T cell receptor excision circles and HIV-1 *2-LTR* episomal DNA to predict AIDS in patients not receiving effective therapy

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Objective: To determine whether improved prediction of AIDS-free survival following HIV-1 seroconversion is achieved by measuring HIV-1 *2-LTR* episomal DNA (*2-LTR*) circles and T cell receptor rearrangement excision circles (TREC), reflecting HIV replication and lymphocyte emigration from the thymus, respectively.

Design: Subanalysis of a cohort of 154 patients with hemophilia who became HIV positive between 1978 and 1985 and were followed prospectively.

Methods: Relative hazards (RH) of AIDS, in the absence of highly effective anti-HIV therapy, were estimated for age, HIV-1 viral load, CD4 lymphocyte count and levels of HIV-1 *2-LTR* circles and TREC [per 10^6 peripheral blood mononuclear cells (PBMC)].

Results: TREC correlated significantly with CD4 cell counts ($r = 0.30$) and age ($r = -0.60$). *2-LTR* circles correlated significantly with HIV-1 viral load ($r = 0.35$). If viral load, CD4 lymphocytes and age were included in a proportional hazards model, the risk of AIDS during a median of 11.6 years of follow-up was increased significantly with fewer TREC (adjusted RH, 2.0 per $\log_{10}$ copies/10^6 PBMC) and more *2-LTR* circles (RH, 1.7 per $\log_{10}$ copies/10^6 PBMC). AIDS prediction with TREC and *2-LTR* circles held for most subgroups defined by median viral load, CD4 lymphocytes and age.

Conclusions: PBMC that have high levels of HIV-1 replication and low levels of recent thymic emigrants are associated with a substantially increased risk of AIDS. It is not known if measurement of either TREC or *2-LTR* circles will complement HIV-1 viral load as an estimation of the risk of AIDS for patients who are receiving highly effective anti-HIV therapy.

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Introduction

Low levels of T cell receptor rearrangement excision DNA circles (TREC) in peripheral blood lymphocytes appear to be associated with and are potentially predictive of AIDS risk among persons with HIV-1 infection [1–3]. TREC are thought to represent a marker of recent thymic emigrants [4], although this is debated [5]. More recently, circular episomal DNA sequences from the two long-terminal repeats (*2-LTR*) of the HIV-1 genome have been identified in lymphocytes [6–10]. These *2-LTR* circles are short lived and occur only when HIV-1 provirus is being actively reverse transcribed. They, therefore, identify infected cells in which HIV-1 is replicating [7], and they have been associated with adverse signs of HIV-1 progression [6–10]. As part of a continuing effort to clarify the biology of HIV-1-related immunosuppression and the risk of AIDS in a population-based cohort, we sought to determine whether TREC and *2-LTR* circles were predictive of AIDS in the era prior to highly active antiretroviral therapy (HAART) and whether such associations were independent of HIV-1 viral load, age and CD4 cell count.

Methods

Patients and laboratory assays

In the Multicenter Hemophilia Cohort Study, HIV-1 viral load in serum during early chronic infection in 165 subjects was shown to be predictive of AIDS risk for more than 10 years in the future [11]. In these same subjects, cryopreserved peripheral blood mononuclear cells (PBMC), separated from blood obtained at the same time or as close as possible to the viral load measurement, were sought for TREC and *2-LTR* analysis. The number of copies of TREC and the *CCR5* gene were quantified by realtime polymerase chain reaction (PCR) using methods described previously [3]. TREC levels were expressed as copies per 10^6 PBMC [3]. *CCR5* was used to determine the denominator number of PBMC. HIV-1 *2-LTR* DNA circles encoding the R-U5-U3 region were quantified by a molecular-beacon-based realtime PCR assay as previously described [12]. HIV-1 viral load was determined with a commercial assay (HIV Amplicor Monitor, Roche Molecular Diagnostics, Branchburg, New Jersey, USA), and CD4 and CD8 cell counts were determined by conventional flow cytometry [11,13].

Statistical analysis

Median and interquartile range (IQR) values were determined. Spearman correlation was used to evaluate the relationships among the variables. Univariate and multivariate Cox proportional hazards models for AIDS risk were constructed with PROC PHREG of the Statistical Analysis System (SAS Institute, Cary, North Carolina, USA) using continuous measures ($\log_{10}$ transformed TREC and *2-LTR* levels and HIV-1 serum viral loads; untransformed age and CD4 cell counts). Univariate models and bivariate models (not presented) were constructed with the TREC and *2-LTR* variables stratified using median values of HIV-1 viral load, age, and CD4 cell count. The cohort was defined and each participant was followed starting with the individual dates of HIV-1 antibody seroconversion. To accommodate late entry (that is, missing participants who progressed to AIDS before they could be tested for TREC/*2-LTR*), each participant entered the Cox models on his or her TREC/*2LTR* date using the 'entry time' option of PROC PHREG. The Cox proportionality assumption was checked with Kaplan–Meier plots (not presented). The log-likelihood test with liberal entry and stay criteria ($P < 0.15$) was used for inclusion and retention in the final multivariate model.

Results

Specimens for TREC and *2-LTR* analysis were available for 161 (98%) of the previously reported 165 subjects. However, because the only specimen for seven subjects was after AIDS onset, the analysis was limited to the remaining 154 subjects (93% of the cohort) whose *2-LTR* and TREC levels were measured at a median of 4.9 years (IQR, 4.1–5.6) after HIV-1 seroconversion.

The analyzed cohort of 154 subjects had a median age of 24 years (IQR 15–32). The distributions of TREC and *2-LTR* levels by HIV-1 viral load, CD4 cell count and age are shown in Fig. 1. The median values were < 10 copies/10^6 PBMC for *2LTR* and 5551 copies/10^6 PBMC for TREC. Median values for the other markers were HIV-1 RNA 35 175 copies/ml, CD4 cell count of 479×10^6 cells/l and CD8 cell count of 713×10^6 cells/l. TREC level (Fig. 1a–c) was correlated significantly with CD4 cell count ($r = 0.30$; $P = 0.0002$) and inversely with age ($r = -0.60$; $P = 0.0001$). *2-LTR* level was significantly correlated with HIV-1 serum viral load ($r = 0.35$; $P = 0.0001$; Fig. 1d).

Fifty-six (36%) of the 154 subjects developed AIDS during a median follow-up of 11.6 years (IQR, 9.3–13.3). In univariate analysis, risk of AIDS was significantly increased with high age [relative hazard (RH) 1.4 per decade], high HIV-1 serum viral load (RH 2.8 per $\log_{10}$ copies/ml), low CD4 cell count (RH 1.2 per 100×10^6 cells/l), low TREC level (RH 3.8 per 10^6 PBMC), and high *2-LTR* level (RH 2.3 per $\log_{10}$ copies). All five of these variables remained significantly

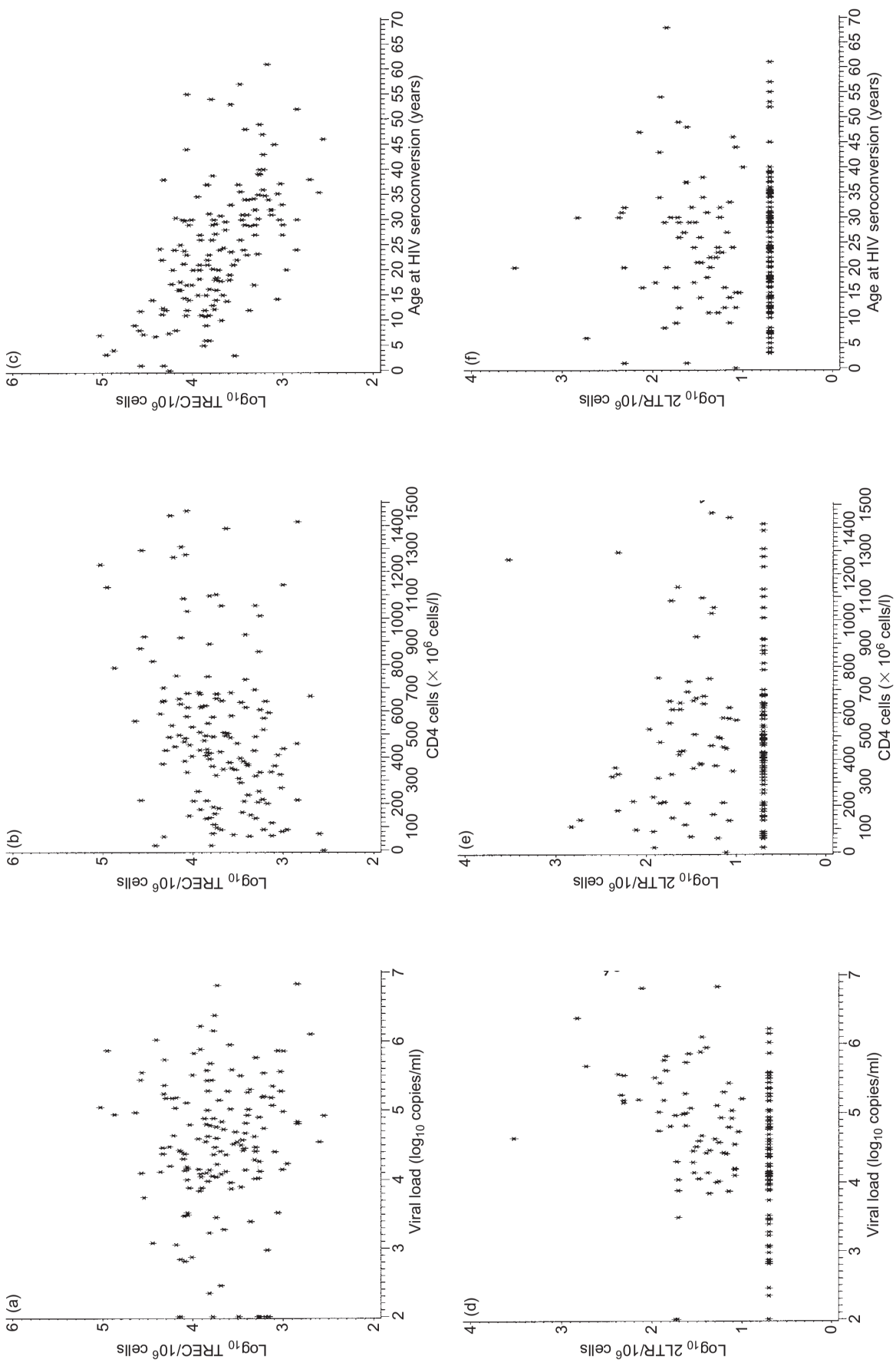

Fig. 1. Quantified levels of T cell receptor rearrangement excision circles (TREC; a–c) and HIV two long-terminal repeats (*2-LTR*) copies (d–e), by HIV serum viral load (a,d), CD4 lymphocyte count (b,e), and age (c,f) among 154 participants in the Multicenter Hemophilia Cohort Study. TREC, *2-LTR*, and HIV viral load were $\log_{10}$ transformed. The lower limits of detection were 10 copies/10^6 PBMC (1.0 $\log_{10}$ copies/10^6 PBMC) for *2-LTR* and 200 copies/10^6 PBMC (2.3 $\log_{10}$ copies/10^6 PBMC) for HIV viral load; the lowest plotted values are halfway between these cutoffs and zero. PBMC, peripheral blood mononuclear cells.

Table 1. AIDS risk by levels of T cell receptor rearrangement excision DNA circles (TREC) and HIV-1 two long-terminal repeats (*2-LTR*) episomal DNA, stratified by other variables among 154 participants in the Multicenter Hemophilia Cohort Study.

	No. developing AIDS	Relative hazard for AIDS (95% CI)	
		Lower TREC[b]	Higher *2-LTR*[b]
For all subjects			
Univariate	56	3.8 (2.2–6.8)	2.3 (1.6–3.2)
Multivariate[a]	56	2.0 (1.0–3.9)	1.7 (1.1–2.8)
By strata			
HIV-1 load			
Greater than median	41	3.1 (1.8–5.5)	1.8 (1.2–2.7)
Median or less	15	3.4 (0.9–13.3)	0.7 (0.1–3.5)
CD4 cell count			
Greater than median	18	4.2 (1.3–13.2)	1.6 (0.8–3.0)
Median or less	38	2.7 (1.4–5.3)	3.2 (1.9–5.4)
Age			
Young: median or less	23	1.8 (0.6–5.2)	1.8 (1.0–3.1)
Old: greater than median	33	5.4 (2.4–12.0)	3.6 (1.9–6.8)

CI, confidence interval.
[a]The multivariate model included age at HIV-1 seroconversion, HIV-1 viral load, TREC and *2-LTR* values (log_{10} transformed and expressed per log_{10}); and CD4 cell count (per 10^6 cells/l) Similar estimates were obtained for TREC (relative hazard 2.2; 95% CI, 1.2–4.3) and *2-LTR* (relative hazard 1.6; 95% CI, 1.1–2.5) in a model allowing different underlying hazards in each stratum defined by median values of HIV-1 viral load, CD4 cell count, and age.
[b]Risk per log_{10} of copies/10^6 peripheral blood mononuclear cells.

related to AIDS risk in multivariate analysis, including HIV-1 viral load (adjusted RH, 2.4), CD4 cell count (adjusted RH, 1.1), age (adjusted RH, 1.3), TREC level [adjusted RH, 2.0; 95% confidence interval (CI), 1.0–3.9), and *2-LTR* level (adjusted RH 1.7; 95% CI, 1.1–2.8). The univariate and multivariate results for TREC and *2-LTR* are presented in Table 1, as are stratified analyses designed to identify subgroups in which TREC and *2-LTR* might be more or less predictive of AIDS risk. Lower TREC values were associated with increased AIDS risk in every subgroup, although this was not statistically significant in young participants (RH 1.8; 95% CI, 0.6–5.2), those who seroconverted at or before 24 years of age. Higher *2-LTR* levels were significantly associated with increased AIDS risk except in the two lowest risk strata: participants with a high CD4 cell count (RH, 1.6; 95% CI, 0.8–3.0) or a low HIV-1 viral load (RH, 0.7; 95% CI, 0.1–3.5).

Discussion

As expected, AIDS risk was increased with high HIV-1 viral load, older age and lower CD4 cell count. AIDS risk was also significantly increased with a low TREC level or a high *2-LTR* level. Our TREC findings confirm those previously reported for a population of hemophiliacs in Greece [3], specifically that AIDS risk in the pre-HAART era was related strongly and inversely to TREC level. In our study, AIDS risk was reduced by half for each log_{10}increment in TREC. The TREC association was independent of the viral load and age, and it complemented traditional CD4 cell testing for estimating AIDS risk.

A new marker of HIV-1 proviral load, *2-LTR* episomal DNA circles, was also evaluated. This represents the number of PBMC that are infected with actively replicating HIV-1. This complements serum or plasma HIV-1 viral load, which reflects the homeostasis between virions shed into and cleared from the circulation. Our finding that high levels of *2-LTR* circles was predictive of AIDS corroborates and extends previous cross-sectional [6,8] and short-term prospective [9,10] studies, lending further evidence that immunodeficiency results from replicating HIV-1 infection.

Our study had several limitations. Serum viral load and *2-LTR* level were highly correlated, which provided face validity. However, as viral load is a strong predictor of AIDS risk, the correlation made it difficult to identify an independent predictive effect of *2-LTR*. In addition, although no violations of the proportional hazards assumption of the models or statistically significant interactions among the variables were identified, our inability to detect *2-LTR* sequences in the cells of many participants emphasizes that the observation of higher AIDS risk with high levels of *2-LTR* must be viewed cautiously. Two other studies have reported that *2-LTR* circles could be detected in approximately half of HIV-1-infected patients receiving no or minimal antiretroviral therapy [9,10], suggesting that our *2-LTR* assay had comparable sensitivity. Finally, sparse data limited our power to perform subgroup and

multivariate analyses. As summarized in Table 1, in each of six predefined subgroups, AIDS risk was elevated with low TREC and, except for participants with low viral loads (< 36 000 copies/ml), with high *2-LTR*.

Substantial increases in TREC level following HAART initiation have been reported in a small number of adults and children, and in some patients these TREC increases correlated with successful clearance of HIV-1 from the plasma and with recovery of cellular responses to a neoantigen [1,2,14,15]. Conversely, among patients receiving HAART with no detectable plasma viral load, detection of *2-LTR* circles recently was associated with productive outgrowth of HIV-1 in culture and with lower CD4 cell counts, both of which are likely to be adverse signs [16]. Nonetheless, it still remains to be seen whether TREC or *2-LTR* levels are prognostic for treatment failure or clinical endpoints among patients receiving effective therapy.

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Appendix

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