

Comparison of Serum TNF- α Levels and HTLV-1 Proviral Load between Patients with HAM/TSP and Asymptomatic HTLV-1 Carriers

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Abstract

Background: Infection with human T-lymphotropic virus type 1 (HTLV-1) remains clinically silent in most of those infected; only a small minority develop HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Tumour necrosis factor alpha (TNF- α) has been proposed as a mediator of this transition, but evidence from peripheral blood is inconsistent, and to our knowledge no such data have been reported from Iraq. **Objective:** To compare serum TNF- α concentrations and HTLV-1 proviral load between patients with HAM/TSP and asymptomatic HTLV-1 carriers. **Materials and Methods:** Cross-sectional study of 33 HTLV-1-seropositive individuals aged 17 to 85 years attending neurology clinics in Najaf, Iraq: 17 with clinically defined HAM/TSP and 16 asymptomatic carriers. Serum TNF- α was measured by sandwich ELISA. Proviral load was quantified by albumin-normalised real-time PCR in 30 participants (14 HAM/TSP, 16 carriers) and expressed as copies per 10^4 peripheral blood mononuclear cells (PBMC). Groups were compared using independent-samples t tests with Welch's correction and Mann-Whitney U tests, proviral load after $\log_{10}$ transformation; sex was compared using Fisher's exact test. Effect estimates are reported with 95% confidence intervals (CI). **Results:** Serum TNF- α did not differ between groups (HAM/TSP 1.57 ± 0.36 pg/mL vs carriers 1.60 ± 0.31 pg/mL; mean difference -0.03 pg/mL, 95% CI -0.27 to 0.21 ; $t = -0.27$, $p = 0.79$; Cohen's $d = -0.09$). Proviral load was approximately twice as high in patients as in carriers (geometric means $1,597$ vs 781 copies/ 10^4 PBMC; ratio 2.05 , 95% CI 0.79 to 5.31 ; $p = 0.14$), a difference in the expected direction that did not reach statistical significance. The groups did not differ significantly in age ($p = 0.20$); the sex distribution differed at borderline significance ($p = 0.057$). **Conclusions:** Serum TNF- α showed no association with HAM/TSP status in this cohort. The confidence interval excludes a large difference, although the precision of that exclusion is itself limited by the analytical range of the assay, since most measured concentrations fell below its lowest calibrator. Proviral load was higher in patients, consistent with previous reports, but the study was too small to establish this difference. These data do not support serum TNF- α as a peripheral marker distinguishing symptomatic from asymptomatic HTLV-1 infection.

Keywords: HTLV-1; HAM/TSP; Tumor necrosis factor alpha; Proviral load; Asymptomatic carriers; Iraq

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a deltaretrovirus of the family Retroviridae and was the first human retrovirus to be identified [1]. Current estimates place the number of people infected worldwide between five and ten million, distributed in clusters of high endemicity rather than uniformly, with recognised foci in the Middle East [2,3]. The

overwhelming majority of infections are clinically silent and persist for life; carriers are typically unaware of their status and may transmit the virus from mother to child, mainly through breastfeeding, by sexual contact, and through contaminated blood products [3]. The structure of the virion and its principal proteins is shown in Figure 1. A small minority of infected individuals develop disease. Adult T-cell leuka-

emia/lymphoma occurs in a few per cent of carriers [4,5], a malignancy in which TNF- α has itself been implicated [6], while a smaller proportion, variously estimated between 0.25% and 4%, develop HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), first described as a distinct clinical entity in 1986 [7]: a chronic inflammatory disorder of the spinal cord characterised by progressive spastic paraparesis, bladder dysfunction and sensory disturbance [8,9]. The determinants of this transition remain incompletely understood, and viral, host-genetic and immunological factors have all been implicated [10–13]. Immunologically, HAM/TSP is characterised by spontaneous proliferation of HTLV-1-infected CD4⁺ T lymphocytes, expansion of Tax-specific CD8⁺ T cells [14], elevated HTLV-1-specific antibody titres in serum and cerebrospinal fluid, and increased local production of pro-inflammatory mediators [9,15,16]. Among these mediators, TNF- α has attracted particular attention: it is a central mediator of systemic inflammation and acts predominantly in a paracrine manner [17,18], and the HTLV-1 Tax protein induces its expression in primary human glial cells and in dendritic cells [19,20].

Whether this compartmentalised inflammatory signal is reflected in peripheral blood is a separate question, and one with practical importance: a serum marker would be far easier to deploy than lumbar puncture in routine clinical assessment. Reports addressing this have been inconsistent, with some studies describing elevated circulating TNF- α in HAM/TSP and others describing reduced or unchanged concentrations [21,22]. To our knowledge, no such data have previously been reported from Iraq. The present study compares serum TNF- α concentrations, together with HTLV-1 proviral load, between patients with HAM/TSP and asymptomatic HTLV-1 carriers attending neurology clinics in Najaf, Iraq.

Materials and Methods

Study design and setting

This was a single-centre, cross-sectional, comparative study of HTLV-1-seropositive individuals attending specialised neurology clinics in Najaf, Iraq. Participants were recruited between 1 January and 5 March 2023. The protocol was reviewed and approved by the Najaf Health Department (approval number 11250, dated 1 January 2023), and written informed consent was obtained from every participant before sample collection. The study was conducted in accordance with the Declaration of Helsinki, and reporting follows the STROBE recommendations for cross-sectional studies.

Participants

Participants were assigned to one of two groups. The HAM/TSP group required a diagnosis established by a consultant neurologist on the basis of history and clinical examination; serological detection of anti-HTLV-1 antibody by line immunoassay (INNO-LIA HTLV I/II Score); and confirmation of infection by real-

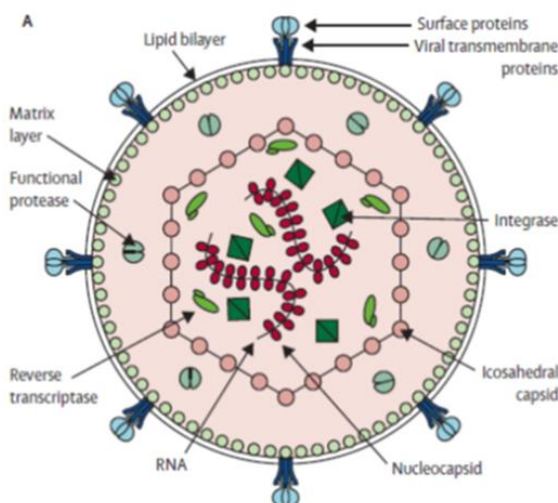


Figure 1. Structure of the HTLV-1 virion and its viral proteins. Reproduced from [8].

time PCR. The asymptomatic carrier group required the same serological and PCR confirmation together with the absence of neurological signs or symptoms on clinical examination. Individuals were excluded if they had received interferon or corticosteroid therapy; if HTLV-1 infection could not be confirmed; if they had concurrent inflammatory or infectious disease, including hepatitis B or C or HIV-1/2; if neurological signs were attributable to a cause unrelated to HTLV-1; if they were immunosuppressed; or if they were pregnant.

Sample size. No formal a priori sample-size calculation was performed. Recruitment was limited by the number of confirmed HTLV-1-seropositive individuals accessible during the study period. Serum TNF- α was measured in 33 participants (17 HAM/TSP, 16 carriers) and proviral load in 30 (14 HAM/TSP, 16 carriers).

Sample collection and PBMC separation

Twelve millilitres of whole blood were drawn from each participant. Six millilitres were transferred into K2-EDTA tubes and mixed gently to prevent clot formation; the remaining six millilitres were collected into gel separator tubes for serum preparation. Peripheral blood mononuclear cells were separated from the EDTA sample by Ficoll density-gradient centrifugation (Cedarlane, Canada) at 400–500 $\times g$ for 25–30 min at 18–20 $^{\circ}C$, then washed two to three times in phosphate-buffered saline (Sigma-Aldrich, Germany) before DNA extraction.

Serum TNF- α assay

Serum TNF- α was measured by a commercial sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions. In brief, calibrator dilutions were added in duplicate to the standard wells and 100 μL of assay buffer in duplicate to the blank wells; 50 μL of assay

buffer followed by 50 μL of serum was added to sample wells, together with 50 μL of diluted biotin conjugate. Plates were covered and incubated for 2 h at room temperature (18–25 $^{\circ}C$) with shaking at 400 rpm. Wells were emptied and washed twice with 400 μL wash buffer, each wash left in the well for 10–15 s before complete aspiration. One hundred microlitres of diluted streptavidin–HRP was added to all wells, including blanks, and plates were incubated for a further 1 h at room temperature before a repeat wash cycle. One hundred microlitres of TMB substrate was added and plates were incubated for 10 min at 18–25 $^{\circ}C$ in the dark. The reaction was stopped with 100 μL stop solution (Figure 2) and optical density was read at 450 nm on an Awareness microplate reader.

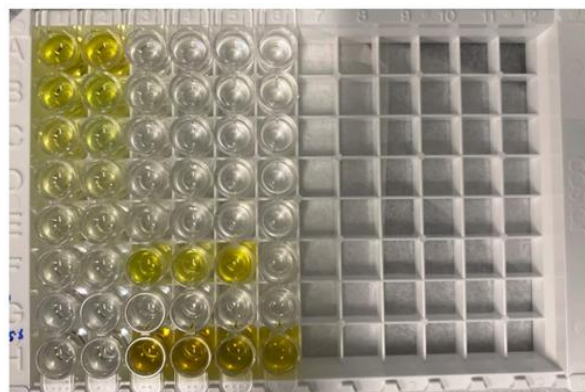


Figure 2. Enzyme-linked immunosorbent assay plate after addition of stop solution; color intensity is proportional to bound TNF- α .

Assay calibration range. The calibrators recorded for the assay spanned 1.56–100.0 pg/mL, with mean optical densities of 0.121 at the lowest calibrator and 1.851 at the highest, and 0.081 for the blank; inter-replicate coefficients of variation of 0.2% to 5.6% are recorded alongside them (Table 1).

Table 1. Calibration data recorded for the TNF- α assay. Whether these are the plate readings or reference values supplied with the assay could not be established from the study records.

Standard	TNF- α (pg/mL)	Mean OD (450 nm)	CV (%)
1	100.0	1.851	0.2
2	50.00	1.004	0.2
3	25.00	0.562	2.1
4	12.50	0.349	2.4
5	6.25	0.207	3.8
6	3.13	0.152	5.6
7	1.56	0.121	5.3
Blank	0.0 (blank)	0.081	not applicable

Proviral load quantification

Genomic DNA was extracted from PBMC using the K2000 Blood DNA Extraction Kit (Genet Bio, Korea). HTLV-1 proviral load was quantified using a commercial real-time PCR kit (Novin Gene HTLV RQ) with parallel amplification of the albumin gene as a cellular reference. HTLV-1 was detected in the FAM channel and albumin in the VIC channel. Cycling comprised initial denaturation at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 52 °C for 30 s and 60 °C for 45 s. A sample was considered positive when a sigmoidal amplification curve with Ct < 40 was obtained in both channels; a sample showing a sigmoidal curve in the VIC channel only, with Ct within the range of the kit standards, was considered negative. Amplification plots for the calibrators and for the study samples are shown in Figure 3. The kit amplifies a conserved region of the HTLV-1 provirus; the primer and probe sequences are proprietary to the manufacturer and are not disclosed in the kit documentation. Proviral load was quantified in peripheral blood mononuclear cells rather than in plasma, in which HTLV-1 nucleic acid is also detectable [23], because the quantity of interest is the proportion of infected cells.

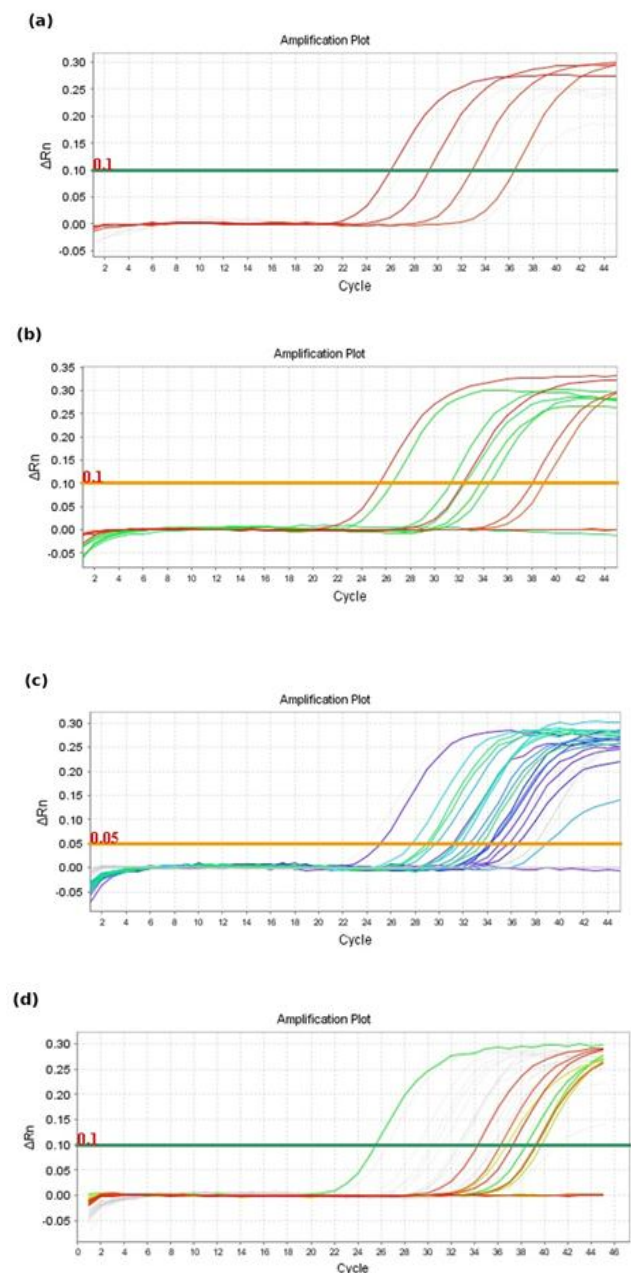


Figure 3. Real-time PCR amplification plots (ΔRn versus cycle). (a) Kit standards, FAM. (b) Standards and controls with study samples. (c) Study samples, VIC (albumin). (d) Study samples, FAM (HTLV-1).

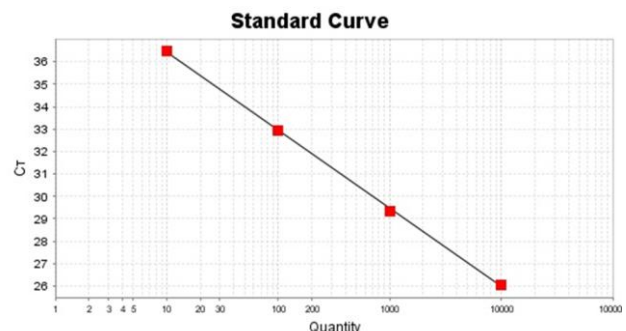


Figure 4. Standard curve for the HTLV-1 calibrators: threshold cycle against nominal copy number, as displayed by the instrument software. The fitted parameters for both the HTLV-1 and the albumin channel are given in the text below.

Derivation of copy numbers. Copy numbers were obtained from the threshold cycle of each sample using the calibration curves constructed from the kit standards (Table 2, Figure 4). For HTLV-1 the curve was $Ct = -3.475 \times \log_{10}(\text{copies}) + 39.890$, with an amplification efficiency of 94.0% and $R^2 = 0.9996$; for albumin it was $Ct = -3.635 \times \log_{10}(\text{copies}) + 41.460$, efficiency 88.4%, $R^2 = 0.9937$. Since each diploid cell carries two albumin copies, proviral load was calculated as twice the ratio of HTLV-1 copies to albumin copies and is expressed as copies per 10^4 PBMC.

Table 2. Threshold cycle values for the HTLV-1 and albumin calibrators, from which the curves given above were fitted.

Standard	Nominal copies/reaction	Ct (HTLV-1)	Ct (albumin)
S1 / C1	10,000	26.08	26.96
S2 / C2	1,000	29.34	30.24
S3 / C3	100	32.92	34.70
S4 / C4	10	36.47	37.59

Statistical analysis

Continuous variables are summarised as mean $\pm$ standard deviation with median and range; proviral load, which spans several orders of magnitude, is summarised as the geometric mean with median and range. Normality was assessed within each group using the Shapiro–Wilk test. Between-group comparisons used the independent-samples t test with Welch's correction, applied as the default rather than conditionally, since it does not assume equal variances and loses little efficiency when

variances are in fact equal. The Mann–Whitney U test is reported alongside every comparison so that conclusions do not rest on distributional assumptions. Proviral load was analysed after $\log_{10}$ transformation, and the between-group contrast is expressed as a ratio of geometric means. Sex was compared using Fisher's exact test. The chi-square test was not used, because expected cell counts were below five in a substantial proportion of cells and because the variables of primary interest are continuous rather than categorical; applying a chi-square test to continuous measurements treats each distinct value as a separate category and yields a statistic without interpretable meaning. Effect estimates are reported with 95% confidence intervals derived from the same Welch model as the corresponding test, together with the standardised effect size (Cohen's d). To aid interpretation of null findings, the smallest standardised difference detectable with 80% power at $\alpha = 0.05$ given the achieved sample sizes is reported; observed post-hoc power is not reported, as it is a deterministic transformation of the p value and adds no information beyond it. Two-sided p values below 0.05 were considered statistically significant. Three sensitivity analyses of the primary outcome are reported. Values below the lowest calibrator were treated as left-censored at that point and compared by a Tobit model fitted by maximum likelihood and by a generalised Wilcoxon test in which the censored observations are tied at the bottom of the ranking; the comparison was also repeated with adjustment for age and sex by analysis of covariance with HC3 heteroscedasticity-consistent standard errors. Analyses were performed in Python 3.12 with NumPy 2.4 and SciPy 1.17; the Tobit likelihood and the HC3 covariance were implemented directly rather than taken from a package.

Results

Participant characteristics

Thirty-three HTLV-1-seropositive individuals were included: 17 with HAM/TSP and 16 asymptomatic carriers. Proviral load records were available for 30 participants (14 HAM/TSP, 16 carriers). The two sets of measurements are held in separate records that carry no common identifier, so it cannot be established from the study documentation which individuals appear in both; three fewer patients have a proviral load record than a TNF- α record. Baseline characteristics are shown in Table 3. Patients with HAM/TSP were on average 7.6 years older than carriers, but the difference did not reach statistical significance and the confidence interval was wide (95% CI -4.2 to 19.4 years; $p = 0.20$). Women predominated in both groups, more markedly among patients (15 of 17, 88.2%, against 9 of 16, 56.3%; men numbered 2 and 7 respectively); this difference was of borderline significance (Fisher's exact $p = 0.057$). The median ages did not differ either (Mann-Whitney $p = 0.27$). The age distribution of the two groups is shown in Figure 5.

Table 3. Characteristics of the study groups. Age compared by Welch's t test and, for the median row, the Mann-Whitney U test; sex by Fisher's exact test.

Characteristic	HAM/TSP (n = 17)	Carriers (n = 16)	P
Age, years, mean $\pm$ SD	50.3 $\pm$ 18.1	42.7 $\pm$ 15.1	0.20
Age, years, median (range)	46 (18–85)	42.5 (17–68)	0.27
Female sex, n (%)	15 (88.2)	9 (56.3)	0.057

Serum TNF- α (primary outcome)

Serum TNF- α concentrations were normally distributed in both groups (Shapiro-Wilk $p = 0.22$ and $p = 0.59$). Mean concentrations were essentially identical: 1.57 ± 0.36 pg/mL in the H

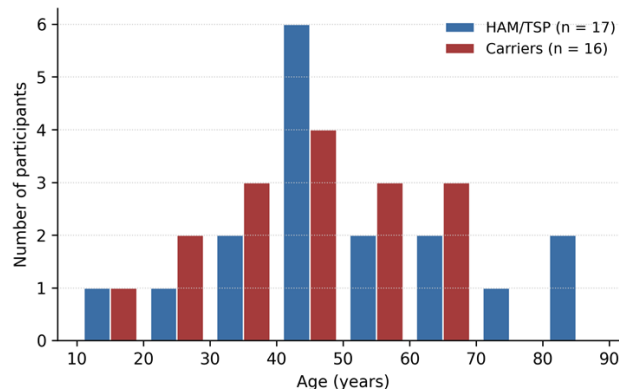


Figure 5. Age distribution by group, in ten-year bands.

AM/TSP group and 1.60 ± 0.31 pg/mL among carriers, with medians of 1.473 (range 0.788–2.298) and 1.598 (1.116–2.112) pg/mL respectively (Table 4). The mean difference of -0.03 pg/mL was not statistically significant (Welch $t = -0.27$, $p = 0.79$), and the Mann-Whitney U test agreed ($U = 130.0$, $p = 0.84$). The 95% confidence interval ran from -0.27 to +0.21 pg/mL and the standardised effect size was -0.09, a magnitude conventionally regarded as negligible. Individual values are plotted in Figure 6, where the two distributions overlap across almost their entire range. Eighteen of the 33 measured concentrations (54.5%) fell below 1.56 pg/mL, the lowest calibrator listed for the assay (Table 1; 11/17 in the HAM/TSP group and 7/16 among carriers). Every observed value, the highest being 2.298 pg/mL, lay below the second-lowest calibrator of 3.13 pg/mL, and the full range spans roughly 0.035 optical density units above the blank. The comparison above is therefore bounded by the resolution of the assay as well as by the sample size, a point developed in Section 5.

HTLV-1 proviral load

Proviral load spanned three orders of magnitude across the cohort, from 7.3 to 8,079 copies/ 10^4 PBMC, corresponding to between 0.07 and 81%

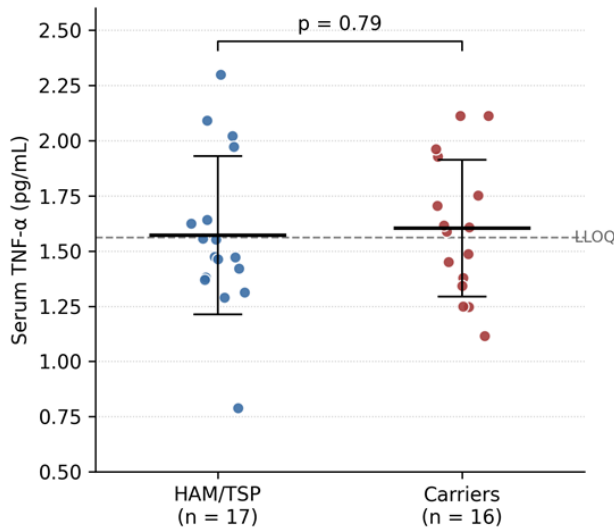


Figure 6. Individual serum TNF- α concentrations. Bars, group mean; whiskers, one standard deviation; dashed line, lowest calibrator listed for the assay, 1.56 pg/mL.

of circulating mononuclear cells carrying integrated provirus. The geometric mean was 1,597 copies/ 10^4 PBMC in patients with HAM/TSP and 781 copies/ 10^4 PBMC among asymptomatic carriers, a ratio of 2.05, with medians of 1,670 (range 270–7,932) and 1,312 (7.3–8,079) copies/ 10^4 PBMC (Table 4). The standardised effect size was 0.55, a magnitude conventionally regarded as moderate. The $\log_{10}$ -transformed values were normally distributed among patients (Shapiro–Wilk $p = 0.29$) but not among carriers ($p = 0.009$), owing to the single lowest observation, which is why the rank-based test and the robustness analysis in Table 5 are reported alongside. The difference lay in the direction reported in previous series but did not reach statistical significance (Welch t on $\log_{10}$ values = 1.54, $p = 0.14$; Mann–Whitney $p = 0.19$), and the confidence interval around the ratio was wide, from 0.79 to 5.31. Individual values are plotted in Figure 7. The conclusion was unaffected by the analytical choices made. Table 5 shows the comparison repeated without transformation, by rank-based testing, and after

exclusion of the extreme observations, the lowest value alone and then the two highest as well; across these specifications the ratio of geometric means remained between 1.5 and 2.1 and the p value between 0.11 and 0.33, and none reached significance. The lowest value is the carrier with 7.3 copies/ 10^4 PBMC and the two highest are the carrier and the patient with 8,079 and 7,932 copies/ 10^4 PBMC.

Table 4. Serum TNF- α and HTLV-1 proviral load. TNF- α as mean $\pm$ SD; proviral load as the geometric mean, analysed on the $\log_{10}$ scale. Welch's t test throughout. CI, confidence interval.

Outcome	n	HAM/TSP	Carriers	Effect (95% CI)	p
Serum TNF- α , pg/mL	17/16	1.57 $\pm$ 0.36	1.60 $\pm$ 0.31	−0.03 (−0.27 to 0.21)	0.79
Proviral load, copies/ 10^4 PBMC	14/16	1,597	781	$\times$ 2.05 (0.79 to 5.31)	0.14

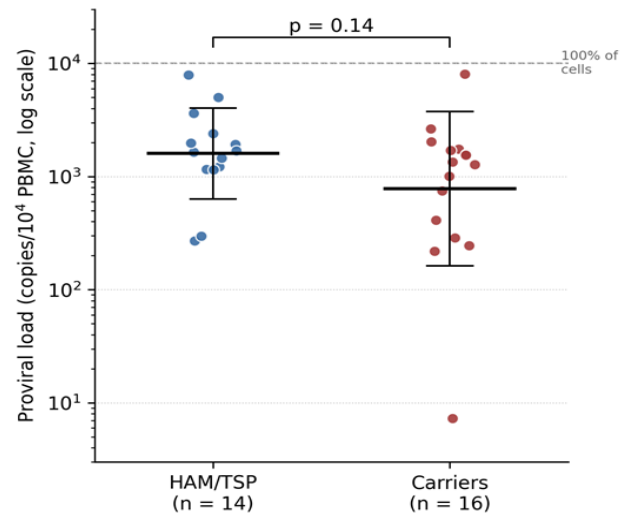


Figure 7. Individual proviral load values, logarithmic scale. Bars, geometric mean; whiskers, one standard deviation of $\log_{10}$ values; dashed line, one provirus copy per cell.

Table 5. Robustness of the proviral load comparison across six analytical specifications.

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Analysis	N (patients vs carriers)	Test statistic	p
Welch t test on $\log_{10}$ values (primary)	14 vs 16	t = 1.544	0.14
Mann-Whitney U test	14 vs 16	U = 144.0	0.19
Welch t test, untransformed values	14 vs 16	t = 0.986	0.33
Welch t test on $\log_{10}$, Lowes t value excluded	14 vs 15	t = 1.139	0.26
Welch t test on $\log_{10}$, two highest values excluded	13 vs 15	t = 1.667	0.11
Welch t test on $\log_{10}$, all three extremes excluded	13 vs 14	t = 1.319	0.20

Exploratory and sensitivity analyses

Across the whole sample, serum TNF- α was unrelated to age (Pearson $r = -0.003$, $p = 0.99$; Spearman $\rho = -0.02$, $p = 0.91$) and did not differ

between men and women (1.53 pg/mL, $n = 9$, vs 1.61 pg/mL, $n = 24$; $p = 0.44$).

Censored-data and adjusted analyses. Because 18 of the 33 TNF- α values fall below the lowest calibrator, the primary comparison was repeated treating those observations as left-censored at 1.56 pg/mL. A Tobit model gave a difference of -0.10 pg/mL (95% CI -0.42 to 0.21 , $p = 0.51$) and a generalised Wilcoxon test for censored data, in which all values below the calibrator are treated as tied at the bottom, gave $U = 116.5$ and $p = 0.45$. The proportion of values falling below the calibrator did not differ between the groups either (11/17 against 7/16; Fisher's exact $p = 0.30$). Because the groups differed by 7.6 years in mean age and by 32 percentage points in the proportion of women, the comparison was also repeated with adjustment for age and sex by analysis of covariance with heteroscedasticity-consistent standard errors, giving a difference of -0.07 pg/mL (95% CI -0.33 to 0.19 , $p = 0.59$). No specification alters the conclusion. The same adjustment could not be applied to proviral load, because the proviral load records carry no age or sex. The age and sex comparisons above were not pre-specified and are reported as exploratory; the censored-data and adjusted analyses are sensitivity analyses of the primary comparison.

Statistical power

Given the achieved sample sizes, the smallest standardised difference detectable with 80% power at $\alpha = 0.05$ was $d = 1.01$ for serum TNF- α (17 versus 16) and $d = 1.06$ for proviral load (14 versus 16). For TNF- α this corresponds to a difference of approximately 0.34 pg/mL, an order of magnitude larger than the difference observed. The study was therefore well powered only to exclude large differences; it cannot exclude small ones.

Individual participant data

Every value reported above can be recomputed from Tables 6 and 7, which list the individual measurements. The two tables come from separate records that carry no common identifier, so they cannot be linked at the level of the individual participant.

Table 6. Individual HTLV-1 proviral load. Copy numbers are twice the ratio of HTLV-1 to albumin copies, each derived from the curves in Table 2.

Code	Group	Ct HTLV-1	Ct albumin	Copies/10 ⁴ PBMC	% of cells
52	HAM/TSP	30.58	27.78	1,647	16.5
54	HAM/TSP	24.66	22.18	2,398	24
55	HAM/TSP	30.67	29.63	5,010	50.1
56	HAM/TSP	25.99	25.46	7,932	79.3
59	HAM/TSP	28.55	22.80	269.7	2.7
61	HAM/TSP	24.51	21.68	1,929	19.3
62	HAM/TSP	27.22	24.55	1,973	19.7
63	HAM/TSP	24.80	22.98	3,627	36.3
65	HAM/TSP	26.15	23.19	1,694	16.9
66	HAM/TSP	33.95	30.84	1,227	12.3
76	HAM/TSP	28.42	24.97	1,162	11.6
78	HAM/TSP	28.64	25.18	1,148	11.5
85	HAM/TSP	26.14	22.94	1,455	14.6
86	HAM/TSP	28.10	22.48	296.7	2.97
51	Carrier	26.42	23.76	2,032	20.3
53	Carrier	26.01	25.51	8,079	80.8
58	Carrier	24.80	21.83	1,751	17.5
60	Carrier	26.37	23.43	1,704	17
64	Carrier	25.02	21.90	1,582	15.8
68	Carrier	29.92	26.77	1,345	13.5
69	Carrier	26.47	23.08	1,278	12.8
70	Carrier	29.28	23.23	218.3	2.18
71	Carrier	34.26	23.07	7.278	0.0728
73	Carrier	25.87	22.08	1,009	10.1

Code	Group	Ct HTLV-1	Ct albumin	Copies/10 ⁴ PBMC	% of cells
75	Carrier	28.87	24.75	750.4	7.5
81	Carrier	28.47	22.81	286.2	2.86
82	Carrier	27.79	22.68	413.6	4.14
83	Carrier	26.28	23.18	1,544	15.4
84	Carrier	29.62	23.77	245.4	2.45
87	Carrier	25.47	23.18	2,641	26.4

Table 7. Individual serum TNF- α concentrations with age and sex. Across both groups there were 24 women and 9 men.

Group	TNF- α (pg/mL)	Age (years)	Sex
HAM/TSP	1.290	72	F
HAM/TSP	1.420	48	F
HAM/TSP	2.020	46	F
HAM/TSP	1.556	26	F
HAM/TSP	1.641	18	F
HAM/TSP	0.788	46	F
HAM/TSP	1.625	50	F
HAM/TSP	1.472	46	F
HAM/TSP	1.972	46	F
HAM/TSP	1.552	61	F
HAM/TSP	2.092	42	F
HAM/TSP	1.380	33	M
HAM/TSP	1.370	82	F
HAM/TSP	1.473	85	M
HAM/TSP	1.463	50	F
HAM/TSP	2.298	65	F
HAM/TSP	1.312	39	F
HAM/TSP, n = 17	mean 1.572	mean 50.3	15 F, 2 M
Carrier	1.752	42	M

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Group	TNF- α (pg/mL)	Age (years)	Sex
Carrier	1.247	33	F
Carrier	2.112	54	F
Carrier	1.588	35	F
Carrier	1.617	17	F
Carrier	1.486	21	M
Carrier	1.927	68	F
Carrier	1.704	60	M
Carrier	1.378	31	M
Carrier	2.112	24	F
Carrier	1.116	52	F
Carrier	1.607	54	F
Carrier	1.250	43	M
Carrier	1.343	62	M
Carrier	1.451	41	F
Carrier	1.962	46	M
Carrier, n = 16	mean 1.603	mean 42.7	9 F, 7 M

Discussion

In this cross-sectional study of Iraqi patients with HTLV-1 infection, serum TNF- α concentrations were indistinguishable between individuals with HAM/TSP and asymptomatic carriers, the observed difference amounting to roughly 2% of the group means. Proviral load was approximately twice as high in patients as in carriers, in the direction reported by others, but the difference did not reach statistical significance in a sample of thirty. The absence of a difference in circulating TNF- α is not necessarily inconsistent with the immunopathological model of HAM/TSP. The evidence most strongly supporting a role for TNF- α derives from the affected tissue and from cerebrospinal fluid rather than from peripheral

blood. TNF- α -positive cells were detected in the cerebrospinal fluid of patients with HAM/TSP three decades ago [24], and more recent work on neuroinflammatory markers has repeatedly found the signal concentrated in the central nervous system compartment: neopterin and CXCL10 are markedly raised in cerebrospinal fluid in HAM/TSP while serum values discriminate far less well [25]. Inflammation localised to the central nervous system compartment need not produce a measurable systemic signal, particularly for a cytokine with a short circulating half-life that acts predominantly in a paracrine manner [17,18]. Our result nevertheless stands against one directly comparable study. Neco and colleagues studied 21 patients with HAM/TSP and 48 asymptomatic carriers in Brazil, and measured cytokines by cytometric bead array in the serum of a subset of that cohort, 16 patients and 27 carriers. They reported higher serum TNF- α in the patient group, alongside raised CXCL9, CXCL10 and IFN- γ [22]. Their serum comparison rests on numbers close to ours, and it is the same comparison, so the disagreement is worth stating plainly rather than leaving implicit. Two differences may bear on it. The first is analytical: their measurements were made by a bead-based assay across the full physiological range, whereas more than half of our ELISA readings fell below the lowest calibrator recorded for the assay, where discrimination between samples is poorest. The second is clinical: the inflammatory profile of HAM/TSP changes with disease activity, which is itself now formally classified [26], so cohorts sampled at different stages need not agree. We cannot distinguish between these explanations with the present data, and we regard the question as open rather than settled by either study. A second study points the other way, and it answers the most obvious objection to our result. Freitas and colleagues measured TNF- α by multiplex bead-

based immunoassay in paired cerebrospinal fluid and serum samples from 20 patients with HAM/TSP and 13 asymptomatic carriers [27]. In serum, TNF- α was quantifiable and was retained in their analysis unlike VILIP-1, which they excluded because more than half of its serum values fell below the limit of detection and they found no difference between the groups. That matters here because the principal objection to our null result is that it might be an artefact of measuring near the foot of the calibration curve. A platform on which serum TNF- α cleared the exclusion threshold reached the same conclusion, which makes the analytical explanation of our finding less likely than the substantive one. A separate observation in the same paper is worth recording: in cerebrospinal fluid, as opposed to serum, Freitas and colleagues found TNF- α below the limit of detection in more than half of their samples and excluded it from further analysis on that ground. That is a different compartment from the one we sampled and so is not a direct parallel to the censoring described in Section 5, but it does suggest that TNF- α lies close to the limits of routine assays in this population wherever it is measured. A third and larger study is consistent with this. Assone and colleagues quantified plasma IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ and TNF by cytometric bead array in 110 people living with HTLV-1, 67 asymptomatic and 43 with HAM/TSP [28]. IFN- γ and IL-17A distinguished the two groups; TNF did not, and was of value instead in predicting the response to corticosteroid therapy. A cohort more than three times the size of ours, measured on the same platform that Neco and colleagues used, therefore also failed to separate patients from carriers on TNF. On the present evidence TNF- α appears to be a poor discriminator of disease status in peripheral blood, whatever its role within the central nervous system. The same conclusion holds prospectively: in a nested

analysis within a Brazilian cohort of 615 asymptomatic individuals followed for 6,673 person-years, plasma TNF at entry, compared between nine incident cases and 59 who remained asymptomatic, did not significantly predict which participants would later develop HAM (odds ratio 0.96, 95% CI 0.92 to 1.01) [29]. Peripheral cytokine profiles in HAM/TSP are in any case not uniformly pro-inflammatory. During chronic HTLV-1 infection, virus-specific T cells can acquire an exhausted phenotype through the induction of co-inhibitory molecules, with a corresponding loss of capacity to produce IL-2, IFN- γ and TNF- α [30]. If the patients in the present study were sampled at a chronic, established stage of disease, an exhausted rather than an activated peripheral phenotype would be the expected finding. This reading is not settled, however. A recent study of 30 patients, 30 carriers and 30 uninfected controls found PD-1 raised on CD4⁺ and CD8⁺ T cells in HAM/TSP while TIM-3 was unchanged, and interpreted the profile, together with elevated IFN- γ , as proinflammatory rather than one of classical exhaustion [31]. Both readings are compatible with an unchanged circulating TNF- α ; they differ in mechanism, and the present data cannot distinguish between them. The proviral load finding is consistent with the established literature. Higher proviral load in symptomatic patients than in asymptomatic carriers is one of the more consistently replicated observations in HTLV-1 research [10,11,32], and the difference observed here runs in the same direction, although the separation between the groups is far from complete [16]. Freitas and colleagues, expressing proviral load as the percentage of infected cells in the same way, reported $8.46\% \pm 6.37\%$ in patients against $4.50\% \pm 3.64\%$ in carriers, a 1.88-fold difference that reached significance at $p = 0.049$ in 33 participants [27]. The two contrasts are not expressed on the same

scale, since ours is a ratio of geometric means from a $\log_{10}$ analysis and theirs a ratio of arithmetic means. Computed the same way as theirs, our difference is 1.46-fold; on the log scale of our primary analysis it is 2.05-fold. Their value falls between the two, so the cohorts from Brazil and from Iraq are of the same order on the size of the effect. Our absolute values are the higher: our geometric means correspond to 16.0% and 7.8% of cells and our arithmetic means to 22.7% and 15.6%, against their 8.46% and 4.50%, an excess of roughly two- to threefold depending on the summary used. Section 5 sets out what may account for that, and none of it bears on the ratio, which is unchanged by any constant factor. That our difference did not reach significance reflects the size of the cohort and the breadth of the biological distribution rather than an absence of effect: with fourteen and sixteen participants only a standardised difference above one could have been detected, and the confidence interval around the observed ratio spans values from a modest reduction to a five-fold increase. The result is best read as compatible with, rather than as evidence for, the established association.

A distinction between association and prediction is worth drawing at this point, because the two are often conflated in this literature. Cross-sectional comparisons of the kind reported here, ours included, generally find higher proviral load in patients than in carriers. Prospective follow-up gives a different picture: in the largest series to date, a nested comparison within a cohort of 615 asymptomatic individuals followed for 6,673 person-years, proviral load at entry did not predict which of them would subsequently develop HAM (odds ratio 1.31, 95% CI 0.92 to 1.88), whereas female sex and low anti-inflammatory cytokines did [29]. A cross-sectional design such as ours can establish an

association with disease status; it cannot speak to prediction, and we make no claim that it does.

The demographic findings are unremarkable. Older age and female sex are established risk factors for HAM/TSP [28], and in prospective follow-up female sex was an independent predictor of incident disease, nine of eleven incident cases occurring in women [29]. The female predominance seen in both of our groups, more marked among patients, is compatible with that pattern and with more efficient male-to-female sexual transmission. The age difference between groups was in the expected direction but was not statistically significant at this sample size.

Limitations

This study has several limitations, set out explicitly because they bear directly on how the findings should be read.

Sample size and statistical power. With 17 patients and 16 carriers for the primary outcome, and 14 versus 16 for proviral load, the study could detect only large standardised differences with conventional power. A negative result in this setting establishes the absence of a large effect, not the absence of any effect. No a priori power calculation was undertaken; recruitment was constrained by the number of confirmed seropositive individuals accessible during the study period.

Analytical range of the cytokine assay. More than half of the measured TNF- α concentrations fell below the lowest calibrator listed for the assay, and every observed value lay below the second-lowest calibrator recorded (Table 1). Values obtained by extrapolation below the lowest standard carry substantially greater uncertainty than values within the calibrated range. Two consequences follow. First, the confidence interval reported for the TNF- α comparison should be read as excluding a large

difference in measured concentration, not as establishing analytical equivalence between the groups. Second, because more than half the observations fall below the lowest calibrator, the data are left-censored at that point. We therefore repeated the comparison by methods appropriate to censored data, and the conclusion is unchanged (Section 3.4). A high-sensitivity assay calibrated to the sub-picogram range, with the calibration data retained, would be needed to address the question properly. We also note that the assay was not identified by manufacturer or catalogue number in the study records, which limits what can be said about its analytical characteristics.

Derivation of proviral load. Copy numbers were calculated from the four-point calibration curves supplied with the PCR kit rather than taken from the instrument software, and the extrapolation this entails is considerable. Twenty-seven of the thirty albumin measurements lie above the highest albumin calibrator of 10,000 copies, the largest by a factor of 27.6, that is $1.44 \log_{10}$ beyond the calibrated range; seven of the thirty HTLV-1 measurements lie above their own top calibrator, by up to 2.7-fold. The albumin copy numbers imply a median of about 53,000 cell equivalents per reaction, corresponding to roughly 350 ng of input DNA, which is above the range a reaction of this kind normally accommodates, so the albumin figures are probably too high. Because albumin is the denominator, an overestimate there would if anything push the calculated proviral loads downward, and the comparison with published cohorts in Section 4 suggests the opposite. A second and simpler possibility should therefore be considered: the calculation assumes the albumin calibrator is expressed in gene copies, which is why it is multiplied by two for the two copies present in a diploid cell. If the calibrator is instead expressed in cell equivalents,

that factor is not required and every value reported here would be halved, which would bring them into the range published elsewhere. We cannot settle this from the information available, and raise it so that it can be checked against the assay documentation.

The relative comparison between groups is unaffected by any constant factor: if albumin copy numbers are inflated uniformly, that factor divides into every proviral load equally and cancels when one group is compared with the other. Nor does variation in the amount of input DNA bias the ratio, because each channel is quantified against its own calibration curve, so a change in input shifts both threshold cycles and is recovered exactly on inversion; the difference in amplification efficiency between the channels (94.0% for HTLV-1, 88.4% for albumin) is accounted for within the calibrated range. Residual error arises from extrapolation beyond the highest albumin calibrator rather than from efficiency as such. Albumin copy numbers did not differ materially between the groups (medians 75,500 and 106,900 copies per reaction; Mann–Whitney $p = 0.42$), so any error of that kind would be expected to act similarly on both. Quantification output taken directly from the instrument software would remove the dependence on extrapolation altogether and is the appropriate way to settle the absolute values.

Unlinked measurements. Serum TNF- α and proviral load were recorded in separate datasets that could not be linked at the individual participant level. It was therefore not possible to examine the within-individual relationship between proviral load and cytokine concentration, which would have been the most informative analysis available from these data.

Absence of an uninfected control group. Both groups comprised HTLV-1–infected individuals. Without seronegative controls it cannot be determined whether TNF- α concentrations in

either group differ from those of the uninfected population; the study addresses only the comparison between symptomatic and asymptomatic infection.

Selection of participants. Participants were identified through the specialised neurology clinics of a single city over a recruitment period of nine weeks, and were not drawn from a population-based screening programme. Iraq is not a recognised endemic area for HTLV-1, so a cohort assembled in this way is likely to be enriched for individuals already under neurological care and for those whose infection had come to clinical attention. Asymptomatic carriers identified through such a pathway may differ systematically from carriers in the general population, most of whom never present. The direction of any resulting bias cannot be determined from these data, and the findings should not be read as representative of HTLV-1 infection in Iraq as a whole.

Cross-sectional design. Measurements were taken at a single time point, so no inference about temporal sequence, and hence no causal inference, can be drawn. Asymptomatic carriers in this cohort may yet develop HAM/TSP, and the cytokine profile of patients may differ between early and established disease.

A single cytokine. Only TNF- α was measured. A broader panel, and in particular the IFN- γ and IL-17A that have discriminated disease status in larger cohorts, would allow a more informative characterisation of the peripheral immune profile than a single analyte can provide.

Conclusion

Serum TNF- α concentrations did not differ between patients with HAM/TSP and asymptomatic HTLV-1 carriers in this Iraqi cohort. The confidence interval excludes a large difference, although the width of that exclusion is itself constrained by the analytical range of the

assay. These data provide no support for the use of circulating TNF- α as a peripheral marker of symptomatic HTLV-1 infection. Proviral load was approximately twice as high in patients as in carriers, consistent with the established literature, but the cohort was too small for the difference to reach statistical significance. The null result for TNF- α should not be read as evidence against a role for the cytokine in the immunopathology of HAM/TSP, for which the supporting evidence rests principally on measurements made within the central nervous system compartment. It indicates rather that the process is not reliably reflected in peripheral blood, at least as measured by a standard-sensitivity assay in a sample of this size. Confirmation will require larger, multicentre cohorts with an uninfected comparison group, high-sensitivity cytokine assays calibrated to the observed concentration range, paired serum and cerebrospinal fluid sampling where ethically justified, and independently validated proviral load quantification. Longitudinal follow-up of asymptomatic carriers would be more informative still, since it is the transition to disease, rather than the established disease state, that a useful marker would need to anticipate.

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Data availability

All individual-level data underlying the analyses are reported in Tables 6 and 7 of this article. No separate supplementary file is required.

Conflicts of interest

The authors declare no conflicts of interest.

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