Biomarkers for risk of developing active tuberculosis in contacts of TB patients: a prospective cohort study

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ABSTRACT Identifying those *Mycobacterium tuberculosis* latent-infected individuals most at risk of developing active tuberculosis (TB) using routine clinical and laboratory tests remains a huge challenge in TB control efforts. We conducted a prospective longitudinal study of clinical and laboratory markers associated with the risk of developing active TB in contacts with latent *M. tuberculosis* infection.

HIV-negative household contacts (n=296) of pulmonary TB patients underwent monitoring of clinical features, full blood cell counts, tuberculin skin text (TST) and chest radiography performed regularly during 18 months of follow-up. Paired statistical tests, a Kaplan–Meier analysis and Cox proportional hazard modelling were performed on variables between contacts progressing or not progressing to active TB.

The appearance of TB disease symptoms in contacts was significantly associated with an elevated peripheral percentage of blood monocytes (adjusted hazard ratio (aHR) 6.25, 95% CI 1.63–23.95; p<0.01), a ⩾14 mm TST response (aHR 5.72, 95% CI 1.22–26.80; p=0.03) and an increased monocyte:lymphocyte ratio (aHR 4.97, 95% CI 1.3–18.99; p=0.03). Among contacts having TST ⩾14 mm, a strong association with risk of progression to TB was found with an elevated blood monocyte percentage (aHR 8.46, 95% CI 1.74–41.22; p<0.01).

Elevated percentage of peripheral blood monocytes plus an elevated TST response are potential biomarkers for identifying contacts of TB patients at highest risk of developing active TB.

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Introduction

Tuberculosis (TB) is an aerosol-borne infectious disease caused by *Mycobacterium tuberculosis* resulting in ~8 million incident TB cases and 1.5 million deaths annually [1, 2]. However, most people with *M. tuberculosis* infection remain asymptomatic and develop what is known as latent *M. tuberculosis* infection (LTBI). The tuberculin skin test (TST) has traditionally been used to identify people with LTBI as they benefit from preventive TB treatment [3]. Approximately one-third of the world's population is thought to have LTBI. Identifying and treating all LTBI cases seems impractical as the disease is concentrated in countries with limited healthcare resources and because of the potential side-effects associated with chemotherapy [4]. Up to 10% of people with LTBI may develop active TB in the decades after infection [5]. The risk is highest in the years immediately following infection and in immunosuppressed individuals (*e.g.* people with HIV infection). Screening for the individuals most at risk of developing active TB from the huge "at-risk" reservoir of LTBI cases is an important goal of TB control efforts worldwide [6]. The prevalence of LTBI in close TB contacts is reported to be high: being a household contact is a serious risk factor associated with progression to active TB [7, 8].

Studies have suggested that the subsequent risk of developing disease in close contacts of TB patients was greatest among initially TST-sensitive individuals [9, 10]. However, TST has known limitations, including cross-reactivity with bacillus Calmette–Guérin (BCG) vaccine and nontuberculous mycobacteria infections [11]. Furthermore, the TST and the more complex interferon-γ release assays mostly identify all prior infections and are poor at predicting LTBI that will subsequently go on to develop active TB [12, 13]. Thus, there is a dire need for simple, sensitive laboratory biomarkers for risk of progression of *M. tuberculosis* infection to active disease [14].

The latent *M. tuberculosis* infection state is contained by an active immune response in the host initiated by the pathogen, permitting a controlled persistence of the organism. Several studies indicate that circulating immune cells are activated and recruited to the *M. tuberculosis*-infected lungs to form the granuloma where the *M. tuberculosis* proliferation is controlled by an active interaction of lymphocytes and infected macrophages [15–20]. In a previous study, we observed that peripheral white blood cell (WBC) subpopulation ratios varied according to TB clinical status in a limited number of BCG-vaccinated individuals from an area with a high TB burden [17]. More recently, observations from an HIV-positive or HIV-exposed population in South Africa also suggest an association between the ratio of circulating immune cells and the risk of TB disease [21, 22]. However, the hypothetical association of the peripheral WBC rates with risk of TB disease progression in HIV-negative at-risk populations requires confirmation. We conducted a prospective longitudinal cohort study to assess the role of peripheral blood WBC subpopulation counts as biomarkers of risk of progression to active disease in an HIV-negative population in a country with a high TB burden.

Methods

Study setting and recruitment

Close household contacts of patients with active pulmonary TB from Antananarivo (Madagascar) were investigated. The index cases of these household contacts were adults (aged >15 years) with newly diagnosed sputum smear positive and *M. tuberculosis* culture confirmed TB recruited from the main TB centre in Antananarivo. The inclusion criteria for the household contacts were age >1 year, living in the same house as the index case for ⩾6 months and asymptomatic for active TB. Asymptomatic was defined as an absence of clinical symptoms and signs characteristic of TB and a negative chest radiograph at entry to the study. Subjects who consented to an HIV test were enrolled and only HIV-negative individuals were included in the study. To investigate baseline responses for *M. tuberculosis* nonexposed individuals, age- and sex-matched community controls without symptoms or signs of TB and no known recent or sustained contact with TB cases were recruited at the post-exposure rabies centre of Institute Pasteur de Madagascar (Antananarivo, Madagascar).

Follow-up and monitoring of the household contacts was performed to detect TB symptoms for up to 18 months after inclusion: a clinical visit every 3 months for the first 6 months, and then a visit every 6 months for the remainder of the 18-month period. Any household contacts developing clinical symptoms suggestive of TB and/or a result of abnormal TST (TST highly positive ⩾14 mm) were referred to the anti-TB centre for additional investigations, including chest radiography. Individuals in whom the diagnosis of TB was confirmed during the study were recorded and treated as TB patients. For all subjects, epidemiological, clinical and bacteriological data were recorded prospectively on individual record forms.

Sample size calculation

The sample size calculation for the study was based on experience gained in an earlier study [23]. Briefly, for equal-sized groups of TB progressors and nonprogressors, it would be possible to detect a difference

corresponding to a log report (average rate progressors/average nonprogressors) equal to 1.82 (consistent with the results of the study cited above) with a power of 90% and using an α error of 0.05. For groups of uneven size (1 *versus* 4 for progressors *versus* nonprogressors), including 20 progressors and 80 (20×4) nonprogressors, the ratio of 1.82 would be detectable with a power of 98% (if α=0.05). Applying this calculation to a power of 90%, it would be possible to detect a ratio of 1.62. In the proposed study in Madagascar, we wanted to include 100 index cases and expected (based on prior work) an average of five of six household contacts per index case.

TST assays

Study physicians performed the TST (10 units; Tuberculin Purified Protein Derivative, Sanofi Pasteur, Gentilly, France) at enrolment and after 3 months (M3) from inclusion. The results were read after 72 h. Cutaneous induration ⩾5 mm in diameter was considered to be a positive TST response and induration of ⩾14 mm was considered to be highly reactive.

Whole blood cell count

Blood samples were drawn at inclusion and after 3 months. Blood samples were obtained from the index cases at inclusion and after completion of their TB treatment (12 months). Venous blood samples were drawn in EDTA anticoagulant Vacutainer tubes and stored at room temperature until the full blood cell (FBC) count was performed, using an ABX Pentra 120 Retic haematology analyser (Horiba ABX SAS, Les Ulis, France), according to the manufacturer's instructions. Absolute cell counts were expressed as cells per litre and percentage value per cell population (absolute cell count/total whole blood cell count × 100%). The FBC count included information on red blood cells, platelets, total and differential WBC, which included neutrophils, eosinophils, basophils, monocytes and lymphocytes. A certified physician validated the formulas.

Statistical analysis

A comparison of the WBC subpopulations between the contacts that developed TB symptoms (symptomatic household contact (sHC)) and the household contacts that remained healthy (hHC) was firstly performed by paired sample analyses. Briefly, for every sHC, two age-matched hHCs were selected as controls for the paired analysis. Comparison using the Wilcoxon test for paired sample analysis was performed. The p-values were adjusted using the Bonferroni method to address multiple comparison concerns. Receiver operating characteristic (ROC) curves were then performed using the paired case–control to define the best FBC cut-off point for sensitivity and specificity. Risk of progression to active disease was estimated by performing survival analyses. The Kaplan–Meier method and the Gehan–Breslow–Wilcoxon test were used to compare the survival curves stratified by WBC rates. Cox proportional hazards models were generated to assess the association between WBC counts and development of active TB. Scaled Schoenfeld residuals were used to test the proportional hazards assumption of the Cox regression models and the Akaike information criteria (AIC) were compared between the models. Statistical tests were performed with R software (www.R-project. org). Tests were two sided and p<0.05 was considered as significant.

Ethical approval

The study was approved by the national ethics committee of the Ministry of Health in Madagascar (authorisation number 038-SANPF/CAB). Participants were enrolled after appropriate counselling and explanation of the study. Only participants who had given their written informed consent were enrolled. Written informed consent was obtained from the legal guardians on the behalf of minors/child participants involved in the study.

Results

Association of TST with risk of developing active TB in contacts

296 HIV-negative TB household contacts of active TB index cases (n=85) and 186 community controls were identified (table 1). Of these contacts, no samples were obtained from six subjects, three declined participation, one was a former TB case, while nine were lost to follow-up. BCG vaccination rate, evidenced by BCG scar, was ~98.54% (table 1). The global sex ratio (male/female) was 0.71.

At the end of the follow-up period, 12 (4.4%) out of the remaining 289 household contacts developed symptoms consistent with active TB and were classified as sHC. The mean age of the sHC group was lower than that of the hHCs (n=277, p=0.03; table 1). Nine (75%) out of 12 sHC were children aged <16 years; however neither younger age nor sex was statistically associated with an elevated risk of developing active TB.

When comparing the proportion of TST-positive responders (cut-off TST >5 mm) no correlation was observed between the TST response and BCG vaccination status and there was no significant association

TABLE 1 Characteristics of the study cohorts

	Index cases	Household contacts		Community controls
		Symptomatic	Healthy	
Subjects	83	12	277	186
Age years	34.7 (16–70)	16.6 (2–47)	22.9 (1–79)	25.3 (3–73)
Sex				
Male	45	5	125	89
Female	38	7	152	97
BCG status				
Yes	66 (79.5)	10 (83.3)	247 (89.2)	170 (91.4)
No	6	1	13	3
Undetermined	11	1	17	13
TST response				
≥14 mm	20 (24.1)	10 (83.3)	123 (40.4)	49 (26.3)
5–14 m	18 (21.7)	0	89 (32.1)	48 (25.8)
<5 mm	7 (8.4)	2 (16.7)	63 (22.7)	89 (47.9)
Undetermined	38	0	2	0

Data are presented as n, mean (range) or n (%). BCG: bacillus Calmette–Guérin; TST: tuberculin skin test.

between overall TST positivity and progression to active TB. However, it appeared that strong TST responders were at higher risk of subsequently developing active TB. 10 (83%) out of the 12 sHCs had a TST induration of ≥14 mm at enrolment (table 1). A TST ≥14 mm was associated with a significant risk of progression to active disease in household contacts (age-adjusted hazard ratio 5.47, 95% CI 1.18–25.33; p=0.03).

Peripheral blood monocyte counts were significantly different between household contacts with different TB outcomes during follow-up

Figure 1a depicts the peripheral leukocyte count at inclusion in all study groups. The subjects with known exposure to *M. tuberculosis* (index cases and household contacts) showed a significantly higher level of leukocytes (p<0.05) than community controls. However, when the WBC counts were stratified into blood cell subpopulations, significant differences according to clinical status were observed.

A severe decrease in the lymphocyte count (p<0.001) (fig. 1c) associated with an increase of both neutrophil (p<0.001) (fig. 1b) and monocyte rates (p<0.05) (fig. 1d) was globally observed in those study participants with active disease compared to the asymptomatic individuals. Furthermore, an elevated monocyte count was observed in the TB contacts compared to the community controls (p<0.001) (fig. 1c). Those contacts that subsequently developed TB displayed a WBC pattern distinct from both the index cases and the other healthy groups (community controls and hHC). The sHC study participants had significantly elevated monocyte counts compared to both the community controls and hHC groups (p<0.001) (fig. 1c).

Moreover, among those individuals with a TST ≥14 mm, the sHC group segregated from the healthy (community controls and hHC) individuals with regards to lymphocyte and neutrophil counts, and segregated from the TB patients with regards to monocyte counts (online supplementary material). Peripheral WBC populations that segregated the sHC (n=12) from the hHC (n=24) paired sample analyses (table 2) showed that both the monocyte and lymphocyte absolute counts were significantly different in the hHC when compared to sHC (p=0.04 and p=0.02, respectively). However, after Bonferroni adjustment, the monocyte percentage was the only significant difference between sHC and hHC (p<0.01) (table 2).

Peripheral WBC counts in TB patients after successful anti-TB treatment

To assess whether the variations observed in the peripheral WBCs were associated with active TB, we also measured WBC counts before and after treatment for TB in the index cases. Consistent with this hypothesis, the total WBC count decreased post-treatment (p<0.01) (fig. 2). In particular, the neutrophil and monocyte fractions in these treated patients were significantly reduced upon completion of treatment when compared to their rates at enrolment (fig. 2). While lymphocyte counts increased slightly, this was not significant in absolute terms (data not shown), but the decline in other cell types meant that the increase was highly significant (p<0.001) (fig. 2) as a percentage of total WBCs. Thus, post-treatment, the index cases showed a pattern (decreasing monocytes and neutrophils and increasing lymphocytes) that suggested that they were moving towards a profile similar to that observed in healthy individuals.
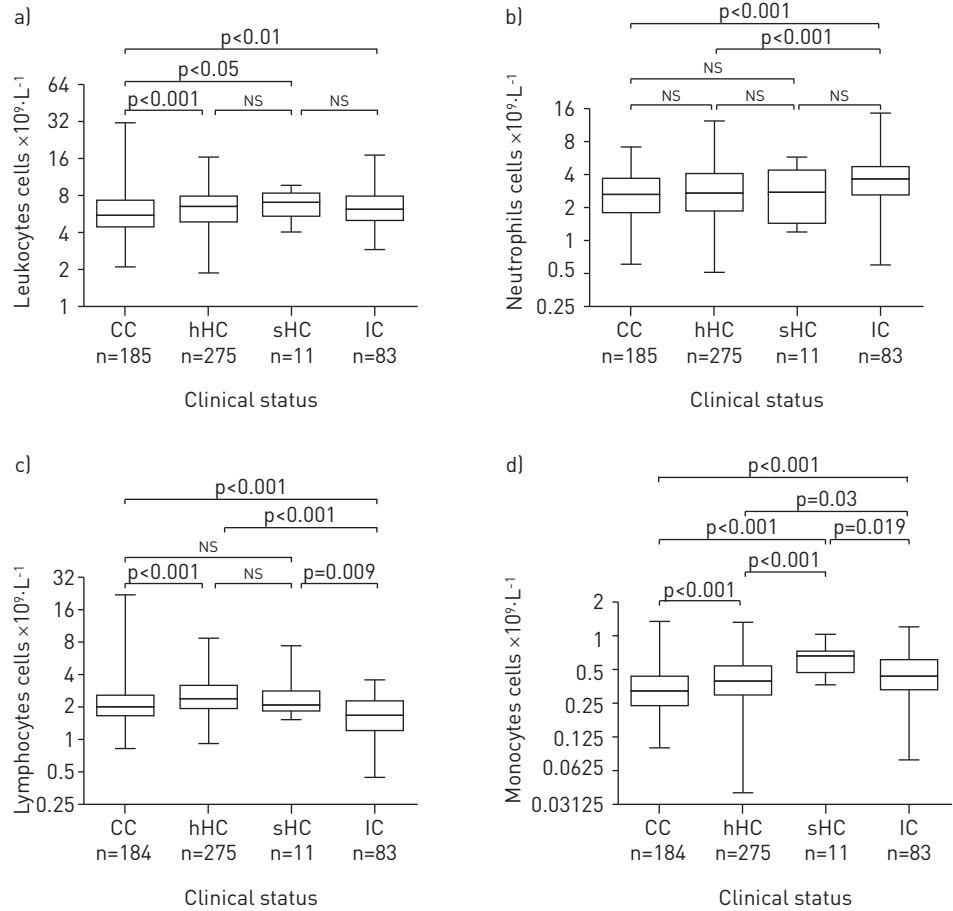
FIGURE 1 Comparison of white blood cell count in the different clinical status groups prior to inclusion in the study. a) Leukocytes; b) neutrophils; c) lymphocytes; d) monocytes. Data are presented as median and ranges of absolute cell counts. CC: community controls; hHC: household contacts that remained healthy; sHC: household contacts that developed tuberculosis (TB) symptoms in the course of follow-up; IC: TB index cases; NS: nonsignificant. Mann–Whitney U-tests were used for the pairwise comparison of groups.

Assessment of risk of developing active TB according to peripheral blood monocyte rate and TST
As the monocyte percentage was the biomarker that showed most significant difference between sHC and hHC (table 2), a ROC analysis was performed to identify a cut-off associated with elevated risk for developing TB in the TB contacts. A cut-off point from the ROC curves of 7.5% monocytes in total

TABLE 2 Descriptive paired analysis of cohorts with regard to the development of tuberculosis symptoms

	sHC	hHC	p-value	Bonferroni correction
Subjects n	12	24		
Leukocytes	7.3 (4.1–9.8)	7.9 (3.2–14.9)	0.39	NS
Neutrophils				
Absolute number	2.7 (1.2–5.9)	3.8 (0.8–10.2)	0.42	NS
Percentage	46.1 (13.0–100.0)	47.0 (12.8–100.0)	0.72	NS
Lymphocytes				
Absolute number	2.2 (1.5–7.4)	2.85 (1.3–8.4)	0.02[#]	0.14
Percentage	37.6 (18.5–77.5)	37.8 (18.5–74.2)	0.47	NS
Monocytes				
Absolute number	0.68 (0.38–1.04)	0.47 (0.19–0.86)	0.04[#]	0.28
Percentage	8.5 (6.4–16.5)	6.1 (2.7–12.5)	<0.001[#]	<0.01[#]

Data are presented as median (range), unless otherwise stated. sHC: symptomatic household contacts; hHC: healthy household contacts; NS: nonsignificant. [#]: statistically significant values.
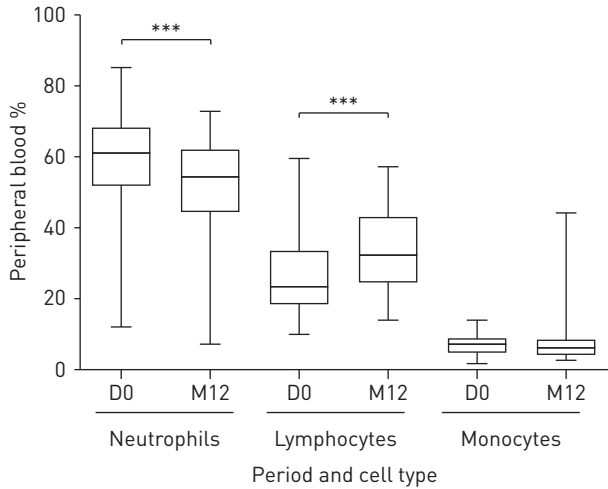
FIGURE 2 Comparison of white blood cell rates in the tuberculosis patients. Data are presented as median and ranges of cell percentages. D0: at inclusion (n=83); M12: after treatment (n=66). Mann–Whitney U-tests were used for the pairwise comparison of groups. ***: p<0.001.

peripheral blood mononuclear cells gave the best separation, and was associated with a sensitivity and specificity of 75% (fig. 3).

TST ⩾14 mm and blood monocyte rate ⩾7.5% in the TB contacts remained associated with the highest risk of developing active TB adjusted for lymphocyte count (table 3). Household contacts with both TST ⩾14 mm and monocyte rate ⩾7.5% were associated with a significantly elevated risk of progressing to active TB (p<0.001 by the log-rank test) (fig. 4). The hazard ratio (HR) for developing TB symptoms in household contacts with TST ⩾14 mm, monocyte:lymphocyte ratio and peripheral blood monocyte rate ⩾7.5% were significantly high (table 3). To address the potential confounding of age and sex, these covariates were included in Cox models. After adjustment for age, sex and blood lymphocyte count, household contacts with both TST ⩾14 mm and peripheral blood monocyte rate ⩾7.5% remained significantly at risk of developing active TB (HR 8.46, 95% CI 1.73–41.22; p<0.01), with the lowest AIC (table 3).

Discussion

Developing tests to determine which individuals with LTBI are at the greatest risk of progressing to active TB would allow the identification and treatment of at-risk individuals and reduce the number of active TB cases. This would be a major step forward for TB control programmes. We conducted this longitudinal cohort study to examine the association of peripheral WBC counts with the risk of TB in HIV-negative TB close household contacts. Our study shows the following findings.

Consistent with other studies [24–26] we confirmed that the prognostic power of a positive TST alone in predicting LTBI progression to active TB disease is low. As TB is endemic in Madagascar and the coverage rate is high for BCG vaccination, a weak TST response may not be specific for the detection of *M. tuberculosis* infection, and it is to be expected that the healthy groups are heterogeneous; potentially
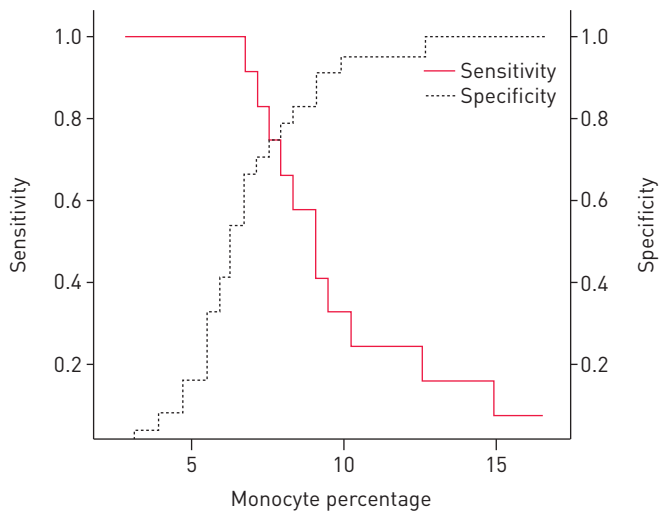

FIGURE 3 Specificity and sensitivity curves according to blood monocyte percentages in the tuberculosis contact population. A cut-off point of 7.5% was obtained.

TABLE 3 Parameters analysed to predict risk of progression to active tuberculosis in household contacts

|	Crude HR (95% CI)	p-value	Adjusted HR (95% CI)	p-value	AIC
Age	0.97 (0.92–1.02)	0.23	0.98 (1.02–1.03)	0.93	
Lymphocytes	0.99 (0.94–1.04)	0.66	0.97 (0.53–1.79)	0.94	
TST ⩾14 mm	5.5 (1.2–25.3)	0.03[#]	5.72 (1.22–26.80)	0.03[#]	
Monocytes ⩾7.5%	6.08 (1.61–22.91)	<0.01[#]	6.25 (1.63–23.95)	<0.01[#]	108.73
Monocyte/lymphocyte ratio	4.49 (1.19–16.94)		4.97 (1.30–18.99)[¶]	0.02[#]	113.39
Monocytes ⩾7.5% + TST ⩾14 mm	8.78 (1.82–42.32)	<0.01[#]	8.46 (1.73–41.22)[+]	<0.01[#]	80.81

n=274. HR: hazard ratio; AIC: Akaike information criterion per Cox model; TST: tuberculin skin test. [#]: statistically significant; [¶]: adjusted for age, sex and TST ⩾14 mm; [+]: adjusted for age, sex and lymphocyte count.

containing both latently infected and uninfected individuals. We therefore used the more stringent definition that a strong TST response with an induration in the TST of ⩾14 mm equated to LTBI [27]. Using this definition, it was also found that a strong TST response (⩾14 mm) was associated with a significantly elevated risk for subsequent progress to TB among contacts, as described previously [28].

Household contacts with LTBI aged <16 years appear to be at slightly higher risk of progressing to active TB in this population than adults. This is in contrast to observations from countries with a low TB burden where the older age group was found to be at higher risk of developing TB [29]. However, this may reflect the different age structure of our cohort, which had fewer elderly individuals, a common pattern in the age structures of developing countries, or different patterns of transmission. Our data suggest that it may be worth extending the World Health Organization recommendation for TB preventive treatment to individuals aged from <5 to <16 years in countries that have a high TB burden and populations of similar age distributions to that studied here.

An increased risk of subsequently developing TB was associated with elevated peripheral blood monocyte rate and TST response ⩾14 mm among TB household contacts in this study. Differences in the peripheral blood cell count have been shown to identify elevated risk of developing active TB in HIV-infected individuals [13, 30]. However, while the monocyte:lymphocyte ratio seemed to give the best predictive value in the HIV-infected individuals, observations from the present study in HIV-negative TB contacts suggested that the peripheral blood monocyte rate alone can identify elevated risk of progression to active TB, and when combined with a strong TST response gave a better risk association than the monocyte: lymphocyte ratio according to the AIC obtained from the different models. The effect of HIV on lymphocyte and leukocyte numbers [31] could explain the differences observed in these HIV-positive and -negative populations.

FIGURE 4 Kaplan–Meier estimates for overall survival from diagnosis of active tuberculosis (TB) symptoms in the TB contacts. The groups were divided based upon peripheral monocyte percentages and tuberculin skin testing.
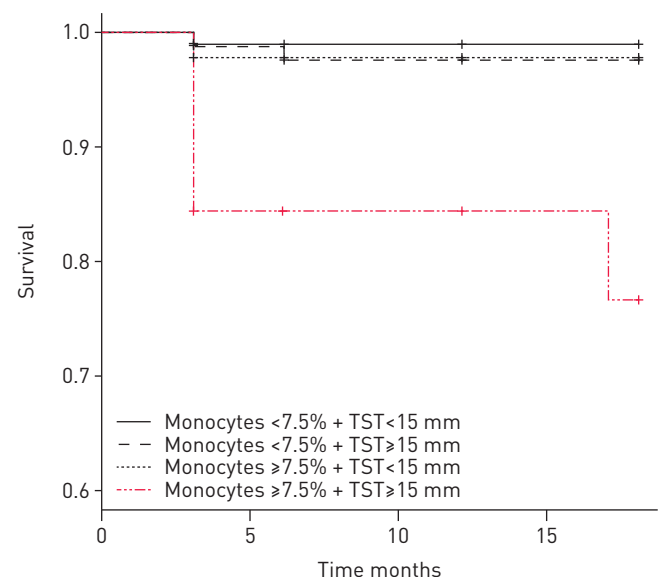
Monocytes are produced in the bone marrow from monoblasts and then circulate in the bloodstream and reside in the spleen. The circulating monocytes are precursors for tissue macrophages that are implicated in defence against a range of microbial pathogens [32, 33]. These cells are actively recruited from the bloodstream to form the lung granuloma that contain *M. tuberculosis* infection, but it has also been established that *M. tuberculosis* can inhibit the host immune response by multiple mechanisms, in particular by inhibiting the phagocytic process and using the phagocytes as ecological niches where the pathogen can replicate [32–35]. Chemokines such as monocyte chemoattractant protein-1 are suggested to be involved in the massive monocyte recruitment to the lung to control *M. tuberculosis* infection [36, 37]. A role for type I interferons in the accumulation of myeloid cell populations in the lung and pulmonary recruitment of inflammatory monocytes that lead to TB disease immunopathology has also been suggested [38–40]. An ongoing infection with an inadequate adaptive immune response against the pathogen may explain the increased percentage of blood monocytes observed in the TB contacts in our study prior to the development of active disease.

It has already been reported that patients with active TB have an increased frequency of peripheral blood monocytes compared to healthy individuals, and this study and others have shown that effective anti-TB chemotherapy can reverse this [17, 41, 42]. The high count of peripheral monocytes also observed in the TB contacts prior to progression to an active TB in the present study is consistent with the hypothesis that this could be a biomarker for progressive TB [42, 43], and our study suggests that this is the case even before the appearance of symptoms.

In summary, combining TST, a technique widely used to assess an *M. tuberculosis* infection in developing countries, and the peripheral blood monocyte count, a technique available in many health centres, may serve as a simple, cheap and practical test to identify those most at risk of progression to TB disease. If confirmed in larger studies and in diverse populations, using both tests in TB contacts could avoid unnecessary treatment and may improve the identification of individuals that need to be prioritised for treatment.

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