, the number of TREC was

significantly lower in HIV-1-infected than in seronegative individuals (*p*<0.001); 79% of the infected participants had values above the 10th percentile of the age-matched normal individuals.

Prognosis of HIV-1 infection

Among the 127 participants without clinical AIDS at study entry, baseline TREC values were lower in the 53 who progressed to AIDS than in those who remained free of AIDS (geometric mean 2843 [95% CI 1468–5504] vs 6560 [4723–9113] TREC per 10⁶ cells; *p*=0.017). Clinical AIDS developed within 1 year of follow-up in all four patients who had fewer than 10 TREC per 10⁶ cells at study entry.

For quartiles of the TREC distribution, ranging from lowest to highest values (figure 3), the cumulative rates of death by 9 years after study entry were 78.6% (95% CI 63.1–90.8), 52.8% (36.3–71.4), 56.5% (26.2–89.8), and 36.5% (21.5–57.4). The corresponding rates for progression to clinical AIDS were 79.6% (56.4–95.2), 53.7% (36.4–73.4), 40.9% (24.6–62.6), and 38.4% (22.0–61.1). The median times to death in these quartiles were 2.5 years, 7.7 years, 8.5 years, and longer than 10 years (log-rank *p*<0.0001); the median times to clinical AIDS were 4.0 years, 8.1 years, longer than 10 years, and longer than 10 years (log-rank *p*=0.009). Figure 3 also shows the proportions alive and free of clinical AIDS for participants with TREC values above or below the 10th percentile of the age-matched uninfected individuals. The progression rates differed significantly between the two groups (log-rank test *p*=0.01 for time to death and *p*=0.006 for time to AIDS).

In univariate analysis, TREC values, concurrent CD4 T-cell count, plasma viral load, and age at HIV-1 seroconversion were each significantly associated with the hazard of death (table). However, after adjustment for the other factors, age at seroconversion was no longer significantly associated. Age at seroconversion was not a significant predictor of progression to AIDS. The time between HIV-1 seroconversion and first collection of blood for mononuclear cells was not significantly associated with either endpoint (data not shown). The inclusion of time between HIV-1 seroconversion and first blood sample as another covariate in the proportional-hazards models did not modify the association between TREC and either endpoint. The predictive value of TREC value was reduced but remained significant after adjustment for concurrent CD4 T-cell count, HIV-1 RNA, and age at seroconversion. Sensitivity analysis with Kaplan-Meier methods and Cox's proportional models and allowance for late entry gave similar results. Further sensitivity analysis to assess the potential bias due to the exclusion of 27 study participants because no peripheral-blood mononuclear cells were available showed that TREC remained a significant prognostic factor of progression to death and AIDS even when the highest possible value of TREC observed in our cohort was assigned to the missing men.

Results from an explanatory analysis for four subgroups, defined by the median values of HIV-1 RNA and CD4 T-cell count suggested that the number of TREC is most informative in individuals with viral loads below the median and CD4 T-cell counts above the median. Within this subgroup, the relative hazard of death for individuals with TREC values below (*n*=10)

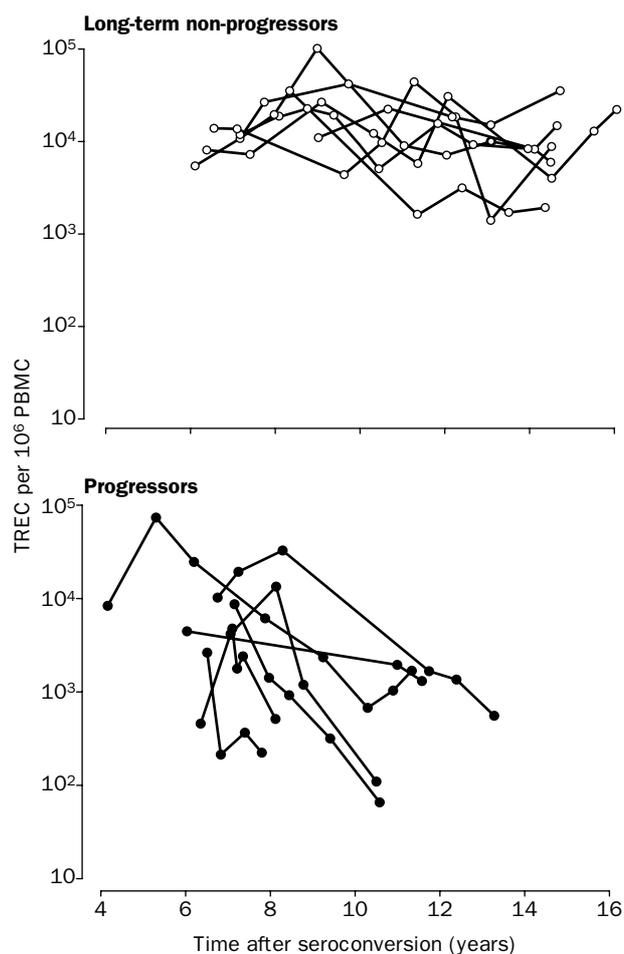


Figure 4: Longitudinal TREC measurements in long-term non-progressors and progressors

the median compared with those who had values above the median ($n=30$) was 3.3 (95% CI 0.7–16.3). The relative risks with TREC in the other three subgroups ranged from 1.0 to 1.3.

Longitudinal trends in TREC values

In the longitudinal study, TREC values were measured in long-term non-progressors and progressors (figure 4). The time since seroconversion of the first TREC value measurement (baseline) was similar in these two sets (median time 7 and 6.5 years, respectively; $p>0.10$). After baseline, TREC value was measured yearly for each individual. The baseline TREC values were significantly higher in long-term non-progressors than in progressors (median 10 231 [range 4854–32 318] vs 4568 [446–32 318] TREC per 10^6 peripheral-blood mononuclear cells; $p=0.047$).

The concentration of TREC remained stable over time in long-term non-progressors but fell in progressors. The median rate of change per year of \log_{10} TREC was -0.027 (IQR -0.046 to 0.008) in long-term non-progressors compared with -0.23 (-0.606 to -0.071) in progressors ($p=0.035$). In absolute scale, the median rate of decrease per year was 6% (-2 to 10) in long-term non-progressors and 41% (15 to 75) in progressors. The rate of change of the \log_{10} TREC values was significantly correlated with the corresponding rate of decrease of CD4 T-cell count (Spearman's $r=0.50$; $p=0.07$). As expected, the rate of decline of CD4 T-cell

count was significantly lower in long-term non-progressors than in progressors (median 21 [IQR 14–37] vs 61 [39–79] cells/year; $p=0.01$).

Discussion

TREC have no function themselves, but they reflect the population of recent thymic emigrants that have not undergone extensive cellular proliferation peripherally. We found that TREC concentration is an important predictor of AIDS and death in HIV-1 disease. By proportional-hazards analysis, we showed that TREC is an independent predictor of the clinical outcome for HIV-1-infected individuals. Age at seroconversion lost its predictive power after adjustment for TREC. Perhaps age at seroconversion reflects thymic function and recent thymic emigrants, which are more accurately measured by TREC. Furthermore, progression to AIDS and death did occur in participants with TREC values similar to those in the HIV-1-uninfected population; this finding suggests that only very low age-adjusted values have clinical significance. We found that TREC concentration may be particularly informative in patients with high CD4 T-cell counts and low HIV-1 RNA loads, who otherwise have the best prognosis. Further studies will be needed to examine whether low TREC values are an earlier warning sign of subsequent fast progression to AIDS and death in this subgroup. Larger studies will be needed for a detailed exploration of interactions of TREC with other prognostic markers.

In our longitudinal study, not only had long-term non-progressors TREC values within the normal range of uninfected individuals, but also these values were maintained for many years after HIV-1 seroconversion. In contrast, progressors had lower values than long-term non-progressors, and the values declined nearly ten times faster. At the rate of TREC decline that we measured in progressors ($-0.23 \log_{10}/\text{year}$), TREC would fall from 10 000 to 50 per 10^6 peripheral-blood mononuclear cells in 10 years. In this study a value of 50 TREC per 10^6 peripheral-blood mononuclear cells indicated extremely high risk of progression to AIDS and death. This finding is consistent with previous results.²⁶ The direct correlation between the gradients of the declines in TREC and CD4 T cells supports the hypothesis that the decrease in recent thymic emigrant cells is causally related to the loss of CD4 T cells.

Contributors

A Hatzakis and L G Kostrikis designed the study and interpreted the results. G Touloumi did the statistical analysis and contributed to the interpretation of the results. R Karanicolas participated in the development of the multiplex molecular-beacon-based real-time PCR technique and did the real-time PCR studies. A Karafoulidou and T Mandalaki were co-principal investigators of the Greek Hemophilia Cohort and supervised the running of the clinical part of the study. C Anastassopoulou did the plasma HIV-1 RNA measurements. L Zhang, J J Goedert, and D D Ho provided substantial input into the original study design and the interpretation of results. L G Kostrikis devised the original hypothesis, developed the multiplex real-time PCR technique and coordinated the study. All investigators participated in the writing of the paper.

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