International Journal of Infectious Diseases 141 (2024) 106999

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Detection of *Mycobacterium tuberculosis* DNA in CD34$^+$ peripheral blood mononuclear cells of adults with tuberculosis infection and disease

Federica Repele[1,#], Tonino Alonzi[1,#], Assunta Navarra[2], Chiara Farroni[1], Andrea Salmi[1], Gilda Cuzzi[1], Giovanni Delogu[3,4], Gina Gualano[5], Vincenzo Puro[2], Gabriella De Carli[2], Enrico Girardi[6], Fabrizio Palmieri[5], Adrian R. Martineau[7], Delia Goletti[1,*]

[1] *Translational Research Unit, National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Rome, Italy*
[2] *Department of Epidemiology, National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Rome, Italy*
[3] *Dipartimento di Scienze Biotecnologiche di Base, Cliniche Intensivologiche e Perioperatorie-Sezione di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy*
[4] *Mater Olbia Hospital, Olbia, Italy*
[5] *Respiratory Infectious Diseases Unit, National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Rome, Italy*
[6] *Scientific Direction, National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Rome, Italy*
[7] *Centre for Immunobiology, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK*

ARTICLE INFO

Article history:
Received 25 January 2024
Revised 22 February 2024
Accepted 4 March 2024

Keywords:
Tuberculosis infection
Mycobacterium tuberculosis
ddPCR
IGRA
Tuberculosis niche
CD34$^+$ cells

ABSTRACT

Objectives: To investigate whether *Mycobacterium tuberculosis* (Mtb) DNA is detected in peripheral blood mononuclear cells (PBMC) of subjects with tuberculosis (TB) or TB infection (TBI) living in a low-burden country.
Methods: We prospectively enrolled 57 patients with TB, 41 subjects with TBI, and 39 controls in Rome, Italy. PBMC were isolated, cluster of differentiation (CD)34$^+$ and CD34$^-$ cells were immunomagnetic separated, DNA was extracted, and digital polymerase chain reaction for IS*6110* and *rpoB* sequences was used to detect Mtb DNA in PBMC subsets and unfractionated PBMC.
Results: We detected Mtb DNA at a low copy number in CD34$^+$ cells in 4o f 30 (13%) patients with TB, 2 of 24 (8%) subjects with TBI, and 1 of 24 (4%) controls. Mtb DNA was detected in unfractionated PBMC in 3 of 51 (6%) patients with TB, 2 of 38 (5%) subjects with TBI, and 2 of 36 (6%) controls. In CD34$^-$ cells, only 1 of 31 (3%) subjects with TBI tested positive for Mtb DNA.
Conclusions: Mtb DNA was detected at low frequencies and levels in the PBMC of subjects with TBI and donors with TB living in a low-burden country. In particular, Mtb DNA was detected more frequently in CD34$^+$ cells, supporting the hypothesis that these cells may represent a Mtb niche. This finding informs biological understanding of Mtb pathogenesis and may support the development of a microbial blood biomarker for Mtb infection.

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) and represents a major public health threat worldwide, with 10.6 million cases and about 1.3 million deaths in 2022 [1]. For TB control and for reducing Mtb transmission, it is crucial to diagnose TB infection (TBI) and identify the subset of infected people at the highest risk of progression from infection to disease, who will benefit the most from preventive therapy [2]. Currently, there is no gold-standard test for TBI diagnosis [3]. Tuberculin skin test (TST) and interferon-γ release assays (IGRA) detect only an immune response associated with TBI and they have a low positive predictive value for progression to disease [4,5]. In addition, a minority of subjects who tested negative on IGRA may also progress to TB [6]. Several new methodologies for TBI diagnosis based on the direct identification of Mtb rather than the host response directed at Mtb have been attempted [7].

* Corresponding author: Tel.: +39 06 55170 906.
E-mail address: delia.goletti@inmi.it (D. Goletti).
# Contributed equally to the study.

https://doi.org/10.1016/j.ijid.2024.106999

Recent studies have suggested that Mtb DNA can be detected in the peripheral blood mononuclear cells (PBMC) of infected subjects using a lytic bacteriophage-based blood assay (Actiphage) and polymerase chain reaction (PCR) in patients with either TB disease (11 of 15, 73%) or TBI (3 of 18, 17%) [8]. Interestingly, two of the three subjects positive for Mtb DNA progressed to TB disease within 7 months, suggesting the potential utility of this methodology in identifying the TB progressors [8]. More recently, the same group showed that an Actiphage test positivity at baseline is associated with the presence of features of incipient TB [9].

Other studies demonstrated that Mtb can be detected in peripheral blood cells, in particular, in the hematopoietic stem cells [10]. It has been shown that Mtb DNA is present in peripheral hematopoietic stem cells ($CD34^+$ cells) of IGRA-positive donors [11]. The Mtb present in $CD34^+$ cells of subjects with TBI was in a dormant state because it was almost unable to form colonies on agar. However, these Mtb-infected $CD34^+$ cells, when they were inoculated via intra-tracheal injection into immunocompromised mice, caused a disseminate TB disease, accompanied by the detection of culturable Mtb bacilli from several mouse tissues, such as the lung, spleen, thymus, and bone marrow [11].

Moreover, Mtb DNA was also detected by droplet digital PCR (ddPCR) in $CD34^+$ and $CD34^-$ cells isolated from asymptomatic adults with recent household or occupational exposure to pulmonary and bovine TB [12]. This study carried out in Ethiopia, a high TB endemic country, led to the detection of Mtb DNA in 156 of 197 (79%) subjects, with a higher prevalence in $CD34^+$ cells (154 of 197, 78%) than $CD34^-$ cells (46 of 197, 23%). Intriguingly, the proportion of Mtb DNA detection did not differ between IGRA-negative and IGRA-positive donors, 77 of 99 (78%) versus 79 of 98 (81%), respectively [12]. The administration of isoniazid preventive therapy was also shown to reduce the proportion of detectable Mtb DNA in a subset of participants living with HIV who received this intervention. These data suggest that new molecular approaches to the detection of microbiological biomarkers of Mtb infection may have a role in TBI diagnosis and treatment monitoring.

However, data are lacking on the ability to detect Mtb DNA in patients with TBI in low TB burden settings. Moreover, the detection of Mtb DNA in PBMC of patients with TB disease has yet to be evaluated. We, therefore, conducted an observational study in Rome, Italy to determine whether Mtb DNA could be detected in the PBMC of patients with TBI or TB disease.

Methods

Study population

This study was approved by the National Institute for Infectious Diseases Lazzaro Spallanzani (INMI) Ethical Committee (approval numbers: 55/2019), and written informed consent was signed by all the donors, all recruited at INMI. Donors were prospectively enrolled between August 2020 and September 2023. Microbiological TB diagnosis was based on molecular and/or cultural positive results from different biological specimens. Clinically diagnosed TB disease was defined based on clinical and radiologic criteria and on the physician's decision to prescribe a full course of TB treatment to the patient that led to full recovery. Histologically, TB was defined based on the pathology examination of the suspected tissues. TBI was defined based on a positive score to QuantiFERON-TB Gold Plus (QFT) (Qiagen) in the absence of clinical, microbiological, and radiological signs of TB disease. Among TBI donors, 20 of 41 (49%) were remote infections, whereas 21 of 41 (51%) were recent infections. Subjects with TB and TBI were enrolled before or within 4 days from treatment start.

The "no TB group" was formed by healthy donors (HDs) and by patients with pneumonia and other diseases enrolled at INMI as presumptive TB, which was excluded by the absence of microbiological evidence of the disease and because the patients recovered with treatments not involving TB drugs.

Cell line and bacterial strains

Human THP-1 cells, a human monocytic leukemia cell line, were cultured as already reported [13]. Cells were grown in RPMI 1640 (Euroclone; Cat. No. ECB9006) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin solution (Euroclone, Cat. No. ECB3000D and ECB3001D, respectively) and 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies Italia; Cat. No. 10270106), previously inactivated at 56°C for 30 minutes. Cells were maintained at 37°C, with a 5% CO_2 humidified atmosphere.

The Mtb strain H37Rv used for THP-1 infection was isolated at the Fondazione Policlinico Gemelli IRCCS, Università Cattolica del Sacro Cuore [14].

THP-1-derived macrophages infection with Mtb

To induce macrophage differentiation, THP-1 cells were treated with 0.1 µg/ml phorbol 12-myristate 13-acetate (Merck Life Science; Cat. No. J63916.MX) for 48 hours. Experiments with Mtb were performed in a biosafety laboratory level 3 following standard safety procedures. THP-1-derived macrophages were infected with Mtb H37Rv at different multiplicities of infection (0.1, 1, and 10) using RPMI supplemented with heat-inactivated 2% fetal bovine serum and 2 mM L-glutamine. A total of 4 hours after infection, cells were washed three times with 1 × phosphate buffered saline (Euroclone; Cat. No. ECB4004L) to remove extracellular bacteria. Cells were then detached with trypsin-ethylenediamine tetraacetic acid solution (Merck Life Science; Cat. No. T3924) and heated at 80°C for 30 minutes. DNA was extracted as described below.

PBMC isolation, separation of $CD34^+$ and $CD34^-$ from PBMC, and flow cytometry

PBMC were isolated on density gradient centrifugation from 21-27 ml of blood using Ficoll (Cedarlane Labs; Cat. No. CL5020-RC). $CD34^+$ and $CD34^-$ cells were separated from PBMC using CD34 MicroBead Ultra-Pure Kits and MS columns (Miltenyi Biotec; Cat. No. 130-100-453 and 130-042-201, respectively) according to the manufacturer's instructions. Effective isolation of $CD34^+$ was confirmed by flow cytometry; Supplementary Figure 1 shows one of three donors tested and not included in our cohort. Cells were stained before and after isolation with allophycocyanin-conjugated anti-human CD34 and BV786-conjugated anti-human CD45 (BD Biosciences; Cat. No. 555824 and 563716, respectively) (gating strategies are shown in Supplementary Figure 1) and acquired on a DxFlex cytometer (Beckman Coulter). Data were analyzed with CytExpert software (Beckman Coulter).

DNA extraction

DNA was extracted from Mtb-infected THP-1-derived macrophages and from three different peripheral cell subpopulations of each donor ($CD34^+$, $CD34^-$ cells, and unfractionated PBMC) using a cetyltrimethylammonium bromide and chloroform-isoamyl alcohol protocol, as already reported [15]. Concentrations of DNA were determined using the NanoDrop 2000c spectrophotometer (ThermoFisher Scientific).

Droplet digital polymerase chain reaction

ddPCR was performed using a QX200 ddPCR System (Bio-Rad Laboratories) to detect two Mtb-specific DNA sequences, the multi-

copy (8-15 copies) insertion sequence (IS)*6110*, and the single-copy gene *rpoB*.

The reaction was performed using 2 × Supermix for probes no dUTP (Bio-Rad Laboratories; Cat. No. 1863024) according to the manufacturer's instructions. The total volume used in each ddPCR procedure was 20 μl. The sequences of primers used for IS*6110* amplification were as follows: forward 5'-AGAAGGCGTACTCGACCTGA-3', reverse 5'-GATCGTCTCGGCTAGTGCAT-3' and probe 5'-AGGCAGGCATCCAACCG-3', which was labeled with HEX at 5' terminal and BHQ1 at 3' terminal. The sequences of primers used for *rpoB* amplification were as follows: forward 5'- CAAAACAGCCGCTAGTCCTAGTC-3', reverse 5'-AAGGAGACCCGGTTTGGC-3', and probe 5'-AGTCGCCCGCAAAGTTCCTCGAA-3', which was marked with FAM at 5' terminal and BHQ1 at 3' terminal (all from Merck Life Science). The amplification was performed using the following conditions: 95°C for 10 minutes for enzyme activation, then 45 cycles each consisting of 30 seconds denaturation at 95°C and of 1 minute primer annealing and extension at 55°C, and a final 95°C for 10 minutes step for droplet stabilization. The temperature ramp rate was 2.5°C/second.

A no-template control and a positive control (Mtb-infected THP-1-derived macrophages) were used in each ddPCR experiment. Data were analyzed using QuantaSoft Analysis software and thresholds were set at amplitudes of 1200 for channel 2 (detecting the HEX-labeled IS*6110* probe) and at 4000 for channel 1 (detecting the FAM-labeled *rpoB* probe) across all the samples to separate positive and negative droplets. ddPCR data were considered valid for droplet counts >10,000/well according to the manufacturer's instructions. The absolute target concentration is calculated by Poisson distribution. ddPCR data are presented as copy number per 20 μl and as number of positive droplets per well. The limits of detection (LODs) were calculated considering the number of positive droplets of the negative controls and the gene copies/20 ul obtained at the lower Mtb gDNA concentration for each gene (i.e. 1 fg for *rpoB* or 0.25 fg for IS*6110*). The LODs for IS*6110* and *rpoB* were calculated using the formula: LOD = limit of blank + 1.645 × $SD_{low\ concentration\ sample}$, where limit of blank = $mean_{blank}$ + 1.645 × SD_{blank} [16].

Statistical analysis

Data were analyzed using GraphPad (GraphPad Prism 9 XML Project) and Stata (StataCorp LLC 2021, Stata Statistical Software: Release 17, College Station, TX, USA). The comparisons between groups were evaluated using the Kruskal-Wallis test, whereas the chi-square test was used for categorical variables.

The Spearman rank correlation coefficient was used to evaluate the correlation between copy number per 20 μl well for IS*6110* compared with *rpoB*.

The proportion of Mtb DNA-positive in $CD34^-$ cells and in PBMC was compared with that obtained in $CD34^+$ cells by test of proportions.

The association of Mtb DNA detection with cell population and diagnosis was investigated using multivariable mixed-effects logistic regression analysis in which, to account for repeated measures on cell population, a random intercept by subjects was used.

On the subset of subjects with QFT test, the association of Mtb DNA detection and QFT status, in each cell populations, was evaluated by Firth logistic regression analysis to obtain finite estimations in case of perfect separation.

Results

Characteristics of the enrolled donors and $CD34^+$ cell isolation

We prospectively enrolled 137 individuals: 57 with TB, 41 with TBI, and 39 with no TB (Table 1). Within the no TB group, 14

Table 1
Demographical and clinical information of enrolled subjects.

Characteristics		TB	TBI	NO TB		Total	p-value
				prTB	Healthy donors		
N (%)		57 (42)	41 (30)	25 (18)	14 (10)	137 (100)	
Age median (interquartile range)		47 (36-61)	53 (34-63)	55 (42-63)	40 (31-45)		0.0312[a]
Female N (%)		21 (37)	16 (39)	13 (52)	10 (71)	60 (44)	0.0887[b]
Origin N (%)	West Europe	21 (37)	23 (56)	16 (64)	14 (100)	74 (54)	
	East Europe	15 (26)	11 (27)	2 (8)	0 (0)	28 (20)	
	Asia	7 (12)	4 (10)	2 (8)	0 (0)	13 (9)	0.0003[b]
	Africa	9 (16)	2 (5)	3 (12)	0 (0)	14 (10)	
	South America	5 (9)	1 (2)	2 (8)	0 (0)	8 (6)	
BCG-vaccinated N (%)		36 (63)	18 (44)	9 (36)	1 (7)	64 (47)	0.0010[b]
HIV-infected N (%)		2 (4)	0 (0)	2 (8)	0 (0)	4 (3)	0.2614[b]
QFT N (%)	Positive	28 (49)	41 (100)	0 (0)	0 (0)	69 (50)	
	Negative	3 (5)	0 (0)	18 (72)	14 (100)	35 (26)	
	Not done	26 (46)	0 (0)	7 (28)	0 (0)	33 (24)	
TB diagnosis N (%)	Microbiological[c]	53 (92)	-	-	-	53 (39)	
	Histological	2 (4)	-	-	-	2 (1)	
	Clinical	2 (4)	-	-	-	2 (1)	
	Pulmonary	44 (77)	-	-	-	44 (32)	
TB localization N (%)	Extrapulmonary	5 (9)	-	-	-	5 (4)	
	Pulmonary and extrapulmonary	8 (14)	-	-	-	8 (6)	
	Pneumonia-no TB	-	-	12 (48)	-	12 (9)	
	Non tuberculosis mycobacteria	-	-	5 (20)	-	5 (4)	
NO TB controls N (%)	COVID-19	-	-	5 (20)	-	5 (4)	
	Adenocarcinoma	-	-	1 (4)	-	1 (1)	
	Lymphoma	-	-	1 (4)	-	1 (1)	
	BCG orchitis	-	-	1 (4)	-	1 (1)	

BCG, Bacille Calmette-Guérin; QFT, QuantiFERON-TB Gold Plus; TB, tuberculosis; TBI, TB infection; prTB, presumptive TB.
[a] Kruskal-Wallis test
[b] chi-square test
[c] microbiological diagnosis is based on molecular and/or cultural positive results.

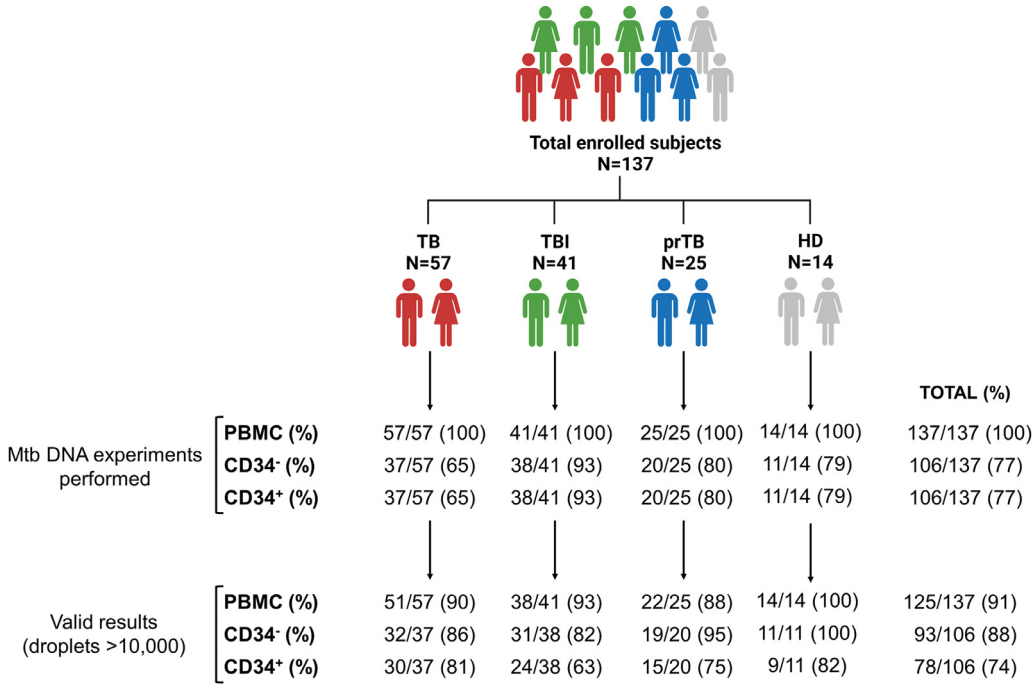

Figure 1. Flowchart of enrolled subjects. Number of total enrolled subjects, number of subjects in which cell selection was performed, and number of valid ddPCR results were obtained. CD, cluster of differentiation; HD, healthy donor; Mtb, *Mycobacterium tuberculosis*; PBMC, peripheral blood mononuclear cells; prTB, presumptive TB; TB, tuberculosis; TBI, TB infection. It was created with BioRender.com.

were HDs, whereas 25 were patients with pneumonia or other diseases, admitted to our hospital as presumptive TB in whom clinical evaluation and microbiological and molecular tests excluded TB diagnosis. Demographic and clinical characteristics are reported in Table 1.

After enrollment, as shown in Figure 1, PBMC isolation was performed in all subjects. Owing to a low number of PBMC obtained, the isolation of $CD34^+$ cells was performed in 37 of 57 (65%) patients with TB, 38 of 41 (93%) subjects with TBI, and 31 of 39 (80%) donors with no TB (20 of 25 of presumptive TB and 11 of 14 of HDs).

A representative example of $CD34^+$ cell isolation from the PBMC of an HD is reported in Supplementary Figure 1. Before magnetic separation, the proportion of $CD34^+$ was 0.14% of the PBMC, whereas after isolation, these cells were 87.23% within the $CD34^+$ fraction and 0.01% within the $CD34^-$ fraction, respectively, confirming what previously showed [12,17]. In the next experiments, because it is known that the $CD34^+$ cells are only 0.01-0.2% of PBMC [18], DNA was extracted from the different cell populations without verifying the cell purity of the populations.

Valid ddPCR data (droplet counts >10,000/well) were obtained for at least 63% of the samples available (Figure 1).

Detection of Mtb DNA

We analyzed the presence of Mtb DNA in the peripheral cell subpopulations by ddPCR detecting two Mtb-specific DNA sequences, IS*6110* and *rpoB*. Supplementary Figure 2 shows some representative amplification plots. Samples were considered positive when the DNA copy number per 20 µL was above the LODs (Methods), which were 4.3 copies/20 µl and 3.6 copies/20 µl, for IS*6110* and *rpoB,* respectively.

As shown in Figure 2, Mtb DNA was detected mainly in the $CD34^+$ cells and in unfractionated PBMC; Mtb DNA was detected in the $CD34^-$ population of only one subject with TBI.

In the $CD34^+$ cells, IS*6110* was detected in 4 of 30 (13%) patients with TB, in 2 of 24 (8%) subjects with TBI, and in 1 of 15 (7%) donors with presumptive TB, whereas it was not detected in HDs. Notably, IS*6110* was also detected in unfractionated PBMC in 2 of 51 (4%) patients with TB, 1 of 38 (3%) subjects with TBI, and 2 of 22 (9%) donors with presumptive TB. IS*6110* was not detected in HDs.

Similar results were obtained by analyzing the *rpoB* gene, which was detected in the $CD34^+$ cells population of patients with TB (3 of 30, 10%), subjects with TBI (2 of 24, 8%), and donors with presumptive TB (1 of 24, 4%). This gene was not detected in $CD34^-$ cells, whereas it was detected in unfractionated PBMC in 1 of 51 (2%) patients with TB, 2 of 38 (5%) subjects with TBI, and 1 of 22 (5%) donors with presumptive TB, respectively.

The logistic regression analysis of these results showed a significant lower probability to detect Mtb DNA in $CD34^-$ cells (odds ratio [OR] 0.09, 0.01-0.84, $p = 0.035$) than $CD34^+$ cells (Table 2). Moreover, although not statistically significant, the probability of detecting Mtb DNA was about two-fold higher in subjects with TB or TBI with respect to donors with no TB (Table 2).

The seven patients with TB positive for Mtb DNA were all microbiologically confirmed. In particular, five were pulmonary TB,

Table 2
Evaluation of the association between Mtb DNA detection and TB status or cell population.

	aOR	95% confidence interval	p-value
NO TB	Ref.		
TB	2.12	0.38 - 11.91	0.395
TBI	1.89	0.31 - 11.66	0.495
$CD34^+$	Ref.		
$CD34^-$	**0.09**	**0.01 - 0.84**	**0.035**
PBMC	0.58	0.17 - 1.93	0.373

aOR, adjusted odds ratio; CD, cluster of differentiation; TB, tuberculosis; PBMC, peripheral blood mononuclear cells. OR was estimated by multivariable mixed-effects logistic regression.

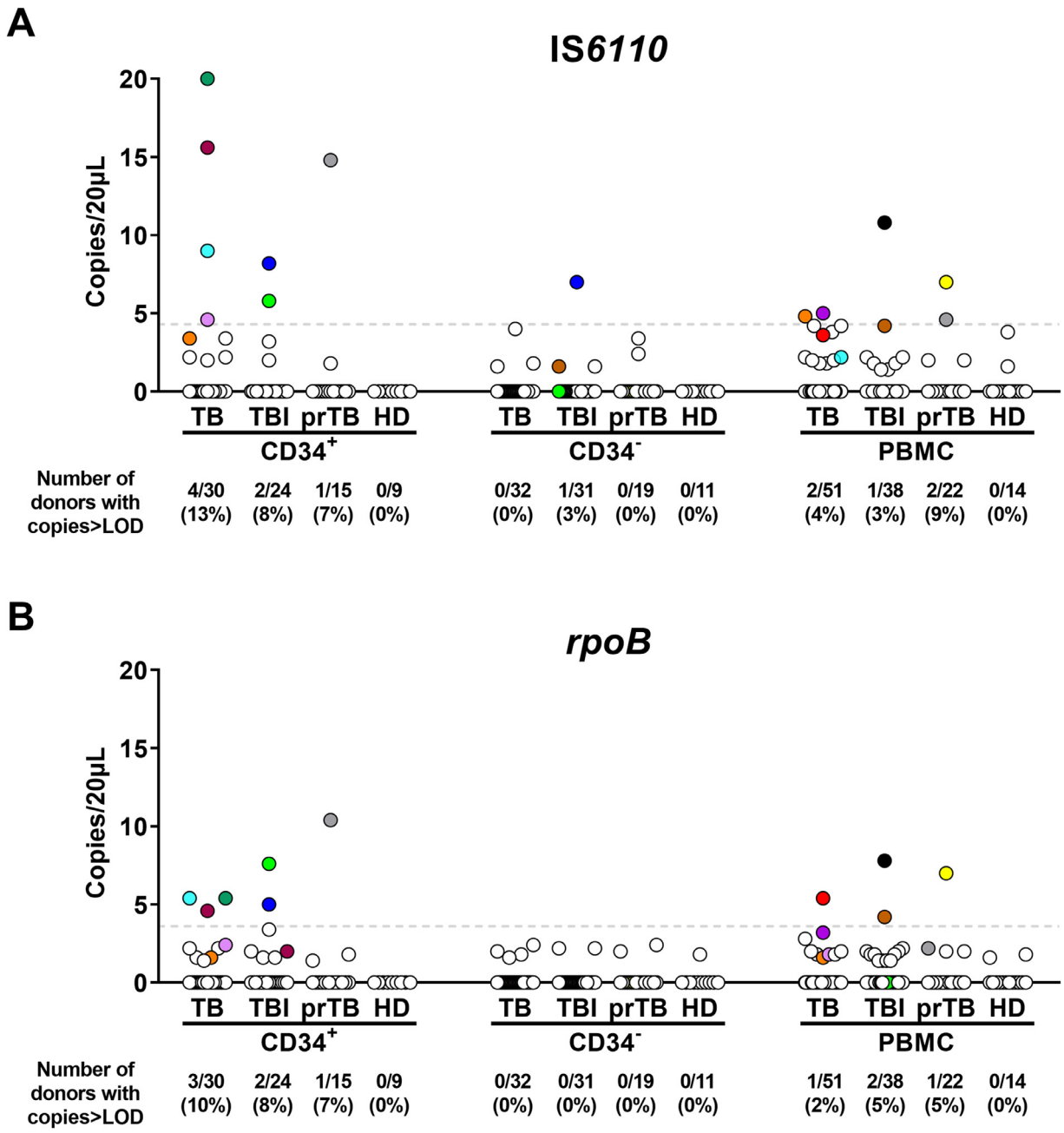

Figure 2. IS*6110* and *rpoB* DNA copy number of peripheral cell subpopulations. IS*6110* (A) and *rpoB* (B) copy number per 20 μl well detected in $CD34^+$ and $CD34^-$ cells and PBMC in patients with TB, subjects with TBI, donors with presumptive TB, and HDs. Dotted lines indicate the LODs for Mtb specific DNA sequences (4.3 copies per 20 µl for IS*6110* and 3.6 copies per 20 µl for *rpoB*). The different colored dots indicate different positive subjects. CD, cluster of differentiation; HD, healthy donors; LOD, limit of detection; PBMC, peripheral blood mononuclear cells; TB, tuberculosis; TBI, TB infection; prTB, presumptive TB.

one was pulmonary and extrapulmonary TB, and one extrapulmonary TB. Of the four donors with TBI positive for Mtb DNA, three were remote infections and one was a recent infection. The two donors with presumptive TB positive for Mtb DNA had a QFT-negative score.

The copy number per 20 µl of IS*6110* and *rpoB* were significantly positively correlated in all peripheral cell populations, with $r = 0.77$ for $CD34^+$ cells, $r = 0.41$ for $CD34^-$ cells, and $r = 0.60$ for unfractionated PBMC ($p < 0.0001$) (Figure 3).

It is worth noting that the number of positive droplets was very low, with counts ranging from 2 to 11 for IS*6110* and from 2 to 7 for *rpoB* (Supplementary Figure 3).

Finally, we evaluated whether the detection of Mtb DNA was associated with QFT status (Supplementary Figure 4). Regardless of the cellular populations, the majority of donors who testing positive for Mtb DNA were scored QFT-positive for IS*6110* (10 of 13, 77%) and *rpoB* (8 of 10, 80%).

As shown in Supplementary Table 1, although not statistically significant, subjects in whom Mtb DNA was detected had a trend to be QFT-positive rather than QFT-negative. This was observed mainly in $CD34^+$ cells (OR 2.48, 95% confidence interval [CI] 0.39-15.85, $p = 0.336$) than in $CD34^-$ cells (OR 1.72, 95% CI 0.07-43.55, $p = 0.743$) and PBMC (OR 1.11, 95% CI 0.23-5.27, $p = 0.896$). No correlation was found between Mtb antigen–stimulated interferon-

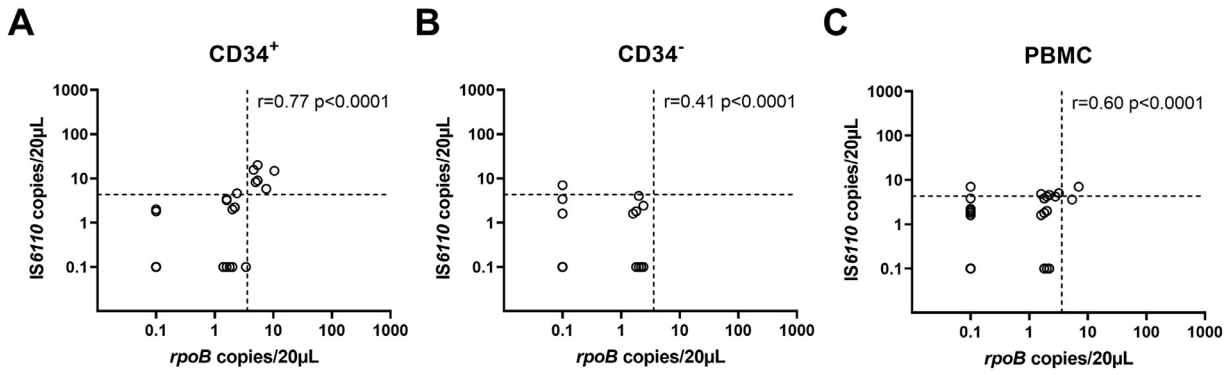

Figure 3. Correlation between IS*6110* and *rpoB* DNA copy number of peripheral cell populations. IS*6110* versus *rpoB* copy number per 20 µl well for $CD34^+$ cells (A), $CD34^-$ cells (B), and PBMC (C). Dotted lines indicate the limit of detection for Mtb-specific DNA sequences (4.3 copies per 20 µl for IS*6110* and 3.6 copies per 20 µl for *rpoB*). Correlation coefficient and p-values were calculated with the Spearman test.
CD, cluster of differentiation; PBMC, peripheral blood mononuclear cells.

γ production of QFT and IS*6110* or *rpoB* copy number per 20 μl (data not shown).

Discussion

In the current study, we demonstrated that Mtb DNA can be detected in peripheral blood cells of subjects with TBI and disease who are attending TB services in a low TB burden country (Italy). Mtb DNA detection was more frequent in the $CD34^+$ cells of patients with TB or TBI, supporting the hypothesis that these cells may serve as a niche for Mtb. These results have potential to underpin development of innovative strategies for TB and TBI diagnosis.

Recently, it was reported that Mtb DNA is detected at high frequencies and levels in asymptomatic TB contacts living in Ethiopia, which is a high TB endemic country [12]. Our study found that Mtb DNA could be also detected in subjects with TBI living in a low TB incidence setting, a country with a considerably lower prevalence than Ethiopia [1]. In addition, we showed the presence of Mtb DNA in patients with TB disease and in donors with presumptive TB. It is important to note that in all positive donors, the Mtb DNA was found at a lower copy number than what was previously found in Ethiopia [12]. This difference may be because of different factors, such as the lower volume of blood used in our experiments versus those in Ethiopia (21-27 ml vs 100 ml), which may have limited the number of Mtb-containing cells. However, we did not find any correlation between the Mtb DNA copy number and the amount of DNA used (data not shown). Another factor may be linked to the low environmental exposure to Mtb in a low burden country such as Italy. This agrees with the low Mtb DNA copy number found in the $CD34^+$ cells of donors living in another Western country (Austria) [11].

IS*6110* and *rpoB* copy numbers were significantly positively correlated in all peripheral cell populations, indicating the robustness of the Mtb detection. In most of the subjects enrolled, as expected, the positive samples for the single-copy gene *rpoB* were also positive for the IS*6110* sequence, which is generally present in Mtb genome in multiple copies. We found two exceptions in the PBMC of one patient with TB and one donor with TBI, in which the *rpoB* identification was not confirmed by the IS*6110* detection. In these two samples, the IS*6110* copy number was just below the LOD, indicating that the low number of positive droplets may be an important issue to consider for a positive or negative score of Mtb DNA detection.

Because Mtb DNA was detected mainly in the $CD34^+$ cells, our results agree with previous data [11,12], further indicating that Mtb, along with other pathogens, affects the blood cell production by the bone marrow. A clear example is the increased myeloid cellularity in the circulation in patients with TB [19]. This is likely because of an alteration of the cytokine milieu controlling hematopoietic stem cells differentiation mechanisms, which is also able to influence the immune response against Mtb [20–24].

Interestingly, we found that Mtb DNA was also detected in a minority of subjects without TB or TBI, in particular, in two patients admitted to the hospital with a presumptive TB. These individuals were QFT-negative and TB disease diagnosis was microbiologically and molecularly excluded. This finding agrees to what was previously reported in Ethiopia, in which the 49% (77 of 156) of Mtb DNA-positive TB contacts were scored QFT-negative [12]. The status of being QFT-negative and Mtb DNA-positive may have different explanations, such as being caused by a recently acquired infection or by the presence of Mtb-specific T cell responses independent of interferon-γ release, known to be present in persistently QFT-negative individuals despite high TB exposure [25].

We also wondered if Bacille Calmette-Guérin (BCG) vaccination could have an impact on Mtb DNA detection. We showed that in the 13 subjects who tested Mtb DNA-positive, six (46%) were not vaccinated with BCG, indicating that BCG vaccination is not responsible for the results obtained.

Regarding the correlation between Mtb DNA detection and TB dissemination, it is difficult to have conclusive data owing to the low number of the patients evaluated with extrapulmonary (5 patients) or extrapulmonary and pulmonary TB (8 patients) localization versus those with a pulmonary localization (44 patients). Further studies are needed to dissect it out.

This study has a few limitations. We did not evaluate the changes in Mtb DNA copy number over time, as previously done [12]. Moreover, donors were followed up only for 14-23 months after the enrollment restraining the ability to further evaluate the TB outcome in those with presumptive TB as well as in TBI. However, although the Actiphage approach reported that Mtb DNA is associated with incipient TB [9] and with TB progression [8], others found that the detection of Mtb DNA in peripheral blood of TB contacts is more likely to represent a quiescent Mtb infection than incipient or subclinical disease [11,26]. Further studies are required to clarify the prognostic significance of detecting Mtb DNA in the blood of asymptomatic people.

In conclusion, our results indicate that Mtb DNA may be detected, although at a low frequency and at a low copy number, in the peripheral blood cells of subjects living in a low TB burden

country. Mtb DNA was found mainly in the CD34$^+$ cell population, reinforcing the hypothesis that these cells represent an Mtb reservoir. Based on the available literature, the presence of an immune response against Mtb (TST-positive or IGRA-positive) does not discriminate between individuals currently infected from those that eradicated the mycobacteria . Therefore, the detection of Mtb DNA in the blood can be considered as an approach to refine this description [25], although more evidence will be needed to demonstrate the clinical value of this test. These results may also underpin future efforts to develop new approaches for the diagnosis of TB and TBI.

Declarations of competing interest

DG reported the following competing interest: PBD Biotech. EG reported the competing interest: research grants from Gilead Sciences and Mylan not related to the present work and speaker fees for Gilead Sciences and ViiV not related to this work. The remaining authors have no competing interest to declare.

Funding

This work was supported by Italian Ministry of Health (Ricerca Corrente, Linea 4) and INAIL (Istituto Nazionale Assicurazione Infortuni sul Lavoro; BRIC-2019 ID 27). TBVAC-HORIZON, funded by the European Union's HORIZON program under Grant No. 101080309. The funders had no impact on any decision-making regarding the manuscript.

Ethical approval

This study was approved by the ethics committee of the National Institute of Infectious Diseases Lazzaro Spallanzani-IRCCS (approval numbers 55/2019).

Acknowledgments

The authors gratefully acknowledge the nurses and the patients who helped conduct this study.

Author contributions

Study conception and design: DG, ARM. Experimental setting and data generation: FR, TA. Technical support for sample processing: AS. Analysis and interpretation of data: FR, TA, AN, ARM, DG. Patient enrollment: GG, FP, DG. Drafting the article: FR, TA, DG. Revising the article critically for important intellectual content and approving the submitted version: all authors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2024.106999.

Transparency Declaration

This article is part of a supplement entitled Commemorating World Tuberculosis Day, March 24th, 2024: "Yes! We Can End TB" published with support from an unrestricted educational grant from QIAGEN Sciences Inc.

References

[1] World Health Organization. Global tuberculosis report, https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023; 2023 [accessed 02 January 2024].

[2] Cantini F, Niccoli L, Capone A, Petrone L, Goletti D. Risk of tuberculosis reactivation associated with traditional disease modifying anti-rheumatic drugs and non-anti-tumor necrosis factor biologics in patients with rheumatic disorders and suggestion for clinical practice. *Expert Opin Drug Saf* 2019;**18**:415–25. doi:10.1080/14740338.2019.1612872.

[3] Goletti D, Delogu G, Matteelli A, Migliori GB. The role of IGRA in the diagnosis of tuberculosis infection, differentiating from active tuberculosis, and decision making for initiating treatment or preventive therapy of tuberculosis infection. *Int J Infect Dis* 2022;**124**:S12–19. doi:10.1016/j.ijid.2022.02.047.

[4] Kontsevaya I, Cabibbe AM, Cirillo DM, DiNardo AR, Frahm N, Gillespie SH, et al. Update on the diagnosis of tuberculosis. *Clin Microbiol Infect* 2023. doi:10.1016/j.cmi.2023.07.014.

[5] Esmail H, Cobelens F, Goletti D. Transcriptional biomarkers for predicting development of tuberculosis: progress and clinical considerations. *Eur Respir J* 2020;**55**:1901957. doi:10.1183/13993003.01957-2019.

[6] Gupta RK, Lipman M, Jackson C, Sitch AJ, Southern J, Drobniewski F, et al. Quantitative IFN-γ release assay and tuberculin skin test results to predict incident tuberculosis. A prospective cohort study. *Am J Respir Crit Care Med* 2020;**201**:984–91. doi:10.1164/rccm.201905-0969OC.

[7] Alonzi T, Repele F, Goletti D. Research tests for the diagnosis of tuberculosis infection. *Expert Rev Mol Diagn* 2023;**23**:783–95. doi:10.1080/14737159.2023.2240230.

[8] Verma R, Swift BMC, Handley-Hartill W, Lee JK, Woltmann G, Rees CED, et al. A novel, high-sensitivity, bacteriophage-based assay identifies low-level Mycobacterium tuberculosis bacteremia in immunocompetent patients with active and incipient tuberculosis. *Clin Infect Dis* 2020;**70**:933–6. doi:10.1093/cid/ciz548.

[9] Kim JW, Bowman K, Nazareth J, Lee J, Woltmann G, Verma R, et al. PET-CT-guided characterisation of progressive, preclinical tuberculosis infection and its association with low-level circulating Mycobacterium tuberculosis DNA in household contacts in Leicester, UK: a prospective cohort study. *Lancet Microbe* 2024;**5**:e119–30. doi:10.1016/S2666-5247(23)00289-6.

[10] Mayito J, Andia I, Belay M, Jolliffe DA, Kateete DP, Reece ST, et al. Anatomic and cellular niches for Mycobacterium tuberculosis in latent tuberculosis infection. *J Infect Dis* 2019;**219**:685–94. doi:10.1093/infdis/jiy579.

[11] Tornack J, Reece ST, Bauer WM, Vogelzang A, Bandermann S, Zedler U, et al. Human and mouse hematopoietic stem cells are a depot for dormant Mycobacterium tuberculosis. *PLOS ONE* 2017;**12**:e0169119. doi:10.1371/journal.pone.0169119.

[12] Belay M, Tulu B, Younis S, Jolliffe DA, Tayachew D, Manwandu H, et al. Detection of Mycobacterium tuberculosis complex DNA in CD34-positive peripheral blood mononuclear cells of asymptomatic tuberculosis contacts: an observational study. *Lancet Microbe* 2021;**2**:e267–75. doi:10.1016/S2666-5247(21)00043-4.

[13] Alonzi T, Petruccioli E, Vanini V, Fimia GM, Goletti D. Optimization of the autophagy measurement in a human cell line and primary cells by flow cytometry. *Eur J Histochem* 2019;**63**:3044. doi:10.4081/ejh.2019.3044.

[14] Romagnoli A, Petruccioli E, Palucci I, Camassa S, Carata E, Petrone L, et al. Clinical isolates of the modern Mycobacterium tuberculosis lineage 4 evade host defense in human macrophages through eluding IL-1β-induced autophagy. *Cell Death Dis* 2018;**9**:624. doi:10.1038/s41419-018-0640-8.

[15] van Soolingen D, de Haas PE, Kremer K. Restriction fragment length polymorphism typing of mycobacteria. *Methods Mol Med* 2001;**54**:165–203. doi:10.1385/1-59259-147-7:165.

[16] Armbruster DA, Pry T. Limit of Blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 2008;**29**:S49–52.

[17] Park GB, Kim MJ, Vasileva EA, Mishchenko NP, Fedoreyev SA, Stonik VA, et al. Echinochrome A promotes ex vivo expansion of peripheral blood-derived CD34+ cells, potentially through downregulation of ROS Production and activation of the Src-Lyn-p110δ pathway. *Mar Drugs* 2019;**17**:526. doi:10.3390/md17090526.

[18] Jelic TM, Estalilla OC, Vos JA, Harvey G, Stricker CJ, Adelanwa AO, et al. Flow cytometric enumeration of peripheral blood CD34+ cells predicts bone marrow pathology in patients with less than 1% blasts by manual count. *J Blood Med* 2023;**14**:519–35. doi:10.2147/JBM.S417432.

[19] Fritschi N, Vaezipour N, Buettcher M, Portevin D, Naranbhai V, Ritz N. Ratios from full blood count as markers for TB diagnosis, treatment, prognosis: a systematic review. *Int J Tuberc Lung Dis* 2023;**27**:822–32. doi:10.5588/ijtld.22.0598.

[20] Moorlag SJCFM, Rodriguez-Rosales YA, Gillard J, Fanucchi S, Theunissen K, Novakovic B, et al. BCG vaccination induces long-term functional reprogramming of human neutrophils. *Cell Rep* 2020;**33**:108387. doi:10.1016/j.celrep.2020.108387.

[21] Mishra A, Singh VK, Actor JK, Hunter RL, Jagannath C, Subbian S, et al. GM-CSF dependent differential control of Mycobacterium tuberculosis infection in human and mouse macrophages: is macrophage source of GM-CSF critical to tuberculosis immunity? *Front Immunol* 2020;**11**:1599. doi:10.3389/fimmu.2020.01599.

[22] Khan N, Downey J, Sanz J, Kaufmann E, Blankenhaus B, Pacis A, et al. M. tuberculosis reprograms hematopoietic stem cells to limit myelopoiesis and impair trained immunity. *Cell* 2020;**183**:752–70 e22. doi:10.1016/j.cell.2020.09.062.

[23] Maceiras AR, Silvério D, Gonçalves R, Cardoso MS, Saraiva M. Infection with hypervirulent Mycobacterium tuberculosis triggers emergency myelopoiesis but not trained immunity. *Front Immunol* 2023;**14**:1211404. doi:10.3389/fimmu.2023.1211404.

[24] Delgobo M, Mendes DA, Kozlova E, Rocha EL, Rodrigues-Luiz GF, Mascarin L, et al. An evolutionary recent IFN/IL-6/CEBP axis is linked to monocyte expansion and tuberculosis severity in humans. *eLife* 2019;**8**:e47013. doi:10.7554/eLife.47013.

[25] Martineau AR, Chandran S, Palukani W, Garrido P, Mayito J, Reece ST, et al. Towards a molecular microbial blood test for tuberculosis infection. *Int J Infect Dis* 2024. doi:10.1016/j.ijid.2024.106988.

[26] Rosenheim J, Abebe M, Belay M, Tulu B, Tayachew D, Tegegn M, et al. Detection of M. tuberculosis DNA in TB contacts' PBMC does not associate with blood RNA signatures for incipient tuberculosis. medRxiv. 06 December 2023. http://dx.doi.org/10.1101/2023.09.26.23296131 [accessed 06 December 2023].